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Squamous cell carcinogenesis of the skin:

Molecular characterization of prognostic biomarkers

MANUEL ANTÓNIO COSTA CAMPOS

Tese de Doutoramento em Medicina e Oncologia Molecular

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Publicações

Ao abrigo do disposto do nº 2, alínea a) do artigo 31º do Decreto-Lei n.º 115/2013 de 7 de Agosto fazem parte integrante desta tese de doutoramento os seguintes trabalhos já publicados:

Artigo I

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Artigo II

Campos, M. A., Lopes, J. M., and Soares, P. 2018. The Genetics of Cutaneous Squamous Cell Carcinogenesis. Eur J Dermatol, 28 (5):597-605.

Artigo III

Campos, M. A., Macedo, S., Fernandes, M., Pestana, A., Pardal, J., Batista, R., Vinagre, J., Sanches, A., Baptista, A., Lopes, J. M.*, and Soares, P*. 2019. TERT Promoter Mutations Are Associated With Poor Prognosis in Cutaneous Squamous Cell Carcinoma. J Am Acad Dermatol, 80 (3):660-669.

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Artigo IV

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When something is important enough,
you do it even if the odds are not
in your favor.

Elon Musk

Table of Contents

Abbreviations

AK	Actinic keratosis
AJCC	American Joint Committee on Cancer
ALT	Alternative lengthening of telomeres
cBCC	Basal cell carcinoma
bp	Base pairs
BRAF	v-Raf murine sarcoma viral oncogene homolog B
BWH	Brigham and Women´s Hospital stating system
CCND1	Cyclin D1
CHVNGE	Centro Hospitalar Vila Nova de Gaia e Espinho
COSMIC	Catalog of Somatic Mutations in Cancer
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ELK1	ETS-Like Gene 1
ELK4	ETS-Like Gene 4
ETS	E-Twenty-six family
FFPE	Formalin-fixed paraffin-embedded
HPV E6	Human papillomavirus protein E6
HRAS	Harvey rat sarcoma viral oncogene homolog
KRAS	Kirsten rat sarcoma viral oncogene homolog
LOH	Loss of heterozygosity
MAPK	Mitogen-activated protein kinase
NMSC	Non-Melanoma Skin Cancer
NRAS	Neuroblastoma RAS viral (v-RAS) oncogene homologue
RAS	Rat sarcoma viral oncogene homolog
RB	Retinoblastoma
RNA	Ribonucleic acid
cSCC	Cutaneous squamous cell carcinoma
SPDV	Portuguese Society of Dermatology and Venereology
TERTp	Tert promoter
TERT	Telomerase Reverse Transcriptase gene
TKI	Tyrosine Kinase Inhibitor
TNM	Tumor, node, metastases
TP53	Tumor protein P53
UVR	Ultraviolet radiation

Abstract

Cutaneous squamous cell carcinomas (cSCC) are the second most common skin cancer in Caucasians. Despite its high frequency, when compared to other squamous cell carcinomas of the lung, head, and neck, cSCC has not received such great interest by the scientific community in the last decades. Its growing incidence, rising costs associated with its management, and the fact that they are responsible for 20% of all cutaneous cancer-related deaths highlight the need for a better understanding of its carcinogenesis.

Microscopic evaluation of a tissue specimen taken from an excised cSCC remains the gold standard for establishing a correct diagnosis. It is expected that in the future, analysis of the molecular characteristics of a tumor will aid in establishing a diagnosis, estimating prognosis, as well as helping in the selection of the most appropriate treatment for patients diagnosed with cSCC. *TP53* mutations have been reported in up to 90% of the cSCC and are considered an early event in cutaneous squamous cell carcinogenesis, but its prognostic significance has not been determined. *RAS* mutations have been described in a low percentage of cases and are considered to participate in the early carcinogenesis of cSCC. *TERTp* mutations were described in a high percentage of invasive cSCCs (25 to 74%) and were suggested to have a more relevant role in tumor progression than initiation. *TERTp* mutations have been described as potential biological predictors of metastasis and/or mortality in several cancer models, but its putative prognostic value in cSCC has not been established. To date, only clinicopathological prognostic markers have been described in cSCC for recurrence and metastasis, and the identification of a reliable, easy-to-perform and clinically useful molecular marker would be of great interest in cSCC prognostication.

This thesis aimed to contribute to the unraveling of cutaneous squamous cell carcinogenesis and to progress towards a better understanding of its epidemiology, molecular etiopathogenesis, and potentially identify biomarkers of prognosis. Paper I was the starting point of our project, where we identified and clinically characterized all cSCC that would be included in further studies. This paper described trends in a segment of the Portuguese population and showed that the incidence of in *situ* and invasive cSCC had rapidly increased between January 2004 and December 2013. A rising age-adjusted incidence was observed in both genders, particularly in the last study period of the study

(16.2/100 000-person year). Paper II revised the current knowledge of cutaneous squamous cell carcinogenesis comprehensively and was an important milestone given that we identified which genetic alterations would be included in our project. Based on this revision, our clinical hypothesis that *TERTp* mutations, *RAS* mutations, and P53 overexpression could turn out to be biomarkers of prognosis led to the development of Paper III and Paper IV. *TERTp* mutations frequency had been previously reported in cSCC (up to 74%), but its prognostic significance had not been established. The desire to define the correct frequency of *TERTp* mutations and a potential association with prognosis in cSCC led us to perform genetic profiling of *TERTp* mutations in a large retrospective series of cSCC and to determine the predictive value of these mutations in prognosis (Paper III). In our series, *TERTp* mutations were detected in 19.4% of *in situ* and 34.7% of invasive cSCC. As in other cancer models, *TERTp* mutations turned out to be an independent predictor of recurrence and metastasis in cSCC. Our study reported that mutated cSCC cases had an 8 times higher risk of recurrence and 16 times higher risk of metastasis than wild-type cases. The appeal to discover additional biomarkers of prognosis in cutaneous squamous cell carcinogenesis led us to study *RAS* mutations and P53 expression (Paper IV). No differences in P53 overexpression between *in situ* and invasive cSCC was observed in our study. *RAS* mutations were present more frequently in invasive than *in situ* cSCC (10.7% vs 3.2%, respectively) and seemed to be a marker of invasiveness since these mutations were more frequently associated with an infiltrative than the expansive pattern of invasion. *RAS* mutations failed to be associated with prognosis, but P53 overexpression was more frequently observed in recurrent and metastatic cases and turned out to be a predictor of recurrence in the univariate analysis. Despite our enthusiastic results, we are aware that larger studies with more recurrent and metastatic cSCC are necessary to confirm the prognostic significance of *TERTp* mutations and P53 overexpression.

In conclusion, we believe our study has contributed to the unraveling of cutaneous squamous cell carcinogenesis and to progress towards a better understanding of its epidemiology in Portugal, molecular etiopathogenesis, and to have potentially identified a novel molecular biomarker of prognosis in cSCC (*TERTp* mutations).

Resumo

Os carcinomas espinocelulares (CEC) são o segundo cancro cutâneo mais comum em Caucasianos. Apesar da sua elevada incidência, os CEC não têm sido alvo de interesse equivalente na comunidade científica quando comparados com outros carcinomas epidermoides (ex: pulmão e da cabeça/pescoço). A incidência crescente, o aumento dos custos associados ao tratamento e o facto de serem responsáveis por 20% de todas as mortes por cancro cutâneo, evidenciam a necessidade de um melhor conhecimento da sua carcinogénese.

O diagnóstico de CEC baseia-se na avaliação microscópica. Espera-se que, no futuro, a análise molecular de um tumor auxilie no seu diagnóstico, na predição do prognóstico, bem como na seleção do tratamento mais adequado em pacientes com CEC. As mutações no *TP53* foram descritas em até 90% dos CEC e são consideradas eventos precoces na sua carcinogénese, embora o seu significado prognóstico ainda não tenha sido esclarecido. As mutações do *RAS* foram descritas numa pequena percentagem de CEC e foram consideradas como eventos precoces na sua carcinogénese. As mutações do promotor da telomerase (*TERTp*) foram descritas numa elevada percentagem de CEC invasores (25 a 74%) e propostas como tendo um papel mais relevante na progressão do que na iniciação tumoral. As mutações do *TERTp* foram descritas como sendo potencialmente preditivas de metástase e/ou mortalidade em vários modelos de cancro, mas o seu valor como marcador de prognóstico em CEC ainda não foi esclarecido. Adicionalmente, apenas foram descritos marcadores clínicopatológicos de prognóstico para o desenvolvimento de recidiva e metástase em CEC. A identificação de um marcador molecular confiável, de fácil determinação e clinicamente útil seria de grande interesse na determinação do prognóstico em pacientes diagnosticados com CEC.

O objetivo desta tese foi contribuir para o melhor conhecimento da carcinogénese dos CEC, concorrendo para o entendimento da sua epidemiologia, patogenia molecular e, potencialmente, permitir a identificação de biomarcadores de prognóstico. O Artigo I foi o ponto de partida do nosso projeto, tendo sido possível a identificação e caracterização clínica dos CEC que viriam a ser incluídos nos estudos subsequentes. Este artigo descreve as tendências epidemiológicas de um segmento da população portuguesa, demonstrando uma

incidência crescente dos CEC (*in situ* e invasores) entre janeiro de 2004 e dezembro de 2013. O estudo revelou uma incidência crescente em ambos os sexos, particularmente no último período de estudo (16,2/100.000 pessoas-ano). Na segunda publicação realizou-se, de forma compreensiva, uma revisão sobre o conhecimento atual da carcinogénese do carcinoma espinocelular da pele. O Artigo II foi um marco importante visto que foram identificadas as alterações genéticas incluídas no nosso projeto. A hipótese de que as mutações do *TERTp*, as mutações do *RAS* e a sobre-expressão da P53 poderiam ser biomarcadores de prognóstico levaram ao desenvolvimento dos Artigo III e IV. As mutações do *TERTp* foram descritas previamente em CEC (até 74%), mas o seu significado prognóstico ainda persiste por estabelecer. Com o objetivo de definir a frequência de mutações do *TERTp* na nossa série e estabelecer uma associação destas mutações com o prognóstico, realizamos a caracterização molecular de uma grande série retrospetiva de CEC (Artigo III). No nosso estudo, as mutações do *TERTp* foram detetadas em 19,4% dos cSCC *in situ* e em 34,7% dos cSCC invasores. Tal como noutros modelos oncológicos, as mutações do *TERTp* revelaram-se um preditor independente de recidiva e metástase em CEC, sendo que os casos mutados apresentaram um risco 8 vezes maior de recidiva e 16 vezes maior de metástase do que os casos não-mutados. O desejo de pesquisar outros biomarcadores de prognóstico levou-nos a estudar as mutações do *RAS* e a expressão de P53 (Artigo IV). No nosso estudo, não foram observadas diferenças na expressão de P53 entre os CEC *in situ* e invasores. As mutações do *RAS* foram detetadas mais frequentemente em CEC invasores do que *in situ* (10,7% *vs* 3,2%, respetivamente) e associaram-se mais frequentemente a um padrão infiltrativo do que expansivo de invasão. As mutações do *RAS* não se associaram ao prognóstico, mas a sobre-expressão da P53 foi observada com maior frequência nos casos recidivantes e metastáticos, revelando valor preditivo de recidiva na análise univariada. Apesar dos nossos resultados encorajadores, estamos conscientes de que estudos com um maior número de CEC recidivantes e metastáticos serão necessários para confirmar o significado prognóstico das mutações do *TERTp* e da sobre-expressão da P53.

Em conclusão, acreditamos que o nosso estudo contribuiu para uma melhor compreensão da carcinogénese dos carcinomas espinocelulares da pele e para um melhor conhecimento da sua epidemiologia em Portugal, da sua patogenia molecular, e para a identificação de um novo biomarcador molecular potencialmente preditivo de prognóstico em CEC (mutações do *TERTp*).

CHAPTER 1

Introduction

Non-melanoma skin cancer (NMSC) represents the most frequently observed malignancy among Caucasians (Staples, Elwood et al. 2006). In individuals with fair skin, approximately 75-80% of these malignancies are basal cell carcinomas (cBCCs) and up to 25% are squamous cell carcinomas (cSCC). Despite this classical distribution of frequencies, one study cited a 1:1 ratio between cBCC and cSCC, suggesting an increased incidence of cSCC in a growing elderly population (Rogers, Weinstock et al. 2015). This thesis will focus mainly on cSCC.

There has been a long pursuit of the etiologic factors associated with skin cancer in general and particularly with cSCC. In 1775, Sir Percivall Pott described in his treatise "*Chirurgic Observations Relative to the Cancer of the Scrotum"* a possible relationship between cSCC and chimney soot exposure (Pott 1974). Later, during the Industrial Revolution, association with arsenic, coal tar, shale oil, and creosote and the development of skin cancer were reported. In the late 1800s, Paul Unna noted a connection that chronically sun-exposed sailors had a higher incidence of skin cancers.

Despite huge advances in oncology and the fact that cSCC is readily accessible to biopsy and consequent histologic/molecular studies, cSCC did not receive such great interest by the scientific community in the last decades.

Epidemiology

Keratinocyte carcinomas (cBCC and cSCC) occur worldwide in all races. Skin cancer incidence is higher than all other cancers combined and, in some countries, it is estimated that one in five individuals will develop skin cancer during their lifetime (over 95% will be NMSC) (Stern 2010). Compared to other squamous cell carcinomas of the lung, head, and neck, there is a lack of epidemiological information, namely in countries like Portugal, where there is a high annual cumulative exposure to ultra-violet radiation (UVR).

In Portugal, cancer registries do not uniformly collect epidemiological information about NMSC. Improving national cancer registries and complete epidemiological information in each country is of maximal importance since cSCC is responsible for 20% of

all cutaneous cancer-related deaths (Rowe, Carroll et al. 1992). The exact incidence of cSCC may be difficult to ascertain due to diagnostic accuracy and diagnostic criteria (e.g. differentiation between actinic keratoses (AK) and *in situ* cSCC). Deriving precise data is hampered by the fact that these neoplasms are not routinely included in cancer registries and they are often treated in private offices. Another limitation of most cancer registries is the fact that they fail to include any subsequent tumors after the first cSCC and to discriminate between cutaneous and mucosal SCC. Although an accurate incidence is difficult to establish, this cancer is considered to be one of the most costly malignancies in populations of European ancestry (Housman, Feldman et al. 2003). It is expectable that the costs associated with cSCC will continue to rise due to the lengthening of the individual lifespan and the aging population.

The most important intrinsic risk factor for NMSC is skin phenotype (Table 1) (Halder and Bridgeman-Shah 1995, Rigel, Friedman et al. 1996), and the most important extrinsic risk factor is UVR. The interaction between the aforementioned intrinsic and extrinsic risk factors is responsible for the highest incidence rates of cSCC in fair-skinned people who have a sun-sensitive phenotype (fair eye, skin and hair color, and skin that is prone to sunburn) (Lomas, Leonardi-Bee et al. 2012). It is known that the average amount of annual UVR correlates with the incidence of skin cancer. There is also a direct relationship between the incidence and latitude; the closer individuals are to the equator, the greater their exposure to UVR. In Australia, the risk of having NMSC at the age of 70 years is 70% for men and 48% for women (Harris, Griffith et al. 2001). Australians, exposed to very high and long-term UVR levels are more likely to develop cSCC than any other populations in countries with more intermittent UVR exposure such as the United Kingdom (Staples, Elwood et al. 2006, Lomas, Leonardi-Bee et al. 2012). Another factor that may influence cSCC incidence is altitude; Switzerland has the highest altitude of all mainland Europe and reportedly the highest cSCC incidence rates, as well as presenting the fastest increase (Lomas, Leonardi-Bee et al. 2012).

Table 1. Influence of skin color on the epidemiology of NMSC.

Characteristic	Lightly pigmented individuals	Darkly pigmented individuals
Annual incidence of NMSC	230 per 100 000	3.4 per 100 000
cBCC:cSCC ratio	4:1	1.1:1

When approaching squamous cell carcinogenesis, although not always evident, we must take into account its classical multistep model: premalignant lesions, AK and *in situ* cSCC/Bowen's disease, invasive carcinoma, and metastatic cSCC.

AK is most often found in fair-skinned individuals, but it can be seen in all races. If AK were considered a malignant neoplasm, it would transform cSCC into the most common cancer in Caucasians. It is estimated that 12% of the population will develop AK, most frequently located in sites with the most cumulative sun exposure (e.g. bald scalp, superior helices of the ears, face, dorsal hands, extensor forearms). AK and cSCC present common risk factors: skin phototypes I/II, significant cumulative sun exposure, prior history of AK, older age, immunosuppression, and male gender (Green 2015). AK is also a marker for increased risk of developing invasive cSCC but the rates of transformation are low and are difficult to ascertain with precision (Chen, Feldman et al. 2005). A systematic review showed that progression rates of AK to invasive cSCC ranged from 0% to 0.075% per lesion-year, with a risk of up to 0.53% per lesion in patients with a history of NMSC (Werner, Sammain et al. 2013). The estimated cumulative lifetime risk among patients with multiple AK is approximately 6% to 10% (Salasche 2000).

In situ cSCC is estimated to evolve to invasive cSCC by up to 5% (Jaeger, Gramkow et al. 1999). Risk factors for *in situ* cSCC are similar to AK and invasive cSCC and include fair skin, long-term sun-damage, radiation exposure, immune compromise, human papillomavirus infection, and chronic injuries (such as chronic lupus erythematous lesions and ulcers) (Kossard and Rosen 1992, Reizner, Chuang et al. 1994, Leibovitch, Huilgol et al. 2005, Hama, Ohtsuka et al. 2006, Moloney, Comber et al. 2006, Drake and Walling 2008, Morton, Birnie et al. 2014). Incidence is higher in the seventh decade of life, and most studies have reported a slight female preponderance (Eedy and Gavin 1987, Jaeger, Gramkow et al. 1999, Hansen, Drake et al. 2008). Many studies report that *in situ* cSCC occur mainly on sun-exposed sites, with the head and neck being the most common location on males (29–54%) (Jaeger, Gramkow et al. 1999, Foo, Lee et al. 2007); however, the lower limbs seem to be affected more frequently in women than in men (Eedy and Gavin 1987, Kossard and Rosen 1992, Brewster, Bhatti et al. 2007).

Invasive cSCC is the second most common skin cancer in Caucasians and the most common skin cancer in immunosuppressed organ transplantation recipients. In immunosuppressed patients, there is a 65- to 250-fold increased incidence of cSCC (Lindelof, Sigurgeirsson et al. 2000, Berg and Otley 2002). The epidemiology of cSCC are similar to those of AK and *in situ* cSCC, with the majority of cSCC occurring on the head,

neck, upper extremities, or shins (Karia, Han et al. 2013). cSCC is found more frequently in men (3:1, male: female) and the incidence increases significantly after 60 years of age (Harris, Griffith et al. 2001). The incidence of cSCC is rising worldwide in all age groups over the last several decades at an estimated 3-10% per year, with >400 000 cases of invasive cSCC diagnosed annually in the US (Karia, Han et al. 2013). There has been a steady rise in invasive cSCC incidence, with reported increases of 50% to 200% over the past 3 decades. In Europe, the incidence has been rising, with an estimated absolute increase of 2000 new cases per year in countries with 4.5-9 million inhabitants (Birch-Johansen, Jensen et al. 2010, Hussain, Sundquist et al. 2010, Carsin, Sharp et al. 2011). This rise in incidence is largely ascribed to a longer lifetime UVR exposure as a result of greater longevity, ozone depletion, and increased voluntary exposure to UVR (Karia, Han et al. 2013). The growing incidence of cSCC will result in a higher demand for medical care related to skin cancer, which has already been estimated to grow 5% annually in Central Europe (de Vries, van de Poll-Franse et al. 2005).

The vast majority of cSCC patients are diagnosed with early-stage disease, and the prognosis is excellent in these cases. Most cSCC are readily treated and cured with surgery. Although mortality is a rarity in cBCC (estimated at 0.12 per 100 000 inhabitants) (Buchanan, De'Ambrosis et al. 2014), occurring primarily in immunocompromised patients and individuals with basal cell nevus syndrome, it represents an important issue in cSCC, especially in the elderly. In adults over 85 years of age, the majority of skin cancer deaths are due to cSCC (Weinstock 1997). The risk of developing metastases from cSCC is generally low, with a 5-year metastatic rate of 5% (Alam and Ratner 2001). Metastasis is predominantly nodal (Kivisaari and Kahari 2013). However, depending on the patient (gender, age, phototype, immune status) (Brunner, Veness et al. 2013, Dacosta Byfield, Chen et al. 2013) and tumor characteristics (depth of invasion, histologic features, location, horizontal size, perineural invasion, tumor recurrence, incomplete excision, multiple tumors) (Rowe, Carroll et al. 1992, Weinberg, Ogle et al. 2007, Brantsch, Meisner et al. 2008, Thompson, Kelley et al. 2016) certain group of patients are at increased risk for metastases (up to 40%) (Alam and Ratner 2001, Gore, Shaw et al. 2016). Weinstock reported an age-adjusted mortality rate for confirmed cases of cSCC in Rhode Island of 0.26/100 000 inhabitants. Mortality is higher in Caucasians and older persons and men have a 3:1 greater risk when compared to women. cSCC located on the ear, lip, and genitalia appears to have a higher risk of death (Weinstock 2006).

Genetics

In cSCC, the microscopic evaluation of a tissue specimen taken from an excised tumor remains the gold standard for establishing the diagnosis. DNA extraction and analysis of transcribed genes and expressed proteins adds important information to the histologic features detected by microscopy. It is expectable that in the future diagnosis and prognostic information, as well as the selection of treatment, will most likely be based upon the combination of histological examination and an analysis of the molecular characteristics of the tumor.

Carcinogenesis is usually considered the process by which cancer arises and progress, and it normally includes multiple step-by-step events (Kinzler and Vogelstein 1996). This so-called multistep process, not always evident in squamous cell carcinogenesis, includes several rate-limiting steps, representing the acquisition of mutations and/or epigenetic modifications, eventually leading to the development of cancer. cSCC arises in somatic cells and is of epithelial origin. Theoretically, the stepwise changes involve an accumulation of errors (mutations) in vital regulatory pathways that control cell division, apoptosis, senescence, cell-cell, and cell-matrix interactions, and cell death. These alterations will provide a selective growth advantage compared to surrounding cells, resulting in tumor growth. Another important factor in tumor progression is decreased efficiency in DNA repair systems and increased genetic instability (Loeb, Bielas et al. 2008). It is important to recall the hallmarks of cancer when approaching the carcinogenesis of a tumor, which includes six traits: self-sufficiency concerning growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion/metastasis (Hanahan and Weinberg 2000).

Despite being called NMSC, cBCC and cSCC are biologically and genetically different and a distinction between both entities has to be made, rendering the term “NMSC” a poor conceptual term. Disruption of the Hedgehog-Patched signaling pathway is closely linked to the development of cBCC while this is not true in cSCC (Hutchin, Kariapper et al. 2005).

TP53

The *TP53*, coding for the Tumor Protein P53 (P53) initially erroneously classified as an oncogene, is a tumor suppressor gene that was first described in 1979. P53 is involved in

a large number of cellular events and is thought of as the "guardian of the genome" (Lane 1992). This gene encodes a protein which contains transcriptional activation, DNA binding, and oligomerization domains. This protein reacts to diverse cellular stresses to regulate the expression of target genes, thereby inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. Alterations often result from mutation, but can also occur due to other processes, such as P53 interaction with viral proteins such as Human papillomavirus protein E6 (HPV E6). HPV E6 binds to P53 and targets it for proteasomal degradation (Munger and Howley 2002).

The *TP53* is the most frequently mutated gene in human cancer and mutations are present both in cBCC (at least in 50% of the cases) and cSCC (up to 90% of the cases) (Brash, Rudolph et al. 1991). *TP53* mutations are considered as an early event in carcinogenesis since they are frequently detected both in AK and *in situ* cSCC (Kramata, Lu et al. 2005) but its prognostic significance has not been ascertained.

RAS

RAS is a proto-oncogene that has been long implicated in the initiation of cSCC carcinogenesis in murine models (Balmain, Ramsden et al. 1984). This proto-oncogene plays a role in different cellular processes (RAS family controls cell growth). Three members of the RAS family (*H-RAS*, *K-RAS*, and *N-RAS*) are mutated in human tumors (Downward 2003).

Data from the Catalog of Somatic Mutations in Cancer (COSMIC) indicates that 21% of cSCC harbor activating mutations in at least 1 of 3 *RAS* genes (9% *H-RAS*, 7% *N-RAS*, and 5% *K-RAS*) (Bamford, Dawson et al. 2004). UV-induced mutations in *RAS* genes were reported in AK and considered to participate in the early carcinogenesis of cSCC (Tsai and Tsao 2004, Ratushny, Gober et al. 2012). In one study, *RAS* mutation was insufficient to initiate carcinogenesis but when the additional genetic alteration was present (blockade of nuclear factor-kB), it induced epidermal tumorigenesis (Dajee, Lazarov et al. 2003). New insights and interest in *RAS* mutations have risen from the increase of frequency of these mutations in patients treated with BRAF-inhibitors for the treatment of advanced melanoma (Su, Viros et al. 2012). Melanoma patients treated with RAF inhibitors develop cSCC in up to 25% (Flaherty, Puzanov et al. 2010, Chapman, Hauschild et al. 2011). The potential mechanism consists of a paradoxical increase in MAPK signaling in the context of mutated or activated *RAS*. Tumors from the cohort of patients treated with an RAF inhibitor were

prone to present higher *RAS* mutations frequency despite similar rates of mutations in other genes in patients treated with non-RAF inhibitors (Oberholzer, Kee et al. 2012). These findings suggest that the development of tyrosine kinase inhibitors (TKI) induced cSCC is not due to a direct mutagenic event of RAF inhibitor therapy but at least in part, due to pro-proliferative interaction between RAF inhibitors and latent *RAS* mutant keratinocytes. The increased incidence of *RAS* mutated cSCC in melanoma patients treated with BRAF-inhibitors has brought curiosity about the potential participation of this genetic alteration in cutaneous squamous cell carcinogenesis. It would be interesting to uncover a possible association between these mutations, other genetic alterations, and clinicopathological characteristics.

Telomeres, telomerase, and immortalization

Telomeres and telomerase represent one of the most fascinating discoveries in modern cell biology. Telomeres are located at the ends of the chromosomes and their main function is to preserve their integrity and maintain genome stability. The importance of the telomeres resides in the fact that they avoid that the DNA damage surveillance mechanisms recognize chromosomal ends (Revenga Arranz, Paricio Rubio et al. 2004, Jafri, Ansari et al. 2016). In humans, telomere length is approximately 10-15 kb and are composed of several repetitions of the TTAGGG sequence (Nandakumar and Cech 2013). Telomerase is a ribonucleoprotein enzymatic complex that is capable of adding TTAGGG repeats. This enzymatic complex is responsible for disrupting the shortening of the telomeres during cell division (Morin 1989). Telomerase is composed of two subunits: a reverse transcriptase heterodimer formed by a noncoding RNA template (TERC, telomerase RNA component) and an enzymatic subunit (TERT, telomerase reverse transcriptase) (Figure 1) (Greider and Blackburn 1985). High telomerase activity in cSCC is reported to vary between 25 and 85% of the cases in different studies, but no association with clinicopathological characteristics was reported (Taylor, Ramirez et al. 1996, Parris, Jezzard et al. 1999). Telomerase was shown to be more active in sun-damaged skin compared to sun-protected sites, which supports that that UVR modifies telomerase activity in the skin (Pellegrini, Maturo et al. 2017). Telomerase activation has been considered to be an early event in squamous cell carcinogenesis, since it was detected not only in invasive cSCC but also in both AK and *in situ* cSCC, and preceded the occurrence of UV-associated *TP53* mutations in the skin (Ueda, Ouhtit et al. 1997).

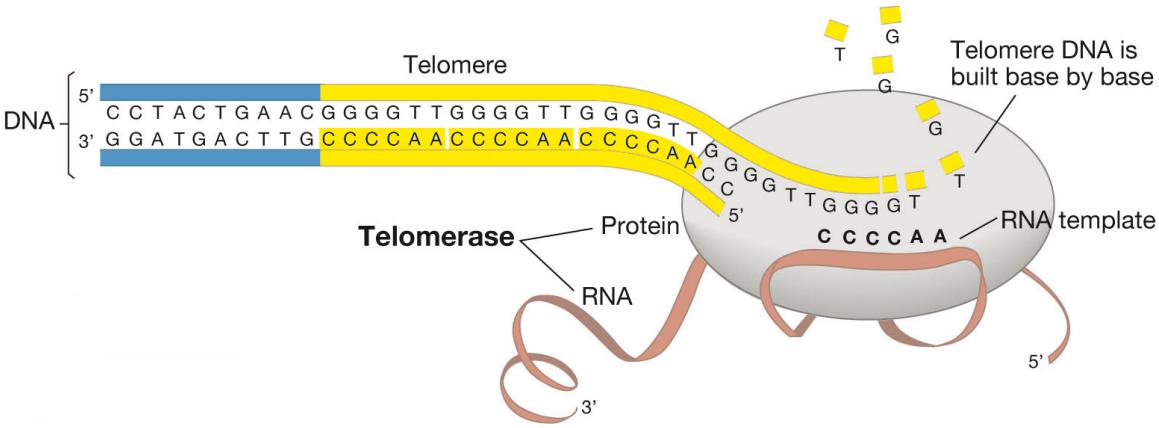

Figure 1. Figure representative of the telomerase complex components. The telomerase complex comprises the TERT (reverse transcriptase subunit) and TERC (RNA component).

It is known that normal somatic cells are not immortal and hold a pre-determined limited number of divisions, the so-called Hayflick limit. Thus cancer, to achieve immortalization, requires telomerase expression/re-expression or another mechanism for telomere maintenance. Currently, we know that there are at least two major pathways that cells can use to maintain telomere lengthening; they either reactivate telomerase which elongates telomeres by adding hexameric 5′-TTAGGG-3′ tandem repeats to the chromosomal ends or take advantage of a non-telomerase dependent mechanism, the alternative lengthening of telomeres (ALT) (Kim, Piatyszek et al. 1994, Cesare and Reddel 2010). Reactivation of telomerase is thought to be present in up to 90% of human cancers and it is known that proliferative cancer cells maintain their telomere length (Kyo, Takakura et al. 2008). The remaining 10% to 15% of human cancers do not have detectable telomerase activity and a subset of such cases to maintain telomere length relies on the ALT mechanism (Cesare and Reddel 2010).

TERT promoter mutations

Mutations in the promoter of the telomerase (*TERTp*) gene were first discovered in melanoma at the beginning of 2013 (Horn, Figl et al. 2013, Huang, Hodis et al. 2013). These two seminal papers were pioneering not only in describing a novel mechanism for telomerase re-activation/re-expression but also reporting an alteration in a noncoding region. The studies although took different approaches reached the same conclusions. In the study of Horn *et al*., a melanoma-prone family was investigated through linkage and by next-

generation sequencing in which a germline disease-segregating mutation was identified in the *TERTp* (Horn, Figl et al. 2013). Huang *et al*. took a different approach, relying wholly on genome sequencing data publicly available, they detected the presence of *TERTp* mutations in several melanoma cases (Huang, Hodis et al. 2013).

The model presented by Horn *et al*. and Huang *et al*. in figure 2 was based on mutations detected in the *TERTp*. The mutations clustered mostly, but not exclusively, in two hotspots that are located at -146 base pairs (bps) and -124 bps distance upstream of the start site (Huang, Hodis et al. 2013). All the mutations correspond to cytidine to thymidine transitions at a dipyrimidine motif indicating a putative signature of UV-induced DNA damage. These mutations created a novel binding consensus for E-Twenty-Six/Ternary Complex Factors (ETS/TCFs) transcription factors that respond to a binding consensus with the sequence CCGGAA (Horn, Figl et al. 2013, Huang, Hodis et al. 2013).

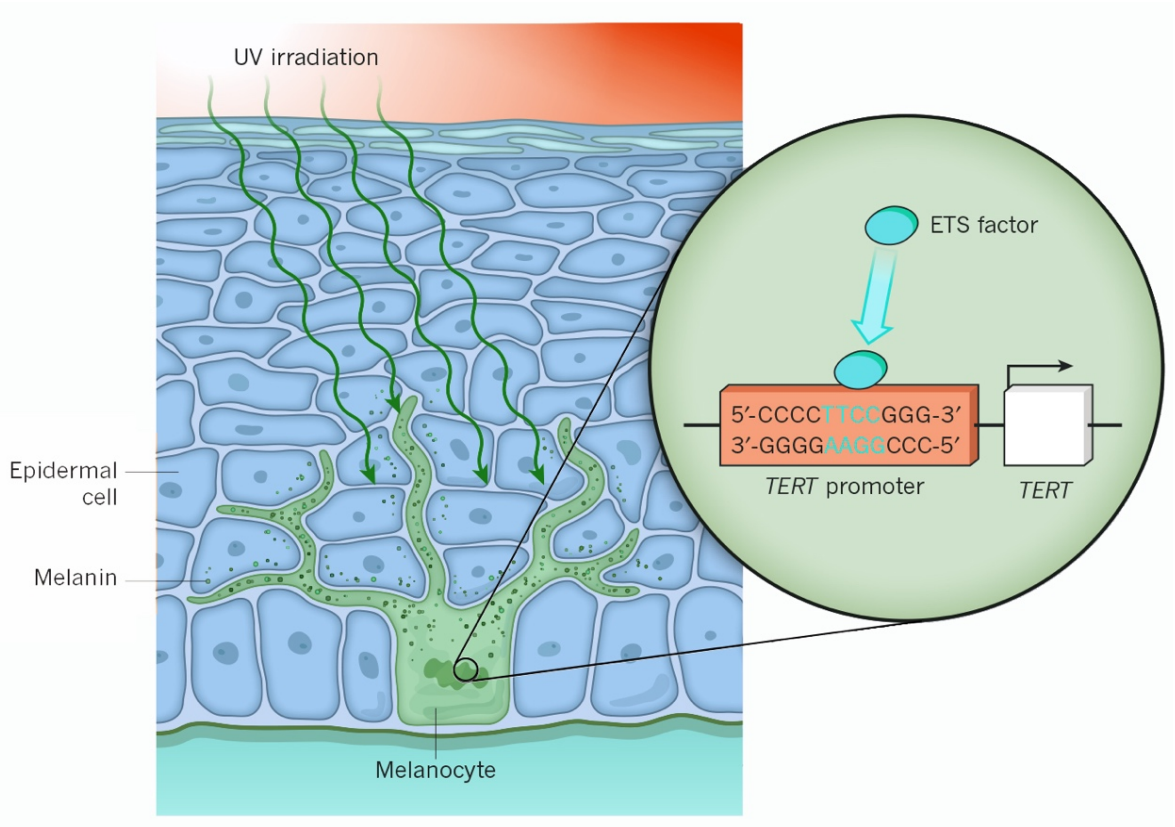

Figure 2. Model of telomerase promoter mutations in melanoma. Reprinted by permission from Macmillan Publishers Ltd: Nature, (Patton and Harrington 2013), copyright (2013).

In vitro, it was reported that the presence of these mutations led to a 2- to 4-fold increase of the *TERTp* activity (Horn, Figl et al. 2013, Huang, Hodis et al. 2013). Since the initial discovery of *TERTp* mutations, researchers have been trying to understand the biological meaning of such alterations and to elucidate how they contribute to human cancer.

The initial findings of such mutations prompted the study of such alterations in other human cancers. For the first time, our group and others reported the presence of recurrent somatic mutations in the *TERTp* in cancers of the central nervous system, hepatocellular carcinoma, thyroid (follicular cell-derived tumors) and tumors originated from tissues with relatively low rates of self-renewal (Killela, Reitman et al. 2013, Liu, Bishop et al. 2013, Nault, Mallet et al. 2013, Vinagre, Almeida et al. 2013). However, *TERTp* mutations were not restricted to the above tumors and, soon, many other studies started reporting the association of *TERTp* mutations in others, such as atypical fibroxanthoma, pleomorphic dermal sarcoma (Griewank, Schilling et al. 2014), bladder cancer (Allory, Beukers et al. 2014, Hurst, Platt et al. 2014), clear cell carcinoma of the ovary (Wu, Ayhan et al. 2014), basal cell carcinoma and squamous cell carcinoma of the skin (Scott, Laughlin et al. 2014). Scott et al. first reported *TERTp* mutation in cSCC, describing a prevalence of 50% (13 out 26 cases) in invasive cSCC and 20% (11 out of 55 cases) of *in situ* cSCC. In this previous study, TERTp was suggested to have a more relevant role in tumor progression than initiation, given that *TERTp* mutations were more frequent in invasive cSCC than *in situ* cSCC (Scott, Laughlin et al. 2014). Since then, additional studies have reported *TERTp* mutations in 25 to 74% of invasive cSCC (Griewank, Murali et al. 2013, Killela, Reitman et al. 2013, Cheng, Kurtis et al. 2015).

TERTp mutations are described as a potential biological predictor of metastasis and/or mortality in melanoma, glioblastoma, medulloblastoma, bladder, and thyroid cancers (Killela, Reitman et al. 2013, Rachakonda, Hosen et al. 2013, Remke, Ramaswamy et al. 2013, Melo, da Rocha et al. 2014, Populo, Boaventura et al. 2014). In cSCC only a small series of cases were evaluated and no information about the putative prognostic value of these changes is available in the literature.

Biomarkers in cSCC

cSCC is a skin cancer of high incidence, associated with significant morbidity and mortality in advanced cases (de Vries, van de Poll-Franse et al. 2005). Nowadays, the challenge faced by clinicians is to identify the cases of cSCC that present the highest risk for recurrence, metastasis, and/or mortality.

Histologic differentiation has for long been described as an indicator of prognosis. The presence of poor differentiation has been associated with higher local recurrence risk (7% *vs* 2%) and higher metastatic risk (7% *vs* 3%) than well-differentiated cSCC (Brantsch, Meisner et al. 2008). Keratoacanthomas and verrucous carcinomas (includes the Buschke-Lowenstein tumor found in the genitalia and groin and epithelioma *cuniculatum* found on the plantar surface of the foot) are two examples of well-differentiated cSCC with an excellent prognosis. On the other hand, desmoplastic cSCC is a highly infiltrative variant, with a 10 times greater risk of recurrence and 6 times greater risk of metastasis (Breuninger, Schaumburg-Lever et al. 1997). A prospective cohort study reported that desmoplastic growth was an independent risk factor for local recurrence in cSCC (hazard ratio of 16.11) (Brantsch, Meisner et al. 2008). Adenosquamous cSCC is another variant that has been reported to have a high risk of local recurrence, metastasis, and death (Azorin, Lopez-Rios et al. 2001). Tumor size is another important variable that has been associated with prognosis. When tumor size is larger than 2.0 cm in diameter recurrence risk doubles and metastasis risk triples when compared to tumors with a diameter of less than 2.0 cm (Friedman 1993). Thompson et al. reported that tumor diameter > 2.0 cm was the risk factor most frequently associated with disease-specific death, with a 19-fold higher risk of death from cSCC when compared to smaller tumors (Thompson, Kelley et al. 2016). The same authors reported that tumor depth was the risk factor most highly associated with recurrence and metastasis. Tumor thickness > 2mm had a 10-fold higher risk of local recurrence and tumors extending beyond the subcutaneous fat had an 11-fold higher risk of metastasis compared to more superficial variants (Thompson, Kelley et al. 2016). One study reported that tumors thinner than 2mm could not metastasize (Brantsch, Meisner et al. 2008). Another risk factor associated with recurrence, metastasis, and disease-specific mortality is a perineural invasion. The presence of perineural invasion was associated with a 47% risk of recurrence and a 35% risk of metastasis (Ross, Whalen et al. 2009, Carter, Johnson et al. 2013). Location on the ear and lips have been described to have a poorer prognosis; cSCC located on the ears and lips presented a local recurrence risk of 19% and 11%, and a

metastatic risk of 9% and 14%, correspondingly. One must not forget that recurrence and metastasis seem to associate, and recurrent cases have a much worsen prognosis. Recurrent cSCC has shown to present a higher risk of regional lymph node metastasis compared with primary tumors (Rowe, Carroll et al. 1992). Some authors argued that the progression of cSCC appears to be stepwise from local recurrence to regional spread and then distant metastasis (Martinez and Cook 2007). cSCC arising from chronic inflammation (e.g. leg ulcer, burn scar, radiation site, discoid lupus) have a reported metastatic risk of 26% (Rowe, Carroll et al. 1992). Lastly, immunosuppressed patients have a higher risk of recurrence (13%) and metastasis (5-8%) in the second year after excision (Martinez, Otley et al. 2003).

As previously described, only clinicopathological prognostic markers have been described in cSCC for recurrence and metastasis (see table 2 for a summary of clinical prognostic markers) (Thompson, Kelley et al. 2016).

Table 2. Summary of Risk Factors and Outcome Associations for cSCC.

	Relative risk of recurrence	Relative risk of metastasis	Disease-specific death
Breslow thickness > 2mm	9.64	10.76	-
Invasion beyond subcutaneous fat	7.61	11.21	4.49
Breslow thickness > 6mm	7.13	6.93	-
Perineural invasion	4.30	2.95	4.06
Diameter > 2 cm	3.22	6.15	19.10
Location on the temple	3.20	2.88	-
Poor differentiation	2.66	4.98	5.65
Location on the ear	-	2.33	4.67
Location on the lip	-	2.28	4.55
Immunosuppression	-	1.59	-

Originated from Thompson et al., with permission.

These prognostic factors contributed to the staging of cSCC. Two of the most frequently used staging systems are the American Joint Committee on Cancer (AJCC) staging system (Farasat, Yu et al. 2011), and the Brigham and Women's Hospital (BWH) staging system (Karia, Jambusaria-Pahlajani et al. 2014). Even though a detailed description of these staging systems is not intended, it is important to understand that these systems have a common goal, to stratify patients into groups where patients have a similar clinical

outcome. This staging helps identify high-risk patients that require further workup, adjuvant radiation, and chemotherapy. The AJCC staging system classifies cases by local tumor burden (T), nodal status (N), and the presence of metastatic disease (M) (see table 3 to 6). More recently, the AJCC introduced the 8th edition of its cancer staging system that is only applied to cSCC located on head and neck skin and vermillion lip (Lydiatt, Patel et al. 2017). Alternatively, the BWH staging system does not include N or M staging criteria (see table 7). These staging systems have been criticized for having low specificity and for being too complicated for use in clinical practice (Warner and Cockerell 2011). The AJCC and BWH staging systems have been described to distinguish poorly to moderately between patients who develop metastases and those who did not (Roscher, Falk et al. 2018), and the 8th Edition AJCC only includes head and neck cSCC, thereby limiting its usefulness (Warner and Cockerell 2011).

Table 3. Definition of cSCC tumor (T) staging system in 7th edition of AJCC manual

Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma *in situ*
T1	Tumor ≤ 2 cm in greatest dimension with <2 high-risk features*
T2	Tumor >2 cm in greatest dimension with or without one additional high-risk feature*, or any size with ≥ 2 high-risk features*
T3	Tumor with invasion of maxilla, mandible, orbit, or temporal bone
T4	Tumor with invasion of skeleton (axial or appendicular) or perineural invasion of skull base
* High-risk features include depth (>2-mm thickness; Clark level ≥IV); perineural invasion; location (primary site ear; primary site nonglabrous lip); and differentiation (poorly differentiated or undifferentiated).	

Table 4. Definition of cSCC nodal (N) staging for 7th edition of AJCC manual

Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in single ipsilateral lymph node, ≤ 3 cm in greatest dimension
N2	Metastasis in single ipsilateral lymph node, >3 cm but not >6 cm in greatest dimension; or in multiple ipsilateral lymph nodes, none >6 cm in greatest dimension; or in bilateral or contralateral lymph nodes, none >6 cm in greatest dimension

N2a	Metastasis in single ipsilateral lymph node, >3 cm but not >6 cm in greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes, none >6 cm in greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes, none >6 cm in greatest dimension
N3	Metastasis in lymph node, > 6 cm in greatest dimension

Table 5. Definition of cSCC distant metastasis (M) staging for 7th edition of AJCC manual

Mx	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Present distant metastasis

Table 6. Final 7th edition AJCC stage grouping for cSCC

Stage	T	N	M
0	In situ	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T3 T1 or T2	N0 or N1 N1	M0 M0
IV	T1, 2 or 3 Any T T4 Any T	N2 N3 Any N Any N	M0 M0 M0 M1

Table 7. BWH tumor staging system for cSCC

Stage	Number of high-risk factors*
T1	0
T2a	1
T2b	2-3
T3	≥4 or bone invasion

*Brigham and Women's Hospital high-risk factors include tumor diameter ≥ 2 cm, poorly differentiated histology, perineural invasion ≥ 0.1 mm, or tumor invasion beyond the subcutaneous fat (excluding bone invasion which automatically upgrades tumor to Brigham and Women's Hospital stage T3).

These observations reveal that current stating systems are unsatisfactory, and there is a need to improve these systems. As in other models (e.g. thyroid) (Haugen, Alexander et al. 2016), we believe that the inclusion of reliable, easy-to-perform, and clinical useful molecular markers would be of great interest in cSCC prognostication.

CHAPTER 2

Aim and objectives

The general aim of my proposed Ph.D. thesis project was to contribute to the unraveling of cutaneous squamous cell carcinogenesis and to progress towards a better understanding of its epidemiology, molecular etiopathogenesis, and potentially identify biomarkers of prognosis.

To achieve this purpose, we divided the work into three main objectives, further divided into specific objectives, as follows:

1. To perform an epidemiological study about trends of cutaneous squamous cell carcinoma in the last 10 years (2004-2013) in a hospital-based registry in the north of Portugal (Paper I).
    a. To perform a descriptive and analytical analysis of the cSCC excised in CHVNGE;
    b. To establish trends (incidence, survival, and mortality) of the patients diagnosed with cSCC in our series;
2. To perform a detailed revision of the information related to all the molecular alterations described in cSCC, and their prognostic and therapeutic implication (Paper II).
    a. To review the reported molecular alterations in cSCC in a comprehensive way, to aid future investigation;
    b. To assess the molecular and genetic alterations according to the main molecular anomalies associated with the development of cSCC.
3. To perform the molecular characterization in a series of cSCC and identify potential markers of prognosis (recurrence and/or metastasis). *TERTp* mutations, *RAS* mutations, and P53 expression were evaluated (Paper III and IV).
    a. To evaluate the frequency of *TERTp* mutations in cSCC and correlate it with clinicopathologic features and patient outcome.
    b. To evaluate P53 expression and *RAS* mutations frequency in cSCC and correlate them with clinicopathological features and patient outcome.

CHAPTER 3

Material and methods

The approaches followed for each study and detailed methodologies are indicated in the articles belonging to the thesis.

CHAPTER 4 Results

Paper I

Trends of Cutaneous Squamous Cell Carcinoma in the Hospital of Gaia (2004-2013)

Campos, M. A., Massa, A., Varela, P., Moreira, A., Sanches, A., Pópulo, H., Soares, P., and Baptista, A. 2018. Trends of Cutaneous Squamous Cell Carcinoma in the Hospital of Gaia (2004-2013). J Port Soc Dermatol, 76 (3):279-286.

Artigo Original

Tendências do Carcinoma Espinocelular Cutâneo no Hospital de Gaia (2004-2013)

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RESUMO – **Introdução:** O carcinoma espinocelular cutâneo é o segundo cancro cutâneo mais comum e a sua incidência tem crescido. O objetivo do nosso estudo foi realizar uma análise descritiva e analítica dos carcinoma espinocelular cutâneo excisados no Centro Hospitalar Vila Nova de Gaia e Espinho (CHVNGE) num período de 10 anos e estabelecer tendências (incidência, sobrevida e mortalidade). **Materiais e Métodos:** A informação foi retrospetivamente recolhida nos Registos Oncológico e Histológico do CHVNGE entre o período de Janeiro de 2004 e Dezembro de 2013. O objetivo do nosso estudo foi descrever as características e tendências (incidência, associação a queratoses actínicas e carcinomas basocelulares, sobrevida e mortalidade) do carcinoma espinocelular cutâneo. **Resultados:** Foram removidas 485 lesões em 380 pacientes (56,1% homens e 43,9% mulheres). 361 pacientes apresentavam doença invasora e 124 doença *in situ*. O serviço de Dermatologia removeu a maioria das lesões (70,4%), seguido pelo serviço de Cirurgia Plástica (16,4%) e Cirurgia Geral (4,7%). A faixa etária ≥ 75 anos foi a mais atingida por carcinoma espinocelular cutâneo em ambos os sexos ($p < 0,001$). A média de idades dos pacientes com carcinoma espinocelular cutâneo invasor foi de 76,7anos (± 11,5), sendo mais elevada no sexo feminino (79,0 *vs* 74,0 anos, $p < 0,001$). A face foi a localização topográfica mais comum (42,1%) nos dois sexos ($p = 0,002$). Houve um aumento da taxa de incidência ajustada à idade em ambos os sexos, particularmente no último período do estudo (16,2/100 000 pessoas). A sobrevida aos 5 anos foi de 98,7%. A idade média do carcinoma espinocelular cutâneo *in situ* foi inferior à da doença invasora (75,5 anos ± 11,3). Dos doentes com carcinoma espinocelular cutâneo *in situ*, 20,6% tinham antecedentes de carcinoma basocelular e as mulheres apresentaram mais queratoses actínicas ($p = 0,040$). A face foi o local mais comum (30,8%). A taxa de incidência de carcinoma espinocelular cutâneo *in situ* aumentou, sendo maior nas mulheres e na faixa etária ≥75 anos. **Conclusão:** Este estudo demonstra um rápido aumento da incidência do carcinoma espinocelular cutâneo numa população portuguesa envelhecida e realça a necessidade de melhorar os registos oncológicos em Portugal.
PALAVRAS-CHAVE – Carcinoma Espinocelular/epidemiologia; Carcinoma Espinocelular/tendências; Neoplasias da Pele.

Trends of Cutaneous Squamous Cell Carcinoma in the Hospital of Gaia (2004-2013)

ABSTRACT *– **Introduction:** Cutaneous squamous cell carcinoma (cSCC) is the second most common skin cancer and its incidence has been rising. The objective of our study was to perform a descriptive and analytical analysis of the cutaneous squamous cell carcinoma excised in the Hospital Center Vila Nova de Gaia e Espinho (CHVNGE) over a period of 10 years and establish trends (incidence, survival and mortality). **Material and Methods:** Information was retrospectively gathered in the CHVNGE, from January*

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2004 to December 2013, using the regional cancer registry and the histopathological registry of the hospital. The aim of this study was to describe the characteristics and trends of cutaneous squamous cell carcinoma (incidence, association with actinic keratosis or basal cell carcinoma, survival and mortality rates). **Results:** *485 cutaneous squamous cell carcinoma were surgically removed in a total of 380 patients (56.1% men and 43.9% women). 361 patients presented invasive cutaneous squamous cell carcinoma and 124 in situ cutaneous squamous cell carcinoma. The Dermatology Department removed 70.4% of the cutaneous squamous cell carcinoma, followed by the Plastics (16.4%) and General Surgery Departments (4.7%). Cutaneous squamous cell carcinoma was more prevalent in the age-group ≥75-years in both sexes ($p < 0.001$).The mean age of invasive cutaneous squamous cell carcinoma was 76.7 years (±11.5 years), women being older than men (79.0 vs 74.0 years, $p < 0.001$). The face was the most common topographic location (42.1%), in both genders ($p < 0.001$). We observed a rising incidence in both genders, particularly in the last study period (16.2/100 000 person-year). The 5-year survival rate was 98.7%. The mean age of in situ cutaneous squamous cell carcinoma was lower than invasive disease (75.5 years ± 11.3). A previous basal cell carcinoma occurred in 20.6% and actinic keratosis were diagnosed more frequently in women ($p = 0.040$). The face was the most common location (30.8%). Incidence rates have risen, particularly in women and age-group ≥ 75-years.* **Conclusion:** *Our study reports a rapid increase of the incidence in an ageing Portuguese population and highlights the importance of improving the existing cancer registries in Portugal.*
KEYWORDS – *Carcinoma, Squamous Cell/epidemiology; Carcinoma, Squamous Cell/trends; Skin Neoplasms.*

INTRODUÇÃO

O carcinoma espinocelular da pele (CEC) é o segundo cancro cutâneo mais comum e a sua incidência tem crescido nas últimas décadas.[1-6] As taxas de incidência, ajustadas à idade para CEC, têm aumentado na Europa e estima-se um aumento absoluto de 2000 casos por ano em países com 4,5-9 milhões de habitantes.[3,4,7] O factor etiológico mais comum é a radiação ultravioleta (UV). O CEC apresenta um modelo de carcinogénese clássico: lesão percursora (queratose actínica), carcinoma *in situ* (doença de Bowen), carcinoma invasor e carcinoma metastizado. Pacientes com múltiplas queratoses actínicas apresentam um risco cumulativo de desenvolver um CEC invasor de 6% a 10% e uma lesão de queratose actínica tem uma taxa de progressão para CEC estimada entre 0,025% a 16% por ano.[8,9] Estudos sugerem que 65% do CEC têm origem em queratoses actínicas.[10] O CEC invasor pode recorrer (3% - 5%) e metastizar (4% - 5%). Embora o CEC localizado tenha um excelente prognóstico, o CEC metastizado está associado a um prognóstico reservado que se reflete numa sobrevida aos 10 anos inferior a 10%. À luz do atual conhecimento, não é possível determinar quais os factores de risco para que uma queratose actínica ou uma doença de Bowen adquira capacidade de progressão e invada a membrana basal, ou um CEC localizado atinja a derme e metastize. Comparativamente a outros carcinomas epidermóides (pulmão, cabeça e pescoço), existe falta de informação sobre a sua epidemiologia, especialmente em países como Portugal, onde existe uma grande possibilidade de exposição UV. É urgente caracterizar o CEC epidemiologicamente e molecularmente, uma vez que é responsável por 20% das mortes associadas a cancro cutâneo.[11]

O objetivo do nosso estudo foi realizar uma análise descritiva e analítica dos CEC excisados no Centro Hospitalar Vila Nova de Gaia e Espinho (CHVNGE) num período de 10 anos e estabelecer tendências (incidência, sobrevida e mortalidade).

MATERIAIS E MÉTODOS

Realizamos uma análise retrospetiva dos pacientes com CEC tratados no CHVNGE com excisão cirúrgica, no período compreendido entre Janeiro de 2004 e Dezembro de 2013. Utilizamos como fonte para identificação dos casos o Registo Oncológico e o Registo Histológico do CHVNGE.

Foi considerado como local topográfico a pele (C44) e utilizamos os seguintes códigos morfológico: 8070 (carcinoma de células escamosas) e 8081 (doença de Bowen).

Os critérios de inclusão compreenderam pacientes imunocompetentes com o diagnóstico histológico de CEC. Foram excluídos da análise biopsias de lesões excisadas, pacientes com doenças hereditárias com risco aumentado de CEC (xeroderma *pigmentosum*, epidermodisplasia *verruciformis* e albinismo), pacientes transplantados e com tratamentos imunossupressores sistémicos. Os CEC recorrentes na mesma localização anatómica foram considerados como uma única lesão. Foram efetuadas duas análises separadas para CEC invasores e CEC *in situ*, podendo o mesmo paciente estar incluído em ambos os grupos, se apresentasse um CEC invasor e um *in situ* em localizações anatómicas distintas.

A idade dos pacientes foi dividida em quatro grupos etários: < 54 anos, 55-64 anos, 65-74 anos e ≥ 75 anos. Os 10 anos do estudo foram divididos em 3 períodos: 2004-2006, 2007-2009 e 2010-2013. A divisão em três períodos teve como objetivo obter uma amostra mais representativa da população, minimizar flutuações por ano e obter uma maior estabilidade durante o cálculo das taxas de incidências. Utilizamos a Classificação Internacional de Doenças e Problemas relacionadas 10ª edição (ICD-10) para classificação da localização, comportamento biológico e grau de diferenciação. A localização anatómica foi categorizada nos seguintes grupos: lábios (C44.0), pálpebras (C44.1), orelha (C44.2), face (C44.3), couro cabeludo/pescoço (C44.4), tronco (C44.5), braços (C44.6), pernas (C44.7) e desconhecido (C44.8 e C44.9).

Foi efetuada uma revisão individual dos processos clínicos de cada paciente e do resultado histológico. As variáveis

clinicas foram: sexo, idade de diagnóstico, localização topográfica, presença de queratose actínica, história pessoal de carcinoma basocelular, presença de metástases, data e motivo do óbito e serviço responsável pela exérese cirúrgica. As variáveis histológicas incluíram: grau de diferenciação, espessura, nível de Clark, presença de ulceração, presença de invasão linfovascular e presença de invasão perineural.

À semelhança de estudos anteriores,[12] decidimos realizar uma análise estatística centrada na lesão e uma análise centrada no paciente. Os motivos para realização destas duas análises prendem-se com o facto de um paciente poder apresentar mais que um CEC e porque outros estudos sugeriram que estas análises refletem de forma mais eficiente a carga de trabalho. Na análise centrada no paciente foi realizada uma avaliação estatística separada para os pacientes com CEC invasor e aqueles com CEC *in situ*. Uma vez que o CHVNGE tem uma das maiores áreas de referenciação em Portugal, com cerca de 700 000 habitantes a referenciarem diretamente para este Serviço de Dermatologia, e o facto das políticas de referenciação não terem sido constantes ao longo dos 10 anos, no cálculo de tendências foram apenas incluídos os pacientes residentes no concelho de Gaia.

A análise estatística foi realizada usando o *software* SPSS versão 22. Os nossos cálculos de incidência e mortalidade foram ajustados à população europeia. A população em cada ano do concelho de Vila Nova de Gaia foi obtida através do Instituto Nacional de Estatística (INE).

RESULTADOS

Foram removidas 485 lesões em 380 pacientes (56,1% homens e 43,9% mulheres). A faixa etária ≥ 75 anos foi a mais comum ($p < 0,001$). Dessas lesões, 361 correspondiam a CEC invasor e 124 lesões a CEC *in situ*. Dos pacientes, 81,8% apresentavam apenas uma lesão e 18,2% duas ou mais lesões (Tabela 1). O Serviço de Dermatologia removeu a maioria das lesões (70,4%), seguido pelo serviço de Cirurgia Plástica (16,4%) e Cirurgia Geral (4,7%).

As características clínico-patológicas encontram-se resumidas na Tabelas 2 e 3. A média de idades, dos pacientes com CEC invasor, foi de 76,7 anos (±11,5), mais elevada no sexo feminino (79,0 ± 11,5 *vs* 74,0 ± 13 anos, $p < 0,001$). A face foi a localização topográfica mais comum (42,1%) em ambos os sexos ($p = 0,002$). Na análise entre géneros, o membro inferior e o membro superior foram os locais mais frequentemente atingidos no sexo feminino, enquanto o lábio, a orelha e o couro cabeludo foram as localizações mais atingidas nos homens. A presença clínica

Tabela 1 - Número de lesões por paciente da nossa série.

Nº lesões	Nº pacientes	%
1	311	81,8
2	50	13,2
3	11	2,9
4	4	1,1
5	2	0,5
6	2	0,5

Tabela 2 - Características clínicas comparativas dos CEC invasores com os CEC *in situ*.

	CEC invasor			CEC *in situ*		
	Mulheres	Homens	*p*	Mulheres	Homens	*p*
Sexo	159 (53,5%)	138 (46,5%)	-	70 (65,4%)	37 (34,6%)	-
Idade média	79,0±10	74,0±12,6	<0,001	77,5±12,5	71,9±2,1	0,023
Grupos etários						
≤54 anos	4 (2,5%)	12 (8,7%)		4 (5,7%)	4 (10,8%)	
55 – 64 anos	6 (3,8%)	14 (10,1%)	<0,001	1 (1,4%)	3 (8,1%)	0,183
65 – 74 anos	29 (18,2%)	40 (29,0%)		16 (22,9%)	10 (27,0%)	
≥ 75 anos	120 (75,5%)	72 (52,2%)		49 (70,0%)	20 (54,1%)	
Localização						
Lábio	7 (4,4%)	17 (12,3%)		1 (1,4%)	0 (0%)	
Pálpebra	6 (3,8%)	3 (2,2%)		2 (2,9%)	0 (0%	
Orelha	4 (2,5%)	17 (12,3%)		2 (2,9%)	4 (10,8%)	
Face	71 (44,7%)	54 (39,1%)		23 (32,9%)	10 (27,0%)	
Couro cabeludo/ pescoço	12 (7,5%)	15 (10,9%)	0,002	2 (2,9%)	5 (13,5%)	-
Tronco						
Membro superior	8 (5,0%)	7 (5,1%)		4 (5,7%)	4 (10,8%)	
Membro inferior	16 (10,1%)	7 (5,1%)		11 (15,7%)	7 (18,9%)	
Indeterminada	26 (16,4%)	11 (7,9%)		25 (35,7%)	5 (13,5%)	
	9 (5,7%)	7 (5,1%)		0 (0%)	2 (5,4%)	
Queratose actínica	90 (56,6%)	64 (46,4%)	0,080	47 (67,1%)	17 (45,9%)	0,040
Carcinoma basocelular prévio	28 (17,6%)	20 (14,5%)	0,684	11 (15,7%)	11 (29,7%)	-

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Tabela 3 - Características histopatológicas dos casos de CEC invasor.

	Mulheres n= 159	Homens n=138	p
Diferenciação Bem Moderadamente Pouco diferenciado Indiferenciado Indeterminado	 84 (52,8%) 38 (23,9%) 11 (6,9%) 1 (0,6%) 25 (15,7%)	 64 (46,3%) 41 (29,7%) 4 (2,9%) 3 (2,2%) 26 (18,8%)	0,757
Espessura (mm)	3,5	3,8	0,683
Nível de Clark I II III IV V	 107 (67,3%) 0 (0%) 15 (9,4%) 23 (14,5%) 14 (8,8%)	 97 (70,3%) 2 (1,4%) 5 (3,6%) 15 (10,9%) 19 (13,8%)	0,057
Ulceração	33 (20,8%)	22 (15,9%)	0,565
Invasão linfovascular	5 (3,1%)	2 (1,3%)	0,796
Invasão perineural	4 (2,5%)	4 (2,9%)	0,824

de queratose actínica foi relatada em 52,2% dos pacientes com CEC invasor, não existindo diferenças entre sexos (p = 0,08). Dos pacientes, 16,2% apresentavam antecedentes de exérese de carcinomas basocelulares.

Os CEC bem diferenciados e moderadamente diferenciados corresponderam a 76,4% dos casos. A maioria dos CEC excisados apresentou nível de Clark I (68,7%). A ulceração foi descrita em 18,5% e apenas 5,1% dos casos apresentaram invasão linfovascular e/ou perineural. As taxas de incidência por grupo etário e por período de estudo foram superiores no grupo etário com mais de 75 anos e verificou-se um aumento significativo ao longo dos 3 períodos (Tabela 4). Uma vez que estamos perante uma população envelhecida e com uma sobre representação dos pacientes com mais de 75 anos, foi calculada a taxa de incidência ajustada à idade. Como apresentado na Tabela 5, houve um aumento das taxas de incidências ajustada à idade em ambos os sexos, particularmente no último período de estudo (16,2/100 000 pessoas). O sexo masculino apresentou um maior incremento da taxa de incidência ajustada à idade

Tabela 4 - Taxas de incidência por grupo etário e período, por 100 000 habitantes.

	CEC invasor			CEC *in situ*		
	2004-2006	2007-2009	2010-2013	2004-2006	2007-2009	2010-2013
≤54 anos	1,9	2,8	0,5	0,4	0,5	2,8
55 – 64 anos	7,6	10,1	7,6	2,5	0	7,6
65 – 74 anos	64,7	46,7	64,7	10,8	10,8	71,9
≥ 75 anos	145,8	186,8	264,3	18,2	68,4	227,9

Tabela 5 - Taxas de incidência ajustadas à idade, por 100 000 habitantes.

	CEC invasor			CEC *in situ*		
	Global	Homens	Mulheres	Global	Homens	Mulheres
2004-2006	13,7	12,6	11,3	1,9	1,1	2,5
2007-2009	13,8	14,4	12,8	1,7	3,3	4,1
2010-2013	16,2	20,4	13,4	16,3	14,2	17,6

nos três triénios (Tabela 5). Durante o período do estudo, ocorreram quatro mortes por CEC invasor com progressão locoregional e metastização ganglionar, correspondendo a uma taxa de sobrevida aos 5 anos de 98,7%.

A idade média do CEC *in situ* foi inferior à da doença invasora (75,5 ± 11,3 anos). As mulheres apresentaram uma idade média superior aos homens (77,5 ± 12,5 *vs* 71,9 ± 2,1; $p = 0,023$). As mulheres com CEC *in situ* apresentaram mais queratoses actínicas (67,1% *vs* 45,9%; $p = 0,040$). Dos pacientes, 20,6% realizaram exérese prévia de um carcinoma basocelular, não havendo diferenças significativas entre géneros. À semelhança dos CEC invasores, o grupo etário ≥75 anos foi o mais prevalente. A face foi o local mais comum (30,8%), sendo que não se verificaram diferenças estatisticamente significativas entre géneros. A taxa de incidência aumentou ao longo dos três períodos de estudo, sendo maior nas mulheres e na faixa etária ≥75 anos (Tabela 4). Houve um aumento das taxas de incidências ajustada à idade em ambos os sexos, particularmente no último período (16,3/100 000 pessoas). O sexo feminino apresentou um maior aumento da taxa de incidência ajustada á idade nos três períodos de estudo (Tabela 5). Não foram registadas mortes por CEC *in situ*.

DISCUSSÃO

O aumento da incidência dos CEC é um problema de saúde pública e é fundamental conhecermos a sua epidemiologia de forma a adequar os recursos (humanos e económicos) nos próximos anos.

O nosso estudo apresenta como limitações, o facto da identificação e recolha de informação ter sido feita a partir de um registo oncológico, onde poderão existir falhas de codificação; existe um viés de seleção uma vez que os carcinomas espinocelulares podem ser tratados em outras instituições de saúde (clínicas privadas, hospitais privados, outros hospitais públicos fora da área de referenciação); a distinção entre tumor primário e recidiva nem sempre ser fácil; o nosso estudo apresenta um *follow-up* curto (10 anos); e a divisão do *follow-up* em 3 períodos obrigou a existência de um período com 4 anos (2010-2013), o que poderá ter contribuído em parte para o aumento da incidência descrita.

À semelhança de outros estudos com latitudes semelhantes, a idade média da nossa população foi comparável (76,7 ± 11,5 *vs* 77,32 ± 11,5).[13] A face foi a localização topográfica mais frequente (42,1%), embora com um predomínio inferior a outro estudo (63,7% em estudo Espanhol[13]). Semelhante a um estudo populacional Holandês, os CEC em mulheres distribuem-se com uma maior frequência nos membros inferiores e superiores, enquanto nos homens o lábio, orelha e couro cabeludo estão descritos como sendo mais frequentemente atingidos.[2] Outro estudo Escocês descreveu que os CEC se apresentam mais frequentemente no pavilhão auricular em homens do que em mulheres (22% *vs* 1%), enquanto que o membro inferior é mais frequentemente atingido no sexo feminino (24% *vs* 3%), à semelhança do que acontece no nosso estudo.[12] Mais de metade dos pacientes apresentava concomitantemente queratoses actínicas, realçando a importância do diagnóstico precoce destas lesões e tratamento com modalidades cirúrgicas (crioterapia, curetagem, eletrocoagulação, laser CO_2, biopsias excisionais) e não cirúrgicas (imiquimod, mebutato de ingenol, diclofenac, 5-fluorouracilo, terapêutica fotodinâmica). A patogénese do CEC tem sido associada a uma exposição solar cumulativa ao longo da vida, traduzida pelo predomínio dos pacientes com faixa etária superior a 75 anos (64,6%). Por sua vez, a patogénese do carcinoma basocelular tem sido associado a uma exposição intermitente de alta intensidade. Uma vez que a exposição UV cumulativa e intermitente são acontecimentos indissociáveis, não nos surpreende que 16,2% dos pacientes apresentaram como antecedentes a exérese de um carcinoma basocelular.

De acordo com outros estudos, a faixa etária ≥ 75 anos apresentou a maior taxa de incidência nos três períodos, com um aumento expressivo a partir dos 65 anos em ambos os sexos (Fig. 1).[12] Os homens apresentaram uma maior taxa de incidência ajustada à idade nos três períodos de estudo, com um amento de 12,6 por 100 000 habitantes no período 2004-2006 para 20,4 por 100 000 habitantes no

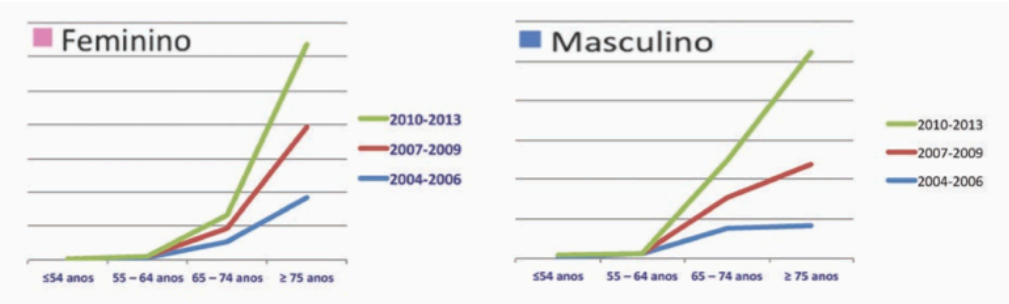

Figura 1 - Tendências da taxa de incidência por grupo etário em ambos os sexos ao longo dos três triénios.

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período de 2010-2013. O maior aumento verificou-se no último intervalo de anos, consistente com outros estudos.[2] Este aumento no sexo masculino da taxa de incidência é consistente com o estudo Holandês que apresentou um aumento da taxa de incidência ajustada à idade entre 1989 e 2008 de 22,2 para 32,4 por 100 000 habitantes, com um incremento mais acentuado no período mais recente.[2] O aumento da taxa de incidência no sexo feminino foi menor ao longo do estudo (11,3 *vs* 12,8 *vs* 13,4 por 100 000 habitantes), ao contrário do estudo Holandês em que a taxa de incidência ajustada à idade mais que duplicou no sexo feminino no mesmo período (8,1 *vs* 17,2 por 100 000 habitantes).[2] Outros estudos corroboram o aumento da taxa de incidência nos últimos anos.[12] A Tabela 6 apresenta a taxa de incidência global em outros países europeus.[13-16] Foram observadas quatro mortes, com uma taxa de sobrevida aos 5 anos de 98,7%. Esta taxa de sobrevida foi superior à relatada no estudo Holandês (92%), provavelmente porque a nossa série incluiu casos menos avançados. Uma justificação para um menor número de CEC avançados poderá dever-se ao facto da grande maioria das lesões serem provenientes do serviço de Dermatologia (diagnóstico mais precoce), apenas serem incluídos doentes imunocompetentes, e porque os casos mais avançados são geralmente encaminhados para o Instituto Português de Oncologia do Porto. Apesar disto, o estudo Holandês admite uma superestimativa da mortalidade devido à maior prevalência de indivíduos submetidos a transplante de órgãos sólidos ou uso de terapia imunossupressora, indivíduos que foram excluídos da nossa análise.

Poucos estudos incidiram sobre a epidemiologia do CEC in situ a nível mundial e o nosso representa o primeiro estudo de tendências em CEC *in situ* a nível nacional. Como esperado a idade média de diagnóstico foi inferior à dos CEC invasores (75,5 ± 11,3 anos *vs* 76,7±11,5 anos). Tal como nos CEC invasores, as mulheres apresentaram uma idade média estatisticamente superior ($p = 0,023$). A distribuição anatómica dos CEC in situ na nossa população não encontrou diferenças estatisticamente significativa entre géneros, ao contrário dos CEC invasores e ao contrário de outros estudos. O estudo escocês reportou uma maior prevalência de lesões localizadas na cabeça e pescoço em homens (52% *vs* 29%) e no pavilhão auricular (11% *vs* <1%). Por sua vez, as lesões no membro inferior foram descritas como sendo muito mais comuns em mulheres (50% *vs* 12%).[12] As mulheres foram diagnosticadas mais frequentemente com queratoses actínicas, o que poderá refletir uma maior preocupação do sexo feminino com saúde e aspeto estético, recorrendo mais precocemente à consulta de Dermatologia e outras especialidades. Outro dado que poderá relacionar-se com a maior preocupação pela saúde do sexo feminino, consiste na maior taxa de incidência ajustada à idade nas mulheres ao longo dos períodos de estudo. As mulheres poderão recorrer mais precocemente à consulta de dermatologia, permitindo o diagnóstico e exérese de lesões num estádio inicial. À semelhança do CEC invasor, é frequente os pacientes desenvolverem carcinomas basocelulares e CEC *in situ* ao longo da sua vida. A faixa etária ≥ 75 anos apresentou uma taxa de incidência superior e esta apresentou um enorme incremento ao longo dos 3 períodos de estudo (18,2 *vs* 68,4 *vs* 227,9). A explicação para o aumento na taxa de incidência no período entre 2010-2013 é difícil de explicar, podendo ter contribuído em parte o fato deste período compreender 4 anos de estudo e as mudanças na política de referenciação (o primeiro Regulamento do Sistema Integrado de Referenciação e de Gestão do Acesso à Primeira Consulta de Especialidade Hospitalar nas Instituições do Serviço Nacional de Saúde (CTH) data de 2008 - Portaria n.º 615/2008 de 11 de Julho, tendo apenas entrado em vigor ao longo do ano de 2009). O estudo escocês apresentou uma taxa de incidência ajustada à idade global de 28,5 por 100 000 no período entre 1992-2003.[12] Estudos antigos dos pacientes residentes no Havai e em Rochester (EUA) apresentaram taxas de incidência globais de 98,8 e 13,8 por 100 000 habitantes, respetivamente, nos períodos entre 1976 e 1984.[17,18]

Tabela 6 - Taxas de incidência global em Portugal e em outros países europeus.

	Anos	CEC invasor
Gaia (Portugal)*	2004-2006 2007-2009 2010-2013	13,7 13,8 16,2
Sória (Espanha)	1998-2000	17,8
Granada (Espanha)	1990-1994	18,2
Inglaterra*	2000-2006	22,65
Suíça	1997	28,9
Irlanda do Norte*	2000-2006	30,6
Zona Este (Escócia)*	1995-1997	34,7

* taxa de incidência ajustada à população europeia

Um estudo Americano alertou para a taxa de incidência crescente de cancros cutâneos não-melanoma nos indivíduos com menos de 40 anos no período entre 1976 e 2003.[19] O *follow-up* curto do nosso estudo poderá ter limitado a observação desta tendência no nosso estudo.

CONCLUSÃO

As taxas de incidência na população de Gaia têm aumentado rapidamente nos últimos anos. É urgente melhorar os registos oncológicos que incluam os carcinomas espinocelulares, de forma a melhorar a alocação de recursos humanos e económicos nos próximos anos. Têm sido realizadas várias campanhas de sensibilização na população, alertando para os malefícios do sol e dos escaldões, contudo pouco esforço tem sido feito para alertar para exposição solar cumulativa, o principal responsável pela patogénese dos CEC. Acreditamos que este estudo traz mais-valias para o conhecimento da epidemiologia oncológica a nível nacional e acreditamos que a SPDV e outras associações (Associação Portuguesa de Cancro Cutâneo, por exemplo) têm e irão continuar a ter um papel preponderante na educação e transmissão de informação para a população.

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Paper II

The genetics of cutaneous squamous cell carcinogenesis

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The genetics of cutaneous squamous cell carcinogenesis

In this review, the current knowledge of cutaneous squamous cell carcinogenesis (cSCC) is outlined based on an appraisal of the different features of cSCC, with particular emphasis on genetic alterations underlying aetiopathogenesis. When appropriate, diagnostic and/or prognostic biomarkers for cSCC are also considered. This review is intended to aid future investigation into the molecular characterization of cSCC.

Key words: carcinogenesis, cutaneous squamous cell carcinoma, cSCC, genetics

Cutaneous squamous cell carcinoma (cSCC) is the second most common cancer in Caucasians with an incidence of about one million cases per year [1]. Recent population studies report that age-standardized incidence rates are rapidly rising with absolute increases of approximately 2,000 new cSCC cases annually in countries with 4.5 to 9 million inhabitants [2-4]. Ultraviolet radiation is the most common causal factor [5]. Other risk factors include fair skin, blue eyes, a history of sunburn during childhood, exposure to ionizing radiation, genodermatosis, organ transplants (with a 65-fold increased risk), and chronically injured or diseased skin. Although the effect of tobacco is not as great as for other SCC, tobacco may double the risk of cSCC [6]. cSCC follows a classic multistep carcinogenesis model: premalignant lesion (actinic keratosis), *in situ* squamous carcinoma/Bowen disease, invasive carcinoma, and metastatic carcinoma. Patients with multiple actinic keratosis (AK) have a 6-10% life-time risk of cSCC [7] and the estimated rate of progression to cSCC for a single AK is reported at 0.025-16% (per year) [8]. Some studies state that 65% cSCC cases arise from AK [9]. cSCC can recur (3-5%) and metastasize (4-5%) [10].

Another entity, keratoacanthoma (KA), has generated controversy since its first description in 1889. Discussion between scholars as to whether this entity is benign or malignant, or whether it corresponds to a well-differentiated cSCC or a distinct entity, has been ongoing for decades. Contrary to cSCC, KA is assumed to originate from the hair follicle, which suggests a benign nature [11, 12], but similar to cSCC, UV radiation is the predominant risk factor [12]. Other risk factors include immunosuppression, skin trauma (*e.g.* surgical procedures, chemical peeling, dermabrasion, cryotherapy, photodynamic therapy or irritation after application of tar and imiquimod), and treatment with BRAF inhibitors and Hedgehog pathway inhibitors [13, 14]. Clinically, it may present as a solitary lesion or as multiple lesions (Ferguson-Smith type) [14, 15]. Histologically, architectural differences and immunohistochemical markers make it possible to differentiate between cSCC and KA.

cSCC most frequently occurs in chronically sun-exposed areas, such as the face (particularly the lip, ear, nose, cheek, and eyelid) and the dorsum of the hands. The head and neck are the most affected areas in males, while the upper limbs followed by the head and neck are the most common locations in females. In order to aid prognostic and appropriate management, cSCC cases are classified based on histological subtype (acantholytic, spindle, verrucous, and desmoplastic), grade of differentiation (well-differentiated, moderately differentiated, poorly differentiated or undifferentiated), tumour depth (maximum vertical diameter), level of dermal invasion (Clarkïs level), and the presence or not of perineural, lymphatic or vascular invasion [16]. Although not optimal for cSCC, to date, staging is based on the TNM system of the 2010 American Joint Committee on Cancer guidelines [17]. Patients with localized cSCC usually have excellent outcome, but metastatic cSCC has a poor prognosis with a 25-35% five-year survival rate and <10% ten-year survival rate [18]. Prognostic cSCC factors associated with the development of metastasis include recurrence, tumours arising from scars, clinical size (>2 cm), histological type (acantholytic, spindle, and desmoplastic subtypes), tumour thickness (>6 mm), horizontal size, poor differentiation, perineural invasion, subcutaneous fat invasion, immunosuppression, and location on the lip, ear and possibly the temple [10, 19]. The epidemiology of cSCC is summarized in *table 1*.

Notwithstanding recent advances, the molecular profile of cSCC is far from clarified. Compared to other SCC (*e.g.*

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Table 1. Epidemiological features of cSCC.

	cSCC
Incidence	About one million cases per year
Anatomical location	Skin
Risk factors	Ultraviolet radiation, fair skin, blue eyes, a history of sunburns during childhood, exposure to ionizing radiation, certain genodermatosis, organ transplants, chronically injured or diseased skin, tobacco
Protective factors	Sun protection
Precursor lesions	Actinic keratosis
Recurrence rate	3-5%
Rate of metastasis	4-5%
Field cancerization	Yes
5-year survival rate	Localized: excellent Metastasized: 25-35%

lung, head and neck), there is little information about the molecular genetics of cSCC. With this in mind, we sought to review the reported molecular alterations in cSCC in a comprehensive way, in order to aid future investigation. In the following review, for the sake of simplicity, the molecular and genetic alterations are considered according to the main molecular anomalies associated with the development of cSCC.

Cell cycle regulation and apoptosis

TP53

The tumour suppressor protein most frequently inactivated in cSCC is p53, encoded by the gene *TP53*, known as the "guardian of the genome" [20]. p53 is a tumour suppressor protein which contains transcriptional activation, DNA binding, and oligomerization domains. This protein responds to diverse cellular stresses to regulate expression of target genes, thereby inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. Anomalies associated with these events often result from mutation of *TP53* but can also occur as a result of interactions between p53 and viral proteins such as HPV E6 [14].
The molecular abnormalities in cSCC are summarized in *table 2* and *figure 1*.
TP53 tumour suppressor gene mutation is the most common and the earliest identified genetic alteration in cSCC. Mutations occur in up to 90% of cSCC cases but less in premalignant lesions (7-48%). The reported variability of the mutation rate in AK is suggested to be due, in part, to the different severity of the lesions investigated [21, 22]. *TP53* mutation seems to be frequent in metastatic cSCC (79%; 24/29 cases) [23]. A more recent targeted sequencing study demonstrated a significantly higher mutation frequency in metastatic tumours compared to primary tumours (85% vs 54%; $p < 0.002$), highlighting the importance of functional p53 as a barrier to cancer progression [24]. Nevertheless, the prognostic impact of these mutations requires clarification.

Retinoblastoma

The retinoblastoma gene (*RB1*) is another major tumour suppressor gene involved in cell cycle regulation. RB1 protein stabilizes constitutive heterochromatin to maintain the overall chromatin structure, and the active hypophosphorylated form of the protein binds transcription factor E2F1 [14] to control gene transcription.
There are few studies on cSCC reporting *RB1* inactivation or RB1 protein loss. In one of the few immunohistochemical (IHC) studies, loss of expression was reported in 8% (2/26) of AK and 16% (7/45) of cSCC cases [25].

Cyclin D1

Cyclin D1 (*CCND1*) accelerates the passage of cells through G1 phase and reduces the requirement for mitogens [17]. This protein is described to participate in tissue organization and differentiation in the early stages of cSCC [26], with overexpression frequently reported in keratinocyte carcinogenesis [25, 27]. One study reported cyclin D1 overexpression in 46% (12/26) of AK and 60% (27/45) of cSCC cases [25]. Another study reported overexpression in 43% (13/30) of Bowen Disease (BD) and 71% (17/24) of cSCC cases [28]. The overexpression of cyclin D1 in premalignant lesions (AK) suggests that it may be an early event in cSCC carcinogenesis. Although studies have reported an increase in expression with increasing histological differentiation in oral SCC, there is a lack of correlation between cyclin D1 overexpression and the degree of differentiation in cSCC [28-30]. One study reported a positive correlation between cyclin D1 overexpression and depth of invasion and metastasis [31]. Larger studies are needed to confirm the prognostic importance of cyclin D1.

Cyclin-dependent kinase inhibitors

These cell cycle inhibitors belong to two main families: the ink4 family (*e.g.* p16 [*CDKN2A*]) and the Cip/Kip family (*e.g.* p21 [*CDKN1A*] and p27 [*CDKN1B*]).
p16 is a specific inhibitor of cyclin-dependent kinases 4 and 6 (*CDK4* and *CDK6*, respectively) [18]. Studies targeting p16 in cSCC are scarce. There are reports of *CDKN2A* alterations (*e.g.* mutations, copy loss, promoter methylation) in 76% cSCC cases and *CDKN2A* mutations in 48% of metastatic cSCC cases [23]. In a study of metastatic cSCC, with a *CDKN2A* mutation frequency of 31% (11/35), *CDKN2A* mutation was associated with disease-specific death ($p = 0.001$) [32]. A more recent study demonstrated a lower rate of mutation (17%) compared to previous studies [24]. Larger studies are needed to confirm p16 as a prognostic biomarker.
To our knowledge, no study has determined the prevalence of overexpression and the mutation rate of Cip/Kip family genes in cSCC.

KNSTRN

KNSTRN encodes the kinetochore localized Astrin/SPAG5 binding protein that assists kinetochore formation during cellular division [19]. In one study, *KNSTRN* mutation was reported in 13% AK and 19% cSCC cases. UV-induced mutation is assumed to occur in premalignant lesions, suggesting that the mutation might be an early event in the

Table 2. Molecular abnormalities in cSCC.

	cSCC		
	Mutations	**Over-expression**	**Down-regulation**
Cell cycle regulation and apoptosis			
TP53	Up to 90%		
RB1			16%
CCND1		60-71%	
CDKN2A	17-48% (metastatic cases)		76%
KNSTRN	19%		
Non-coding promoter mutations			
TERTp	Up to 50%		
Terminal differentiation			
NOTCH1	Up to 82%		
FBXW7			
TP63			
RIPK4	24% (metastatic cases)		
EGFR and other TKRs			
EGFR	2.5-3%	Up to 73%	
PIK3CA	10%		
HRAS	11-13%		
KRAS	10 %		
RASA1	13%		
Adhesion, invasion, and microenvironment molecules			
CDH1			85%
FAT1	44%		

development of cSCC [33]. Still, these results were not confirmed based on a large series of cSCC. *KNSTRN* mutation was also not observed in other studies [23, 24]. Since *KNSTRN* mutations may represent a potential target for new drugs, more studies are necessary to clarify the prevalence and role of *KNSTRN* mutation in cSCC.

Non-coding telomerase (TERT) promoter mutations and stemness

Telomerase (*TERT*) is a ribonucleoprotein complex that synthesizes telomeric DNA (TTAGGG hexamers) which is required to maintain telomere length [34]. *TERT* promoter (*TERTp*) mutations increase telomere length and stability, allowing cancer cells to divide and avoid senescence or apoptosis. Lately, recurrent somatic mutations in the *TERTp*, which affect the catalytic subunit of telomerase, have been described in several cancer models (melanomas, basal cell carcinomas, squamous cell carcinomas, cancers of the central nervous system, and bladder and thyroid cancers [follicular cell-derived]) [35-40].

Some studies have reported *TERTp* mutations in SCC at different locations. Scott *et al*. reported *TERTp* mutation in 50% (13/26) of cSCC and 20% (11/55) of BD cases [38]. *TERT*p mutations were more frequent in cSCC than in BD ($p = 0.019$), suggesting a more relevant role in tumour progression than initiation [38]. Poorly differentiated cSCC is reported to harbour more *TERTp* mutations, but the small size of the series analysed limited the statistical significance of the study [38]. Additional studies have reported *TERTp* mutations in 25-50% of cases [37, 40]. Importantly, in the future, *TERTp* mutations might be used as biological predictors of metastasis and mortality [41], as is the case for melanoma, glioblastoma, medulloblastoma, bladder, and thyroid cancers [40, 42-45]. Larger studies are necessary to ascertain whether *TERTp* mutations have prognostic value for cSCC.

Terminal differentiation factors and retinoid receptors

NOTCH and associated factors

The Notch signalling pathway is involved in the regulation of self-renewal, cell cycle exit, and cell survival [46-48]. Additionally, Notch activity can suppress HPV E6 and E7 protein expression [49].

NOTCH1 and *NOTCH2* mutations have been described in 75% of cSCC cases [49]. Initially, these mutations were reported to occur following homozygous *TP53* mutation, which suggested a more relevant role in tumour progression than initiation [49]. A more recent exome sequencing study reported an 82% mutation rate (with mutation identified in normal skin in 70% cases), identifying *NOTCH1* and *NOTCH2* mutation as an early event in squamous cell carcinogenesis [50].

Based on a whole-exome sequencing study of aggressive cSCC, the frequency of *NOTCH1* and *NOTCH2* mutations appears to be similar (>50%), and more than 30% mutations exhibit loss of function. In another cohort

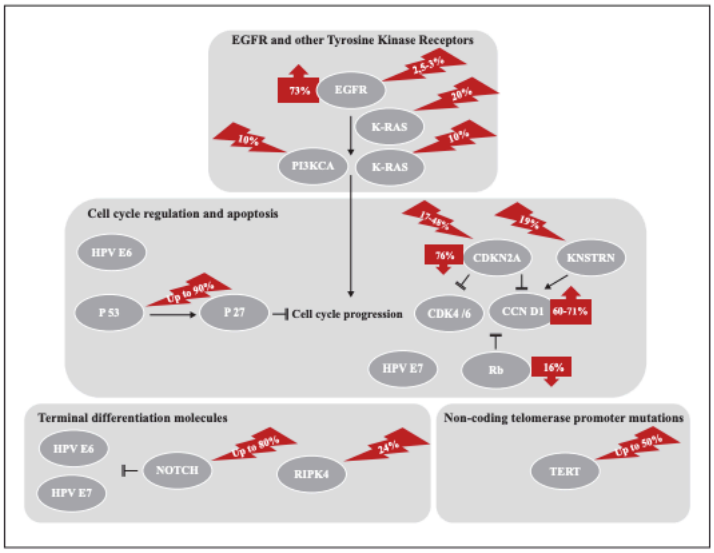

Figure 1. Illustration of the main oncogenic pathways involved in cSCC carcinogenesis. The red lightning symbol indicates the reported incidence of mutations for each oncogene or tumour suppressor gene in cSCC. Upward red arrow indicates overexpression and downward red arrow indicates downregulation in cSCC.

of patients with metastatic cSCC, *NOTCH1/2/4* mutations were reported in 69% of cases [23]; most of the *NOTCH* mutations were missense mutations not previously reported. Another targeted sequencing study of metastatic cSCC reported *NOTCH* mutations in 66% cases [24]. If confirmed, these results would make *NOTCH* mutations the most prevalent genetic alteration in cSCC, however, the frequent identification of these mutations in adjacent normal skin precludes its prognostic value.
A next-generation sequencing study reported that, in addition to *NOTCH1* alterations, *FBXW7* alterations were present in 7% of SCC cases at different locations [51]. *FBXW7* is part of the ubiquitin ligase complex that mediates *NOTCH1* degradation [52], thus constituting an alternative mechanism of *NOTCH* inactivation.

TP63

TP63 is a member of the p53 family and plays a central role in the development of the stratified epithelium, such as the epidermis [53, 54]. This gene may have antagonist roles in cSCC, in contrast to other SCC (*e.g.* head and neck squamous cell carcinoma [HNSCC]). In HNSCC, for example, p63 overexpression is frequent (>95%) and is associated with increased patient survival. On the other hand, p63 expression may be a strong predictor of poor differentiation in non-melanoma skin cancer [55]. In a targeted sequencing study of metastatic cSCC, *TP63* was amplified in 24% (7/29) of cases [23]. However, there are no specific studies addressing *TP63* genetic alterations in cSCC or its putative prognostic value.

RIPK4

An exome-sequencing study identified, for the first time, a potential driver gene in cSCC: *RIPK4* [56]. RIPK4 protein is a serine/threonine protein kinase that interacts with protein kinase C-delta, which is required for keratinocyte differentiation [57]. Based on a genomic analysis of metastatic cSCC, seven cases (24%) with recurrent *RIPK4* mutations were reported [23], two of which were truncating, suggesting recurrent inactivation of the gene. More studies are necessary to ascertain the relevance of *RIPK4* in metastatic cSCC.

Epidermal growth factor receptor and tyrosine kinase receptor pathways

Epidermal growth factor receptor (EGFR) activation or overexpression leads to upstream signalling of both MAPK and PI3K pathways, and is involved in proliferation and evasion of apoptosis [58].
EGFR-activating mutations are rare and have been reported in 2.5-3% of cSCC cases [59, 60]. On the other hand, EGFR

overexpression is described in 43% (9/21) to 73% (30/41) of cSCC cases [61, 62]. EGFR inhibitors (*i.e.* erlotinib and getinib) and EGFR antibodies (*i.e.* cetuximab and panitumumab) are widely used for lung SCC and a need to refine subsets of advanced cSCC that are likely to respond to EGFR therapy is needed. Future research is therefore necessary to clarify this premise.

PI3K pathway

PIK3CA encodes a positive regulator of the PI3K signalling pathway. PI3K is a lipid kinase that converts plasma membrane PIP2 to PIP3 [63] and activates multiple cellular pathways, namely mTOR.
In contrast to other SCC (*i.e.* HNSCC), PI3K pathway mutations do not appear to have a relevant role in cSCC carcinogenesis. Based on an exome sequencing study of cSCC, 10% of cSCC cases presented with *PIK3CA* mutation. These mutations included two inactivating mutations but so far no mutations have been found within the classic hotspot (E545, H1047) [56]. Based on an exome-targeted analysis of metastatic cSCC, oncogenic activation of the RAS/RTK/PI3K pathway was reported in 45% cases and significantly correlated with worse progression-free survival [23]. Although this pathway appears to be important in HNSCC and lung squamous cell carcinoma (LSCC), its role in cSCC is not yet established [56, 64].
In cSCC, no mutations were reported in *PTEN*, which encodes a negative regulator of the PI3K signalling pathway and switches PIP3 to PIP2 [63].

MAPK pathway

RAS oncogenes play a role in different cellular processes (the RAS family controls cell growth and the RHO family controls the actin cytoskeleton). Three members of the RAS family (*HRAS*, *KRAS* and *NRAS*) are reported to be frequently mutated in human tumours [65].
RAS mutations appear to be rare in cSCC. *KRAS* mutation has been reported in 10% cSCC cases [66]. Exome-level sequencing of eight primary cSCC revealed mutation in *HRAS* in one case (13%) [64]. Another exome sequencing study reported an overall activating *RAS* mutation frequency of 11% [50]. Nevertheless, an increased level of RAS with active GTP was described in cSCC, suggesting the possibility that RAS activation in cSCC may also result from upstream stimulation (tyrosine kinase receptor activation), as reported in breast carcinoma [59]. In a cohort of patients with metastatic cSCC, oncogenic activation of the RTK/RAS/PI3K pathway was reported in 45% of cases and significantly correlated with worse progression free-survival [23]. *BRAF* gene mutations are rare events in cSCC [67].
It is important to mention the paradoxical effect observed in melanoma patients treated with tyrosine kinase inhibitors (TKI) concerning the increase in mutated *RAS* in cSCC. In fact, melanoma patients treated with RAF inhibitors develop keratoacanthomas (KA) or cSCC in up to 25% cases [68, 69]. The potential mechanism consists of paradoxical increase in MAPK signalling within the context of mutated or activated *RAS*. Tumours from a cohort of patients treated with a RAF inhibitor were prone to *RAS* mutations despite similar rates of total mutations in patients treated with non-RAF inhibitors [70]. These findings suggest that development of TKI-induced cSCC is not due to a direct mutagenic event associated with RAF inhibitor therapy, but rather due, at least in part, to pro-proliferative interaction between RAF inhibitors and latent *RAS* mutant keratinocytes.

RASA1

RASA1 belongs to a family of RAS GTPase activating proteins, many of which appear to be implicated as tumour suppressors in cancer because they function as negative regulators of the pro-oncogene *RAS* [71]. The role of *RASA1* in cancer has not been clearly defined, despite its frequent inactivation by mutation in many tumour types [72].
Based on an exome sequencing study of cSCC, *RASA1* mutation was reported in 13% of cases [56].

Adhesion, invasion and microenvironmental factors

E-cadherin complex

E-cadherin (*CDH1*) and catenins are key proteins of the adhesion complex at adherent junctions that link neighbouring epithelial cells [73].
There are reports of E-cadherin promoter hypermethylation in 6/7 (85%) cSCC, 4/8 (50%) *in situ* cSCC, 4/9 (44%) AK, and 2/9 (22%) non-neoplastic skin cases. In non-melanoma skin cancer (NMSC), downregulation of E-cadherin is associated with increased tumour invasiveness, an increased potential for distant metastasis, and advanced-stage cSCC [74].

FAT1 gene

The *FAT1* gene is an orthologue of the Drosophila fat gene, which encodes a tumour suppressor essential for controlling cell proliferation during Drosophila development. The gene product is a member of the cadherin superfamily and is expressed at high levels in a number of foetal epithelia [75].
A whole-exome study reported *FAT1* gene mutation in 17/39 (43.6%) aggressive cSCC cases, but without prognostic impact [56].

Matrix metalloproteinases

Matrix metalloproteinases (MMP) are zinc-dependent endopeptidases that can degrade many extracellular matrix proteins [76]. Immunohistochemical expression of MMP2 and MMP9 is associated with cutaneous squamous carcinogenesis and is a potential marker for invasion and progression [77].

Angiogenic and inflammatory factors

Hypoxia leads to an increased production of proangiogenic factors and diminished production of antiangiogenic factors. Proangiogenic factors include: vascular

endothelial growth factor (*VEGF*), platelet-derived growth factor (*PDGF*), fibroblast growth factors 1 and 2 (*FGF1* and *FGF2*, respectively), and interleukin 8 (*IL-8*), among others [78].
There are reports relating VEGFA overexpression to lymphatic metastasis in mouse models of cSCC [79]. There are no clinical studies relating VEGFA to cSCC prognosis. In cSCC, there appears to be high expression of COX-2 in premalignant and malignant lesions [80], and COX-2 expression increases during progression of the tumour [81].

Other features

Aneuploidy

Aneuploidy, although not a hallmark of malignancy, is more frequent in malignant than benign tumours, and is associated with tumour progression. For cSCC, there are few studies evaluating DNA ploidy, and in some, aneuploidy has been suggested to be significantly associated with a risk of metastasis, however, this association remains to be clarified [82].

Epigenetic alterations

Epigenetic alterations cause modifications in DNA domains involved in the control of gene expression. These alterations include DNA methylation, histone acetylation, phosphorylation, ubiquitination, and sumoylation [83].
In a recent epigenetic study of cSCC, no widespread difference in methylation pattern was reported and *FRZB* was identified as a potential epigenetic predictor of metastasis, however, no significant difference was observed when protein expression was compared between metastatic and non-metastatic cSCC [84]. *KMTC2* is a member of the ASC2/NCOA6 complex (ASCOM) with histone methylation activity and is involved in transcriptional co-activation/regulation. There are reports of inactivating *KMT2C* mutations in several cancers, including leukaemia and carcinomas of the stomach, bladder, and breast. A report on exome sequencing for cSCC described inactivating mutations in *KMT2C* (15/39 [38%] cases); patients with *KMT2C* mutations presented significant shorter periods of recurrent free survival, a shorter time to recurrence, and a trend to develop bone metastasis; these data support a role for *KMT2C* in the aggressive behaviour of cSCC [56]. A more recent targeted sequencing study reported a high mutation rate of epigenetic regulators, such as *KMTD2* (8/12 [67%] cases), *KAT6A* (4/12 [33%] cases), *KMTC2* (7/12 [58%] cases), *SETD2* (6/12 [50%] cases), *ARID2* (2/12 [17%] cases), *TET2* (1/12 [8%] cases), *KDM6A* (1/12 [67%] cases), and *CREBBP* (2/12 [17%] cases) [24]. Larger studies are needed to confirm the prognostic value of these alterations.

Human papillomavirus

Human Papillomavirus (HPV) is a double-stranded DNA virus that infects the squamous epithelium. HPV genotypes are classified into five genera: α, β, γ, μ, and ν, based on the degree of sequence similarity. HPV can be subdivided into low and high risk, depending on the malignant progression potential of the associated lesion. High-risk mucosal HPVs cause almost all cases of cervical cancer, and are also associated with a significant fraction of other anogenital tract cases, as well as oropharyngeal cancers [85]. The mechanism of oncogenesis is ascribed to viral proteins E6 (which binds to p53, rendering it a target for proteasomal degradation) and E7 (which binds to RB1, rendering it a target for proteasomal degradation), leading to a loss of tumour suppressor genes that inhibit cell cycle progression [86].
HPV 5 and 8 have been reported in 90% of cSCC cases as a rare genetic disease termed "epidermodysplasia verruciformis" (EV). The association of HPV 5 and 8 with cSCC in EV patients led to their classification as "possibly carcinogenic" [87]. β HPV are also the likely aetiological agents of cSCC that arises in chronically immunosuppressed patients. The association between HPV infection and cSCC development in immunocompetent patients remains controversial [87]. While β HPV genomes are frequently detected in cSCC specimens, they are also often found on healthy skin of non-EV individuals [88, 89]. Epidemiological studies have demonstrated that the prevalence of β HPV in AK is higher than in cSCC suggesting that β HPV may play a role during the initial stages of carcinogenesis [90, 91]. Despite these initial reports, HPV transcription in cSCC has not been identified in recent high-throughput sequencing studies [92, 93]. A possible explanation resides in the fact that the majority of β HPV E7 and E6 proteins, including those of HPV 5 and 8, do not have the ability to destabilize p53 and RB1.

Keratoacanthoma

Based on array comparative genomic hybridization, it has been possible to successfully discriminate between KA and cSCC in 85% of cases, leading to the assumption that these are two distinct entities [94]. Molecular identification of mutations in *TGF1* (which encodes TGFß) in KA of Ferguson-Smith type (85-90%) and its absence in cSCC suggests the existence of a distinct pathogenic pathway [95, 96]. Despite the identification of *TGF1* mutations, larger studies are required to establish this mutation as an unequivocal molecular marker in KA.

Conclusion and future perspectives

In an era of predictive biomarkers and patients stratified for therapy, in which new drugs with various molecular targets are being developed, a comprehensive understanding of the molecular basis of cSCC is of outstanding importance, especially for patients with metastatic disease in which prognosis is poor and effective therapies are lacking. Despite improvements in surgery, chemotherapy, radiotherapy, and supportive care, overall survival has not markedly improved for patients with advanced cSCC. Current chemotherapy treatments for cSCC are not targeted, but instead primarily platinum-based treatments (cisplatin and carboplatin) with concurrent radiation are used. Options for recurrent/metastatic cSCC remain very limited and despite significant improvements in targeted treatment for other skin cancers (*e.g.* melanoma and basal cell carcinoma), currently there is no targeted therapy approved for cSCC. Our

increasing knowledge of molecular alterations concerning cSCC opens new avenues in the design of more efficient targeted therapies.

Retinoids act on retinoid acid receptors, mediate epidermal growth factor genes, and inhibit dermal microvascular endothelial cells and neutrophil migration. These have been used successfully to prevent the development of cSCC in immunosuppressed patients after renal transplantation [49-51], however, the use of retinoids in chemoprevention of cSCC in immunocompetent patients was not approved by the FDA [52, 53]. New interest in the use of retinoids in chemoprevention has emerged for patients with development of cSCC following treatment with BRAF inhibitors for metastatic melanoma (7-31%) [54]. At this moment in time, no targeted therapy is approved for advanced cSCC. Few case reports have demonstrated that cetuximab presents a better response rate compared to conventional chemotherapy in patients with metastatic cSCC. We believe that the new monoclonal antibodies (transtuzumab, pertuzumab, onartuzumab, and cixutumumab) and tyrosine kinase inhibitors (geftinib, erlotinib, lapatinib, and afatinib), that target EGFR and other members of the EGFR family, may play a role in the therapy of cSCC, but predictive biomarkers in prospective clinical trials are needed [97]. The *PIK3A* gene is mutated in cSCC, making this pathway an attractive target for therapeutic inhibition (developed molecules include GDC-0941, PX-866, NVP-BKM120, and NVPBYL719). *TP53* alterations are present in the majority of cSCC cases, thus the ability to selectively target tumours with decreased p53 activity could have major implications for these patients. Several experimental strategies have been undertaken to target tumour cells, leading to wild-type p53 activation and restoration (*e.g.* RITA, nutlins, mdm2-inhibitors, and benzodiazepinedione) or mutant p53 reactivation (CDB3, c-terminal peptides, and CP-31398) [98]. *In vitro* studies have suggested that NOTCH inhibitors (gamma-secretase inhibitors and monoclonal antibodies) may play a role in cancer treatment, although no study has so far included cSCC [99]. TERT-targeted therapies (*e.g.* GRNI63, T-oligo, DN-hTERT, BIBRI532, BRACOI9, RHPS4, and telomestatin) are a promising treatment option in cSCC, since a majority of the tumours present with *TERTp* mutations. However, the clinical testing of some of these molecules has been hampered due to the toxic characteristics of the drugs [39]. Overexpression of programmed cell death protein 1/programmed death-ligand 1 (*PD-1/PD-L1*) has been described in cSCC [100]. In this context, immune checkpoint antibodies (*e.g.* ipilimumab, pembrolizmumab, and nivolumab) that block the *PD-1/PD-L1* pathway have been reported in advanced unresectable or metastatic cSCC [101-105]. Patients treated with these anti-*PD-1* inhibitors showed a partial response with a favourable side-effect profile, suggesting that these treatments may represent a promising new therapeutic option for advanced-stage cSCC.

We believe that the next few years will reveal a development of biologic therapies which efficiently target these genetic alterations and improve the survival of patients with cSCC. ■

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Paper III

TERT promoter mutations are associated with poor prognosis in cutaneous squamous cell carcinoma

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ORIGINAL ARTICLE

TERT promoter mutations are associated with poor prognosis in cutaneous squamous cell carcinoma

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Background: Telomerase reverse transcriptase gene (*TERT*) promoter (*TERT*p) mutations have been reported as potential predictors of poor prognosis in several cancers, but the prognostic value of *TERT*p mutations for cutaneous squamous cell carcinoma (cSCC) has not been determined.

Objective: To evaluate the frequency of *TERT*p mutations and correlate it with clinicopathologic features and patient outcome.

Methods: We performed genetic profiling of *TERT*p mutations in a retrospective series of cSCCs. The predictive value of *TERT*p mutations and clinicopathologic parameters were assessed by using logistic regression models.

Results: A total of 152 cSCCs from 122 patients were analyzed for *TERT*p mutations; the mutation rate was 31.6% (48 of 152), and it was higher in invasive cSCC (42 of 121 [34.7%]) than in in situ cSCC (6 of 31 [19.4%]). Age older than 75 years (odds ratio [OR], 14.84; $P = .013$] and *TERT*p mutation (OR, 8.11; $P = .002$) were independent predictors of local recurrence. *TERT*p mutation (OR, 15.89; $P = .022$) was independently associated with higher risk of lymph node metastasis.

Limitations: The restricted number of metastatic cases.

Conclusion: *TERT*p mutations may prove to be a molecular biomarker with prognostic significance in invasive cSCC, but larger studies are needed. (J Am Acad Dermatol https://doi.org/10.1016/j.jaad.2018.08.032.)

Key words: biomarker; cutaneous squamous cell carcinoma; metastases; outcome; prognosis; prognostic biomarker; recurrence; squamous cell carcinoma; *TERT*; *TERT* promoter mutation.

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Cutaneous squamous cell carcinoma (cSCC) is the second most common cancer in whites.[1] Ultraviolet radiation is the most common causal factor, and cSCC occurs most frequently in chronically sun-exposed areas, such as the face.[2] cSCC carcinogenesis includes premalignant lesions, actinic keratosis, in situ squamous carcinoma/Bowen disease, invasive carcinoma, and metastatic cSCC carcinoma, although a multistep model is not always present.[3] cSCC can recur and metastasize, and metastatic cases have a poor prognosis, with a 5-year survival rate of 25% to 35% and a 10-year survival rate less than a 10%.[4-6] Some clinicopathologic prognostic markers have been proposed in cSCC for local recurrence and metastasis (tumor thickness >6 mm; invasion beyond subcutaneous fat; perineural invasion; tumor size >2 cm; poor differentiation; and localization in the temple, lip, and ear).[7]

CAPSULE SUMMARY

- Patients with telomerase reverse transcriptase gene (*TERT*) promoter–mutated cutaneous squamous cell carcinoma (have higher risks for local recurrence and lymph node metastases.
- In the future, *TERT* promoter mutation may be included in the prognostic assessment of patients with cutaneous squamous cell carcinoma.

Telomerase reverse transcriptase gene (*TERT*), a ribonucleoprotein complex that synthesizes telomeric DNA (TTAGGG hexamers), is responsible for maintaining telomere length.[8] *TERT* promoter (*TERT*p) mutations create binding sites for E-twenty-six family transcription factors that result in telomerase expression and increasing telomere length and stability, allowing cancer cells to divide and preventing senescence or apoptosis. Recurrent somatic *TERT*p mutations have been found in a high percentage of melanomas, cancers of the central nervous system, bladder cancers, and thyroid cancers (follicular cell-derived).[9-13] In cutaneous carcinomas,[13,14] an ultraviolet light–induced damage signature has been attributed to *TERT*p mutations (cytidine-to-thymidine transitions at dipyrimidine motifs).[9,10] These *TERT*p mutations have been described as a potential biologic predictor of metastasis and/or mortality in melanoma, glioblastoma, medulloblastoma, and bladder and thyroid cancers.[12,15-18] However, in cSCC, only small series of cases were evaluated and no information about the putative prognostic value of these changes is available in the literature.

In this study, we assessed *TERT*p mutations in a large series of cSCCs and correlated these mutations with clinicopathologic features and patients outcome.

MATERIALS AND METHODS

Patient selection, sample selection, and clinicopathologic characterization

All the procedures described in this study were in accordance with national and institutional ethical standards and were previously approved by local ethical review committees. A more detailed description of methods is available in the Supplemental Methods (available at http://www.jaad.org).

A descriptive and statistical analysis of all consecutive cSCCs surgically removed at Centro Hospitalar Vila Nova de Gaia e Espinho between January 2004 and December 2013 was performed. The inclusion criteria included immunocompetence, histologic diagnosis of cSCC, and available follow-up data. The exclusion criteria included lesions in patients with genetic diseases associated with increased risk of cSCC (eg, xeroderma pigmentosum, epidermodysplasia verruciformis, and albinism). None of the cases in this retrospective series were treated with Mohs micrographic surgery. A total of 184 histologic specimens were revised by pathologists with experience in cutaneous neoplasms (J.M.L., J.P., and M.F.). Tumors were categorized according to the protocol for examination of specimens of the College of American Pathologists and the American Joint Committee on Cancer guidelines.[19] Representative tumor areas were marked by the pathologists on hematoxylin and eosin–stained slides to perform manual microdissection.

For statistical analysis, age at diagnosis was categorized as being in 1 of 2 groups according to the mean age (≤75 vs >75 years), and topographic locations were classified according to the *International Statistical Classification of Diseases and Related Health Problems, Tenth Revision*,[20] and according to sun exposure. Specimens were classified as in situ cSCC or invasive cSCC according to histologic subtype. In addition to College of American Pathologists protocol variables, other recorded categories were pattern of invasion and presence of intratumoral and peritumoral infiltrate. The T stage of each tumor was classified according to the correlative tumor, node, and metastasis classification. As in other studies,[21,22] recurrence was defined as the development of a histologically confirmed cSCC in the same topographic area in

Abbreviations used:

cSCC: cutaneous squamous cell carcinoma
OR: odds ratio
*TERT*p: telomerase reverse transcriptase gene promoter

addition to being identified by the assisting dermatologist as recurrence. Progression-free survival was defined as the time until diagnosis of recurrence and/or metastasis. Progression-free survival and overall follow-up times were recorded in months.

DNA extraction and mutation analysis

DNA was retrieved from 10-μm cuts of formalin-fixed paraffin-embedded tissue samples after careful microdissection. A DNA extraction kit (Citogene, Citomed, Odivelas, Portugal) was used according to the manufacturer's instructions. Polymerase chain reaction was performed with Qiagen Multiplex kit (Qiagen, Hilden, Germany) using the recommended settings.[11] Direct sequencing reaction was performed with the BigDye Terminator Kit (Perkin-Elmer, Foster City, CA), and the fragments were run in an ABI prism 3100 Genetic Analyzer (Perkin-Elmer). Independent polymerase chain reaction amplification/sequencing was performed for both positive and inconclusive (not confirmed as positive or negative) samples. Mutations were detected by using Mutation Surveyor DNA variant analysis software (Softgenetics, State College, PA) and matched with reference sequences from GenBank. Sequences were obtained from base pairs −270 to −50 upstream of the start (ATG) codon, which include the recurrent *TERT*p mutations described in other cancers.[9,10] The described *TERT*p mutations evaluated included the following: −124C>T, −146C>T, tandem −124/−125CC>TT, and tandem −138/139CC>TT.

Statistical analysis

Statistical analysis was conducted with SPSS software (version 24.0, IBM Corp, Armonk, NY). Descriptive statistics, the chi-square test, Fisher exact test, and Student *t* test (unpaired, 2-tailed) were used when appropriate. The predictive value of *TERT*p and other variables for recurrence, metastasis, and progression-free survival was assessed by using univariate and multivariate logistic regression models. In the regression models, all the variables that were significantly associated with the specified outcome in the univariate model were included in the multivariate analysis. Confidence intervals were calculated with 95% coverage. Survival curves were plotted by the Kaplan-Meier method with the log-rank statistics. Results were considered statistically significant at P less than .05. The significance level was adjusted by Bonferroni correction (with 0.05 divided by the number of performed comparisons for each dependent variable) when multiple comparisons were performed (results displayed in Supplemental Table I (available at http://www.jaad.org).

RESULTS

Of the 184 histologically characterized cases, we were unable to determine *TERT*p status in 32 cases owing to the small size and/or low quality of the samples. In total, 152 lesions from 122 patients were analyzed for *TERT*p mutations. Of these, 31 corresponded to in situ cSCC and 121 to invasive cSCC. The overall frequency of *TERT*p mutations was 31.6% (48 of 152 cases). *TERT*p mutations were present in 6 of 31 in situ cSCCs (19.4%) and 42 of 121 invasive cSCCs (34.7%). The following mutations were detected: −124 (G>A mutation in 26 of 48 cases [54.2%]), −146 (G>A mutation in 18 of 48 cases [37.5%]), and tandem mutation at position −124/−125 in 4 cases (8.3%). The mutations were mutually exclusive.

Relationship between *TERT*p mutations and clinicopathologic features

Table I presents the clinicopathologic features and the frequency of *TERT*p mutations in the series. Clinicopathologic factors and their association with *TERT*p mutations are presented in Supplemental Table I.

We analyzed all the cSCCs and observed that *TERT*p mutations were present in the face, trunk, and upper and lower limb. *TERT*p mutations were more frequent in invasive cSCCs than in in situ cSCCs, although the difference was not statistically significant.

In in situ cSCCs, men showed a higher frequency of *TERT*p mutations (in 5 of 15 [33.3%]) than women did (in 1 of 16 [6.3%]). Of the 3 in situ cSCCs that recurred, none of had *TERT*p mutations.

In invasive cSCCs, *TERT*p mutation was associated with a larger maximum tumor thickness (4.8 ± 4.1 mm vs 3.4 ± 2.2 mm [P = .050]), and tumors with thicker than 6 mm displayed a higher frequency of *TERT*p mutations (in 10 of 19 cases [52.6%] vs 29 of 96 cases [30.2%] cases [P = .059]), although neither association reached statistical significance after adjustment of the P values with Bonferroni correction. Despite the difference not reaching statistical significance, tumors with *TERT*p mutations were larger than tumors with wild-type *TERT*p (2.3 ± 1.8 cm vs 1.6 ± 1.2 respectively

Table I. Clinicopathologic features and the frequency of *TERT*p mutations

Feature	All lesions	In situ cSCC	Invasive cSCC
No. of cases	152	31	121
Age at diagnosis, y, mean ± SD	76.8 ± 11.8	78.5 ± 7.0	76.5 ± 12.7
Male	73.8 ± 11.7	77.8 ± 6.8	73.0 ± 12.3
Female	80.8 ± 10.8	78.4 ± 7.8	81.6 ± 11.6
Sex, n (%)			
Male	87 (57.2)	15 (48.4)	72 (59.5)
Female	65 (42.8)	16 (51.6)	49 (40.5)
Sun exposure, n (%)			
Chronic	103 (67.8)	11 (35.5)	92 (76.0)
Intermittent	43 (28.3)	18 (58.1)	25 (20.7)
Undetermined	6 (3.9)	2 (6.5)	4 (3.3)
Localization, n (%)			
Face	97 (63.8)	9 (29.0)	88 (72.7)
Trunk	8 (5.3)	4 (12.9)	4 (3.3)
Upper limb	21 (13.8)	6 (19.4)	15 (12.4)
Lower limb	20 (13.2)	10 (32.3)	10 (8.3)
Undetermined	6 (3.9)	2 (6.5)	4 (3.3)
Follow-up, mo, mean ± SD	42.9 ± 28.8	39.7 ± 22.0	43.7 ± 30.3
Progression-free survival, mo, mean ± SD	39.8 ± 29.3	38.4 ± 22.2	40.1 ± 30.9
Adverse outcome, n (%)			
No	129 (84.9)	28 (90.3)	101 (83.5)
Yes	23 (15.1)	3 (9.7)	20 (16.5)
Recurrence, n (%)			
No	132 (86.8)	28 (90.3)	104 (86.0)
Yes	20 (13.2)	3 (9.7)	17 (14.0)
Metastases, n (%)			
No	144 (94.7)	31 (100)	113 (93.4)
Yes	8 (5.3)	0	8 (6.6)
*TERT*p mutations, n (%)			
Wild-type	104 (68.4)	25 (80.6)	79 (65.3)
Mutation	48 (31.6)	6 (19.4)	42 (34.7)
Procedure, n (%)			
Biopsy	34 (22.4)	6 (19.4)	28 (23.1)
Excision	118 (77.6)	25 (80.6)	93 (76.9)
Maximum tumor size, cm, mean ± SD	1.9 ± 1.5	1.9 ± 1.2	1.9 ± 1.5
Maximum tumor size, n (%)			
<2 cm	69 (45.4)	10 (32.3)	59 (48.8)
≥2 cm	39 (25.7)	9 (29.0)	30 (24.8)
Cannot be assessed	44 (28.9)	12 (38.7)	32 (26.4)
Peripheral margins, mm, mean ± SD	2.3 ± 2.9	1.6 ± 1.5	2.5 ± 3.1
Deep margins, mm, mean ± SD	2.6 ± 2.4	3.0 ± 1.7	2.5 ± 2.5
Ulceration, n (%)			
No	53 (34.9)	14 (45.2)	39 (32.2)
Yes	91 (59.9)	15 (48.4)	76 (62.8)
Undetermined	8 (5.3)	2 (6.5)	6 (5.0)
Actinic keratosis, n (%)			
No	50 (32.9)	6 (19.4)	44 (36.4)
Yes	92 (60.5)	25 (80.6)	67 (55.4)
Undetermined	10 (6.6)		10 (8.3)
Invasion, n (%)			
Noninvasive	31 (20.4)		
Invasive	121 (79.6)		
Histologic type, n (%)			
Acantholytic			9 (7.4)
Verrucous			2 (1.7)
NOS			110 (90.9)

Continued

Table I. Cont'd

Feature	All lesions	In situ cSCC	Invasive cSCC
Histologic grade, n (%)			
Well differentiated			43 (35.5)
Moderately/poorly differentiated			74 (61.2)
Cannot be assessed			4 (3.3)
Pattern of invasion, n (%)			
Expansive			66 (54.5)
Infiltrative			51 (42.1)
Cannot be assessed			4 (3.3)
Level of invasion, n (%)			
Papillary dermis			36 (29.8)
Reticular dermis			53 (43.8)
Subcutaneous tissue			25 (20.7)
Cannot be assessed			7 (5.8)
Maximum tumor thickness, mm, n (%)			3.8 ± 3.1
<6 mm			96 (79.3)
≥6 mm			19 (15.7)
Cannot be assessed			6 (5.0)
Intratumoral infiltrate, n (%)			
Moderate/intense			13 (10.7)
Few/absent			108 (89.3)
Peritumoral infiltrate, n (%)			
Moderate/intense			68 (56.2)
Few/absent			53 (43.8)
Lymphovascular invasion, n (%)			
Not present			116 (95.9)
Present			5 (4.1)
Perineural invasion, n (%)			
Not present			118 (97.5)
Present			3 (2.5)

cSCC, Cutaneous squamous cell carcinoma; *NOS*, not otherwise specified; *TERT*p, telomerase reverse transcriptase gene promoter.

[P = .068]). *TERT*p mutations were significantly more frequent in cases that recurred (in 13 out of 17 cases [76.5%] vs in 29 of 104 cases [27.9%] [P < .001]). *TERT*p mutations were also more common in metastatic cases (in 7 of 8 cases [87.5%]) than in nonmetastatic cases (35 of 113 cases [31.0%]), but statistical analysis was precluded owing to the small number of wild-type cases with metastasis (n = 1).

Relationship between *TERT*p mutation and outcome

In this analysis, we included only invasive cSCCs (n = 121). The mean follow-up time of the patients was 43.7 plus or minus 30.3 months (range, 6-156 months).

A total of 17 cases (14.0%) and 8 cases (6.6%) presented recurrence and metastasis (all of which were lymph node metastasis), respectively, during follow-up. Table II presents the main characteristics of the cases with adverse outcomes.

Regression modeling was performed for factors associated with an adverse outcome (recurrence or metastases) in invasive cSCC (Table III). When the factors associated with the risk of recurrence were analyzed, age older than 75 (odds ratio [OR], 13.19; P = .014], absence of ulceration (OR, 2.96; P = .048), and *TERT*p mutation (OR, 8.41; P = .001) were identified to be predictors in the univariate analysis. When the aforementioned factors were included in the multivariate analysis, *TERT*p mutation (OR, 8.11; P = .002) and age older than 75 years (OR, 14.84; P = .013] were identified as independent predictors of recurrence. As shown in Table II, 82.4% of the cases that recurred were located on the face. When facial and extrafacial lesions were compared, no statistical difference was observed between the mean values of their surgical margins (mean peripheral margins of 2.5 ± 3.5 vs 2.7 ± 1.8, respectively, and mean deep margins of 2.6 ± 2.7 vs 2.6 ± 2.1, respectively). Peripheral margins (OR, 1.12; P = .121) and deep margins (OR, 0.92; P = .924) were not associated with recurrence in the univariate analysis. Despite the fact that the differences between margins did not achieve significance in the univariate

Table II. Features of invasive cSCC in patients with recurrence and/or lymph node metastasis

Case	Patient age, y/sex	Location	AO	PFS, mo	TERTp mutation	Max, cm	PM/DM, mm	HT	HG	TT, mm	Peritumoral infiltrate	FU, mo	Status at FU
1	85/F	Ear	R	22	WT	2	3.5/3.0	NOS	Mod	4	Mod-int	56	NED
2	78/M	Forehead	R	16	WT	1.8	2.0/1.5	NOS	Mod	1.5	Few-abs	22	DNR
3	80/M	Ear	R	35	Mut	5	4.0/0.8	NOS	Mod	9	Mod-int	80	NED
4	81/M	Ear	R + Ms	11	Mut	1.5	4.0/8.0	Acan	Mod	7	Few-abs	36	DOD
5	83/M	Forehead	R + Ms	9	Mut	NA	0/0	NOS	Poor	NA	Few-abs	34	DNR
6	81/M	Ear	R	32	Mut	0.9	3.0/2.0	NOS	Well	2	Mod-int	41	NED
7	80/F	Arm	R + Ms	28	Mut	2.5	4.5/2.0	NOS	Well	2	Mod-int	96	NED
8	82/F	Hand	R + Ms	38	Mut	1.2	4.0/0.1	NOS	Mod	5	Few-abs	58	NED
9	75/M	Arm	R	39	Mut	NA	0.5/0.2	NOS	Mod	3	Few-abs	56	NED
10	80/M	Temple	R	25	Mut	1.1	1.5/2.0	NOS	Mod	4	Few-abs	38	NED
11	86/M	Ear	Ms	3	WT	NA	0/0	NOS	Mod	2	Few-abs	12	DOD
12	87/M	Cheek	R	7	WT	1.5	1.5/4.0	NOS	Mod	4	Few-abs	24	DNR
13	87/F*	Cheek	R	12	Mut	1	4.0/4.0	NOS	Mod	5	Mod-int	36	NED
14	87/F*	Forehead	R	12	Mut	0.8	2.0/3.0	NOS	Mod	1.5	Mod-int	36	NED
15	88/F	Nose	R	16	Mut	0.6	1.5/2.0	NOS	Mod	2	Few-abs	24	NED
16	82/F	Nose	R	20	WT	0.9	2.5/0.5	NOS	Poor	3	Mod-int	62	NED
17	80/M	Ear	R	8	Mut	3	0/0	NOS	Well	4	Few-abs	12	DNR
18	86/M	Ear	Ms	6	Mut	NA	0/0.2	NOS	Mod	5	Few-abs	12	DNR
19	86/F	Nose	Ms	10	Mut	2.5	3.0/5.0	NOS	Mod	11	Few-abs	24	NED
20	84/M	Scalp	R + Ms	3	Mut	NA	21.0/1.0	NOS	Mod	18	Few-abs	30	NED

Acan, Acantholytic; *AO*, adverse outcome; *cSCC*, cutaneous squamous cell carcinoma; *DNR*, death not related; *DOD*, dead of disease; *F*, female; *Few-abs*, few-absent; *FU*, follow-up; *HG*, histologic grade; *HT*, histologic type; *M*, male; *Max*, maximum tumor size; *Mod*, moderately differentiated; *Mod-int*, moderate-intense; *Ms*, lymph node metastasis; *Mut*, mutated; *NA*, not available/analyzed; *NED*, no evidence of disease; *NOS*, not otherwise specified; *PFS*, progression-free survival; *PM/DM*, peripheral margins and deep margins; *Poor*, poorly differentiated; *R*, recurrence; *SD*, standard deviation; *Sub*, subcutaneous tissue; *TERT*p, telomerase reverse transcriptase gene promoter; *TT*, tumor thickness; *Well*, well-differentiated; *WT*, wild-type.
*Tumor taken from the same patient on the same day.

analysis, we included margins in the multivariate analysis because lower margins may be a confounder in relation to *TERT*p mutation prognostic value in recurrence. After adjustment for margins, *TERT*p mutation (OR, 6.75; $P = .004$) and age older than 75 years (OR, 13.00; $P = .022$) continued to be independent predictors of recurrence in the multivariate analysis.

When we analyzed predictors of metastasis, univariate analysis demonstrated that few or absent peritumoral lymphocytes (OR, 10.20; $P = .033$), subcutaneous tissue invasion (OR, 5.46; $P = .034$), peripheral margins (OR, 1.16; $P = .048$), and *TERT*p mutation (OR, 15.60; $P = .012$) were associated with a higher likelihood of metastasis. In the multivariate analysis, only *TERT*p mutation (OR, 15.89; $P = .022$) was independently associated with a higher risk of metastasis.

The Kaplan-Meier survival analysis revealed that *TERT*p mutation (Fig 1, *A* and *B*) and age older than 75 years (Fig 1, *C* and *D*) were associated with a shorter time for recurrence (log rank $P < .001$ and $P < .001$) and a shorter time for metastasis to occur (log rank $P = .002$ and $P = .007$). Absent or few peritumoral lymphocytes (Fig 1, *E*) and subcutaneous tissue invasion (Fig 1, *F*) were associated with a shorter time for metastasis occurrence (log rank $P = .007$ and $P = .014$).

DISCUSSION

The most important added value provided by the present study is the finding that patients with mutated *TERT*p invasive cSCC have a substantially higher risk of adverse outcome (recurrence and metastasis). Our results are in accordance with those of previous studies in other cancers, in which *TERT*p mutation was a significant predictor of poor prognosis.[15,18,23] The usefulness of *TERT*p mutations as a prognostic marker is particularly relevant because invasive cSCC is highly frequent, a small percentage of such carcinomas behave aggressively, and there is a lack of good prognostic indicators in this setting.

Concerning invasive cSCC, we report a lower mutation rate (34.7%) than those reported in most previously published studies (50.0%-74.1%); however, those studies were conducted with a limited number of samples (5-37 cases).[13,14,24,25] Because our study included a larger consecutive series of cases in a hospital, we believe that it represents a

Table III. Predictive factors for recurrence and lymph node metastasis

	Recurrence				Metastasis			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
Factor	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Mean age, y								
≤75	1	**.014**	1	**.013**	NA	NA		
>75	13.19 (1.69-103.19)		14.84 (1.77-124.17)					
Ulceration								
Yes	1	**.048**	1	.116	1	.196		
No	2.96 (1.01-8.68)		2.69 (0.78-9.21)		2.78 (0.59-13.11)			
Level of invasion								
Dermis	1	.741			1	**.034**	1	.163
Subcutaneous tissue	0.80 (0.21-3.05)				5.46 (1.14-26.28)		3.95 (0.57-27.30)	
Peritumoral infiltrate								
Moderate-intense	1	.184			1	**.033**	1	.054
Few-absent	2.03 (0.72-5.74)				10.20 (1.21-85.68)		9.69 (0.96-97.75)	
Peripheral margins*	1.12 (0.97-1.28)	.121			1.16 (1.00-1.35)	**.048**	1.10 (0.90-1.33)	.352
*TERT*p								
Wild-type	1	**.001**	1	**.002**	1	**.012**	1	**.022**
Mutation	8.41 (2.53-27.90)		8.11 (2.22-29.59)		15.60 (1.85-131.65)		15.89 (1.48-170.70)	

Parameters with significant results in the univariate analysis of 1 of the adverse outcomes (recurrence or metastasis) are included. None of the other clinicopathologic features were associated with outcome in the univariate analysis. Not displayed are facial location and deep margins that were not associated with recurrence and metastasis in the univariate analysis. Boldface indicates statistical significance.

CI, Confidence interval; *NA*, not applicable (no metastasis occurred in patients age 75 years or younger); *OR*, odds ratio; *TERT*p, telomerase reverse transcriptase gene promoter.

*Peripheral margins were analyzed in the model as a continuous variable.

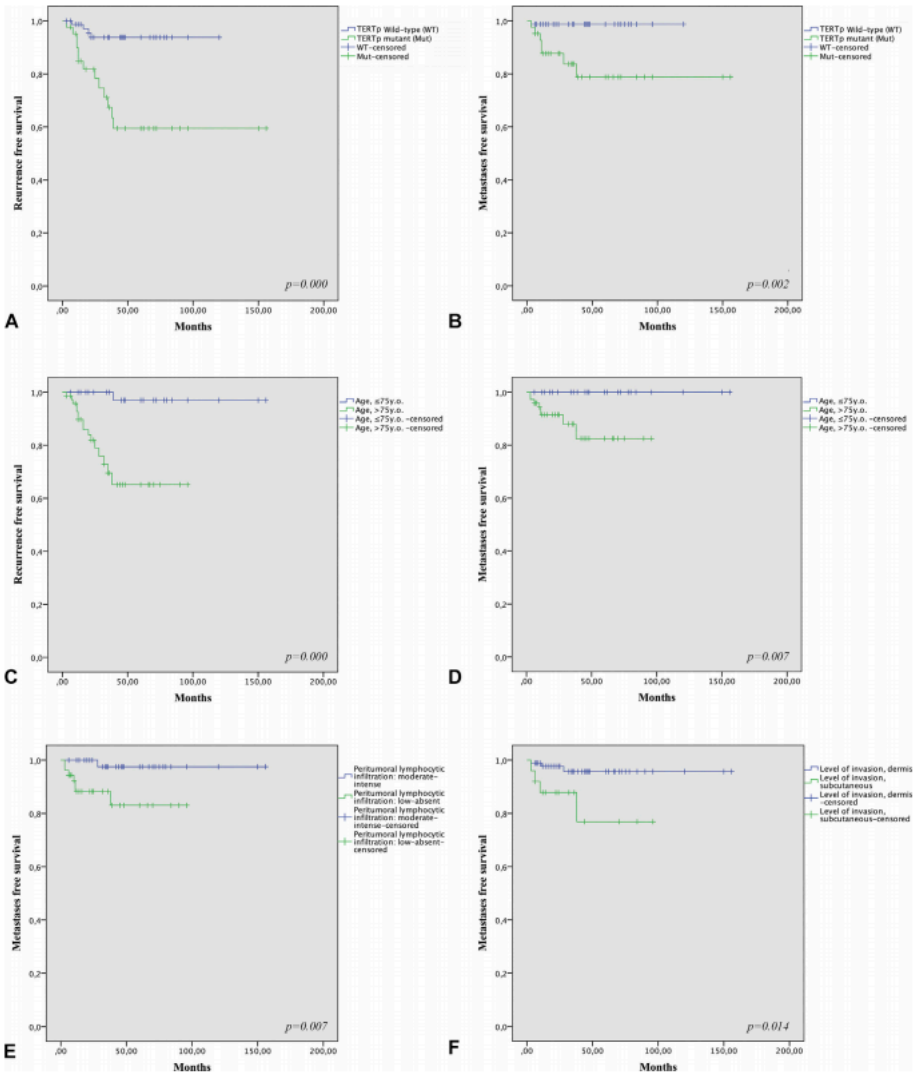

Fig 1. Kaplan-Meier curves for recurrence-free survival and metastasis-free survival of invasive cutaneous squamous cell carcinoma, respectively, according to telomerase reverse transcriptase gene (*TERT*) promoter (*TERT*p) status (**A** and **B**) and age (**C** and **D**). Kaplan-Meier curves for metastasis-free survival of invasive cutaneous squamous cell carcinoma according to peritumoral lymphocytic infiltration (**E**) and level of invasion (**F**). *MUT*, Mutation; *WT*, wild type.

more accurate estimation of *TERT*p mutation in cSCC. Our study indicates that *TERT*p mutation may be more frequent in invasive cSCC than in in situ cSCC, although studies with a larger number of in situ cases are necessary to confirm this premise.

As in melanoma[15,23] *TERT*p-mutated, invasive cSCCs presented a larger maximum tumor thickness than wild-type *TERT*p cases did, although the difference in our study was not statistically significant. Our results also showed a higher (but not statistically significantly so) frequency of *TERT*p mutations in tumors thicker than 6 mm, which is a parameter that has been described as being associated with a higher risk of metastasis.[21]

The role of *TERT*p mutation in early cutaneous squamous cell carcinogenesis remains to be clarified because our study revealed a higher rate of *TERT*p mutation in in situ cSCCs (19.4%) than the previously reported rate (9.1%),[13] even though recurrent in situ cSCCs did not have this mutation in our series. Thus, studies including cases of normal-appearing skin, actinic keratosis, in situ cSCCs, and invasive cSCCs are warranted to clarify the role of *TERT*p mutation in the putative pathogenic model(s) of cSCC.

Our rate of recurrence of invasive cSCC (14.0%) is within the range of those rates reported in previous studies (3.0%-16.0%).[21,22,26-28] We observed a lymph node metastasis rate (6.6%) slightly higher than that reported in the literature (3.7%-4.6%).[21,22] The fact that all cases were drawn from a hospital that often assists patients with more advanced disease may in part explain the differences in the aforementioned reported rates. Most of the recurrent invasive cSCC cases were located on the face, which is an anatomic region where larger clearance margins are difficult to attain. Despite this, facial location and mean margins were not associated with recurrence.

With regard to other prognostic factors in this series, age older than 75 years was an independent predictor of recurrence in invasive cSCC, as identified in previous studies.[22,28-30] Our results also indicated that little or no peritumoral infiltration was associated with metastasis in the univariate analysis and with a shorter time until this adverse outcome occurred. In other tumor models (including melanoma), the absence of or a reduced number of lymphocytes (without addressing the different subsets by immunohistochemistry) is an independent parameter associated with adverse prognosis.[31-33] Invasion of the subcutaneous tissue, which is a classic risk factor for recurrence and metastasis of cSCC,[7] was a predictor of metastasis in the univariate analysis and was associated with a shorter time until metastization. Despite these results, when adjusted for other variables, few or absent peritumoral infiltrates and invasion of the subcutaneous tissue failed to be independent prognostic predictors.

We are aware that our series lacks a substantial number of metastatic cSCC samples with longer follow-up. Nevertheless, we evaluated a consecutive series and found that the prevalence of advanced cSCC in our cohort is actually slightly higher when compared with the frequency reported by others. Another limitation is that there could be a potential selection bias because not all initially selected cases had histologic specimens available.

We conclude that *TERT*p mutations may be potential markers for aggressive behavior in cSCC because they may be more frequently observed in invasive cSCC than in situ cSCC and were associated with recurrence and metastasis in invasive cSCC. Moreover, recurrence and metastasis in invasive cSCC were likely to occur sooner in cases with *TERT*p mutation. As in other cancers,[34] the inclusion of *TERT*p mutation in management guidelines should be considered in cSCC.

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SUPPLEMENTAL METHODS

Patient selection, sample selection, and clinicopathologic characterization

All the procedures described in this study were in accordance with national and institutional ethical standards and were previously approved by the local ethical review committees. According to Portuguese law, informed consent is not required for retrospective studies.

A descriptive and statistical analysis of all consecutive cutaneous squamous cell carcinomas (cSCCs) surgically removed at Centro Hospitalar Vila Nova de Gaia e Espinho (CHVNGE) between January 2004 and December 2013 was performed. The inclusion criteria included immunocompetence, a histologic diagnosis of cSCC, and available follow-up data. The exclusion criteria included lesions in patients with genetic diseases associated with increased risk of cSCC (eg, xeroderma pigmentosum, epidermodysplasia verruciformis, and albinism). None of the cases in this retrospective series were treated with Mohs micrographic surgery. We identified and gathered available formalin-fixed paraffin-embedded tissue samples from the pathology department of Centro Hospitalar Vila Nova de Gaia e Espinho. A total of 184 histologic specimens were examined by pathologists with experience in cutaneous neoplasms (J.M.L., J.P., and M.F.). Tumors were characterized on the basis of the protocol for examination of specimens of the College of American Pathologists (CAP) and the American Joint Committee on Cancer guidelines.[S1,S2] The included College of American Pathologists criteria were as follows: procedure, tumor site, tumor size, histologic type, histologic grade, thickness of the tumor, status of surgical (peripheral and deep) margins of the excised tumors, lymphovascular invasion, perineural invasion, lymph node status, and pathologic staging (pathologic tumor, node, and metastasis stating). We also included additional criteria such as type of infiltration (expansive or infiltrative), presence of ulceration, peritumoral and intratumoral lymphocytic infiltrate, presence of actinic keratosis in adjacent epidermis, and presence of ulceration in the histologic specimens. We used transected tumor biopsy specimens of 26 cases (5 in situ cSCCs and 21 invasive cSCCs) to evaluate pristine primary tumors, without alterations derived from subsequent complete re-excision of each tumor. There was no evidence of invasive components in any of the re-excised specimens of the in situ cSCC cases (data not shown). Similarly, the values of the parameters evaluated in the invasive cSCC cases did not differ. Importantly, none of these 26 cases were used to evaluate the impact of surgical margins, except for the other parameters (as stated in the Results and Tables I-III) whenever they were adequately assessed. Representative tumor areas were marked by the pathologists on hematoxylin and eosin–stained slides to carry out manual microdissection.

Age at diagnosis was recorded and categorized into 2 groups according to the mean age (≤75 years vs >75 years) for statistical analyses. Topographic locations of the specimens were classified according to *International Statistical Classification of Diseases and Related Health Problems, Tenth Revision*[S3] (including lips, eyelid, ear, face, scalp/neck, trunk, upper limb, lower limb, and undetermined). The topographic locations were then grouped into 5 locations for statistical analyses, including the face (including the neck), trunk, upper limb, lower limb, and not specified. Moreover, topographic locations were subdivided into chronically sun-exposed (scalp/neck, face, ears, eyelids, and hands) and intermittently sun-exposed (trunk, upper limb, lower limbs, and feet) locations. Because 82.4% of the recurrent cases and 75.0% of the metastatic cases were from invasive cSCCs located on the face, topographic location was further divided into facial lesions and extrafacial lesions for statistical purposes. Specimens were classified as in situ cSCC or invasive cSCCs, with the latter category subdivided by histologic type into acantholytic, spindle cell, verrucous, pseudovascular, adenosquamous, and not otherwise specified. Histologic grade was classified as well differentiated, moderately differentiated, poorly differentiated, and undifferentiated. Pattern of invasion was classified as expansive or infiltrative. The tissue level of the tumors was classified as invading the papillary dermis, invading the reticular dermis, or invading the subcutaneous tissue and beyond. Maximum tumor thickness was measured in millimeters and categorized as being in 1 of 2 groups (<6 mm vs >6 mm), in which only invasive cSCCs were included. Peripheral and deep surgical margins were measured in millimeters. The presence of ulceration and actinic keratosis was identified. Actinic keratosis was identified in the adjacent normal epidermis. Intratumoral and peritumoral infiltrates were classified as moderate/intense and few/absent. The presence of lymphovascular and perineural invasion was annotated. The T stage of each tumor was classified according to the correlative tumor, node, and metastasis classification. As in previous studies,[S4,S5] recurrence was defined as the development of a histologically confirmed cSCC in the same topographic area in addition to being identified by the assisting dermatologist as

recurrence. Progression-free survival was defined as the time until diagnosis of recurrence and/or metastasis. Progression-free survival and overall follow-up times were recorded in months.

DNA extraction and mutation analysis

DNA from the formalin-fixed paraffin-embedded tissue samples was retrieved from 10-μm cuts after careful microdissection. A DNA extraction kit (Citogene, Citomed, Odivelas, Portugal) was used according to the manufacturer's instructions. The extracted DNA was quantified with a Nanodrop N-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and stored at −20°C. Polymerase chain reaction (PCR) was performed with a Qiagen Multiplex Kit (Qiagen, Hilden, Germany) using the recommended settings for Q solution DNA amplification. Genomic DNA (25-100 ng) was amplified by PCR under the following cycling conditions: 30 seconds at 95°C; 90 seconds at 62°C, and 20 seconds at 72°C for 40 cycles. The primers used can be found in a previous publication by our group.[S6] Direct sequencing reaction was performed with the BigDye Terminator Kit (Perkin-Elmer, Foster City, CA), and the fragments were run in an ABI prism 3100 Genetic Analyzer (Perkin-Elmer). The sequencing reaction was performed in a forward direction, and an independent PCR amplification/sequencing was performed in both a forward and reverse direction for positive and inconclusive (not confirmed as positive or negative) samples. Mutations were detected by using Mutation Surveyor DNA variant analysis software (Softgenetics, State College, PA) and matched with reference sequences from GenBank. Sequences evaluated corresponded to the loci in the TERTp ranging from −270 to −50 base pairs upstream of the TERT gene start codon that include the recurrent TERTp mutations described in other cancers.[S7,S8] The described *TERT*p mutations evaluated included the following: −124C>T, −146C>T, tandem −124/−125CC>TT and tandem −138/139CC>TT.

Statistical analysis

Statistical analysis was conducted with SPSS software (version 24.0, IBM Corp, Armonk, NY). The results were expressed as a percentage or mean plus or minus SD. Statistical analysis was performed on both the whole series of cSCCs and 2 different groups of cSCCs (in situ and invasive cSCCs). The chi-square test, Fisher exact test, and Student *t* test (unpaired, 2-tailed) were used when appropriate. The predictive value of *TERT*p mutations and other variables (age, sex, sun exposure, localization, presence of ulceration, presence of actinic keratosis, tumor size, histologic type, histologic grade, pattern of invasion, level of invasion, maximum tumor thickness, intratumoral infiltrate, peritumoral infiltrate, lymphovascular invasion, and perineural invasion) for recurrence, metastasis, and progression-free survival were assessed by using univariate and multivariate logistic regression models. Survival curves were plotted by the Kaplan-Meier method with the log-rank statistics. In the regression models, all the variables that were significantly associated with the specified outcome in the univariate model were included in the multivariate analysis. Confidence intervals were calculated with 95% coverage. Results were considered statistically significant at P less than .05. The significance level was adjusted by Bonferroni correction (with 0.05 divided by the number of performed comparisons for each dependent variable) when assessing *TERTp* mutations according to different clinicopathologic and molecular characteristics (results displayed in Supplemental Table I; available at http://www.jaad.org), as multiple comparisons were performed.

SUPPLEMENTAL REFERENCES

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STATISTICAL ANALYSIS PLAN

Telomerase reverse transcriptase gene (*TERT*) promoter (*TERTp*) mutations are associated with poor prognosis in cutaneous squamous cell carcinoma (cSCC).

Study objectives

Assess *TERTp* mutations, clinicopathologic features, and outcome in a series of cSCCs.

Primary objective

Assess *TERTp* mutations in a large series of cSCCs and correlate these mutations with clinicopathologic features and patients' outcome (recurrence or metastasis).

Study design

Retrospective study (genetic profiling of *TERTp* mutations in a retrospective series of cSCCs).

Study population

Consecutive cSCCs surgically removed at Centro Hospitalar Vila Nova de Gaia and Espinho between January 2004 and December 2013.

Definition of population for analysis

The study population included cSCCs that were surgically removed at Centro Hospitalar Vila Nova de Gaia and Espinho.

Statistical methodology

Statistical procedures. Continuous variables were summarized by mean and standard deviation. Categoric data were summarized as the number and percentage of subjects in each category.

The comparison of groups was performed by using parametric tests, such as *t* tests for continuous variables and the chi-square test (or Fisher exact test) for categoric variables or the equivalent nonparametric test, as appropriate.

A logistic regression model was used to find associations between clinicopathologic features, *TERTp* status, and adverse outcome, controlling for possible confounding variables. In the regression models, all the variables significantly associated with the specified outcome in the univariate model were included in the multivariate analysis.

Kaplan-Meier survival curves were presented to investigate the effect of *TERTp* mutation and other variables in progression-free survival.

All statistical tests were performed under a 2-sided significance level of 5%. The significance level was adjusted by Bonferroni correction (with 0.05 divided by the number of comparisons performed for each dependent variable) when assessing *TERTp* mutations according to different clinicopathologic and molecular characteristics (results displayed in Supplemental Table I; available at http://www.jaad.org), as multiple comparisons were performed.

Supplemental Table I. Clinicopathologic and molecular associations with *TERT*p mutations in cSCC

	All lesions				In situ cSCC				Invasive cSCC			
Clinicopathologic feature	**Total**	***TERT*p WT**	***TERT*p Mut**	***P* value**	**Total**	***TERT*p WT**	***TERT*p Mut**	***P* value**	**Total**	***TERT*p WT**	***TERT*p Mut**	***P* value**
No. of cases, n (%)	152	104 (68.4%)	48 (31.6%)		31	25 (80.6%)	6 (19.4%)		121	79 (65.3%)	42 (34.7%)	
Age at diagnosis, mean ± SD	76.9 ± 11.7	77.2 ± 11.4	75.9 ± 12.6	.544	78.5 ± 7.0	78.6 ± 7.4	76.2 ± 6.9	.476	76.5 ±12.7	76.8 ± 12.5	75.9 ± 13.3	.726
Male	73.9 ± 11.6	73.1 ± 11.7	75.1 ± 11.8	.451	77.8 ± 6.8	78.7 ± 6.6	76.0 ± 7.7	.490	73.0 ± 12.3	71.8 ± 12.3	74.9 ± 12.5	.314
Female	80.8 ± 10.7	81.8 ± 9.3	77.7 ± 14.5	.189	78.4 ± 7.8	78.5 ± 8.1	77.0	.863	81.6 ± 11.6	83.3 ± 9.5	77.7 ± 15.0	.124
Sex, n (%)				.119				NA				.435
Male	87 (57.2)	55 (52.9)	32 (66.7)		15 (48.4)	10 (40.0)	5 (83.3)		72 (59.5)	45 (57.0)	27 (64.3)	
Female	65 (42.8)	49 (47.1)	16 (33.3)		16 (51.6)	15 (60.0)	1 (16.7)		49 (40.5)	34 (43.0)	15 (35.7)	
Sun exposure, n (%)				NA				NA				NA
Chronic	103 (67.8)	67 (64.4)	36 (75.0)		11 (35.5)	9 (36.0)	2 (33.3)		92 (76.0)	58 (73.4)	34 (81.0)	
Intermittent	43 (28.3)	32 (30.8)	11 (22.9)		18 (58.1)	14 (56.0)	4 (66.7)		25 (20.7)	18 (22.8)	7 (16.7)	
Undetermined	6 (3.9)	5 (4.8)	1 (2.1)		2 (6.5)	2 (8.0)	0		4 (3.3)	3 (3.8)	1 (2.4)	
Localization, n (%)				NA				NA				NA
Face	97 (63.8)	64 (61.5)	33 (68.8)		9 (29.0)	7 (28.0)	2 (33.3)		88 (72.7)	57 (72.2)	31 (73.8)	
Trunk	8 (5.3)	3 (2.9)	5 (10.4)		4 (12.9)	1 (4.0)	3 (50.0)		4 (3.3)	2 (2.5)	2 (4.8)	
Upper limb	21 (13.8)	14 (13.5)	7 (14.6)		6 (19.4)	6 (24.0)	0		15 (12.4)	8 (10.1)	7 (16.7)	
Lower limb	20 (13.2)	18 (17.3)	2 (4.2)		10 (32.3)	9 (36.0)	1 (16.7)		10 (8.3)	9 (11.4)	1 (2.4)	
Undetermined	6 (3.9)	5 (4.8)	1 (2.1)		2 (6.5)	2 (8.0)	0		4 (3.3)	3 (3.8)	1 (2.4)	
Localization, n (%)*				.506				NA				.942
Extrafacial	49 (33.6)	35 (35.4)	14 (29.8)		20 (69.0)	16 (69.6)	4 (66.7)		29 (24.8)	19 (25.0)	10 (24.4)	
Face	97 (66.4)	64 (64.6)	33 (70.2)		9 (31.0)	7 (30.4)	2 (33.3)		88 (75.2)	57 (75.0)	31 (75.6)	
Procedure, n (%)				.252				NA				.092
Biopsy	34 (22.4)	26 (25.0)	8 (16.7)		6 (19.4)	4 (16.0)	2 (33.3)		28 (23.1)	22 (27.8)	6 (14.3)	
Excision	118 (77.6)	78 (75.0)	40 (83.3)		25 (80.6)	21 (84.0)	4 (66.7)		93 (76.9)	57 (72.2)	36 (85.7)	
Maximum tumor size, cm, mean ± SD	1.9 ± 1.5	1.7 ± 1.2	2.2 ± 1.8	.149	1.9 ± 1.2	2.0 ± 1.2	1.8 ± 1.5	.765	1.9 ± 1.5	1.6 ± 1.2	2.3 ± 1.8	.068
Maximum tumor size, n (%)				.489				NA				.384
<2 cm	69 (45.4)	47 (66.2)	22 (59.5)		10 (32.3)	8 (53.3)	2 (50.0)		59 (48.8)	39 (69.6)	20 (60.6)	
≥2 cm	39 (25.7)	24 (33.8)	15 (40.5)		9 (29.0)	7 (46.7)	2 (50.0)		30 (24.8)	17 (30.4)	13 (39.4)	
Cannot be assessed	44 (28.9)				12 (38.7)				32 (26.4)			
Peripheral margins, mm, mean ± SD	2.3 ± 2.9	2.0 ± 2.5	2.9 ± 3.4	.125	1.6 ± 1.5	1.6 ± 1.5	1.5 ± 1.3	.900	2.5 ± 3.1	2.2 ± 2.8	3.0 ± 3.6	.191
Deep margins, mm, mean ± SD	2.6 ± 2.4	2.5 ± 2.3	2.7 ± 2.5	.627	3.0 ± 1.7	2.6 ± 1.5	4.8 ± 1.0	.017	2.5 ± 2.5	2.5 ± 2.5	2.5 ± 2.5	.963
Ulceration, n (%)				.173				NA				.389
No	53 (34.9)	32 (33.0)	21 (44.7)		14 (45.2)	9 (39.1)	5 (83.3)		39 (32.2)	23 (31.1)	16 (39.0)	
Yes	91 (59.9)	65 (67.0)	26 (55.3)		15 (48.4)	14 (60.9)	1 (16.7)		76 (62.8)	51 (68.9)	25 (61.0)	
Undetermined	8 (5.3)				2 (6.5)				6 (5.0)			

Continued

Supplemental Table I. Cont'd

	All lesions				In situ cSCC				Invasive cSCC			
Clinicopathologic feature	**Total**	***TERT*p WT**	***TERT*p Mut**	***P* value**	**Total**	***TERT*p WT**	***TERT*p Mut**	***P* value**	**Total**	***TERT*p WT**	***TERT*p Mut**	***P* value**
Actinic keratosis, n (%)				.750				NA				.553
No	50 (32.9)	35 (36.1)	15 (33.3)		6 (19.4)	5 (20.0)	1 (16.7)		44 (36.4)	30 (41.7)	14 (35.9)	
Yes	92 (60.5)	62 (63.9)	30 (66.7)		25 (80.6)	20 (80.0)	5 (83.3)		67 (55.4)	42 (58.3)	25 (64.1)	
Undetermined	10 (6.6)								10 (8.3)			
Invasion, n (%)				.101								
Noninvasive	31 (20.4)	25 (24.0)	6 (12.5)									
Invasive	121 (79.6)	79 (76.0)	42 (87.5)									
Histologic type, n (%)												NA
Acantholytic									9 (7.4)	6 (7.6)	3 (7.1)	
Verrucous									2 (1.7)	2 (2.5)	0	
NOS									110 (90.9)	71 (89.9)	39 (92.9)	
Histologic grade, n (%)												.275
Well differentiated									43 (35.5)	31 (40.3)	12 (30.0)	
Moderately/poorly differentiated									74 (61.2)	46 (59.7)	28 (70.0)	
Cannot be assessed									4 (3.3)			
Histologic grade, n (%)												NA
Well/moderately differentiated									106 (87.6)	70 (90.9)	36 (90.0)	
Poorly differentiated									11 (9.1)	7 (9.1)	4 (10.0)	
Cannot be assessed									4 (3.3)			
Pattern of invasion, n (%)												.960
Expansive									66 (54.5)	43 (56.6)	23 (56.1)	
Infiltrative									51 (42.1)	33 (43.4)	18 (43.9)	
Cannot be assessed									4 (3.3)			
Level of invasion, n (%)												.667
Papillary dermis									36 (29.8)	23 (30.7)	13 (33.3)	
Reticular dermis									53 (43.8)	37 (49.3)	16 (41.0)	
Subcutaneous tissue									25 (20.7)	15 (20.0)	10 (25.6)	
Cannot be assessed									7 (5.8)			
Level of invasion, n (%)												.490
Dermis									89 (73.6)	60 (80.0)	29 (74.4)	
Subcutaneous tissue									25 (20.7)	15 (20.0)	10 (25.6)	
Cannot be assessed									7 (5.8)			
Maximum tumor thickness, n (%)									3.8 ± 3.1	3.4 ± 2.2	4.8 ± 4.1	.050
<6 mm									96 (79.3)	67 (88.2)	29 (74.4)	.059
≥6 mm									19 (15.7)	9 (11.8)	10 (25.6)	
Cannot be assessed									6 (5.0)			

Continued

Supplemental Table I. Cont'd

	All lesions				In situ cSCC				Invasive cSCC			
Clinicopathologic feature	**Total**	***TERT*p WT**	***TERT*p Mut**	***P* value**	**Total**	***TERT*p WT**	***TERT*p Mut**	***P* value**	**Total**	***TERT*p WT**	***TERT*p Mut**	***P* value**
Intratumoral infiltrate, n (%)												NA
Moderate-intense									13 (10.7)	8 (10.1)	5 (11.9)	
Few-absent									108 (89.3)	71 (89.9)	37 (88.1)	
Peritumoral infiltrate, n (%)												.816
Moderate-intense									68 (56.2)	45 (57.0)	23 (54.8)	
Few-absent									53 (43.8)	34 (43.0)	19 (45.2)	
Lymphovascular invasion, n (%)												NA
Not present									116 (95.9)	75 (94.9)	41 (97.6)	
Present									5 (4.1)	4 (5.1)	1 (2.4)	
Perineural invasion, n (%)												NA
Not present									118 (97.5)	78 (98.7)	40 (95.2)	
Present									3 (2.5)	1 (1.3)	2 (4.8)	
Recurrence, n (%)								NA				**<.001**
No					28 (90.3)	22 (88.0)	6 (100.0)		104 (86.0)	75 (94.9)	29 (69.0)	
Yes					3 (9.7)	3 (12.0)	0		17 (14.0)	4 (5.1)	13 (31.0)	
Metastasis, n (%)												NA
No					31 (100)				113 (93.4)	78 (98.7)	35 (83.3)	
Yes					0				8 (6.6)	1 (1.3)	7 (16.7)	
Progression-free survival, mo, mean ± SD	39.8 ± 29.3	38.3 ± 25.8	43.0 ± 35.8	.418	38.4 ± 22.2	34.7 ± 19.8	53.8 ± 26.7	.057	40.1 ± 30.9	39.4 ± 27.4	41.4 ± 36.9	.736
Follow-up, mo, mean ± SD	42.9 ± 28.8	39.7 ± 25.5	49.8 ± 34.1	.071	39.7 ± 22.0	36.3 ± 19.9	53.8 ± 26.7	.080	43.7 ± 30.3	40.8 ± 27.0	49.3 ± 35.3	.144

Values in bold are statistically significant with the following *P* value cutoffs after Bonferroni correction: *P* less than .003 for all lesions and in situ cSCC and *P* less thasn .002 for invasive cSCC. An unpaired Student *t* test was used to compare means in continuous variables; chi-square and Fisher exact tests were applied to evaluate a possible association between categoric variables. *cSCC*, Cutaneous squamous cell carcinoma; *Mut*, mutated; *NA*, not applicable (the chi-square or Fisher exact test is not applicable because more than 20% of the expected counts are less than 5); *SD*, standard deviation; *TERTp*, telomerase reverse transcriptase gene promoter; *WT*, wild type.
*Cases of undetermined location were excluded (n = 6).

Paper IV

Prognostic significance of *RAS* mutations and P53 expression in Cutaneous Squamous Cell Carcinomas

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Prognostic Significance of RAS Mutations and P53 Expression in Cutaneous Squamous Cell Carcinomas

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Abstract: *TP53* is considered the most commonly-altered gene in cutaneous squamous cell carcinoma (cSCC). Conversely, *RAS* mutations have been reported in a low percentage of cSCC. The objective of our study was to evaluate the frequency of p53 expression and *RAS* mutations in cSCC and correlate them with clinicopathological features and patient outcome. We performed immunohistochemistry for p53 and genetic profiling for *RAS* mutations in a retrospective series of cSCC. The predictive value of p53 expression, *RAS* mutations, and clinicopathological parameters was assessed using logistic regression models. The overall frequency of *RAS* mutations was 9.3% (15/162), and 82.1% of the cases (133/162) had p53 overexpression. *RAS* mutations rate was 3.2% (1/31) of in situ cSCCs and 10.7% (14/131) of invasive cSCCs. *RAS* mutations were more frequently associated with an infiltrative than an expansive pattern of invasion ($p = 0.046$). p53 overexpression was a predictor of recurrence in the univariate analysis. Our results indicate that *RAS* mutations associate with features of local aggressiveness. Larger studies with more recurrent and metastatic cSCCs are necessary to further address the prognostic significance of p53 overexpression in patients' risk stratification.

Keywords: *RAS*; p53; mutation; expression; prognostic biomarker; prognosis; biomarker; cutaneous squamous cell carcinoma; recurrence; metastases; outcome

1. Introduction

Cutaneous squamous cell carcinoma (cSCC) is the second-most-common skin cancer in Caucasians and cumulative ultraviolet radiation is considered the major ethiopathogenic factor [1,2]. cSCC carcinogenesis includes premalignant lesions (actinic keratosis (AK) and in situ squamous carcinoma/Bowen's disease), invasive, and metastatic cSCCs, although a multistep model is not always detected [3]. Some studies state that 65% of cSCCs arise from AK [3]. cSCCs most frequently occur in chronically sun-exposed areas such as the face (particularly the lip, ear, nose, cheek, and eyelid)

and the dorsum of the hands. In order to assist prognostics, cSCCs are classified based on their histological subtype (e.g., acantholytic, spindle, verrucous, and desmoplastic), grade of differentiation (well differentiated, moderately differentiated, poorly differentiated, or undifferentiated), tumor depth (maximum vertical thickness), level of dermal invasion (Clark's level) and the presence/absence of perineural, lymphatic, or vascular invasion [4]. Even though not optimal for cSCCs, to date staging is based on the TNM (Tumor, Node, Metastasis) system of the 2010 American Joint Committee on Cancer (AJCC) guidelines [5] and for head and neck cutaneous squamous cell carcinoma the recent 8th edition of the AJCC [6]. cSCCs can recur (3–5%) and metastasize (4–5%) [7]. Patients with localized cSCCs usually have an excellent outcome but for metastatic cSCCs a poorer prognosis is observed with survival rates of 25–35% (five-year survival rate) and less than 10% (ten-year survival rate) [8–10]. Only clinicopathological prognostic markers have been reported in cSCC for recurrence (tumor thickness > 2 mm and >6 mm, invasion beyond subcutaneous fat, perineural invasion, tumor size > 2 cm, and poor differentiation and location in the temple) and metastasis (tumor thickness > 2 mm and >6 mm, invasion beyond subcutaneous fat, perineural invasion, tumor size > 2 cm, poor differentiation, immunosuppression, and location in the temple, lip, and ear) [11]. We recently reported the association of *TERT* promoter mutations with worse prognosis (recurrence and metastasis) but we admit that its putative prognostic significance still needs to be established in larger series [12].

The *TP53* gene encodes a nuclear transcription factor that is usually involved in the negative regulation of the cell cycle and in promoting apoptosis and is frequently impaired during tumor progression [13–15]; it has been considered the most-commonly-mutated gene in squamous cell carcinoma. Immunohistochemical expression of p53 has for a long time been a matter of debate in cSCC [16]. p53 overexpression varies greatly among different studies (15–92%) [17] and was associated with either wild type or mutated cases [18,19]. It is suggested that the immunopositivity of p53 is not a surrogate marker of *TP53* mutation [18,19] and that p53 overexpression seems to be precocious in chronic sun-exposed skin, sometimes preceding genomic instability [17,20]. Several mechanisms were suggested to regulate p53 expression. MDM2 has been hypothesized as a strong modulator of p53 ubiquitination and its modulation could result in increased p53 expression [21,22]. Another potential mechanism for p53 overexpression is aberrant p53 protein accumulation due to tetrameric proteins formed by wild type and mutant p53 proteins, the well-known dominant negative effect [23]. The prognostic impact of p53 overexpression in cSCC demands further clarification.

RAS is a small GTPase that activates the mitogen-activated protein kinases (MAPKs) and other signaling pathways involved in cell survival, proliferation, and apoptosis. *RAS* mutations were reported in a low percentage of cSCCs (<13%) [24–27]. A significant subset of patients treated with either the multikinase inhibitor sorafenib or the *BRAF* V600E inhibitors, vemurafenib and dabrafenib, rapidly develop cSCCs harboring *H-RAS* mutations [28]. This evidence points to the fact that these inhibitors give rise to paradoxical activation of the MAPK pathway, which in turn cooperates with mutations in other key oncogenes and tumor suppressors such as *H-RAS* and *TP53* [29]. These recent data have drawn attention to the role of *RAS* mutations in cSCC carcinogenesis.

In vivo studies revealed that a germline *TP53* mutation and activated *H-RAS* act synergistically to enhance tumor progression [30]. Taking into account the possible interplay between *TP53* and *RAS*, we assessed p53 overexpression, *H-RAS*, and *K-RAS* mutations in a large series of cSCCs and correlated these alterations with clinicopathological features and patients´ outcome.

2. Materials and Methods

2.1. Patient Selection, Sample Selection, and Clinicopathological Characterization

All the procedures reported in this study were in accordance with national and institutional ethical standards and were approved by the Local Ethical Review Committees of the Centro Hospitalar Vila Nova de Gaia e Espinho (CHVNGE) (ethical permit number 182-2014-3 with the title "Carcinogénese

do carcinoma espinocelular da pele" attributed to M.A.C.). According to Portuguese law, informed consent is not required for retrospective studies.

The descriptive and statistical analysis refers to all the consecutive cSCCs surgically removed at CHVNGE within the time period between January 2004 and December 2013. For the inclusion criteria, we selected immunocompetent patients with a histological diagnosis of cSCC and with available follow-up data. Exclusion criteria were applied to patients with genetic diseases that conferred increased risk of cSCC, such as xeroderma pigmentosum, epidermodysplasia verruciformis, and albinism. None of the cases of this retrospective series was subjected to Mohs micrographic surgery. Cases with available formalin-fixed paraffin-embedded tissues (FFPE) were retrieved from the Pathology Department of CHVNGE. One hundred and eighty-four histological specimens were reclassified by pathologists experienced in cutaneous neoplasms (J.M.L., J.P., and M.F.). Tumors were evaluated based on the protocols from the College of American Pathologists (CAP) and the American Joint Committee on Cancer (AJCC) guidelines [31,32]. The evaluated CAP criteria were tumor site and size, histological type and grade, thickness, status of surgical (superficial and deep) margins of the excised cSCC, lymph-vascular and perineural invasion, lymph node status, and pathological staging (pTNM). We included further criteria, such as the pattern of infiltration (expansive or infiltrative), presence of ulceration, peritumoral and intratumoral lymphocytic infiltrate, and the presence of AK in adjacent skin. Transected tumor biopsies of 32 cases (4 in situ cSCCs and 28 invasive cSCCs) were genetically and immunohistochemically profiled to evaluate primary tumors without alterations (e.g., fibrosis) derived from subsequent complete re-excision of previously-biopsied tumors. No evidence of invasive components was detected in any of the re-excised specimens of the in situ cSCCs. Similarly, the values of the parameters evaluated in the invasive cSCC cases did not differ (data not shown). None of the 32 cases was used to evaluate the impact of surgical margins, except for the other parameters (as stated in the Results and Tables), whenever they were adequately assessed. Representative areas were selected by the pathologists in hematoxylin and eosin slides to continue with microdissection.

Age at diagnosis was registered and stratified into two groups according to the mean age (<80 years vs. ≥80 years) for statistical analyses. Topographic locations of the tumors were classified according to the International Statistical Classification of Diseases and Related Health Problems 10th Revision (including lips, eyelid, ear, face, scalp/neck, trunk, upper limb, lower limb, and not specified) [33]. The topographic locations were stratified into five locations, namely, the face (including the neck), trunk, upper limb, lower limb, and not specified. Topographic locations were additionally subdivided into chronically sun-exposed (scalp/neck, face, ears, eyelids, and hands) and intermittently sun-exposed (trunk, upper limb, lower limbs, and feet) locations. The tumors were divided into in situ and invasive cSCCs; the latter were subdivided by histologic type (acantholytic, spindle cell, verrucous, pseudo vascular, adenosquamous, and not otherwise specified (NOS)). The histological grade was classified in well-, moderate-, or poorly-differentiated and undifferentiated. Pattern of invasion was divided in expansive or infiltrative. The tissue level of the tumor invasion was classified as invading the papillary dermis, the reticular dermis, the subcutaneous tissue, or beyond. The maximum tumor thickness of invasive cSCCs was split into two groups <6 and ≥6 mm; distance to the nearest superficial and deep surgical margins were also measured in mm. The presence of ulceration and actinic keratosis was annotated; actinic keratosis was evaluated in the adjacent skin. Intra- and peritumoral lymphocytic infiltrate was classified as moderate–intense or few–absent. The presence of lymphovascular and perineural invasion was annotated. "T" of each tumor was classified according to the TNM classification. Recurrence was defined as the development of a histologically-confirmed cSCCs in the same topographic area in addition to being identified by the assisting dermatologist as recurrence, as in previous studies [7,34]. Progression-free survival (PFS) was defined as the time until diagnosis of recurrence and/or metastasis; PFS and overall follow-up are presented in months.

2.2. DNA Extraction and Mutation Analysis

DNA was extracted from 10-µm cuts of FFPE tissues followed by microdissection. The DNA extraction kit (Citogene®, Citomed, Portugal) was used in accordance with manufacturer's protocol. PCRs were performed with PromegaGoTaq® G2 Flexi DNA polymerase (Promega, Southampton, UK) and with the recommended settings. Sanger sequencing was achieved using the BigDye Terminator Kit (Perkin-Elmer, CA, USA) and with the fragments running in an ABI (Applied Biosystems) prism 3100 Genetic Analyzer (Perkin-Elmer, CA, USA). Independent PCR amplification and sequencing were performed for both positive and inconclusive (not confirmed as positive or negative) samples. Sequencing analysis targeted exons 1 and 2 of *H-RAS* and *K-RAS*. The sequencing reactions were performed in a sense direction for exon 1 and in anti-sense direction for exon 2. Mutations were evaluated and classified using the Mutation Surveyor DNA variant analysis software (Softgenetics, PA, USA) and were matched to reference control sequences from GenBank.

2.3. Immunohistochemistry Protocol and Analysis

Sections, 4-µm in thickness, from the FFPE blocks, were used for immunohistochemical studies. Specimen tissues were deparaffinized and rehydrated. The antigen retrieval was performed on a steamer for 45 min using citrate buffer pH 6.0 from Thermo Scientific (TA-050-CBX). Endogenous peroxidase was blocked, and a non-specific binding blockage from Thermo Scientific (TA-125-PQB) was also used. Sections were incubated with the primary antibody, anti-p53 antibody (1:700) from Leica (NCL-L-p53-DO7) using a diluent from Thermo Scientific (TA-125-ADQ), during 60 min at room temperature. Then, a biotinylated goat polyvalent secondary antibody was used from Thermo Scientific (TP-125-BN). Finally, the chromogenic detection was performed with 3,3'-diaminobenzidine (Dako, K3468) reaction and counterstained with Mayer's hematoxylin. Negative controls underwent a similar procedure, with the exclusion of the primary antibody.

Each slide underwent digital acquisition using a D-Sight Fluo 2.0 digital microscope (A. Menarini Diagnostics, Florence, Italy). Tumor area to be analyzed was manually selected on the digital slide by one researcher (M.A.C.) until at least 2000 tumor cells were included. Whenever the whole tumor specimen could not be selected, the invasive front of the tumor was preferentially included. The same digital slide was scanned with a validated automated scanning system (VISIA Imaging s.r.l. software version 2.5.0.1, Italy) [35]. The software automatically established the overall percentage of positive cells and the percentage of cells that disclosed absent staining (0), weak staining (1+), moderate staining (2+), and strong staining (+3). The software additionally calculated an "h-index score" (sum of the percentage of positive cells * staining intensity). The h-index score (h-score) is a continuous variable that represents the mean score of positivity and intensity. p53 expression was considered as normal (wild type) when few/scattered dispersed cells disclosed immunostaining, and abnormal when overexpression was present (see Figure 1). p53 overexpression was quantified using an h-score that had been previously reported as a valid approach for routine immunohistochemical quantification [36,37]. H-score was defined by the software as 1+ (h-score < 100), 2+ (h-score 100–200), and 3+ (h-score 200–300). The expression in non-tumor cells (e.g., epidermis, follicular, and adnexal glandular epithelium, and mesenchymal cells) present in each case was used as an internal control for wild type p53 expression. A certified dermatopathologist (J.M.L.) performed an internal validation of our series without prior consultation or recourse to clinical, or previously-annotated p53 h-score data, as reported in other studies [38], and confirmed an agreement between this new method and routine standard immunohistochemistry analysis (data not shown).

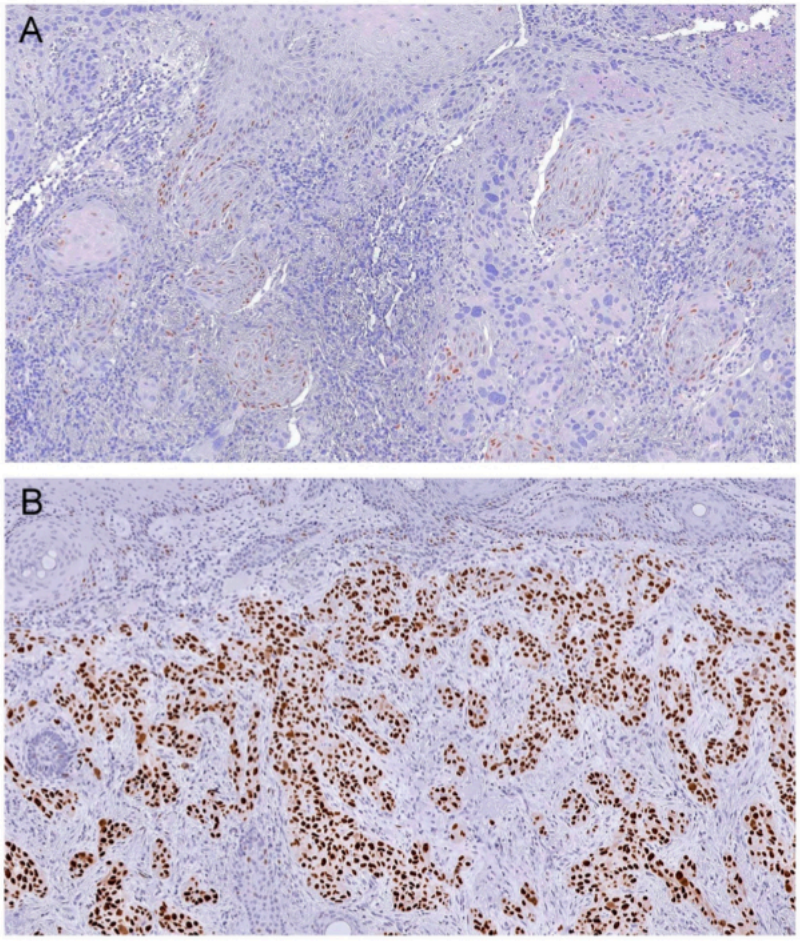

Figure 1. Representative images of immunoexpression of p53 in cutaneous squamous cell carcinomas (cSCCs) for (**A**) wild type expression and (**B**) overexpression. Original magnification 100×.

2.4. Statistical Analysis

Continuous variables were described by the mean and standard deviation (SD); the categorical variables were described by the absolute frequency and the relative frequency. Differences in proportions were tested with the chi-square test or the Fisher's exact test, as appropriate. Differences between two independent samples were assessed with the t test for continuous variables. Analysis of variance (ANOVA) was used to compare continuous variables between independent samples. The predictive value of *RAS* mutation, p53 expression, and other parameters for recurrence, metastasis, and PFS was assessed using univariate and multivariate logistic regression models. In the logistic regression models, all the parameters that were significantly associated with the outcome in the univariate model were included in the multivariate analysis. The odds ratio (OR) and respective 95% confidence intervals

(CI) were estimated in the regression models. The results were considered statistically significant at $p < 0.05$. The statistical analysis was conducted with software Statistical Package for the Social Sciences (SPSS) version 24.0 (SPSS Inc., Chicago, IL, USA).

3. Results

Of the 184 histologically-characterized cases, *RAS* and p53 status was not determined in 22 cases due to the small size and/or low quality of the tumor samples. For the remaining 162 lesions, excised from 128 patients, we analyzed *RAS* mutations and p53 expression, Table 1. Of these, 31 were in situ cSCCs and 131 invasive cSCCs, Table 1. The overall frequency of *RAS* mutations was 9.3% (15 out 162 cases) and the mean p53 overexpression was 1+ h-score (91.6 ± 5.9), Table 1. p53 overexpression was observed in 82.1% of the cases (74.2% of the in situ and 84.0% of the invasive cSCCs), Table 1. None of the cases had null-type expression, Table 1. The mean p53 h-score of in situ cSCCs was 109.6 ± 16.3 and 87.9 ± 6.2 in invasive cSCCs. 146 out of 162 cases (90.1%) had available *TERTp* mutation status previously published by our group and were included when appropriate [12], Table 1.

Table 1. Clinicopathological features, frequency of *RAS* mutations, and p53 expression.

		All Tumors	In Situ cSCCs	Invasive cSCCs
Clinical and molecular features	**Number of cases**	162	31	131
	Age at diagnosis (mean (±SD))	77.6 ± 12.2	79.5 ± 7.4	77.1 ± 13.0
	Male	74.9 ± 12.2	78.8 ± 6.3	74.1 ± 12.9
	Female	81.6 ± 11.0	80.3 ± 8.6	82.0 ± 11.7
	Gender (*n* (%))			
	Male	97 (59.9)	16 (51.6)	81 (61.8)
	Female	65 (40.1)	15 (48.4)	50 (38.2)
	Sun exposure (*n* (%))			
	Chronic	110 (67.9)	11 (35.5)	99 (75.6)
	Intermittent	49 (30.2)	19 (61.3)	30 (22.9)
	Undetermined	3 (1.9)	1 (3.2)	2 (1.5)
	Location (*n* (%))			
	Face	108 (66.7)	10 (32.3)	98 (74.8)
	Trunk	9 (5.6)	5 (16.1)	4 (3.1)
	Upper limb	20 (12.3)	4 (12.9)	16 (12.2)
	Lower limb	22 (13.6)	11 (35.5)	11 (8.4)
	Not specified	3 (1.9)	1 (3.2)	2 (1.5)
	Follow-up (months)	41.6 ± 28.9	38.9 ± 21.5	42.2 ± 30.3
	Progression-free survival (months)	38.7 ± 29.2	37.6 ± 21.7	38.9 ± 30.7
	Recurrence			
	No	142 (87.7)	28 (90.3)	114 (87.0)
	Yes	20 (12.3)	3 (9.7)	17 (13.0)
	Metastases			
	No	154 (95.1)	31 (100)	123 (93.9)
	Yes	8 (4.9)	0	8 (6.1)
	***p53* immunohistochemistry**			
	Cells counted	4224,4 ± 2223.7	3041,1 ± 1066.0	4497,5 ± 2332.1
	Mean h-score	91.6 ± 5.9	109.6 ± 16.3	87.9 ± 6.2
	Wild type	29 (17.9)	8 (25.8)	21 (16.0)
	Overexpression (h-score 1+)	78 (48.1)	12 (38.7)	66 (50.4)
	Overexpression (h-score 2+)	45 (27.8)	8 (25.2)	37 (28.2)
	Overexpression (h-score 3+)	10 (6.2)	3 (9.7)	7 (5.3)
	***RAS* mutations**			
	Wild type	147 (90.7)	30 (96.8)	117 (89.3)
	Mutation	15 (9.3)	1 (3.2)	14 (10.7)
	***HRAS* mutations**			
	Wild type	149 (92.0)	30 (96.8)	119 (90.8)
	Mutation	13 (8.0)	1 (3.2)	12 (9.2)
	***KRAS* mutations**			
	Wild type	160 (98.8)	31 (100)	129 (98.5)
	Mutation	2 (1.2)	0 (0)	2 (1.5)

Table 1. *Cont.*

		All Tumors	In Situ cSCCs	Invasive cSCCs
	***TERTp* mutations**			
	Wild type	98 (60.5)	21 (67.7)	77 (58.8)
	Mutation	48 (29.6)	6 (19.4)	42 (32.1)
	Maximum tumor size			
	<2 cm	75 (46.3)	12 (38.7)	63 (48.1)
	≥2 cm	39 (24.1)	9 (29.0)	30 (22.9)
	Not assessed	48 (29.6)	10 (32.3)	38 (29.0)
	Superficial margins (mm)	2.1 ± 2.8	1.7 ± 1.8	2.2 ± 2.9
	Deep margins (mm)	2.4 ±2.3	3.3 ± 1.7	2.2 ± 2.4
	Ulceration			
	No	53 (32.7)	11 (35.5)	42 (32.1)
	Yes	101 (62.3)	19 (61.3)	82 (62.6)
	Undetermined	8 (4.9)	1 (3.2)	7 (5.3)
	Actinic Keratosis			
	No	56 (34.6)	6 (19.4)	50 (38.2)
	Yes	96 (59.3)	25 (80.6)	71 (54.2)
	Undetermined	10 (6.6)		10 (7.6)
	Invasion			
	Non-invasive	31 (19.1)		
	Invasive	131 (80.9)		
	Histologic type			
	Acantholytic			10 (7.6)
	Spindle cell			1 (0.8)
	Verrucous			2 (1.5)
	Bowenoid			1 (0.8)
	Not otherwise specified (NOS)			117 (89.3)
Maximum tumor thickness	**Histological grade**			
	Well differentiated			46 (35.1)
	Moderately differentiated			68 (51.9)
	Poorly differentiated			13 (9.9)
	Not assessed			4 (3.1)
	Pattern of invasion			
	Expansive			70 (53.4)
	Infiltrative			57 (43.5)
	Not assessed			4 (3.1)
	Level of invasion			
	Papillary dermis			39 (29.8)
	Reticular dermis			60 (45.8)
	Subcutaneous tissue			26 (19.8)
	Not assessed			6 (4.6)
	Maximum tumor thickness			3.8 ± 3.0
	Maximum tumor thickness			
	< 6 mm			103 (78.6)
	≥ 6 mm			22 (16.8)
	Not assessed			6 (4.6)
	Intratumoral infiltrate			
	Moderate–intense			13 (9.9)
	Few–absent			118 (90.1)
	Peritumoral infiltrate			
	Moderate–intense			74 (56.5)
	Few–absent			57 (43.5)
	Lymphovascular invasion			
	Not present			126 (96.2)
	Present			5 (3.8)
	Perineural invasion			
	Not present			128 (97.7)
	Present			3 (2.3)

3.1. Relationship between RAS Mutations, P53 Expression, and Clinicopathological Features

Table 1 presents the clinicopathological features, the frequency of *RAS* mutations, and p53 expression in the series. Clinicopathological factors and their association with *RAS* mutations and p53 expression are presented in Supplementary Tables S1–S3.

We analyzed all cSCCs and observed that *RAS* mutations were present in lesions on the face, trunk, and upper and lower limbs. *RAS* mutations were more frequent in male than in female patients but without statistical significance. The *RAS* mutations did not associate with any clinicopathological feature of in situ cSCCs.

p53 overexpression was higher in women and on locations intermittently sun-exposed, but without statistical significance. p53 h-score was significantly higher in in situ cSCCs located on the lower limbs compared to other locations (161.8 ± 78.7 vs. 69.4 ± 49.9, respectively; $p = 0.002$). The three in situ cSCCs that recurred were wild type for *RAS* mutations and had p53 overexpression.

The *RAS* mutations were more frequently associated with an infiltrative than an expansive pattern (10 out of 57 (17.5%) vs. 4 out of 70 (5.7%), respectively; $p = 0.046$) in invasive cSCCs. Despite not reaching statistical significance, tumors with *RAS* mutations had more lymphovascular invasion than tumors with wild type *RAS* (2 out of 5 (40.0%) vs. 12 out of 126 (9.5%), respectively; $p = 0.088$). The *RAS* mutations were not associated with recurrence and metastasis of cSCC. p53 h-score was higher in recurrent than in non-recurrent tumors (118.8 ± 78.0 vs. 82.6 ± 61.5, respectively; $p = 0.039$).

RAS mutations and p53 overexpression were not associated with *TERTp* mutations in this series of cSCCs (see Supplementary Tables S1–S3).

3.2. Relationship between RAS Mutation, P53 Expression, and Outcome

For this analysis, we only included invasive cSCCs ($n = 131$). The mean follow-up time (± SD) of the patients was 42.2 ± 30.3 months (range 6–156 months).

Seventeen cases (13.0%) and 8 cases (6.1%) presented recurrence and/or metastasis, respectively (all were lymph node metastasis), during follow-up of patients. Supplementary Table S4 presents the main features of the cases with adverse outcomes.

A regression model was performed for parameters associated with an adverse outcome (recurrence or metastases) in invasive cSCCs (Table 2). When analyzing the parameters associated with the risk of recurrence, age > 80 years (OR 16.00; $p = 0.008$), presence of ulceration (OR 2.92; $p = 0.049$), and p53 overexpression (OR 1.01; $p = 0.045$) were identified as predictors in the univariate analysis. When the aforementioned parameters were included in the multivariate analysis, only age > 80 years (OR 12.17; $p = 0.019$) was identified as an independent predictor of recurrence.

When analyzing predictors of metastasis, the univariate analysis revealed that invasion of the subcutaneous tissue (OR 5.82; $p = 0.028$), distance to the nearest superficial margin (OR 1.18; $p = 0.026$), maximum tumor thickness (OR 1.25; $p = 0.011$), and few or absent peritumoral lymphocytes (OR 10.22; $p = 0.032$) were associated with a higher likelihood of metastasis. In the multivariate analysis, none of the aforementioned parameters was associated with a higher risk of metastasis of cSCCs.

Table 2. Predictive factors for recurrence and lymph node metastasis.

	Recurrence				Metastasis			
	Univariate Analysis		Multivariate Analysis		Univariate Analysis		Multivariate Analysis	
	Odds Ratio (OR) (95% CI)	*p*	OR (95% CI)	*p*	OR (95% CI)	*p*	OR (95% CI)	*p*
Mean age (years)								
< 80	1	**0.008**	1	**0.019**	NA	NA		
≥ 80	16.00 (2.05–124.71)		12.17 (1.51–97.82)					
Ulceration								
No	1	**0.049**	1	0.261	1	0.196		
Yes	2.92 (1.00–8.51)		1.97 (0.60–6.41)		2.77 (0.59–13.01)			
Level of invasion								
Dermis	1	0.829			1	**0.028**	1	0.315
Subcutaneous tissue	0.86 (0.23–3.29)				5.82 (1.21–27.89)		2.68 (0.39–18.27)	
RAS								
Wild type	1	0.878			1	0.864		
Mutation	1.13 (0.23–5.57)				1.21 (0.14–10.62)			
***p53* overexpression ***	1.01 (1.00–1.02)	**0.045**	1.01 (1.00–1.02)	0.145	1.01 (1.00–1.02)	0.281		
Superficial margins *	1.14 (1.00–1.31)	0.059			1.18 (1.02–1.37)	**0.026**	1.03 (0.85–1.25)	0.753
Max. tumor thickness *	1.09 (0.94–1.27)	0.238			1.25 (1.05–1.40)	**0.011**	1.17 (0.90–1.51)	0.247
Peritumoral infiltrate								
Moderate–intense	1	0.178			1	**0.032**	1	0.069
Few–absent	2.04 (0.72–5.74)				10.22 (1.22–85.65)		8.00 (0.85–75.30)	

RAS mutations and parameters with significant results in the univariate analysis in one of the adverse outcomes (recurrence or metastasis). All other clinicopathological features were not associated with outcome in the univariate analysis. NA, no metastasis occurred in patients <80 years of age. * p53 overexpression, superficial margins, and maximal tumor thickness were analyzed in the model as a continuous variable.

4. Discussion

In the present study, we intended to evaluate the putative prognostic value of p53 expression and *RAS* mutations in cSCCs, since it remains a matter of controversy.

Our study indicates that *RAS* mutation seems to be more frequent in invasive rather than in situ cSCCs but studies with a larger number of in situ tumors will be pivotal to validate this hypothesis. When addressing invasive cSCCs, we report a similar *RAS* mutation rate (10.7%) in agreement with most of the previously-published studies (up to 13%) [26,39–41]. *RAS* mutations were more-frequently associated with an infiltrative than an expansive pattern of invasion, suggesting that these mutations might enhance tumor progression. A putative mechanism for a more infiltrative tumor front has been reported, suggesting that *RAS* mutations cooperate in modifying the epithelial-to-mesenchymal transition [42,43]. In our series, we observed a higher frequency of lymphovascular invasion in *RAS* mutated cSCCs, although not reaching significance.

As previously reported, no differences in p53 immunohistochemistry were observed in non-invasive and invasive cSCCs in the present study [16]. p53 overexpression detected (84%) is within the range of previous reports (15–92%) of invasive cSCCs [44–47]. No association was noted between p53 overexpression and clinicopathological features.

Our recurrence rate (13.0%) in invasive cSCCs is within the range of previously-reported studies (3.0–16.0%) [7,34,48–50]. In contrast with the reported studies, we observed a slightly higher lymph node metastasis rate (6.1% vs. 3.7–5.2%) [7,34,51,52]. The fact that 74.8% of invasive cSCCs were located on the face, including locations with higher metastatic risk, may in part explain the differences in the aforementioned reported rates.

RAS mutations were not associated with cSCC prognosis in our study. As previously reported by our group, older age and *TERTp* mutations turned out to be independent predictors of recurrence in invasive cSCCs [12]. In our study, ulceration was detected to be a predictor of recurrence in the univariate analysis. Although ulceration has been described as a risk factor for recurrence in melanoma [53], previous studies have failed to establish this premise in cSCCs [54,55]. Noteworthy, p53 overexpression was a predictor of recurrence in the univariate analysis, but previous studies failed to establish a prognostic significance [56]. When adjusted for other co-variables, the presence of ulceration and p53 overexpression failed to be independent predictors of recurrence in our study. Despite not being an independent predictor of recurrence, we must highlight that 16 out of 17 invasive cSCCs recurred and disclosed p53 overexpression. Further studies with a larger number of recurrent invasive cSCCs are warranted to ascertain the role of p53 overexpression as a putative biomarker of recurrence.

Invasion of the subcutaneous tissue and maximal tumor thickness are established risk factors for metastasis of cSCCs and were predictors of metastasis in our univariate analysis [11]. An association with metastasis and few or absent peritumoral lymphocytic infiltrate was detected in the univariate analysis and with a shorter time until adverse outcome. In other tumor models (e.g., melanoma), the absence or reduced number of lymphocytes (let alone the different subsets by immunohistochemistry) is an independent parameter associated with adverse prognosis [57–59]. Notwithstanding these results, when adjusted for other co-variables, invasion of the subcutaneous tissue, maximal tumor thickness, and few or absent peritumoral infiltrate failed to be independent prognostic predictors in our series of cSCCs. Importantly, p53 overexpression was detected in all metastatic cSCCs in our series. The limited number of metastases in our series might have hampered p53 overexpression prognostic significance in predicting metastasis of cSCCs.

The role of *RAS* mutations and p53 overexpression remains to be clarified in early cutaneous squamous cell carcinogenesis. *RAS* mutation rate did not differ when comparing in situ with invasive cSCCs and none of the three cases that recurred disclosed *RAS* mutation. p53 immunostaining was not significantly different in invasive and in situ cSCCs, but the three in situ cSCCs that recurred disclosed p53 overexpression. As in invasive cSCCs, the role of p53 overexpression as a marker of recurrence

might be considered for in situ cSCCs, even though studies with a higher number of recurrent cases are mandatory.

The synergistic interaction between *RAS* mutation and p53 described in a mice model of cSCC was not confirmed in our series (see Supplementary Tables S5 and S6).

Several studies have suggested that cSCCs arising in lower extremities have distinctive features, including an increased frequency in women, as opposed to cSCCs in other localizations where there is a male preponderance [60,61]. Lower extremities are reported as the most common site for cSCCs in African Americans, suggesting a pathogenesis less reliant on chronic sun damage [62]. In our series, in situ cSCCs localized in the lower limbs were significantly more common in female than in male patients, but this difference was not detected in invasive cSCCs. Remarkably, we observed that in situ cSCCs located in the lower limbs had a significantly higher p53 h-score compared to other locations. To our knowledge, our results have not been previously described and support the hypothesis that in situ cSCCs might have distinct etiopathogenesis. Further studies and larger series will be important to cover the two major pitfalls we detected in this study, the lack of a considerable number of metastatic cSCCs with an extensive follow-up of patients, and the absence of *TP53* mutational genotyping to correlate with the p53 expression; as reported by Köbel and colleagues, it is not possible to predict the presence of *TP53* mutation based on its immunoexpression, since wild type and mutated cases can partially give rise to similar staining patterns [63].

5. Conclusions

Our results indicate that *RAS* mutations associate with features of local aggressiveness, but do not seem to be independent prognostic markers of outcome in patients affected by cSCC. All the metastatic cSCCs had p53 overexpression, and p53 overexpression was an independent predictor of recurrence in the univariate analysis. Larger studies with more recurrent and metastatic cSCCs are necessary to confirm the putative prognostic significance of p53 overexpression in patients' risk stratification.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4425/11/7/751/s1, Table S1: Clinicopathological and molecular associations with RAS mutations in cSCC, Table S2: Clinicopathological and molecular associations with p53 overexpression, Table S3: Clinicopathological and molecular associations with p53 overexpression (mean h-score), Table S4: Clinicopathological and molecular associations with p53 overexpression (mean h-score) of cSCC with adverse features, Table S5: Features of cSCC with RAS mutated and with p53 overexpression, Table S6: Clinicopathological and molecular combinations between RAS status and p53 expression.

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CHAPTER 5 Discussion and Conclusions

We proposed to perform a clinically driven research project in one of the most common cancers worldwide. Despite its frequency, we felt that there was a lack of epidemiological and molecular studies on cSCC. We began our investigation in 2014, a period where it was not possible to ascertain why an actinic keratosis or a Bowen's disease acquired the ability to invade and break the basement membrane, and what were the molecular risk factors that triggered a localized cSCC enter the dermis, and eventually metastasize. The rationale for this study was that although large information is available about squamous cell carcinomas in areas other than the skin, there is a lack of information about the epidemiology, risk factors, and biological behavior of cSCC compared to other squamous cell carcinomas (lung, head, and neck) and compared to other skin cancers (e.g. melanoma).

Notwithstanding recent advances, there was an obvious need for the identification of molecular markers that could be used at the time of excision of the primary tumor for evaluation of the risk of recurrence and metastasis. In an era of predictive biomarkers and patient stratification for therapy, where new drugs with various molecular targets are being developed, if these biomarkers could be identified and targeted by novel therapies, we could be upon an extraordinary opportunity to challenge skin cancer progression.

The main aim of the proposed work was to provide a better understanding of the epidemiology of cSCC in a Portuguese Hospital and identify potential clinical and molecular prognostic factors that could assist in patients' risk stratification. As being a dermatologist, I felt there was a need to estimate the magnitude of cSCC incidence in my hospital, and a further attempt to identify biomarkers that could help my daily practice.

The first step of our project was to identify and clinically characterize all cSCC excised from my Dermatology Department. Studies had demonstrated a worldwide increase in cSCC incidence, but there is a lack of studies reporting the frequency of cSCC in Portugal. To our knowledge, this was the first study that described trends in a segment of the Portuguese population and showed that the incidence of in *situ* and invasive cSCC had rapidly increased between January 2004 and December 2013. We were able to compare the demographics of our patients with other series and established that our data were concordant

with previous studies. We observed a rising age-adjusted incidence in both genders, particularly in the last study period of the study (16.2/100 000-person year). Patients more than 75 years old were more frequently affected by cSCC in both male and female patients, highlighting the importance of cumulative UVR in its etiopathogenesis and the impact of this carcinoma in the elderly Portuguese population. This study also highlighted the need to improve hospital cancer registries, namely the need to improve the registry of recurrent and metastatic cases. A variable that was not included was the cancer-specific death due to cSCC that limited substantially the calculation of the mortality rate of our cohort. Additionally, to have an improved understanding of the epidemiology of a segment of the Portuguese population, Paper I was a valuable milestone since we were able to identify the cases of cSCC excised in our Hospital and gather FFPE tissues for further revision. Although we initially included 485 cSCC excised from 380 patients, a substantial part of these tumors had unavailable paraffin blocks. The Pathology Department of CHVNGE did not perform exclusively the histologic evaluation of all cSCC specimens. During the study period, various private pathology departments collaborated with the institution, limiting our capability of collecting all histologic blocks and creating a lack of a common histologic protocol for characterization of our cases. Given the lack of uniformity in the histological description and based on our wish to include additional histological variables, the available FFPE tissues were collected and revised by a certified Dermatopathologist and two experienced pathologists.

After establishing our study cases, there was a need to identify which genetic alterations would be included in our project. In Paper II we revised the current knowledge of cutaneous squamous cell carcinogenesis comprehensively, with particular emphasis on genetic alterations underlying etiopathogenesis. cSCC is known to carry more mutations than other common malignancies (5 times more than lung cancer and 4 times more than in melanoma) (Cancer Genome Atlas Research 2012, Pickering, Zhou et al. 2014). This high mutation rate in cSCC compelled us to be very purposeful when choosing which molecular alterations were going to be evaluated. Early inactivation of both alleles of *TP53* is a known genetic alteration, observed in up to 90% of cSCC (Brash 2006, Ratushny, Gober et al. 2012), making this genetic alteration mandatory to include in our study. Following the inactivation of *TP53*, a marked expansion in genetic alterations is known to take place and we would like to emphasize some of these alterations. *CDKN2A* alterations (e.g. mutations, copy loss, promoter methylation) were reported in 76% of cSCC cases (Li, Hanna et al. 2015). Cyclin D1 (CCND1) overexpression is described in squamous cell carcinogenesis in

up to 71% (Bito, Ueda et al. 1995, Jensen, Prasad et al. 2010, Shen, Xu et al. 2014). Regardless of conflicting data, *KNSTRN* mutations were reported in 19% of invasive cSCC (Brantsch, Meisner et al. 2008, Li, Hanna et al. 2015, Yilmaz, Ozer et al. 2017). Telomerase activity has for long been described to be elevated in cSCC leading to the immortalization of tumor cells (Parris, Jezzard et al. 1999). More recently, *TERTp* mutations were described in up to 74% of cSCC (Griewank, Murali et al. 2013, Killela, Reitman et al. 2013, Scott, Laughlin et al. 2014, Cheng, Kurtis et al. 2015). Loss of function mutations of *NOTCH-1* and *NOTCH-2* genes have been noted in up to 82% of cSCC emphasizing the importance of *NOTCH* genes as tumor suppressors in these epithelial malignancies (Talora, Cialfi et al. 2005, Wang, Sanborn et al. 2011). *EGFR*-activating mutations and *RAS* mutations were reported in a lower percentage of cases (2.5-3% and less than 13%, respectively) (Pierceall, Goldberg et al. 1991, Grandis, Chakraborty et al. 1998, Leffell 2000, Durinck, Ho et al. 2011, South, Purdie et al. 2014). Even though many genetic alterations have been described, none of them have successfully been established as a prognostic biomarker. The clinical hypothesis that some of these genetic alterations could turn to be biomarkers of prognosis led to the development of Paper III and Paper IV.

The discovery by Horn *et al*. and Huang *et al*. in melanoma of *TERTp* mutations in a non-coding region supported the hypothesis that these promoter mutations could function as important events, contributing to tumorigenesis through telomerase dysregulation, and subsequently leading to a positive growth selection (Horn, Figl et al. 2013, Huang, Hodis et al. 2013). In melanoma where *TERTp* mutations were originally described, Huang *et al*. reported a frequency of about 70%, making *TERTp* mutations the most frequent genetic event in melanoma. Despite this high frequency of mutation, *TERTp* mutations may have been overestimated. In the study from Horn *et al*., a smaller frequency was reported (33% for primary tumors) and our group confirmed, in two subsequent studies, that the most acceptable frequency values are around 20 to 30% in primary tumors, and can rise in metastatic lesions (Horn, Figl et al. 2013, Vinagre, Almeida et al. 2013, Populo, Boaventura et al. 2014, Vinagre, Pinto et al. 2014). *TERTp* mutations are the most frequent genetic event in several cancers, e.g. non-invasive bladder cancer, hepatocarcinoma, and glioblastomas (Killela, Reitman et al. 2013, Vinagre, Almeida et al. 2013, Vinagre, Pinto et al. 2014, Batista, Cruvinel-Carloni et al. 2016). One year after the first description of *TERTp* mutation in melanoma, its presence was reported by Scott et al. both *in situ* (20%) and invasive cSCC (~50%), in a limited series of cases. Later, other studies reported a mutation rate as high as 74% in invasive cSCC (Griewank, Murali et al. 2013, Killela, Reitman et al. 2013, Cheng,

Kurtis et al. 2015). None of the aforementioned studies reported a prognostic significance for *TERTp* mutations, as for melanoma, glioblastoma, medulloblastoma, bladder, and thyroid cancers (Killela, Reitman et al. 2013, Rachakonda, Hosen et al. 2013, Remke, Ramaswamy et al. 2013, Melo, da Rocha et al. 2014, Populo, Boaventura et al. 2014). *TERTp* mutations had only been studied in a small number of cSCC cases (maximum of 37 cases per study, with 115 cases in all studies). As in melanoma, we believed that a possible overestimation of *TERTp* mutations could be occurring. The appeal to define the correct frequency of *TERTp* mutations and a potential association with prognosis in cSCC led us to perform genetic profiling of *TERTp* mutations in a large retrospective series of cSCC and to determine the predictive value of these mutations in prognosis (Paper III). We established the mutational status of *TERTp* mutations in the largest consecutive series of cases drown from a hospital (152 cases from 122 patients). As in melanoma, our mutation rate is lower than previously reported, but we believe our result represents a more accurate estimation of *TERTp* mutation in both *in situ* and invasive cSCC (19.4% and 34.7%, respectively). Our study portrayed that *TERTp* mutations were more frequent in invasive than *in situ* cSCC, suggesting that these mutations could facilitate the ability to invade and break the basement membrane, favoring tumor progression. In our series, *TERTp* mutation failed to associate with classical risk factors for recurrence and metastasis (tumor thickness > 6 mm, invasion beyond subcutaneous fat, perineural invasion, tumor size > 2 cm, poor differentiation, localization in the temple, lip, and ear). Even though they did not associate with classical risk factors for adverse outcomes, *TERTp* mutations were more frequent in cases that recurred and metastasized. The association with recurrence and metastasis led us to clarify if *TERTp* mutations could be a predictive prognostic factor in cSCC. *TERTp* mutations turned out to be an independent predictor of recurrence and metastasis in our study. Mutated cSCC cases had an 8 times higher risk of recurrence and 16 times higher risk of metastasis than wild-type cases. We were able to point TERTp mutations as a significant predictor of prognosis, likewise in previous studies in other cancer models (Griewank, Murali et al. 2014, Melo, da Rocha et al. 2014, Populo, Boaventura et al. 2014). The importance of such a breakthrough was highlighted in an editorial from JAAD where the author promoted *TERTp* mutations to front-runner status in molecular cutaneous oncology prognostication (Heymann 2019). The usefulness of *TERTp* mutations as a prognostic marker is particularly relevant because invasive cSCC is the second most common cancer in Caucasians, and although a small percentage of such carcinomas behave aggressively, cSCC is responsible for most skin cancer-specific deaths in the elderly (Weinstock 1997). The current prognostic systems are

based on clinicopathological characteristics and have been considered to weakly distinguish patients who partake in adverse outcomes (Roscher, Falk et al. 2018). Furthermore, overall survival has not markedly improved in patients with advanced cSCC. The possibility of *TERTp* mutations being a robust molecular prognostic biomarker for cSCC opens avenues for better stratification of patient risk for adverse outcomes.

The desire to unravel additional biomarkers of prognosis in cutaneous squamous cell carcinogenesis drove us to study additional genetic alterations in cSCC (Paper IV). For a long time, *TP53* was considered the most frequently mutated gene in cSCC, but its prognostic impact had not been established (Brash, Rudolph et al. 1991). It was clear that the inclusion of P53 in our carcinogenesis model was mandatory since the beginning of this thesis. Regardless of our wish to determine the presence of *TP53* mutations in our series, we were unable to perform this purpose for the following reasons: DNA was retrieved from FFPE tissues in which the quality and quantities of DNA available were limited; *TP53* is a large gene with 11 exons, and the initial determination of *TERTp* mutations in our series precluded enough DNA for *TP53* mutation detection. Taking into account the previous limitations we decided to perform immunohistochemistry for P53 in our series with an innovative digital acquisition microscope and an automated scanning system. Even though *RAS* mutations were described as a rare event in cSCC (less than 13%) (Pierceall, Goldberg et al. 1991, Leffell 2000, Durinck, Ho et al. 2011, South, Purdie et al. 2014), we decided to include this genetic alteration since in murine models it was suggested that *RAS* could act synergistically with P53 to enhance tumor progression (Zhang, Yao et al. 2005). Furthermore, the increased frequency of *RAS* mutations in cSCC from patients treated with BRAF-inhibitors for the treatment of advanced melanoma (Su, Viros et al. 2012) suggested undisclosed roles of *RAS* in the etiopathogenesis of cSCC. Also, *RAS* mutations were extensively studied in the late 90s, but recent studies did not include this alteration. If we take for example thyroid medullary carcinomas were *RAS* mutations were initially undetected or described in low frequency in the 90s (Moley, Brother et al. 1991, Fenton, Anderson et al. 1999, Bockhorn, Frilling et al. 2000), but in the next two decades turned out to be an important alteration, motivated us to include this mutation in our analysis (Goutas, Vlachodimitropoulos et al. 2008, Moura, Cavaco et al. 2009, Lyra, Vinagre et al. 2014). Our study revealed no differences in P53 overexpression between *in situ* and invasive cSCC. *RAS* mutations were present more frequently in invasive than *in situ* cSCC (10.7% *vs* 3.2%, respectively), despite not reaching significance. *RAS* mutations seemed to be a marker of invasiveness since these mutations were more frequently associated with an infiltrative than

the expansive pattern of invasion, and *RAS* mutated cases had a higher frequency of lymphovascular invasion than wild-type cases, despite the later did not reach statistical significance. *RAS* mutations were not associated with prognosis in our study, but P53 overexpression was more frequently observed in recurrent and metastatic cases and turned out to be a predictor of recurrence in the univariate analysis. Despite failing to be an independent prognostic predictor of recurrence in the multivariate analysis, our study described for the first time a possible significance of P53 overexpression in prognosis. Larger studies, with more recurrent and metastatic cases, are necessary to confirm this assumption. In 90.1% of the cases in Paper IV, *TERTp* mutation status was available from previously published Paper III. Thus, we tried to establish a possible association between these alterations and prognosis. Despite our attempt, no prognostic association was noted between *TERTp* mutations, *RAS* mutations, and P53 overexpression. We concluded that larger studies with more recurrent and metastatic cSCC are necessary to check the prognostic significance of P53 overexpression in patients' risk stratification.

Despite not corresponding to our main objectives, we demonstrated prognostic significance for some classical clinicopathological features in our series. We believe the fact that classical risk factors associated with prognosis strengthened our studies comparability. Older age was an independent predictor of recurrence in invasive cSCC, as identified in previous studies (Eroglu, Berberoglu et al. 1996, Kyrgidis, Tzellos et al. 2010, Schmults, Karia et al. 2013, Diaz-Corpas, Morales-Suarez-Varela et al. 2015). Invasion of the subcutaneous tissue and tumor thickness >6mm, classical risk factors for recurrence, and metastasis of cSCC (Thompson, Kelley et al. 2016), were predictors of metastasis in the univariate analysis and were associated with a shorter time until a metastasis.

A limited number of recurrent and metastatic cases was a known limitation of our study. Nevertheless, we evaluated a consecutive series and found that the prevalence of advanced cSCC in our cohort is slightly higher when compared to the frequency reported by others. Another limitation is that we assessed the expression of P53 and have not defined the presence and relationship with its mutational status. Another issue is that there could be a potential selection bias since not all initially selected cases in Paper I had histological specimens available for use in Paper III and IV.

In conclusion, we believe our study has contributed to an improved understanding of cutaneous squamous cell carcinogenesis. Our investigation enabled us to a better comprehension of the epidemiology of cSCC in Portugal; to establish a well-characterized clinicopathological series of cSCC; to discover a novel molecular prognostic biomarker

(*TERTp* mutation); to indicate that *RAS* mutations may associate with features of local aggressiveness; and that P53 overexpression could potentially turn out to be a marker of recurrence and metastasis.

CHAPTER 6

Future perspectives

Despite the advances in cutaneous squamous cell carcinogenesis, there are still molecular alterations that need to be investigated to understand the complete multistep model of cSCC and establish new molecular prognostic biomarkers. I would like to obtain an answer or, at least, try to get an answer if *NOTCH*, *KNSTRN*, *CDKN2A* mutations play a role in our series of cSCC.

I believe that our series is robust but needs to include more recurrent and metastatic cases. The possibility of including more cases from other hospitals in the North of Portugal could be an option.

The possibility of confirming our enthusiastic results of *TERTp* mutations in a larger series, with more advanced cSCC, will help to establish *TERTp* mutations as a definite prognostic biomarker. I believe the inclusion of these mutations in existing staging systems will help to stratify patient risk, and ultimately save lives.

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