Carcinoma papilar da tireoide: análise genotípica e fenotípica para identificação de biomarcadores de agressividade clínico-patológica

Papillary thyroid carcinoma: identification of biomarkers of clinico-pathological aggressiveness through genotypic and phenotypic analysis

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Porto – 2012

Dissertação de candidatura ao grau de Doutor em Medicina apresentada à Faculdade de Medicina da Universidade do Porto

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Aqui deixo os meus sinceros **Agradecimento**s a todos os que me ajudaram a superar este desafio. A gratidão é muito sentida e devida a muitas pessoas.

Ao Professor Manuel Sobrinho Simões, agradeço a sua imensa generosidade, a exigente e paciente orientação, a partilha da sua visão apaixonada do Mundo.

À Professora Paula Soares, quero agradecer o incentivo, as críticas rigorosas, e por me dar, tantas vezes, o benefício da dúvida.

À Professora Fátima Carneiro, agradeço o incentivo e a disponibilização de meios para a concretização dos trabalhos apresentados.

Ao Professor José Manuel Cameselle Teijeiro, agradeço por me mostrar que, na tireoide, as coisas mais preciosas são tão pequenas que quase não se veem.

À Dr.ª Joana Santos e ao *Grupo da Tireoide*, quero agradecer as discussões acesas e todas as vezes que me mostraram que era preciso estudar muito.

À *Genzyme* e à *Fundação Calouste Gulbenkian*, agradeço o apoio financeiro concedido aos meus projetos.

À Dr.ª Maria Emília Paiva e ao Professor José Manuel Lopes, quero agradecer as críticas impiedosas.

Aos restantes elementos do Serviço de Anatomia Patológica do Centro Hospitalar de São João, agradeço o respeito pelos meus silêncios, e a todos quantos conseguiram relevar o meu mau feitio.

À Carla, agradeço as noites brancas despendidas na construção das bases de dados e o apoio, que só as irmãs sabem dar.

À minha Mãe e ao meu Pai, por tudo.

Ao António e ao Argos, agradeço por me fazerem acreditar, todos os dias, que a vida pode ser mais doce.

Ao abrigo do Decreto-Lei nº388/70, fazem parte integrante desta dissertação os seguintes trabalhos:

- Trabalhos publicados que integram a temática principal da Tese

Eloy C, Santos J, Soares P, Sobrinho-Simões M
"The preeminence of growth pattern and invasiveness and the limited influence of *BRAF* and *RAS* mutations in the occurrence of papillary thyroid carcinoma lymph node metastases"
Virchows Archiv 2011; 459: 265-276

Eloy C, Santos J, Soares P, Sobrinho-Simões M
"Intratumoural lymph vessel density is related to presence of lymph node metastases and separates encapsulated from infiltrative papillary thyroid carcinoma"
Virchows Archiv 2011; 459: 595-605

- Trabalho submetido para publicação que integra a temática principal da Tese

Eloy C, Santos J, Cameselle-Teijeiro J, Soares P, Sobrinho-Simões M
"TGF-beta/Smad pathway and *BRAF* mutation play different roles in circumscribed and infiltrative papillary thyroid carcinoma"
(aceite para publicação na revista *Virchows Archiv*)

- Trabalhos publicados que constituem estudos periféricos à temática central da Tese, cujos resultados são debatidos na respetiva Discussão

Sobrinho-Simões M, Eloy C, Vinagre J, Soares P
"Molecular pathology of thyroid tumors: diagnostic and prognostic relevance"
International Journal of Surgical Pathology 2010; 18: 209S-212S

Sobrinho-Simões M, Eloy, C, Magalhães J, Lobo C, Amaro T
"**Follicular thyroid carcinoma**"
*Modern Patholo*gy 2011; 24: S10-S18

Eloy C, Vinagre J, Cameselle-Teijeiro J, Paiva ME, Soares P, Sobrinho-Simões M
"Tumour-in-tumour of the thyroid with basaloid differentiation. A lesion with a solid cell nest neoplastic component?"
International Journal of Surgical Pathology 2011; 19: 276-280

Cruz J, Eloy C, Aragués JM, Vinagre J, Sobrinho-Simões M
"Small-cell (basaloid) thyroid carcinoma: a neoplasm with a solid cell nest histogenesis?"
International Journal of Surgical Pathology 2011; 19: 620-626

Em cumprimento do disposto no referido Decreto-Lei declara que, nos trabalhos em que é primeira autora, participou ativamente na recolha e no estudo do material, tendo redigido os textos com a colaboração dos outros autores. Nos trabalhos em que é segunda autora, colaborou com os outros autores no estudo do material, na redação dos textos e na respetiva documentação.
A dissertação inclui, também, resultados ainda não publicados.

Abbreviations

ATC/UTC – Anaplastic thyroid carcinoma/undifferentiated thyroid carcinoma
BRAF – v-raf murine sarcoma viral oncogene homolog B1
BRAF mutation – *BRAF* V600E point mutation
CD – Cluster designation
CPTC – Classic papillary thyroid carcinoma
D2-40 – Podoplanin
DNA – Deoxyribonucleic acid
E-FVPTC – Encapsulated follicular variant of papillary thyroid carcinoma
EMT – Epithelial-to-mesenchymal transition
FA – Follicular adenoma
FNAB – Fine-needle aspiration biopsy
FTC – Follicular thyroid carcinoma
FVPTC – Follicular variant of papillary thyroid carcinoma
LVD – Lymph vessel density
LYVE-1 – Lymph vessel endothelial hyaluronan receptor-1
MAPK – Mitogen-activated protein kinase
N-RAS mutation – *N-RAS* Q61R point mutation
NTRK – Neurotrophic tyrosine receptor kinase
P-Smad2/3 – Phosphorylated Smad2 and/or Smad3
PAX8 – Paired box gene 8
PC-FVPTC – Poorly circumscribed follicular variant of papillary thyroid carcinoma
PC-PTC – Poorly circumscribed papillary thyroid carcinoma
PDTC – Poorly differentiated thyroid carcinoma
PPARgamma – Peroxisome proliferation-activated receptor gamma
PTC – Papillary thyroid carcinoma
PTC**-M** – Papillary thyroid carcinoma without lymph node metastases
PTC**+M** – Papillary thyroid carcinoma with lymph node metastases
RAS – Rat sarcoma viral oncogene
RET – Rearranged during transfection
Smad2 – Mothers against decapentaplegic homolog 2
Smad3 – Mothers against decapentaplegic homolog 3
Smad4 – Mothers against decapentaplegic homolog 4
Smad7 – Mothers against decapentaplegic homolog 7
TGF-beta – Transforming growth factor-beta
VEGFR – Vascular endothelial growth factor receptor
WC-PTC – Well circumscribed papillary thyroid carcinoma
WHO – World Health Organization

The abbreviations of Portuguese words used in Resumo are explained in the text itself.

Ao meu Avô Joviano
(*in memoriam*)

Foreword

Variability is probably the single word which better characterizes papillary thyroid carcinoma (PTC). Diagnostic subtleties in PTC and the multiple morphological variants of PTC can be a challenge to the Pathologist but can also constitute a source of confusion for the Clinician and his Patient whose expectations can be flawed by an unpredictable course of the disease.

To avoid disproportionate treatments and surprising outcomes, an extra effort has been made to search for personalized management and treatment of patients with thyroid cancer, often based upon molecular findings frequently lacking a precise morphological and topographical characterization. The growing knowledge on the molecular profile of tumours has contributed to a deeper understanding of cancer biology; despite this, we disagree with those who announce the end of Surgical Pathology, relaying their faith into sophisticated molecular technologies with little or no input from the histopathological standpoint.

Taking this into consideration, and using PTC as a model, we decided to characterize exhaustively from the clinico-pathological standpoint a series of cases with long term follow up which were screened for molecular alterations in the elements of the pro-proliferative mitogen-activated protein kinase pathway and for topographic differences in the expression of the elements of the growth suppressive Transforming growth factor-beta/Smad-dependent pathway. The series of PTCs was composed by cases of classic and follicular variant of PTC. The restriction of the analysis to these two variants, the most frequent after papillary microcarcinoma, was forced by the low number of available cases of less frequent variants, assuring also that the conclusions of the study would not be limited in their application to a marginal group of rare cases. In addition, we decided to consider tumour size as a continuous variable, without separating papillary microcarcinoma into a distinct category. This latter decision reflects the acknowledgment of the influence of time over cancer progression: the evaluation of the time factor and the disclosure of the hidden natural history configure, nowadays, major problems in cancer research.

As a consequence of the limitations imposed by the availability of cases, our series of PTC included cases with lymph node metastases and cases without metastatic disease or recurrence, lacking a significant representation of cases with distant metastases and fatal cases. The evaluation of aggressiveness based upon lymph node metastases is debatable as lymph node metastases do not have an important impact over patients' survival. Despite this, the occurrence of lymph node metastases is, beyond any doubt, a manifestation of malignancy (a condition that sometimes is not very obvious in PTC), an important cause of morbidity and a determinant of additional treatment.

The series of PTC cases was selected from the Cancer Registry of the Pathology Department of Centro Hospitalar de São João after reviewing all filed cases of thyroid tumours. The results of this review allowed also the identification of a set of problems that may provide clues to future studies oriented to the understanding of PTC biology. Part of this series and some of the identified problems are being studied by the Consortium composed by Centro Hospitalar de São João, IPATIMUP and Instituto Português de Oncologia Francisco Gentil do Porto within the project "Identificação de fatores prognósticos e de seleção terapêutica em carcinomas diferenciados da tireoide" sponsored by Calouste Gulbenkian Foundation.

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I Introduction

I.1 Thyroid: from the first stages of development until the senescent morphological and functional tissue

The thyroid gland is an endocrine organ located in the neck that is responsible for the production and controlled release of thyroid hormones. The thyroid hormones are essential to fetal development and regulation of many metabolic processes.
The thyroid median *primordium* derives from the endoderm and appears at the foramen *cecum* of the tongue by the 26th day of fetal life [1]. From this cephalic location, the median *primordium*, descends to its final position anteriorly to the 2nd- 4th traqueal ring and expands laterally to form the lateral lobes [2]. At this stage, by the 9th- 10th week of fetal life, the thyroid parenchyma is organized into solid and trabecular structures with small *lumina* where thyroglobulin positive colloid starts to be produced [2]. The well-formed follicles that constitute the functional units of the thyroid appear two weeks later [2]. The follicles are composed by cuboid follicular cells that face a colloid filled lumen and are surrounded by a basement membrane.
Concurrently, at 6th-7th week of fetal life, the two thyroid lateral *primordia* derived from the ultimobranchial bodies (IVth -Vth branchial pouch complex) are integrated in the lateral lobes of the thyroid carrying the neural crest derived C cells and the endoderm derived main cells [1,2]. The C cells are neuroendocrine cells responsible for the production of calcitonin – a protein involved in the phospho-calcium homeostasis – and are difficult to distinguish from follicular cells as they often integrate the follicles. The main cells can be detected in nearly 78% of fetal thyroids, under the appearance of isolated cells or nests [3] – the so called solid cell nests. The solid cell nests are normal elements of the adult thyroid occurring in up to 68% of adult thyroids [4] that can harbor C cells together with the main cells. The solid cell nests can become hyperplastic [5] and are thought to represent nests of putative thyroid stem cells [6]. Other intra-thyroid tissues, presumably of branchial pouch origin, include islands of mature cartilage and ectopic thymus, parathyroid and salivary gland [7-9].
The normal adult thyroid with all its components is organized in lobules separated by fibrous septa conveying nervous bundles and vessels, including a rich lymphatic network that coalesce in the peri-thyroid soft tissue condensation and drain to cervical and mediastinic lymph nodes.
The follicular cell, under a hostile environment like a chronic inflammatory setting, and/or in senescent *status*, may acquire particular phenotypes. Examples of these particular phenotypes include the oncocytic transformation and the squamous and ductal metaplasia of the follicular epithelium [10]. The oncocytic phenotype results from the accumulation of cytoplasmatic mitochondria and can occur in benign and malignant tumour cells [11]. Similarly, with age, the colloid tends to coalesce in globular formations and to accumulate calcium oxalate crystals.

I.2 Papillary thyroid carcinoma (PTC) and related thyroid diseases

I.2.1 PTC

Only a small percentage of the very common thyroid diseases are malignant. Clinically evident thyroid cancer accounts for approximately 1% of all malignant tumours in developed countries [12] and for about 2% in Portugal [13, 14]. In the USA, for instance, the incidence of thyroid cancer is ~9/100 000 *per* year [15]. The incidence rate of thyroid cancer have been increasing over the last years in most countries including Portugal (**TableI.1**), while the mortality rates due to these tumours has decreased [12, 14].
The number of new cases of papillary thyroid carcinoma (PTC) *per* year increased 56% in Portugal, from 2001 to 2005 [13, 14, 16, 17] and, nowadays, represents 80 to 90% of all thyroid cancers [14]. PTC is the most frequent endocrine malignancy, even in the pediatric group [12].
Although it can occur in a wide range of ages, PTC is more frequent in women in the 5th-6th decade of life [12], predominantly as a sporadic disease, with only 5% of cases developing in a familial setting [18]. Linkage studies suggest that familial PTC is a heterogeneous disorder caused by more than one susceptibility gene [19, 20]. Regarding the influence of environmental factors, the exposure to radiation in general and to radioactive iodine in particular appears to be the most closely related to the occurrence of PTC, as it is illustrated by the striking incidence in children exposed to radioactive fallout [21]. The relative frequency of

PTC is also greater in regions of adequate or high dietary iodine intake compared to regions of iodine deficiency [12].

Table I.1 Incidence rates of thyroid cancer *per* 100 000 habitants in Portugal (A) and , specifically, in the North of Portugal (B)[13, 14, 16, 17]

	Year 2001			Year 2005		
	Males	Females	Total	Males	Females	Total
A	2.5	10.5	6.6	4.0	15.4	9.9
B	2.7	14.4	8.8	5.7	24.1	15.2

PTC usually presents as a cold nodule or mass in the thyroid or as a cervical adenopathy. Additionally, numerous clinically silent, incidentally found PTCs are detected in image studies of the neck performed by other reasons, at autopsy and in surgically removed glands for benign conditions [12, 22].
In the preoperative setting, the ultrasound examination of the thyroid nodule followed by fine-needle aspiration biopsy (FNAB) constitute the most effective procedure to diagnose PTC and to separate it from benign thyroid nodules which do not need surgery [23]. In reference centers, the diagnostic accuracy of FNAB reaches 90% [24].
In the surgical specimen, PTC may assume the appearance of an infiltrative whitish mass or mimic a follicular adenoma (FA) with a well circumscribed, often encapsulated, periphery. Moreover, PTCs can be multicentric and are often very small, becoming difficult to identify.
Together with follicular thyroid carcinoma (FTC), PTC forms the group of the well differentiated thyroid carcinomas disclosing follicular cell differentiation [12]. The diagnosis of PTC relies on the distinctive nuclear features of tumour cells that include enlargement, elongation and overlapping, clear-empty appearance, contours irregularity, prominence of grooves and nuclear pseudo-inclusions. The determinant nature of these nuclear features underlies the success of the cytological diagnosis in FNAB.
The architectural organization of PTC is variable as is illustrated by the 16 variants recognized by the World Health Organization (WHO) in 2004 [12]. If one excludes the incidentally found papillary microcarcinomas which by definition measures less than 1cm, the classic PTC (CPTC) and the follicular variant of PTC (FVPTC) are the most frequently diagnosed variants [12]. CPTC and FVPTC present distinct cyto-architectural features [12], distinct molecular alterations [25-27] and distinct biological/clinical behaviour [25, 28, 29] (*see below*). CPTC discloses, predominantly, an infiltrative often multicentric growth pattern, a papillary or mixed papillary/follicular architecture together with occasional cystic formations and psammoma bodies, while FVPTC presents a variable growth pattern and a monotonous follicular/trabecular architecture that may be composed by medium or large size follicles (*see below*).
The majority of CPTC and FVPTC cases evolve favourably, and the patients have a 10-year survival up to 98% [1, 30]. Despite the generally good prognosis, PTCs may give rise to recurrences as well as to lymph node and distant metastases, sometimes many years after diagnosis [31].
The most frequent molecular alterations detected in PTC occur in mitogenic-activated protein kinase (MAPK) pathway elements and include *BRAF* and *RAS* mutations, and *RET/PTC* and *NTRK* rearrangements [12, 32].

I.2.2 Benign and malignant diseases related to PTC

PTC related inflammatory diseases include Hashimoto thyroiditis and Graves' disease that are autoimmune disorders afflicting predominantly middle age women.
Hashimoto thyroiditis is a disease characterized by the production of auto-antibodies against thyroglobulin and other follicular cell antigens [1]. Morphological features of Hashimoto thyroiditis include extensive lymphocytic infiltration of the thyroid with germinal center formation and oncocytic transformation of the follicular epithelium. "Complications" of Hashimoto thyroiditis include lymphoma [33] and PTC [34]. It has been suggested that Hashimoto thyroiditis represents a precursor stage of PTC [35] due to the occurrence of scattered PTC-type nuclei and *RET/PTC* translocations in Hashimoto thyroiditis [36]. The probable *passenger* nature of *RET/PTC* translocations [37] and the absence of *BRAF* mutations in Hashimoto thyroiditis [38] argue against the possibility of Hashimoto thyroiditis play a major role in PTC occurrence.
Graves' disease is characterized by the production of auto-antibodies against specific domains of the thyroid

stimulating hormone receptor [1]. Morphologic features of Graves' disease include hyperplastic follicles with prominent papillary formations that may be difficult to distinguish from PTC. Although well established in Hashimoto thyroiditis, the increased incidence of PTC in patients with Graves' disease is still debatable [1].
The papillary aspect of the follicular epithelium can also occur in **nodular goiter** and be confused with concurrent and incidental PTC.
Follicular adenoma (FA) is defined as a benign encapsulated tumour that shows evidence of follicular differentiation and lacks capsular and/or vascular invasion and nuclear features typical of PTC [12]. If PTC nuclear features occur focally or are doubtful, the differential diagnosis between benign and malignant tumour can be very difficult. In this situation, it may be advisable to classify the lesion as **well differentiated tumour of uncertain malignant potential** [12, 39]. The FA diagnostic is one of exclusion and therefore it is difficult to make in a precise manner without the recommended complete analysis of the tumour capsule. This turns impossible its diagnosis at the preoperative setting (FNAB) and very difficult in most frozen section examinations. An encapsulated follicular tumour with images of capsular and/or vascular invasion but without nuclear features typical of PTC is a **follicular carcinoma** (FTC) [12]. Whenever the PTC nuclear features are focal or doubtful in an encapsulated and clearly invasive tumour, the designation of **well differentiated carcinoma - not otherwise specified** is advised [12, 39]. Nowadays, FTC accounts for less than 5-10% of the clinically evident thyroid carcinomas [40, 41], occurring mainly in females at the 5^{th}-6^{th} decades of life and rarely in childhood. FTC may be associated to low iodine intake [42] or to a history of nodular goiter, in some cases developed under a genetic background [43]. In FTC, the development of multicentricity and regional lymph node metastases are much less frequent than in PTC, while blood borne metastases to lungs and bones occur mainly in widely invasive and angio-invasive FTC [44]. Widely invasive FTC may be morphologically difficult to distinguish from poorly differentiated thyroid carcinoma (PDTC), both carrying a long term mortality approaching 50%. On the other hand, minimally invasive, non angio-invasive FTC may be difficult to distinguish from FA and carries very low long term mortality [12, 45]. If one cannot separate FTC from FA, the use of the **follicular tumour of uncertain malignant potential** designation is advisable [12, 39]. It is useless to search for molecular biomarkers in this setting, as some molecular features such as chromosome instability [46, 47], *RAS* mutations [48] and *PAX8/PPARgamma* [49] can be detected in both FTC and FA. Most of these molecular alterations are also shared by the FVPTC [48, 50]. As stated above, whenever encapsulated, the FVPTC can also be very difficult to distinguish from minimally invasive FTC, as the morphologic criteria depend only in the appreciation of the subjective PTC nuclear features [12]. The widespread and occasionally not precise utilization of the so-called nuclear criteria for diagnosing PTC is contributing to the decreasing incidence of FTC and to the increased incidence of FVPTC since it's the recognition as an entity [1, 12, 41].
Poorly differentiated carcinoma (PDTC) and **anaplastic/undifferentiated thyroid carcinoma** (ATC/UTC) are tumours with a poor and very poor prognosis, respectively. These carcinomas are thought to arise from well differentiated carcinomas, PTC and FTC [12, 51]. This assumption is based upon the frequent recognition of a well differentiated tumour component within or in contiguity with PDTC and ATC/UTC. ATC/UTC tends to occur in old patients and typically have p53 mutations. Both PDTC and ATC/UTC share molecular alterations with PTC (e.g *BRAF* and *RAS* mutations) and with FTC (e.g *RAS* mutations)[51,52].
PTC can exceptionally coexist with other common and rare tumours of the thyroid, including **medullary thyroid carcinoma** (the malignant C cell neoplasm) [53], **mucoepidermoid carcinoma** [54] and **squamous cell carcinoma** [55].

I.3 PTC: in the limits of morphology

I.3.1 Clinico-pathological features related to aggressiveness

There are several clinical and/or morphological features of PTC that have been frequently, though not always, associated to a less favourable prognosis, namely advanced age at time of diagnosis [29, 31, 56], male gender [31], tumour size above 1 cm [29, 57], extra-thyroid extension [29, 56, 58, 59], multicentricity [29], absence of total encapsulation [29, 58, 60], vascular invasion [29] and distant metastases [29, 56]. The presence of lymph node metastases is not fully accepted as a prognostic factor, and it is not associated to an increased mortality rate despite being determinant for the type of surgical approach and radioactive iodine

therapy [29, 56]. Whenever lymph node metastases are found at presentation they are predictive of an increased risk of subsequent loco-regional recurrence [61].
The acknowledgement of the aforementioned clinico-pathological prognostic factors led to several staging or scoring systems. These systems provide good, and frequently, quite equivalent prognostic information [62] (**Table I.2**). Unfortunately, based upon the scores built with these systems, 15% of tumour recurrences and over 10% of cancer deaths occur in patients with carcinomas initially classified as low risk [63, 64].
One of the most controversial items considered in some of these systems is tumour grading. Akslen and LiVolsi [65, 66] have established a grading score for PTC that highlighted, for the first time, the importance of vascular invasion together with nuclear atypia and tumour necrosis [65, 66]. Nowadays, the tumour grading is epitomized by the recognition that many *high grade* carcinomas actually correspond to PDTCs [51, 67].

Table I.2 Some of the clinico-pathological staging/scoring systems used in the evaluation of PTC prognosis.

Prognostic variable	Patient factors		Tumour factors					Surgical factors
Staging/scoring system	Age	Gender	Size	Grade	DNA ploidy	Extra-thyroid extension	Distant metastases	Incomplete resection
EORTC[68] Byar *et al*, 1979	+	+	-	-	-	+	+	-
AGES[69] Hay *et al*, 1987	+	-	+	+	-	+	+	-
AMES[70] Cady *et al*, 1988	+	+	+	-	-	+	+	-
DAMES[71] Pasieka *et al*, 1992	+	+	+	-	+	+	+	-
MACIS[72] Hay *et al*, 1993	+	-	+	-	-	+	+	+
SAG[65] Akslen, 1993	+	+	-	+	-	-	-	-
TNM 7th edition[73] Brierley *et al*, 2009	+	-	+	-	-	+	+	-

+ factor included in the score, - factor not included in the score

Part of the controversies on the prognostic value of the above mentioned features reflects the variable composition of the different series. This holds particularly true regarding the relative proportion of CPTC and FVPTC in general, and of the encapsulated subtype of FVPTC in particular [60, 74].Most cases of CPTC are poorly circumscribed, display a peripheral invasive front and give frequently rise to lymph node metastases, whereas cases of FVPTC are frequently encapsulated [12]. In the absence of unequivocal signs of capsular or vascular invasion, encapsulated FVPTCs follow an extremely benign course [60]. There is also a relatively frequent poorly circumscribed/ infiltrative subtype of FVPTC that, like CPTC, is prone to give rise to lymph node metastases [25, 75] (*see below*).
It is also acknowledged that other histological variants of PTC display relative specific patterns of invasiveness and metastisation [12]. For instance, the diffuse sclerosing variant and the diffuse/multinodular subtype of FVPTC that occur in young patients, frequently metastasize both to regional lymph vessels and to distant organs, and respond to radioactive iodine therapy [76-79]. These variants should be separated, for prognosis and therapy prediction, from the locally aggressive tall cell and columnar cell variants of PTC which occur in elderly patients and respond poorly to radioactive iodine therapy [76, 77, 80, 81].

I.3.2 Vessels and metastatic pattern

One of the reasons that justify the attempts to distinguish PTC from FTC relies on the different preferential routes of metastatic spread associated with these two thyroid cancer histotypes [12]. While PTC have a tendency to give rise to lymph node metastases *via* lymphatic vessels, FTC metastasize mainly *via* blood vessels to lungs and bones [1, 12, 82].
Although traditionally included in the PTC group [12], the FVPTC encompasses a subtype of encapsulated tumours which tend to share features with both CPTC and FTC [25, 28, 75]. Besides the rare and aggressive diffuse/multinodular subtype of FVPTC [79], cases of FVPTC may be classified according to their peripheral

growth pattern in two categories: infiltrative (or poorly circumscribed) subtype (PC-FVPTC) and encapsulated subtype (E-FVPTC) [75]. The PC-FVPTCs share most of the morphological features of CPTC, give frequently rise to lymph node metastases and disclose *BRAF* V600E mutation in 26% of the cases [26, 75], whereas the E-FVPTCs seem morphologically related to FA and minimally invasive FTC, often disclose *RAS* mutations and *PAX8/PPARgama* rearrangements [26, 48, 83] and follow an extremely benign course in the absence of unequivocal signs of capsular or vascular invasion [45, 60]. In the presence of vascular invasion, the E-FVPTCs tend to give rise, like FTC, to blood borne metastases [75, 79, 84].
The peculiar metastatic properties of the FVPTC subtypes are not fully understood and may be related to angiogenesis. Several groups have reported an increased expression of factors promoting the formation of new blood vessels (hemangiogenesis) in PTC in comparison with normal thyroid tissue [85, 86]; such factors are variably correlated with tumour size [86, 87], increased microvessel count using CD31 [88], increased risk of recurrence and decreased disease free survival [87, 89].
The expression of lymphangiogenic factors (vascular endothelial growth factor-C and vascular endothelial growth factor-D) appears to be increased in PTC in comparison with normal thyroid tissue [85, 86, 90, 91], benign lesions [85, 86, 90, 91] and FTC [92]. The increased expression of lymphangiogenic factors in PTC is related with increased intratumoural and peritumoural lymph vessel density (LVD) [90, 91, 93]. The association between the expression of lymphangiogenic factors in PTC and the occurrence of lymph node metastases was observed by some groups [86, 91, 93-95] whereas others did not detect such association [85, 90].
In the past, the endothelial markers used to identify vessels, like factor VIII, CD31, CD34 and von Willebrand factor, highlighted both lymphatic and blood vessels [87, 88, 96-99]. The situation has changed with the finding of lymphatic vessel specific immunomarkers [100, 101], that have been used to determine the intratumoural and the peritumoural LVD in several thyroid tumour types. The best known of these lymphatic endothelium specific markers are the D2-40 (podoplanin) [81, 90, 102, 103], the LYVE-1 (lymph vessel endothelial hyaluronan receptor) [85, 102, 104] and the VEGFR-3 (Vascular endothelial growth factor receptor-3) [91, 93, 94].
Intratumoural LVD has been found to be higher in PTC than in FA [85, 90], and higher in FVPTC than in FA or FTC [103]. Some authors reported an association between increased PTC intratumoural LVD and male gender [104], multifocal disease [104], invasiveness [94] and occurrence of lymph node metastases [91, 93, 94, 104], in contrast to other groups that did not find any association between PTC intratumoural LVD and the above mentioned features [85, 90].
The values for peritumoural LVD were similar in PTC, FTC and FA in the series of Giorgadze *et al* [103], while in the series of Garcia *et al* [90] FTC cases disclosed higher peritumoural LVD than PTC. There are also conflicting results regarding the association between PTC peritumoural LVD and the occurrence of lymph node metastases [90, 93].

I.4 PTC: beyond morphology

I.4.1 Transforming growth factor-beta (TGF-beta) and the Smad-dependent pathway

The infiltrative growth pattern of most non encapsulated forms of PTC is mainly the result of increased motility and tumour cells' invasiveness [105, 106, 107], which are features related to the so called epithelial-to-mesenchymal transition (EMT). At PTC´s infiltrative periphery, the neoplastic cells display loss of polarity/cohesiveness [107], decreased expression of beta-catenin and E-cadherin at the cell membrane [108], increased nuclear expression of STAT3 [Couto *et al*, unpublished results], increased cytoplasmatic expression of vimentin [107], fibronectin 1[105], met [109], ret [110], cytokeratin 5/6 [111] and of galectin-3 [112], and increased nuclear expression of cyclin D1[113].
The above mentioned alterations at the infiltrative edge of the tumours reflect probably the cross-talk between tumour cells and cellular and non-cellular elements of the stroma, in part mediated by integrin-related epithelial-to-stromal connections which are enhanced at PTC´s periphery [114]. Dendritic cells were found to be located at the tumour´s invasion edge in a small series of PTC [115] and, together with lymphocytes, appear to be decreased in PTCs with distant metastases [116], while increased density of tumour-associated macrophages in poorly differentiated carcinomas correlate with invasion and decreased survival [117].

EMT is partially regulated by **Transforming growth factor-beta** (TGF-beta) in PTC cell lines [118-121] and in animal models [122]. TGF-beta is one of the members of the TGF-beta superfamily and occurs in 3 isoforms – TGF-beta1, TGF-beta2 and TGF-beta3 – that function *via* the same membrane receptor signaling system [123]. In TGF-beta/Smad-dependent pathway, the activated TGF-beta receptor complex propagates the signal inside the cell through the phosphorylation of receptor regulated **Smad2** and **Smad3** downstream proteins localized in the cytoplasm [123-125]. Phosphorylated Smad2 and Smad3 form heteromeric complexes with **Smad4** and re-localize to the nucleus where they regulate gene transcription [123-125]. Inhibitory **Smad7** acts in an opposite manner and antagonizes TGF-beta/Smad-dependent signaling, inducing TGF-beta receptor degradation and thus preventing Smad2 and Smad3 phosphorylation [124, 125]. TGF-beta/Smad-dependent pathway activation induces major growth suppressive effects on epithelial cells in general [125], and on animal [126-128] and human [129, 130] thyroid cell lines, as well as on some PTC cell lines [118, 121], in particular. TGF-beta is also related with inhibition of migration and invasiveness in some PTC cell lines [118].
The expression of TGF-beta, Smad2, Smad3, and Smad7 has been detected in multinodular goiters [131-133] and in FAs of some series [133, 134], but not in other series of thyroid lesions where TGF-beta was the only element searched for [135].
In tumours, TGF-beta expression has been detected in 52-100% of PTC cases [131, 133-136] and, whenever specifically studied at the tumour´s periphery, was associated to the occurrence of extra-thyroid extension and lymph node metastases [119]. Similarly, phosphorylated Smad2/Smad3 (P-Smad2/3), has been detected in every case of PTC [133], while Smad4 is expressed in 75% of PTC cases [133] and in every PTC cell line [137, 138]. Smad7 expression has been detected in 80% of PTCs and in PTC cell lines [133, 137]
In tumour cells, particularly when exhibiting an EMT phenotype characterized by prominent mesenchymal-type features [105], the response to TGF-beta growth suppressive effect can be partially or totally abrogated *via* interferences in the signaling pathways that mediate its action [119, 122, 105, 139-141]. TGF-beta anti-proliferative effects were reported as attenuated in cases of multinodular goiter [141] and ATC/UTC cell lines [142], as well as in rat thyroid cell lines [143]. At the EMT, the TGF-beta altered cell response has been associated to the development of pro-oncogenic effects, namely through the inhibition of cell adhesion and enhancement of motility [125, 144].
Besides playing a functional role in tumour cell migration, invasion and EMT *in vitro*, TGF-beta and high TGF-beta/Smad-dependent pathway activity are associated with invasion, nodal metastization and *BRAF* mutation in PTCs [119].
It has been reported that oncogenic *BRAF* stimulates TGF-beta secretion *in vitro* [119]. *BRAF* is thought to play a major role in extracellular matrix remodeling [144] and is required for TGF-beta-induced EMT during progression from PTC to poorly differentiated carcinoma in an animal model [122]. It has also been shown that *BRAF* suppresses the expression of E-cadherin, which is a typical finding at the infiltrative edge of poorly circumscribed PTCs, and that this effect can be abrogated with both TGF-beta and MAPK inhibitors [119].

I.4.2 *BRAF, RAS* and the mitogen-activated protein kinase (MAPK) pathway

BRAF and *RAS* proto-oncogene point mutations are two of the most common structural genetic alterations in PTC. Other genetic alterations also leading to activation of the intra-cellular mitogen-activated protein kinase (MAPK) pathway are the chromosomal rearrangements of receptor tyrosine kinase genes (*RET* and *NTRK*)[12].
The activation of the MAPK pathway has been implicated in the regulation of transcription factors that control genes related to cell proliferation, differentiation and survival [145].
The ***BRAF*** gene codifies a protein from the serine-treonin family that is a critical effector of the MAPK pathway [32]. The most frequent activating *BRAF* mutation results from the transversion of a thymine to an adenine at nucleotide 1799 in exon 15, substituting a valine for a glutamic acid at codon 600 (V600E)[146, 147]. Besides V600E which occurs in nearly 90% of *BRAF* mutated thyroid cancers, other rare mutations and even some rare rearrangements have been described [147].
BRAF mutations have been detected in 7% to 83% of PTC [26, 27, 32, 48, 52, 56, 58, 74, 146-153].
The detection of the V600E mutation in FNAB specimens is a powerful adjunct for the diagnosis of PTC and has increased the specificity of PTC cytological diagnosis to almost 100% [154-156] but, unfortunately, the

sensitivity of such detection remains low due to the high percentage of non *BRAF* mutated PTCs [59, 147, 157].
The preoperative prognostic value of *BRAF* mutation detection in PTC remains controversial [58, 155, 158] partly reflecting the variable composition of the different series. *BRAF* mutations are present in 40-54% of CPTC [52, 74, 146, 151, 153] and in 7-29% of FVPTC [26, 48, 52, 74, 151, 153].
BRAF mutations have been associated to the classic PTC phenotype [52, 74, 151], advanced age at time of diagnosis [52, 56, 74], male gender[152], tumour size above 1cm [153], extra-thyroid extension[52, 154, 159], absence of total encapsulation [26, 154], vascular invasion[56], lymph node metastases [58, 149, 150], distant metastases [150], recurrence [154] and higher tumour stages [56, 150]. Basolo *et al* [59] have shown that in small PTC´s there is a significant association between the presence of *BRAF* mutation and tumour invasiveness and nodal metastization [59].
The most important argument against the value of the preoperative detection of the *BRAF* mutation stems from the relative high percentage of mutated papillary microcarcinomas that have an excellent prognosis [57, 58, 158] even if some of them tend to be locally infiltrative and carry a high rate of lymph node metastases [58, 59, 155, 160].
The practical value of the *BRAF* V600E detection for therapeutic purposes in metastatic follicular cell derived carcinomas with down regulation of sodium-iodine symporter and thyroid peroxidase that frequently cease to respond to radioactive iodine is very promising[156, 158, 161].
RAS proto-oncogenes (*H-RAS*, *K-RAS* and *N-RAS*) are key regulators of growth and differentiation that also play a role in the canonic MAPK pathway [32]. Point mutations of the GTP domain are the most frequent mechanism of oncogenic activation of *RAS*. In the setting of PTC, the most frequent alteration of this group of proto-oncogenes is the activating *N-RAS* mutation at codon 61 of exon 2 (Q61R) [162, 163].
In thyroid tumours, *RAS* mutations are more frequent in FA and FTC [1] and occur in 0 to 43% of PTC [26, 27, 32, 48, 83, 162, 163], more often in FVPTC (25-43%) [26, 27, 48, 83] than in CPTC (0-23%) [27, 48, 83, 162].
RAS mutations have been associated to large tumour size [48], encapsulation [26, 84, 159], vascular invasion [83], extra-thyroid extension [159], poorly differentiated areas [83] and low rate of lymph node metastases [83, 159]. *BRAF* and *RAS* mutations appear to be alternative events in PTC [148].
The proto-oncogene ***RET*** codifies a tyrosine-kinase receptor that is located at the cell membrane and is also implicated in the activation of the MAPK pathway [32]. The oncogenic rearrangements *RET/PTC* (more often *RET/PTC1* and *RET/PTC3*) are the result of double stands breaks in DNA of chromosome 10. The incidence of *RET/PTC* rearrangements is 60% in PTCs of children and young patients (much higher than in adults), reaching 80% in patients with PTC developed in the setting of nuclear fallout [164].
The ***NTRK*** gene codifies a high affinity receptor that triggers the activation of MAPK pathway and can be constitutively activated after rearrangements with other genes. *NTRK* rearrangements occur in less than 10% of PTCs [12].

II Aims

The biological and clinical behaviour of PTC is extremely variable and is still difficult to predict. The numerous clinico-pathological and molecular factors with putative but not definitive interest to the management of patients with PTC is one of the two reasons that justify the difficulties of the Pathologist for writing a pathological report and the doubts of the Clinician about its interpretation. The second reason emerges from the impact of immunohistochemical and molecular factors over the fragile diagnostic criteria of malignancy, which are starting to interfere with the established classification of thyroid tumours.
The aims of this Thesis reflect the clinico-pathological inspiration of the work and spring from the two reasons mentioned above.

Structural aim

To review all cases of thyroid tumours filed in the Cancer Registry of the Pathology Department of Centro Hospitalar de São João.

Specific aims

First specific aim To evaluate the relative contribution of the clinico-pathological features of the tumours and of their molecular profiles to the development of PTC aggressive behaviour.

Second specific aim To identify genetic and epigenetic alterations of PTC that may be used as biomarkers of invasion and metastization in the daily practice of diagnostic Anatomic Pathology.

To achieve the **structural** aim we addressed the following problems:

1. Selection of a representative series of PTC cases.

2. Identification of problems in thyroid cancer diagnosis and prognosis that should be approached in future studies. A particular interest was devoted to the neoplastic components coexistent with PTC with influence over the patients' outcome.

To achieve the **first specific** aim we addressed the following problems:

1. Clarification of the differential contribution of the clinico-pathological features to the development of lymph node metastases in classic PTC (CPTC) and follicular variant of PTC (FVPTC). The role played by intratumoural and peritumoural lymph vessel density was particularly addressed.

2. Clarification of the differential contribution of *BRAF* (V600E) and *N-RAS* (Q61R) mutations to the development of lymph node metastases in CPTC and FVPTC, and of the association of such mutations to other clinico-pathological features.

3. Clarification of the morphological and molecular basis underlying the differences in the biological and clinical behaviour of encapsulated FVPTC (E-FVPTC) and poorly circumscribed FVPTC (PC-FVPTC).

To achieve the **second specific** aim we addressed the following problems:

1. Disclosure of the relationship between the expressions of TGF-beta, phosphorylated Smad2/Smad3, Smad4 and inhibitory Smad7 and some clinico-pathological features associated to PTC aggressiveness.

2. Disclosure of the topographic differences of TGF-beta expression in CPTC and FVPTC cases with and without genetic alterations.

III.1 Identification of the thyroid tumours filed in the Cancer Registry of the Pathology Department of Centro Hospitalar de São João

The first step in the identification of all cases of thyroid tumours (malignant tumours and tumours of uncertain malignant potential) filed in the Cancer Registry of the Pathology Department of Centro Hospitalar de São João, was the gathering of the pathology reports and the slides of the cases. All thyroid tumours submitted to histological examination were identified from 1978 to 2006 (29 years). Clinical data were collected from the reports and further complemented with the available follow up data retrieved from the clinical files of each patient. The available survival data were unfortunately, largely incomplete. All available slides, corresponding to 75% of the recorded cases, were reviewed and the major tumour of each case was classified according to the 2004 WHO criteria [12].

III.2 Selection of a representative series of PTC cases

A representative series of 75 PTC cases, including a group of 42 CPTCs and a group of 33 FVPTCs, was selected from the cases previously retrieved from the Cancer Registry of the Pathology Department of Centro Hospitalar de São João.
All recorded PTCs with papillary and/or follicular architecture and available filed material were considered for selection. The slides were reviewed independently by two pathologists (CE and MSS) and the tumours were classified as CPTC and FVPTC, irrespective of size. The cases with doubtful PTC features, with features typical of other PTC variants and/or with unknown follow up were excluded.
Both groups integrated cases with lymph node metastases (**+M**), together or not with distant metastases, and cases without metastases and long term follow up (**-M**). The PTC-**M** subgroup included all the CPTC and FVPTC cases with at least 10 years of follow up and image examination and/or cytological data confirmative of the absence of metastases in the lymph nodes, lungs and bones. The PTC**+M** subgroup included more recent cases of CPTC and FVPTC with available material representative of the tumour and of its lymph node metastases that were either present at the time of surgery or developed during follow up. Three cases disclosed, together with lymph node metastases, lung metastases detected in image studies.
None of the patients of the PTC**+M** subgroup died from the disease (this information has limited value due to the short follow up of most of the cases).

III.2.1 Tumour samples and pathological classification

All samples had been fixed in formalin and included in paraffin. A paraffin block representative of the tumour and adjacent non tumoural tissue of each case was selected.
All the tumours with papillary pattern in over 5% of the tumour sampled area were classified as CPTC, whereas those tumours with a predominant follicular pattern and papillary pattern in 5% or less of the tumour sampled area were classified as FVPTC. Small tumours with 1cm or less in diameter were also classified according to their growth pattern as CPTC or FVPTC.
Well circumscribed tumours (WC-PTC) were those with expansive growth pattern without a well formed capsule or limited by a capsule with no or minimal invasion, and infiltrative/poorly circumscribed tumours (PC-PTC) were those with infiltrative growth pattern or with a widely invaded capsule. The designation of encapsulated tumour was exclusively applied to FVPTCs totally limited by a usually thin capsule with no or minimal invasion (E-FVPTC). Under the designation of multicentricity, all multifocal lesions, regardless of representing multicentric tumours or intra-thyroidal disseminated carcinomas, were included. Extra-thyroid extension was considered to be present whenever macroscopic extra-thyroid extension was observed. Lymph vessel invasion was considered to be present whenever at least one image of unequivocal invasion of a lymph vessel was observed. Venous invasion, from here foreword designated vascular invasion, was considered to be present whenever at least one image of unequivocal venous vessel invasion was observed. The presence of oncocytic cells was considered whenever they accounted for more than 10% of the tumour cells.
The clinical and available follow up data, together with the pathological features of the tumoural and non tumoural thyroid tissue of each case, were registered in an informatic supported database.
The analyses of the tumour samples performed in this study were authorized by the Ethics Committee of Centro Hospitalar de São João.

III.3 Immunohistochemical analysis

The selected series of 75 PTC cases was studied by immunohistochemistry.
Immunohistochemical staining was performed using streptovidin-biotin technique. Sections of 2μm thickness were cut from the selected paraffin blocks and placed on coated slides. The slides were dried, deparaffinized in Clear Rite (Richard - Allan, Thermo Scientific) and hydrated in sequential gradients of ethanol. For antigen retrieval, sections were immersed in 10mM sodium citrate buffer, pH 6.0 and then heated for 15 minutes. Endogenous peroxidase was blocked with 3% hydrogen peroxidase in methanol (Panreac) for 10 minutes in humid chamber and then Avidin blocking and Biotin blocking was performed (Lab Vision, Thermo Scientific). In order to block unspecific bounding to hydrophobic epitopes, the sections were immersed in Ultra V Block (Lab Vision, Thermo Scientific) for 10 minutes at room temperature. Slides were incubated overnight at 4ºC with the following primary antibodies:

- **D2-40** monoclonal antibody in a 1:25 dilution (D2-40, Signet Laboratories, Dedham, MA, USA)
- **TGF-beta1/2** rabbit polyclonal antibody (from here foreword designated as TGF-beta) in a 1:50 dilution (TGF-beta1(V), Santa Cruz Biotechnology, CA) against a peptide in the C-terminus of precursor and mature human TGF-beta1 and TGF-beta2 ligands
- **P-Smad2/3** rabbit polyclonal antibody in a 1:100 dilution (P-Smad2/3 (Ser 423/425), Santa Cruz Biotechnology, CA) against C-terminus of both phosphorylated Smad2 and Smad3
- **Smad4** monoclonal mouse antibody in a 1:100 dilution (Smad4 (B-8), Santa Cruz Biotechnology, CA)
- **Smad7** mouse monoclonal antibody in a 1.100 dilution (Smad7 (Z8-B), Santa Cruz Biotechnology, CA)

Then, slides were incubated with biotinylated secondary goat antibody and streptavidin-peroxidase complex (Lab Vision, Thermo Scientific) for 10 minutes at room temperature. The sections were developed with DAB solution (DAKO Corporation) according to the manufacturer's instructions, counterstained with hematoxylin (J.T.Baker, Deventer, Holland) for 2 minutes, dehydrated in sequential gradients of ethanol, immersed in Clear Rite solution, mounted with Mounting medium (Richard - Allan, Thermo Scientific) and coverslipped with Entellan (Merck). Adequate positive and negative controls in tissue microarrays were used.

III.3.1 Assessment of the immunohistochemical expression of D2-40 and determination of lymph vessel density (LVD)

The expression of D2-40 was searched in 61 tumours of the series including E-FVPTCs (n=14) and PC-PTCs (n=49). The remaining cases (n=14) had either insufficient representation of tumour or the tumour comprised post fine-needle aspiration tracts, previously associated to an increased LVD [103]).
D2-40 expression was considered positive whenever cytoplasmatic staining of endothelial cells of lymphatic vessels was observed. The evaluation of D2-40 expression was performed by counting positive D2-40 structures with morphological features of lymphatic vessels that were clearly separated from adjacent vessels and from other soft tissue components, as previously described [165]. Packed vessels were assumed as constituting one lymphatic unit. Analysis was performed at x200 magnification in the areas of highest vascularity (hot spots). The LVD of each case corresponds to the average obtained in counting 10 hot spot fields. The evaluation of D2-40 expression and lymph vessel counting was performed blindly in intratumoural and peritumoural areas of all 61 cases, except in 3 FVPTC cases in which the remaining non neoplastic thyroid tissue was insufficiently represented. The intratumoural lymph vessels were defined as vessels within the tumour area, whereas the peritumoural lymph vessels included vessels both in the capsule and in the remaining non tumoural thyroid tissue.

III.3.2 Assessment of the immunohistochemical expression of TGF-beta

The TGF-beta expression in the tumour cells was evaluated in the center and in the periphery of all carcinomas (CPTC and FVPTC) and in the respective remaining thyroid tissue, which was used as an internal control tissue, in order to reduce the influence of fluctuations of the physiological expression in each case. TGF-beta expression detected in cells of the control tissue was considered as the basal expression.
TGF-beta expression in the tumour cells located in the center of each tumour was compared with the expression in the respective control tissue and the TGF-beta expression in the tumour cells located in the

periphery of each tumour was compared with the expression in the respective center of the tumour. The mean expressions observed in each location were also compared. The center of the tumour comprised the bulk of the tumour, i.e., the whole neoplastic tissue except the subcapsular areas and/or the invasive front. The periphery of the tumour comprised the rim of tumour cells underneath the capsule or at the expansive periphery in WC-PTC, and in the rim of tumour cells in the invasive front in PC-PTC. TGF-beta expression was cytoplasmatic and was graded in 4 categories that were considered to represent a sort of semi-quantitative steps of number of positive cells and immunostaining intensity:

- Negative 0 (weak staining in less than 25% of cells)
- Positive 1 (weak staining in at least 25% of cells or moderate intensity in less than 25% of cells)
- Positive 2 (moderate intensity in at least 25% of cells or intense staining in less than 25% of cells)
- Positive 3 (intense staining in at least 25% of cells)

III.3.3 Assessment of the immunohistochemical expression of Smad2/3, Smad4 and Smad7

The expression of phosphorylated Smad2/3 (P-Smad2/3), Smad4 and Smad7 was both nuclear and cytoplasmatic and was evaluated in all CPTC and FVPTC tumours and in the respective remaining thyroid tissue, which was used as an internal control tissue and considered as the basal expression. The expression of P-Smad2/3, Smad4 and Smad7 in the center of the tumour and at the periphery were considered too difficult to distinguish and, therefore, assumed as similar. The expression was graded in 2 categories regarding each sub-cellular location (cytoplasmatic or nuclear) separately:

- Negative (weak staining in less than 25% of cells)
- Positive (weak, moderate or intense staining in at least 25% of cells)

III.4 Molecular analysis

All but 10 cases of the selected PTCs were screened for *BRAF* and *N-RAS* mutations.
Genomic DNA was extracted from 10μm sections of 65 tumours, including 34 CPTC and 31 FVPTC (the remaining 10 cases had insufficient or inadequate available material). Slides were microscopically examined, marked and dissected under microscopic observation. The obtained material was deparaffinized in xylene, washed in ethanol and rehydrated. DNA extraction was performed using the Genomic DNA Purification Kit (Gentra Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. Sequences of *BRAF* (exon 15) and *N-RAS* (exon 2) genes were amplified using primer pairs listed in the **Table III.1**, using a programmable thermocycler (MyCycler, Bio-Rad,USA). The PCR mixture (25μl) contained 2.5μl x10 PCR Buffer, 0.8mM dNTPs (Fermentas), 0.07μg each primer, 0.1-0.2 μg genomic DNA, 0.5 UTaq DNA Polymerase (Go Taq Flexi DNA Polymerase, Promega Corporation) and 2.5mM magnesium chloride (Promega Corporation). After 10 minutes of initial denaturation, the PCR mixtures were subjected to 35 cycles of denaturation for 30 seconds at 95ºC, annealing according to the amplicon and extension for 45 (*BRAF*) and 40 (*N-RAS*) seconds at 72ºC. A final extension period of 10 minutes at 72ºC was performed. PCR products were submitted to electrophoresis in agarose gel and purified by enzymatic treatment. Purified samples were sequenced on both strands as follows: 3μl purified PCR product was added to 7μl sequencing reaction containing 3.4μl Sequencing Buffer (Applied Biosystems, Forester City, CA), 30ng primer and 0.6μl Big Dye Terminator (Applied Biosystems). Sequencing was performed in an ABI Prism 3100 Genetic Analyser (Applied Biosysytems). Mutated samples were re-extracted, re-amplified and re-sequenced to confirm the result.

Table III.1 Primers and PCR conditions.

Gene	Exon	Primer sequence	Length (bp)	PCR annealing temperature (ºC)	PCR annealing time (seconds)
BRAF	15	F: TCATAATGCTTGCTCTGATAGGA R: GGCCAAAAATTTAATCAGTGGA	224	58	40
N-RAS	2	F: GATTCTTACAGAAAACAAGT R: CCTTCGCCTGTCCTCATGTATT	119	57	35

III.5 Statistical analysis

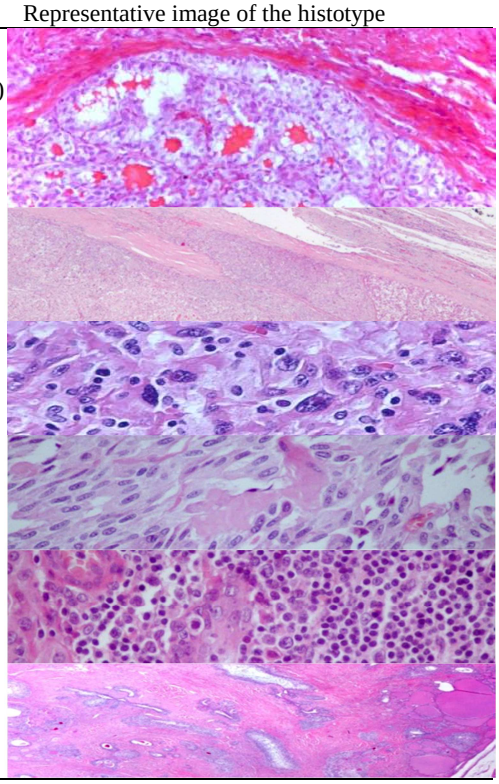
The χ^2 test with Yates' correction and the Students't test (unpaired) were used in the statistical analysis. Two values were considered significantly different when $p<0.05$ and suggestively different when $p<0.1$.

IV Results

IV.1 Identification of the thyroid tumours filed in the Cancer Registry of the Pathology Department of Centro Hospitalar de São João

In the 29-year period covered by this study, 1237 cases of primary thyroid tumours were identified in the Cancer Registry of the Pathology Department of Centro Hospitalar de São João.
Table IV.1 summarizes the distribution of the cases *per* tumour histotype.

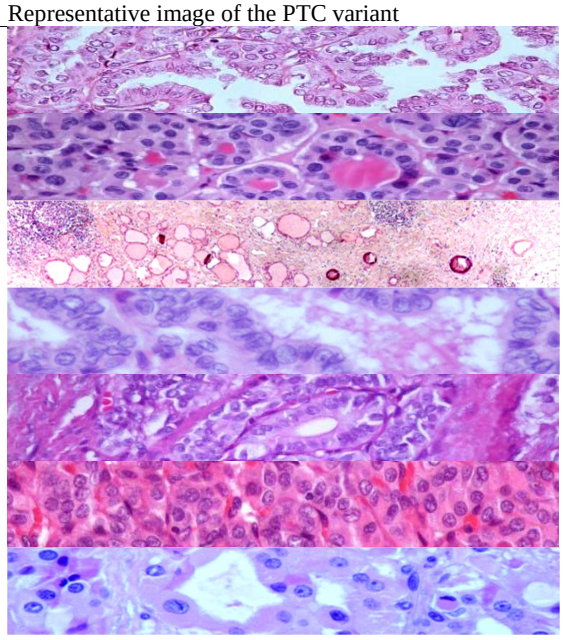
Table IV.1 Distribution of the 1237 cases of primary thyroid tumours *per* each tumour histotype

Diagnosis	%(n)	Representative image of the histotype
Well differentiated carcinoma and well differentiated tumour	94.4(1168)	
Papillary thyroid carcinoma	89.3(1043)	
Follicular thyroid carcinoma	10.4(122)	
WDTUMP	0.3(3)	
Poorly differentiated carcinoma	1.2(15)	
Undifferentiated carcinoma	0.8(10)	
Medullary carcinoma	3.1(38)	
Lymphoma	0.4(5)	
Mucoepidermoid carcinoma	0.1(1)	

WDTUMP – well differentiated tumour of uncertain malignant potential

The number of cases with a well differentiated carcinoma diagnosis has increased over the years, particularly due to an increased number of PTC cases, both of PTCs larger than 1cm and of PTCs with 1cm or less in their largest dimension, while the number of FTC cases remained persistently low over the years.
The overall percentage of PTC cases with tumour size (largest dimension of the largest tumour) above 1.0cm (56.8%) was slightly higher than the percentage of cases with 1.0cm or less (43.2%) (**Table IV.2**).
Of the 1043 PTC cases, 86.9% (n=906) were diagnosed in women and 13.1% (n=137) in men, with a mean age at the diagnosis of 46.4 years (median 47.0 years, range 12 – 85 years) and of 47.5 years (median 49 years, range 11 – 84 years), respectively. The 1043 PTC cases were sub-classified irrespective of size, according to the WHO criteria [12]. The FVPTC (n=411) was the most frequent variant followed by CPTC (n=377); the remaining tumours (n=255) belonged to other variant categories, were not classified with precision or were not classified due to absence of respective filed material (**Table IV.2**).
From the CPTC cases, 23.1% (87 out of 377) developed lymph node metastases and 2.3% (9 out of 377) developed distant metastases to the lungs and/or bones at the time of diagnosis or later. One or more recurrences were identified in 6.1% (23 out of 377) of CPTC. From the FVPTC cases, 2.9% (12 out of 411) developed lymph node metastases and 1.0% (4 out of 411) developed distant metastases to the lungs and/or bones at the time of diagnosis or later. One or more recurrences were identified in 2.7% (11 out of 411) of FVPTC. The development of distant metastases and recurrences was similar in CPTC and FVPTC cases, and the occurrence of lymph node metastases was more frequent in CPTC cases than in FVPTC cases ($p<0.001$).
In the group of cases displaying lymph node and/or blood borne metastases there was not a single case of well differentiated tumour of uncertain malignant potential, nor any case of minimally invasive, non angio-invasive FTC or encapsulated, noninvasive FVPTC (*see* ***Paper 5*** *for further details*).

Table IV.2 Sub-classification of the 1043 PTCs

PTC variants*	% (n)	Representative image of the PTC variant
Classic (CPTC)	36.1(377)	
Follicular (FVPTC)	39.4(411)	
Diffuse sclerosing	1.1(11)	
Tall cell	0.6(6)	
Columnar cell	0.2(2)	
Solid	4.0(42)	
Oncocytic	5.2(54)	
Unclassified/unknown	13.4(140)	

*In 435 cases the largest tumour measured 1cm or less in their largest dimension

In the 29 year period of the study, 5 cases of PTC with a poorly differentiated carcinoma component were identified and included in the 15 cases of poorly differentiated carcinoma discriminated in **Table IV.1**. Out of the five patients, two developed recurrences (40%), one had distant metastases (20%), one died from the disease (20%) and the remaining patient was lost for follow up.

Besides the thyroid tumours listed in **Table IV.1**, it was also possible to identify two cases of PTC coexisting with particular basaloid neoplasms (*see **Papers 6** and **7** for further details*), one of which was seen in consultation. Finally, we have also reviewed a case of metastases from ductal breast carcinoma within a pre-existing PTC.

IV.2 Clinico-pathological features of the selected series of PTC cases

The clinico-pathological features of the 75 selected cases, including 42 CPTCs and 33 FVPTCs, are summarized in **Tables IV.3** and **IV.4**, respectively.
CPTC and FVPTC cases had similar clinico-pathological features except different frequencies of poorly circumscribed growth pattern (83.3% of CPTC and 54.5% of FVPTC; p=0.010) and vascular invasion (28.6% of CPTC and 57.6% of FVPTC; p=0.018).

IV.2.1 Classic PTC (CPTC)

The 42 CPTC cases were 23 CPTC-**M** (54.8%) with a mean follow up time of 19.9±2.5 years and 19 CPTC+**M** (45.2%) with a mean follow up time of 5.5±3.2 years. The CPTC-**M** cases included 20 females (87.0%) with a mean age of 41.5±15.7 years and 3 males (13.0%) with a mean age of 49.3±21.4 years. The patients with CPTC-**M** underwent partial thyroidectomy (lobectomy or lobectomy plus isthmectomy) in 60.9% of cases and total thyroidectomy in 34.8% of cases; in 4.3% of the cases the precise extension of the surgery was not known. The CPTC+**M** cases included 15 females (78.9%) with a mean age of 38.1±19.1 years and 4 males (21.1%) with a mean age of 22.3±14.0 years. The patients with CPTC+**M** underwent total thyroidectomy with or without lymph node dissection in 73.7% and 26.3% of cases, respectively.
CPTC-**M** tumours presented less often poorly circumscribed growth pattern (69.6% of CPTC-**M** and 100.0% of CPTC+**M**; p=0.010), extra-thyroid extension (13.0% of CPTC-**M** and 47.4% of CPTC+**M**; p=0.020) and multicentricity (26.1% of CPTC-**M** and 63.2% of CPTC+**M**; p=0.028) than CPTC+**M** (**Table IV.5**).

CPTC-**M** and CPTC+**M** tumours were not significantly different concerning gender, age, size, presence of capsule or capsular invasion, vascular invasion (**Table IV.5**) and not also concerning necrosis (21.7% and 21.1%, respectively), intra-tumoural inflammation (60.9% and 63.2%, respectively), oncocytic cells (43.5% and 26.3%, respectively) and mitotic index (0.70 and 0.53 mitoses *per* 10 high power fields, respectively). The lymphocytic-rich stroma was detected only in two CPTC-**M** tumours (**Table IV.3**). In the adjacent thyroid parenchyma there were no significantly different frequencies of focal or diffuse Hashimoto type thyroiditis in CPTC-**M** and CPTC+**M** cases (47.8% and 57.9%, respectively), nor of benign follicular pathology (30.4% and 15.8%, respectively) (**Table IV.3**).

Table IV.3 Clinico-pathological features of the CPTC cases

Case	Gender	Age (years)	Follow up (years)	Tumour size (cm)	Capsule	Capsular invasion	Growth pattern	Multicentricity	EE	Vascular invasion	Necrosis	Mitotic index[a]	Oncocytic cells	Stroma type	II	Thyroiditis[b]	BFP	Surgery type	At the time of diagnosis		
																			pTNM	Stage	LNM
1	M	58	17	0.8	+	+	PC	-	-	-	-	1	-	C	-	f	-	PT	T1aN0M0	I	-
2	F	66	19	1.5	+	+	WC	-	-	-	-	1	-	C	+	-	NG	T	T1bN0M0	I	-
3	F	31	21	3.5	+	+	PC	+	-	-	+	0	-	P	-	-	FA	PT	T2N0M0	I	-
4	F	59	21	0.6	+	+	PC	+	-	-	+	1	+	-	-	-	NG	PT	T1aN0M0	I	-
5	F	37	21	1.0	+	-	WC	+	-	-	-	1	+	L	+	f	-	T	T1aN0M0	I	-
6	F	35	18	0.3	+	+	PC	-	-	-	-	0	-	P	-	-	NG	PT	T1aN0M0	I	-
7	F	40	18	1.5	-	NA	PC	-	-	-	-	0	+	L	+	+	-	T	T1bN0M0	I	-
8	F	38	18	0.5	-	NA	PC	-	-	-	-	0	-	C	-	-	NG	PT	T1aN0M0	I	-
9	M	25	20	1.2	-	NA	PC	-	-	+	+	0	-	C	-	-	-	PT	T1bN0M0	I	-
10	F	30	21	0.6	-	NA	PC	+	-	-	-	2	+	C	+	f	-	PT	T1aN0M0	I	-
11	F	40	20	2.2	-	NA	WC	-	-	-	-	0	+	-	+	f	-	T	T2N0M0	I	-
12	F	50	20	3.0	+	+	WC	-	-	-	+	0	-	C	+	f	-	PT	T2N0M0	II	-
13	F	25	17	2.1	+	+	WC	-	-	-	+	0	-	P	+	-	-	T	T2N0M0	I	-
14	F	45	22	4.5	+	-	WC	+	-	-	-	1	+	-	-	-	-	PT	T3N0M0	III	-
15	F	23	24	2.5	-	NA	PC	-	-	-	-	1	-	P	+	-	NG	ND	T2N0M0	I	-
16	M	65	24	2.3	-	NA	PC	-	-	-	-	0	-	C	+	f	-	PT	T2N0M0	II	-
17	F	21	19	2.0	-	NA	PC	-	-	+	-	3	-	C	+	f	-	PT	T1bN0M0	I	-
18	F	79	23	2.8	+	+	PC	-	+	-	-	0	-	C	+	f	-	PT	T3N0M0	III	-
19	F	65	16	3.5	-	NA	PC	-	+	-	-	1	+	P	+	f	NG	T	T3N0M0	III	-
20	F	28	24	2.5	-	NA	PC	-	-	+	-	2	+	C	-	-	-	T	T2N0M0	I	-
21	F	28	20	2.0	-	NA	PC	-	-	+	-	1	-	C	-	-	-	PT	T1bN0M0	I	-
22	F	43	18	1.5	+	+	WC	+	-	+	-	0	+	C	+	-	-	PT	T1bN0M0	I	-
23	F	47	16	4.5	-	NA	PC	-	+	+	-	1	+	P	+	f	-	T	T3N0M0	III	-
24	F	28	5	0.9	+	+	PC	-	-	-	-	0	+	-	-	-	-	T	T1aN1M0	I	+
25	F	21	6	1.2	+	+	PC	-	-	-	+	0	+	C	+	+	NG	TL	T1bN1M0	I	+
26	F	22	14	1.9	-	NA	PC	+	-	-	-	1	-	-	-	-	-	TL	T1bN1M0	I	+
27	F	30	3	1.3	+	+	PC	-	+	+	-	0	-	P	+	+	-	TL	T3N1M0	I	+
28	F	56	3	0.7	-	NA	PC	+	-	-	-	0	-	-	+	-	NG	TL	T1aN1M0	III	+
29	F	18	3	1.5	-	NA	PC	+	+	+	-	1	-	C	+	f	-	T	T3N1M0	I	+
30	M	22	4	1.1	-	NA	PC	+	-	-	-	1	-	P	-	+	-	TL	T1bN1M0	I	+
31	F	22	6	0.8	-	NA	PC	-	-	-	+	0	-	C	+	-	-	TL	T1aN1M0	I	+
32	F	56	3	0.8	-	NA	PC	+	-	-	-	0	-	P	-	-	-	TL	T1aN1M0	III	+
33	M	42	5	1.6	-	NA	PC	+	+	-	+	0	+	P	+	f	-	TL	T3N1M0	I	+
34	F	38	7	3.0	+	+	PC	+	-	+	-	1	-	P	+	+	-	T	T2N1M0	I	+
35	F	35	5	1.7	+	+	PC	-	-	+	-	0	-	C	+	f	-	TL	T1bN1M0	I	+
36[c,d]	M	11	9	6.0	-	NA	PC	+	+	+	-	0	-	C	+	f	-	TL	T4aN1M0	II	+
37[c,d]	F	56	4	2.4	-	NA	PC	-	+	-	-	1	+	C	+	-	-	TL	T3N1M0	IVC	+
38	F	83	1	7.5	-	NA	PC	-	+	-	+	1	+	-	+	-	-	T	T4aN1M0	IVA	+
39	F	18	4	3.6	-	NA	PC	+	-	+	-	0	-	C	-	-	-	TL	T2N1M0	I	+
40	M	14	4	2.5	-	NA	PC	+	+	-	-	1	-	P	-	f	-	TL	T3N1M0	I	+
41	F	32	9	1.1	-	NA	PC	-	+	-	-	1	-	C	-	f	NG	T	T3N1M0	I	+
42	F	56	10	7.5	-	NA	PC	+	+	-	-	2	-	C	+	f	-	TL	T3N1M0	III	+

N number, *M* male, *F* female, + presence, - absence, *WC* well circumscribed, *PC* poorly circumscribed, *EE* extra-thyroid extension, *C* cellular, *P* poorly cellular, *L* lymphocyte rich, *II* intra-tumoural inflammation, *f* focal, *BFP* benign follicular pathology in the adjacent thyroid tissue, *NG* nodular goiter, *FA* follicular adenoma, *LNM* lymph node metastases, *PT* partial thyroidectomy, *T* total thyroidectomy, *TL* total thyroidectomy with lymph node dissection, *ND* not determined, *NA* not applicable.

[a] Number of mitoses *per* 10 high power fields (400x), [b] Thyroiditis was considered + whenever Hashimoto thyroiditis features were present, [c] Cases with concurrent distant metastases, [d] Cases with recurrence(s).

Table IV.4 Clinico-pathological features of the FVPTC cases

Case	Gender	Age (years)	Follow up (years)	Tumour size (cm)	Capsule	Capsular invasion	Growth pattern	Multicentricity	EE	Vascular invasion	Necrosis	Mitotic index	Oncocytic cells	Stroma type	II	Thyroiditis	BFP	Surgery type	At the time of diagnosis		LNM
																			pTNM	Stage	
1	F	32	22	2.0	+	+	WC	-	-	+	-	2	+	P	+	f	-	PT	T1bN0M0	I	-
2	F	26	21	1.8	-	NA	PC	+	-	+	-	0	-	P	-	-	-	PT	T1bN0M0	I	-
3	F	59	19	0.5	+	-	WC	-	-	-	+	0	+	-	+	-	NG	T	T1aN0M0	I	-
4	F	51	18	1.4	+	-	WC	-	-	-	-	0	+	C	+	-	NG	T	T1bN0M0	I	-
5	F	36	18	0.5	-	NA	PC	-	-	-	-	0	-	C	+	-	NG	PT	T1aN0M0	I	-
6	F	39	15	1.0	-	NA	WC	+	-	-	-	1	-	-	-	-	NG	PT	T1aN0M0	I	-
7	F	48	13	0.6	+	-	WC	-	-	-	-	0	-	P	-	+	NG	PT	T1aN0M0	I	-
8	F	33	20	0.2	-	NA	PC	-	-	-	-	0	-	C	+	+	FA	T	T1aN0M0	I	-
9	F	30	15	0.5	-	NA	WC	-	-	-	-	0	-	-	-	-	NG	PT	T1aN0M0	I	-
10	F	21	21	0.6	-	NA	PC	-	-	-	-	0	-	-	-	f	NG	T	T1aN0M0	I	-
11	F	37	18	0.3	-	NA	PC	-	-	+	-	0	-	P	-	-	NG	PT	T1aN0M0	I	-
12	F	28	21	2.8	-	NA	WC	+	-	+	-	0	-	P	-	-	-	T	T2N0M0	I	-
13	F	34	20	3.5	+	-	WC	-	-	-	-	0	-	P	-	f	-	PT	T2N0M0	I	-
14	M	61	22	4.0	+	+	WC	-	-	+	-	0	-	-	-	-	-	T	T2N0M0	II	-
15	M	35	20	3.5	+	+	WC	-	-	+	-	0	-	-	-	-	-	PT	T2N0M0	I	-
16	F	50	20	2.5	+	+	WC	-	-	-	-	1	-	-	-	-	-	PT	T2N0M0	II	-
17	F	47	23	3.0	-	NA	PC	-	-	-	-	0	+	-	-	-	-	PT	T2N0M0	II	-
18	F	49	14	3.0	+	+	WC	+	-	+	-	0	-	-	-	f	NG	T	T2N0M0	II	-
19	F	35	21	1.7	-	NA	PC	+	-	+	-	1	-	P	+	f	NG	PT	T1bN0M0	I	-
20	F	32	13	4.7	-	NA	PC	+	-	+	-	0	+	-	-	-	-	T	T3N0M0	I	-
21	F	52	13	2.5	+	+	WC	+	-	-	-	1	-	P	-	-	NG	T	T2N0M0	II	-
22	F	66	13	2.5	+	+	WC	+	-	-	+	0	+	P	-	f	-	PT	T2N0M0	II	-
23	F	58	13	1.5	-	NA	PC	+	-	+	-	1	+	C	+	-	NG	PT	T1bN0M0	I	-
24	F	54	10	1.8	-	NA	PC	+	-	+	-	0	+	P	+	f	NG	PT	T1bN0M0	I	-
25	M	45	20	4.0	+	+	WC	-	-	-	-	0	+	-	-	-	-	T	T2N0M0	II	-
26 [d]	M	57	3	9.5	+	+	PC	-	+	+	-	6	-	-	-	-	-	TL	T3N0M0	III	+
27	F	56	3	0.8	+	+	PC	+	-	+	-	0	-	P	+	-	-	TL	T1aN1M0	III	+
28	M	21	4	2.5	-	NA	PC	-	+	+	-	1	-	C	+	-	-	TL	T4aN1M0	I	+
29	F	20	14	1.0	-	NA	PC	+	-	+	-	1	-	-	+	+	-	TL	T1aN1M0	I	+
30	F	49	4	2.2	+	+	PC	-	-	+	-	1	-	-	-	-	-	T	T2N1M0	III	+
31	F	43	10	4.0	-	NA	PC	+	+	+	-	1	-	-	+	f	-	TL	T4aN1M0	I	+
32	F	43	10	1.5	-	NA	PC	+	+	+	-	0	-	C	+	-	-	TL	T3N1M0	I	+
33 [c,d]	M	63	7	1.8	+	+	PC	-	-	+	-	0	-	-	-	-	FA	T	T1bN0M0	I	+

N number, *M* male, *F* female, + presence, - absence, *WC* well circumscribed, *PC* poorly circumscribed, *EE* extra-thyroid extension, *C* cellular, *P* poorly cellular, *L* lymphocyte rich, *II* intra-tumoural inflammation, *f* focal, *BFP* benign follicular pathology in the adjacent thyroid tissue, *NG* nodular goiter, *FA* follicular adenoma, *LNM* lymph node metastases, *PT* partial thyroidectomy, *T* total thyroidectomy, *TL* total thyroidectomy with lymph node dissection, *ND* not determined, *NA* not applicable.

[a] Number of mitoses *per* 10 high power fields (400x), [b] Thyroiditis was considered + whenever Hashimoto thyroiditis features were present,

[c] Cases with concurrent distant metastases, [d] Cases with recurrence(s).

IV.2.2 Follicular variant of PTC (FVPTC)

The 33 FVPTC cases were 25 FVPTC-**M** (75.8%) with a mean follow up time of 17.7±3.7 years and 8 FVPTC+**M** (24.2%) with a mean follow up time of 6.9±4.1 years. The FVPTC-**M** cases included 22 females (88.0%) with a mean age of 41.7±12.2 years and 3 males (12.0%) with a mean age of 47.0±13.1 years. The patients with FVPTC-**M** underwent partial thyroidectomy (lobectomy or lobectomy plus isthmectomy) in 60.0% of cases or total thyroidectomy in 40.0% of cases. The FVPTC+**M** cases included 5 females (62.5%) with a mean age of 42.2±13.5 years and 3 males (37.5%) with a mean age of 47.0±22.7 years. The patients with FVPTC+**M** underwent total thyroidectomy with or without lymph node dissection in 75.0% and 25.0% of cases, respectively.

FVPTC-**M** tumours were significantly less often poorly circumscribed than FVPTC+**M** (40.0% of FVPTC-**M** and 100.0% of FVPTC+**M**; p=0.004), presented significantly less often extra-thyroid extension (0.0% of FVPTC-**M** and 50.0% of FVPTC+**M**; p=0.002) and vascular invasion (44.0% of FVPTC-**M** and 100.0% of FVPTC+**M**; p=0.010).

FVPTC-**M** and FVPTC+**M** displayed different mean mitotic indexes (0.28 and 1.25 mitoses/10 high power fields, respectively; *p*=0.031). FVPTC-**M** cases presented significantly more often (56.0%) benign follicular pathology in the adjacent thyroid parenchyma than FVPTC+**M** tumours (12.5%)(*p*=0.046). In the subgroup of tumours with 1 cm or less, all the FVPTC-**M** had associated benign follicular pathology (8 out of 8), while none of the FVPTC+**M** had associated benign follicular pathology (0 out of 2). A similar difference was observed in the subgroup of tumours larger than 1cm (14 out of 25 and 1 out of 8, respectively)(*p*=0.022).
FVPTC-**M** and FVPTC+**M** were not significantly different concerning gender, age, presence of capsule or capsular invasion, multicentricity (**Table IV.5**), nor necrosis (8.0% and 0.0%, respectively), intra-tumoural inflammation (32.0% and 62.5%, respectively), presence of oncocytic cells (36.0% and 0.0%, respectively), focal or diffuse Hashimoto type thyroiditis (36.0% and 25.0%, respectively) and stroma types (**Table IV.4**).

IV.2.3 PTC cases with lymph node metastases (PTC+M) *versus* PTC cases without lymph node metastases (PTC-M)

Table IV.5 summarizes the differences between the 27 PTC+**M** and the 48 PTC-**M**.
PTC+**M** cases presented more often a poorly circumscribed growth pattern (*p*<0.0001), extra-thyroid extension (*p*<0.0001) and larger size of the tumours in the group with 1cm or less (*p*=0.019) than PTC-**M** tumours. PTC+**M** tumours tended to be more often multicentric than PTC-**M** tumours (*p*=0.051).
When comparing poorly circumscribed CPTC+**M** and CPTC-**M** tumours the former were more often multicentric than the latter (57.9% and 18.8%, respectively; *p*=0.036) (**Table IV.6**). Similarly, poorly circumscribed FVPTC+**M** tumours displayed more often extra-thyroid extension than FVPTC-**M** (50.0% and 0.0%, respectively; *p*=0.023) (**Table IV.6**).

Table IV.5. Comparison of clinico-pathological features of CPTC and FVPTC cases with and without lymph node metastases (LNM)

PTC Variant	Features	With LNM	Without LNM	*p* value
CPTC	Gender (Female:Male)	15:4	20:3	
	Age, years(mean±sd)	34.7±19.0	42.5±16.2	
	Tumour size of tumours >1,cm (mean±sd)	2.9±2.3	2.5±1.0	
	Tumour size of tumours ≤1,cm (mean±sd)	0.8±0.1	0.6±0.2	>0.1
	Capsule (absence/total)	0.74	0.52	
	Capsule invasion (presence/total)	1.00	0.82	
	Vascular invasion (presence /total)	0.32	0.26	
	Multicentricity (presence/total)	0.63	0.26	
	Growth pattern (poorly circumscribed/total)	1.00	0.70	**<0.05**
	Extra-thyroid extension (presence/total)	0.47	0.13	
FVPTC	Gender (Female:Male)	5:3	22:3	
	Age, years (mean±sd)	44.0±16.1	42.3±12.1	
	Tumour size of tumours >1,cm (mean±sd)	3.6±3.0	2.7±1.0	>0.1
	Capsule (absence/total)	0.50	0.52	
	Capsule invasion (presence/total)	1.00	0.67	
	Multicentricity (presence/total)	0.50	0.40	
	Tumour size of tumours ≤1,cm (mean±sd)	0.9±0.1	0.5±0.2	**0.1-0.05**
	Vascular invasion (presence /total)	1.00	0.44	
	Growth pattern (poorly circumscribed/total)	1.00	0.40	**<0.05**
	Extra-thyroid extension (presence/total)	0.50	0.00	
CPTC + FVPTC	Gender (Female:Male)	20:7	42:6	
	Age, years (mean±sd)	37.5±18.4	42.4±14.1	
	Tumour size of tumours >1,cm (mean±sd)	3.1±2.4	2.6±1.0	>0.1
	Capsule (absence/total)	0.67	0.52	
	Capsule invasion (presence/total)	1.00	0.74	
	Vascular invasion (presence /total)	0.52	0.35	
	Multicentricity (presence/total)	0.59	0.33	**0.1-0.05**
	Tumour size of tumours ≤1,cm (mean±sd)	0.8±0.1	0.6±0.2	
	Growth pattern (poorly circumscribed/total)	1.00	0.51	**<0.05**
	Extra-thyroid extension (presence/total)	0.48	0.06	

Table IV.6 Comparison of clinico-pathological features of poorly circumscribed CPTC and poorly circumscribed FVPTC cases with and without lymph node metastases (LNM)

PTC Subtype	Features	With LNM	Without LNM	*p* value
PC-CPTC	Multicentricity (presence/total)	0.58	0.19	**0.036**
	Extra-thyroid extension (presence/total)	0.47	0.19	0.152
	Vascular invasion (presence /total)	0.32	0.31	>0.999
PC-FVPTC	Multicentricity (presence/total)	0.50	0.50	>0.999
	Extra-thyroid extension (presence/total)	0.50	0.00	**0.023**
	Vascular invasion (presence /total)	1.00	0.67	0.206

IV.3 D2-40 expression in lymph vessels and evaluation of lymph vessel invasion and LVD in the selected series of PTC cases

The clinico-pathological features and the LVD data on the 29 cases of FVPTC separated into E-FVPTC (n=12) and PC-FVPTC (n=17) are summarized in **Table IV.7**. The respective data on the 32 cases of CPTC are summarized in **Table IV.8**.

TableIV.7 Clinico-pathological features of the 29 cases of FVPTC (12 cases of E-FVPTC and 17 cases of PC-FVPTC) and respective intratumoural and peritumoural LVD

	Case	Gender	Age (years)	Tumour size (cm)	M	EE	LI	VI	LNM	I-LVD	P-LVD
E-FVPTC	1	F	32	2.0	-	-	-	+	-	0.0	8.1
	4[a]	F	51	1.4	-	-	-	-	-	0.0	15.4
	6[b]	F	39	1.0	+	-	-	-	-	0.6	11.5
	7[a]	F	48	0.6	-	-	-	-	-	0.0	14.1
	9[b]	F	30	0.5	-	-	-	-	-	0.0	14.2
	12[b]	F	28	2.8	+	-	-	+	-	0.0	6.8
	13[a]	F	34	3.5	-	-	-	-	-	0.0	9.8
	14	M	61	4.0	-	-	-	+	-	0.0	12.7
	15	M	35	3.5	-	-	-	+	-	0.0	18.0
	16	F	50	2.5	-	-	-	-	-	0.0	10.3
	21	F	52	2.5	+	-	-	-	-	0.0	14.2
	22	F	66	2.5	+	-	-	-	-	0.0	18.2
PC-FVPTC	2	F	26	1.8	+	-	-	+	-	0.0	11.5
	5	F	36	0.5	-	-	-	-	-	0.5	10.1
	10	F	21	0.6	-	-	-	-	-	4.8	9.7
	11	F	37	0.3	-	-	-	+	-	0.0	8.6
	17	F	47	3.0	-	-	-	-	-	0.0	9.8
	20	F	32	4.7	+	-	-	+	-	1.0	21.0
	23	F	58	1.5	+	-	+	+	-	0.3	9.8
	24	F	54	1.8	+	-	+	+	-	0.2	10.0
	25	M	45	4.0	-	-	-	-	-	0.1	9.4
	26	M	57	9.5	-	+	+	+	+	4.4	14.0
	27	F	56	0.8	+	-	+	+	+	1.3	1.5
	28	M	21	2.5	-	+	-	+	+	2.0	ND
	29	F	20	1.0	+	-	-	+	+	6.0	21.0
	30	F	49	2.2	-	-	-	+	+	3.9	15.0
	31	F	43	4.0	+	+	-	+	+	0.1	ND
	32	F	43	1.5	+	+	-	+	+	4.3	ND
	33[c]	M	63	1.8	-	-	-	+	+	0.0	10.8

M male, *F* female, + presence, - absence, *M* multicentricity, *EE* extra-thyroid extension, *LI* lymph vessel invasion, *VI* vascular invasion, *LNM* lymph node metastases, *I* intratumoural, *P* peritumoural, *ND* not determined. [a] Cases without capsular invasion, [b] Cases with a very thin and irregular capsule, [c] Case with concomitant distant metastases.

TableIV.8 Clinico-pathological features of the 32 cases of CPTC and respective intratumoural and peritumoural LVD

Case	Gender	Age (years)	Tumour size (cm)	M	EE	LI	VI	LNM	I-LVD	P-LVD
1	M	58	0.8	-	-	-	-	-	0.0	7.0
3	F	31	3.5	+	-	-	-	-	0.2	9.8
6	F	35	0.3	-	-	+	-	-	0.3	8.1
7	F	40	1.5	-	-	-	-	-	0.4	8.7
9	M	25	1.2	-	-	-	+	-	2.0	10.9
10	F	30	0.6	+	-	-	-	-	0.0	7.9
15	F	23	2.5	-	-	+	-	-	0.9	15.8
16	M	65	2.3	-	-	-	-	-	0.0	16.5
17	F	21	2.0	-	-	+	+	-	0.0	14.1
18	F	79	2.8	-	+	-	-	-	0.0	16.0
19	F	65	3.5	-	+	+	-	-	0.0	8.3
20	F	28	2.5	-	-	-	+	-	0.0	12.0
21	F	28	2.0	-	-	-	+	-	0.0	14.1
23	F	47	4.5	-	+	+	+	-	5.1	9.3
24	F	28	0.9	-	-	-	-	+	0.2	13.0
25	F	21	1.2	-	-	+	-	+	0.1	19.3
26	F	22	1.9	+	-	+	-	+	0.7	18.0
27	F	30	1.3	-	+	-	+	+	0.5	14.7
28	F	56	0.7	+	-	+	-	+	1.0	10.3
29	F	18	1.5	+	+	-	+	+	0.0	12.0
30	F	22	0.8	-	-	-	-	+	0.2	12.5
32	F	56	0.8	+	-	+	-	+	0.7	13.4
33	M	42	1.6	+	+	-	-	+	2.2	3.7
34	F	38	3.0	+	-	-	+	+	0.0	14.3
35	F	35	1.7	-	-	-	+	+	1.4	11.7
36[a]	M	11	6.0	+	+	+	+	+	0.4	14.3
37[a]	F	56	2.4	-	+	-	-	+	1.5	11.5
38	F	83	7.5	-	+	-	-	+	1.0	11.3
39	F	18	3.6	+	-	+	+	+	3.1	13.3
40	M	14	2.5	+	+	+	-	+	2.0	14.0
41	F	32	1.1	-	+	-	-	+	0.4	7.3
42	F	56	7.5	+	+	-	-	+	0.6	20.0

M male, *F* female, + presence, - absence, *M* multicentricity, *EE* extra-thyroid extension, *LI* lymph vessel invasion, *VI* vascular invasion, *LNM* lymph node metastases, *I* intratumoural, *P* peritumoural, *ND* not determined. [a] Cases with concomitant distant metastases.

IV.3.1 Clinico-pathological features, including lymph vessel invasion, of encapsulated FVPTC (E-FVPTC) cases *versus* poorly circumscribed FVPTC (PC-FVPTC) and CPTC cases

E-FVPTC cases presented significantly less often vascular invasion (p=0.029) and lymph node metastases (p=0.009) than PC-FVPTC cases (**Table IV.9**). None of the E-FVPTC cases presented extra-thyroid extension, lymph vessel invasion or lymph node metastases while in cases with infiltrative growth pattern (PC-FVPTC *plus* CPTC), occurrence of extra-thyroid extension was observed in 32.7% of the cases, lymph vessel invasion was also observed in 32.7% of the cases, and lymph node metastases were detected in 75.0% of the cases (p=0.026, p=0.014 and p=0.0006, respectively) (**Tables IV.7, IV.8** and **IV.9**).

TableIV.9 Clinico-pathological features, including lymph vessel invasion, of PTC cases

Features		E-FVPTC n(%)	PC-FVPTC n(%)	CPTC n(%)	FVPTC *vs* CPTC *(p)*	E-FVPTC *vs* PC-FVPTC *(p)*	E-FVPTC *vs* PC-FVPTC+CPTC *(p)*
Gender	Female	10 (83.3)	13 (76.5)	26 (81.3)	>0.999	>0.999	>0.999
	Male	2 (16.7)	4 (23.5)	6 (18.7)			
Age	>45 years	6 (50.0)	7(41.2)	10 (31.3)	0.302	0.716	0.342
	≤45 years	6 (50.0)	10 (58.8)	22 (68.7)			
Tumour size	>1.0 cm	9 (75.0)	12 (70.6)	25 (78.1)	0.767	>0.999	>0.999
	≤1.0 cm	12 (25.0)	5 (29.4)	7 (21.9)			
M		4 (33.3)	8 (47.1)	12 (37.5)	0.798	0.703	0.749
EE		0 (0.0)	4 (23.5)	12 (37.5)	**0.045**	0.121	**0.026**
LI		0 (0.0)	4 (23.5)	12 (37.5)	**0.094**	**0.059**	**0.014**
VI		4 (33.3)	13 (76.5)	11 (34.4)	**0.075**	**0.029**	0.520
LNM		0 (0.0)	8 (47.1)	18 (56.3)	**0.038**	**0.009**	**0.0006**

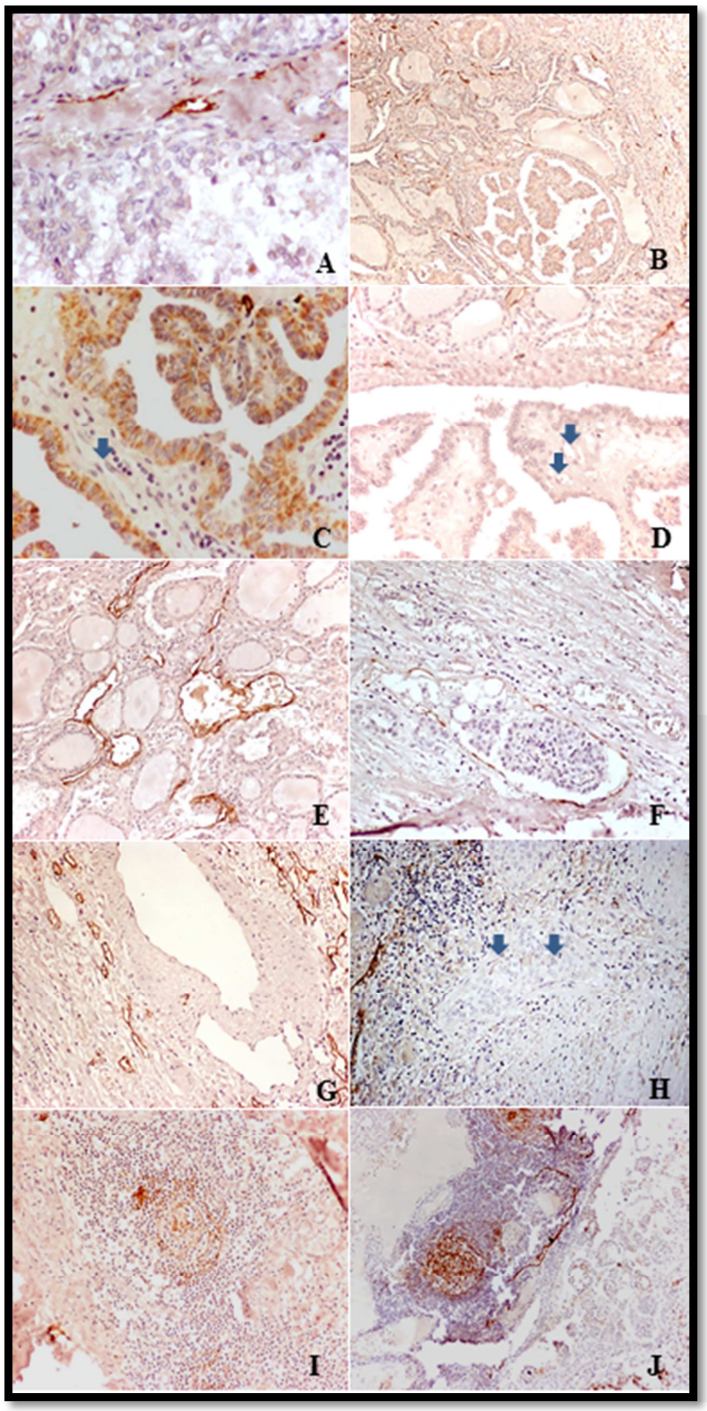
M multicentricity, *EE* extra-thyroid extension, *LI* lymph vessel invasion, *VI* vascular invasion, *LNM* lymph node metastases

In cases without extra-thyroid extension, the occurrence of lymph vessel invasion was related to lymph node metastases (p=0.022).

IV.3.2 D2-40 expression in lymph vessels

The D2-40 expression in the endothelial cells in intratumoural location highlighted small thin-walled lymph vessels with tiny lumina located in the dependence of fibrous septa arising from the tumour capsule (**FigureIV.1A**) or amongst tumour cells (**FigureIV.1B**). The D2-40 positive lymph vessels were found less often in the center of the tumours than in the periphery, particularly at the invasive front of the infiltrative tumours.

In both FVPTC subgroups and in the CPTC group, there was, besides the subset of lymph vessels with D2-40 expression, a majority of small thin-walled, D2-40 negative, nearly collapsed capillary vessels of uncertain origin, and ectatic lymph vessels in the remaining thyroid tissue. Such D2-40 negative capillary vessels were morphologically indistinguishable from vessels with D2-40 expression (**FigureIV.1C** and **D**).



D2-40 expression in the peritumoural thyroid tissue highlighted thin-walled ectatic lymph vessels (**FigureIV.1E**) in all cases. Peritumoural lymph vessels with D2-40 expression showed, in some cases displaying infiltrative growth, neoplastic thrombi within their lumina (**FigureIV.1F**). Neoplastic thrombi were not observed within the intratumoural thin-walled vessels without D2-40 expression.

All the blood vessels with an organized wall surrounded by a smooth muscle layer in which the presence of vascular invasion was searched, did not disclose D2-40 expression in the endothelial cells (**FigureIV.1G**). Neoplastic thrombi were also detected in peritumoural thick walled vessels (**FigureIV.1H**).

Complex networks of lymph vessels expressing D2-40 were detected in the peritumoural tissue in association with lymphoid aggregates and in perithyroidal lymph nodes with and without metastases (**FigureIV.1I** and **J**).

Figure IV.1

D2-40 expression in the endothelial cells.
A – D2-40 positive intratumoural lymph vessels in a fibrous septum (case23 CPTC, 400x); B – D2-40 positive intratumoural lymph vessels between tumour cells at the invasive front (case39 CPTC, 100x); C – Intratumoural lymph vessels D2-40 expression and thin walled vessels without D2-40 expression(arrow) (case39 CPTC, 400x); D – Intratumoural thin walled vessels without D2-40 expression (arrows) (case39 CPTC, 200x); E – D2-40 positive peritumoural lymph vessels (case16 FVPTC, 100x); F – D2-40 positive intratumoural lymph vessel with neoplastic thrombus (case26 FVPTC, 200x); G – Peritumoural medium sized vein without D2-40 expression surrounded by D2-40 positive small lymph vessels (case13 FVPTC, 200x); H – D2-40 negative peritumoural vein with neoplastic thrombus (case24 FVPTC, 200x); I – Complex network of D2-40 positive lymph vessels in the peritumoural tissue associated with a lymphoid aggregate (case16 FVPTC, 100x) and J – Complex network of D2-40 positive lymph vessels in a perithyroidal lymph node with PTC metastases (case37 CPTC, 100x).

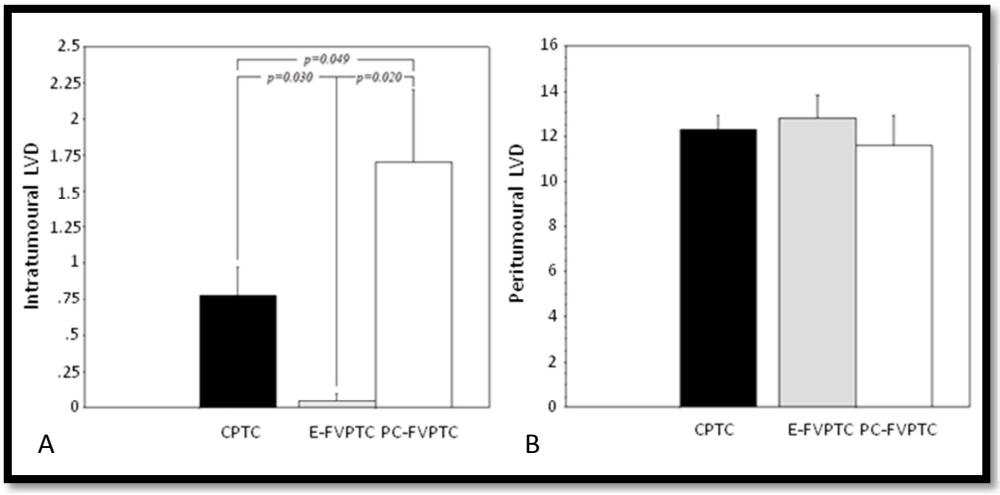


Figure IV.2

Comparison between the LVD in the FVPTC group (E-FVPTC and PC-FVPTC) and in the CPTC group.
A – Graphic representing the mean intratumoural LVD in the three PTC groups; B – Graphic representing the mean peritumoural LVD in the three PTC groups.

IV.3.3 Intratumoural LVD

In 11 out of 12 cases of E-FVPTC (91.7%) not a single intratumoural D2-40 stained vessel was observed (**Table IV.7**). Case 6 of the E-FVPTC subgroup was the only one with D2-40 positive intratumoural lymph vessels and did not show signs of extra-thyroid extension, lymph vessel invasion or vascular invasion, nor had given rise to lymph node metastases (**Table IV.7**). At variance with E-FVPTC, the majority of PC-FVPTC cases (76.5%) had intratumoural D2-40 positive lymph vessels. The mean intratumoural LVD was significantly higher in PC-FVPTC (1.7±2.1) and in CPTC (0.8±1.1) than in E-FVPTC (0.05) (p=0.020 and p=0.030, respectively) (**Figure IV.2A**). It was also higher in PC-FVPTC than in CPTC (p=0.049) (**Figure IV.2A**).
The mean intratumoural LVD was significantly higher in tumours with extra-thyroid extension (p=0.041), lymph vessel invasion (p=0.026) and lymph node metastases (p=0.008) (**TableIV.10**). In FVPTC cases, the mean intratumoural LVD was significantly higher in PC-FVPTC cases with lymph node metastases (p=0.047) (**TableIV.10**).

IV.3.4 Peritumoural LVD

The mean peritumoural LVD was similar in FVPTC (E-FVPTC: 12.8±3.6 and PC-FVPTC: 11.6±5.0, p=0.501) and in CPTC (12.3±3.7)(p=0.888) (**Figure IV.2B**). In all PTC groups, the mean peritumoural LVD was significantly higher than the mean intratumoural LVD (p<0.0001), and it was not significantly associated with any clinico-pathological or molecular feature, namely lymph vessel invasion (p=0.626) or lymph node metastases (p=0.288).

TableIV.10 Relationship between the intratumoural LVD and the morphological features of FVPTC (E-FVPTC and PC-FVPTC) and CPTC cases

Features		E-FVPTC		PC-FVPTC		CPTC		PC-FVPTC+CPTC		PTC	
		mean±sd	*p*	mean±sd	*p*	mean±sd	*p*	mean±sd	*p*	mean±sd	*p*
M	Present	0.2±0.3	0.170	1.7±2.3	0.930	0.9±1.0	0.616	1.2±1.6	0.696	1.0±1.5	0.560
	Absent	0.0±0.0		1.7±2.1		0.7±1.2		1.0±1.6		0.8±1.4	
EE	Present	-	-	2.7±2.1	0.289	1.1±1.5	0.156	1.5±1.7	0.181	1.5±1.7	**0.041**
	Absent	0.1±0.2		1.4±2.1		0.6±0.8		0.9±1.5		0.7±1.3	
LI	Present	-	-	2.4±2.6	0.363	1.2±1.5	0.104	1.6±1.9	0.135	1.6±1.9	**0.026**
	Absent	0.1±0.2		1.4±1.9		0.5±0.7		0.6±1.3		0.6±1.2	
VI	Present	0.0±0.0	0.506	1.8±2.1	0.715	1.1±1.7	0.192	1.5±1.9	**0.078**	1.3±1.8	**0.052**
	Absent	0.1±0.2		1.4±2.3		0.6±0.7		0.7±1.0		0.6±1.0	
LNM	Present	-	-	2.8±2.2	**0.047**	0.9±0.9	0.532	1.5±1.6	**0.084**	1.5±1.6	**0.008**
	Absent	0.1±0.2		0.8±1.5		0.6±1.4		0.7±1.4		0.5±1.2	

M multicentricity, *EE* extra-thyroid extension, *LI* lymph vessel invasion, *VI* vascular invasion, *LNM* lymph node metastases

IV.4 Immunohistochemical expression of elements of the TGF-beta/Smad-dependent pathway in the selected series of PTC cases

Tables IV.11 and **IV.12** summarize the immunohistochemical expression of TGF-beta, P-Smad2/3, Smad4 and Smad7 in the 42 CPTC cases and in the 33 FVPTC, respectively.

TableIV.11. Clinico-pathological and immunohistochemical features of the CPTC cases

Case	Gender	Age (years)	Tumour size (cm)	Growth pattern	Oncocytic cells	Stroma type	II	Multicentricity	EE	Vascular invasion	Lymph vessel invasion	I-LVD	Intensity of expression			P-Smad2/3 C (n/c)	P-Smad2/3 CT (n/c)	Smad4 C (n/c)	Smad4 CT (n/c)	Smad7 C (n/c)	Smad7 CT (n/c)	LNM
													TGF-beta C	TGF-beta P	TGF-beta CT							
1	M	58	0.8	PC	-	C	-	-	-	-	-	0.0	3	2	1	+/-	-/+	-/+	-/+	+/+	-/+	-
2	F	66	1.5	WC	-	C	+	-	-	-	ND	ND	2	2	1	+/+	+/-	-/+	+/+	+/+	+/-	-
3	F	31	3.5	PC	-	P	-	+	-	-	-	0.2	3	3	2	+/+	+/-	-/+	+/+	+/+	+/-	-
4	F	59	0.6	PC	+	-	-	+	-	-	ND	ND	2	2	1	+/+	+/-	-/+	+/+	+/+	+/-	-
5	F	37	1.0	WC	+	L	+	+	-	-	ND	ND	3	3	2	+/-	+/+	-/+	-/+	+/+	+/+	-
6	F	35	0.3	PC	-	P	-	-	-	-	+	0.3	2	3	0	+/-	+/-	+/+	+/+	+/-	+/-	-
7	F	40	1.5	PC	+	L	+	-	-	-	-	0.4	3	3	1	+/+	+/-	-/+	-/+	+/-	+/-	-
8	F	38	0.5	PC	-	C	-	-	-	-	ND	ND	2	2	0	+/+	+/+	-/+	-/+	+/+	+/-	-
9	M	25	1.2	PC	-	C	-	-	-	+	-	2.0	2	1	1	+/+	+/-	-/+	-/+	+/+	+/-	-
10	F	30	0.6	PC	+	C	+	+	-	-	-	0.0	2	3	2	+/+	+/-	-/+	+/+	-/+	-/+	-
11	F	40	2.2	WC	+	-	+	-	-	-	ND	ND	1	2	2	+/+	+/-	-/+	-/+	+/+	+/+	-
12	F	50	3.0	WC	-	C	+	-	-	-	ND	ND	1	1	0	+/-	+/-	-/+	-/+	+/-	+/-	-
13	F	25	2.1	WC	-	P	+	-	-	-	ND	ND	1	1	1	+/+	+/-	-/+	+/+	+/+	+/-	-
14	F	45	4.5	WC	+	-	-	+	-	-	ND	ND	2	3	2	+/-	+/+	+/+	-/+	+/+	+/-	-
15	F	23	2.5	PC	-	P	+	-	-	-	+	0.9	3	3	2	+/+	+/+	-/+	+/+	+/+	+/+	-
16	M	65	2.3	PC	-	C	+	-	-	-	-	0.0	3	3	2	+/+	+/+	-/+	+/+	+/+	+/+	-
17	F	21	2.0	PC	-	C	+	-	-	+	+	0.0	1	2	0	+/+	+/-	-/+	+/+	+/+	+/-	-
18	F	79	2.8	PC	-	C	+	-	+	-	-	0.0	3	3	1	+/+	-/-	-/+	-/+	+/+	+/-	-
19	F	65	3.5	PC	+	P	+	-	+	-	+	0.0	1	2	0	+/+	+/-	-/+	+/-	+/-	+/-	-
20	F	28	2.5	PC	+	C	-	-	-	+	-	0.0	3	3	0	+/+	+/+	-/+	+/+	+/+	+/-	-
21	F	28	2.0	PC	-	C	-	-	-	+	-	0.0	3	3	0	+/+	+/-	-/+	+/+	+/+	+/+	-
22	F	43	1.5	WC	+	C	+	+	-	+	ND	ND	1	1	0	+/+	+/-	-/+	+/+	+/+	+/+	-
23	F	47	4.5	PC	+	P	+	-	+	+	+	5.1	1	3	1	+/+	+/-	-/+	+/+	+/+	+/-	-
24	F	28	0.9	PC	+	-	-	-	-	-	-	0.2	1	2	1	+/+	+/-	+/+	+/+	+/+	+/-	+
25	F	21	1.2	PC	+	C	+	-	-	-	+	0.1	1	2	2	+/+	+/+	+/+	+/+	-/+	+/-	+
26	F	22	1.9	PC	-	-	-	+	-	-	+	0.7	1	1	1	+/+	+/-	-/+	+/+	+/+	+/-	+
27	F	30	1.3	PC	-	P	+	-	+	+	-	0.5	2	3	2	+/+	+/-	-/+	+/+	-/+	+/-	+
28	F	56	0.7	PC	-	-	+	+	-	-	+	1.0	3	3	2	+/+	+/-	-/+	+/+	-/+	+/-	+
29	F	18	1.5	PC	-	C	+	+	+	+	-	0.0	3	3	2	+/+	+/-	+/+	+/+	-/+	+/-	+
30	M	22	1.1	PC	-	P	-	+	-	-	-	0.2	2	3	2	+/-	+/-	+/+	+/+	-/-	+/-	+
31	F	22	0.8	PC	-	C	+	-	-	-	ND	ND	1	2	2	+/+	+/-	-/+	+/+	-/+	+/-	+
32	F	56	0.8	PC	-	P	-	+	-	-	+	0.7	1	2	1	+/+	+/-	-/+	+/+	-/+	+/+	+
33	M	42	1.6	PC	+	P	+	+	+	-	-	2.2	2	3	3	+/+	+/-	-/+	+/+	-/+	+/-	+
34	F	38	3.0	PC	-	P	+	+	-	+	-	0.0	1	1	2	+/+	+/-	-/+	+/+	-/+	+/-	+
35	F	35	1.7	PC	-	C	+	-	-	+	-	1.4	0	2	2	+/+	+/-	-/+	+/+	-/+	+/-	+
36[a,b]	M	11	6.0	PC	-	C	+	+	+	+	+	0.4	1	2	0	+/+	+/-	-/+	+/+	+/-	+/-	+
37[a,b]	F	56	2.4	PC	+	C	+	-	+	-	-	1.5	1	2	2	+/+	+/-	-/+	+/+	-/+	+/-	+
38	F	83	7.5	PC	+	-	+	-	+	-	-	1.0	0	3	ND	+/+	ND	-/+	ND	-/+	ND	+
39	F	18	3.6	PC	-	C	-	+	-	+	+	3.1	2	2	1	+/+	+/-	+/+	+/+	-/+	+/-	+
40	M	14	2.5	PC	-	P	-	+	+	-	+	2.0	1	2	1	+/+	+/-	+/+	+/+	-/-	+/-	+
41	F	32	1.1	PC	-	C	-	-	+	-	-	0.4	0	2	1	+/-	+/-	-/+	+/+	+/-	+/-	+
42	F	56	7.5	PC	-	C	+	+	+	-	-	0.6	1	2	1	+/+	+/-	-/+	+/+	-/+	+/-	+

M male, *F* female, + presence, - absence, *WC* well circumscribed, *PC* poorly circumscribed, *C* cellular, *P* poorly cellular, *L* lymphocyte rich, *II* intra-tumoural inflammation, *EE* extra-thyroid extension, *I-LVD* intratumoural lymph vessel density,*C* expression in the center of the tumour, *P* expression in the tumour periphery, *CT* expression in the control tissue, *n* nuclear expression, *c* cytoplasmatic expression, *LNM* lymph node metastases, *ND* not determined. [a] Cases with concurrent distant metastases, [b] Cases with recurrence(s).

Table IV.12. Clinico-pathological and immunohistochemical features of the FVPTC cases

Case	Gender	Age (years)	Tumour size (cm)	Growth pattern	Oncocytic cells	Stroma type	II	Multicentricity	EE	Vascular invasion	Lymph vessel invasion	I-LVD	Intensity of expression			P-Smad2/3 C (n/c)	P-Smad2/3 CT (n/c)	Smad4 C (n/c)	Smad4 CT (n/c)	Smad7 C (n/c)	Smad7 CT (n/c)	LNM
													TGF-beta C	TGF-beta P	TGF-beta CT							
1	F	32	2.0	WC	+	P	+	-	-	+	-	0.0	2	0	2	+/+	+/-	-/+	+/+	+/+	+/-	-
2	F	26	1.8	PC	-	P	-	+	-	+	-	0.0	2	3	1	+/+	+/-	-/+	+/+	+/+	+/+	-
3	F	59	0.5	WC	+	-	+	-	-	-	ND	ND	0	0	0	+/-	+/-	-/+	+/-	+/-	+/-	-
4	F	51	1.4	WC	+	C	+	-	-	-	-	0.0	2	3	1	+/+	+/-	+/+	+/+	+/+	+/-	-
5	F	36	0.5	PC	-	C	+	-	-	-	-	0.5	1	1	1	+/+	+/-	+/+	+/+	+/-	+/-	-
6	F	39	1.0	WC	-	-	-	+	-	-	-	0.6	1	1	0	+/-	+/-	+/+	+/+	+/+	+/-	-
7	F	48	0.6	WC	-	P	-	-	-	-	-	0.0	1	0	0	+/+	+/+	+/+	+/+	+/-	+/-	-
8	F	33	0.2	PC	-	C	+	-	-	-	ND	ND	3	3	0	+/+	+/+	+/+	+/+	+/+	+/-	-
9	F	30	0.5	WC	-	-	-	-	-	-	-	0.0	1	1	0	+/+	+/+	-/+	+/+	+/-	+/-	-
10	F	21	0.6	PC	-	-	-	-	-	-	-	4.8	1	3	1	+/-	+/-	-/+	+/+	+/-	+/-	-
11	F	37	0.3	PC	-	P	-	-	-	+	-	0.0	1	1	1	+/-	+/-	-/+	+/+	+/-	+/-	-
12	F	28	2.8	WC	-	P	-	+	-	+	-	0.0	2	3	0	+/+	+/-	-/+	+/+	+/+	+/+	-
13	F	34	3.5	WC	-	P	-	-	-	-	-	0.0	0	0	0	+/-	+/-	+/+	-/+	+/-	+/-	-
14	M	61	4.0	WC	-	-	-	-	-	+	-	0.0	2	2	2	+/+	+/-	-/+	+/+	+/+	+/-	-
15	M	35	3.5	WC	-	-	-	-	-	+	-	0.0	1	1	1	+/+	+/-	-/+	+/+	+/-	+/-	-
16	F	50	2.5	WC	-	-	-	-	-	-	-	0.0	1	1	1	+/-	+/-	-/+	-/+	+/-	+/-	-
17	F	47	3.0	PC	+	-	-	-	-	-	-	0.0	2	3	0	+/-	+/-	+/+	+/+	+/+	+/-	-
18	F	49	3.0	WC	-	-	-	+	-	+	ND	ND	2	1	1	+/+	+/+	-/+	+/+	+/+	+/-	-
19	F	35	1.7	PC	-	P	+	+	-	+	ND	ND	3	3	1	+/+	+/-	-/+	+/+	+/+	+/-	-
20	F	32	4.7	PC	+	-	-	+	-	+	-	1.0	2	3	2	+/+	+/-	+/+	+/+	+/+	+/-	-
21	F	52	2.5	WC	-	P	-	+	-	-	-	0.0	1	1	2	+/-	+/-	+/+	+/+	+/-	+/-	-
22	F	66	2.5	WC	+	P	-	+	-	-	-	0.0	1	1	0	+/+	+/-	+/+	+/+	+/-	+/-	-
23	F	58	1.5	PC	+	C	+	+	-	+	+	0.3	0	1	1	+/+	+/-	-/+	+/+	+/+	+/-	-
24	F	54	1.8	PC	+	P	+	+	-	+	+	0.2	1	2	1	+/+	+/+	-/+	+/+	+/+	+/-	-
25	M	45	4.0	WC	+	-	-	-	-	-	-	0.1	1	1	1	+/-	+/-	-/+	+/+	+/-	+/-	-
26[b]	M	57	9.5	PC	-	-	-	-	+	+	+	4.4	2	2	1	+/+	+/+	+/+	+/+	-/+	+/-	+
27	F	56	0.8	PC	-	P	+	+	-	+	+	1.3	1	2	2	+/+	+/-	+/+	+/+	-/-	+/-	+
28	M	21	2.5	PC	-	C	+	-	+	+	-	2.0	1	2	0	+/+	+/-	-/+	+/+	-/+	+/-	+
29	F	20	1.0	PC	-	-	+	+	-	+	-	6.0	0	1	2	+/-	+/-	-/+	+/+	-/-	+/-	+
30	F	49	2.2	PC	-	-	-	-	-	+	-	3.9	0	1	0	+/-	+/-	-/+	+/+	-/-	+/-	+
31	F	43	4.0	PC	-	-	+	+	+	+	-	0.1	0	1	0	+/-	+/-	-/+	+/+	-/-	+/-	+
32	F	43	1.5	PC	-	C	+	+	+	+	-	4.3	1	3	1	+/-	+/-	-/+	+/+	+/-	+/-	+
33[a,b]	M	63	1.8	PC	-	-	-	-	-	+	-	0.0	1	3	1	+/-	+/-	-/+	+/+	-/+	+/-	+

M male, *F* female, + presence, - absence, *WC* well circumscribed, *PC* poorly circumscribed, *C* cellular, *P* poorly cellular, *EE* extra-thyroid extension, *I-LVD* intratumoural lymph vessel density, *C* expression at the center of the tumour, *P* expression at the tumour periphery, *CT* expression in the control tissue, *n* nuclear expression, *c* cytoplasmatic expression, *LNM* lymph node metastases, *ND* not determined. [a] Cases with concurrent distant metastases, [b] Cases with recurrence(s).

IV.4.1 CPTC *versus* FVPTC

IV.4.1.1 TGF-beta expression

TGF-beta expression was observed in all but two FVPTC-**M** cases and was detected in the center of 88.0% of tumours (95.2% of CPTC and 81.8% of FVPTC), at the periphery of 94.7% of tumours (100% of CPTC and 87.9% of FVPTC) and in the control tissues in 73.0% of cases (78.6% of CPTC and 63.3% of FVPTC).
In comparison with the respective control tissue, the center of the carcinomas disclosed basal or overexpression of TGF-beta in 83.8% of cases. The TGF-beta expression at the periphery of each tumour was similar or higher than the TGF-beta expression in the center in all but one CPTC-**M** and three FVPTC-**M** cases (94.7%) (**Tables IV.11** and **IV.12**) (**Figure IV.3**).
The mean intensity of TGF-beta expression in the center of the tumours, in comparison with the respective control tissue, was much higher in PTC-**M** than in PTC+**M** ($p<0.0001$).The mean intensity of TGF-beta expression at the periphery, compared with the respective center of the tumours, was significantly higher in

PTC+**M** than in PTC-**M** (p=0.0001) and was also significantly higher in tumours with poorly circumscribed growth pattern (p<0.0007) and extra-thyroid extension (p=0.002) (**Table IV.13**).

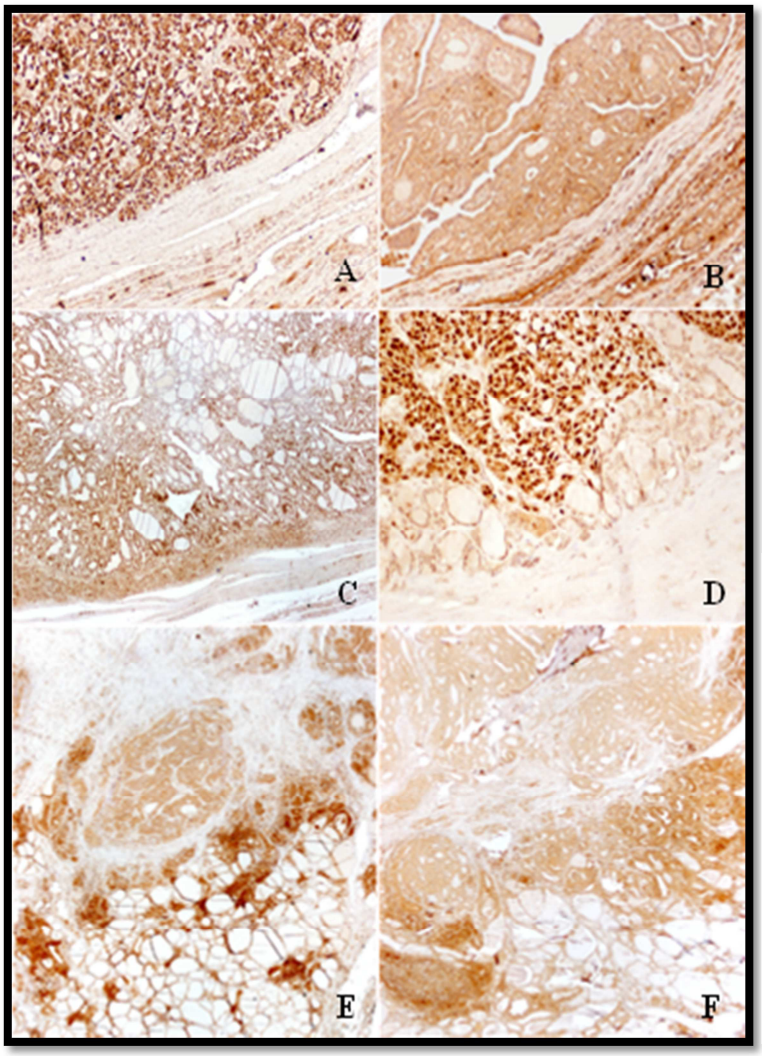


Figure IV.3

TGF-beta expression at the center and at the periphery of the tumours.
A- Well circumscribed FVPTC-M with similar expression at the center and at the periphery (case 14; 200x); B – Well circumscribed FVPTC-M with increased expression at the periphery (case12, 200x); C – Poorly circumscribed FVPTC+M with lung metastases and increased expression at the periphery (case 33, 200x); D – Well circumscribed FVPTC-M with decreased expression at the periphery (case18, 200x); E – Poorly circumscribed CPTC+M with increased expression at the periphery (case33, 40x) and F – Poorly circumscribed CPTC-M with increased expression at the periphery (case17,40x).

IV.4.1.2 Smad 2/3 expression

P-Smad2/3 nuclear expression was observed in all tumours (**Figure IV.4A** and **B**) and in all but two control tissues of the CPTC-**M** group (97.3%) (**Figure IV.4C**).
P-Smad2/3 cytoplasmatic expression was observed in 72.0% of tumours and in 18.9% of control tissues. In comparison to the respective control tissue, P-Smad2/3 had a basal level or was underexpressed in the cytoplasm of tumour cells in 43.2% of the cases. Tumours with basal or underexpression of cytoplasmatic P-Smad2/3 belonged more frequently to the group of FVPTC than to the CPTC group (60.6% and 29.3%, respectively; p=0.010) (**Tables IV.11** and **IV.12**).

IV.4.1.3 Smad4 expression

Smad4 nuclear expression was observed in 26.7% of tumours and 85.1% of control tissues (**Figure IV.4D, E** and **F**). Smad4 had a basal level or was overexpressed in the nuclei of tumour cells in 39.2% of the cases in comparison with the respective control tissue. Vascular invasion was significantly less frequent in tumours with basal or overexpressed nuclear Smad4 (p=0.004), particularly in the FVPTC group (p=0.003) (**Table IV.13**).
Smad4 cytoplasmatic expression was observed in all tumours and in all but two control tissues of the PTC-**M** group (97.3%) and was not significantly associated to any clinical or pathological feature.

IV.4.1.4 Smad7 expression

Smad7 nuclear expression was observed in 69.3% of tumours and in all but two control tissues of the CPTC-**M** group (97.3%) (**Figure IV.4G, H** and **I**).
Smad7 had a basal level or was overexpressed in the nuclei of the tumour cells in 71.6% of tumours in comparison with the respective control tissue. There was a significant association between basal to overexpressed Smad7 in the nuclei of the tumour cells and well circumscribed growth pattern (p=0.0001), unicentric CPTC (p=0.048), absence of vascular invasion in FVPTC (p=0.013), absence of extra-thyroid extension in FVPTC (p=0.023) and absence of lymph node metastases in both CPTC and FVPTC ($p<0.0001$) (**Table IV.13**).
Smad7 cytoplasmatic expression was observed in 66.7% of tumours and in 14.9% of the control tissues. Smad7 had a basal level or was underexpressed in the cytoplasm of the tumour cells in 48.6% of cases in comparison with the respective control tissue, both in CPTC and FVPTC. Smad7 cytoplasmatic expression was not significantly associated to any clinical or pathological feature.

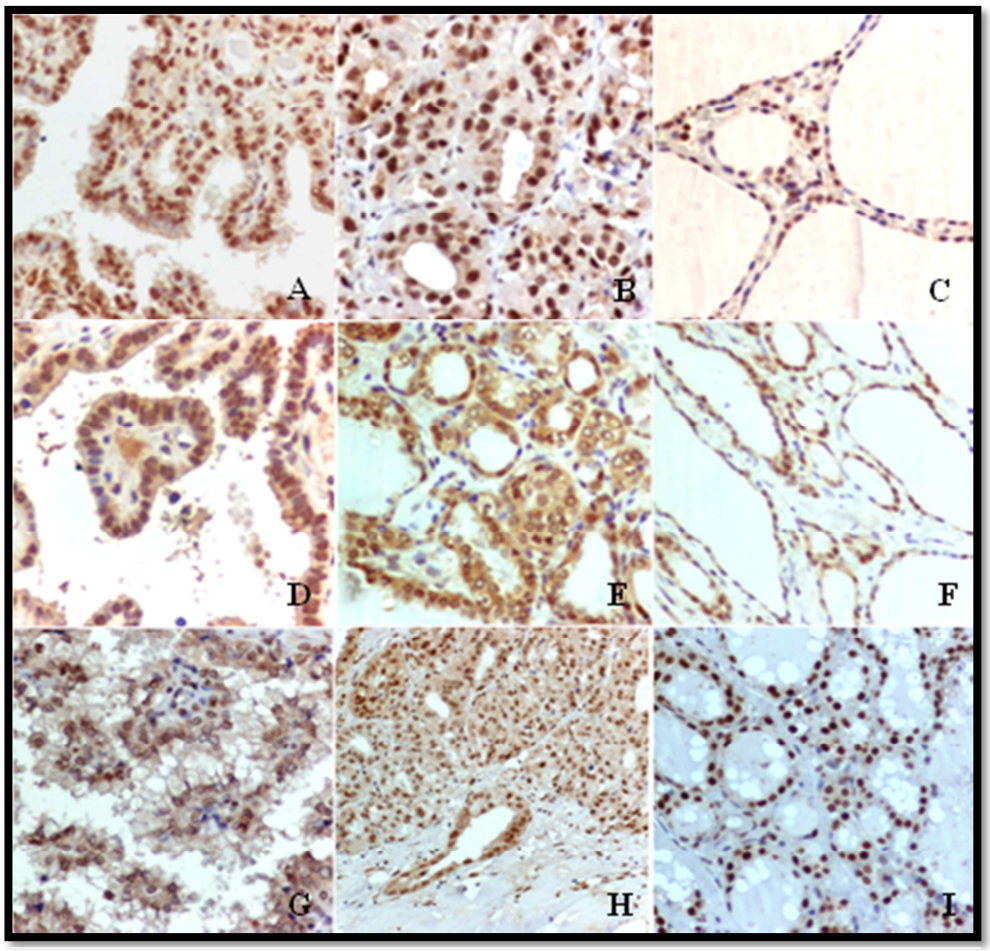


Figure IV.4

Expression of P-Smad2/3: A – Papillary areas of the center of the tumour (case 26 of CPTC group, 200x), B –Follicular areas of the center of the tumour (case 28 of FVPTC group, 400x) and C – Control tissue (case 2 of CPTC group, 400x).
Expression of Smad4: D – Papillary areas of the center of the tumour (case 39 of CPTC group, 200x), E – Follicular areas of the center of the tumour (case 27 of FVPTC group, 200x) and F– Control tissue (case 27 of FVPTC group, 200x).
Expression of Smad7: G – Papillary areas of the center of the tumour (case 9 of CPTC group, 400x), H – Follicular areas of the periphery of the tumour (case 1 of FVPTC group, 100x) and I – Control tissue (case 1 of FVPTC group; 200x).

Table IV.13 Comparison between the immunohistochemical patterns of and the PTC features.

	Features		TGF-beta intensity of expression (mean±sd)				Overexpression or basal nuclear expression (%)			
			C-CT	*p*	P-C	*p*	Smad4	*p*	Smad7	*p*
CPTC+FVPTC	**GP**	PC	0.5±1.2	0.839	0.7±0.7	**0.0007**	33.3	0.198	58.8	**0.0001**
		WC	0.4±0.7		0.1±0.5		52.2		100.0	
	M	Present	0.3±0.9	0.217	0.5±0.6	0.630	35.5	0.635	61.3	0.120
		Absent	0.6±1.2		0.5±0.8		41.8		79.1	
	EE	Present	0.3±0.9	0.409	1.0±0.7	**0.002**	26.7	0.377	40.0	**0.008**
		Absent	0.5±1.1		0.4±0.7		42.4		79.7	
	VI	Present	0.4±1.2	0.719	0.6±0.8	0.259	19.4	**0.004**	61.3	0.120
		Absent	0.5±1.0		0.4±0.7		53.5		79.1	
	LNM	Present	-0.2±0.9	**<0.0001**	0.9±0.6	**0.0001**	30.8	0.325	19.2	**<0.0001**
		Absent	0.8±1.0		0.3±0.7		43.8		100.0	
CPTC	**GP**	PC	0.6±1.2	0.787	0.6±0.7	0.362	35.3	0.401	58.8	**0.075**
		WC	0.5±0.8		0.3±0.5		57.1		100.0	
	M	Present	0.4±0.7	0.393	0.5±0.5	0.757	35.3	0.753	47.1	**0.048**
		Absent	0.7±1.3		0.5±0.8		31.7		79.2	
	EE	Present	0.2±1.0	0.233	1.0±0.6	**0.006**	27.3	0.478	45.5	0.140
		Absent	0.7±1.2		0.3±0.7		43.3		73.3	
	VI	Present	0.8±1.4	0.449	0.5±0.9	0.945	25.0	0.305	58.3	0.719
		Absent	0.4±1.0		0.5±0.6		44.8		69.0	
	LNM	Present	-0.2±0.9	**<0.0001**	0.8±0.6	**0.009**	33.3	0.540	22.2	**<0.0001**
		Absent	1.1±1.0		0.3±0.7		43.5		100.0	
FVPTC	**GP**	PC	0.4±1.2	0.812	0.9±0.7	**<0.0001**	29.4	0.296	58.8	**0.007**
		WC	0.4±0.7		0.0±0.5		50.0		100.0	
	M	Present	0.2±1.2	0.383	0.6±0.7	0.310	35.7	>0.999	78.6	>0.999
		Absent	0.5±0.8		0.4±0.8		42.1		78.9	
	EE	Present	0.5±0.6	0.824	1.0±0.8	0.148	25.0	>0.999	25.0	**0.023**
		Absent	0.4±1.0		0.4±0.7		41.4		86.2	
	VI	Present	0.2±1.0	0.224	0.7±0.7	**0.077**	15.8	**0.003**	63.2	**0.013**
		Absent	0.6±1.0		0.2±0.7		71.4		100.0	
	LNM	Present	0.1±1.0	**0.091**	1.1±0.6	**0.004**	25.0	0.431	12.5	**<0.0001**
		Absent	0.6±1.0		0.3±0.7		44.0		100.0	

GP Growth pattern, *M* multicentricity, *EE* extra-thyroid extension, *VI* vascular invasion, *LNM* lymph node metastases, *C* expression at the center of the tumour, *P* expression at the tumour periphery, *CT* expression in the control tissue.

IV.4.2 Well circumscribed PTC (WC-PTC) *versus* poorly circumscribed PTC (PC-PTC)

Globally, the difference between the mean TGF-beta expression in the center of the tumours and in the control tissue was similar in WC-PTC and in PC-PTC (p=0.839) whereas the difference between the mean TGF-beta expression in the periphery of the tumours and in the center of the tumours was much higher in PC-PTC than in WC-PTC (p=0.0007) (**Table IV.13**).

In 63.6% of WC-PTC cases (14 out of 22) the TGF-beta expression was nearly homogeneous, absent or low/moderate, throughout the tumour. In PC-PTC, the PTC-**M** cases disclosed a significantly more intense expression of TGF-beta in the center of the tumours than in the control tissue (p<0.0001), whereas in PTC+**M** cases the cells in the periphery of the tumours presented a significantly more intense expression of TGF-beta than those in the center of the tumours (p=0.017) (**Table IV.14**).

Smad4 nuclear expression in WC-PTC subgroup was detected only in tumours without vascular invasion (p=0.005) (**Table IV.14**), whereas in PC-PTC, Smad4 nuclear expression was not significantly associated to any clinical, pathological or molecular features.

Table IV.14 Comparison between the immunohistochemical patterns of expression in well and poorly circumscribed PTCs.

	Features		Increment in TGF-beta intensity of expression (mean±sd)				Overexpression or basal nuclear expression (%)				Underexpression or basal cytoplasmatic expression (%)	
			T	*p*	P	*p*	Smad4	*p*	Smad7	*p*	P-Smad2/3	*p*
WC PTC	**VI**	Present	0.7±0.8	0.376	0.0±0.6	0.641	0.0	**0.005**	100.0	>0.999	16.7	**0.069**
		Absent	0.4±0.7		0.1±0.5		70.6		100.0		64.7	
PC PTC	**M**	Present	0.1±0.9	**0.053**	0.7±0.6	0.935	26.1	0.381	47.8	0.167	21.7	**0.025**
		Absent	0.8±1.3		0.7±0.9		39.3		67.9		53.6	
	EE	Present	0.3±0.9	0.398	1.0±0.7	**0.048**	26.7	0.746	40.0	0.119	26.7	0.348
		Absent	0.6±1.3		0.6±0.7		50.0		66.7		44.4	
	LNM	Present	-0.2±0.9	**<0.0001**	0.9±0.6	**0.017**	30.8	0.771	19.2	**<0.0001**	34.6	0.573
		Absent	1.2±1.1		0.4±0.8		36.0		100.0		44.0	

VI vascular invasion, *M* Multicentricity, *EE* extra-thyroid extension, *LNM* lymph node metastases, *T* expression at the center of the tumour, *P* expression at the tumour periphery,

IV.5 Results of the molecular analysis and correlation with the clinico-pathological and immunohistochemical data of the selected series of PTC cases

Tables IV.15 and **IV.16** summarize the result of the molecular analysis and the clinico-pathological features of the CPTC and of the FVPTC cases, respectively.

TableIV.15 Clinico-pathological, immunohistochemical and molecular features of the CPTC cases

Case	Gender	Age (years)	Tumour size (cm)	Growth pattern	Multicentricity	EE	Vascular invasion	Lymph vessel invasion	I -LVD	LNM	Intensity of expression			P-Smad2/3 C (n/c)	P-Smad2/3 CT (n/c)	Smad4 C(n/c)	Smad4 CT (n/c)	Smad7 C (n/c)	Smad7 CT (n/c)	*BRAF*	*N-RAS*
											TGF-beta C	TGF-beta P	TGF-beta CT								
1	M	58	0.8	PC	-	-	-	-	0.0	-	3	2	1	+/-	-/+	-/+	-/+	+/+	-/+	ND	ND
2	F	66	1.5	WC	-	-	-	ND	ND	-	2	2	1	+/+	+/-	-/+	+/+	+/+	+/-	ND	ND
3	F	31	3.5	PC	+	-	-	-	0.2	-	3	3	2	+/+	+/-	-/+	+/+	+/+	+/-	-	-
4	F	59	0.6	PC	+	-	-	ND	ND	-	2	2	1	+/+	+/-	-/+	+/+	+/+	+/-	-	-
5	F	37	1.0	WC	+	-	-	ND	ND	-	3	3	2	+/-	+/+	-/+	-/+	+/+	+/+	ND	ND
6	F	35	0.3	PC	-	-	-	+	0.3	-	2	3	0	+/-	+/-	+/+	+/+	+/-	+/-	ND	ND
7	F	40	1.5	PC	-	-	-	-	0.4	-	3	3	1	+/+	+/-	-/+	-/+	+/-	+/-	V600E	-
8	F	38	0.5	PC	-	-	-	ND	ND	-	2	2	0	+/+	+/+	-/+	-/+	+/+	+/-	ND	ND
9	M	25	1.2	PC	-	-	+	-	2.0	-	2	1	1	+/+	+/-	-/+	-/+	+/+	+/-	-	-
10	F	30	0.6	PC	+	-	-	-	0.0	-	2	3	2	+/+	+/-	-/+	+/+	-/+	-/+	V600E	-
11	F	40	2.2	WC	-	-	-	ND	ND	-	1	2	2	+/+	+/-	-/+	-/+	+/+	+/+	-	-
12	F	50	3.0	WC	-	-	-	ND	ND	-	1	1	0	+/-	+/-	-/+	-/+	+/-	+/-	V600E	-
13	F	25	2.1	WC	-	-	-	ND	ND	-	1	1	1	+/+	+/-	-/+	+/+	+/+	+/-	V600E	-
14	F	45	4.5	WC	+	-	-	ND	ND	-	2	3	2	+/-	+/+	+/+	-/+	+/+	+/-	V600E	Q61R
15	F	23	2.5	PC	-	-	-	+	0.9	-	3	3	2	+/+	+/+	-/+	+/+	+/+	+/+	-	-
16	M	65	2.3	PC	-	-	-	-	0.0	-	3	3	2	+/+	+/+	-/+	+/+	+/+	+/+	-	-
17	F	21	2.0	PC	-	-	+	+	0.0	-	1	2	0	+/+	+/-	-/+	+/+	+/+	+/-	V600E	-
18	F	79	2.8	PC	-	+	-	-	0.0	-	3	3	1	+/+	-/-	-/+	-/+	+/+	+/-	-	-
19	F	65	3.5	PC	-	+	-	+	0.0	-	1	2	0	+/+	+/-	-/+	+/-	+/-	+/-	V600E	-
20	F	28	2.5	PC	-	-	+	-	0.0	-	3	3	0	+/+	+/+	-/+	+/+	+/+	+/-	-	-
21	F	28	2.0	PC	-	-	+	-	0.0	-	3	3	0	+/+	+/-	-/+	+/+	+/+	+/+	V600E	-
22	F	43	1.5	WC	+	-	+	ND	ND	-	1	1	0	+/+	+/-	-/+	+/+	+/+	+/+	-	-
23	F	47	4.5	PC	-	+	+	+	5.1	-	1	3	1	+/+	+/-	-/+	+/+	+/+	+/-	V600E	-
24	F	28	0.9	PC	-	-	-	-	0.2	+	1	2	1	+/+	+/-	+/+	+/+	+/+	+/-	V600E	-
25	F	21	1.2	PC	-	-	-	+	0.1	+	1	2	2	+/+	+/+	+/+	+/+	-/+	+/-	-	-
26	F	22	1.9	PC	+	-	-	+	0.7	+	1	1	1	+/+	+/-	-/+	+/+	+/+	+/-	-	-
27	F	30	1.3	PC	-	+	+	-	0.5	+	2	3	2	+/+	+/-	-/+	+/+	-/+	+/-	-	-
28	F	56	0.7	PC	+	-	-	+	1.0	+	3	3	2	+/+	+/-	-/+	+/+	-/+	+/-	-	-
29	F	18	1.5	PC	+	+	+	-	0.0	+	3	3	2	+/+	+/-	+/+	+/+	-/+	+/-	-	-
30	M	22	1.1	PC	+	-	-	-	0.2	+	2	3	2	+/-	+/-	+/+	+/+	-/-	+/-	-	-
31	F	22	0.8	PC	-	-	-	ND	ND	+	1	2	2	+/+	+/-	-/+	+/+	-/+	+/-	ND	ND
32	F	56	0.8	PC	+	-	-	+	0.7	+	1	2	1	+/+	+/-	-/+	+/+	-/+	+/+	ND	ND
33	M	42	1.6	PC	+	+	-	-	2.2	+	2	3	3	+/+	+/-	-/+	+/+	-/+	+/-	-	-
34	F	38	3.0	PC	+	-	+	-	0.0	+	1	1	2	+/+	+/-	-/+	+/+	-/+	+/-	V600E	-
35	F	35	1.7	PC	-	-	+	-	1.4	+	0	2	2	+/+	+/-	-/+	+/+	-/+	+/-	V600E	-
36[a,b]	M	11	6.0	PC	+	+	+	+	0.4	+	1	2	0	+/+	+/-	-/+	+/+	+/-	+/-	-	-
37[a,b]	F	56	2.4	PC	-	+	-	-	1.5	+	1	2	2	+/+	+/-	-/+	+/+	-/+	+/-	-	-
38	F	83	7.5	PC	-	+	-	-	1.0	+	0	3	ND	+/+	ND	-/+	ND	-/+	ND	V600E	-
39	F	18	3.6	PC	+	-	+	+	3.1	+	2	2	1	+/+	+/-	+/+	+/+	-/+	+/-	-	-
40	M	14	2.5	PC	+	+	-	+	2.0	+	1	2	1	+/+	+/-	+/+	+/+	-/-	+/-	-	-
41	F	32	1.1	PC	-	+	-	-	0.4	+	0	2	1	+/-	+/-	-/+	+/+	+/-	+/-	V600E	-
42	F	56	7.5	PC	+	+	-	-	0.6	+	1	2	1	+/+	+/-	-/+	+/+	-/+	+/-	ND	ND

M male, *F* female, + presence, - absence, *WC* well circumscribed, *PC* poorly circumscribed, *EE* extra-thyroid extension, *I-LVD* Intratumoural lymph vessels density, *LNM* lymph node metastases, *C* expression at the center of the tumour, *P* expression at the tumour periphery, *CT* expression at the control tissue, *n* nuclear expression, *c* cytoplasmatic expression, *ND* not determined. [a] Cases with concurrent distant metastases, [b] Cases with recurrence(s).

Table IV.16 Clinico-pathological, immunohistochemical and molecular features of the FVPTC cases

Case	Gender	Age (years)	Follow-up (years)	Tumour size (cm)	Growth pattern	Multicentricity	EE	Vascular invasion	Lymph vessel invasion	I-LVD	LNM	Intensity of expression			P-Smad2/3 C (n/c)	P-Smad2/3 CT (n/c)	Smad4 C (n/c)	Smad4 CT (n/c)	Smad7 C (n/c)	Smad7 CT (n/c)	*BRAF*	*N-RAS*
												TGF-beta C	TGF-beta P	TGF-beta CT								
1	F	32	22	2.0	WC	-	-	+	-	0.0		2	0	2	+/+	+/-	-/+	+/+	+/+	+/-	-	-
2	F	26	21	1.8	PC	+	-	+	-	0.0	-	2	3	1	+/+	+/-	-/+	+/+	+/+	+/+	V600E	-
3	F	59	19	0.5	WC	-	-	-	ND	ND	-	0	0	0	+/-	+/-	-/+	+/-	+/-	+/-	-	-
4	F	51	18	1.4	WC	-	-	-	-	0.0	-	2	3	1	+/+	+/-	+/+	+/+	+/+	+/-	-	-
5	F	36	18	0.5	PC	-	-	-	-	0.5	-	1	1	1	+/+	+/-	+/+	+/+	+/-	+/-	V600E	-
6	F	39	15	1.0	WC	+	-	-	-	0.6	-	1	1	0	+/-	+/-	+/+	+/+	+/+	+/-	-	Q61R
7	F	48	13	0.6	WC	-	-	-	-	0.0	-	1	0	0	+/+	+/+	+/+	+/+	+/-	+/-	-	-
8	F	33	20	0.2	PC	-	-	-	ND	ND	-	3	3	0	+/+	+/+	+/+	+/+	+/+	+/-	-	-
9	F	30	15	0.5	WC	-	-	-	-	0.0	-	1	1	0	+/+	+/+	-/+	+/+	+/-	+/-	-	-
10	F	21	21	0.6	PC	-	-	-	-	4.8	-	1	3	1	+/-	+/-	-/+	+/+	+/-	+/-	-	-
11	F	37	18	0.3	PC	-	-	+	-	0.0	-	1	1	1	+/-	+/-	-/+	+/+	+/-	+/-	-	-
12	F	28	21	2.8	WC	+	-	+	-	0.0	-	2	3	0	+/+	+/-	-/+	+/+	+/+	+/+	-	-
13	F	34	20	3.5	WC	-	-	-	-	0.0	-	0	0	0	+/-	+/-	+/+	-/+	+/-	+/-	-	-
14	M	61	22	4.0	WC	-	-	+	-	0.0	-	2	2	2	+/+	+/-	-/+	+/+	+/+	+/-	V600E	-
15	M	35	20	3.5	WC	-	-	+	-	0.0	-	1	1	1	+/+	+/-	-/+	+/+	+/-	+/-	-	Q61R
16	F	50	20	2.5	WC	-	-	-	-	0.0	-	1	1	1	+/-	+/-	-/+	-/+	+/-	+/-	-	-
17	F	47	23	3.0	PC	-	-	-	-	0.0	-	2	3	0	+/-	+/-	+/+	+/+	+/+	+/-	-	Q61R
18	F	49	14	3.0	WC	+	-	+	ND	ND	-	2	1	1	+/+	+/+	-/+	+/+	+/+	+/-	-	-
19	F	35	21	1.7	PC	+	-	+	ND	ND	-	3	3	1	+/+	+/-	-/+	+/+	+/+	+/-	ND	ND
20	F	32	13	4.7	PC	+	-	+	-	1.0	-	2	3	2	+/+	+/-	+/+	+/+	+/+	+/-	-	-
21	F	52	13	2.5	WC	+	-	-	-	0.0	-	1	1	2	+/-	+/-	+/+	+/+	+/-	+/-	-	-
22	F	66	13	2.5	WC	+	-	-	-	0.0	-	1	1	0	+/+	+/-	+/+	+/+	+/-	+/-	-	-
23	F	58	13	1.5	PC	+	-	+	+	0.3	-	0	1	1	+/+	+/-	-/+	+/+	+/+	+/-	V600E	-
24	F	54	10	1.8	PC	+	-	+	+	0.2	-	1	2	1	+/+	+/+	-/+	+/+	+/+	+/-	-	-
25	M	45	20	4.0	WC	-	-	-	-	0.1	-	1	1	1	+/-	+/-	-/+	+/+	+/-	+/-	-	-
26[b]	M	57	3	9.5	PC	-	+	+	+	4.4	-	2	2	1	+/+	+/+	+/+	+/+	-/+	+/-	-	-
27	F	56	3	0.8	PC	+	-	+	+	1.3	+	1	2	2	+/+	+/-	+/+	+/+	-/-	+/-	-	-
28	M	21	4	2.5	PC	-	+	+	-	2.0	+	1	2	0	+/+	+/-	-/+	+/+	-/+	+/-	-	-
29	F	20	14	1.0	PC	+	-	+	-	6.0	+	0	1	2	+/-	+/-	-/+	+/+	-/-	+/-	ND	ND
30	F	49	4	2.2	PC	-	-	+	-	3.9	+	0	1	0	+/-	+/-	-/+	+/+	-/-	+/-	-	-
31	F	43	10	4.0	PC	+	+	+	-	0.1	+	0	1	0	+/-	+/-	-/+	+/+	-/-	+/-	-	-
32	F	43	10	1.5	PC	+	+	+	-	4.3	+	1	3	1	+/-	+/-	-/+	+/+	+/-	+/-	V600E	-
33[a,b]	M	63	7	1.8	PC	-	-	+	-	0.0	+	1	3	1	+/-	+/-	-/+	+/+	-/+	+/-	-	-

M male, *F* female, + presence, - absence, *WC* well circumscribed, *PC* poorly circumscribed, *EE* extra-thyroid extension, *I-LVD* Intratumoural lymph vessels density, *LNM* lymph node metastases, *C* expression at the center of the tumour, *P* expression at the tumour periphery, *CT* expression at the control tissue, *n* nuclear expression, *c* cytoplasmatic expression, *ND* not determined. [a] Cases with concurrent distant metastases, [b] Cases with recurrence(s).

IV.5.1 Correlation between the results of the molecular analysis and the clinico-pathological data, including the lymph vessels profile

Overall, the *BRAF* V600E mutation occurred in 19 out of 65 PTC cases (29.2%) and the *N-RAS* Q61R mutation occurred in 4 out of 65 PTC tumours (6.2%).

The frequency of *BRAF* mutation in CPTC group was significantly higher than in FVPTC group (41.2% and 16.1%, respectively)(p=0.032), and occurred in 13 out of 42 PTC-**M** tumours (31.0%) and in 6 out of 23 PTC+**M** tumours (26.1%). The *N-RAS* mutation occurred only in PTC-**M** tumour (**Tables IV.15** and **IV.16**).

In the CPTC group, 9 out of 18 CPTC-**M** (50.0%) and 5 out of 16 CPTC+**M** (31.3%) displayed *BRAF* mutation. The *BRAF* V600E mutation was detected in one case of E-FVPTC (6.7%) and four cases of PC-FVPTC (25.0%) (**Table IV.16** and **Figure IV.5**). The *N-RAS* mutation was detected in 1 out of 34 CPTC tumours (2.9%); it was a Q61R mutation in a well circumscribed encapsulated CPTC-**M** tumour (1 out of 18 – 5.6%) with a concurrent *BRAF* V600E mutation.

In the FVPTC group, the *BRAF* V600E mutation was detected in four FVPTC-**M** (16.7%) and one FVPTC+**M** (14.3%). *N-RAS* Q61R mutation was detected in 3 out of 31 cases (9.7%) all of which were

FVPTC-**M** tumours (3 out of 24 – 12.5%). The *N-RAS* Q61R mutation was detected in two cases of E-FVPTC (13.3%) and one case of PC-FVPTC (6.3%) (**Tables IV.15** and **IV.16; Figure IV.5**).

The presence of *BRAF* mutation in CPTC was significantly associated to female gender (p=0.031)(14 females and 0 males) (**Table IV.17**). In FVPTC cases, the mean intratumoural LVD was significantly lower in E-FVPTC cases with wild type *N-RAS* (p=0.016). Case 6 of the E-FVPTC subgroup was the only one with D2-40 positive intratumoural lymph vessels and was a *N-RAS* mutated tumour that did not show signs of extra-thyroid extension, lymph vessel invasion or vascular invasion, nor had given rise to lymph node metastases (**Table IV.16**).

The presence of *BRAF* and *N-RAS* mutation was not significantly associated to any other clinical or morphological feature (**Tables IV.15, IV.16** and **IV.17**).

Table IV.17 Comparison of molecular features of CPTC and FVPTC cases

PTC Variant	Features	*BRAF* Wild type	*BRAF* V600E	*p* value
CPTC	Gender (female:male)	14:6	14:0	**0.031**
	Age, years(mean±sd)	35.2±19.0	40.5±16.9	0.41
	Tumour size, cm (mean±sd)	2.2±1.2	2.7±1.9	0.294
	Number of tumours (>1cm/total)	0.90	0.86	>0.999
	Capsule (absence/total)	0.70	0.57	0.487
	Capsule invasion (presence/total)	1.00	0.83	>0.999
	Growth pattern (poorly circumscribed/total)	0.90	0.79	0.627
	Multicentricity (presence/total)	0.55	0.29	0.079
	Extra-thyroid extension (presence/total)	0.35	0.29	>0.999
	Vascular invasion (presence /total)	0.35	0.33	>0.999
	Lymph vessels invasion (presence/total)	0.44	0.27	0.448
	Intratumoural LVD (mean±sd)	0.9±1.0	0.8±1.5	0.771
	Lymph node metastases (presence/total)	0.55	0.36	0.315
	pT (T3+T4/total)	0.35	0.36	>0.999
	Stage III+IV/total	0.15	0.29	0.410
FVPTC	Gender (Female:Male)	21:5	4:1	>0.999
	Age, years (mean±sd)	43.5±12.5	44.8±14.8	0.837
	Tumour size,cm (mean±sd)	2.4±1.9	1.9±1.3	0.576
	Number of tumours (>1cm/total)	0.62	0.80	>0.999
	Capsule (absence/total)	0.73	0.94	0.172
	Capsule invasion (presence/total)	0.73	1.00	>0.999
	Growth pattern (poorly circumscribed/total)	0.46	0.80	0.333
	Multicentricity (presence/total)	0.35	0.60	0.350
	Extra-thyroid extension (presence/total)	0.23	0.20	>0.999
	Vascular invasion (presence /total)	0.50	0.80	0.345
	Lymph vessels invasion (presence/total)	0.13	0.20	>0.999
	Intratumoural LVD (mean±sd)	0.8±1.5	1.0±1.8	0.778
	Lymph node metastases (presence/total)	0.23	0.20	>0.999
	pT (T3+T4/total)	0.15	0.20	>0.999
	Stage III+IV/total	0.12	0.00	>0.999
CPTC + FVPTC	Gender (Female:Male)	35:11	18:1	0.093
	Age, years (mean±sd)	39.9±16.0	41.6±16.1	0.689
	Tumour size, cm (mean±sd)	2.3±1.6	2.5±1.7	0.647
	Number of tumours (>1cm/total)	0.80	0.84	0.740
	Capsule (absence/total)	0.54	0.63	0.589
	Capsule invasion (presence/total)	0.81	0.86	>0.999
	Growth pattern (poorly circumscribed/total)	0.65	0.79	0.379
	Multicentricity (presence/total)	0.43	0.32	0.418
	Extra-thyroid extension (presence/total)	0.33	0.32	>0.999
	Vascular invasion (presence /total)	0.43	0.47	0.791
	Lymph vessels invasion (presence/total)	0.26	0.25	>0.999
	Intratumoural LVD (mean±sd)	0.8±1.3	0.9±1.6	0.993
	Lymph node metastases (presence/total)	0.37	0.32	0.780
	pT (T3+T4/total)	0.24	0.32	0.547
	Stage III+IV/total	0.13	0.21	0.461

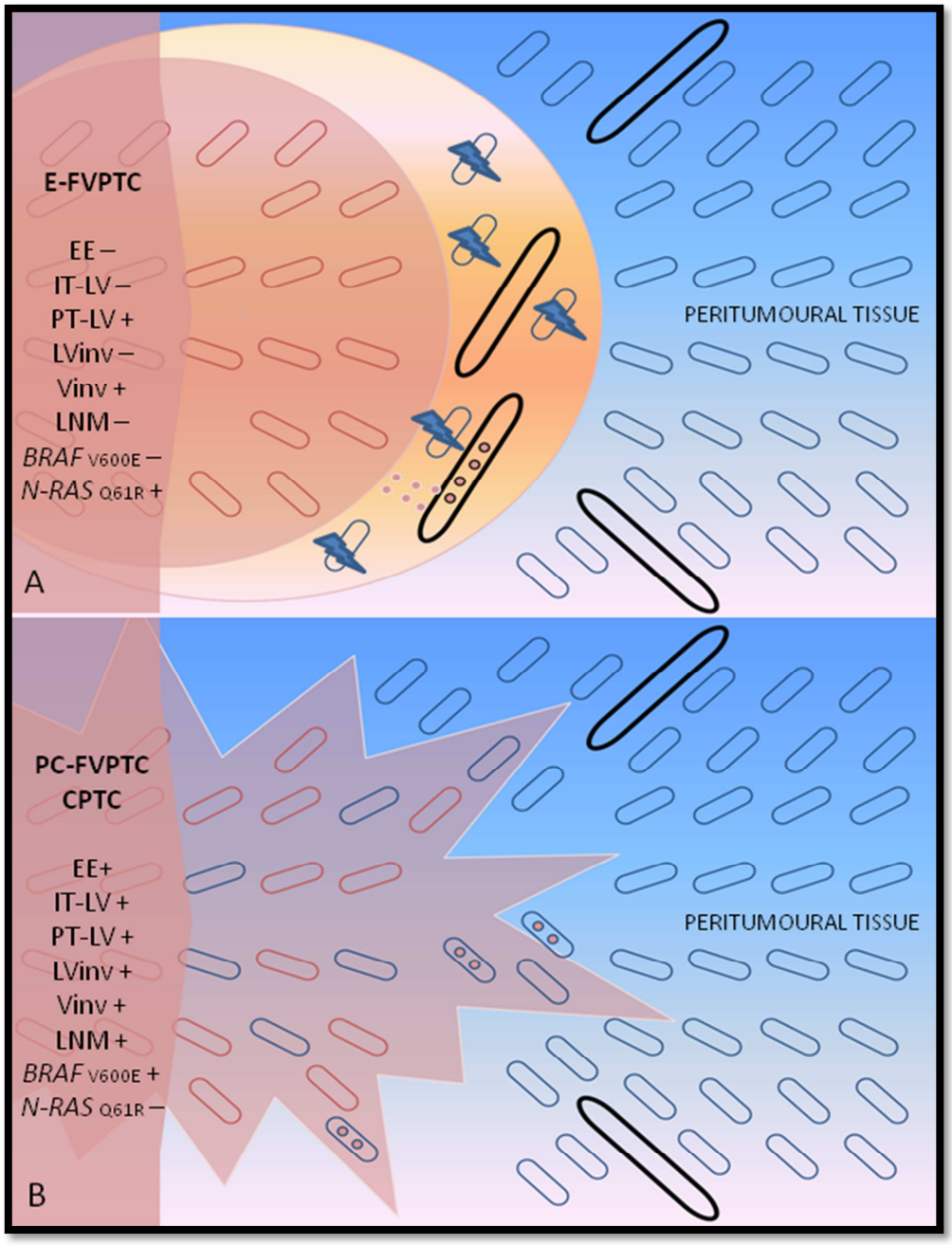


Figure IV.5

Summary of the most impressive findings concerning the distinction between E-FVPTC (A) and PC-FVPTC+CPTC (B).
Red vessels – D2-40 negative intratumoural thin walled vessels; Blue vessels – D2-40 positive intratumoural and peritumoural lymph vessels; Black vessels – thick walled veins; Blue thunder over blue vessel – collapsed D2-40 positive lymph vessel; Pink spots – neoplastic thrombi; EE – extra-thyroid extension; IT-LV – D2-40 positive intratumoural lymph vessels; PT-LV – peritumoural lymph vessels; LVinv – lymph vessel invasion; Vinv – vascular invasion, LNM – lymph node metastasis).

IV.5.2 Correlation between the results of the molecular analysis and the immunohistochemical expression of elements of TGF-beta/Smad-dependent pathway

The *BRAF* V600E mutation was detected in 20.0% of WC-PTC including 4 WC-PTC cases disclosing similar or higher TGF-beta expression in the periphery than in the center, while none of the 3 WC-PTCs with a lower TGF-beta expression in the periphery than in the center were *BRAF* mutated tumours. The group of WC-PTC cases with similar or higher TGF-beta expression at the periphery than in the center had similar clinico-pathological features and no lymph node metastases, independently of the *BRAF* mutational *status* (**Tables IV.15 and IV.16**).
The *BRAF* mutation was detected in 15 PC-PTCs (33.3%) disclosing similar or higher TGF-beta expression in the periphery than in the center. PC-PTC cases with similar or higher TGF-beta expression at the periphery than in the center disclosed lymph node metastases in 40.0% of *BRAF* mutated tumours and in 56.7% of tumours with wild-type *BRAF* (**Tables IV.15 and IV.16**).

The **Table IV.18** summarizes the result of the molecular analysis and the immunohistochemical expression of elements of TGF-beta/Smad-dependent pathway in CPTC and FVPTC.

Table IV.18 Comparison between the immunohistochemical patterns of expression of the elements of TGF-beta/Smad-dependent pathway and the PTC molecular profile.

	Features		Increment in TGF-beta intensity of expression (mean±sd)				Underexpression or basal cytoplasmatic expression (%)	
			C-CT	*p*	P-C	*p*	P-Smad2/3	*p*
CPTC+FVPTC	***BRAF***	V600E	0.2±1.2	0.295	0.8±0.8	**0.029**	22.2	**0.053**
		Wild type	0.5±1.0		0.4±0.7		50.0	
	N-RAS	Q61R	0.8±1.0	0.532	0.5±0.6	0.965	75.0	0.302
		Wild type	0.5±1.0		0.5±0.7		40.0	
CPTC	***BRAF***	V600E	0.3±1.3	0.562	0.8±0.8	**0.048**	23.1	>0.999
		Wild type	0.6±1.1		0.4±0.6		25.0	
	N-RAS	Q61R	0.0	0.694	1.0	0.525	100.0	0.242
		Wild type	0.5±1.2		0.5±0.7		21.9	
FVPTC	***BRAF***	V600E	0.0±0.7	0.255	0.8±0.8	0.324	20.0	**0.060**
		Wild type	0.5±0.9		0.4±0.8		69.2	
	N-RAS	Q61R	1.0±1.0	0.239	0.3±0.6	0.720	66.7	>0.999
		Wild type	0.4±0.9		0.5±0.8		60.7	

C expression at the center of the tumour, *P* expression at the tumour periphery, *CT* expression at the control tissue.

The mean intensity of TGF-beta expression at the periphery, compared with the respective center of the tumours, was significantly higher in *BRAF* mutated PTCs when CPTC and FVPTC are considered together (p=0.029) or when the comparison is restricted to CPTC (p=0.048) (**Table IV.18**).
Tumours with basal or underexpression of P-Smad2/3 cytoplasmatic expression tended to have more often wild type *BRAF* than mutated *BRAF* (p=0.053) (**Table IV.18**). The same trend was observed when this comparison was restricted to FVPTC (p=0.060) (**Table IV.18**).
BRAF V600E mutation and *N-RAS* Q61R mutation were not significantly associated to Smad4 or Smad7 expression in CPTC nor in FVPTC (**Tables IV.15 and IV.16**).

V Discussion

As in other worldwide centers [41, 167-169], the number of PTC diagnosis recorded in the Cancer Registry of the Pathology Department of Centro Hospitalar de São João (Porto, North of Portugal) has increased over the last 29 years encompassing both small and large tumours, probably reflecting not only a real increment in the number of PTC cases but also changes in health care guidelines/politics. Such changes, like the widespread use of thyroid ultrasound, the preoperative cytological diagnosis and the more extensive sampling of the surgical specimens during pathological examination, appear to have contributed to the detection of asymptomatic and small PTCs [12], as expressed in the trend towards decreased mean tumour size of PTCs archived in our Cancer Registry.

At variance with the increasing incidence of thyroid cancer, the mortality due to these tumours has decreased [12]. In our files, the large majority of PTC follows an indolent clinical behaviour and the rates of recurrence and distant metastases are globally low in comparison with other series on record [56]. These low rates probably reflect a number of facts: deficient information on the follow up of the patients, increasing number of incidentally found small and/or very small tumours and inclusion in the group of carcinomas of the encapsulated form of PTC previously diagnosed as follicular adenoma.

The identification of areas with peculiar features topographically associated to PTC or the identification of metastasis into preexisting PTC appear to constitute unusual settings with putative impact in the patients' outcome that justify the systematic need for an appropriate sampling of the tumours. In our series, as in others, the coexistence of well differentiated PTC with areas of neoplastic progression towards poorly differentiated carcinoma was associated to a poorer outcome in comparison to strictly well differentiated PTCs [62, 67]. Conversely, the coexistence of PTC and basaloid neoplasms represents a rare finding with unknown consequences to the natural history of PTC. The two reported cases of basaloid neoplasms associated to PTC expressed diffusely p63, like the main cells of solid cell nests and, in one case, the basaloid neoplasm shared with the PTC component the same *N-RAS* mutation. The morphological and immunophenotypic features of, at least, such basaloid neoplasms (Eloy et al, unpublished observations) are similar to those observed in the solid cell nests. Solid cell nests can occur in topographic continuity with papillary microcarcinoma sharing with it a *BRAF* molecular alteration [169], conveying the hypothesis of a histogenetic relationship between solid cell nests and PTC (*see **Papers 6** and **7** for further discussion*).

Besides some clinically aggressive cases of PTC with or without coexistent particular histopathologic components, our files also record strictly well differentiated PTCs (more frequently CPTC than FVPTC cases) displaying signs of clinico-pathological aggressiveness, namely lymph node metastases.

Predictive factors of PTC metastatic capacity and of local aggressive behaviour still remain incompletely established.

In the study of our series of 75 PTCs (discussed from here foreword) we found that, both in CPTC and in FVPTC, extra-thyroid extension and poorly circumscribed growth pattern were the clinical and morphological features most closely associated to lymph node metastases. In fact, all the PTCs metastatic to the lymph nodes were poorly circumscribed, whereas all well circumscribed PTCs did not give rise to lymph node metastases.

In the CPTC group, multicentricity was significantly associated to lymph node metastases, particularly in subgroup of poorly circumscribed tumours. In the poorly circumscribed tumours of the FVPTC group, extra-thyroid extension was the most important feature associated to lymph node metastases. In FVPTC cases, vascular invasion and mitotic index were also significantly associated to lymph node metastases, although the latter may reflect the existence of a single case with 6 mitoses *per* 10 high power fields.

FVPTC tumours measuring 1cm or less and arising in a gland with benign pathology other than thyroiditis were less often associated to lymph node metastases than solitary FVPTC. This difference probably reflects our biased sample of small tumours that was composed by incidentally found PTCs in patients operated for benign pathology (nodular goiter or FA). In our series, this difference was also observed in tumours larger than 1cm. The low frequency of lymph node metastases from PTC arising together with concurrent benign pathology other than thyroiditis has not been reported before to the best of our knowledge, and needs confirmation in larger series.

Our results support the assumption that encapsulated, non angio-invasive FVPTC carry an excellent prognosis even when the patients underwent partial thyroidectomy [27, 28, 45, 60, 170] (*see **Paper 4***). The emerging importance of the growth pattern of PTC on the outcome of the patients justifies the need for a complete sampling of the periphery of the tumours during the pathological examination of the surgical specimen, as it is recommended in the setting of encapsulated follicular tumours [12].

The peculiar metastatic properties of the FVPTC subtypes (E-FVPTC and PC-FVPTC) are not fully understood and may be related to lymphangiogenesis or, at least, to the abundance of lymph vessels in both settings. The search for intratumoural lymph vessels disclosed a clear cut difference between E-FVPTC and PC-FVPTC and CPTC. D2-40 positive lymph vessels were not detected within 11 out of 12 tumours of the E-FVPTC group, a result that fits with the absence of lymph vessel invasion and lymph node metastases in E-FVPTC. A very low frequency of identifiable intratumoural lymph vessels had been previously reported by other groups in FA [85, 90, 103] and FTC [85, 103].

The rarity of extra-thyroid extension, intratumoural lymph vessels, lymph vessel invasion and lymph node metastases in E-FVPTC, together with the differences on the molecular features i.e. the scarcity of *BRAF* V600E mutation [26] and prominence of *RAS* mutations [26], also confirmed in the present study (*see below*), contributes to separate E-FVPTC from PC-FVPTC and to approach the former to minimally invasive FTC [75, 84] (*see **Papers 4** and **5** for further discussion*). It remains to be clarified whether the absence of intratumoural D2-40 positive lymph vessels in most E-FVPTCs, as well as in other follicular patterned tumours, reflects a low capacity of incorporation (with or without proliferation) of lymphatic vessels since the first steps of tumourigenesis, or their disappearance by a collapsing mechanism (or other mechanism) secondary to the increased intratumoural pressure that may occur during the neoplastic development of encapsulated tumours [100].

The results we have obtained contribute also to show that PC-FVPTC cases share most of the features of CPTC cases [75] (*see **Paper 4***). This is particularly obvious regarding the poorly circumscribed peripheral growth pattern which is frequently or occasionally accompanied by vascular invasion and extra-thyroid extension, occurrence of *BRAF* V600E mutation (*see below*), abundance of intratumoural lymphatic vessels, lymph vessel invasion and lymph node metastases.

The significant association we observed between intratumoural LVD and occurrence of lymph node metastases confirms the data reported by several groups [91, 93, 94, 104] and contradicts others [85, 90]. In our series, lymph vessel invasion appears to be the second strongest factor associated with the occurrence of lymph node metastases after extra-thyroid extension. The relation between high intratumoural LVD determined by D2-40 and lymph node metastases is indirectly confirmed by the absence of nodal metastasis in E-FVPTC cases that do not have intratumoural lymphatic vessels despite the abundance of peritumoural lymph vessels.

The abundance of D2-40 positive peritumoural lymph vessels was similar in the three groups of PTC (E-FVPTC, PC-FVPTC and CPTC) as it had been previously reported by Giorgadze *et al* [103] in a series of follicular patterned lesions. Peritumoural LVD was not significantly related to any clinico-pathological feature, namely the occurrence of lymph vessel invasion or lymph node metastasis, thus supporting the results of Garcia *et al* [90]. The mean peritumoural LVD determined by D2-40 expression was higher than intratumoural LVD as previously reported [90] and was similar in all groups of PTC, thus implying that such feature does not appear to be modulated by the tumour histotype.

In our series, as in others [104], all lymph vessels disclosing neoplastic thrombi in their lumina expressed D2-40, suggesting that the intratumoural D2-40 negative vessels may constitute less efficient pathways for PTC dissemination. It remains to be clarified if the D2-40 expression in such cases is induced by the presence of the neoplastic thrombi or if intratumoural D2-40 vessels, mainly located at the invasive front, as observed by Hall *et al* [104] is the result of the invasion of peritumoural tissues with subsequent incorporation of the originally peritumoural D2-40 positive lymph vessels in the bulk of the tumour. The latter hypothesis is favoured by the association we found between extra-thyroid extension and intratumoural high LVD. In this scenario, the absence of an invasive front, as well as the presence of a well constituted capsule, could prevent the recruitment of D2-40 positive peritumoural lymph vessels and their subsequent invasion by the neoplastic cells, as suggested by the nearly absence of D2-40 positive intratumoural lymph vessels in E-FVPTC of our series.

The prominence of peritumoural small sized veins, including capsular veins presenting luminal neoplastic trombi, point to the role played by vascular (venous) invasion. Vascular (venous) invasion was observed in 26 out of 61 cases of PTC, but in such cases the invaded veins did not express D2-40 in the endothelial cells, arguing against the hypothesis that D2-40 expression may be induced in endothelial cells by neoplastic thrombi. The neoplastic thrombi seen in the thick walled veins may result from invasion of small intratumoural vessels or from the direct invasion of such thick walled blood vessels which present a well-developed muscle layer probably able to resist the effect of the intratumoural pressure over the vessels of the

capsule [100, 171]. The increment in the intratumoural interstitial pressure is thought to be partially due to the functionally impaired lymphatic system in tumours, thus leading to subsequent problems of fluids and macromolecules [172]. In our series, the number of cases with distant metastases (n=3) is too low (data not shown) to allow the clarification of the role played by D2-40 positive and negative vessels in the occurrence of blood borne metastasization.

Our findings highlight the role played by intratumoural lymphangiogenesis in lymph node metastization at variance with the well acknoledged importance of intratumoural angiogenesis in the metastatic potential of FTC and PTC [85, 87, 97-99]. While in CPTC the most important pathway of initial dissemination appears to be *via* intratumoural lymph vessels, in encapsulated follicular patterned tumours the invasion *via* veins of the capsule or outside the tumour is aparantly necessary to give rise to blood borne metastasis [1, 82].

Our study highlights the role of intratumoural lymph vessels in PTC nodal metastization and reinforces the importance of distinguishing E-FVPTC from PC-FVPTC regarding invasiveness.

The tumour cells' invasiveness is a feature related to the EMT that develops at the periphery of the tumours, in their invasive front [105-107]. It is known that the neoplastic cells at the tumour periphery differ substantially from those in the bulk of the tumours regarding the expression of several structural and functional proteins (e.g. cytokeratins, vimentin, beta-catenin, E-cadherin, fibronectin 1, TGF-beta) [105, 107,108,111,119].

The role of TGF-beta in thyroid cell signaling and in thyroid carcinogenesis, like in many other models, is very complex and profoundly influenced by the cellular and tissue context [119], thus being better evaluated in tissue samples than in cell lines. In the present study, we observed signs of the dual role of TGF-beta regarding the co-existence in the same tumour of signs of activation and of inhibition of TGF-beta/Smad-dependent pathway according to the location of the neoplastic cells.

The tumour suppressive effect of TGF-beta/Smad-dependent pathway that had been firmly established in thyroid cell lines [126-130] and in some PTC cell lines [118,121], was found to be abrogated at the periphery of PTC [105, 119] where increased vimentin expression and loss of E-cadherin with increased motility and invasiveness configure the EMT invasive phenotype [107]. We observed, particularly in the poorly circumscribed tumours, that TGF-beta expression was stronger in the invasive front than in the central regions of the tumours, and that an increased TGF-beta expression at the periphery of the tumours was associated to the occurrence of extra-thyroid extension and lymph node metastases, as previously reported by Riesco-Eizaguirre *et al* [119].

Our subdivision of PTCs into well and poorly circumscribed carcinomas (WC-PTC and PC-PTC, respectively) goes further than the usual division into CPTC and FVPTC and provides interesting results: in WC-PTC (no infiltrative features and no lymph node metastases) the mean increment of TGF-beta immunoexpression from the center to the periphery was very low in comparison with PC-PTC, in line with the above mentioned association between TGF-beta expression at the periphery and invasiveness, also reported by other authors [119].

We detected TGF-beta and P-Smad2/3 expressions in the center of all cases of WC-PTC and PC-PTC and control tissues (except in nine TGF-beta negative cases), as it had been reported previously [131, 133, 135, 136], thus highlighting the existence of signs of functioning TGF-beta/Smad-dependent pathway in PTC.

Smad4 nuclear expression is considered as indicative of functioning TGF-beta/Smad-dependent pathway [133,137,139] but the nuclear translocation of phosphorylated Smad2 in response to TGF-beta, does not require the presence of Smad4 [123], as our results also indicate. In the present study, all tumours expressed Smad4 in the cytoplasm of the neoplastic cells, like it had been observed by Matsuo *et al* [133] and Cerutti *et al* [137], whereas Smad4 nuclear expression was detected in 27% of tumours. In WC-PTC, Smad4 nuclear expression was detected only in cases without vascular invasion. The nuclear expression of Smad4 in PTC cells had not been detected previously to the best of our knowledge. The percentage of cases with Smad4 nuclear expression may be related with an impaired nuclear translocation of Smad2/3-Smad4 complexes as a sort of end result of Smad4 mutations that were detected in another series in 21% of PTCs [173]. Unfortunately, the absence of available material for the molecular search of Smad4 mutations in the present series did not allow the confirmation or the rejection of this hypothesis. Whatever the mechanisms involved in the process, we found that signs of functioning TGF-beta/Smad-dependent pathway in the center of the tumours, namely basal or overexpression of TGF-beta and nuclear Smad4, were associated to low vascular invasiveness and absence of lymph node metastases, respectively.

Smad7 nuclear expression has been associated to loss of TGF-beta/Smad-dependent pathway inhibition [174] and was found to be at basal or overexpressed levels in 72% of the cases of the present series, a percentage similar to that reported in another series of PTC [133]. We found that Smad7 nuclear expression was associated to low neoplastic invasiveness, namely well circumscribed growth pattern and unicentricity in CPTC, and to absence of extra-thyroid extension and vascular invasion in FVPTC. More importantly, Smad7 nuclear expression was associated to absence of lymph node metastases both in CPTC and FVPTC.

The encapsulated, non-angio-invasive FVPTC is the prototype of a carcinoma with an extremely benign course [60]. In the present series, and in almost every series on record, most WC-PTCs display a capsule and are apparently protected from the cascade of TGF-beta oncogenic effects occurring at the periphery of PC-PTC. The WC-PTC that do not disclose invasive front nor EMT features (independently of the occurrence of *BRAF* mutation), nor lymph node metastases, often present a nearly homogeneous TGF-beta expression throughout the tumour.

At variance with TGF-beta, *BRAF* mutation has been found to promote growth and cell invasiveness [52].In one animal model of PTC, the oncogenic *BRAF* was found to cooperate with TGF-beta in the induction of migration and invasion, disrupting the suppressive effects of TGF-beta/Smad-dependent signaling pathway [122]. Basolo *et al* [59] have shown that in small PTCs there is a significant association between the presence of *BRAF* mutation and tumour invasiveness and nodal metastization [59]. In fact, besides the recognized putative contribution of the detection of *BRAF* mutation to the preoperative diagnosis of PTC and the emerging evidence of its influence over the PTC response to radioiodine therapy [161], the data on *BRAF* mutated cell lines and experimental models, as well as on the observational data on human PTC, do not prove that *BRAF* mutation *per se* is a major prognostic factor, remaining to be clarified how does *BRAF* mutation increases tumour cells invasiveness. Moreover, the association between *BRAF* mutation and some pathological features with clinical significance are incompletely established [27, 52, 56, 58, 74, 149-154, 159].

In our series, *BRAF* V600E mutation was less frequently detected than in most series on record [26, 27, 32, 48, 52, 56, 58, 74, 147-153]. It was significantly more frequent in CPTC than in FVPTC cases, an observation consistent with the results of Trovisco *et al* [74], as well as those of many other series [52, 151]. The *BRAF* V600E mutation was detected in 7% of WC-FVPTC and 25% of PC-FVPTC.

At variance with the results reported by others, we did not observe in our series a significant association between *BRAF* mutation and poor circumscription [26, 154], tumour size above 1cm [153], extra-thyroid extension [52, 154, 159], vascular invasion [56] and lymph node metastases [58, 149, 154]. In the present series, the occurrence of *BRAF* mutation was not also significantly associated with intratumoural LVD. None of the 3 patients of our series with distant metastases had a *BRAF* mutated tumour in contrast with the results of Namba *et al* [150].

In our series there was not a significant association between the presence of *BRAF* mutation and lymph node metastases. This result fits with those of some studies on record [74, 147, 151] and contradicts others [56, 58, 154]. Instead, *BRAF* mutation was strongly associated to the CPTC group, whose cases were more often poorly circumscribed than FVPTC cases. Within each group, no additional significant associations to other morphological features related to aggressiveness were found.

BRAF V600E mutated CPTCs were significantly more often found in females than in males, in contrast with the previously observed higher frequency in males [152] or similar frequency in both genders [25, 60, 74, 151].

Although the mean age of PTC patients with and without the *BRAF* V600E mutation was not significantly different in CPTC and FVPTC groups, we did not find a single patient younger than 20 years with a mutated *BRAF* tumour, thus confirming that *BRAF* mutation is rarely present in PTCs of young patients [52, 56, 74]. In most series, *BRAF* mutated PTCs tend to occur in older patients than PTCs without the *BRAF* mutation and to be rarely found in children or young adults. [56, 74] The inverse holds true for *RET/PTC* rearrangements which are frequently detected in PTC occurring in children and adolescents [175, 176].

At variance with *RET/PTC* rearrangements that can be detected in cases of nodular goiters/follicular adenomas and Hashimoto thyroiditis displaying large, clear and irregular nuclei [36], *BRAF* mutations have not been detected in benign lesions of the thyroid [177] except in exceedingly rare cases [74, 148]. The reason for the absence of *BRAF* V600E mutation in benign lesions and in PTCs of children and adolescents remains unknown. It is tempting to speculate that *BRAF* mutation *per se* may induce senescence in thyroid cells as it has been observed in benign melanocytic lesions [178, 179]. Following this hypothesis, the

tumourigenic effect of *BRAF* mutation would only be seen whenever the mutation is accompanied by synergic mutations and/or epigenetic changes in other pathways [123, 179]. We are advancing that the *BRAF* mutation may be tumourigenic for the human thyroid only in relatively late stages of PTC development (in normal thyroid and in early neoplastic stages the mutation might induce a senescent phenotype). The results recently published by Franco *et al* [180] in a mouse model support the hypothesis that such synergies may be obtained in early stages of the neoplastic development and can be mediated by cooperation with the thyrotrophin receptor signaling pathway [180]. *In vitro* and *in vivo* studies are necessary to test the aforementioned hypotheses that are also important from a prognostic standpoint.

The putative relationship between alterations of the TGF-beta/Smad-dependent pathway and *BRAF* mutation is difficult to evaluate in an observational study of human tumours like the present one. Despite acknowledging this limitation we think our findings suggest the existence of a synergic influence of TGF-beta over *BRAF* mutated neoplastic cells in the periphery of the tumours.

BRAF V600E mutation was more frequently detected in the subset of tumours with higher increment of TGF-beta expression at the periphery. This finding concurs with the results of Riesco-Eizaguirre *et al* [119] and suggests that, at the periphery of the tumours, the association of high levels of TGF-beta expression with *BRAF* mutation and invasiveness, as previously described in animal models [122], PTC cell lines and PTCs [119], reflects a synergic interaction that may potentiate the oncogenic effect of *BRAF* mutation. In the present series, the occurrence of *BRAF* mutation did not significantly alter, *per se*, the frequency of lymph node metastases, thus highlighting the complexity of the process and reinforcing the importance of growth pattern and invasion for the end result of the neoplastic development process.

Considering that the increased expression of TGF-beta at the periphery of the tumours may functionally articulate with *BRAF* V600E mutation [122], it remains unclear the reason underlying this particular topographic immunoexpression of TGF-beta. Since *BRAF* mutation occurs, at least in most *BRAF* positive cases, in every tumour cell, the TGF-beta expression increment should be detected, in case it was a direct result of the *BRAF* mutation, throughout the whole neoplastic tissue. It is likely that this increment of TGF-beta at the periphery of the tumours is influenced by the crosstalk between the neoplastic cells and cellular and non-cellular stromal elements, such as tumour-associated macrophages [117, 122], dendritic cells [115], lymphocytes [116] and/or basement membrane components [122]. A similar interaction with *BRAF* has been recently described with STAT3 which is highly expressed in the periphery of *BRAF* mutated PTCs and has been found to result from the crosstalk between neoplastic cells and stromal elements [Couto *et al*, unpublished results]. The aforementioned need of co-existing factors in the setting of *BRAF* oncogenic mutation provides an explanation for the low prevalence of *BRAF* positive PTCs in young patients [74], even if one assume that *BRAF* mutation may be an initiating event.

In our series we also observed that FVPTCs with basal or underexpression of P-Smad2/3 tended to be more often *BRAF* mutated carcinomas, although the low number of *BRAF* mutated FVPTCs weakens any conclusions on these results.

Other interactions between TGF-beta signaling pathways and MAPK pathway have been advanced, namely through oncogenic *RAS* that is thought to be required for the disruption of TGF-beta-growth suppressive effects in cell lines [60, 174, 181]. In our series, the *N-RAS* mutation was detected in 6% of the tumours, a prevalence that fits within the interval of previously reported data in other PTC series [25, 26, 31, 48, 83, 148, 162, 163]. The *N-RAS* mutation was detected in the single case of the E-FVPTC group with intratumoural lymph vessels, but it does not seem appropriate to draw any conclusions on the putative relationship between *N-RAS* mutation and lymphangiogenesis from an anecdotic case. We found no significant association of *N-RAS* Q61R mutation with any other clinical or morphological feature, but these negative results may reflect the low number of mutated cases in our series.

In one case (case 14 of the CPTC group) there was coexistence of *BRAF* and *N-RAS* mutation demonstrating that, although rarely, *BRAF* and *RAS* mutations can coexist in the same tumour [148]. It remains to be verified if the two mutations coexist in the same cells or correspond to distinct clones, as demonstrated in melanomas [182].

Our results confirm the prognostic prominence of staging over cytological and molecular features, and the major importance of architecture (encapsulated *versus* infiltrative growth pattern) and topography (different immunohistochemical characteristics of tumour cells according to their peripheral or central location) in the understanding of PTC development.

Taking into consideration all the data we have obtained we conclude that nodal metastases are associated to poorly circumscribed, locally invasive PTCs with high intratumoural lymph vessel density; such metastatic PTCs exhibit low levels of nuclear Smad7 and a peripheral EMT phenotype displaying TGF-beta overexpression, regardless of the occurrence of *BRAF* mutation.

The main conclusions of the Thesis, according to the proposed Aims, are the following:

Structural aim To review all cases of thyroid tumours filed in the Cancer Registry of the Pathology Department of Centro Hospitalar de São João

1. **Selection of a representative series of PTC cases.**

    - A series of 75 PTC cases was selected and considered representative of PTCs in the North of Portugal. We think that such cases are also representative of the general tendencies observed in other series on record. Despite this, we cannot rule out the possibility that our results and conclusions may reflect some uncontrolled geographic, institutional and/or sampling biases.

2. **Identification of problems in thyroid cancer diagnosis and prognosis that should be approached in future studies.** ***A particular interest was devoted to the neoplastic components coexistent with PTC with influence over the patients' outcome.***

    - In the group of thyroid tumours displaying lymph node and/or blood borne metastases there was not a single case of well differentiated tumour of uncertain malignant potential, neither any case of minimally invasive, non angio-invasive follicular thyroid carcinoma, nor any case of encapsulated, non invasive follicular variant of PTC. In the future, studies regarding the role of the tumour capsule and the clarification of the malignant potential of encapsulated PTCs should be carried out.

    - The identification of poorly differentiated areas in PTCs and the identification of metastases over preexisting thyroid tumours constitute unusual settings with negative impact over the patients´ outcome. In the future, an attempt should be made to separate PTC cases that progress to poorly differentiated carcinomas from those that do not progress. The environmental conditions that favour the occurrence of metastatic disease in thyroid tumours should also be explored.

    - The identification of a basaloid component occasionally displaying solid cell nest features that may coexist with PTC constitutes an unusual finding with unknown impact over the patients' outcome. In the future, an attempt should be made to clarify the putative role of solid cell nests in PTCs neoplastic development.

Specific aims

First specific aim To evaluate the relative contribution of the clinico-pathological features of the tumours and of their molecular profiles to the development of PTC aggressive behaviour.

1. **Clarification of the differential contribution of the clinico-pathological features to the development of lymph node metastases in classic PTC (CPTC) and follicular variant of PTC (FVPTC).** ***The role played by intratumoural and peritumoural lymph vessel density was particularly addressed.***

    - The clinico-pathological features more closely associated to the occurrence of lymph node metastases, both in CPTC and FVPTC, are extra-thyroid extension and poorly circumscribed growth pattern.

    - Encapsulated, non angio-invasive FVPTCs carry an excellent prognosis even when the patients undergo partial thyroidectomy. This and the aforementioned conclusion justify the

recommendation of routine examination of the whole periphery of well circumscribed thyroid nodules with PTC nuclear features.

- Intratumoural lymph vessel density correlates with the occurrence of extra-thyroid extension, lymph vessel invasion and lymph node metastases in PTC.

- Peritumoural lymph vessel density is not significantly associated to any clinico-pathological or molecular feature of PTC.

2. **Clarification of the differential contribution of *BRAF* (V600E) and *N-RAS* (Q61R) mutations to the development of lymph node metastases in CPTC and FVPTC, and of the association of such mutations to other clinico-pathological features.**

    - *BRAF* mutation is more frequent in CPTC than in FVPTC in contrast with *N-RAS* mutation which is more frequent in FVPTC than in CPTC.

    - Our results do not support the existence of an association between *BRAF* mutation and the development of lymph node metastases in CPTC nor in FVPTC.

    - Our results do not also support the existence of an association between *N-RAS* mutation and the development of lymph node metastases in CPTC nor in FVPTC but the series is too small to allow a definitive conclusion on this issue.

    - Our results do not support the existence of an association between *BRAF* mutation and poor circumscription, extra-thyroid extension, tumour size, lymph vessel invasion and vascular invasion. This and the aforementioned conclusion do not support the practical usefulness of preoperative routine detection of the *BRAF* mutation in order to predict an aggressive phenotype.

    - Our results do not support the existence of an association between *N-RAS* mutation and poor circumscription, extra-thyroid extension, tumour size, lymph vessel invasion and vascular invasion. This and the aforementioned results on *N-RAS* do not support the practical usefulness of preoperative routine detection of *N-RAS* mutations regarding the prediction of an aggressive phenotype but, again, the series is too small, with a low number of *N-RAS* mutated cases, to allow a definitive conclusion on this issue.

3. **Clarification of the morphological and molecular basis underlying the differences in the biological and clinical behaviour of encapsulated FVPTC (E-FVPTC) and poorly circumscribed FVPTC (PC-FVPTC).**

    - E-FVPTC differs from PC-FVPTC regarding the rarity of extra-thyroid extension, intratumoural lymph vessels, lymph vessel invasion and lymph node metastases, as well as regarding the scarcity of *BRAF* V600E mutation and prominence of *RAS* mutations; the latter features contribute to approach E-FVPTC to minimally invasive FTC.

    - PC-FVPTC shares most of the features of CPTC, particularly regarding the poorly circumscribed peripheral growth pattern, occasionally accompanied by vascular invasion and extra-thyroid extension, occurrence of *BRAF* V600E mutation, abundance of intratumoural lymphatic vessels, frequent lymph vessel invasion and frequent occurrence of lymph node metastases.

Second specific aim To identify genetic and epigenetic alterations of PTC that may be used as biomarkers of invasion and metastization in the daily practice of diagnostic Anatomic Pathology.

1. **Disclosure of the relationship between the expressions of TGF-beta, phosphorylated Smad2/Smad3, Smad4 and inhibitory Smad7 and some clinico-pathological features associated to PTC aggressiveness.**

    - Lymph node metastases are not detected in well circumscribed PTCs (WC-PTCs) regardless the presence of immunoexpression for TGF-beta, phosphorylated Smad2/Smad3, Smad4 and Smad7.

    - Increased cytoplasmatic expression of TGF-beta at the periphery of poorly circumscribed PTCs (PC-PTCs) is associated to morphological features of invasiveness, featuring the so-called epithelial-to-mesenchymal transition (EMT), and to lymph node metastases.

    - The nuclear expression of Smad7 is more frequent in WC-PTCs than in PC-PTCs and is associated with unicentricity and absence of extra-thyroid extension, vascular invasion and nodal metastases.

    - Smad7 is a marker of favourable evolution that may be routinely used to predict the occurrence of lymph node metastases. Our results indicate that preoperative detection of Smad7 nuclear expression should be evaluated in a forthcoming study as it may constitute a valuable tool for risk stratification of patients with PTC.

    - Smad4 nuclear expression in WC-PTC is detected only in tumours without vascular invasion.

2. **Disclosure of the topographic differences of TGF-beta expression in CPTC and FVPTC cases with and without genetic alterations**.

    - In PTC, there are signs of the dual role of TGF-beta regarding the co-existence, in the same tumour, of signs of activation and inhibition of TGF-beta/Smad-dependent pathway according to the location of the neoplastic cells. TGF-beta levels of expression in the neoplastic cells are related to topography and invasiveness, thus being better evaluated in tissue samples, where epithelial-stromal relations are preserved, than in cell lines.

    - Increased cytoplasmatic expression of TGF-beta in the periphery of PC-PTC is associated with the occurrence of *BRAF* V600E mutation which does not significantly alter, *per se*, the frequency of lymph node metastases, thus highlighting the complexity of the process and reinforcing the prognostic importance of growth pattern and invasion.

    - The role of *BRAF* V600E mutation in PTC prognosis depends upon the synergic interactions with other factors, namely TGF-beta overexpression at the periphery of the tumours, and probably reflects the influence of the crosstalk between the neoplastic cells and cellular and non-cellular stromal elements. The need of co-existing factors interacting with *BRAF* mutation provides an explanation for the rarity of *BRAF* mutated PTCs in young patients.

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VIII Resumo

A incidência do carcinoma papilar da tireoide (CPT) tem aumentado nos últimos anos, enquanto o carcinoma folicular da tireoide (CFT) tem sido cada vez menos diagnosticado em todo o Mundo.
O CPT dá origem, frequentemente, a metástases ganglionares *via* invasão e disseminação através de vasos linfáticos, enquanto o CFT metastiza principalmente *via* vasos sanguíneos para os pulmões e para os ossos. A variante folicular do CPT (VFCPT) compreende o subtipo mal circunscrito/infiltrativo (VFCPT-MC), que partilha a maioria das características do CPT clássico (CPTC), e o subtipo encapsulado (VFCPT-E) que parece estar relacionado com o CFT minimamente invasor.
Os fatores de prognóstico indicadores de agressividade clinico-patológica permanecem incompletamente estabelecidos, em parte devido à diferente constituição das séries publicadas no que se refere à proporção relativa de CPTC e dos subtipos de VFCPT.
Algumas características clinico-patológicas do CPT, tal como a ocorrência de mutações dos genes *BRAF* e *RAS*, ainda não constituem marcadores de agressividade completamente aceites. Por outro lado, foi recentemente descrita uma associação entre a ativação da via Smad-dependente do TGF-beta e invasão local, metastização ganglionar e mutação do *BRAF* em CPT.
No presente estudo clínico-patológico, recolhemos todos os casos de tumores da tireoide (tumores malignos e tumores de potencial maligno incerto) registados no Registo Oncológico do Serviço de Anatomia Patológica do Centro Hospitalar de São João, entre 1978 e 2006, e desse conjunto selecionámos uma série de 75 casos de CPTC e VFCPT, incluindo VFCPT-E e VFCPT-MC, que foram divididos em casos de CPT bem circunscrito (CPT-BC) (n=22) e CPT mal circunscrito (CPT-MC) (n=53), de acordo com as características dos seus limites. Avaliámos as características morfológicas dos tumores, nomeadamente a densidade de vasos linfáticos (DVL) determinada pela expressão de D2-40, a expressão imuno-histoquímica de TGF-beta e dos elementos da via Smad-dependente do TGF-beta (Smad2/Smad3 fosforilado, Smad4 e Smad7), e o estado mutacional do *BRAF* e do *N-RAS* nos tumores, com o objetivo de procurar esclarecer a contribuição destas características para a ocorrência de metástases ganglionares.
O número de casos com o diagnóstico de carcinoma bem diferenciado (CPT e CFT) registados no Registo Oncológico do Serviço de Anatomia Patológica do Centro Hospitalar de São João tem vindo a aumentar ao longo dos anos devido a um aumento do número de casos de CPT, enquanto o número de casos de CFT tem permanecido baixo. Identificámos 1043 casos de CPT que foram subclassificados, de acordo com os critérios da Organização Mundial de Saúde, independentemente do seu tamanho. A VFCPT foi a variante mais frequente (411 casos) seguida do CPTC (377 casos). Dos casos de CPTC, 23% desenvolveram metástases ganglionares, 2% desenvolveram metástases à distância e 6% tiveram recidivas. Dos casos de VFCPT, 3% desenvolveram metástases ganglionares, 1% desenvolveram metástases à distância e 3% tiveram recidivas.
No período de 29 anos (1978 – 2006), identificámos cinco casos de doentes com CPT com componente de carcinoma pouco diferenciado que evoluíram de forma desfavorável, e dois casos de doentes com CPT que coexistia com um componente basaloide, cujo significado clinico a longo prazo permanece desconhecido.
Na nossa série de 75 CPT, a mutação V600E do *BRAF* foi detetada em 29% dos tumores, 41% dos casos de CPTC e 16% dos casos de VFCPT, enquanto a mutação Q61R do *N-RAS* foi detetada em 6% dos tumores, 3% dos casos de CPTC e 10% dos casos de VFCPT. A mutação V600E do *BRAF* foi detetada em 7% dos casos de VFCPT-E e 25% dos casos de VFCPT-MC. A mutação do *BRAF* foi significativamente mais frequente em CPTC e em mulheres, e foi detetada apenas em doentes com mais de 20 anos, sugerindo um efeito tumorigénico tardio no desenvolvimento do CPT. A mutação do *BRAF* não se associou a qualquer das outras características estudadas relacionáveis com agressividade.
As características morfológicas mais relacionadas com a ocorrência de metástases ganglionares foram a extensão extra-tireoideia e o padrão de crescimento mal circunscrito, tanto em CPTC como em VFCPT. Outras características adicionais significativamente associadas a metástases ganglionares foram a multicentricidade no CPTC e a invasão vascular na VFCPT. Nenhum dos casos de VFCPT-E apresentou extensão extra-tireoideia, invasão vascular linfática, nem metástases ganglionares, ao contrário do sucedido com os casos de CPTC e VFCPT-MC. Apenas um caso de VFCPT-E (8%) apresentou vasos linfáticos intratumorais que expressavam D2-40, em contraste com a sua presença em 77% dos casos de VFCPT-MC.
A DVL intratumoral determinada pela expressão de D2-40 correlacionou-se com a ocorrência de extensão extra-tireoideia, invasão vascular linfática e metástases ganglionares no CPT. Em oposição com a DVL intratumoral, a DVL peritumoral não se associou a nenhuma das características clinico-patológicas ou moleculares do CPT, sendo semelhante em CPTC, VFCPT-E e VFCPT-MC.

Não foram detetadas metástases ganglionares em nenhum dos casos de CPT-BC independentemente da presença de expressão de TGF-beta, Smad2/Smad3, Smad4 e Smad7, ou da ocorrência de mutação do *BRAF*.
O aumento da expressão citoplasmática de TGF-beta na periferia dos CPT-MC associou-se a características morfológicas de invasão, configurando a chamada transição epitélio-mesenquimatosa, e à presença de metástases ganglionares, assim como à ocorrência de mutação do *BRAF* cuja presença não altera, por si, a frequência de metástases ganglionares.
A expressão nuclear de Smad7 foi mais frequente em CPT-BC que em CPT-MC e associou-se a unicentricidade e ausência de extensão extra-tireoideia, invasão vascular e metástases ganglionares.
Os nossos resultados demonstram a importância (e a absoluta necessidade) de uma amostragem tão extensa quanto possível (e de uma observação detalhada) da periferia das peças cirúrgicas de CPT. Demonstram também a maior importância do padrão de crescimento infiltrativo das neoplasias e dos sinais de invasão extra-tireoideia em relação à presença de mutações do *BRAF* no desenvolvimento de metástases ganglionares tanto em CPTC como em VFCPT.
Os resultados do perfil de vasos linfáticos em CPT sublinham o papel dos vasos linfáticos intratumorais na metastização ganglionar do CPT e reforçam a importância em distinguir a VFCPT-E da VFCPT-MC no que diz respeito à invasão, padrão metastático e perfil molecular.
Finalmente, concluímos que as metástases ganglionares estão significativamente associadas a CPT mal circunscritos com elevada densidade de vasos linfáticos intratumurais; os CPT com metástases ganglionares têm baixos níveis de expressão nuclear de Smad7 e uma transição epitélio-mesenquimatosa periférica com sobre-expressão de TGF-beta, independentemente da presença de mutação do *BRAF*.

IX Abstract

The incidence of papillary thyroid carcinoma (PTC) has been rising in the last years while follicular thyroid carcinoma (FTC) is being diagnosed less and less frequently everywhere.
PTC gives frequently rise to nodal metastases *via* lymphatic vessels while FTC metastasizes mainly *via* blood vessels to lung and bones. The follicular variant of PTC (FVPTC) encompasses the poorly circumscribed/infiltrative subtype (PC-FVPTC), which shares most of the features of classic PTC (CPTC), and the encapsulated subtype (E-FVPTC) which appears to be related to minimally invasive FTC.
Prognostic factors indicative of PTC clinico-pathological aggressive behaviour remain incompletely established in part due to the different composition of the series on record regarding the relative proportion of CPTC and FVPTC subtypes.
Several clinico-pathological features of PTC, together with the occurrence of *BRAF* and *RAS* mutations, are still not fully accepted as markers of aggressiveness. On the other hand, TGF-beta/Smad-dependent pathway activity has been recently associated with local invasion, nodal metastization and *BRAF* mutated PTCs.
In the present clinico-pathological study we collected all cases of thyroid tumours (malignant tumours and tumours of uncertain malignant potential) from the Cancer Registry of the Pathology Department of Centro Hospitalar de São João, from 1978 to 2006, and selected a series of 75 CPTC and FVPTC cases, including E-FVPTC and PC-FVPTC, which were divided into cases of well circumscribed PTC (WC-PTC) (n=22) and poorly circumscribed PTC (PC-PTC) (n=53), according to the characteristics of their borders. We evaluated the morphological features of the tumours, namely intratumoural and peritumoural lymph vessels density (LVD) determined by D2-40 immunoexpression, the immunoexpression of TGF-beta and TGF-beta/Smad-dependent pathway elements (phosphorylated Smad2/Smad3, Smad4 and Smad7) and the *BRAF* and *N-RAS* mutational *status* of the tumours, in an attempt to clarify the contribution of these features for the occurrence of nodal metastases.
The number of cases with a diagnosis of well differentiated carcinoma (PTC and FTC) recorded in the Cancer Registry of the Pathology Department of Centro Hospitalar de São João has increased over the years due to an increased number of PTC cases, while the number of FTC cases remained persistently low over the years. We identified 1043 PTC cases that were sub-classified, irrespective of size, accordind to the WHO criteria. The FVPTC (n=411) was the most frequent variant followed by CPTC (n=377). From the CPTC cases, 23% developed lymph node metastases, 2% developed distant metastases and 6% had recurrences. From the FVPTC cases, 3% developed lymph node metastases, 1% developed distant metastases and 3% had recurrences.
In the 29 year period (1978 – 2006), we identified five cases of PTC with a poorly differentiated component that followed an aggressive clinical course and two cases of PTC coexisting with a particular basaloid component with unknown impact over the patients' long term outcome.
In our series of 75 PTCs, *BRAF* V600E mutation was detected in 29% of tumours, 41% of CPTC and 16% of FVPTC, whereas *NRAS* Q61R mutation was detected in 6% of tumours, 3% of CPTC and 10% of FVPTC. The *BRAF* V600E mutation was detected in 7% of E-FVPTC and 25% of PC-FVPTC. *BRAF* mutation was significantly more frequent in the CPTC group and in females, and it was detected only in patients older than 20 years, suggesting a late tumourigenic effect in the development of PTC. *BRAF* mutation was not significantly associated to any of the other studied features related to aggressiveness.
The morphological features most closely related to the occurrence of nodal metastases were extra-thyroid extension and poorly circumscribed growth pattern, in both CPTC and FVPTC. Additional features significantly associated to nodal metastases were multicentricity in CPTC and vascular invasion in FVPTC. None of the E-FVPTC cases presented extra-thyroid extension, lymph vessel invasion or nodal metastases, at variance with CPTC and PC-FVPTC cases. Only one case of E-FVPTC (8%) had intratumoural D2-40-stained vessels in contrast to their presence in 77% of the cases of PC-FVPTC. Intratumoural LVD determined by D2-40 expression correlated with the occurrence of extra-thyroid extension, lymph vessel invasion and lymph node metastases in PTC cases. At variance with intratumoural LVD, peritumoural LVD was not associated with any clinico-pathological or molecular feature, being similar in CPTC, E-FVPTC and PC-FVPTC.
Nodal metastases were not detected in any WC-PTC regardless the presence of immunoexpression for TGF-beta, Smad2/Smad3, Smad4 and Smad7, and of the occurrence of *BRAF* mutation.
Increased cytoplasmatic expression of TGF-beta at the periphery of PC-PTC was associated to morphological features of invasiveness, featuring the so-called epithelial-to-mesenchymal transition (EMT),

and presence of nodal metastases, as well as to the occurrence of *BRAF* mutation which did not significantly alter, *per se*, the frequency of nodal metastases.
The nuclear expression of Smad7 was more frequent in WC-PTCs than in PC-PTCs and was associated with unicentricity and absence of extra-thyroid extension, vascular invasion and nodal metastases.
Our results emphasize the importance of an extensive sampling and detailed observation of PTC surgical specimens; they also highlight the importance of infiltrative growth pattern and invasiveness over the presence of *BRAF* mutation in CPTC and FVPTC for the development of nodal metastases.
The results on the lymph vessels profile of PTC stress the role of intratumoural lymph vessels in PTC nodal metastization and reinforce the importance of distinguishing E-FVPTC from PC-FVPTC regarding invasiveness, metastatic pattern and molecular profile.
Finally, we conclude that nodal metastases are associated to poorly circumscribed, locally invasive PTCs with high intratumoural lymph vessel density; such metastatic PTCs exhibit low levels of nuclear Smad7 and a peripheral EMT phenotype displaying TGF-beta overexpression, regardless of the occurrence of *BRAF* mutation.

Paper 1

Eloy C, Santos J, Soares P, Sobrinho-Simões M

"The preeminence of growth pattern and invasiveness and the limited influence of *BRAF* and *RAS* mutations in the occurrence of papillary thyroid carcinoma lymph node metastases"

Virchows Archiv 2011; 459: 265-276

Virchows Arch (2011) 459:265–276
DOI 10.1007/s00428-011-1133-7

ORIGINAL ARTICLE

The preeminence of growth pattern and invasiveness and the limited influence of *BRAF* and *RAS* mutations in the occurrence of papillary thyroid carcinoma lymph node metastases

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Received: 7 June 2011 / Revised: 12 July 2011 / Accepted: 12 July 2011 / Published online: 28 July 2011
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Abstract Prognostic factors indicative of papillary thyroid carcinoma (PTC) aggressive behaviour remain incompletely established partially due to the different composition of the series on record regarding the relative proportion of classic PTC (CPTC) and follicular variant PTC (FVPTC) subtypes. Several clinico-morphological features of PTC, together with the occurrence of *BRAF* mutations, are still not fully accepted as markers of aggressiveness. In the present clinico-pathological study of a series of 75 CPTC and FVPTC cases, we evaluated the relative contribution of the morphological features of the tumours and their *BRAF* and *N-RAS* status for the occurrence of nodal metastases. The morphological features most closely related to the occurrence of nodal metastases were extra-thyroid extension and poorly circumscribed growth pattern, in both CPTC and FVPTC. Additional features significantly associated to nodal metastases were multicentricity in the CPTC and vascular invasion in the FVPTC group. *BRAF* V600E mutation was detected in 29% of tumours, 41% of CPTC and 16% of FVPTC; *N-RAS* Q61R mutation was detected in 6% of tumours, 3% of CPTC and 10% of FVPTC. *BRAF* mutation was significantly more frequent in the CPTC group and in females, and it was detected only in patients older than 20 years, suggesting a late tumourigenic effect in the development of PTC. *BRAF* mutation was not significantly associated to any of the other studied features related to aggressiveness or nodal metastases. These results highlight the importance of infiltrative growth pattern and invasiveness over the presence of *BRAF* mutation in classic and follicular variant PTC for the development of nodal metastases.

Keywords Papillary thyroid carcinoma · *BRAF* · RAS · Staging · Prognosis

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Introduction

Papillary thyroid carcinoma (PTC) is the most frequent malignant endocrine neoplasia encompassing 16 variants in the last WHO classification [1]. If one excludes the incidentally found papillary microcarcinomas, the classic PTC (CPTC) and the follicular variant of PTC (FVPTC) are the most frequently diagnosed variants. CPTC and FVPTC present distinct cytoarchitectural features [1], molecular alterations [2–4] and biological behaviour [2,

5, 6]. The majority of CPTC and FVPTC cases evolve favourably, and the patients have a 10-year survival up to 98% [1, 7]; the outcome is particularly favourable in patients with encapsulated tumours [8–10]. Despite the (very) good prognosis, PTCs may give rise to recurrences and/or metastases, sometimes many years after diagnosis [11].

There are several clinical and/or morphological features of PTC that have been frequently, though not always, associated to a less favourable prognosis, namely advanced age at time of diagnosis [6, 11, 12], male gender [11], tumour size above 1 cm [6, 13], extra-thyroid extension [6, 12, 14, 15], multicentricity [6], absence of total encapsulation [6, 8, 9, 14], vascular invasion [6] and distant metastases [6, 12]. The presence of lymph node metastases is not fully accepted as a prognostic factor, and it is not associated to an increased mortality rate despite being determinant for the type of surgical approach and radioactive iodine therapy [6, 12].

Part of the controversies on the prognostic value of the above-mentioned features reflects the variable composition of the different series. This holds particularly true regarding the relative proportion of CPTC and FVPTC in general and of the encapsulated subtype of FVPTC in particular [8, 16]. The variable percentages of CPTC and FVPTC in the different series also contribute to explain the variation of the prevalence of *BRAF* mutations from 7% to 83% [3, 4, 12, 14, 16–26] and that of *RAS* mutations from 0 to 43% [3, 4, 18, 24, 26–29].

BRAF is a critical effector of the canonic mitogen-activated protein kinase (MAPK) pathway having the V600E as its most frequent mutation; this mutation is present in 40–54% of CPTC [16, 17, 21, 23, 25] and in 7–29% of FVPTC [3, 16, 21, 23, 25, 26]. *BRAF* mutations have been associated to the classic PTC phenotype [16, 21, 23], advanced age at time of diagnosis [12, 16, 23], male gender [22], tumour size above 1 cm [25], extra-thyroid extension [23, 30, 31], absence of total encapsulation [3, 30], vascular invasion [12], lymph node metastases [14, 19, 30], distant metastases [20], recurrence [30] and higher tumour stages [12, 20].

RAS is an oncogene that also plays a role in the canonic MAPK pathway and is mutated in 0–23% of CPTC [4, 26–28] and in 25–43% of FVPTC [3, 4, 26, 27]. *RAS* mutations have been associated to large tumour size [26], encapsulation [3, 27, 31], vascular invasion [27], extra-thyroid extension [31], poorly differentiated areas [27] and low rate of lymph node metastases [27, 31]. *BRAF* and *RAS* mutations appear to be alternative events in PTC [18].

In the present clinico-pathological study of a series of 75 CPTC and FVPTC cases, we evaluate the relative contribution of the morphological features of the tumours (namely growth pattern and invasiveness) and their *BRAF* and *N-RAS* status for the occurrence of lymph node metastases.

Materials and methods

Tumour samples

A series of 75 PTC cases was retrieved from the Cancer Registry of the Pathology Department of Hospital São João: 42 CPTC and 33 FVPTC. Both CPTC and FVPTC groups included cases with lymph node metastases (+M), together or not with distant metastases, and cases without metastases and long-term follow-up (−M). The PTC−M subgroup included all the CPTC and FVPTC cases with at least 10 years of follow-up, available stored material representative of the tumour and imagiological and/or cytological data confirmative of the absence of metastases in the lymph nodes, lungs and bones. The PTC+M subgroup included more recent cases of CPTC and FVPTC with available material representative of the tumour and its lymph node metastases present at the time of surgery or developed during follow-up. Three cases disclosed, together with lymph node metastases, lung metastases detected in image studies (cases 36 and 37 of CPTC+M subgroup and case 33 of FVPTC+M subgroup). Both in cases with and without lymph node metastases, there were tumours larger than 1 cm and small tumours with 1 cm or less in diameter that were classified according to their growth pattern as CPTC or FVPTC. None of the patients of the PTC+M subgroup died from the disease (this information has limited value due to the short follow-up of most of the cases).

All samples had been fixed in formalin and included in paraffin. Clinical data were collected from the reports and clinical files; H&E slides were retrieved from the archives and reviewed independently by two pathologists (CE and MSS), and the tumours were classified using the WHO criteria [1]. Cases with doubtful PTC features were excluded. Well-circumscribed tumours were those with expansive pattern of growth without capsule or limited by a capsule with no or minimal invasion, and poorly circumscribed tumours were those with infiltrative pattern of growth or with a widely invaded capsule. Vascular invasion was considered to be present whenever at least one image of unequivocal venous vessel invasion was observed. Extra-thyroid extension was considered to be present when macroscopic extra-thyroid extension was observed. Under the designation of multicentricity, all multifocal lesions, regardless of representing multicentric tumours or intra-thyroidal disseminated carcinomas, were included. The presence of oncocytic cells was considered whenever they accounted for more than 10% of the tumour cells. Clinical and morphological features of the major tumour of each case were registered in an informatic supported database.

Mutation screening

Genomic DNA was extracted from 10-μm sections of 65 tumours: 34 CPTC and 31 FVPTC (the remaining cases have insufficient available material). Slides were microscopically examined, marked and dissected under microscopic observation. The obtained material was deparaffinized in xylene, washed in ethanol and rehydrated. DNA extraction was performed using the Genomic DNA Purification Kit (Gentra Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. Sequences of *BRAF* (exon 15) and *N-RAS* (exon 2) genes were amplified using primer pairs listed in Table 1, using a programmable thermocycler (MyCycler, Bio-Rad, USA). The PCR mixture (25 μl) contained 2.5 μl ×10 PCR buffer, 0.8 mM dNTPs (Fermentas), 0.07 μg each primer, 0.1–0.2 μg genomic DNA, 0.5 U Taq DNA Polymerase (Go Taq Flexi DNA Polymerase, Promega Corporation) and 2.5 mM magnesium chloride (Promega Corporation). After 10 min of initial denaturation, the PCR mixtures were subjected to 35 cycles of denaturation for 30 s at 95°C, annealing according to the amplicon and extension for 45 (*BRAF*) and 40 s (N-*RAS*) at 72°C. A final extension period of 10 min at 72°C was performed. PCR products were submitted to electrophoresis in agarose gel and purified by enzymatic treatment. Purified samples were sequenced on both strands as follows: 3 μl purified PCR product was added to 7 μl sequencing reaction containing 3.4 μl Sequencing Buffer (Applied Biosystems, Forester City, CA), 30 ng primer and 0.6 μl Big Dye Terminator (Applied Biosystems). Sequencing was performed in an ABI Prism 3100 Genetic Analyser (Applied Biosystems). Mutated samples were re-extracted, re-amplified and re-sequenced to confirm the result.

Statistical analysis

The results are expressed as absolute values, percentages and/or mean ± standard deviation. The χ^2 test with Yates correction and Student's *t* test (unpaired) were used in the statistic analysis. Two values were considered significantly different whenever $p<0.05$.

Results

Tables 2 and 3 summarize the clinical, morphological and molecular features of the 42 CPTC cases and those of the 33 FVPTC cases, respectively. CPTC and FVPTC cases had similar clinical and morphological features except different frequencies of poorly circumscribed tumours (83.3% of CPTC and 54.5% of FVPTC, $p=0.010$) and vascular invasion (28.6% of CPTC and 57.6% of FVPTC, $p=0.018$) (Tables 4 and 5).

CPTC

The 42 CPTC cases were 23 CPTC−M (54.8%) with a mean follow-up time of 19.9±2.5 years and 19 CPTC+M (45.2%) with a mean follow-up time of 5.5±3.2 years. The CPTC−M cases included 20 females (87.0%) with a mean age of 41.5±15.7 years and 3 males (13.0%) with a mean age of 49.3±21.4 years. The patients with CPTC−M underwent partial thyroidectomy (lobectomy or lobectomy plus isthmectomy) in 60.9% of cases and total thyroidectomy in 34.8% of cases; in 4.3% of the cases, the precise extension of the surgery was not known. The CPTC+M cases included 15 females (78.9%) with a mean age of 38.1±19.1 years and 4 males (21.1%) with a mean age of 22.3±14.0 years. The patients with CPTC+M underwent total thyroidectomy with or without lymph node dissection in 73.7% and 26.3% of cases, respectively.

CPTC−M tumours presented less often poorly circumscribed growth pattern (69.6% of CPTC−M and 100.0% of CPTC+M, $p=0.010$), extra-thyroid extension (13.0% of CPTC−M and 47.4% of CPTC+M, $p=0.020$) and multicentricity (26.1% of CPTC−M and 63.2% of CPTC+M, $p=0.028$) than CPTC+M (Table 4).

CPTC−M and CPTC+M cases were not significantly different concerning gender, age, size, presence of capsule or capsular invasion, vascular invasion (Table 4) and not also concerning necrosis (21.7% and 21.1%, respectively), intra-tumoural inflammation (60.9% and 63.2%, respectively), oncocytic cells (43.5% and 26.3%, respectively) and mitotic index (0.70 and 0.53 mitoses/10 HPF, respectively). The lymphocyte-rich stroma was detected only in two CPTC−M tumours (Table 2). In the adjacent thyroid

Table 1 Primers and PCR conditions

Gene	Exon	Primer sequence	Length (bp)	PCR annealing temperature (°C)	PCR annealing time (seconds)
BRAF	15	F:TCATAATGCTTGCTCTGATAGGA R: GGCCAAAAATTTAATCAGTGGA	224	58	40
N-RAS	2	F: GATTCTTACAGAAAACAAGT R: CCTTCGCCTGTCCTCATGTATT	119	57	35

Table 2 Clinical, morphological and molecular features of the CPTC cases

N	Gender	Age (years)	Follow-up (years)	Tumour size (cm)	Capsule	Capsular invasion	Growth pattern	Multicentricity	EE	Vascular invasion	Necrosis	Mitotic index[a]	Oncocytic cells	Stroma type	II	Thyroiditis[b]	BFP	Surgery type	At the time of diagnosis		*BRAF*	*N-RAS*	LNM
																			pTNM	Stage			
1	M	58	17	0.8	+	+	P	−	−	−	−	1	−	C	−	f	−	PT	T1aN0M0	I	ND	ND	−
2	F	66	19	1.5	+	+	W	−	−	−	−	1	−	C	+	−	NG	T	T1bN0M0	I	ND	ND	−
3	F	31	21	3.5	+	+	P	+	−	−	+	0	−	Pc	−	−	FA	PT	T2N0M0	I	−	−	−
4	F	59	21	0.6	+	+	P	+	−	−	+	1	+	−	−	−	NG	PT	T1aN0M0	I	−	−	−
5	F	37	21	1.0	+	−	W	+	−	−	−	1	+	L	+	f	−	T	T1aN0M0	I	ND	ND	−
6	F	35	18	0.3	+	+	P	−	−	−	−	0	−	Pc	−	−	NG	PT	T1aN0M0	I	ND	ND	−
7	F	40	18	1.5	−	NA	P	−	−	−	−	0	+	L	+	+	−	T	T1bN0M0	I	V600E	−	−
8	F	38	18	0.5	−	NA	P	−	−	−	−	0	−	C	−	−	NG	PT	T1aN0M0	I	ND	ND	−
9	M	25	20	1.2	−	NA	P	−	−	+	+	0	−	C	−	−	−	PT	T1bN0M0	I	−	−	−
10	F	30	21	0.6	−	NA	P	+	−	−	−	2	+	C	+	f	−	PT	T1aN0M0	I	V600E	−	−
11	F	40	20	2.2	−	NA	W	−	−	−	−	0	+	−	+	f	−	T	T2N0M0	I	−	−	−
12	F	50	20	3.0	+	+	W	−	−	−	+	0	−	C	+	f	−	PT	T2N0M0	II	V600E	−	−
13	F	25	17	2.1	+	+	W	−	−	−	+	0	−	Pc	+	−	−	T	T2N0M0	I	V600E	−	−
14	F	45	22	4.5	+	−	W	+	−	−	−	1	+	−	−	−	−	PT	T3N0M0	III	V600E	Q61R	−
15	F	23	24	2.5	−	NA	P	−	−	−	−	1	−	Pc	+	−	NG	ND	T2N0M0	I	−	−	−
16	M	65	24	2.3	−	NA	P	−	−	−	−	0	−	C	+	f	−	PT	T2N0M0	II	−	−	−
17	F	21	19	2.0	−	NA	P	−	−	+	−	3	−	C	+	f	−	PT	T1bN0M0	I	V600E	−	−
18	F	79	23	2.8	+	+	P	−	+	−	−	0	−	C	+	f	−	PT	T3N0M0	III	−	−	−
19	F	65	16	3.5	−	NA	P	−	+	−	−	1	+	Pc	+	f	NG	T	T3N0M0	III	V600E	−	−
20	F	28	24	2.5	−	NA	P	−	−	+	−	2	+	C	−	−	−	T	T2N0M0	I	−	−	−
21	F	28	20	2.0	−	NA	P	−	−	+	−	1	−	C	−	−	−	PT	T1bN0M0	I	V600E	−	−
22	F	43	18	1.5	+	+	W	+	−	+	−	0	+	C	+	−	−	PT	T1bN0M0	I	−	−	−
23	F	47	16	4.5	−	NA	P	−	+	+	−	1	+	Pc	+	f	−	T	T3N0M0	III	V600E	−	−
24	F	28	5	0.9	+	+	P	−	−	−	−	0	+	−	−	−	−	T	T1aN1M0	I	V600E	−	+
25	F	21	6	1.2	+	+	P	−	−	−	+	0	+	C	+	+	NG	TL	T1bN1M0	I	−	−	+
26	F	22	14	1.9	−	NA	P	+	−	−	−	1	−	−	−	−	−	TL	T1bN1M0	I	−	−	+
27	F	30	3	1.3	+	+	P	−	+	+	−	0	−	Pc	+	+	−	TL	T3N1M0	I	−	−	+
28	F	56	3	0.7	−	NA	P	+	−	−	−	0	−	−	+	−	NG	TL	T1aN1M0	III	−	−	+
29	F	18	3	1.5	−	NA	P	+	+	+	−	1	−	C	+	f	−	T	T3N1M0	I	−	−	+
30	M	22	4	1.1	−	NA	P	+	−	−	−	1	−	Pc	−	+	−	TL	T1bN1M0	I	−	−	+
31	F	22	6	0.8	−	NA	P	−	−	−	+	0	−	C	+	−	−	TL	T1aN1M0	I	ND	ND	+
32	F	56	3	0.8	−	NA	P	+	−	−	−	0	−	Pc	−	−	−	TL	T1aN1M0	III	ND	ND	+
33	M	42	5	1.6	−	NA	P	+	+	−	+	0	+	Pc	+	f	−	TL	T3N1M0	I	−	−	+
34	F	38	7	3.0	+	+	P	+	−	+	−	1	−	Pc	+	+	−	T	T2N1M0	I	V600E	−	+
35	F	35	5	1.7	+	+	P	−	−	+	−	0	−	C	+	f	−	TL	T1bN1M0	I	V600E	−	+
36	M	11	9	6.0	−	NA	P	+	+	+	−	0	−	C	+	f	−	TL	T4aN1M0	II	−	−	+[c]
37	F	56	4	2.4	−	NA	P	−	+	−	−	1	+	C	+	−	−	TL	T3N1M0	IVC	−	−	+[c]
38	F	83	1	7.5	−	NA	P	−	+	−	+	1	+	−	+	−	−	T	T4aN1M0	IVA	V600E	−	+
39	F	18	4	3.6	−	NA	P	+	−	+	−	0	−	C	−	−	−	TL	T2N1M0	I	−	−	+
40	M	14	4	2.5	−	NA	P	+	+	−	−	1	−	Pc	−	F	−	TL	T3N1M0	I	−	−	+

Table 2 (continued)

N	Gender	Age (years)	Follow-up (years)	Tumour size (cm)	Capsule	Capsular invasion	Growth pattern	Multicentricity	EE	Vascular invasion	Necrosis	Mitotic index[a]	Oncocytic cells	Stroma type	II	Thyroiditis[b]	BFP	Surgery type	At the time of diagnosis		*BRAF*	*N-RAS*	LNM
																			pTNM	Stage			
41	F	32	9	1.1	–	NA	P	–	+	–	–	1	–	C	–	F	NG	T	T3N1M0	I	V600E	–	+
42	F	56	10	7.5	–	NA	P	+	+	–	–	2	–	C	+	F	–	TL	T3N1M0	III	ND	ND	+

N number, *M* male, *F* female, + presence, – absence, *W* well circumscribed, *P* poorly circumscribed, *EE* extra-thyroid extension, *C* cellular, *Pc* poorly cellular, *L* lymphocyte rich, *II* intra-tumoural inflammation, *f* focal, *BFP* benign follicular pathology in the adjacent thyroid tissue, *NG* nodular goitre, *FA* follicular adenoma, *ND* not determined, *NA* not applicable, *LNM* lymph node metastases, *PT* partial thyroidectomy, *T* total thyroidectomy, *TL* total thyroidectomy with lymph node dissection

[a] Number of mitoses per 10 high power fields (×400)

[b] Thyroiditis was considered + whenever Hashimoto thyroiditis features were present

[c] Cases with concurrent distant metastases

parenchyma, there were no significantly different frequencies of focal or diffuse Hashimoto type thyroiditis in CPTC−M and CPTC+M cases (47.8% and 57.9%, respectively), nor of benign follicular pathology (30.4% and 15.8%, respectively) (Table 4).

FVPTC

The 33 FVPTC cases were 25 FVPTC−M (75.8%) with a mean follow-up time of 17.7±3.7 years and 8 FVPTC+M (24.2%) with a mean follow-up time of 6.9±4.1 years. The FVPTC−M cases included 22 females (88.0%) with a mean age of 41.7±12.2 years and 3 males (12.0%) with a mean age of 47.0±13.1 years. The patients with FVPTC−M underwent partial thyroidectomy (lobectomy or lobectomy plus isthmectomy) in 60.0% of cases or total thyroidectomy in 40.0% of cases. The FVPTC+M cases included five females (62.5%) with a mean age of 42.2±13.5 years and three males (37.5%) with a mean age of 47.0±22.7 years. The patients with FVPTC+M underwent total thyroidectomy with or without lymph node dissection in 75.0% and 25.0% of cases, respectively.

FVPTC−M tumours were significantly less often poorly circumscribed than FVPTC+M (40.0% of FVPTC−M and 100.0% of FVPTC+M, p=0.004), presented significantly less often extra-thyroid extension (0.0% of FVPTC−M and 50.0% of FVPTC+M, p=0.002) and vascular invasion (44.0% of FVPTC−M and 100.0% of FVPTC+M, p=0.010).

FVPTC−M and FVPTC+M displayed different mean mitotic indexes (0.28 and 1.25 mitoses/10 HPF, respectively; p=0.031). FVPTC−M cases presented significantly more often (56.0%) benign follicular pathology in the adjacent thyroid parenchyma than FVPTC+M tumours (12.5%) (p= 0.046). In the subgroup of tumours with 1 cm or less, all the FVPTC−M had associated benign follicular pathology (8 out of 8), while none of the FVPTC+M had associated benign follicular pathology (0 out of 2). A similar difference was observed in the subgroup of tumours larger than 1 cm (14 out of 25 and 1 out of 8, respectively) (p=0.022).

FVPTC−M and FVPTC+M were not significantly different concerning gender, age, presence of capsule or capsular invasion, multicentricity (Table 4), nor necrosis (8.0% and 0.0%, respectively), intra-tumoural inflammation (32.0% and 62.5%, respectively), presence of oncocytic cells (36.0% and 0.0%, respectively), focal or diffuse Hashimoto type thyroiditis (36.0% and 25.0%, respectively) and stroma types (Table 4).

PTC+M versus PTC−M

Table 4 summarizes the differences between the 27 PTC+M and the 48 PTC−M. PTC+M cases presented more often a

Table 3 Clinical, morphological and molecular features of the FVPTC cases

N	Gender	Age (years)	Follow-up (years)	Tumour size (cm)	Capsule	Capsular invasion	Growth pattern	Multicentricity	EE	Vascular invasion	Necrosis	Mitotic index[a]	Oncocytic cells	Stroma type	I I	Thyroiditis[b]	BFP	Surgery type	At the time of diagnosis		*BRAF*	*N-RAS*	LNM
																			pTNM	Stage			
1	F	32	22	2.0	+	+	W	−	−	+	−	2	+	Pc	+	f	−	PT	T1bN0M0	I	−	−	−
2	F	26	21	1.8	−	NA	P	+	−	+	−	0	−	Pc	−	−	−	PT	T1bN0M0	I	V6ooE	−	−
3	F	59	19	0.5	+	−	W	−	−	−	+	0	+	−	+	−	NG	T	T1aN0M0	I	−	−	−
4	F	51	18	1.4	+	−	W	−	−	−	−	0	+	C	+	−	NG	T	T1bN0M0	I	−	−	−
5	F	36	18	0.5	−	NA	P	−	−	−	−	0	−	C	+	−	NG	PT	T1aN0M0	I	V6ooE	−	−
6	F	39	15	1.0	−	NA	W	+	−	−	−	1	−	−	−	−	NG	PT	T1aN0M0	I	−	Q61R	−
7	F	48	13	0.6	+	−	W	−	−	−	−	0	−	Pc	−	+	NG	PT	T1aN0M0	I	−	−	−
8	F	33	20	0.2	−	NA	P	−	−	−	−	0	−	C	+	+	FA	T	T1aN0M0	I	−	−	−
9	F	30	15	0.5	−	NA	W	−	−	−	−	0	−	−	−	−	NG	PT	T1aN0M0	I	−	−	−
10	F	21	21	0.6	−	NA	P	−	−	−	−	0	−	−	−	f	NG	T	T1aN0M0	I	−	−	−
11	F	37	18	0.3	−	NA	P	−	−	+	−	0	−	Pc	−	−	NG	PT	T1aN0M0	I	−	−	−
12	F	28	21	2.8	−	NA	W	+	−	+	−	0	−	Pc	−	−	−	T	T2N0M0	I	−	−	−
13	F	34	20	3.5	+	−	W	−	−	−	−	0	−	Pc	−	f	−	PT	T2N0M0	I	−	−	−
14	M	61	22	4.0	+	+	W	−	−	+	−	0	−	−	−	−	−	T	T2N0M0	II	V6ooE	−	−
15	M	35	20	3.5	+	+	W	−	−	+	−	0	−	−	−	−	−	PT	T2N0M0	I	−	Q61R	−
16	F	50	20	2.5	+	+	W	−	−	−	−	1	−	−	−	−	−	PT	T2N0M0	II	−	−	−
17	F	47	23	3.0	−	NA	P	−	−	−	−	0	+	−	−	−	−	PT	T2N0M0	II	−	Q61R	−
18	F	49	14	3.0	+	+	W	+	−	+	−	0	−	−	−	f	NG	T	T2N0M0	II	−	−	−
19	F	35	21	1.7	−	NA	P	+	−	+	−	1	−	Pc	+	f	NG	PT	T1bN0M0	I	ND	ND	−
20	F	32	13	4.7	−	NA	P	+	−	+	−	0	+	−	−	−	−	T	T3N0M0	I	−	−	−
21	F	52	13	2.5	+	+	W	+	−	−	−	1	−	Pc	−	−	NG	T	T2N0M0	II	−	−	−
22	F	66	13	2.5	+	+	W	+	−	−	+	0	+	Pc	−	f	−	PT	T2N0M0	II	−	−	−
23	F	58	13	1.5	−	NA	P	+	−	+	−	1	+	C	+	−	NG	PT	T1bN0M0	I	V6ooE	−	−
24	F	54	10	1.8	−	NA	P	+	−	+	−	0	+	Pc	+	f	NG	PT	T1bN0M0	I	−	−	−
25	M	45	20	4.0	+	+	W	−	−	−	−	0	+	−	−	−	−	T	T2N0M0	II	−	−	−
26	M	57	3	9.5	+	+	P	−	+	+	−	6	−	−	−	−	−	TL	T3N0M0	III	−	−	+
27	F	56	3	0.8	+	+	P	+	−	+	−	0	−	Pc	+	−	−	TL	T1aN1M0	III	−	−	+
28	M	21	4	2.5	−	NA	P	−	+	+	−	1	−	C	+	−	−	TL	T4aN1M0	I	−	−	+
29	F	20	14	1.0	−	NA	P	+	−	+	−	1	−	−	+	+	−	TL	T1aN1M0	I	ND	ND	+
30	F	49	4	2.2	+	+	P	−	−	+	−	1	−	−	−	−	−	T	T2N1M0	III	−	−	+
31	F	43	10	4.0	−	NA	P	+	+	+	−	1	−	−	+	f	−	TL	T4aN1M0	I	−	−	+
32	F	43	10	1.5	−	NA	P	+	+	+	−	0	−	C	+	−	−	TL	T3N1M0	I	V6ooE	−	+
33	M	63	7	1.8	+	+	P	−	−	+	−	0	−	−	−	−	FA	T	T1bN0M0	I	−	−	+[c]

N number, *M* male, *F* female, + presence, − absence, *W* well circumscribed, *P* poorly circumscribed, *EE* extra-thyroid extension, *C* cellular, *Pc* poorly cellular, *L* lymphocyte rich, *II* intra-tumoural inflammation, *f* focal, *BFP* benign follicular pathology in the adjacent thyroid tissue, *NG* nodular goitre, *FA* follicular adenoma, *ND* not determined, *NA* not applicable, *LNM* lymph node metastases, *PT* partial thyroidectomy, *T* total thyroidectomy, *TL* total thyroidectomy with lymph node dissection

[a] Number of mitoses per 10 high power fields (×400)

[b] Thyroiditis was considered + whenever Hashimoto thyroiditis features were present

[c] Cases with concurrent distant metastases

Table 4 Comparison of clinical and morphological features of CPTC and FVPTC cases with and without lymph node metastases (LNM)

PTC variant	Features	With LNM	Without LNM	*p* value
CPTC	Gender (female/male)	15:4	20:3	>0.1
	Age, years (mean ± SD)	34.7±19.0	42.5±16.2	
	Tumour size of tumours >1, cm (mean ± SD)	2.9±2.3	2.5±1.0	
	Tumour size of tumours ≤1, cm (mean ± SD)	0.8±0.1	0.6±0.2	
	Capsule (absence/total)	0.74	0.52	
	Capsule invasion (presence/total)	1.00	0.82	
	Vascular invasion (presence/total)	0.32	0.26	
	Multicentricity (presence/total)	0.63	0.26	<0.05
	Growth pattern (poorly circumscribed/total)	1.00	0.70	
	Extra-thyroid extension (presence/total)	0.47	0.13	
FVPTC	Gender (female/male)	5:3	22:3	>0.1
	Age, years (mean ± SD)	44.0±16.1	42.3±12.1	
	Tumour size of tumours >1, cm (mean ± SD)	3.6±3.0	2.7±1.0	
	Capsule (absence/total)	0.50	0.52	
	Capsule invasion (presence/total)	1.00	0.67	
	Multicentricity (presence/total)	0.50	0.40	
	Tumour size of tumours ≤1, cm (mean ± SD)	0.9±0.1	0.5±0.2	0.1–0.05
	Vascular invasion (presence/total)	1.00	0.44	<0.05
	Growth pattern (poorly circumscribed/total)	1.00	0.40	
	Extra-thyroid extension (presence/total)	0.50	0.00	
CPTC + FVPTC	Gender (female/male)	20:7	42:6	>0.1
	Age, years (mean ± SD)	37.5±18.4	42.4±14.1	
	Tumour size of tumours >1, cm (mean ± SD)	3.1±2.4	2.6±1.0	
	Capsule (absence/total)	0.67	0.52	
	Capsule invasion (presence/total)	1.00	0.74	
	Vascular invasion (presence/total)	0.52	0.35	
	Multicentricity (presence/total)	0.59	0.33	0.1–0.05
	Tumour size of tumours ≤1, cm (mean ± SD)	0.8±0.1	0.6±0.2	<0.05
	Growth pattern (poorly circumscribed/total)	1.00	0.54	
	Extra-thyroid extension (presence/total)	0.48	0.06	

poorly circumscribed growth pattern ($p<0.0001$), extra-thyroid extension ($p<0.0001$) and larger size of the tumours in the group with 1 cm or less ($p=0.019$) than PTC−M tumours. PTC+M tumours tended to be more often multicentric than PTC−M tumours ($p=0.051$).

When comparing poorly circumscribed CPTC+M and CPTC−M tumours, the former were more often multicentric than the latter (57.9% and 18.8%, respectively; $p=0.036$) (Table 6). Similarly, poorly circumscribed FVPTC+M tumours displayed more often extra-thyroid extension than FVPTC−M (50.0% and 0.00%, respectively; $p=0.023$) (Table 6).

Mutational analysis: BRAF and N-RAS

Overall, the *BRAF* V600E mutation occurred in 19 out of 65 PTC cases (29.2%), and the *N-RAS* Q61R mutation occurred in 4 out of 65 PTC tumours (6.2%). The frequency of *BRAF* mutation in the CPTC group was significantly higher than in the FVPTC group (41.2% and 16.1%, respectively) ($p=0.032$), and occurred in 13 out of 42 PTC−M tumours (31.0%) and in 6 out of 23 PTC+M tumours (26.1%). The *N-RAS* mutation occurred only in PTC−M tumours (Tables 2 and 3).

In the CPTC group, 9 out of 18 CPTC−M (50.0%) and 5 out of 16 CPTC+M (31.3%) displayed *BRAF* mutation. The presence of *BRAF* mutation in CPTC was significantly associated to female gender ($p=0.031$) (14 females and 0 males) (Table 5). *N-RAS* mutation was detected in 1 out of 34 CPTC tumours (2.9%); it was a Q61R mutation in a well-circumscribed encapsulated CPTC−M tumour (1 out of 18–5.6%).

In the FVPTC group, the *BRAF* V600E mutation was detected in four FVPTC−M (16.7%) and one FVPTC+M (14.3%). *N-RAS* Q61R mutation was detected in 3 out of 31 cases (9.7%); all of such cases were FVPTC−M tumours (3 out of 24–12.5%).

Table 5 Comparison of molecular features of CPTC and FVPTC cases

PTC variant	Features	*BRAF* wild type	*BRAF* V600E	*p* value
CPTC	Gender (female/male)	14:6	14:0	0.031
	Age, years (mean ± SD)	35.2±19.0	40.5±16.9	0.41
	Tumour size, cm (mean ± SD)	2.2±1.2	2.7±1.9	0.294
	Number of tumours (>1 cm/total)	0.90	0.86	>0.999
	Capsule (absence/total)	0.70	0.57	0.487
	Capsule invasion (presence/total)	1.00	0.83	>0.999
	Growth pattern (poorly circumscribed/total)	0.90	0.79	0.627
	Multicentricity (presence/total)	0.55	0.29	0.079
	Extra-thyroid extension (presence/total)	0.35	0.29	>0.999
	Vascular invasion (presence/total)	0.35	0.33	>0.999
	Lymph node metastases (presence/total)	0.55	0.36	0.315
	pT (T3 + T4/total)	0.35	0.36	>0.999
	Stage III + IV/total	0.15	0.29	0.410
FVPTC	Gender (female/male)	21:5	4:1	>0.999
	Age, years (mean ± SD)	43.5±12.5	44.8±14.8	0.837
	Tumour size, cm (mean ± SD)	2.4±1.9	1.9±1.3	0.576
	Number of tumours (>1 cm/total)	0.62	0.80	>0.999
	Capsule (absence/total)	0.73	0.94	0.172
	Capsule invasion (presence/total)	0.73	1.00	>0.999
	Growth pattern (poorly circumscribed/total)	0.46	0.80	0.333
	Multicentricity (presence/total)	0.35	0.60	0.350
	Extra-thyroid extension (presence/total)	0.23	0.20	>0.999
	Vascular invasion (presence/total)	0.50	0.80	0.345
	Lymph node metastases (presence/total)	0.23	0.20	>0.999
	pT (T3 + T4/total)	0.15	0.20	>0.999
	Stage III + IV/total	0.12	0.00	>0.999
CPTC + FVPTC	Gender (female/male)	35:11	18:1	0.093
	Age, years (mean ± SD)	39.9±16.0	41.6±16.1	0.689
	Tumour size, cm (mean ± SD)	2.3±1.6	2.5±1.7	0.647
	Number of tumours (>1 cm/total)	0.80	0.84	0.740
	Capsule (absence/total)	0.54	0.63	0.589
	Capsule invasion (presence/total)	0.81	0.86	>0.999
	Growth pattern (poorly circumscribed/total)	0.65	0.79	0.379
	Multicentricity (presence/total)	0.43	0.32	0.418
	Extra-thyroid extension (presence/total)	0.33	0.32	>0.999
	Vascular invasion (presence/total)	0.43	0.47	0.791
	Lymph node metastases (presence/total)	0.37	0.32	0.780
	pT (T3 + T4/total)	0.24	0.32	0.547
	Stage III + IV/total	0.13	0.21	0.461

The presence of *BRAF* and *N-RAS* mutation was not significantly associated to any other clinical or morphological feature (Tables 2, 3, 5 and 6).

Discussion and conclusions

Prognostic factors indicative of PTC metastatic capacity or aggressive behaviour still remain incompletely established. The same holds true regarding the prognostic value of *BRAF* V600E mutation per se, as well as the association between *BRAF* mutation and some pathological features with clinical significance [3, 12, 14, 16, 19–23, 25, 30, 31].

In the present study, we found that, both in CPTC and in FVPTC, extra-thyroid extension and poorly circumscribed growth pattern, rather than *BRAF* V600E mutation, were the pathological features most closely associated to lymph node metastases. In fact, all the PTCs metastatic to the lymph nodes were poorly circumscribed, whereas all well-circumscribed tumours were non-metastatic to the lymph nodes.

In the CPTC group, multicentricity was significantly associated to lymph node metastases, particularly in the

Table 6 Comparison of clinical and morphological features of poorly circumscribed CPTC and FVPTC cases with and without lymph node metastases (LNM)

PTC variant	Features	With LNM	Without LNM	*p* value
CPTC	Multicentricity (presence/total)	0.58	0.19	0.036
	Extra-thyroid extension (presence/total)	0.47	0.19	0.152
	Vascular invasion (presence/total)	0.32	0.31	>0.999
	BRAF V600E mutation	0.31	0.46	0.466
	N-RAS mutation	0.00	0.00	>0.999
FVPTC	Multicentricity (presence/total)	0.50	0.50	>0.999
	Extra-thyroid extension (presence/total)	0.50	0.00	0.023
	Vascular invasion (presence/total)	1.00	0.67	0.206
	BRAF V600E mutation	0.14	0.33	0.585
	N-RAS mutation	0.00	0.11	>0.999

subgroup of poorly circumscribed tumours. In the poorly circumscribed tumours of the FVPTC group, extra-thyroid extension was the most important feature associated to lymph node metastases. In FVPTC cases, vascular invasion and mitotic index were also significantly associated to lymph node metastases, although the latter may reflect the existence of a single case with six mitoses/ten HPF.

FVPTC tumours measuring 1 cm or less and arising in a gland with benign pathology other than thyroiditis were less often associated to lymph node metastases than solitary FVPTC. This difference probably reflects our biased sample of small tumours that was composed by incidentally found PTCs in patients operated on for benign pathology (nodular goitre or follicular adenoma). In our series, this difference was also observed in tumours larger than 1 cm. The low frequency of lymph node metastases from PTC arising together with concurrent benign pathology other than thyroiditis has not been reported before to the best of our knowledge and needs confirmation in larger series.

Our results support the assumption that encapsulated, non-angioinvasive FVPTCs carry an excellent prognosis even when the patients underwent partial thyroidectomy [4, 5, 8–10, 32, 33].

In our series, *BRAF* V600E mutation was less frequently detected than in most series on record [3, 4, 12, 14, 16–26]. It was significantly more frequent in CPTC than in FVPTC cases, an observation consistent with the results of Trovisco et al. [16], as well as those of many other series [21, 23].

BRAF V600E mutated CPTCs were significantly more often found in females than in males, in contrast with the previously observed higher frequency in males [22], or similar frequency in both genders [8, 9, 16, 21].

Although the mean age of PTC patients with and without the *BRAF* V600E mutation was not significantly different in CPTC and FVPTC groups, we did not find a single patient younger than 20 years with a mutated *BRAF* tumour, thus confirming that *BRAF* mutation is rarely present in PTCs of young patients [12, 16, 23]. In most series, *BRAF* mutated PTCs tend to occur in older patients than PTCs without the *BRAF* mutation and to be rarely found in children or young adults [12, 16]. The inverse holds true for RET/PTC rearrangements which are frequently detected in PTC occurring in children and adolescents [34, 35].

At variance with RET/PTC rearrangements that can be detected in cases of nodular goitres/follicular adenomas and Hashimoto thyroiditis displaying large, clear and irregular nuclei [36], *BRAF* mutations have not been detected in benign lesions of the thyroid [37] except in exceedingly rare cases [16, 18]. The reason for the absence of *BRAF* V600E mutation in benign lesions and in PTCs of children and adolescents remains unknown. It is tempting to speculate that *BRAF* mutation per se may induce senescence in thyroid cells as it has been observed in benign melanocytic lesions [38, 39]. Following this hypothesis, the tumourigenic effect of *BRAF* mutation would only be seen whenever the mutation is accompanied by synergic mutations and/or epigenetic changes in other pathways [39, 40]. We are advancing that the *BRAF* mutation may be tumourigenic for the human thyroid only in relatively late stages of PTC development (in normal thyroid and in early neoplastic stages, the mutation might induce a senescent phenotype). The results recently published by Franco et al. [41] in a mouse model support the hypothesis that the aforementioned synergies may be obtained in early stages via a TSH effect. Franco et al. [41] demonstrated that *BRAF* tumourigenic early effect can be mediated by cooperation with the thyrotrophin receptor signalling pathway [41]. In vitro and in vivo studies are necessary to test the aforementioned hypotheses that are also important from a prognostic standpoint (see below).

At variance with the results reported by others, we did not observe in our series a significant association between *BRAF* mutation and poor circumscription [3, 30], tumour size above 1 cm [25], extra-thyroid extension [23, 30, 31], vascular invasion [12] and lymph node metastases [14, 19, 30]. None of the three patients of our series with distant metastases had a *BRAF* mutated tumour in contrast with the results of Namba et al. [20].

In our series, the *N-RAS* mutation was detected in 6% of the tumours, a prevalence that lies within the interval of previously reported data in other PTC series [3, 4, 18, 24, 26–29]. We found no significant association of *N-RAS* Q61R mutation with any clinical or morphological feature, but these negative results may reflect the low number of mutated cases in our series. In one case (case 14 of the

CPTC group), there was coexistence of *BRAF* and *N-RAS* mutation demonstrating that, although rarely, *BRAF* and *RAS* mutations can coexist in the same tumour [18]. It remains to be verified if the two mutations coexist in the same cells or correspond to distinct clones, as demonstrated in melanomas [42].

In our series, there is not a significant association between the presence of *BRAF* mutation and lymph node metastases. This result fits with those of some studies on record [16, 17, 21] and contradict others [12, 14, 30]. *BRAF* mutation was strongly associated to the CPTC group, whose cases were more often poorly circumscribed than FVPTC cases. Within each group, no additional significant associations to other morphological features related to aggressiveness were found.

The detection of the V600E mutation in fine needle aspiration biopsy (FNAB) specimens is a powerful adjunct for the diagnosis of PTC and has increased the specificity of PTC cytological diagnosis to almost 100% [30, 43, 44], but unfortunately, the sensitivity of such detection remains low due to the high percentage of non *BRAF* mutated PTCs [15, 45, 46].

The practical value of the *BRAF* V600E detection for therapeutic purposes in metastatic follicular cell-derived carcinomas with sodium–iodine symporter and thyroid peroxidase down regulation not responding to radioactive iodine is also very promising [44, 46–49].

The preoperative prognostic value of such detection in PTC remains controversial [14, 43, 47]. The most important argument against such value stems from the relatively high percentage of papillary microcarcinomas that have an excellent prognosis [13, 14, 47] even if some of them tend to be locally infiltrative and carry a high rate of lymph node metastases [14, 15, 43, 50].

Basolo et al. [15] have shown that in small PTCs, there is a significant association between the presence of *BRAF* mutation and tumour invasiveness and nodal metastization [15]. These findings are very important from a pathological standpoint and support the oncobiological role attributed to activating *BRAF* mutations in cell lines [51] and animal models [41]. The issue of tumour invasiveness is crucial in most CPTCs which tend to be poorly circumscribed and in the infiltrative (poorly circumscribed) subtype of FVPTC [52]; both tend to give rise frequently to lymph node metastases even when the primary tumours are very small [50]. It remains to be clarified how *BRAF* mutation increases tumour cell invasiveness.

The data on *BRAF* mutated cell lines and experimental models, as well as the observational data on PTC on record, do not prove that *BRAF* mutation per se is a major prognostic factor in human PTC. Larger series, with long-term follow-up combining a thorough clinical and morphological evaluation with the detection of *BRAF* V600E and using multivariate analyses, are necessary to clarify the independent prognostic significance of the mutation. Similar studies are also needed to find a way of combining the clinico-imaging features of a given tumour with the FNAB detection of the *BRAF* V600E for deciding the best surgical approach [47].

Acknowledgements This work was partially supported by the grant Prof. Edward Limbert SPEND/GENZYME–Year 2007. Part of the data reported in this study were obtained in the project "Identificação de factores prognósticos e de selecção terapêutica em carcinomas diferenciados da tireoide", which is supported by Fundação Calouste Gulbenkian. IPATIMUP is an Associate Laboratory of the Portuguese Ministry of Science, Technology and Higher Education and is partly supported by the Fundação para a Ciência e Tecnologia.

Conflict of interest The authors declare no conflict of interest.

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Paper 2

Eloy C, Santos J, Soares P, Sobrinho-Simões M

"Intratumoural lymph vessel density is related to presence of lymph node metastases and separates encapsulated from infiltrative papillary thyroid carcinoma"

Virchows Archiv 2011; 459: 595-605

Virchows Arch (2011) 459:595–605
DOI 10.1007/s00428-011-1161-3

ORIGINAL ARTICLE

Intratumoural lymph vessel density is related to presence of lymph node metastases and separates encapsulated from infiltrative papillary thyroid carcinoma

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Received: 13 September 2011 / Revised: 18 October 2011 / Accepted: 19 October 2011 / Published online: 12 November 2011
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Abstract Papillary thyroid carcinoma (PTC) gives frequently rise to nodal metastases via lymphatic vessels while follicular thyroid carcinoma (FTC) metastasises mainly via blood vessels to lung and bones. The follicular variant of PTC (FVPTC) encompasses the infiltrative subtype (I-FVPTC), which shares most of the features of classic PTC (CPTC), and the encapsulated subtype (E-FVPTC), which appears to be related to minimally invasive FTC. In an attempt to contribute to the understanding of the aforementioned differences, we evaluated intratumoural and peritumoural lymph vessels density (LVD), using the immunomarker D2-40 in a series of E-FVPTC, I-FVPTC, and CPTC with known *BRAF* and *RAS status*. None of the E-FVPTC cases presented extra-thyroid extension, lymph vessel invasion or nodal metastases, at variance with I-FVPTC and CPTC cases. The *BRAF* V600E mutation was detected in 8.3% of E-FVPTC, 25.0% of I-FVPTC and in 40.7% of CPTC, while *N-RAS* Q61R mutation was detected only in 10.3% of FVPTC cases. Only one case of E-FVPTC (8.3%) had intratumoural D2-40-stained vessels in contrast to their presence in 76.5% of the cases of I-FVPTC. Intratumoural LVD determined by D2-40 expression correlated with the occurrence of extra-thyroid extension, lymph vessel invasion and lymph node metastases in PTC cases. At variance with intratumoural LVD, peritumoural LVD was not associated with any clinic-pathological or molecular feature, being similar in E-FVPTC, I-FVPTC and CPTC. Our study highlights the role of intratumoural lymph vessels in PTC nodal metastisation and reinforces the importance of distinguishing E-FVPTC from I-FVPTC regarding invasiveness, metastatic pattern and molecular profile.

Keywords Papillary thyroid carcinoma · Lymphangiogenesis · D2-40 · Prognosis, lymph node metastasis

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Introduction

One of the reasons that justify the attempts to distinguish papillary thyroid carcinoma (PTC) from follicular thyroid carcinoma (FTC) relies on the different preferential routes of metastatic spread associated with these two thyroid cancer histotypes [1]. While PTC has a tendency to give rise to lymph node metastases via lymphatic vessels, FTC metastasises mainly via blood vessels to lung and bones [1–3].

Although traditionally included in the PTC group [1], the follicular variant of PTC (FVPTC) encompasses a subtype of encapsulated tumours, which tend to share

features with both classic PTC (CPTC) and FTC [4–7]. Besides the rare and aggressive multinodular subtype of FVPTC [8], cases of FVPTC may be classified according to their peripheral growth pattern in two categories: infiltrative (or poorly circumscribed) subtype (I-FVPTC) and encapsulated subtype (E-FVPTC) [4, 9]. The I-FVPTCs share most of the morphological features of CPTC, give frequently rise to lymph node metastases and disclose *BRAF* V600E mutation in 26% of the cases [4, 10], whereas the E-FVPTCs seem morphologically related to minimally invasive FTC, often disclose *RAS* mutations and *PAX8/PPARgama* rearrangements [10–12] and follow an extremely benign course in the absence of unequivocal signs of capsular or vascular invasion [13, 14]. In the presence of vascular invasion, the E-FVPTCs tend to give rise, like FTC, to blood-borne metastases [4, 9, 15].

The peculiar metastatic properties of the FVPTC subtypes are not fully understood and may be related to angiogenesis. Several groups have reported an increased expression of factors promoting the formation of new blood vessels (hemangiogenesis) in PTC in comparison with normal thyroid tissue [16, 17]; such factors are variably correlated with tumour size [17, 18], increased microvessel count using CD31 [19], increased risk of recurrence and decreased disease-free survival [18, 20].

The expression of lymphangiogenic factors (VEGF-C and VEGF-D) appears to be increased in PTC in comparison with normal thyroid tissue [16, 17, 21, 22], benign lesions [16, 17, 21, 22] and FTC [23]. The increased expression of lymphangiogenic factors in PTC is related with increased intratumoural and peritumoural lymph vessel density (LVD) [21, 22, 24]. The association between the expression of lymphangiogenic factors in PTC and the occurrence of lymph node metastases was observed by some groups [17, 22, 24–26], whereas others did not detect such association [16, 21].

In the past, the endothelial markers used to identify vessels, like factor VIII, CD31, CD34 and von Willebrand factor, highlighted both lymphatic and blood vessels [18, 19, 27–30]. The situation has changed with the finding of lymphatic vessel specific immunomarkers [31, 32], which have been used to determine the intratumoural and the peritumoural LVD in several thyroid tumour types, such as the lymphatic endothelium-specific markers D2-40 (podoplanin) [3, 21, 33, 34], LYVE-1 (lymph vessel endothelial hyaluronan receptor) [16, 33, 35] and VEGFR-3/flt-4 (VEGF receptor-3) [22, 24, 25].

The intratumoural LVD has been found to be higher in PTC than in follicular adenoma (FA) [16, 21] and higher in FVPTC than in FA or FTC [34]. Some authors reported an association amongst increased PTC intratumoural LVD and male gender [35], multifocal disease [35], invasiveness [25] and occurrence of lymph node metastases [22, 24, 25, 35], in contrast to other groups that did not find any association between PTC intratumoural LVD and the above mentioned features [16, 21].

The peritumoural LVD was similar in PTC, FTC and FA in the series of Giorgadze et al. [34], while in the series of Garcia et al. [21], FTC cases disclosed higher peritumoural LVD than PTC. There are conflicting results regarding the association between PTC peritumoural LVD and the occurrence of lymph node metastases [21, 24].

Amongst the molecular alterations associated with PTC, *BRAF* V600E mutation has been variably associated with the occurrence of vascular invasion [36, 37] and lymph node metastases [37–40], while *RAS* mutations have been related to the occurrence of vascular invasion [12] and scarcity of lymph node metastases [12].

The aim of the present study was to evaluate the intratumoural and peritumoural LVD using the immunomarker D2-40 in a series of thoroughly analysed 29 cases, encompassing 12 cases of E-FVPTC and 17 cases of I-FVPTC, and to compare the data thus obtained with those of a similarly analysed series of 32 cases of CPTC.

As ultimate goals we intended to find if there were, firstly, any relationship between the levels of intratumoural and peritumoural LVD and the three histo(sub)types of PTC, and, secondly, any relationship between the aforementioned factors (LVD and histo(sub)types) and the clinico-pathological and molecular features of the cases with a special emphasis on their nodal metastatic *status*.

Materials and methods

Tumour samples

A series of 61 PTC cases were retrieved from the Cancer Registry of the Pathology Department of Hospital São João: 29 FVPTC and 32 CPTC. Both FVPTC and CPTC groups included cases with lymph node metastases and cases without metastases and, at least, 10 years of follow-up. None of the patients died from the disease. Small tumours with 1 cm or less in diameter that were classified according to their growth pattern as FVPTC or CPTC were included in FVPTC and CPTC groups, respectively.

All samples had been fixed in formalin and embedded in paraffin. Clinical data were collected from the reports and clinical files; haematoxylin and eosin slides were retrieved from the archives and reviewed independently by two pathologists (CE and MSS), and the tumours were classified using the WHO criteria [1]. FVPTC cases were classified as E-FVPTC whenever the tumour was totally surrounded by a capsule with or without signs of focal invasion and as I-FVPTC whenever the tumours displayed an infiltrative (poorly circumscribed) growth pattern. All

CPTC cases were either totally or partially poorly circumscribed, i.e. disclosed at least one area of infiltrative growth pattern. Vascular invasion was considered to be present whenever at least one image of unequivocal venous vessel invasion was observed within the capsule or outside the tumour mass. Lymph vessel invasion was considered to be present whenever at least one image of unequivocal invasion of lymph vessel was observed regardless of being or not stained by D2-40.

Mutation screening

Genomic DNA was extracted from 10-μm sections of 55 tumours: 28 FVPTC and 27 CPTC (the remaining six cases had insufficient available material).The detection of *BRAF* (exon 15) and *N-RAS* (exon 2) gene mutations was performed as previously described [37]. Sequences of *BRAF* and *N-RAS* genes were amplified using the primer pairs previously reported [34]. PCR products were submitted to electrophoresis in agarose gel and purified by enzymatic treatment. Purified samples were sequenced on both strand, and mutated samples were re-extracted, amplified and sequenced to confirm the result.

Immunohistochemistry

Immunohistochemical staining was performed using streptavidin–biotin technique. Sections of 2-μm thickness were cut from paraffin blocks and placed on coated slides. The slides were dried, deparaffinised in Clear Rite (Richard-Allan, Thermo Scientific) and hydrated in sequential gradients of ethanol. For antigen retrieval, sections were immersed in 10 mM sodium citrate buffer, pH 6.0 and then heated for 15 min. Endogenous peroxidase was blocked with 3% hydrogen peroxidase in methanol (Panreac) for 10 min in humid chamber, and then avidin blocking and biotin blocking were performed (Lab Vision, Thermo Scientific). In order to block unspecific bounding to hydrophobic epitopes, the sections were immersed in Ultra V Block (Lab Vision, Thermo Scientific) for 10 min at room temperature. Slides were incubated overnight at 4°C with D2-40 monoclonal antibody (D2-40, Signet laboratories, Dedham, MA, USA) in a 1:25 dilution.

Then, slides were incubated with biotinylated secondary goat antibody and streptavidin–peroxidase complex (Lab Vision, Thermo Scientific) for 10 min at room temperature. The sections were developed with DAB solution (DAKO Corporation) according to the manufacturer's instructions and counterstained with haematoxylin (J.T.Baker, Deventer, Holland). Adequate positive and negative controls in tissue microarrays were used.

Assessment of lymph vessel density

D2-40 expression was considered positive whenever cytoplasmatic staining of endothelial cell of lymphatic vessels was observed. The evaluation of expression was performed by counting positive D2-40 structures with morphological features of lymphatic vessels, clearly separated from adjacent vessels and from other soft tissue components, as previously described [41]. Packed vessels were assumed as constituting a single lymphatic unit. Analysis was performed at ×200 magnification in the areas of highest vascularity (hot spots). The LVD of each case corresponds to the average obtained in counting 10 hot spot fields. The evaluation of D2-40 expression and lymph vessel counting was performed blindly in intratumoural and peritumoural areas of all FVPTC and CPTC cases except in three FVPTC cases in which the remaining non neoplastic thyroid tissue was insufficiently represented. The intratumoural lymph vessels were defined as vessels within the tumour area, whereas peritumoural lymph vessels included vessels in both the capsule and in the remaining non tumoural thyroid tissue.

Statistical analysis

The results are expressed as absolute values, percentages and/or mean ± standard deviation. The χ^2 test with Yates correction and the Students' t test (unpaired) were used in the statistic analysis. Two values were considered significantly different whenever $p<0.05$ and suggestively different when $p<0.10$.

Results

The clinico-pathological and molecular features and the LVD data on the 29 cases of FVPTC separated into E-FVPTC ($n=12$) and I-FVPTC ($n=17$) are summarised in Table 1. The respective data on the 32 cases of CPTC are summarised in Table 2.

Clinico-pathological and molecular features

The comparison of the clinico-pathological and molecular features of the PTC groups is summarised in Table 3.

FVPTC and CPTC groups differ regarding extra-thyroid extension ($p=0.045$) and lymph node metastases ($p=0.038$), which were more prevalent in CPTC. FVPTC cases tended to have more often vascular invasion ($p=0.075$), and less often lymph vessel invasion ($p=0.094$) and *BRAF* V600E mutation ($p=0.080$) than CPTC cases (Table 3).

E-FVPTC cases presented significantly less often vascular invasion ($p=0.029$) and lymph node metastases ($p=0.009$) than I-FVPTC cases (Table 3). None of the E-FVPTC cases presented extra-thyroid extension, lymph vessel invasion or lymph node metastases while in cases with infiltrative growth pattern (I-FVPTC *plus* CPTC), occurrence of extra-thyroid extension was observed in 32.7% of the cases, lymph vessel invasion was also

Table 1 Clinical, morphological and molecular features of the 29 cases of FVPTC (12 cases of E-FVPTC and 17 cases of I-FVPTC) and respective intratumoural and peritumoural LVD

Case	Gender	Age (years)	Tumour size (cm)	M	EE	LI	VI	LNM	*RAS*	*BRAF*	I-LVD	P-LVD
E-FVPTC												
1	F	32	2.0	−	−	−	+	−	−	−	0.0	8.1
2[a]	F	51	1.4	−	−	−	−	−	−	−	0.0	15.4
3	F	39	1.0	+	−	−	−	−	Q61R	−	0.6	11.5
4[a]	F	48	0.6	−	−	−	−	−	−	−	0.0	14.1
5	F	30	0.5	−	−	−	−	−	−	−	0.0	14.2
6	F	28	2.8	+	−	−	+	−	−	−	0.0	6.8
7[a]	F	34	3.5	−	−	−	−	−	−	−	0.0	9.8
8	M	61	4.0	−	−	−	+	−	−	V600E	0.0	12.7
19	M	35	3.5	−	−	−	+	−	Q61R	−	0.0	18.0
10	F	50	2.5	−	−	−	−	−	−	−	0.0	10.3
11	F	52	2.5	+	−	−	−	−	−	−	0.0	14.2
12	F	66	2.5	+	−	−	−	−	−	−	0.0	18.2
I-FVPTC												
13	F	26	1.8	+	−	−	+	−	−	V600E	0.0	11.5
14	F	36	0.5	−	−	−	−	−	−	V600E	0.5	10.1
15	F	21	0.6	−	−	−	−	−	−	−	4.8	9.7
16	F	37	0.3	−	−	−	+	−	−	−	0.0	8.6
17	F	47	3.0	−	−	−	−	−	Q61R	−	0.0	9.8
18	F	32	4.7	+	−	−	+	−	−	−	1.0	21.0
19	F	58	1.5	+	−	+	+	−	−	V600E	0.3	9.8
20	F	54	1.8	+	−	+	+	−	−	−	0.2	10.0
21	M	45	4.0	−	−	−	−	−	−	−	0.1	9.4
22	M	57	9.5	−	+	+	+	+	−	−	4.4	14.0
23	F	56	0.8	+	−	+	+	+	−	−	1.3	1.5
24	M	21	2.5	−	+	−	+	+	−	−	2.0	ND
25	F	20	1.0	+	−	−	+	+	ND	ND	6.0	21.0
26	F	49	2.2	−	−	−	+	+	−	−	3.9	15.0
27	F	43	4.0	+	+	−	+	+	−	−	0.1	ND
28	F	43	1.5	+	+	−	+	+	−	V600E	4.3	ND
29[b]	M	63	1.8	−	−	−	+	+	−	−	0.0	10.8

M male, *F* female, + presence, − absence, *M* multicentricity, *EE* extra-thyroid extension, *LI* lymph vessel invasion, *VI* vascular invasion, *LNM* lymph node metastases, *I* intratumoural, *P* peritumoural, *ND* not determined

[a] Cases without capsular invasion, [b] case with concomitant distant metastases

[b] Case with concomitant distant metastases

observed in 32.7% of the cases, and lymph node metastases were detected in 75.0% of the cases (p=0.026, p=0.014 and p=0.0006, respectively; Tables 1, 2 and 3).

The only clinico-pathological or molecular feature significantly associated with the occurrence of lymph node metastases was the presence of extra-thyroid extension (p= 0.0004; Tables 1 and 2). In cases without extra-thyroid extension, the occurrence of lymph vessel invasion was related to lymph node metastases (p=0.022).

The *BRAF* V600E mutation was detected in one case of E-FVPTC (8.3%), four cases of I-FVPTC (25.0%) and in 11 cases of CPTC (40.7%; Tables 1 and 2).

The *N-RAS* Q61R mutation was detected only in FVPTC cases (10.3%), encompassing two cases of E-FVPTC and one case of I-FVPTC (Tables 1 and 2).

D2-40 expression in intratumoural and peritumoural lymph vessels

The D2-40 expression in the endothelial cells in intratumoural location highlighted small thin-walled lymph vessels with tiny lumina located in the dependence of fibrous septa arising from the tumour capsule (Fig. 1a) or amongst tumour cells (Fig. 1b). The D2-40 positive lymph

Table 2 Clinical, morphological and molecular features of the 32 cases of CPTC and respective intratumoural and peritumoural LVD

Case	Gender	Age (years)	Tumour size (cm)	M	EE	LI	VI	LNM	*RAS*	*BRAF*	I-LVD	P-LVD
1	M	58	0.8	−	−	−	−	−	ND	ND	0.0	7.0
2	F	31	3.5	+	−	−	−	−	−	−	0.2	9.8
3	F	35	0.3	−	−	+	−	−	ND	ND	0.3	8.1
4	F	40	1.5	−	−	−	−	−	−	V600E	0.4	8.7
5	M	25	1.2	−	−	−	+	−	−	−	2.0	10.9
6	F	30	0.6	+	−	−	−	−	−	V600E	0.0	7.9
7	F	23	2.5	−	−	+	−	−	−	−	0.9	15.8
8	M	65	2.3	−	−	−	−	−	−	−	0.0	16.5
9	F	21	2.0	−	−	+	+	−	−	V600E	0.0	14.1
10	F	79	2.8	−	+	−	−	−	−	−	0.0	16.0
11	F	65	3.5	−	+	+	−	−	−	V600E	0.0	8.3
12	F	28	2.5	−	−	−	+	−	−	−	0.0	12.0
13	F	28	2.0	−	−	−	+	−	−	V600E	0.0	14.1
14	F	47	4.5	−	+	+	+	−	−	V600E	5.1	9.3
15	F	28	0.9	−	−	−	−	+	−	V600E	0.2	13.0
16	F	21	1.2	−	−	+	−	+	−	−	0.1	19.3
17	F	22	1.9	+	−	+	−	+	−	−	0.7	18.0
18	F	30	1.3	−	+	−	+	+	−	−	0.5	14.7
29	F	56	0.7	+	−	+	−	+	−	−	1.0	10.3
20	F	18	1.5	+	+	−	+	+	−	−	0.0	12.0
21	F	22	0.8	−	−	−	−	+	ND	ND	0.2	12.5
22	F	56	0.8	+	−	+	−	+	ND	ND	0.7	13.4
23	M	42	1.6	+	+	−	−	+	−	−	2.2	3.7
24	F	38	3.0	+	−	−	+	+	−	V600E	0.0	14.3
25	F	35	1.7	−	−	−	+	+	−	V600E	1.4	11.7
26[a]	M	11	6.0	+	+	+	+	+	−	−	0.4	14.3
27[a]	F	56	2.4	−	+	−	−	+	−	−	1.5	11.5
28	F	83	7.5	−	+	−	−	+	−	V600E	1.0	11.3
29	F	18	3.6	+	−	+	+	+	−	−	3.1	13.3
30	M	14	2.5	+	+	+	−	+	−	−	2.0	14.0
31	F	32	1.1	−	+	−	−	+	−	V600E	0.4	7.3
32	F	56	7.5	+	+	−	−	+	ND	ND	0.6	20.0

M male, *F* female, + presence, − absence, *M* multicentricity, *EE* extra-thyroid extension, *LI* lymph vessel invasion, *VI* vascular invasion, *LNM* lymph node metastases, *I* intratumoural, *P* peritumoural, *ND* not determined

[a] Case with concomitant distant metastases

vessels were found less often at the centre of the tumours than in the periphery, particularly at the invasive front of the infiltrative tumours.

In both FVPTC subgroups and in the CPTC group, there was, besides the subset of lymph vessels with D2-40 expression, a majority of small thin-walled, nearly collapsed, D2-40 negative capillary vessels of uncertain origin, and ectatic lymph vessels in the remaining thyroid tissue. Such D2-40 negative capillary vessels were morphologically indistinguishable from vessels with D2-40 expression (Fig. 1c, d).

D2-40 expression in the peritumoural thyroid tissue highlighted thin-walled ectatic lymph vessels (Fig. 1e) in all cases. Peritumoural lymph vessels with D2-40 expression showed, in some cases displaying infiltrative growth, neoplastic thrombi within their lumina (Fig. 1f). Neoplastic thrombi were not observed within the intratumoural thin-walled vessels without D2-40 expression.

All the blood vessels with an organised wall surrounded by a smooth muscle layer, in which the presence of vascular invasion was searched, did not disclose D2-40 expression in the endothelial cells (Fig. 1g). Neoplastic thrombi were also detected in peritumoural thick-walled vessels (Fig. 1h).

Complex networks of lymph vessels expressing D2-40 were detected in the peritumoural tissue in association with

Table 3 Clinical, morphological and molecular features of PTC cases

Features	E-FVPTC n(%)	I-FVPTC n(%)	CPTC n(%)	FVPTC vs CPTC *(p)*	E-FVPTC vs I-FVPTC *(p)*	E-FVPTC vs I-FVPTC+CPTC *(p)*
Gender				>0.999	>0.999	>0.999
Female	10 (83.3)	13 (76.5)	26 (81.3)			
Male	2 (16.7)	4 (23.5)	6 (18.7)			
Age				0.302	0.716	0.342
>45 years	6 (50.0)	7(41.2)	10 (31.3)			
≤45 years	6 (50.0)	10 (58.8)	22 (68.7)			
Tumour size				0.767	>0.999	>0.999
>1.0 cm	9 (75.0)	12 (70.6)	25 (78.1)			
≤1.0 cm	12 (25.0)	5 (29.4)	7 (21.9)			
M	4 (33.3)	8 (47.1)	12 (37.5)	0.798	0.703	0.749
EE	0 (0.0)	4 (23.5)	12 (37.5)	0.045	0.121	0.026
LI	0 (0.0)	4 (23.5)	12 (37.5)	0.094	0.059	0.014
VI	4 (33.3)	13 (76.5)	11 (34.4)	0.075	0.029	0.520
LNM	0 (0.0)	8 (47.1)	18 (56.3)	0.038	0.009	0.0006
RAS Q61R	2 (16.7)	1 (6.3)	0 (0.0)	0.236	0.355	0.117
BRAF V600E	1 (8.3)	4 (25.0)	11 (40.7)	0.080	0.560	0.147

M multicentricity, *EE* extra-thyroid extension, *LI* lymph vessel invasion, *VI* vascular invasion, *LNM* lymph node metastases

lymphoid aggregates and in perithyroidal lymph nodes with and without metastases (Fig. 1i, j).

Intratumoural LVD

In 11 out of 12 cases of E-FVPTC (91.7%), not a single intratumoural D2-40 stained vessel was observed (Table 1). Case 3 of the E-FVPTC subgroup was the only one with D2-40-positive intratumoural lymph vessels and was a *N-RAS* mutated tumour that did not show signs of extra-thyroid extension, lymph vessel invasion or vascular invasion, nor had given rise to lymph node metastases (Table 1). At variance with E-FVPTC, the majority of I-FVPTC cases (76.5%) had intratumoural D2-40 positive lymph vessels.

The mean intratumoural LVD was significantly higher in I-FVPTC (1.7±2.1) and in CPTC (0.8±1.1) than in E-FVPTC (0.05; p=0.020 and p=0.030, respectively; Fig. 2a). It was also higher in I-FVPTC than in CPTC (p=0.049; Fig. 2a).

The mean intratumoural LVD was significantly higher in tumours with extra-thyroid extension (p=0.041), lymph vessel invasion (p=0.026) and lymph node metastases (p=0.008; Table 4). In FVPTC cases, the mean intratumoural LVD was significantly higher in I-FVPTC cases with lymph node metastases (p=0.047) and significantly lower in E-FVPTC cases with wild-type *N-RAS* (p=0.016; Table 4).

Peritumoural LVD

The mean peritumoural LVD was similar in FVPTC (E-FVPTC, 12.8±3.6; I-FVPTC, 11.6±5.0, p=0.501) and in CPTC (12.3±3.7; p=0.888; Fig. 2b). In all PTC groups, the mean peritumoural LVD was significantly higher than the mean intratumoural LVD (p<0.0001), and it was not significantly associated with any clinico-pathological or molecular feature, namely lymph vessel invasion (p=0.626) or lymph node metastases (p=0.288).

Figure 3 highlights the most impressive differences observed between E-FVPTC and I-FVPTC+CPTC.

Discussion and conclusions

The results we have obtained show the different meaning of peritumoural and intratumoural lymph vessels. The abundance of D2-40 positive peritumoural lymph vessels was similar in the three groups of PTC (E-FVPTC, I-FVPTC and CPTC) as it had been previously reported by Giorgadze et al. [34] in their series of follicular patterned lesions. Peritumoural LVD was not related to any clinico-pathological feature, namely the occurrence of lymph vessel invasion or lymph node metastasis, thus supporting the results of Garcia et al. [21]. The mean peritumoural LVD determined by D2-40 expression was higher than intratumoural LVD as previously reported [21] and was similar in all groups of PTC cases, thus implying that such feature does not appear to be modulated by the tumour histotype.

In contrast to the abovementioned peritumoural findings, the search for intratumoural lymph vessels disclosed a clear cut difference between E-FVPTC and I-FVPTC and CPTC. D2-40 positive lymph vessels were not detected within 11 out of 12 tumours of the E-FVPTC group, a result that fits

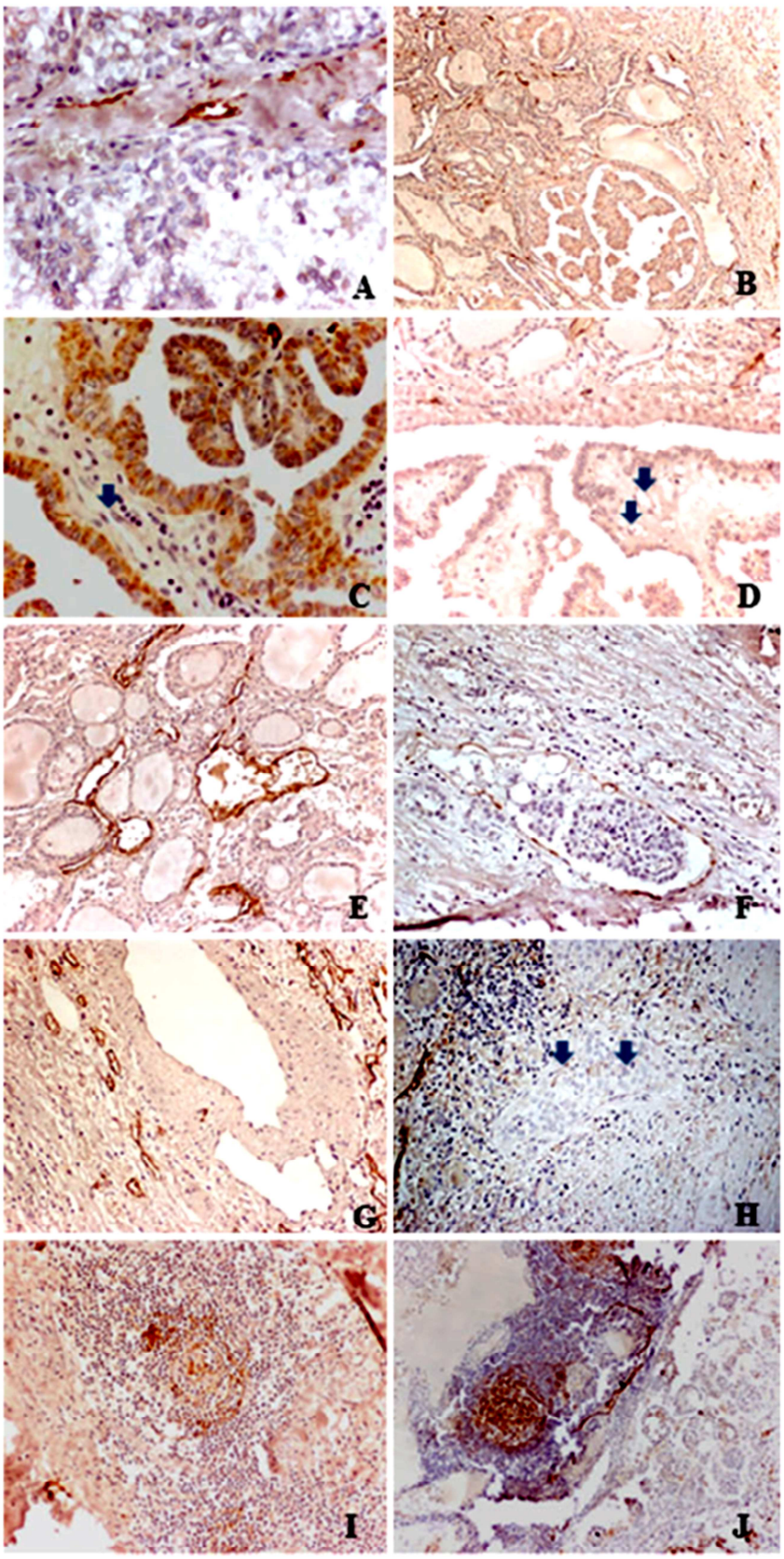


Fig. 1 D2-40 expression in the endothelial cells. **a** D2-40-positive intratumoural lymph vessels in a fibrous septum (case 14 CPTC, ×400); **b** D2-40-positive intratumoural lymph vessels between tumour cells at the invasive front (case 29 CPTC, ×100); **c** intratumoural lymph vessels D2-40 expression and thin walled vessels without D2-40 expression(*arrow*) (case 29 CPTC, ×400); **d** intratumoural thin walled vessels without D2-40 expression (*arrows*) in this area (case 29 CPTC, 200x); **e** D2-40-positive peritumoural lymph vessels (case 10 FVPTC, ×100); **f** D2-40-positive intratumoural lymph vessel with neoplastic thrombus in the lumen (case 22 FVPTC, ×200); **g** peritumoural medium sized vein without D2-40 expression surrounded by D2-40-positive small lymph vessels (case 7 FVPTC, ×200); **h** D2-40-negative peritumoural vein with neoplastic thrombus in the lumen (case 20 FVPTC, ×200); **i** complex network of D2-40-positive lymph vessels in the peritumoural tissue associated with a lymphoid aggregate (case 10 FVPTC, ×100) and **j** complex network of D2-40-positive lymph vessels in a perithyroidal lymph node with PTC metastases (case 27 CPTC, ×100)

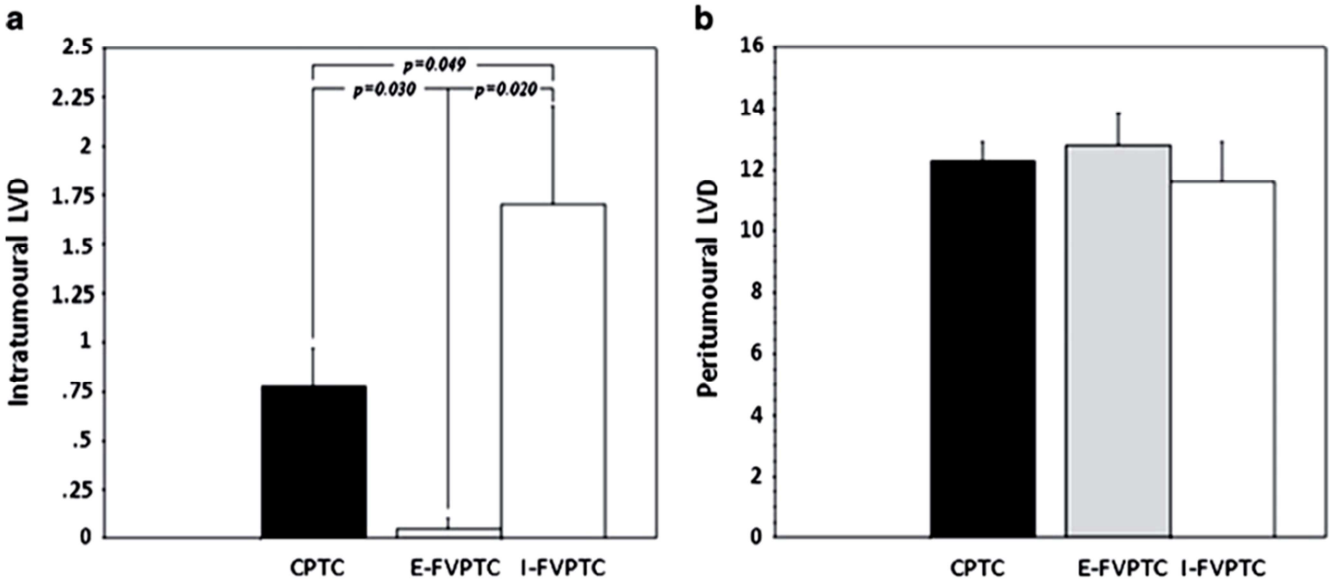


Fig. 2 Comparison between the LVD in the FVPTC group (E-FVPTC and I-FVPTC) and in the CPTC group. **a** Graphic representing the mean intratumoural LVD in the three PTC groups; **b** Graphic representing the mean peritumoural LVD in the three PTC groups

with the absence of lymph vessel invasion and lymph node metastases in E-FVPTC. A very low frequency of identifiable intratumoural lymph vessels had been reported by other groups in FA [16, 21, 34] and FTC [16, 34].

Table 4 Relationship between the intratumoural LVD and the morphological and molecular features of FVPTC (E-FVPTC and I-FVPTC) and CPTC cases

Features	E-FVPTC		I-FVPTC		CPTC		I-FVPTC+CPTC		PTC	
	Mean±SD	*p* value	Mean±SD	*p* value	Mean±SD	*p* value	Mean±SD	*p* value	Mean±SD	*p* value
M		0.170		0.930		0.616		0.696		0.560
Present	0.2±0.3		1.7±2.3		0.9±1.0		1.2±1.6		1.0±1.5	
Absent	0.0±0.0		1.7±2.1		0.7±1.2		1.0±1.6		0.8±1.4	
EE		–		0.289		0.156		0.181		0.041
Present	–		2.7±2.1		1.1±1.5		1.5±1.7		1.5±1.7	
Absent	0.1±0.2		1.4±2.1		0.6±0.8		0.9±1.5		0.7±1.3	
LI		–		0.363		0.104		0.135		0.026
Present	–		2.4±2.6		1.2±1.5		1.6±1.9		1.6±1.9	
Absent	0.1±0.2		1.4±1.9		0.5±0.7		0.6±1.3		0.6±1.2	
VI		0.506		0.715		0.192		0.078		0.052
Present	0.0±0.0		1.8±2.1		1.1±1.7		1.5±1.9		1.3±1.8	
Absent	0.1±0.2		1.4±2.3		0.6±0.7		0.7±1.0		0.6±1.0	
LNM		–		0.047		0.532		0.084		0.008
Present	–		2.8±2.2		0.9±0.9		1.5±1.6		1.5±1.6	
Absent	0.1±0.2		0.8±1.5		0.6±1.4		0.7±1.4		0.5±1.2	
RAS		0.016		0.439		–		0.468		0.404
Q61R	0.3±0.4		0.0±0.0		–		0.0		0.2±0.3	
Wild type	0.0±0.0		1.5±1.9		0.9±1.2		1.1±1.5		0.9±1.4	
BRAF		0.779		0.852		0.771		0.600		0.993
V600E	0.0		0.0		0.8±1.5		0.9±1.6		0.9±1.6	
Wild-type	0.1±0.2		1.5±1.9		0.9±1.0		1.2±1.5		0.9±1.0	

M multicentricity, *EE* extra-thyroid extension, *LI* lymph vessel invasion, *VI* vascular invasion, *LNM* lymph node metastases

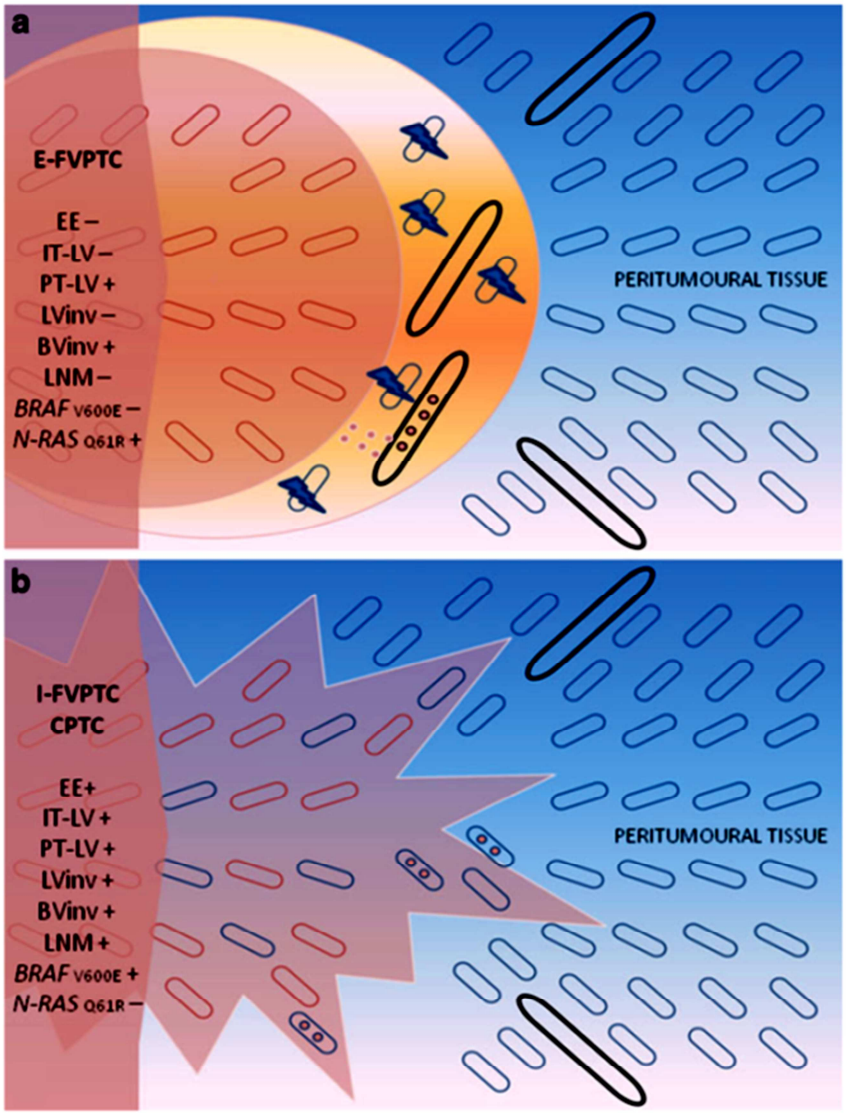


Fig. 3 Summary of the most impressive findings concerning the distinction between E-FVPTC (**a**) and I-FVPTC+CPTC (**b**) (*red vessels* D2-40-negative intratumoural thin-walled vessels; *blue vessels* D2-40 positive intratumoural and peritumoural lymph vessels; *black vessels* thick-walled veins; *blue thunder over blue vessel* collapsed D2-40-positive lymph vessel; *pink spots* neoplastic thrombi; *EE* extra-thyroid extension; *IT-LV* D2-40-positive intratumoural lymph vessels; *PT-LV* peritumoural lymph vessels; *LVinv* lymph vessel invasion; *BVinv* blood vessel invasion, *LNM* lymph node metastasis)

Together with the differences on the molecular features, i.e. the scarcity of *BRAF* V600E mutation [10] and prominence of *RAS* mutations [10], also confirmed in the present study, the rarity of extra-thyroid extension, intratumoural lymph vessels, lymph vessel invasion and lymph node metastases in E-FVPTC contributes to separate E-FVPTC from I-FVPTC and to approach the former to minimally invasive FTC [4, 9, 15]. It remains to be clarified whether the absence of intratumoural D2-40 positive lymph vessels in most E-FVPTCs, as well as in other follicular patterned tumours, reflects a low capacity of incorporation (with or without proliferation) of lymphatic vessels since the first steps of tumourigenesis, or their disappearance by collapsing mechanism (or by other mechanism) secondary to the increased intratumoural pressure, during the neoplastic development of encapsulated tumours [31].

The results we have obtained contribute also to show that I-FVPTC cases share most of the features of CPTC cases [4, 9]. In our series, this is particularly obvious regarding the poorly circumscribed peripheral growth pattern occasionally accompanied by vascular invasion and extra-thyroid extension, occurrence of *BRAF* V600E mutation, abundance of intratumoural lymphatic vessels, frequent lymph vessel invasion and frequent occurrence of lymph node metastases.

The significant association we observed between intratumoural LVD and occurrence of lymph node metastases confirms the data reported by several groups [22, 24, 25, 35] and contradicts others [16, 21]. In our series, lymph vessel invasion appears to be the second strongest factor associated with the occurrence of lymph node metastases after extra-thyroid extension. The relation between high intratumoural LVD determined by D2-40 and lymph node metastases is indirectly confirmed by the absence of nodal metastasis in E-FVPTC cases that do not have intratumoural lymphatic vessels despite the abundance of peritumoural lymph vessels.

There is not enough evidence in the literature to link mutations of oncogenes such as *BRAF* and *N-RAS* with proliferation of blood and/or lymph vessels. In the present series, the occurrence of *BRAF* mutation was not significantly associated with intratumoural LVD. The *N-RAS* mutation was detected in the only case of the E-FVPTC group with intratumoural lymph vessels, but it does not seem appropriate to draw any conclusions on the putative relationship between *N-RAS* mutation and lymphangiogenesis from just a single case.

In our series, as in others [35], all lymph vessels disclosing neoplastic thrombi in their lumina expressed D2-40, suggesting that the intratumoural D2-40 negative vessels may constitute less efficient pathways for PTC dissemination. It remains to be clarified if the D2-40 expression in such cases induced by the presence of the neoplastic thrombi or if intratumoural D2-40 vessels, mainly located at the invasive front, as observed by Hall et al. [35], is the result of the invasion of peritumoural tissues with subsequent incorporation of the originally peritumoural D2-40 positive lymph vessels in the bulk of the tumour. The latter hypothesis is favoured by the association we found between extrathyroid extension and intratumoural high LVD. In this scenario, the absence of an invasive front, as well as the presence of a well constituted capsule, could prevent the recruitment of D2-40 positive peritumoural lymph vessels and their invasion, as suggested by the nearly absence of D2-40 positive intratumoural lymph vessels in E-FVPTC of our series.

The peritumoural small sized veins, including capsular veins that presented luminal neoplastic trombi, point to unequivocal vascular invasion. Vascular invasion was observed in 26 out of 61 cases of PTC, but such invaded veins did not express D2-40 in the endothelial cells, arguing against the hypothesis that the expression of D2-40 may be induced in endothelial cells by neoplastic thrombi. The neoplastic thrombi seen in the thick-walled vessels may result from invasion of small intratumoural vessels or from the direct invasion of such thick-walled blood vessels, which present a well-developed muscle layer probably able to resist the effect of the intratumoural pressure over the capsule [31, 42]. The increment in the intratumoural interstitial pressure is thought to be partially due to the functionally impaired lymphatic system in tumours, with respect to fluid and macromolecules [43]. In our series, the number of cases with distant metastases is too low (data not shown) to allow the clarification of the role played by D2-40 positive and negative vessels in the occurrence of blood borne metastasising.

These findings highlight the role played by intratumoural lymphangiogenesis in lymph node metastisation at variance with the controversial importance of intratumoural angiogenesis in the metastatic potential of FTC and PTC [16, 18, 28–30]. While in CPTC, the most important pathway of initial dissemination appears to be via intratumoural lymph vessels, in encapsulated follicular patterned tumours, the invasion via veins of the capsule or outside the tumour is necessary to predict blood-borne metastasis [3, 44].

Summing up, intratumoural LVD determined by D2-40 expression correlates with the occurrence of extra-thyroid extension, lymph vessel invasion and lymph node metastases in PTC. Intratumoural D2-40 stained vessels are rare in E-FVPTC. At variance with intratumoural LVD, the peritumoural LVD is not associated with any clinical, morphological or molecular feature and is similar in E-FVPTC, I-FVPTC and CPTC. Our study highlights the role of intratumoural lymph vessels in PTC nodal metastisation and reinforces the importance of distinguishing E-FVPTC from I-FVPTC regarding invasiveness, metastatic pattern and molecular profile.

Acknowledgments This work was partially supported by a grant from Prof. Edward Limbert SPEND/GENZYME–Year 2007. Part of the data reported in this study were obtained in the project "Identificação de factores prognósticos e de selecção terapêutica em carcinomas diferenciados da tireoide", which is supported by Fundação Calouste Gulbenkian. IPATIMUP is an Associate Laboratory of the Portuguese Ministry of Science, Technology and Higher Education, and is partly supported by the Fundação para a Ciência e Tecnologia.

Conflict of interest The authors declare no conflict of interest.

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Paper 3

Eloy C, Santos J, Cameselle-Teijeiro J, Soares P, Sobrinho-Simões M
"TGF-beta/Smad pathway and BRAF mutation play different roles in circumscribed and infiltrative papillary thyroid carcinoma"
(aceite para publicação na revista *Virchows Archiv*)

TGF-beta/Smad pathway and *BRAF* mutation play different roles in circumscribed and infiltrative papillary thyroid carcinoma

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ABSTRACT

Poorly circumscribed growth pattern, extra-thyroid extension and high intratumoural lymph vessel density are significantly associated to nodal metastatization in papillary thyroid carcinoma (PTC). It was also shown that TGF-beta/Smad-dependent pathway activity is associated with local invasion, nodal metastatization and *BRAF* mutated PTCs.

We analyzed the immunoexpression of TGF-beta, Smad2/Smad3, Smad4, and Smad7 in a series of 42 cases of classic PTC and 33 cases of follicular variant of PTC with known clinico-pathological and follow-up data, as well as *BRAF* and *RAS status.* The 75 PTCs were divided into poorly circumscribed (PCPTC) (n=53) and well circumscribed (WCPTC) (n=22) according to their borders.

Nodal metastases were not detected in any WCPTC regardless the presence of immunoexpression for TGF-beta, Smad2/Smad3, Smad4 and Smad7, and occurrence of *BRAF* mutation (in 20% of WCPTCs).

Increased cytoplasmatic expression of TGF-beta at the periphery of PCPTC was associated to morphological features of invasiveness, featuring the so-called epithelial-to-mesenchymal transition (EMT), and presence of nodal metastases, as well as to the occurrence of *BRAF* mutation which did not significantly alter, *per se*, the frequency of nodal metastases.

The nuclear expression of Smad7 was more frequent in WCPTCs than in PCPTCs and was associated with unicentricity and absence of extra-thyroid extension, vascular invasion and nodal metastases.

We conclude that nodal metastases are associated to poorly circumscribed, locally invasive PTCs that exhibit low levels of nuclear Smad7 and a peripheral EMT phenotype displaying TGF-beta overexpression, regardless of the occurrence of *BRAF* mutation.

Key words: Papillary thyroid carcinoma; BRAF; TGF-beta; Smad7; prognosis.

INTRODUCTION

The large majority of papillary thyroid carcinomas (PTC) evolve favourably [1] although a subset of cases may progress adversely leading to the development of recurrences and/or metastases [1-3].

Most cases of classic PTC (CPTC) are poorly circumscribed, display a peripheral invasive front and give frequently rise to lymph node metastases [1], whereas many cases of the follicular variant of PTC (FVPTC) are encapsulated, sharing some of the clinico-pathological features of follicular carcinoma such as vascular invasion and distant metastases, as well as its most frequent molecular alterations, *RAS* mutations and *PAX8/PPARgamma* rearrangements [4-7]. There is also a poorly circumscribed/ infiltrative subtype of FVPTC that, like CPTC, discloses a high intratumoural lymph vessel density and is prone to give rise to lymph node metastases [5,6,8].

It has been shown that *BRAF* V600E mutation is particularly prevalent in CPTC, as well as in PTC with a prominent papillary growth pattern such as Warthin-type and tall cell variant of PTC [9], in contrast to its low prevalence in FVPTC [9-11].

In a recent clinico-pathological study of 75 cases of PTC (42 CPTC and 33 FVPTC) we observed that poorly circumscribed/ infiltrative growth pattern and extra-thyroid extension, rather than the presence of *BRAF* V600E, were significantly associated with the occurrence of lymph node metastases [11]. The infiltrative growth pattern of most non-

encapsulated forms of PTC is mainly the result of increased motility and of tumour cell´s invasiveness [12-14] which are features related to the so called epithelial-to-mesenchymal transition (EMT). At PTC´s infiltrative periphery, the neoplastic cells display loss of polarity/cohesiveness [14], decreased expression of beta-catenin and E-cadherin at the cell membrane [14], increased nuclear expression of STAT3[Couto *et al*, unpublished results], increased cytoplasmatic expression of vimentin [15], fibronectin 1[12], met [16], ret [17], cytokeratin 5/6 [18] and galectin-3 [19], and increased nuclear expression of cyclin D1[20].

The above mentioned alterations at the infiltrative edge of the tumours reflect probably the cross-talk between tumour cells and cellular and non-cellular elements of the stroma, in part mediated by integrin-related epithelial-to-stromal connections which are enhanced at PTC´s periphery [21]. Dendritic cells were found to be located at the tumour´s invasion edge in a small series of PTC [22] and, together with lymphocytes, appear to be decreased in PTCs with distant metastases [23], while increased density of tumour-associated macrophages in poorly differentiated carcinomas correlate with invasion and decreased survival [24].

EMT is partially regulated by transforming growth factor beta (TGF-beta) in PTC cell lines [25-28] and in animal models [29]. TGF-beta is one of the members of the TGF-beta superfamily and occurs in 3 isoforms – TGF-beta1, TGF-beta2 and TGF-beta3 – that function *via* the same membrane receptor signaling system [30]. In TGF-beta/Smad-dependent pathway, the activated TGF-beta receptor complex propagates the signal inside the cell through the phosphorylation of receptor regulated Smad2 and Smad3 downstream proteins localized in the cytoplasm [30-32]. Phosphorylated Smad2 and Smad3 form heteromeric complexes with Smad4 and re-localize to the nucleus where they regulate gene transcription [30-32]. Inhibitory Smad7 acts in an opposite manner and antagonizes TGF-beta/Smad-dependent signaling, inducing TGF-beta receptor degradation and thus preventing Smad2 and Smad3 phosphorylation [31, 32]. TGF-beta/Smad-dependent pathway activation induces major growth suppressive effects on epithelial cells in general [32], and on animal [33-35] and human [36, 37] thyroid cell lines, as well as on some PTC cell lines [25,28], in particular. TGF-beta is also related to inhibition of migration and invasiveness in some PTC cell lines [25].

The expression of TGF-beta, Smad2, Smad3 and Smad7 have been detected in multinodular goiters [38-40] and in follicular adenomas of some series [40,41], but not in other series of thyroid lesions where TGF-beta was the only element searched [42].

In tumours, TGF-beta expression has been detected in 52-100% of PTC cases [38,40-43] and, whenever specifically studied at the tumour´s periphery, was associated to the occurrence of extra-thyroid extension and lymph node metastases [26]. Similarly, phosphorylated Smad2/Smad3(P-Smad2/3), has been detected in every case of PTC [40], while Smad4 is expressed in 75% of PTC cases [40] and in every PTC cell line [44,45]. Smad7 expression has been detected in 80% of PTCs and PTC cell lines [40,44].

In tumour cells, particularly when exhibiting an EMT phenotype characterized by mesenchymal-type features [12], the response to TGF-beta growth suppressive effect can be partially or totally abrogated *via* interferences in the signaling pathways that mediate its action [26,29,37,46-48]. TGF-beta anti-proliferative effects were reported as attenuated in cases of multinodular goiter [49], in anaplastic carcinoma cell lines [50], as well as in rat thyroid cell lines [51]. At the EMT, the TGF-beta altered cell response has been associated to the development of pro-oncogenic effects, namely through the inhibition of cell adhesion and enhancement of motility [32,52].

Besides playing a functional role in tumour cell migration, invasion and EMT *in vitro*, TGF-beta and high TGF-beta/Smad-dependent pathway activity are associated with invasion, nodal metastasizing and *BRAF* mutated PTCs [26]. It has been reported that oncogenic *BRAF* stimulates TGF-beta secretion *in vitro* [26]. *BRAF* is thought to play a major role in extracellular matrix remodeling [52] and is required for TGF-beta-induced EMT during progression from PTC to poorly differentiated carcinoma in an animal model [29]. Finally, it has been shown that *BRAF* suppresses the expression of E-cadherin, which is a typical finding at the infiltrative edge of poorly circumscribed PTCs, and that this effect can be abrogated with both TGF-beta and MAPK inhibitors [26].

We decided to analyze the immunohistochemical expression of TGF-beta1 and TGF-beta2 and their downstream phosphorylated Smad2/Smad3 and Smad4 elements, and of the inhibitory Smad7, in well circumscribed and poorly circumscribed PTCs (WCPTC and PCPTC, respectively). The study was made using the previously analyzed series of 75 carcinomas in which *BRAF* and *N-RAS status* had been characterized [11], in order to clarify the putative relationship between functioning TGF-beta/Smad-dependent pathway and *BRAF* mutation in the two PTC subtypes: WCPTC and PCPTC.

MATERIAL AND METHODS

Part of the clinico-pathological data of the 75 cases has been previously reported [8,11].

Tumour samples

A series of 75 PTC cases was retrieved from the Cancer Registry of the Pathology Department of Hospital de São João: 42 CPTC and 33 FVPTC. Both groups (CPTC and FVPTC) included cases with lymph node metastases (+M) (mean follow-up of 5.9 years; range 1 to 14 years) and cases without metastases and long term follow-up (-M) (mean follow-

up of 18.8 years; range 10 to 24 years). Three cases (two CPTC and one FVPTC) disclosed, together with lymph node metastases, lung metastases (Tables 1 and 2). Both groups included tumours larger than 1cm and small tumours, 1cm or less in diameter, that were classified according to their pattern of growth as CPTC or FVPTC. None of the patients died from the disease.
All samples had been fixed in formalin and included in paraffin. Clinical data were collected from the pathology reports and clinical files. H&E slides were retrieved from the archives and reviewed independently by two pathologists (CE and MSS) and the tumours were classified using the WHO criteria [1]. Cases with doubtful PTC features were excluded. Due to their scarcity, variants of PTC such as tall cell, columnar cell and sclerosing diffuse variant were not included. Well circumscribed carcinomas (WCPTC) were those with expansive growth pattern along the whole periphery and those limited by a capsule with none or only minimal focal invasion. Poorly circumscribed carcinomas (PCPTC) were those with infiltrative growth pattern or with an extensively invaded capsule. Vascular invasion was considered to be present whenever one image of unequivocal venous vessel invasion was observed.

Mutation screening

The detection of *BRAF* (exon 15) and *N-RAS* (exon 2) mutations were performed as previously described [11] in 10 μm sections of 65 tumours: 34 CPTC and 31 FVPTC. The remaining 10 cases had insufficient available material for performing the molecular study.

Immunohistochemistry

Immunohistochemical staining was performed using streptavidin-biotin technique as previously described [8]. Briefly, slides were incubated overnight at 4ºC with TGF-beta1/2 rabbit polyclonal antibody (from here on designated as TGF-beta) in a 1:50 dilution (TGF-beta1(V), Santa Cruz Biotechnology, CA) against a peptide in the C-terminus of precursor and mature human TGF-beta1 and TGF-beta2 ligands; P-Smad2/3 rabbit polyclonal antibody in a 1:100 dilution (P-Smad2/3 (Ser 423/425), Santa Cruz Biotechnology, CA) against C-terminus of both phosphorylated Smad2 and Smad3; Smad4 monoclonal mouse antibody in a 1:100 dilution (Smad4 (B-8), Santa Cruz Biotechnology, CA) and Smad7 mouse monoclonal antibody in a 1:100 dilution (Smad7 (Z8-B), Santa Cruz Biotechnology, CA). Then, slides were incubated with biotinylated secondary goat antibody and streptavidin-peroxidase complex (Lab Vision, Thermo Scientific) for 10 minutes at room temperature. Tissue microarrays with adequate positive and negative controls were used in every set of stainings.

Semiquantitative assessment of TGF-beta and Smads expression

The TGF-beta expression in the tumour cells was evaluated in the center and in the periphery of all carcinomas (CPTC and FVPTC) and in the respective remaining thyroid tissue, which was used as an internal control tissue, in order to reduce the influence of fluctuations of the physiological expression in each case. TGF-beta expression detected in cells of the control tissue was considered as the basal expression.
TGF-beta expression in the tumour cells located in the center of each tumour was compared with the expression in the respective control tissue and the TGF-beta expression in the tumour cells located in the periphery of each tumour was compared with the expression in the respective center of the tumour. The mean expressions observed in each location were also compared. The center of the tumour comprised the bulk of the tumour, i.e., the whole neoplastic tissue except the subcapsular areas and/or the invasive front. The periphery of the tumour comprised the rim of tumour cells underneath the capsule or at the expansive periphery in WCPTC and in the invasive front in PCPTC. TGF-beta expression was cytoplasmatic and was graduated in 4 categories that were considered to represent continuous and crescent immunostaining intensity: negative 0 (weak staining in less than 25% of cells), positive 1 (weak staining in at least 25% of cells or moderate intensity in less than 25% of cells), positive 2 (moderate intensity in at least 25% of cells or intense staining in less than 25% of cells) and positive 3 (intense staining in at least 25% of cells).
The expression of P-Smad2/3, Smad4 and Smad7 was nuclear and cytoplasmatic and was evaluated in all CPTC and FVPTC tumours and in the respective remaining thyroid tissue, which was used as an internal control tissue and considered the basal expression. The expression of P-Smad2/3, Smad4 and Smad7 in the center of the tumour and at the periphery were considered too difficult to distinguish and, therefore, assumed as similar. The expression was graduated in 2 categories: negative (weak staining in less than 25% of cells) and positive (weak, moderate or intense staining in at least 25% of cells), regarding each subcellular location (nuclear or cytoplasmatic) separately.

Statistical analysis

The results are expressed as absolute values, percentages and/or mean±standard deviation. The χ^2 test with Yates correction and the Students‘t test (unpaired) were used in the statistical analysis. Two values were considered significantly different whenever $p<0.05$ and suggestively different whenever $p<0.1$.

RESULTS

1. Clinico-pathological and molecular features

CPTC and FVPTC groups displayed similar features except the prevalence of poorly circumscribed tumours (83.3% of CPTC and 54.4% of FVPTC, p=0.010), vascular invasion (26.6% of CPTC and 57.6% of FVPTC, p =0.018) and *BRAF* V600E mutation (41.2% of CPTC and 16.1% of FVPTC, p =0.032). A detailed description of the clinico-pathological and molecular features of the 75 cases has been previously reported [8,11].

2. CPTC versus FVPTC

2a. TGF-beta expression

TGF-beta expression was observed in all but two FVPTC-M cases and was detected in the center of 88.0% of tumours (95.2% of CPTC and 81.8% of FVPTC), at the periphery of 94.7% of tumours (100% of CPTC and 87.9% of FVPTC) and in the control tissues in 73.0% of cases (78.6% of CPTC and 63.3% of FVPTC).
In comparison with the respective control tissue, the center of the carcinomas disclosed basal or overexpression of TGF-beta in 83.8% of cases. The TGF-beta expression at the periphery of each tumour was similar or higher than the TGF-beta expression in the center in all but one CPTC-M and three FVPTC-M cases (94.7%) (Tables 1 and 2) (Fig1).
The mean intensity of TGF-beta expression in the center of the tumours, in comparison with the respective control tissue, was much higher in PTC-M than in PTC+M (p<0.0001).The mean intensity of TGF-beta expression at the periphery, compared with the respective center of the tumours, was significantly higher in PTC+M than in PTC-M (p=0.0001) and was also significantly higher in tumours with poorly circumscribed growth pattern (p<0.0007), extra-thyroid extension (p=0.002) and *BRAF* V600E mutation (p=0.029).
The supplemental table summarizes the result of the comparison between CPTC and FVPTC.

2b. P-Smad2/3 expression

P-Smad2/3 nuclear expression was observed in all tumours (Figs. 2A and 2B) and in all but two control tissues of the CPTC-M group (97.3%) (Fig. 2C).
P-Smad2/3 cytoplasmatic expression was observed in 72.0% of tumours and in 18.9% of control tissues. In comparison to the respective control tissue, P-Smad2/3 had a basal level or was underexpressed in the cytoplasm of tumour cells in 43.2% of the cases. Tumours with basal or underexpression of cytoplasmatic P-Smad2/3 belonged more frequently to the group of FVPTC than to the CPTC group (60.6% and 29.3%, respectively; p=0.010) and tended to have more often wild type *BRAF* than mutated *BRAF* (p=0.053) (Tables 1 and 2). P-Smad2/3 expression was not significantly associated to any other clinical, pathological or molecular features.

2c. Smad4 expression

Smad4 nuclear expression was observed in 26.7% of tumours and 85.1% of control tissues (Figs. 2D, 2E and 2F). Smad4 had a basal level or was overexpressed in the nuclei of tumour cells in 39.2% of the cases in comparison with the respective control tissue. Vascular invasion was significantly less frequent in tumours with basal or overexpressed nuclear Smad4 (p=0.004), particularly in the FVPTC group (p=0.003).
Smad4 cytoplasmatic expression was observed in all tumours and in all but 2 control tissues of the PTC-M group (97.3%).

2d. Smad7 expression

Smad7 nuclear expression was observed in 69.3% of tumours and in all but two control tissues of the CPTC-M group (97.3%) (Figs. 2G, 2H and 2I).
Smad7 had a basal level or was overexpressed in the nuclei of the tumour cells in 71.6% of tumours in comparison with the respective control tissue. There was a significant association between basal to overexpressed Smad7 in the nuclei of the tumour cells and well circumscribed growth pattern (p=0.0001), unicentric CPTC (p=0.048), absence of vascular invasion in FVPTC (p=0.013), absence of extra-thyroid extension in FVPTC (p=0.023) and absence of lymph node metastases in both CPTC and FVPTC (p<0.0001).
Smad7 cytoplasmatic expression was observed in 66.7% of tumours and in 14.9% of the control tissues. Smad7 had a basal level or was underexpressed in the cytoplasm of the tumour cells in 48.6% of cases in comparison with the

respective control tissue, both in CPTC and FVPTC. Smad7 cytoplasmatic expression was not significantly associated to any clinical, pathological or molecular feature.

3. WCPTC versus PCPTC

None of the 22 WCPTC had lymph node metastases while 50.9% of the 53 PCPTC had given rise to lymph node metastases.
Globally, the difference between the mean TGF-beta expression in the center of the tumours and in the control tissue was similar in WCPTC and in PCPTC (p=0.839) whereas the difference between the mean TGF-beta expression in the periphery of the tumours and in the center of the tumours was much higher in PCPTC than in WCPTC (p=0.0007).
In 63.6% of WCPTC cases (14 out of 22) the TGF-beta expression was nearly homogeneous, absent or low/moderate, throughout the tumour. In PCPTC, the PTC-M cases disclosed a significantly more intense expression of TGF-beta in the center of the tumours than in the control tissue (p<0.0001), whereas in PTC+M cases the cells in the periphery of the tumours presented a significantly more intense expression of TGF-beta than those in the center of the tumours (p=0.017) (Table 3).
The *BRAF* V600E mutation was detected in 20.0% of WCPTC including 4 WCPTC cases disclosing similar or higher TGF-beta expression in the periphery than in the center, while none of the 3 WCPTCs with a lower TGF-beta expression in the periphery than in the center were *BRAF* mutated tumours. The group of WCPTC cases with similar or higher TGF-beta expression at the periphery than in the center had similar clinico-pathological features and no lymph node metastases, independently of the *BRAF* mutational *status* (Tables 1 and 2).
The *BRAF* mutation was detected in 15 PCPTCs (33.3%) disclosing similar or higher TGF-beta expression in the periphery than in the center. PCPTC cases with similar or higher TGF-beta expression at the periphery than in the center disclosed lymph node metastases in 40.0% of *BRAF* mutated tumours and in 56.7% of tumours with wild-type *BRAF*.
Smad4 nuclear expression in WCPTC subgroup was detected only in tumours without vascular invasion (p=0.005) (Table 3), whereas in PCPTC, Smad4 nuclear expression was not significantly associated to any clinical, pathological or molecular features.

DISCUSSION AND CONCLUSIONS

The results we have obtained confirm the prognostic prominence of staging over cytological and molecular features, and the major importance of architecture (expansive *versus* infiltrative growth pattern) and topography (different immunohistochemical characteristics of tumour cells according to their peripheral or central location) in the understanding of PTC development.
It is known that the neoplastic cells at the tumour periphery differ substantially from those in the bulk of the tumours regarding the expression of several structural and functional proteins (e.g. cytokeratins, vimentin, beta-catenin, E-cadherin, fibronectin 1, TGF-beta) [12,15,18,26].
The role of TGF-beta in thyroid cell signaling and in thyroid carcinogenesis, like in many other models, is very complex and profoundly influenced by the cellular and tissue context [26], thus being better evaluated in tissue samples than in cell lines. In the present study, we observed signs of the dual role of TGF-beta regarding the co-existence in the same tumour of signs of activation and of inhibition of TGF-beta/Smad-dependent pathway according to the location of the neoplastic cells.
The tumour suppressive effect of TGF-beta/Smad-dependent pathway that had been firmly established in thyroid cell lines [33-37] and in some PTC cell lines [25, 28], was found to be abrogated at the periphery of PTC [12,26], where increased vimentin expression and loss of E-cadherin with increased motility and invasiveness configure an EMT invasive phenotype [12]. We observed, particularly in the poorly circumscribed tumours, that TGF-beta expression was stronger in the invasive front than in the central regions of the tumours, and that an increased TGF-beta expression at the periphery of the tumours was associated to the occurrence of extra-thyroid extension and lymph node metastases, as previously reported by Riesco-Eizaguirre *et al* [26].
Our subdivision of PTCs into well and poorly circumscribed carcinomas (WCPTC and PCPTC, respectively) goes further than the usual division into CPTC and FVPTC and provides interesting results: in WCPTC (no infiltrative features and no lymph node metastases) the mean increment of TGF-beta immunoexpression from the center to the periphery was very low in comparison with PCPTC, in line with the above mentioned association between TGF-beta expression at the periphery and invasiveness, also reported by other authors [26].
We detected TGF-beta and P-Smad2/3 expressions in the center of all cases of WCPTC and PCPTC and control tissues (except in nine TGF-beta negative cases), as it had been reported previously [38,40,42, 43], thus highlighting the existence of signs of functioning TGF-beta/Smad-dependent pathway in PTC.
Smad4 nuclear expression is considered as indicative of a functioning TGF-beta/Smad-dependent pathway [40,44,45], but the nuclear translocation of phosphorylated Smad2 in response to TGF-beta, does not require the presence of Smad4 [30], as our results also indicate. In the present study, all tumours expressed Smad4 in the cytoplasm of the neoplastic

cells, like it had been observed by Matsuo *et al* [40] and Cerutti *et al* [44], whereas Smad4 nuclear expression was detected in 27% of tumours. In WCPTC, Smad4 nuclear expression was detected only in cases without vascular invasion. The nuclear expression of Smad4 in PTC cells had not been detected previously to the best of our knowledge. The percentage of cases with Smad4 nuclear expression may be related with an impaired nuclear translocation of Smad2/3-Smad4 complexes as a sort of end result of Smad4 mutations that were detected in another series in 21% of PTCs [53]. Unfortunately, the absence of available material for the molecular search of Smad4 mutations in the present series did not allow the confirmation or the rejection of this hypothesis. Whatever the mechanisms involved in the process, we found that signs of functioning TGF-beta/Smad-dependent pathway in the center of the tumours, namely basal or overexpression of TGF-beta and nuclear Smad4, were associated to low vascular invasiveness and absence of lymph node metastases, respectively.

Smad7 nuclear expression has been associated to loss of TGF-beta/Smad-dependent pathway inhibition [54] and was found to be at basal or overexpressed levels in 72% of the cases of the present series, a percentage similar to that reported in another series of PTC [40]. We found that Smad7 nuclear expression was associated to low neoplastic invasiveness, namely to well circumscribed growth pattern, and to unicentricity in CPTC and to absence of extra-thyroid extension and vascular invasion, in FVPTC. More importantly, Smad7 nuclear expression was associated to absence of lymph node metastases both in CPTC and FVPTC.

The encapsulated, non-angioinvasive FVPTC is the prototype of a carcinoma with an extremely benign course [55] and discloses a very low intratumoural lymph vessel density [8]. In the present series, and in almost every series on record, most WCPTCs display a capsule and are apparently protected from the cascade of TGF-beta oncogenic effects occurring at the periphery of PCPTC. The WCPTCs that do not disclose invasive front and EMT features (independently of the occurrence of *BRAF* mutation), nor lymph node metastases, present often a nearly homogeneous TGF-beta expression throughout the tumour.

At variance with TGF-beta, *BRAF* mutation has been found to promote growth and cell invasiveness [56]. The putative relationship between alterations of the TGF-beta/Smad-dependent pathway and *BRAF* mutation is difficult to evaluate in an observational study like the present one. Despite acknowledging this limitation we think our findings suggest the existence of a synergic influence of TGF-beta over *BRAF* mutated neoplastic cells in the periphery of the tumours.

BRAF V600E mutation was more frequently detected in the subset of tumours with higher increment of TGF-beta expression at the periphery. This finding concurs with the results of Riesco-Eizaguirre *et al* [26] and suggests that, at the periphery of the tumours, the association of high levels of TGF-beta expression with *BRAF* mutation and invasiveness, as previously described in animal models [29], PTC cell lines and PTCs [26], reflects a synergic interaction that may potentiate the oncogenic effect of *BRAF* mutation [11]. In the present series, the occurrence of *BRAF* mutation did not significantly alter, *per se*, the frequency of lymph node metastases, thus highlighting the complexity of the process and reinforcing the importance of growth pattern and invasion.

Considering that the increased expression of TGF-beta at the periphery of the tumours may functionally articulate with *BRAF* V600E mutation [29], it remains unclear the reason underlying this particular topographic immunoexpression of TGF-beta. Since the *BRAF* mutation occurs, at least in most *BRAF* positive cases, in every tumour cell, the TGF-beta expression increment should be detected, in case it was a direct result of the *BRAF* mutation, throughout the whole neoplastic tissue. It is likely that this increment of TGF-beta at the periphery of the tumours is influenced by the crosstalk between the neoplastic cells and cellular and non-cellular stromal elements, such as tumour-associated macrophages [24,29], dendritic cells [22], lymphocytes [23] and basement membrane components [29]. A similar interaction with *BRAF* has been recently described with STAT3 which is highly expressed in the periphery of *BRAF* mutated PTCs and has been found to result from the crosstalk between neoplastic cells and stromal elements [Couto *et al*, unpublished results]. The aforementioned need of co-existing factors in the setting of *BRAF* oncogenic mutation provides an explanation for the low prevalence of *BRAF* positive PTCs in young patients [11].

Other interactions between TGF-beta signaling pathways and MAPK pathway have been advanced, namely through oncogenic *RAS* that is thought to be required for the disruption of TGF-beta-growth suppressive effects in cell lines [51]. In our series, *N-RAS* Q61R mutation occurred in 6% of cases without any significant association with pathological signs of invasiveness but the number of positive cases is too small to allow any definite conclusion on this issue.

Summing up, the signs of functioning TGF-beta Smad-dependent signaling pathway in the central areas of the tumours are associated to low invasiveness and absence of lymph node metastases while, at the periphery, TGF-beta expression is associated to invasiveness and occurrence of lymph node metastases, both in CPTC and in cases of the poorly circumscribed form of FVPTC. Our findings support the assumption that TGF-beta acts synergically with *BRAF* and probably with other cellular and molecular stromal factors at the periphery of the tumours, leading to neoplastic progression and acquisition of invasive properties by cancer cells.

Our results indicate that Smad7 nuclear expression in the center of the tumours appears to be a marker of favourable evolution, unrelated to the occurrence of *BRAF* mutation. We suggest that preoperative detection of Smad7 nuclear expression should be evaluated by fine-needle aspiration in a large series of PTCs with thorough clinical, imaging and follow-up data, as it may constitute a valuable tool for risk stratification of patients.

We conclude that nodal metastases are associated to poorly circumscribed, locally invasive PTCs that exhibit low levels of nuclear Smad7 and a peripheral EMT phenotype displaying TGF-beta overexpression, regardless of the occurrence of *BRAF* mutation.

Disclosure/Conflict of interests

The authors declare no conflict of interest.

Acknowledgements

This work was partially supported by the grant Prof. Edward Limbert SPEND/GENZYME – Year 2007. Part of the data reported in this study was obtained in the project "Identificação de factores prognósticos e de selecção terapêutica em carcinomas diferenciados da tireoide," which is supported by Fundação Calouste Gulbenkian. IPATIMUP is an Associate Laboratory of the Portuguese Ministry of Science, Technology and Higher Education, and is partly supported by the Fundação para a Ciência e Tecnologia. J. C-T was supported by grant PS09/02050-FEDER from Instituto de Salud Carlos III, Ministry of Science and Innovation, Spain.

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Table1. Clinico-pathological, molecular and immunohistochemical features of the CPTC cases

Case number	Gender	Age (years)	Follow-up (years)	Tumour size (cm)	Growthpattern	Multicentricity	Extra-thyroid extension	Vascular invasion	*BRAF*	*N-RAS*	Intensity of expression			P-Smad2/3 C (n/c)	P-Smad2/3CT (n/c)	Smad4 C (n/c)	Smad4 CT (n/c)	Smad7 C (n/c)	Smad7 CT (n/c)	LNM
											TGF-beta C	TGF-beta P	TGF-beta CT							
1	M	58	17	0.8	PC	-	-	-	ND	ND	3	2	1	+/-	-/+	-/+	-/+	+/+	-/+	-
2	F	66	19	1.5	WC	-	-	-	ND	ND	2	2	1	+/+	+/-	-/+	+/+	+/+	+/-	-
3	F	31	21	3.5	PC	+	-	-	-	-	3	3	2	+/+	+/-	-/+	+/+	+/+	+/-	-
4	F	59	21	0.6	PC	+	-	-	-	-	2	2	1	+/+	+/-	-/+	+/+	+/+	+/-	-
5	F	37	21	1.0	WC	+	-	-	ND	ND	3	3	2	+/-	+/+	-/+	-/+	+/+	+/+	-
6	F	35	18	0.3	PC	-	-	-	ND	ND	2	3	0	+/-	+/-	+/+	+/+	+/-	+/-	-
7	F	40	18	1.5	PC	-	-	-	V600E	-	3	3	1	+/+	+/-	-/+	-/+	+/-	+/-	-
8	F	38	18	0.5	PC	-	-	-	ND	ND	2	2	0	+/+	+/+	-/+	-/+	+/+	+/-	-
9	M	25	20	1.2	PC	-	-	+	-	-	2	1	1	+/+	+/-	-/+	-/+	+/+	+/-	-
10	F	30	21	0.6	PC	+	-	-	V600E	-	2	3	2	+/+	+/-	-/+	+/+	-/+	-/+	-
11	F	40	20	2.2	WC	-	-	-	-	-	1	2	2	+/+	+/-	-/+	-/+	+/+	+/+	-
12	F	50	20	3.0	WC	-	-	-	V600E	-	1	1	0	+/-	+/-	-/+	-/+	+/-	+/-	-
13	F	25	17	2.1	WC	-	-	-	V600E	-	1	1	1	+/+	+/-	-/+	+/+	+/+	+/-	-
14	F	45	22	4.5	WC	+	-	-	V600E	Q61R	2	3	2	+/-	+/+	+/+	-/+	+/+	+/-	-
15	F	23	24	2.5	PC	-	-	-	-	-	3	3	2	+/+	+/+	-/+	+/+	+/+	+/+	-
16	M	65	24	2.3	PC	-	-	-	-	-	3	3	2	+/+	+/+	-/+	+/+	+/+	+/+	-
17	F	21	19	2.0	PC	-	-	+	V600E	-	1	2	0	+/+	+/-	-/+	+/+	+/+	+/-	-
18	F	79	23	2.8	PC	-	+	-	-	-	3	3	1	+/+	-/-	-/+	-/+	+/+	+/-	-
19	F	65	16	3.5	PC	-	+	-	V600E	-	1	2	0	+/+	+/-	-/+	+/-	+/-	+/-	-
20	F	28	24	2.5	PC	-	-	+	-	-	3	3	0	+/+	+/+	-/+	+/+	+/+	+/-	-
21	F	28	20	2.0	PC	-	-	+	V600E	-	3	3	0	+/+	+/-	-/+	+/+	+/+	+/+	-
22	F	43	18	1.5	WC	+	-	+	-	-	1	1	0	+/+	+/-	-/+	+/+	+/+	+/+	-
23	F	47	16	4.5	PC	-	+	+	V600E	-	1	3	1	+/+	+/-	-/+	+/+	+/+	+/-	-
24	F	28	5	0.9	PC	-	-	-	V600E	-	1	2	1	+/+	+/-	+/+	+/+	+/+	+/-	+
25	F	21	6	1.2	PC	-	-	-	-	-	1	2	2	+/+	+/+	+/+	+/+	-/+	+/-	+
26	F	22	14	1.9	PC	+	-	-	-	-	1	1	1	+/+	+/-	-/+	+/+	+/+	+/-	+
27	F	30	3	1.3	PC	-	+	+	-	-	2	3	2	+/+	+/-	-/+	+/+	-/+	+/-	+
28	F	56	3	0.7	PC	+	-	-	-	-	3	3	2	+/+	+/-	-/+	+/+	-/+	+/-	+
29	F	18	3	1.5	PC	+	+	+	-	-	3	3	2	+/+	+/-	+/+	+/+	-/+	+/-	+
30	M	22	4	1.1	PC	+	-	-	-	-	2	3	2	+/-	+/-	+/+	+/+	-/-	+/-	+
31	F	22	6	0.8	PC	-	-	-	ND	ND	1	2	2	+/+	+/-	-/+	+/+	-/+	+/-	+
32	F	56	3	0.8	PC	+	-	-	ND	ND	1	2	1	+/+	+/-	-/+	+/+	-/+	+/+	+
33	M	42	5	1.6	PC	+	+	-	-	-	2	3	3	+/+	+/-	-/+	+/+	-/+	+/-	+
34	F	38	7	3.0	PC	+	-	+	V600E	-	1	1	2	+/+	+/-	-/+	+/+	-/+	+/-	+
35	F	35	5	1.7	PC	-	-	+	V600E	-	0	2	2	+/+	+/-	-/+	+/+	-/+	+/-	+
36	M	11	9	6.0	PC	+	+	+	-	-	1	2	0	+/+	+/-	-/+	+/+	+/-	+/-	+*
37	F	56	4	2.4	PC	-	+	-	-	-	1	2	2	+/+	+/-	-/+	+/+	-/+	+/-	+*
38	F	83	1	7.5	PC	-	+	-	V600E	-	0	3	ND	+/+	ND	-/+	ND	-/+	ND	+
39	F	18	4	3.6	PC	+	-	+	-	-	2	2	1	+/+	+/-	+/+	+/+	-/+	+/-	+
40	M	14	4	2.5	PC	+	+	-	-	-	1	2	1	+/+	+/-	+/+	+/+	-/-	+/-	+
41	F	32	9	1.1	PC	-	+	-	V600E	-	0	2	1	+/-	+/-	-/+	+/+	+/-	+/-	+
42	F	56	10	7.5	PC	+	+	-	ND	ND	1	2	1	+/+	+/-	-/+	+/+	-/+	+/-	+

M-male, F-female, + presence, - absence, WC-well circumscribed, PC-poorly circumscribed, ND-not determined, C-expression in the center of the tumour, P-expression in the tumour periphery, CT-expression in the control tissue, n- nuclear, c-cytoplasmatic, LNM-lymph node metastases. * Cases with concurrent distant metastases.

Table 2. Clinico-pathological, molecular and immunohistochemical features of the FVPTC cases

Case number	Gender	Age (years)	Follow-up (years)	Tumour size (cm)	Growth pattern	Multicentricity	Extra-thyroid extension	Vascular invasion	*BRAF*	*N-RAS*	Intensity of expression			P-Smad2/3 C (n/c)	P-Smad2/3 CT (n/c)	Smad4 C (n/c)	Smad4 CT (n/c)	Smad7 C (n/c)	Smad7 CT (n/c)	LNM
											TGF-beta C	TGF-beta P	TGF-beta CT							
1	F	32	22	2.0	WC	-	-	+	-	-	2	0	2	+/+	+/-	-/+	+/+	+/+	+/-	-
2	F	26	21	1.8	PC	+	-	+	V600E	-	2	3	1	+/+	+/-	-/+	+/+	+/+	+/+	-
3	F	59	19	0.5	WC	-	-	-	-	-	0	0	0	+/-	+/-	-/+	+/-	+/-	+/-	-
4	F	51	18	1.4	WC	-	-	-	-	-	2	3	1	+/+	+/-	+/+	+/+	+/+	+/-	-
5	F	36	18	0.5	PC	-	-	-	V600E	-	1	1	1	+/+	+/-	+/+	+/+	+/-	+/-	-
6	F	39	15	1.0	WC	+	-	-	-	Q61R	1	1	0	+/-	+/-	+/+	+/+	+/+	+/-	-
7	F	48	13	0.6	WC	-	-	-	-	-	1	0	0	+/+	+/+	+/+	+/+	+/-	+/-	-
8	F	33	20	0.2	PC	-	-	-	-	-	3	3	0	+/+	+/+	+/+	+/+	+/+	+/-	-
9	F	30	15	0.5	WC	-	-	-	-	-	1	1	0	+/+	+/+	-/+	+/+	+/-	+/-	-
10	F	21	21	0.6	PC	-	-	-	-	-	1	3	1	+/-	+/-	-/+	+/+	+/-	+/-	-
11	F	37	18	0.3	PC	-	-	+	-	-	1	1	1	+/-	+/-	-/+	+/+	+/-	+/-	-
12	F	28	21	2.8	WC	+	-	+	-	-	2	3	0	+/+	+/-	-/+	+/+	+/+	+/+	-
13	F	34	20	3.5	WC	-	-	-	-	-	0	0	0	+/-	+/-	+/+	-/+	+/-	+/-	-
14	M	61	22	4.0	WC	-	-	+	V600E	-	2	2	2	+/+	+/-	-/+	+/+	+/+	+/-	-
15	M	35	20	3.5	WC	-	-	+	-	Q61R	1	1	1	+/+	+/-	-/+	+/+	+/-	+/-	-
16	F	50	20	2.5	WC	-	-	-	-	-	1	1	1	+/-	+/-	-/+	-/+	+/-	+/-	-
17	F	47	23	3.0	PC	-	-	-	-	Q61R	2	3	0	+/-	+/-	+/+	+/+	+/+	+/-	-
18	F	49	14	3.0	WC	+	-	+	-	-	2	1	1	+/+	+/+	-/+	+/+	+/+	+/-	-
19	F	35	21	1.7	PC	+	-	+	ND	ND	3	3	1	+/+	+/-	-/+	+/+	+/+	+/-	-
20	F	32	13	4.7	PC	+	-	+	-	-	2	3	2	+/+	+/-	+/+	+/+	+/+	+/-	-
21	F	52	13	2.5	WC	+	-	-	-	-	1	1	2	+/-	+/-	+/+	+/+	+/-	+/-	-
22	F	66	13	2.5	WC	+	-	-	-	-	1	1	0	+/+	+/-	+/+	+/+	+/-	+/-	-
23	F	58	13	1.5	PC	+	-	+	V600E	-	0	1	1	+/+	+/-	-/+	+/+	+/+	+/-	-
24	F	54	10	1.8	PC	+	-	+	-	-	1	2	1	+/+	+/+	-/+	+/+	+/+	+/-	-
25	M	45	20	4.0	WC	-	-	-	-	-	1	1	1	+/-	+/-	-/+	+/+	+/-	+/-	-
26	M	57	3	9.5	PC	-	+	+	-	-	2	2	1	+/+	+/+	+/+	+/+	-/+	+/-	+
27	F	56	3	0.8	PC	+	-	+	-	-	1	2	2	+/+	+/-	+/+	+/+	-/-	+/-	+
28	M	21	4	2.5	PC	-	+	+	-	-	1	2	0	+/+	+/-	-/+	+/+	-/+	+/-	+
29	F	20	14	1.0	PC	+	-	+	ND	ND	0	1	2	+/-	+/-	-/+	+/+	-/-	+/-	+
30	F	49	4	2.2	PC	-	-	+	-	-	0	1	0	+/-	+/-	-/+	+/+	-/-	+/-	+
31	F	43	10	4.0	PC	+	+	+	-	-	0	1	0	+/-	+/-	-/+	+/+	-/-	+/-	+
32	F	43	10	1.5	PC	+	+	+	V600E	-	1	3	1	+/-	+/-	-/+	+/+	+/-	+/-	+
33	M	63	7	1.8	PC	-	-	+	-	-	1	3	1	+/-	+/-	-/+	+/+	-/+	+/-	+*

M-male, F-female, + presence, - absence, WC-well circumscribed, PC-poorly circumscribed, ND-not determined, C-expression in the center of the tumour, P-expression in the tumour periphery, CT-expression in the control tissue, n-nuclear, c-cytoplasmatic, LNM-lymph node metastases. * Case with concurrent distant metastases.

Table3. Comparison between the immunohistochemical patterns of expression at the center and at the periphery of the tumours in well and poorly circumscribed PTCs (WCPTC and PCPTC).

	Features	TGF-beta intensity of expression (mean±sd)				Smad4 nuclear expression (%)		Smad7 nuclear expression (%)	
		C-CT	*p*	P-C	*p*	C-CT	*p*	C-CT	*p*
WCPTC	VI		0.376		0.641		**0.005**		>0.999
	Present	0.7±0.8		0.0±0.6		0.0		100.0	
	Absent	0.4±0.7		0.1±0.5		70.6		100.0	
PCPTC	Multicentricity		**0.053**		0.935		0.381		0.167
	Present	0.1±0.9		0.7±0.6		26.1		47.8	
	Absent	0.8±1.3		0.7±0.9		39.3		67.9	
	EE		0.398		**0.048**		0.746		0.119
	Present	0.3±0.9		1.0±0.7		26.7		40.0	
	Absent	0.6±1.3		0.6±0.7		50.0		66.7	
	LNM		**<0.0001**		**0.017**		0.771		**<0.0001**
	Present	-0.2±0.9		0.9±0.6		30.8		19.2	
	Absent	1.2±1.1		0.4±0.8		36.0		100.0	

EE-extra-thyroid extension, VI – vascular invasion, C - expression in the center of the tumour, P – expression in the tumour periphery, CT- expression in the control tissue, LNM-lymph node metastases.

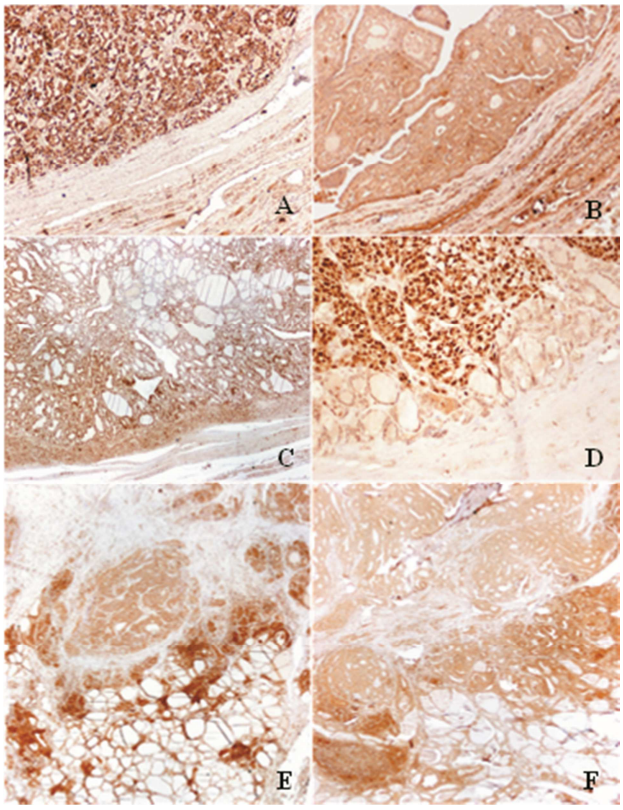


Figure 1. TGF-beta expression at the center and at the periphery of the tumours. A- Well circumscribed *BRAF* mutated FVPTC-M with similar expression at the center and at the periphery (case 14, 200x); B – Well circumscribed wild type *BRAF* FVPTC-M with increased expression at the periphery (case12, 200x); C – Poorly circumscribed wild type *BRAF* FVPTC+M with lung metastases and increased expression at the periphery (case 33, 200x); D – Well circumscribed wild type *BRAF* FVPTC-M with decreased expression at the periphery (case18, 200x); E – Poorly circumscribed wild type *BRAF* CPTC+M with increased expression at the periphery (case33, 40x) and F – Poorly circumscribed *BRAF* mutated CPTC-M with increased expression at the periphery (case17, 40x).

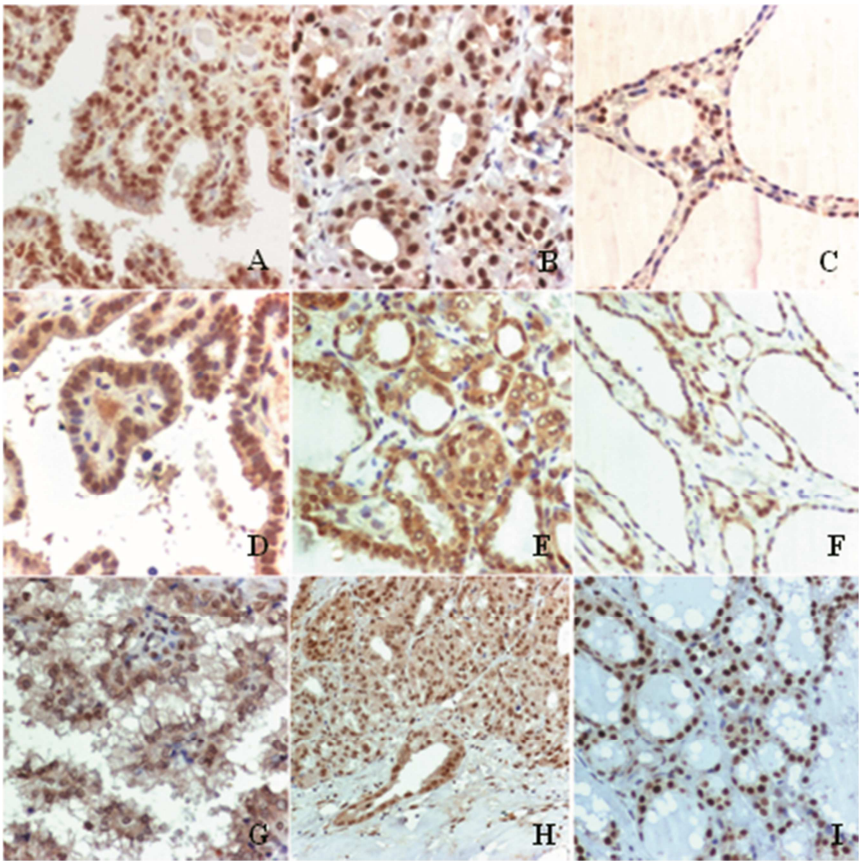


Figure 2. Expression of P-Smad2/3: A – Papillary areas of the center of the tumour (case 26 of CPTC group, 200x), B –Follicular areas of the center of the tumour (case 28 of FVPTC group, 400x) and C – Control tissue (case 2 of CPTC group, 400x). Expression of Smad4: D – Papillary areas of the center of the tumour (case 39 of CPTC group, 200x), E – Follicular areas of the center of the tumour (case 27 of FVPTC group, 200x) and F– Control tissue (case 27 of FVPTC group, 200x). Expression of Smad7: G – Papillary areas of the center of the tumour (case 9 of CPTC group, 400x), H – Follicular areas of the periphery of the tumour (case 1 of FVPTC group, 100x) and I – Control tissue (case 1 of FVPTC group, 200x).

Supplementary table. Comparison between the immunohistochemical patterns of expression at the center and at the periphery of the tumours and the PTC features in CPTC and FVPTC.

	Features		TGF-beta intensity of expression (mean±sd)				Overexpression or basal expression in the nucleus of tumour cells (%)			
			C-CT	*p*	P-C	*p*	Smad4	*p*	Smad7	*p*
CPTC+FVPTC	Growth pattern	PC	0.5±1.2	0.839	0.7±0.7	**0.0007**	33.3	0.198	58.8	**0.0001**
		WC	0.4±0.7		0.1±0.5		52.2		100.0	
	Multicentricity	Present	0.3±0.9	0.217	0.5±0.6	0.630	35.5	0.635	61.3	0.120
		Absent	0.6±1.2		0.5±0.8		41.8		79.1	
	EE	Present	0.3±0.9	0.409	1.0±0.7	**0.002**	26.7	0.377	40.0	**0.008**
		Absent	0.5±1.1		0.4±0.7		42.4		79.7	
	VI	Present	0.4±1.2	0.719	0.6±0.8	0.259	19.4	**0.004**	61.3	0.120
		Absent	0.5±1.0		0.4±0.7		53.5		79.1	
	BRAF	V600E	0.2±1.2	0.295	0.8±0.8	**0.029**	27.8	0.273	88.9	0.117
		Wild type	0.5±1.0		0.4±0.7		43.5		67.4	
	RAS	Q61R	0.8±1.0	0.532	0.5±0.6	0.965	75.0	0.291	100.0	0.566
		Wild type	0.5±1.0		0.5±0.7		36.7		71.7	
	LNM	Present	-0.2±0.9	**<0.0001**	0.9±0.6	**0.0001**	30.8	0.325	19.2	**<0.0001**
		Absent	0.8±1.0		0.3±0.7		43.8		100.0	
CPTC	Growth pattern	PC	0.6±1.2	0.787	0.6±0.7	0.362	35.3	0.401	58.8	**0.075**
		WC	0.5±0.8		0.3±0.5		57.1		100.0	
	Multicentricity	Present	0.4±0.7	0.393	0.5±0.5	0.757	35.3	0.753	47.1	**0.048**
		Absent	0.7±1.3		0.5±0.8		31.7		79.2	
	EE	Present	0.2±1.0	0.233	1.0±0.6	**0.006**	27.3	0.478	45.5	0.140
		Absent	0.7±1.2		0.3±0.7		43.3		73.3	
	VI	Present	0.8±1.4	0.449	0.5±0.9	0.945	25.0	0.305	58.3	0.719
		Absent	0.4±1.0		0.5±0.6		44.8		69.0	
	BRAF	V600E	0.3±1.3	0.562	0.8±0.8	**0.048**	30.8	0.719	84.6	0.132
		Wild type	0.6±1.1		0.4±0.6		40.0		55.0	
	RAS	Q61R	0.0	0.694	1.0	0.525	100.0	0.364	100.0	>0.999
		Wild type	0.5±1.2		0.5±0.7		34.4		65.6	
	LNM	Present	-0.2±0.9	**<0.0001**	0.8±0.6	**0.009**	33.3	0.540	22.2	**<0.0001**
		Absent	1.1±1.0		0.3±0.7		43.5		100.0	
FVPTC	Growth pattern	PC	0.4±1.2	0.812	0.9±0.7	**<0.0001**	29.4	0.296	58.8	**0.007**
		WC	0.4±0.7		0.0±0.5		50.0		100.0	
	Multicentricity	Present	0.2±1.2	0.383	0.6±0.7	0.310	35.7	>0.999	78.6	>0.999
		Absent	0.5±0.8		0.4±0.8		42.1		78.9	
	EE	Present	0.5±0.6	0.824	1.0±0.8	0.148	25.0	>0.999	25.0	**0.023**
		Absent	0.4±1.0		0.4±0.7		41.4		86.2	
	VI	Present	0.2±1.0	0.224	0.7±0.7	**0.077**	15.8	**0.003**	63.2	**0.013**
		Absent	0.6±1.0		0.2±0.7		71.4		100.0	
	BRAF	V600E	0.0±0.7	0.255	0.8±0.8	0.324	20.0	0.368	100.0	0.553
		Wild type	0.5±0.9		0.4±0.8		46.2		76.9	
	RAS	Q61R	1.0±1.0	0.239	0.3±0.6	0.720	66.7	0.558	100.0	>0.999
		Wild type	0.4±0.9		0.5±0.8		39.3		78.6	
	LNM	Present	0.1±1.0	**0.091**	1.1±0.6	**0.004**	25.0	0.431	12.5	**<0.0001**
		Absent	0.6±1.0		0.3±0.7		44.0		100.0	

EE – extra-thyroid extension, VI – vascular invasion, LNM – lymph node metastases, PC – poorly circumscribed, WC – well circumscribed, C - expression in the center of the tumour, P – expression in the tumour periphery, CT- expression in the control tissue.

Paper 4

Sobrinho-Simões M, Eloy C, Vinagre J, Soares P
"Molecular pathology of thyroid tumors: diagnostic and prognostic relevance"
International Journal of Surgical Pathology 2010; 18: 209S-212S

Molecular Pathology of Thyroid Tumors: Diagnostic and Prognostic Relevance

International Journal of Surgical Pathology
Supplement to 18(3) 209S–212S
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DOI: 10.1177/1066896910370886
http://ijsp.sagepub.com
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Introduction

It is difficult to find a more promising field than thyroid oncology to prove the diagnostic usefulness of molecular pathology in its broadest sense. Immunohistochemistry and in situ hybridization methods epitomize the type of molecular pathology we cherish, as surgical pathologists, in our daily routine.

Immunohistochemistry allows a clear cut distinction of primary tumors from metastatic lesions and, within the former, the separation of follicular-cell from C-cell derived tumors. In other words, just by staining for thyroglobulin, TTF1, and calcitonin, we are able to categorize almost every tumor in the thyroid.[1] On the other hand, the frequent occurrence of RET and TRK rearrangements in papillary thyroid carcinomas (PTC)[1,2] and of PAX8-PPARγ rearrangements in follicular patterned lesions[3-5] highlight the usefulness of in situ hybridization in thyroid oncology.

Besides the aforementioned "positive" aspects of a topographically clever molecular approach to thyroid tumor pathology, a number of other genetic alterations have also been closely linked to thyroid tumorigenesis: RET mutations in hereditary and sporadic medullary carcinoma,[6] high prevalence of RAS mutations in follicular patterned-tumors[7,8] a fairly specific BRAF mutation in PTC,[9] and p53 mutations in less differentiated carcinomas.[10]

Despite the substantial advances made in the clarification of the etiopathogenesis of thyroid tumors, one still misses molecular markers of malignancy and prognosis. So far, these problems have been addressed unsuccessfully with high throughput approaches at the genomic, transcriptomic, and proteomic level. The failure of this strategy, together with recent data on the many thousands of mutations found by massive sequencing in single tumor cases,[11,12] have stimulated many groups to go back to a combined strategy using a detailed histopathological characterization as the basis where immunohistochemical and molecular data are anchored.

Several articles of the June 2008 issue of *Endocrinology and Metabolism Clinics of North America* edited by Ezzat and Asa[13] review extensively the etiopathogenic, diagnostic, prognostic, and therapeutic relevance of the molecular features of thyroid tumors. Having this in the back of our mind we decided to focus this short review on some problems of molecular pathology that are (or may become) of practical interest to surgical pathologists.

Diagnosis of Hereditary and Sporadic Medullary Thyroid Carcinoma and Mixed Medullary–Follicular (Papillary) Carcinoma

Besides the importance of searching for calcitonin and carcinoembryonic antigen immunoreactivity in any peculiar thyroid tumor, and to look (always) for RET germline mutations whenever a diagnosis of medullary thyroid carcinoma (MTC) is made, there are a number of situations where molecular methods may be useful in the MTC setting.

The diagnosis of mixed medullary–follicular (papillary) carcinoma, which should not be confused with MTC with entrapped follicles,[14] may be firmly established if both neoplastic components are present in metastatic foci and/or if the characteristic genetic alterations of MTC and follicular carcinoma or PTC are detected in microdissected samples of the primary tumor.

Regarding the diagnosis of incipient malignancy we are not aware of any molecular marker that allows the definitive differential diagnosis between nodular C-cell hyperplasia and micro-MTC, a problem whose frequency has been increasing in recent years mainly because of the exhaustive, in depth

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study of surgical specimens. The utilization of a numerical threshold, such as more than X calcitonin-positive cells, to justify jumping from nodular C-cell hyperplasia to micro-MTC does not seem to make sense. In our institute we require that the micronodule of C-cells have its own stroma[1,15] for diagnosing a micro-MTC but this is not always easy to apply and, moreover, our series is too small and has too short a follow-up to allow any definitive conclusion. Additional data on the evolution of many such cases with detailed morphologic and molecular characterization are necessary to predict the clinical behavior of small C-cell lesions.

Further molecular studies will be necessary to identify the genetic cause(s) of hereditary MTC not associated to RET germline mutations.[1,6,13] Among other candidates, we think there is enough evidence to claim that mtDNA alterations and alterations in nuclear genes encoding mitochondrial proteins (eg, SDHD) play a role in RET-negative C-cell hyperplasia and may modulate the occurrence of MTC.[16-18]

A last point refers to the problems raised by the occurrence of PTC in patients affected by hereditary MTC. This holds true mainly for papillary microcarcinomas, which are known to be extremely frequent in the general population, thus making it difficult to advance a common etiopathogenesis for hereditary MTC and PTC. Taking into consideration the involvement of RET alterations (mutations and rearrangements) in both tumor types, we have always been puzzled by the rarity of the aforementioned coexistence, as well as by the rarity of mixed medullary–papillary carcinomas.[19] According to Melillo et al,[20] RET point mutations can behave as dominant oncogenes for thyroid follicular cells as they do for C-cells; however, their transforming activity in follicular cells is rather moderate, thus explaining the rare association of hereditary MTC with PTC.[20]

Differential Diagnosis of Encapsulated Follicular Patterned Tumors

This is a field where molecular markers are badly needed because everybody recognizes the limitations of using capsular invasion to separate a follicular adenoma/nodular adenomatous nodule from a minimally invasive follicular carcinoma, and to rely on the nuclear characteristics to separate a benign nodule from an encapsulated follicular variant of PTC (FVPTC).[1] We and other groups have extensively reviewed the limitations of the existing molecular markers as indicators of malignancy in such borderline cases using classic histopathology as a frame,[1,13,17,21] and because there have not been (yet?) good news from genome-wide studies,[13] we will just emphasize here a couple of practical points.

1. The identification of aneuploidy, RAS mutations, and PAX8-PPARγ rearrangements cannot be used to separate a benign tumor from a follicular carcinoma because they are also found in the former, though less frequently than in follicular carcinoma.[1,3-5,7,8,21] Curiously, the aforementioned 3 "markers" appear to carry significant prognostic information whenever a diagnosis of follicular carcinoma has been achieved via the demonstration of vascular invasiveness.[1,13,17]
2. The diagnostic problems raised by the FVPTC are restricted to its encapsulated subtype because the poorly circumscribed and the multinodular/diffuse subtypes are easily diagnosed in almost every instance (see the article "Hot Topics in Papillary Thyroid Carcinoma" in this issue). The differential diagnosis between follicular adenoma/follicular carcinoma and the encapsulated subtype of FVPTC resides in the identification of typical PTC nuclei and on the evaluation of their number and distribution in the latter lesions. These problems are reflected in the high interobserver variability when diagnosing FVPTC, even among experts in thyroid pathology.[22] Immunohistochemistry for cytokeratin 19, galectin-3, HBME1, fibronectin, and syalilated and nonsyalilated histo-blood antigens did not provide consistent results, especially when applied to borderline, difficult cases.[1,21-24] The same holds true for the molecular markers that have been tested so far.[13,17,21] The highly prevalent BRAF V600E mutation is usually detected in PTCs exhibiting prominent papillary growth pattern thus turning the efforts to its detection in FVPTC almost useless.[1,9,21,25] Regardless of the immunohistochemical and molecular findings, the retrospective data obtained so far indicate that encapsulated FVPTC without signs of invasion follow a benign course even after being treated by lobectomy only[26,27] (Sobrinho-Simões et al, unpublished observations, 2010).
3. The vascular invasive, encapsulated FVPTC—like follicular carcinoma—tends to give rise to lung and/or bone metastases.[28] The FVPTC shares many of the molecular features of follicular carcinoma (frequent aneuploidy, frequent RAS mutations, frequent PAX8-PPARγ rearrangements)[8]; it remains to be seen whether or not such molecular features will carry prognostic informations in FVPTC as they do in follicular carcinoma.[8,29]

Diagnosis of Poorly Differentiated and Undifferentiated Carcinoma

Despite the large amount of molecular data that have been reported in association with poorly differentiated carcinomas (mutations of RAS, BRAF, and CTNNB1, the gene encoding for β-catenin), and undifferentiated (anaplastic) carcinomas

(p53 and P13K mutations), none of these genetic alterations have been found (yet?) to play a role from a diagnostic standpoint.[13,17] In contrast to this, many of the aforementioned alterations are thought to constitute promising therapeutic targets.[30]

Declaration of Conflicting Interests

The author(s) declared no conflicts of interest with respect to the authorship and/or publication of this article.

Funding

Part of the data reported in this study was obtained in the Project "Identificação de factores prognósticos e de selecção terapêutica em carcinomas diferenciados da tireoide," which is supported by Fundação Calouste Gulbenkian.

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Paper 5

Sobrinho-Simões M, Eloy, C, Magalhães J, Lobo C, Amaro T
"**Follicular thyroid carcinoma**"
*Modern Patholo*gy 2011; 24: S10-S18

MODERN PATHOLOGY (2011) 24, S10–S18

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Follicular thyroid carcinoma

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Follicular thyroid carcinoma is being diagnosed less and less frequently despite the increasing incidence of well-differentiated thyroid carcinomas everywhere. This review will discuss the reasons underlying such an observation focusing on the evolution of the morphological and immunohistochemical diagnostic criteria of follicular thyroid tumors. It will address the differential diagnosis between follicular carcinoma and three tumor types—follicular adenoma, follicular variant of papillary carcinoma and poorly differentiated carcinoma—as well as the problems raised by the newly described categories of follicular tumors: follicular tumor of uncertain malignant potential, well-differentiated tumor of uncertain malignant potential and well-differentiated carcinoma, not otherwise specified. Finally, the prognostic and therapeutic significance of some promising molecular biomarkers will be discussed within the frame of the aforementioned histopathological classification.
Modern Pathology (2011) **24,** S10–S18; doi:10.1038/modpathol.2010.133

Keywords: diagnosis; follicular thyroid carcinoma; molecular biomarkers; prognosis

In the respective chapter of the *WHO Classification of Tumours of Endocrine Organs*, follicular thyroid carcinoma (FTC) is defined as 'A malignant epithelial tumor showing follicular cell differentiation and lacking the diagnostic nuclear features of papillary thyroid carcinoma' (PTC).[1] This definition is almost identical to the definition advanced by Rosai *et al*[2] in the AFIP fascicle on *Tumors of the Thyroid Gland*: 'Follicular carcinoma is a malignant epithelial tumor showing evidence of follicular differentiation and not belonging to any of the other distinctive types of thyroid malignancy.'

Besides the point on follicular cell differentiation that does not usually raise any major difficulty, the two crucial issues of both definitions reside in the need to demonstrate malignancy and to exclude the presence of nuclear features typical of PTC.[1–3]

In this context, malignancy is equivalent to invasiveness (capsular and/or vascular penetration), which may be difficult to disclose with certainty.[1–3] The situation is even worse regarding the tendency to overclassify the nuclear features of many follicular growth-patterned lesions as suggestive or indicative of those of the PTC nuclei; this trend is leading to an overdiagnosis of the follicular variant of PTC (FVPTC) and to an underdiagnosis of FTC.[1,4]

The utilization of more strict criteria for recognizing capsular and vascular invasion, and the aforementioned trend toward overdiagnosing FVPTC has led, together with the increasing number of PTC diagnoses (namely small or very small lesions), to a progressive reduction in the percentage of FTCs among well-differentiated thyroid carcinomas.[1–4] For instance, the percentage of FTCs among well-differentiated carcinomas decreased from 28.1% in the period 1983–1992 to 6.5% in the period 1993–2007 in a very large series from Greece.[5] In Japan, Kakudo *et al*[6] recently reported that FTCs represented 9.7% among well-differentiated and moderately differentiated carcinomas. In a multi-institutional study, we are performing in Porto, FTCs composed of 'normal' follicular cells or oncocytic cells represent ~5% of clinically evident well-differentiated carcinomas (Magalhães *et al*, unpublished results). At present, in most US institutions, the frequency of FTC is even lower than that reported in the aforementioned series.

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Received 28 June 2010; accepted 28 June 2010

Regardless of the differences from series to series, it is generally acknowledged that *bona fide* FTC is nowadays a relatively rare thyroid tumor in contrast to its high frequency in the series of the first half of last century.[1–4]

The prognosis of FTC depends on several factors: age of the patients, size and staging of the tumors, completeness of surgery and responsiveness to radioactive iodine.[1–3,7] It also depends on the degree of invasiveness of tumors: minimally invasive carcinomas carry a much better prognosis than do widely invasive carcinomas.[1–3,7] The prognostic importance of angioinvasion has been recently acknowledged.[1,7,8] The prognostic meaning of tissue and cell differentiation remains controversial, although it is generally accepted that FTCs composed of oncocytic cells and FTCs with a solid, trabecular or insular growth pattern respond less well to radioactive iodine and may carry a worse outcome.[1–3,7,9,10] The coexistence of less well-differentiated areas in some FTCs raises the problem of the differential diagnosis between FTC and poorly differentiated thyroid carcinoma (PDTC).[1,7,11]

The above-mentioned diagnostic and prognostic issues will be addressed in this review using the chapter on follicular carcinoma of the WHO book,[1] not only as a frame but also as a starting point for consolidating the classic criteria and discussing some recently advanced proposals. The molecular features will be integrated in the respective clinicopathological sections.

Macroscopy and invasiveness

Most FTCs are encapsulated solid tumors with a gray tan to brown color at the cut surface (Figure 1a). Minimally invasive FTCs are macroscopically indistinguishable from FA, except for capsular characteristics; the capsule tends to be thicker and more irregular in FTC than in FA.[1–3,7] This is such an important feature that provided one is not dealing with a widely invasive FTC, the hypothesis of FTC should be ruled out if the tumor has no capsule or just a thin one. Widely invasive FTCs may occur as partially encapsulated tumors with extensive penetration of the capsule or as multinodular, bulking tumors without a capsule, occasionally showing permeation of the thyroid veins (Figure 1b).[1–3,7]

FTCs usually occur as single tumors. Multifocality and recurrence in the residual parenchyma after partial thyroidectomy are uncommon. The aneuploid pattern and the various molecular alterations detected in FA and FTC[12–15] support the assumption that the development of such tumors is the end product of multiple oncogenic steps, thus justifying their usual appearance as single (unicentric) neoplasms.[15] At variance with FTCs, PTCs occur frequently as multicentric neoplasms, which may be clonally independent from each other;[15] taking this together with the fact that almost every PTC is

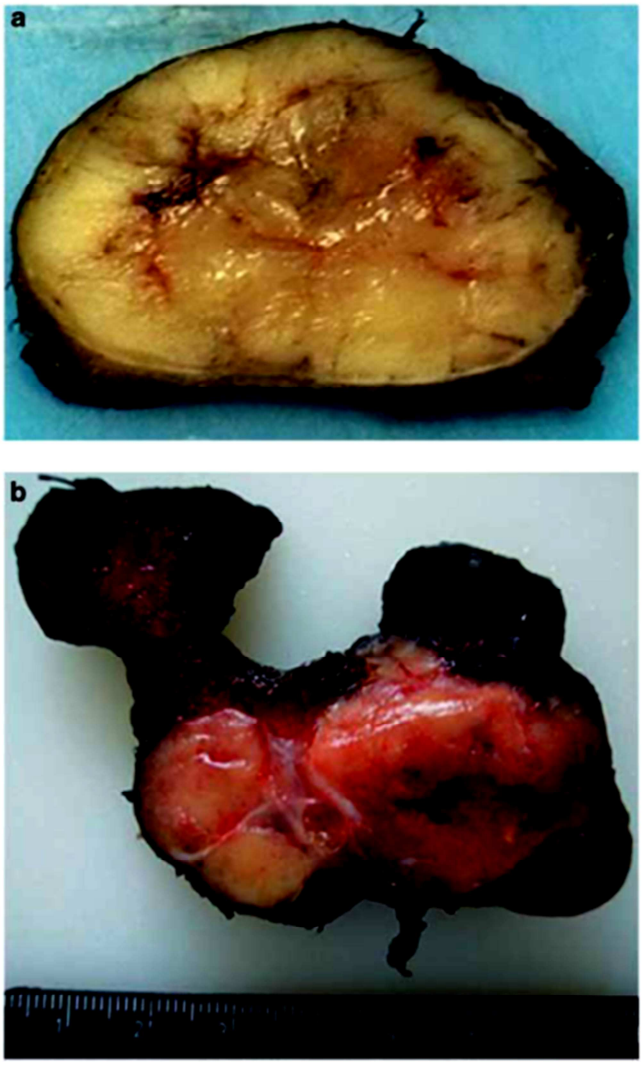


Figure 1 Macroscopic appearance of the two main types of follicular thyroid carcinoma. (**a**) Minimally invasive follicular thyroid carcinoma. (**b**) Widely invasive follicular thyroid carcinoma.

diploid or quasi-diploid and display microsatellite stability, it is tempting to advance that, in contrast to FTC, PTC tumorigenesis reflects the end product of a few oncogenic steps.[15–17]

The classification of FTCs in two categories—minimally and widely invasive—has always created a sense of a 'pathological' gap: what about tumors that are 'simply' invasive? This gap has been filled with the creation of a third group of FTCs, the angioinvasive carcinomas.[1,7] FTC is at present classified as 'minimally invasive without angioinvasion,' 'minimally invasive with angioinvasion' and 'widely invasive.' Most authors agree that for practical (prognostic and therapeutic) purposes, it is crucial to separate minimally invasive, not angioinvasive, FTCs which carry an excellent prognosis from angioinvasive FTCs regardless of whether it falls in the category of widely invasive FTCs.[1–3,7,18–20]

We believe that it is not worthwhile trying to count the number of vessels invaded by neoplastic cells in an attempt to establish a threshold separating two groups of prognostically different angioinvasive FTCs. Nikiforova *et al*[21] have shown an association between the *PAX8-PPARγ* rearrangement and very angioinvasive FTC ('overtly' invasive FTC), and a similar association was found by our group in FVPTC.[22] These findings reinforce the molecular similitude of FA/FTC and FVPTC (see below) and support the putative pathogenic importance of the *PAX8-PPARγ* rearrangement in different types of follicular-patterned tumors, but there is not (yet?) enough evidence to use the detection of the *PAX8-PPARγ* rearrangement for prognostic purposes. This is because first, *PAX8-PPARγ* rearrangements are relatively frequent in follicular adenomas,[23] and second, it is not known whether its presence carries prognostic information independently from vascular invasion.

In daily practice, we always try to detect at least one unequivocal focus of vascular invasion and, whenever this purpose is achieved, a diagnosis of angioinvasive FTC is made.[24] Incidentally, whenever one identifies a focus of vascular invasion, several others are usually detected in the same section or in deeper sections.

The morphological criteria for identifying, for sure, signs of vascular invasion have been thoroughly described by LiVolsi,[3] Rosai *et al*[2] and Rosai,[7] as well as in the WHO book.[1] Although we agree with Mete and Asa[8] about the ideal criteria for diagnosing vascular invasion (tumor cells invading a vessel wall associated with the thrombus adherent to intravascular tumor), we diagnose vascular invasion if we detect well-preserved neoplastic issue within a vein (Figure 2). Whenever facing the dilemma 'vascular invasion or not,' we do not rely on any immunostaining of endothelial cells; we search the additional sections of the capsule and stick to the following rule: If we do not detect any unequivocal signs of venous permeation, we diagnose the tumor as nonangionvasive (see below the point on 'follicular tumors of uncertain malignant potential').

Histology and differential diagnosis

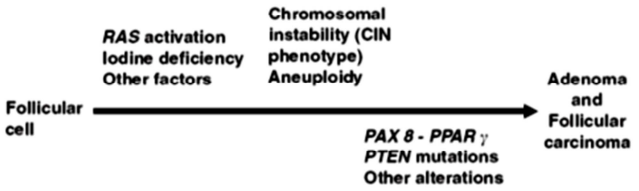
Histologically, FTCs display variable morphology ranging from small/medium-sized follicles containing colloid to trabecular or solid growth pattern (Figure 2). The latter tends to be more frequent in widely invasive FTCs, but they can also occur in minimally invasive FTCs. It is exceedingly rare for a FTC to be composed of macrofollicles; in case one faces a capsular and/or vascular invasive follicular tumor composed of large follicles, with or without colloid, it is mandatory to look for the nuclear characteristics of neoplastic cells because the most likely diagnosis will be a (macro) FVPTC.

The cytological aspects can also vary a lot from case to case, and even within each case, without any clear-cut distinction between minimally and widely invasive FTCs. The same holds true regarding FA and adenomatous nodules of multinodular goiters which may present, either throughout the nodule or in some foci marked as cyto-arquitectural atypias. In other words, neither architectural nor cytological atypia are reliable criteria of malignancy.[1–3,7,24] This and the need to identify unequivocal signs of invasion to make a diagnosis of FTC, renders fine-needle aspiration cytology and frozen sections useless or almost useless in these settings.[1–3,7]

Differential Diagnosis between FA/Adenomatous Nodule and Minimally Invasive FTC

Despite the huge amount of information on proliferation, apoptosis, cell-cycle differentiation and stromal markers obtained at the immunohistochemical level,[1,15,25–31] the differential diagnosis between FA/adenomatous nodule and minimally invasive FTC still depends on the identification of histological signs of invasiveness.[1–3,7,24] The search for such signs should be made in numerous H&E-stained sections involving the capsule of the tumor.[1–3,7,24] In our hands, as stressed above, immunostaining of capsular vessels does not provide useful diagnostic data. There is also no immunohistochemical or molecular feature that may be used as a reliable marker of invasion.[1,24,28]

The most prominent molecular features of FTC are the prominence of aneuploidy and the high prevalence of *RAS* mutations and of *PAX8-PPARγ* rearrangements, the latter as a result of a 2;3 translocation.[12–15,32–34]



All the aforementioned molecular features (such as aneuploidy, *RAS* mutations, *PAX8-PPARγ* rearrangements, etc.) are also frequently observed in FA,[12–15,22,23,32–35] and are therefore almost useless for diagnostic purposes. In addition, the molecular approaches for diagnosing malignancy in follicular tumors using high-throughput technologies (DNA microarrays, massive sequencing, etc.) did not provide diagnostically sound results.[36–38] The interesting preoperative diagnostic findings reported by Cerutti *et al*[39] have not been confirmed in other series, and the same holds true for other studies.[38]

Taking into consideration the recently reported complex landscapes of somatic rearrangements in human breast cancer genomes[40] and the more than

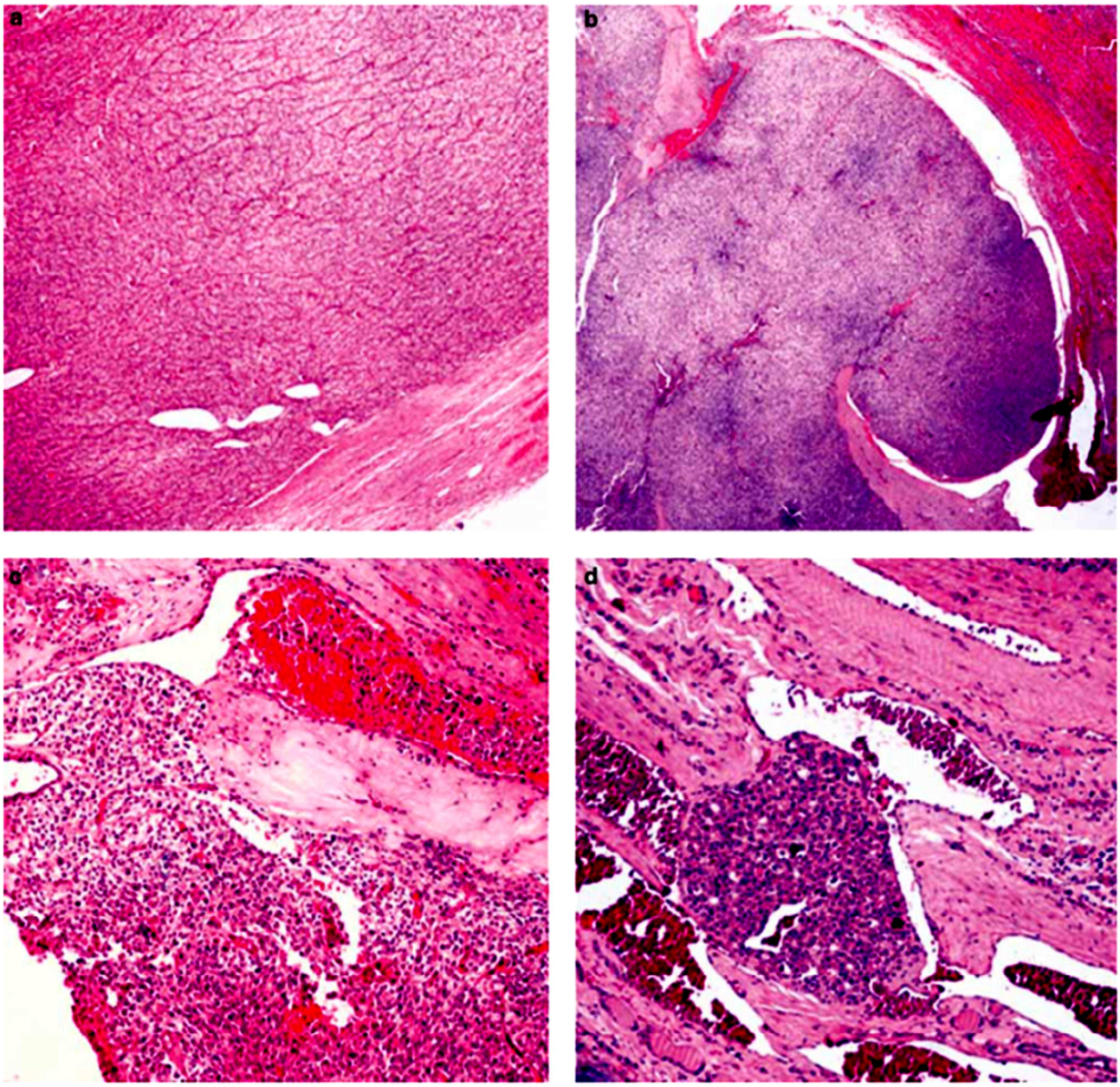


Figure 2 Histological appearance of a minimally invasive, angioinvasive, follicular thyroid carcinoma. (**a**) Microfollicular/solid pattern of growth of the neoplasia surrounded by a thick capsule (HE, ×4), (**b**) displaying, focally, capsular invasion (HE, ×4) and (**c** and **d**) vascular invasion (HE, ×100).

20 000 or 30 000 somatic mutations detected in individual human cancers,[41,42] it may be anticipated that rather than searching for mutations by massive sequencing, it seems more sensible to progress in the diagnosis and prognosis of thyroid tumors by an integrated histological and molecular approach.[28] The morphological frame is crucial for the topographical characterization of any tumor; this holds true particularly for follicular tumors of the thyroid as they epitomize the most important cancer properties: invasiveness in general and vascular invasiveness in particular. Besides 'topography,' the search for serum biomarkers may prove diagnostically useful in this setting. Despite our skepticism on the usefulness of high-throughput technologies in the differential diagnosis between FA and FTC, we anticipate that such methods will provide, in the future, meaningful information for predicting the risk of metastization and the sites of metastization.

For the time being, the histological analysis of exhaustive samples of the tumor capsule is thus necessary to make the differential diagnosis in borderline cases, namely by the identification of vascular invasiveness; if it is not possible to find unequivocal signs of invasiveness, then the designation of 'Follicular tumor of uncertain malignant

potential' advanced by Williams[43] is appropriate.[1,7,24] We realize that the utilization of the term 'uncertain malignant potential' in a pathology report creates a big problem to clinicians. Such a term should be used as rarely as possible, not only because of its 'uncertainty' but also because it is not yet associated with any firmly established management protocol. Such diagnosis should only be made in cases in which one has a thorough evaluation of the whole capsule of the tumor, thus ruling out its utilization in most consultancy cases. The detection of several immunohistochemical and molecular markers has not proved to be diagnostically useful in this setting.[26,44]

In our institutions, follicular tumors of uncertain malignant potential are treated conservatively. Moreover, we think, like Rosai *et al*,[2] Rosai,[7] and other authors[19,20] that follicular tumors in which capsular invasion is the sole sign of malignancy should also be treated conservatively (see below), but these issues need to be addressed in Multidisciplinary Consensus Conferences to produce management protocols.

Differential Diagnosis between Minimally Invasive FTC and Encapsulated FVPTC

At present, this is the most frequent reason for consultancy in thyroid pathology. A few years ago, it used to be the identification of signs of capsular and/or vascular invasion, whereas nowadays it is 'Whether or not the nuclear characteristics of the neoplastic cells support the diagnosis of PTC.'[1–3,7,24,45]

The diagnosis of encapsulated FVPTC has probably become the hottest topic in thyroid pathology mainly because of the too-liberal utilization of the PTC nuclear criteria. It should be stressed that this dilemma is restricted to the encapsulated type of FVPTC as the other two types of FVPTCs (such as poorly circumscribed and multinodular/diffuse types) do not usually raise diagnostic problems.[21,24,45]

We have been stressing, since 2002, the existence of the aforementioned three types of FVPTCs.[46] These types apparently have different natural histories and display quite different patterns of metastization.[15,22,24,34,46,47] Poorly circumscribed/infiltrative FVPTCs metastasize, like conventional PTCs, to regional lymph nodes;[1–3,44,46] encapsulated FVPTCs tend to give rise, whenever displaying angioinvasiveness, to lung and bone metastases without concurrent nodal metastases;[47,48] and multinodular/diffuse FVPTCs are characterized by the frequent metastatic involvement of bones and lungs together with lymph nodes.[49] The peculiar blood-born metastatic pattern of some cases of FVPTCs had been highlighted many years ago by Rosai and colleagues[50] and Rosai.[51]

Besides the low frequency and type of the *BRAF* mutation,[34,52,53] there are other molecular features of FVPTCs in general, and of encapsulated FVPTCs in particular, that differ from those of conventional PTCs. Some of those features resemble the molecular alterations that are characteristic of FA and FTC.[1] E-cadherin immunoexpression in encapsulated FVPTCs resembles that of follicular tumors and normal thyroid rather than that of conventional PTCs.[54,55] The frequency of *RAS* mutations is much higher in FVPTCs than in conventional PTCs,[22,56,57] thus also contributing to approach the FVPTC to FA and FTC.[22,51] Similar prevalences of *RAS* mutations were detected in FA (33%), FTC (22%) and FVPTC (25%).[22] Roque *et al*[58] described, in 2001, using conventional cytogenetics, the t(2;3) (q12;p25) in one case of FVPTC, and Wreesmann *et al*[59] using immunohistochemistry demonstrated, in 2004, overexpression of *PPARγ* in three cases of FVPTCs. Castro *et al*[22] detected the presence of *PAX8-PPARγ* rearrangement in 37% of the cases of encapsulated FVPTCs, 33% of FAs and 45% of FTCs, in contrast to its absence in conventional PTCs.[54,55] The results obtained by Castro *et al*[22] in FVPTCs probably reflect the stringent criteria used in the selection of cases of FVPTCs, excluding all tumors with a follicular/papillary growth pattern.

Taken together, the aforementioned data support the conclusion that FVPTCs show many genetic differences from conventional PTCs[22,59,60] and suggest that at least a subset of FVPTC that apparently corresponds to the encapsulated type of FVPTC shares some of the molecular features of FTC.[22,57] The same holds true in FTC and FVPTC arising in *struma ovarii* cases.[61]

It remains to be seen whether the subset of FVPTC displaying genetic alterations of the FTC type encompasses cases particularly prone to give rise to lung and/or bone metastases. It also remains unclarified whether cases of encapsulated FVPTC lacking signs of vascular invasion metastasize to the lungs or bones regardless of whether having the *PAX8-PPARγ* rearrangement or other genetic alterations. The results obtained so far in our retrospective study of 239 cases of well-differentiated thyroid carcinomas diagnosed in the Hospital S. João and Porto Cancer Institute, from 1978 to 2003, which displayed nodal and/or blood born metastases, has shown that in this group there was not a single case of follicular tumor of uncertain malignant potential or a well-differentiated tumor of uncertain malignant potential, nor any case of minimally invasive, non angioinvasive FTC or encapsulated, noninvasive FVPTC (Magalhães *et al*, unpublished results).

The problem of disclosing the malignant potential of encapsulated, nonangioinvasive FVPTC and, based on such evaluation, to decide the best therapy for such cases is similar to the discussion on the clinical malignancy and therapy selection for minimally invasive FTC with capsular invasion alone (see above). The evidence on record suggests that for both situations, lobectomy or lobectomy plus isthmectomy will be enough.[18,20,62,63]

Summing up, there are two separate problems in the diagnosis of encapsulated tumors with follicular architecture: to decide whether minor nuclear changes of the PTC type justify a diagnosis of FVPTC and to decide whether a minor degree of capsular penetration justifies a diagnosis of malignancy.[43] For the moment and although there is no additional evidence from the molecular pathology side, we agree with Williams and the Chernobyl Study Group[43] that it is more appropriate to recognize the difficulty in deciding whether the nuclei are typical than to arbitrarily place well-differentiated encapsulated tumors with a follicular architecture, in which minor nuclear changes are the only indicator of a PTC, in a definite malignant or a definite benign category.[43] Like Williams[43] and many other authors in Europe,[1,7,24,26,44,46] we refer to these tumors simply as 'well differentiated' without specifying either follicular or papillary, and use the terms 'well-differentiated tumor of uncertain malignant potential' when there is no definite evidence of invasion, and 'well-differentiated carcinoma, NOS' when invasion is present.[1,24,64] The term 'well-differentiated tumor of uncertain malignant potential' should be used as rarely as possible and should never be considered as a substitute for adequate sampling of any follicular-patterned thyroid tumor.[1,65] In our experience, and in that of other groups,[19,20,62,63] these tumors are adequately treated with a conservative approach (lobectomy or lobectomy plus isthmectomy), but one still needs firmly established management protocols. Although we do not sympathize with the designation 'well-differentiated carcinoma, NOS,' we use it occasionally for two main reasons: some follicular-patterned carcinomas of the thyroid are difficult to characterize because they have intermediate nuclei or different types of nuclei, and the treatment is similar for 'conventional PTC,' 'angioinvasive FTC' and 'angioinvasive, well-differentiated carcinoma, NOS.'

Differential Diagnosis between Widely Invasive FTC and Poorly Differentiated Thyroid Carcinoma

In the WHO book,[64] PDTC has been recognized as a histopathological entity. The absence of clear diagnostic criteria for PDTC has been recently solved by Volante *et al*[11] who described such criteria and advanced an algorithmic diagnostic approach for these tumors.

Despite the aforementioned developments in the diagnosis of PDTCs, it may be difficult to separate them from FTCs with a predominant trabecular/insular/solid growth pattern.[11,60,61] The same holds true regarding the separation of PDTCs from PTCs predominantly composed of trabecular, insular or solid areas whenever the nuclei of neoplastic cells superficially resemble the PTC nuclei.[11,64,65]

In both instances (DD with PTC and FTC), the foci of necrosis and high mitotic rate may be used in individual cases as signs suggestive of PDTC but should never be considered as absolute criteria.[11,64,65] The evaluation of the nuclear features is crucial whenever one faces the differential diagnosis with PTC; if the nuclei in the 'poorly differentiated' areas are of the PTC type, then we stick to the diagnosis of PTCs with trabecular or solid areas because the prognosis is much better than that of PDTCs.[11,64]

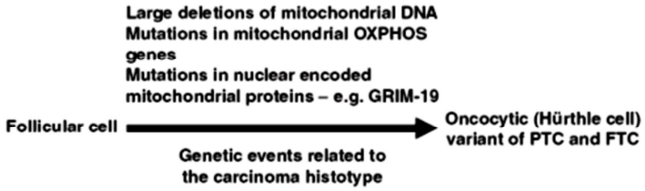
From a pragmatic standpoint, the separation of PDCs from FTCs only matters when one is dealing with a minimally invasive neoplasm, as widely invasive FTCs carry a prognosis that is similar to that of PDTCs and should be treated just as radically as them.[11,64,65]

Oncocytic (Hürthle Cell) Variant of FTC

Tumors with oncocytic (Hürthe cell) features may be benign (oncocytic variant of FA) or malignant (oncocytic variant of PTC and oncocytic variant of FTC). Medullary and poorly differentiated carcinomas may also display oncocytic features.[9,10,66,67]

Hürthle cells are stuffed with the mitochondria with morphological, functional and genetic abnormalities.[9,10,66,67] The accumulation of the mitochondria may be due to a primary alteration of mitochondrial DNA or of nuclear DNA that encode mitochondrial enzymes.[9,10,68,69] Regardless of the mechanism(s) involved in the process, a deficient mitochondrial function leads to an increased number of mitochondria through the stimulation of mitochondrial proliferation.[9,10,68,69]

It has been shown that most of the typical molecular features of conventional PTCs and FTCs are also present in their oncocytic counterpart.[15,35,52,68,69] For practical purposes, the oncocytic appearance is believed to represent a phenotype that is superimposed to the genotypic and histopathological features of thyroid tumors.



The criteria used in the diagnosis of the oncocytic variants of PTCs and FTCs are those used in the diagnosis of conventional tumors, ie, mainly the nuclear characteristics in PTCs and the signs of capsular and/or vascular invasion in FTCs. The prognosis of patients with the oncocytic variant of PTCs or FTCs is similar to that of patients with the respective conventional carcinomas provided the age of the patients and the staging of the tumors are comparable.[1,69–71] From a clinical standpoint, the negative aspect of oncocytic carcinomas of the thyroid is their lesser ability to trap iodine,

thus rendering them less responsive to radioactive iodine.[1,10,72,73]

Although it is now widely accepted that many oncocytic tumors of the thyroid are benign (nodular goiters and FA with oncocytic features), one should keep in mind that the percentage of oncocytic tumors displaying signs of invasiveness is higher than that occurring in tumors without oncocytic features. That is the reason why one should search actively for capsular and vascular invasion, and for the PTC nuclei, whenever dealing with any oncocytic tumor that looks benign at a first glance.[1–3,7,65]

Acknowledgements

This review is partly based on a clinico-pathological study supported by Fundação Calouste Gulbenkian (Project: 'Identificação de factores de prognóstico e de selecção terapêutica em carcinomas diferenciados da Tireoide'). The support of the Consortium IPATIMUP—IPO-Porto is also gratefully acknowledged.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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Paper 6

Eloy C, Vinagre J, Cameselle-Teijeiro J, Paiva ME, Soares P, Sobrinho-Simões M
"Tumour-in-tumour of the thyroid with basaloid differentiation. A lesion with a solid cell nest neoplastic component?"
International Journal of Surgical Pathology 2011; 19:276-280

International Journal of Surgical Pathology
19(2) 276–280
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DOI: 10.1177/1066896910393506
http://ijs.sagepub.com
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Tumor-in-Tumor of the Thyroid With Basaloid Differentiation: A Lesion With a Solid Cell Nest Neoplastic Component?

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Abstract

This study describes an encapsulated thyroid tumor having 3 different concentric appearances in a 70-year-old man. The most peripheral neoplasm showed features of a microfollicular adenoma; the intermediate lesion displayed typical features of the follicular variant of papillary thyroid carcinoma (FVPTC); and the main, central neoplasm, showed a basaloid configuration. At variance with the other neoplasms, the latter component exhibited high mitotic activity. Necrosis, angio-invasion, or capsular invasions were not observed. The centrally located neoplasm was immunoreactive for several cytokeratins, p63, carcinoembryonic antigen, and galectin-3, and negative for thyroid transcription factor 1, thyroglobulin, calcitonin, CD5, and CK20, featuring a phenotype similar to that of thyroid solid cell nests. A *N-RAS* mutation was found both in the basaloid and in the FVPTC components. The clinicopathological and immunohistochemical data ruled out the alternative possibilities of intrathyroidal metastasis and tumor from ectopic (thymic, parathyroid, or salivary gland) tissues.

Keywords

thyroid, tumor-in-tumor, follicular variant of papillary thyroid carcinoma, basaloid carcinoma, solid cell nests, *N-RAS*

Introduction

Tumor-in-tumor phenomena rarely occur in the thyroid. In some instances, they reflect tumor-to-tumor metastases, mainly to follicular adenoma (FA) or to follicular variant of papillary thyroid carcinoma (FVPTC).[1] Primary PTC can also grow in the core of a FA exhibiting a tumor-in-tumor pattern.[2]

First described by Getzowa[3] in 1907, solid cell nests (SCNs) of the thyroid are thought to represent remnants of the ultimobranchial body.[4] SCNs usually display a solid growth pattern, occasionally associated with mucin-filled cystic structures. SCNs are composed of single or multiple clusters of main cells (squamoid, with oval nuclei and eosinophilic cytoplasm) and rare C cells.[5] The main cells of SCN display a basal/stem cell phenotype, characterized by the immunohistochemical expression of p63 and basal cytokeratins in the absence of differentiation markers such as thyroid transcription factor 1 (TTF-1), thyroglobulin, and calcitonin.[6]

Cases of SCN hyperplasia have been previously described either as microscopic lesions composed of multiple small nests[7] or as a single giant SCN.[8] To the best of our knowledge no reports of neoplasms of SCN have been reported to date. Cameselle-Teijeiro et al[7] described an SCN in close contact with a papillary microcarcinoma sharing a *BRAF* V600E mutation; this finding led the authors to suggest the existence of a histogenetic relationship between SCN and PTC. We are not aware of other reports of SCN-in-tumor phenomenon.

Case

Clinical Features

A 70-year-old man with smoking habits and type 2 diabetes was admitted to São João Hospital because of nontoxic

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nodular goiter without associated complains. There was neither a history of radiation exposure nor familial history of any type of thyroid pathology. Thyroid function tests were unremarkable. Ultrasound examination revealed a solid and cystic nodule in the right thyroid lobe measuring 2.5 cm in largest dimension, which was submitted to fine-needle aspiration biopsy (FNAB). Cytological examination confirmed the partial cystic nature of the nodule, with a limited representation of cells. A total thyroidectomy was performed. Six months after surgery, the patient is alive and well. Computed tomography scan and magnetic resonance imaging of the head and neck region were negative for neoplasia in other organs and no enlarged lymph nodes were detected. Scintigraphy did not show metastatic disease. The patient is currently under treatment with radioactive iodine.

Macroscopic and Histologic Findings

The total thyroidectomy specimen weighted 38 g. There was a 3.2 × 3.0 × 2.6 cm whitish encapsulated nodule in the upper pole of the right lobe (Figure 1A), and a brownish encapsulated 2.0 cm nodule in the left lobe. The material was processed according to routine practice with formalin fixation and paraffin embedding for hematoxylin and eosin (H&E), immunohistochemical, and in situ hybridization (ISH) workup. Immunohistochemistry included antibodies for cytokeratins (clone AE1/AE3, Cell Marque, Rocklin, CA), cytokeratin (CK) 5 (XM26, Neomarkers, Fremont, CA), CK7 (OV-TL 12/30, Cell Marque), CK19 (A53-B, Neomarkers), CK20 (CK20, Novocastra, Newcastle-upon-Tyne, UK), thyroid transcription factor 1 (TTF-1; 8G7G3/1, Invitrogen, Carlsbad, CA), thyroglobulin (DAK-Tg6, Dako, Glostrup, Denmark), calcitonin (O83, Dako), chromogranin A, (chromogranin A, Zymed, Carlsbad, CA) synaptophysin (SP11, Neomarkers), p53 (DO-7, Dako), p63 (4A4, Neomarkers), carcinoembryonic antigen (CEA; Polyclonal, Dako), S100 protein (Polyclonal, Dako), smooth muscle actin (HHF35, Neomarkers), vimentin (V9, Dako), galectin-3 (9C4, Novocastra), CD5 (4C7, Novocastra), and Ki-67 (SP6, Neomarkers) as previously reported.[9] Primary antibodies were detected using a peroxidase-coupled polymer system (EnVision FLEX system, Dako). ISH for thyroglobulin and calcitonin was also performed on 4-µm thick paraffin sections using a commercial single-stranded DNA probe (CEM-0016, Histosonda Thyroglobulin, Cenbimo, Lugo, Spain, and CEM-0017, Histosonda Calcitonin, Cenbimo), following the manufacturer's protocol.

Histologically, the nodule in the right lobe was an encapsulated tumor-in-tumor composed of 3 lesions morphologically different (Figures 1B to 1E and 2). The most peripheral lesion, which showed features of FA with a microfollicular growth pattern, was present in nearly 90% of the nodule circumference and, occasionally, also between the intermediate and the main central lesion (Figures 1C and 1D). There were no signs of capsular or vascular invasion. The intermediate lesion displayed the typical features of FVPTC[10] and was present in approximately 50% of the nodule circumference (Figures 1C and 1E). The third (central) lesion constituted the bulk of the nodule and presented a solid pattern of growth with scattered tubular structures filled with a PAS (periodic acid Schiff) diastase–resistant material. This central lesion was composed of monotonous epithelioid cells with oval nuclei and eosinophilic cytoplasm, and exhibited a prominent peripheral palisading (Figures 2A to 2C). At variance with the other lesions, in which we did not detect any mitosis, the central lesion had 4 mitoses *per* 10/high-power field (Figure 2C). We neither observed any colloid-type material nor evidence of necrosis

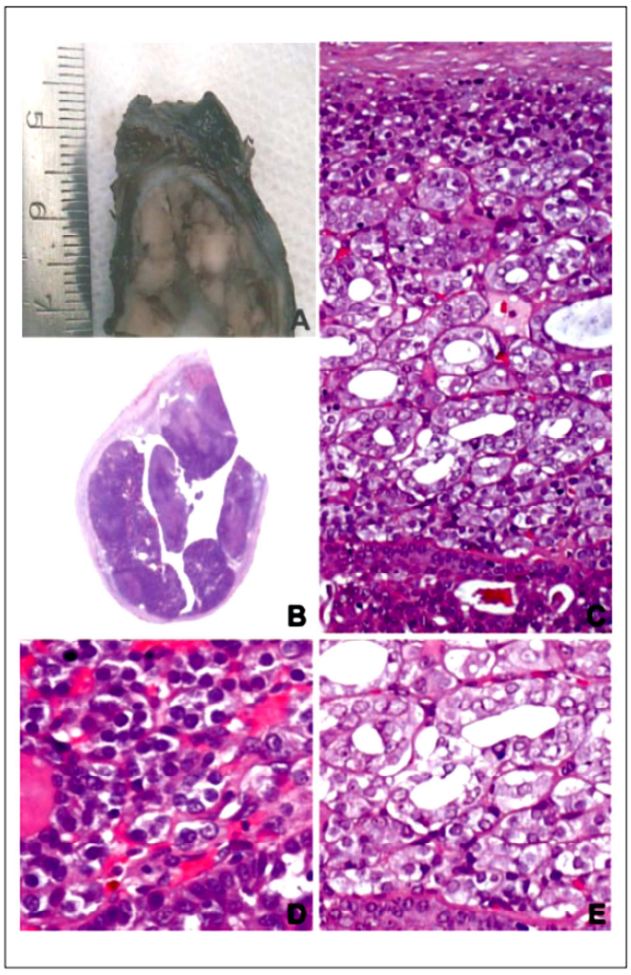


Figure 1. Basaloid tumor-in-follicular variant of papillary thyroid carcinoma (FVPTC)
Gross (A), panoramic (B), and microscopic (C) appearances of the nodule. In figure C the capsule and 3 layers can be seen (from top to bottom): capsule, follicular adenoma (FA), FVPTC, and central lesion (200×). At a higher magnification (400×), follicular adenoma (D) and FVPTC (E) can be seen, in both cases adjacent to the central lesion.

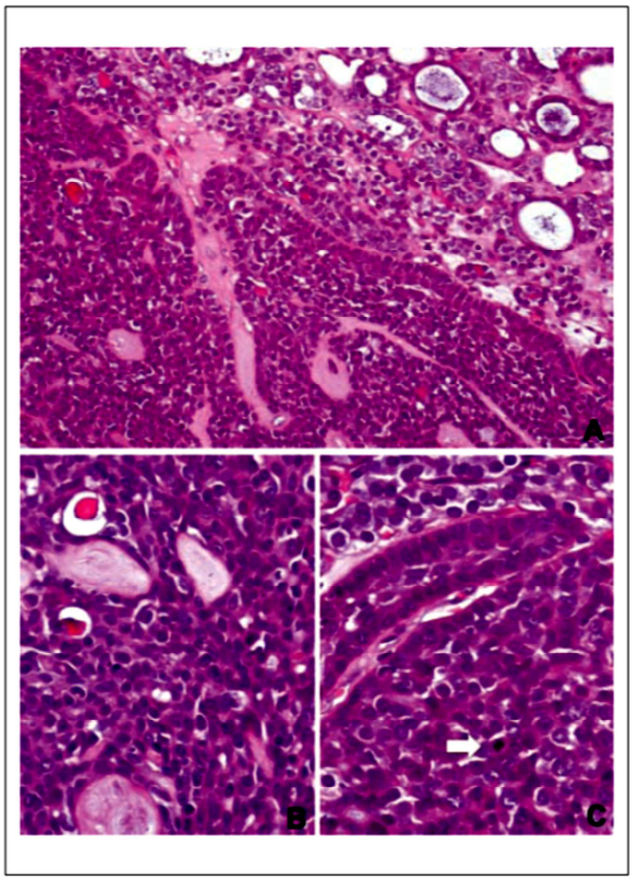


Figure 2. Histological features of the basaloid tumor (central lesion)
A peripheral palisade organization consisting of basaloid cells can be seen (A, 200×) along with cystic formations (B, 400×) and mitosis (C, 400×, arrow).

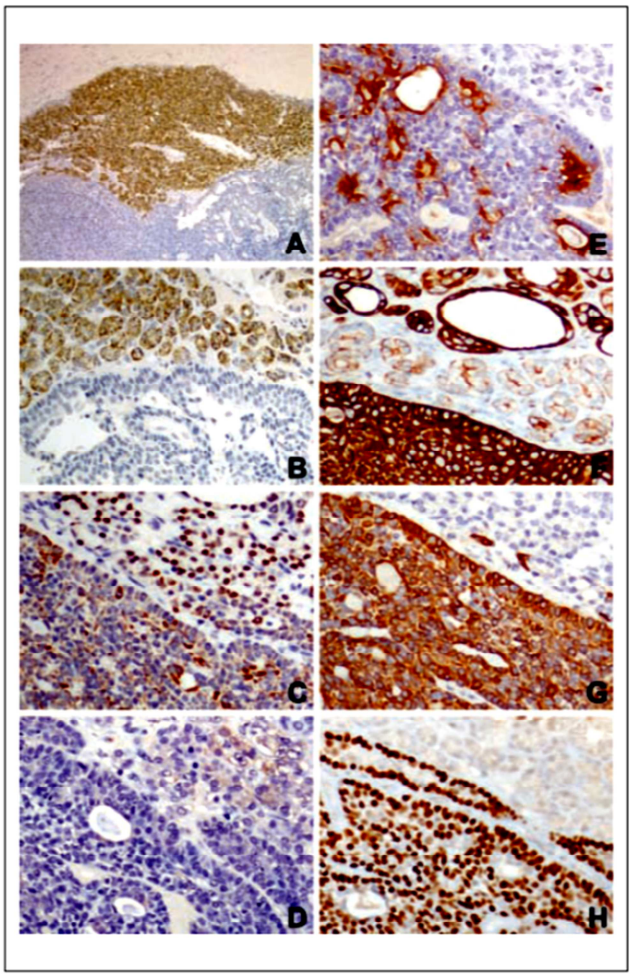


Figure 3. In situ hybridization (ISH)
ISH for thyroglobulin showing differential expression in the nodule (from top to bottom): capsule, follicular adenoma (FA), follicular variant of papillary thyroid carcinoma (FVPTC), and central lesion (A, 50×). ISH for thyroglobulin was positive in the FVPTC and negative in the central lesion (B, 200×). Immunohistochemical profile of interface between the FVPTC and the central lesion for thyroid transcription factor 1 (TTF-1; C, 200×), calcitonin (D, 200×), carcinoembryonic antigen (E, 200×), CK19 (F, 200×), CK5 (G, 200×), and p63 (H, 200×).

or vascular invasion in the central lesion. The nodule in the left lobe was a follicular adenoma. The remaining thyroid parenchyma was apparently normal.

ISH for thyroglobulin and calcitonin performed in the main lesion did not detect any positivity in the central component (Figures 3A and 3B). The results of the immunostains are summarized in Table 1 and shown in Figures 3C to 3H.

Mutations of *BRAF* and *N-RAS* were searched in the FVPTC, in the central lesion, and in the normal appearing thyroid parenchyma of the left lobe as previously described.[7] Polymerase chain reaction amplification of hot spot regions of these genes showed a Q61R *N-RAS* mutation in the exon 2 of both the FVPTC and the central lesion; no *N-RAS* mutations were detected in the normal appearing thyroid parenchyma. The study was not possible, for technical reasons, in the FA. No mutations were identified in the exon 15 hot spot of *BRAF* gene. *RET/PTC* translocations were searched in the central lesion as previously described,[11] but the results were inconclusive because of technical reasons.

Discussion

Tumor-to-tumor metastasis is a rare phenomenon that should be considered as a possibility in the presence of complex thyroid lesions showing biphasic or triphasic morphological and immunohistochemical features.[1] The occurrence of FVPTC apparently within a FA has been documented by several authors but the same does not hold true with the central lesion seen in our case. The possibility of this central lesion representing a metastasis in a preexisting thyroid tumor was made unlikely by the negative clinical and radiologic findings and by the follow, which did not reveal the

Table 1. Immunohistochemical Study of the 3 Lesions

	Differential Expression		
Primary Antibody	FA	FVPTC	Central Lesion
Cytokeratins AE1AE3	F	+	F
Cytokeratin 5	−	−	+
Cytokeratin 7	+	+	+
Cytokeratin 19	−	+	+
Cytokeratin 20	−	−	−
TTF-1	+	+	−
Thyroglobulin	+	+	−
Calcitonin	−	−	−
Chromogranin A	−	−	−
Synaptophysin	−	−	−
p53	−	−	−
p63	−	F	+
CEA	−	−	+[a]
S100 protein	−	−	−
SMA	−	−	−
Vimentin	F	+	−
Galectin 3	−	F	F
CD5	−	−	−
Ki-67	<1%	<1%	5%

Abbreviations: FA, follicular adenoma; FVPTC, follicular variant of papillary thyroid carcinoma; +, diffuse positive; −, diffuse negative; F, focal positivity; TTF-1, thyroid transcription factor 1; CEA, carcinoembryonic antigen; SMA, smooth muscle actin.
[a]Expression in luminal surface of the cysts.

existence of any neoplasia outside the thyroid gland. The alternative hypothesis of a poorly differentiated or undifferentiated thyroid carcinoma was ruled out by the absence of immunoreactivity for TTF-1, thyroglobulin, and calcitonin.[10] Furthermore, the morphological and immunohistochemical features (including the p53 negativity) do not support the diagnosis of poorly differentiated or undifferentiated carcinoma.[10]

Having ruled out the above-listed hypotheses, we evaluated the possibility of the central lesion representing a tumor arising from ectopic intrathyroid tissue. Salivary gland,[12] parathyroid,[13] and thymus[14] ectopic tissue located in the thyroid parenchyma have been reported, as well as rare thyroid neoplasias thought to be originated from such ectopic tissues.[15-17] Although there were basaloid cells with peripheral palisading suggesting a basal cell tumor of salivary gland type, the negativity for S100 protein, smooth muscle actin and vimentin, as well as the absence of any remnants of normal salivary gland tissue do not support this hypothesis. The histological appearance and the absence of chromogranin A do not fit an intrathyroidal parathyroid adenoma. Finally, our tumor did not have features of any of the thymus-derived tumors described by Chan and Rosai.[18]

A primary tumor-in-tumor with unusual features was finally considered, after having excluded the other possibilities. The morphologic features of the central lesion are similar to those observed in SCN main cells. The cells of both lesions are epithelioid/basaloid, with oval nuclei and eosinophilic cytoplasm, and displaying in both instances a solid pattern with cystic/tubular structures filled with a mucin-like PAS diastase positive material. The immunohistochemical profile observed in the central lesion is also similar to that of SCN: positivity for AE1AE3, CK5, CK7, CK19, p63, CEA, and galectin 3, and negativity for TTF-1, thyroglobulin, calcitonin, and CK20.[5,6,19] In "normal" SCN, as well as in multifocal SCN hyperplasia, C-cells are usually present in the periphery of the lesion,[5,7] but they were absent in the present case as well as in a case of "giant" SCN previously reported.[8] Taking all this into consideration, we concluded that the central lesion of the nodule had SCN features and may represent the first case of SCN tumor reported to date.

The histological and immunohistochemical features of the tumor here reported contrast with the features of the so-called ultimobranchial thyroid tumors arising in cattle, which appear to be a distinct entity displaying thyroglobulin–calcitonin positive cells.[20] In the setting of human pathology, SCN tumors may represent the fifth category of the family of thymic and branchial pouch–derived tumors in soft tissues of the neck and thyroid gland previously reported as a unifying scheme by Chan and Rosai.[18]

The morphologic features of the lesion (monomorphic and highly proliferative) and the presence of a mutation of the *N-RAS* oncogene support our assumption that this lesion should be considered a neoplasm rather than a hyperplasia. Its malignant potential remains to be established as the patient follow-up is too short.

The putative relationship between the basaloid central lesion and the FVPTC also remains to be clarified, as well as between the latter and the FA. The fact that the basaloid component was surrounded by an FVPTC and shared with it a Q61R *N-RAS* mutation supports a histogenetic relation between these 2 lesions.[7] An analogous finding was reported by Cameselle-Teijeiro et al,[7] who reported a *BRAF* mutation in a case of SCN hyperplasia with a contiguous papillary microcarcinoma, the mutation being present in both components.

Assuming that the central lesion has been originated from SCN it remains to be understood its basaloid features instead of an SCN appearance. We do not know whether the basaloid features may represent an unusual "differentiation switch" from preexisting SCN or indicate that the basaloid lesion may be the result of the neoplastic development (caused by the *N-RAS* mutation?) of branchial pouch stem cells before they evolve to full blown SCN cells. Both hypotheses remain unproven and further immunohistochemical and molecular studies of hyperplastic and neoplastic SCN lesions (especially those associated with thyroid tumors)

are needed to better understand the genesis and natural history of these unusual lesions.

Acknowledgments

The authors thank Professor Antonio Cardesa, Professor Hans-Konrad Müller-Hermelink, Professor Andreas Rosenwald, and Professor Juan Rosai for helping in the study of the case and in the review of the literature.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research and/or authorship of this article:

This work was partially supported by grant PS09/02050-FEDER from the Ministry of Science and Innovation (Instituto de Salud Carlos III), Spain.

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Paper 7

Cruz J, Eloy C, Aragués JM, Vinagre J, Sobrinho-Simões M
"Small-cell (basaloid) thyroid carcinoma: a neoplasm with a solid cell nest histogenesis?"
International Journal of Surgical Pathology 2011; 19: 620-626

International Journal of Surgical Pathology
19(5) 620–626
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DOI: 10.1177/1066896911405320
http://ijs.sagepub.com
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Small-Cell (Basaloid) Thyroid Carcinoma: A Neoplasm With a Solid Cell Nest Histogenesis?

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Abstract

In the past, undifferentiated (anaplastic) carcinoma of the thyroid included a small-cell variant with either a diffuse or a compact morphology. After the mid-1980s, with the advance of immunohistochemistry, almost all those rare tumors with the former characteristic were reclassified as low-grade lymphomas and those with the latter characteristic as small-cell variants of medullary carcinoma and poorly differentiated ("insular") carcinoma. Since then, no primary thyroid small-cell carcinoma has been reported in the literature, with the exception of a case of a small-cell (neuroendocrine) carcinoma, an exceedingly rare neoplasm akin to medullary carcinoma, with expression of neuroendocrine markers but lacking calcitonin immunoreactivity. Here, the authors report a primary small-cell carcinoma of the thyroid displaying a basaloid appearance and lacking any signs of neuroendocrine or C-cell differentiation.

Keywords

thyroid gland, small cell carcinoma, basaloid cell carcinoma, solid cell nest

Introduction

Small-cell malignancies of the thyroid gland are infrequent. Rare cases of small-cell carcinomas have been reported in the past, but many had an unsatisfactory clinical and imaging workup for exclusion of primary tumors outside the thyroid. With immunohistochemical reevaluation, nearly all those putative primary small-cell thyroid carcinomas proved to be low-grade lymphomas, poorly differentiated ("insular") carcinomas, and small-cell variants of medullary carcinomas, as concluded by several authors like Carcangiu et al[1] and Rosai et al[2]; similar results and conclusions were published later in works reported by Schmid et al[3] and Wolf et al.[4]

Case Report

Clinical and Pathological Findings

A 42-year-old female was referred to an endocrinology consultation for a cervical anterior tumor detected one month earlier. Clinical and ultrasound examination led to the conclusion that it was a thyroid gland tumor, and the patient was submitted to fine-needle aspiration biopsy, which suggested a follicular tumor. The remaining physical examination was normal. Thyroid function tests and calcitonin and calcium serum values were also within normal limits.

An elective total thyroidectomy with no resection difficulties was performed, without cervical lymphadenectomy because no lymphadenopathies were detected during surgical exploration. The surgical specimen weighed 25 g. In the upper pole of the left lobe, of 50 x 20 x 25 mm, a well circumscribed, lobulated, whitish and firm tumour of 25 x 20 x 15 mm was detected. The

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right lobe, of 50 x 15 x 20 mm, and the isthmus were both macroscopically unremarkable. The external glandular surface was bosselated and integrous.

Histological examination showed an unencapsulated neoplasm with a nesting growth pattern consisting of well-defined, variable sized insulae as well as trabeculae and solid areas. The periphery of the neoplasm displayed pushing borders, and invasive foci of the adjacent parenchyma were seen. The neoplasm was composed of small uniform (basaloid) cells with regular, round nuclei with fine chromatin, indistinct nucleoli, and scanty cytoplasm with ill-defined boundaries in a hyaline stroma background. An "eye-catching" histological finding was the presence of scattered cysts of various sizes and lacunae containing amorphous material, with calcified and mummified ghost and shadow cells (pilomatrixoma like) and cellular debris, within the neoplastic nodules. Additionally, epidermoid-like areas and coexisting follicular foci with papillary-like nuclear features were found. "Crunch" artifact foci were also observed. Overall mitotic activity was low, and necrotic areas were not found. Peritumoral vascular invasion was prominent (Figures 1A-1D).

Immunohistochemistry

Immunohistochemistry was performed on 4-μm representative sections after antigen retrieval using the standard avidin–biotin immunoperoxidase detection technique. Positive controls were included in each slide run with satisfactory results.

We observed a strong and diffuse immunoreactivity of the neoplastic cells with the antibodies to p63, CK19, CK8/18, AE1/AE3, and CAM5.2; a concurrent expression of CEA and 34βE12 was also detected in epidermoid foci. Immunoreactivity for TTF-1, thyroglobulin, or calcitonin was not observed in the neoplastic cells. No immunoreactivity for CK5, CK7, sm-actin, chromogranin A, synaptophysin, NSE, GCDFP15, HMB45, or S100 protein was detected. P53 immunoreactivity was seen in less than 10% of the tumoral cells. The proliferative index indirectly evaluated by Mib1/Ki67 was about 2% (Figures 2A-2I).

Ultrastructural Analysis

The ultrastructural evaluation was performed in a representative tissue sample retrieved from the paraffin block; ultrathin sections were mounted on copper grids and stained with uranyl acetate and lead citrate.

The ultrastructural study showed epithelial cells linked by desmosomes and with bundles of tonofilaments. Neurosecretory granules were not observed (Figures 3A and 3B).

Molecular Data

Mutations of *BRAF*, *HRAS*, and *NRAS* were sought in the tumor and in the morphologically normal-appearing thyroid parenchyma. The polymerase chain reaction amplification of "hotspot" regions of these genes (BRAFV600, HRASQ61, and NRASQ61) did not identify any mutation.

Additional Studies

The diagnosis of primary or metastatic small-cell carcinoma of the thyroid led to exhaustive clinical and imaging evaluations of the patient in order to find or rule out a primary tumor outside the thyroid gland, although there were no symptoms or signs of such an existence. Two months after surgery, head and neck, thoracic, abdominal, and pelvic CTs were performed, and no tumoral lesions were detected. An upper-digestive tract endoscopy and a total colonoscopy were done with no evidence of lesions. Breast ultrasound and mammographic studies did not detect any tumoral lesion either. The PET-CT scan with 18F-FDG and 7mCi (259MEq) activity, performed 4 months after surgery, did not disclose any hypermetabolic focus indicative of a possible primary tumor. No cutaneous lesions were identified.

The patient had an uneventful recovery and did not receive any further therapy, apart from thyroid hormone replacement. She is asymptomatic, with no signs of local recurrence or distant metastatic disease 16 months after surgery.

Discussion

The thyroid gland can harbor metastases from distant malignancies, and secondary involvement by small-cell carcinoma, although rare, is no exception. In our case, thorough clinical, laboratory, and imaging investigations were performed, and no tumoral lesions outside the thyroid gland were identified.

Rare cases of small-cell malignancies of the thyroid gland were reported in the past, some of them with questionable exclusions of primaries outside the thyroid.

Until the 1980s, *small-cell carcinoma* of the thyroid gland was considered an independent entity, with a compact and a diffuse variant, and was included as a subgroup of undifferentiated carcinoma. The so-called small-cell, compact type anaplastic (undifferentiated) carcinoma, originally reported by Meissner and Lahey[5] in 1948, was histologically described as an undifferentiated carcinoma composed of small cells forming clusters and strands in a hyaline stroma, with frequent mitoses. The possibility that some of the reported cases could be variants of medullary carcinoma was already raised in the early 1960s by several

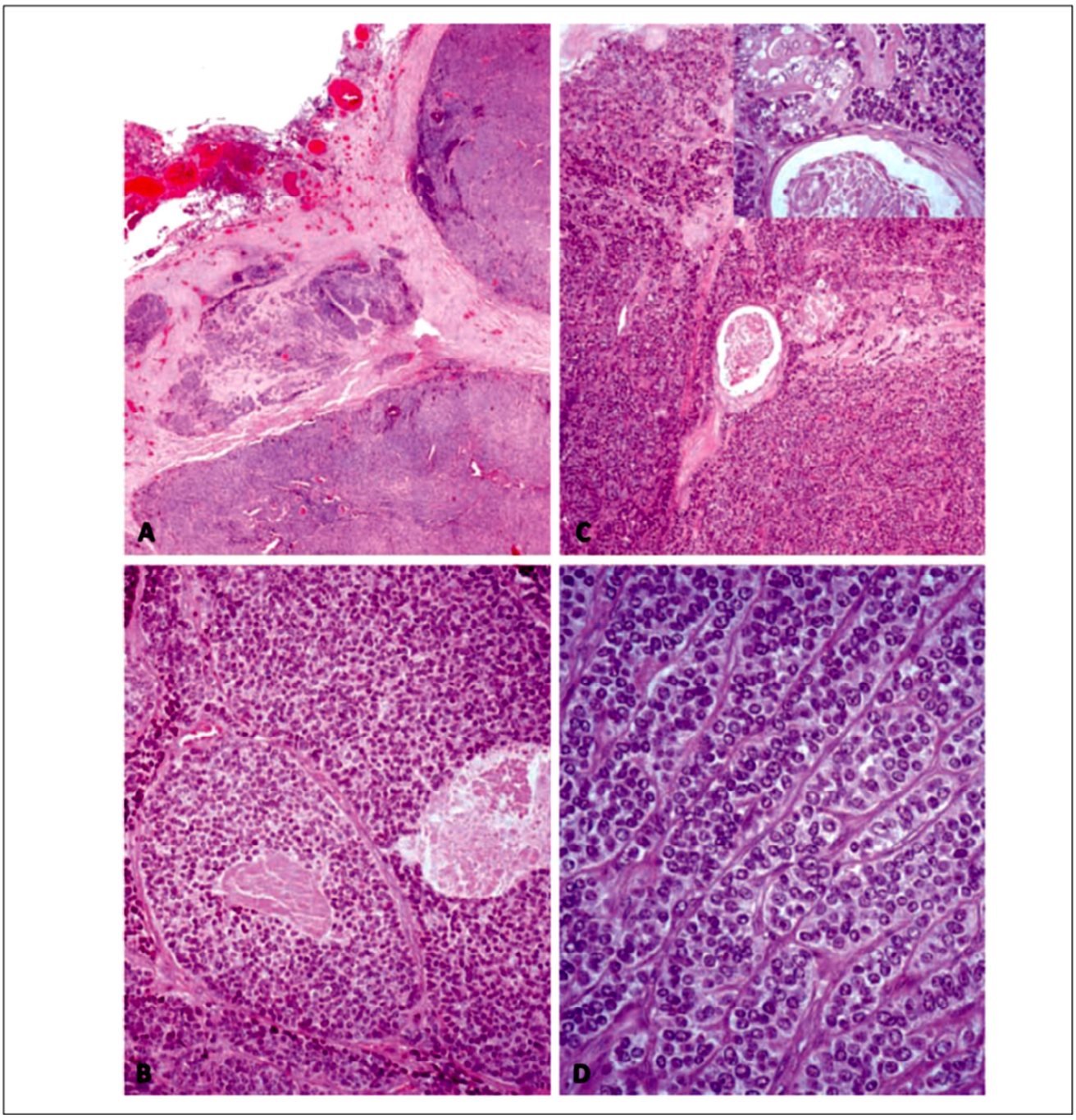


Figure 1. Histological aspects of the neoplasia: A. Periphery of the neoplasia and multinodular growth pattern (hematoxylin and eosin [H&E], 20×). B. Nested pattern areas with lacunae and microcysts (H&E, 200×). C. Solid pattern with focal epidermoid differentiation (H&E, 100×) contiguous to follicular arranged cells with papillary thyroid carcinoma nuclear features (inset). D. Trabecular areas composed of small monomorphic cells (H&E, 400×)

authors. In 1962, Meissner and Phillips[6] described a diffuse type of small-cell carcinoma of the thyroid gland, but the available microscopic illustrations do not allow the exclusion of a lymphoid neoplasm; the presence of occasional follicles within the tumor, a feature that was considered as supporting its origin from a preexisting differentiated thyroid carcinoma, could also be interpreted as entrapped follicular structures by the neoplastic cells.[6,7]

Gonçalves et al[8] described in 1978 the ultrastructural features of two cases that were thought to represent diffusely growing anaplastic small-cell carcinomas of the thyroid, mainly based on the presence of intercellular junctions.

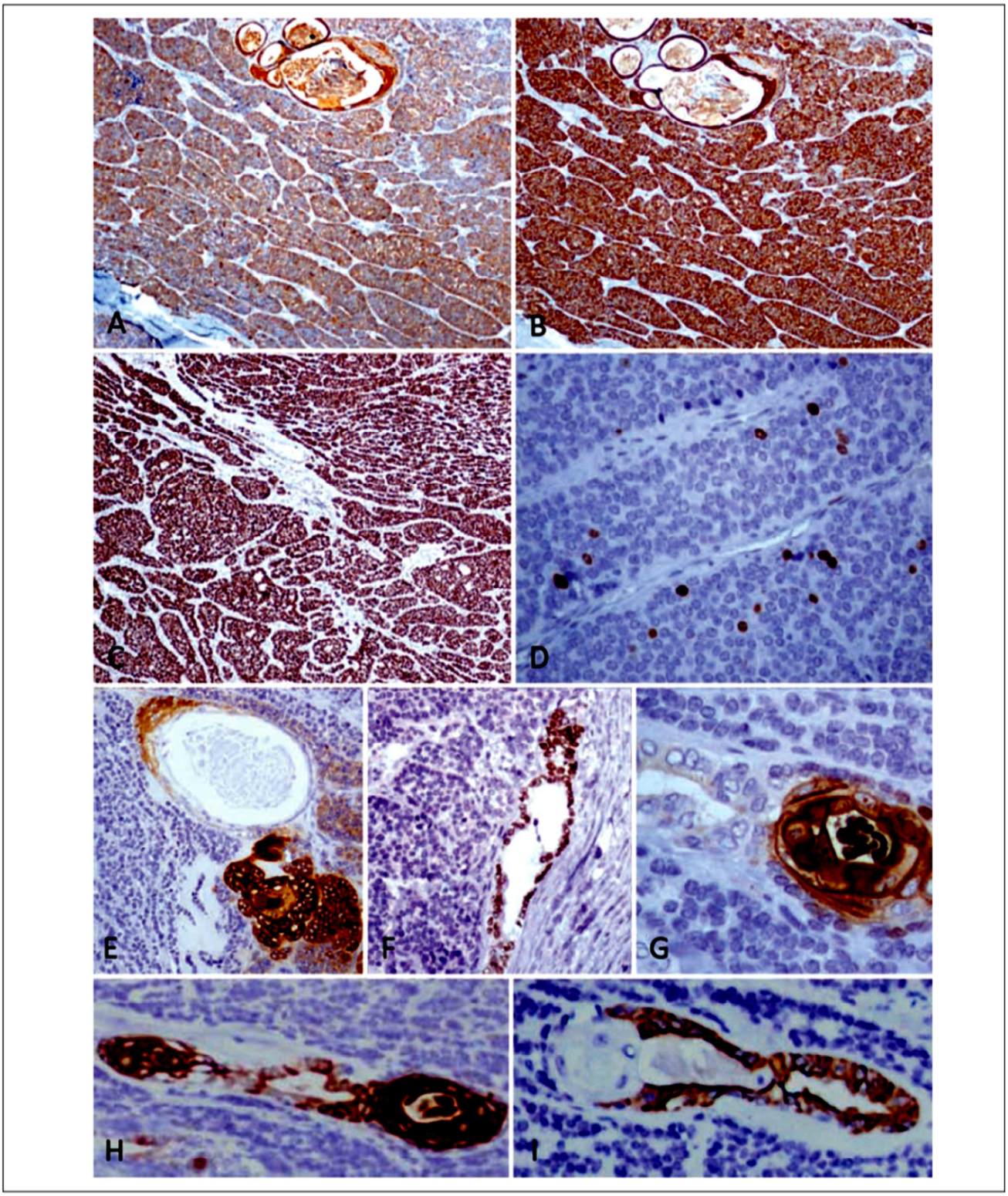


Figure 2. Immunohistochemical profile: A. Global staining for cytokeratins (AE1/AE3, 200×). B. CK19 expression (200×). C. p63 Immunoreactivity of the neoplastic cells (200×). D. Ki-67 labeling index (400×). E. Thyroglobulin highlighting the PTC-like foci (200×). F. TTF-1 highlighting the PTC-like foci (200×). G. CEA staining the epidermoid component (400×). H. High-weight cytokeratins (34βE12) staining the epidermoid component (400×). I. CK7 highlighting the Papillary Thyroid Carcinoma (PTC)-like foci (400×)

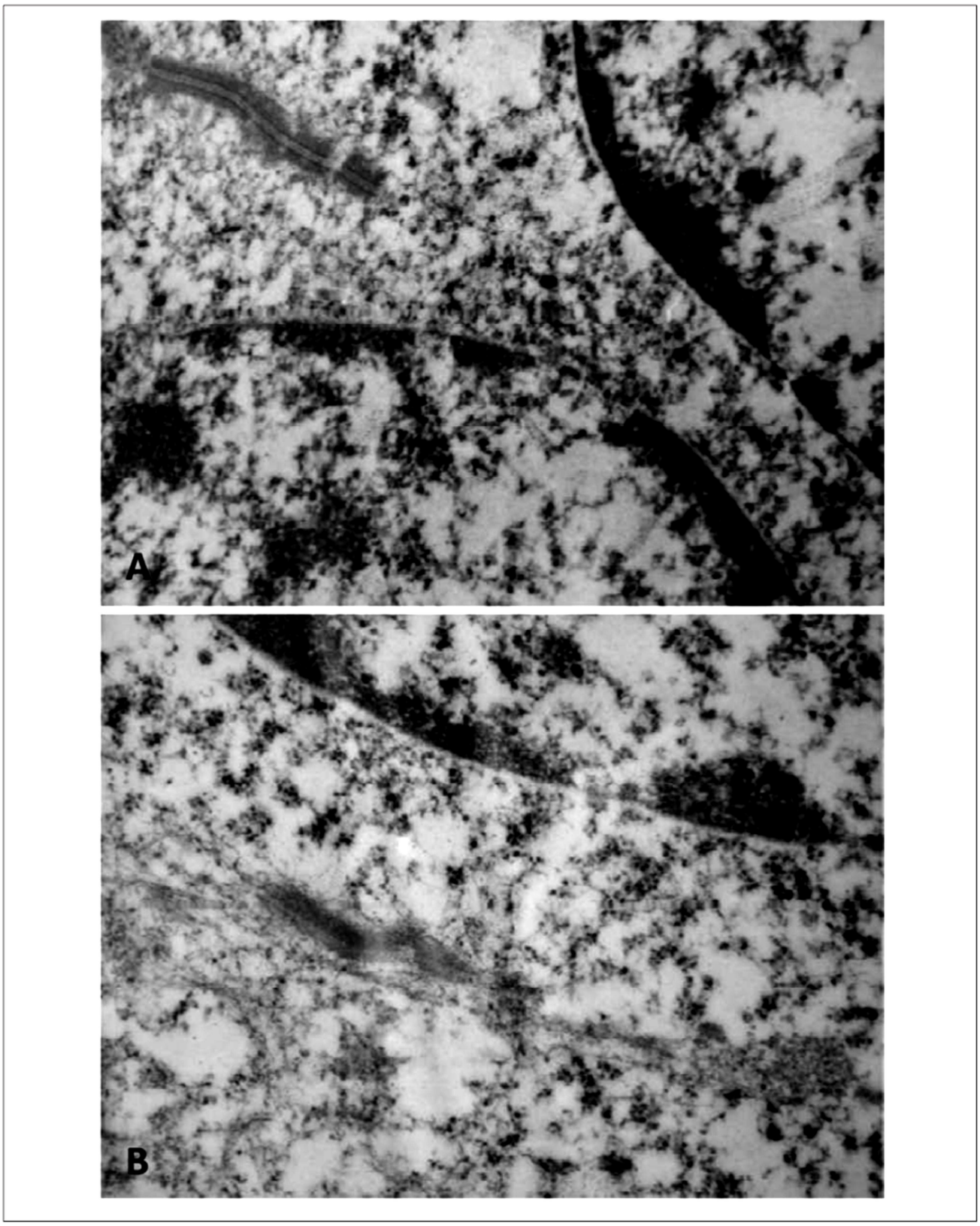


Figure 3. Ultrastructural study of the neoplastic cells: A. Two cell membranes attached by a desmosome (lead citrate and uranyl acetate, 24 000×). B. Tonofilaments in the cytoplasm of a neoplastic cell (lead citrate and uranyl acetate, 30 000×)

The immunohistochemical study performed years later showed that the case in which there were areas of differentiated carcinoma corresponded to a solid type of poorly differentiated carcinoma, and the other was a lymphoma (Sobrinho-Simões, M, unpublished observations, 1992).

In 1980, Luna et al[9] published a case of a densely packed, small, round-cell tumor with an extensive solid pattern; the presence of cell junctions detected by electron microscopy together with the absence of C-cell secretory granules led to the diagnosis of small-cell carcinoma of the thyroid.[9]

The widespread use of immunohistochemistry has led to major advances in this field. Nearly all the reported cases of diffuse and compact types of "small-cell" anaplastic carcinomas were found to be, after reevaluation, non-Hodgkin small B-cell lymphomas, poorly differentiated (insular) carcinomas, or small-cell variants of medullary carcinomas.[1]

Wolf et al[4] studied, using immunohistochemical techniques, a large series of undifferentiated small-cell thyroid neoplasms, originally diagnosed as small-cell carcinomas and lymphomas in the preimmunohistochemical era. These authors concluded that the vast majority were lymphoid malignancies, including those cases previously submitted as small-cell carcinomas. In only one case, with morphological features similar to lung small-cell carcinoma, was an immunohistochemical epithelial profile detected, but no additional studies were done, nor were any studies for excluding a primary tumor outside the thyroid performed. Wolf et al concluded that at variance with many other organs, primary small-cell carcinoma of the thyroid might not exist.

Schmid et al[3] also studied a large series of undifferentiated carcinomas of the thyroid, including nine cases originally classified as small-cell subtype. After the immunohistochemical study, five of these small-cell tumors were reclassified as poorly differentiated carcinomas or undifferentiated carcinomas and three as lymphomas, and one case was found to be a metastasis from a lung primary. These authors also concluded that true small cell carcinoma of the thyroid must be a very rare tumor.[3]

Finally, Rosai et al[2] achieved concordant results and recommended that the term undifferentiated small-cell carcinoma should be discarded.

Medullary thyroid carcinomas (MTCs) can show areas with diffuse, trabecular, and compact patterns of growth composed of cells with scanty cytoplasm and round to ovoid hyperchromatic nuclei. Such areas may be predominant or form a small component of the MTC, but they are usually a part of an otherwise common MTC. Mendelsohn et al[10] designated these neoplasms as small-cell variants of MTCs. In such tumors, the neoplastic "small" cells display calcitonin immunoreactivity; positivity for neuroendocrine markers such as chromogranin A, NSE, and synaptophysin is also detected, and frequently, there is CEA immunoreactivity. Furthermore, the tumor cells also show TTF-1 expression. In our case, typical areas of MTCs were not observed, nor any amyloid deposits. The immunohistochemical study ruled out definitively the diagnosis of MTC.

Some authors consider the existence of a small-cell "neuroendocrine" carcinoma, based on the findings of two cases reported by Eusebi et al.[11] This type of neoplasm, thought to belong to the same category of small-cell variant of MTC, is exceedingly rare and exhibits neuroendocrine markers and CEA positivity but lacks demonstrable C-cell differentiation. These calcitonin-free tumors, which are more frequent in familial settings with coexistent C-cell hyperplasia, are regarded as the least differentiated examples of tumors with C-cell histogenesis—having lost the capacity to secret calcitonin—and behave very aggressively clinically. Both carcinomas reported by Eusebi et al[11] met the morphological criteria for "oat-cell" carcinoma and displayed a high mitotic index. In our opinion, the study excluding a primary outside the thyroid gland could have been more exhaustive because in one of the cases the autopsy revealed bilateral subpleural nodules and metastatic liver deposits.[11] Eusebi et al[11] advanced the proposition that the two tumors should be separated from the small-cell variant of MTC and suggested the term *oat cell* carcinoma of the thyroid. Although the morphological features of our case are similar to one of the cases reported by Eusebi et al, we did not find any immunohistochemical or ultrastructural features supporting the existence of neuroendocrine differentiation.

Our case also has some histological similarities to some of the neoplasms described by Carcangiu et al[12] as poorly differentiated ("insular") carcinoma; however, the complete absence of thyroglobulin and TTF-1 immunoreactivity precludes this diagnosis.

When examining for the first time the peculiar microscopic characteristics of the present case, our impression was that we were probably facing a metastatic tumor from a primary carcinoma located outside the thyroid, but this possibility has been ruled out after an exhaustive study of the patient.

Poorly differentiated thyroid carcinoma, small-cell variant of medullary carcinoma, and small-cell "neuroendocrine" carcinoma were considered as diagnostic hypotheses for the present tumor, but as discussed above, none appears to be a suitable diagnosis. Undifferentiated (anaplastic) carcinoma was also excluded because those neoplasms are composed of spindle, pleomorphic, and epithelioid undifferentiated cells. This neoplasm does not also have CASTLE or thymic carcinoma morphology.

The peculiar and unusual histological appearance of our case—namely, the combination of small-cell cytology

with basaloid-type growing pattern and epidermoid foci—are features consistent with basaloid/epidermoid differentiation. The vacuolar and small cystic structures and the lacunae with amorphous hyaline material, different from those found in poorly differentiated (insular) carcinomas, have a close morphological resemblance to the intracytoplasmic, microvacuolar, and microfollicular structures commonly found in solid cell nests of the thyroid gland.[13] Furthermore, our case has a distinct and unique basal-type immunoprofile among thyroid tumors. In one of the most comprehensive studies published on this subject, Reis-Filho et al[14] demonstrated p63, CEA, AE1/AE3, CAM5.2, 34βE12, and CK19 expression and lack of immunoreactivity for TTF-1, thyroglobulin, and calcitonin in solid cell nests of the thyroid. These results supported previously published data, and suggest a basal/stem-cell phenotype of solid cell nests.[15] Putting together the aforementioned characteristics of our case and of solid nests, it is tempting to suggest that main cells of solid cell nests may be a possible origin of the small-cell (basaloid) carcinoma presented here.

A primary thyroid carcinoma with such morphological, immunohistochemical, and ultrastructural features does not meet the diagnostic criteria for inclusion into any of the nosologic groups of thyroid neoplasms on record. Thus, we suggest the designation *small-cell thyroid carcinoma*, adding the qualifier *basaloid*, for this unique primary tumor of the thyroid that, we hypothesize, may have derived from the main cells of solid cell nests.

In our case, the presence of conspicuous vascular invasion could be a sign of probable aggressive clinical behavior; whether or not a total thyroidectomy is a sufficient therapeutic approach for this case or if it should have been complemented by external radiation therapy and additional systemic multimodality therapies is disputable.

To the best of our knowledge, this is the first primary small-cell basaloid carcinoma of the thyroid without neuroendocrine differentiation that has been reported in the literature.

Acknowledgments

The authors would like to thank Professor Juan Rosai for kindly reviewing the case and suggesting the designation of small-cell (basaloid) carcinoma of the thyroid for this neoplasm. The authors are also grateful to Professor Stirling Carpenter for assistance with the ultrastructural study and Dr Diogo Cruz for bibliographic support.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

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