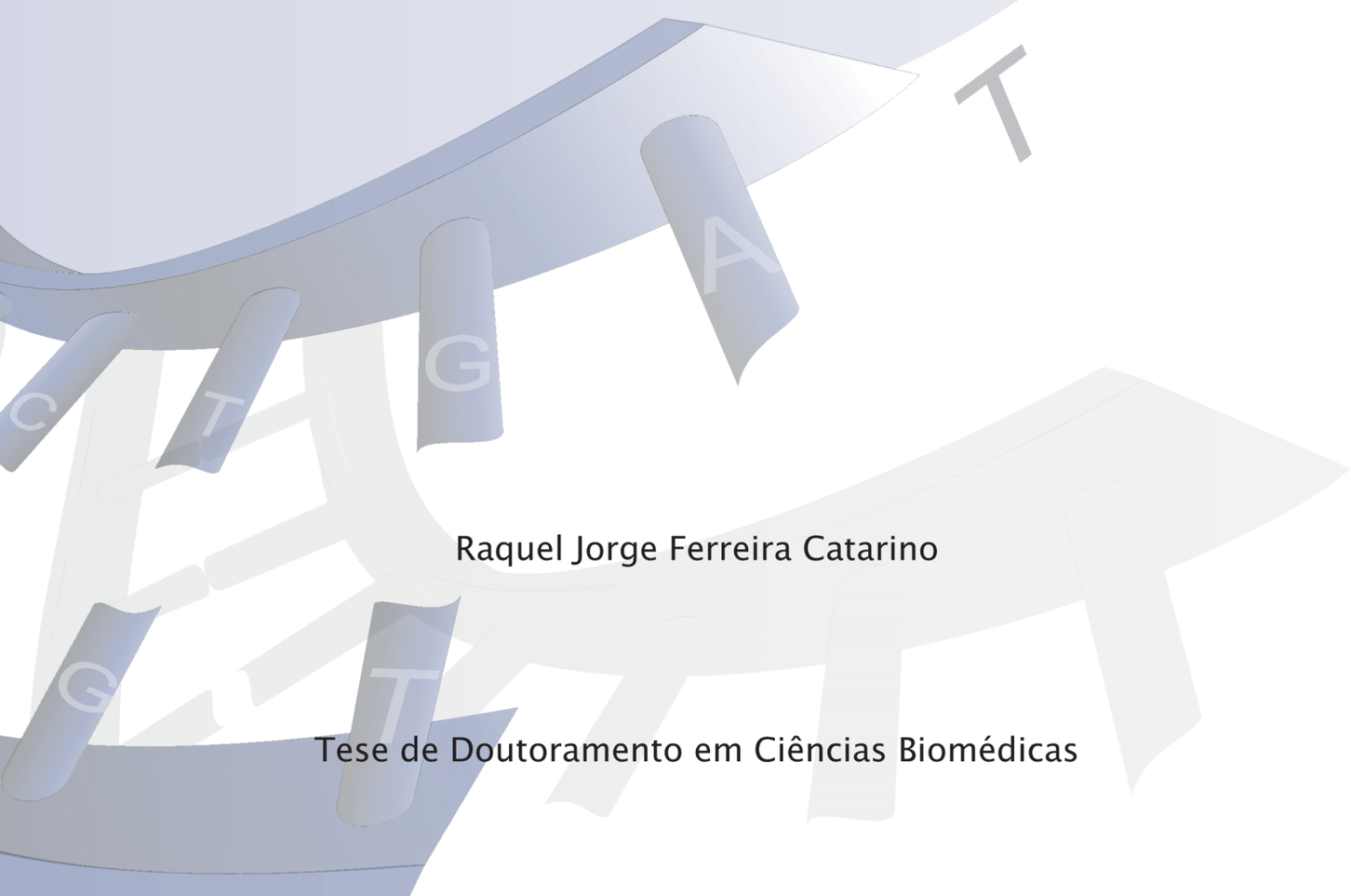
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Circulating DNA levels and cyclin D1 and telomerase genetic variability in cancer patients: improvements for cancer diagnosis and treatment response



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Circulating DNA levels and cyclin D1 and telomerase genetic variability in cancer patients: improvements for cancer diagnosis and treatment response

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Aos meus pais...

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A todos, reitero o meu apreço e a minha eterna gratidão.

LIST OF ABBREVIATIONS

ALT	Alternative length of telomeres
aOR	Adjusted odds ratio
AR	Attributable proportion
BC	Breast cancer
CCND1	Cyclin D1
CDK	Cyclin dependent kinases
CYP	Cytochrome P450 superfamily
EBV	Epstein-barr virus
fcDNA	Free circulating DNA
GST	Glutathione S-transferase family
HLA	Human Leucocyte Antigen
HPV	Human Papillomavirus
HSIL	High-grade squamous intraepithelial lesion of the cervix
hTERT	Catalytic subunit of human telomerase reverse transcriptase
hTR	RNA template of human telomerase reverse transcriptase
KRAS	v-Ki-Ras2-Kirsten rat sarcoma viral oncogene homolog
LSIL	Low-grade squamous intraepithelial lesion of the cervix
NFkB	Nuclear factor kappa B
NPC	Nasopharyngeal carcinoma
NSCLC	Non-small cell lung cancer
OR	Odds ratio
OVCC	Oncogenic virus-associated cervical cases
OVNC	Oncogenic virus-associated nasopharyngeal cases
PCR	Polymerase chain reaction
PGx	Pharmacogenomics
pRB	Retinoblastoma protein
PRF	Percentage of risk factor
RFLP	Restriction Fragment Lenght Polymorphism
RNS	Reactive nitrogen species
ROS	Reactive oxigen species
UCNT	Undifferentiated carcinoma of nasopharyngeal type
WTOD	Waiting time for onset of disease

LIST OF PUBLICATIONS

According to the art. 8 of the Decree-Law nº 388/70, the present Thesis has already produced the following publications in scientific peer-reviewed journals:

Catarino R, Coelho A, Araújo A, Gomes M, Nogueira A, Lopes C, Medeiros R. "Circulating DNA: diagnostic tool and predictive marker for overall survival of NSCLC patients". Cancer Prevention Research – *Submitted*

Catarino R, Araújo A, Coelho A, Gomes M, Nogueira A, Lopes C, Medeiros R. "Cyclin D1 polymorphism in Non-Small Cell Lung Cancer". Eur J Cancer – *Submitted*

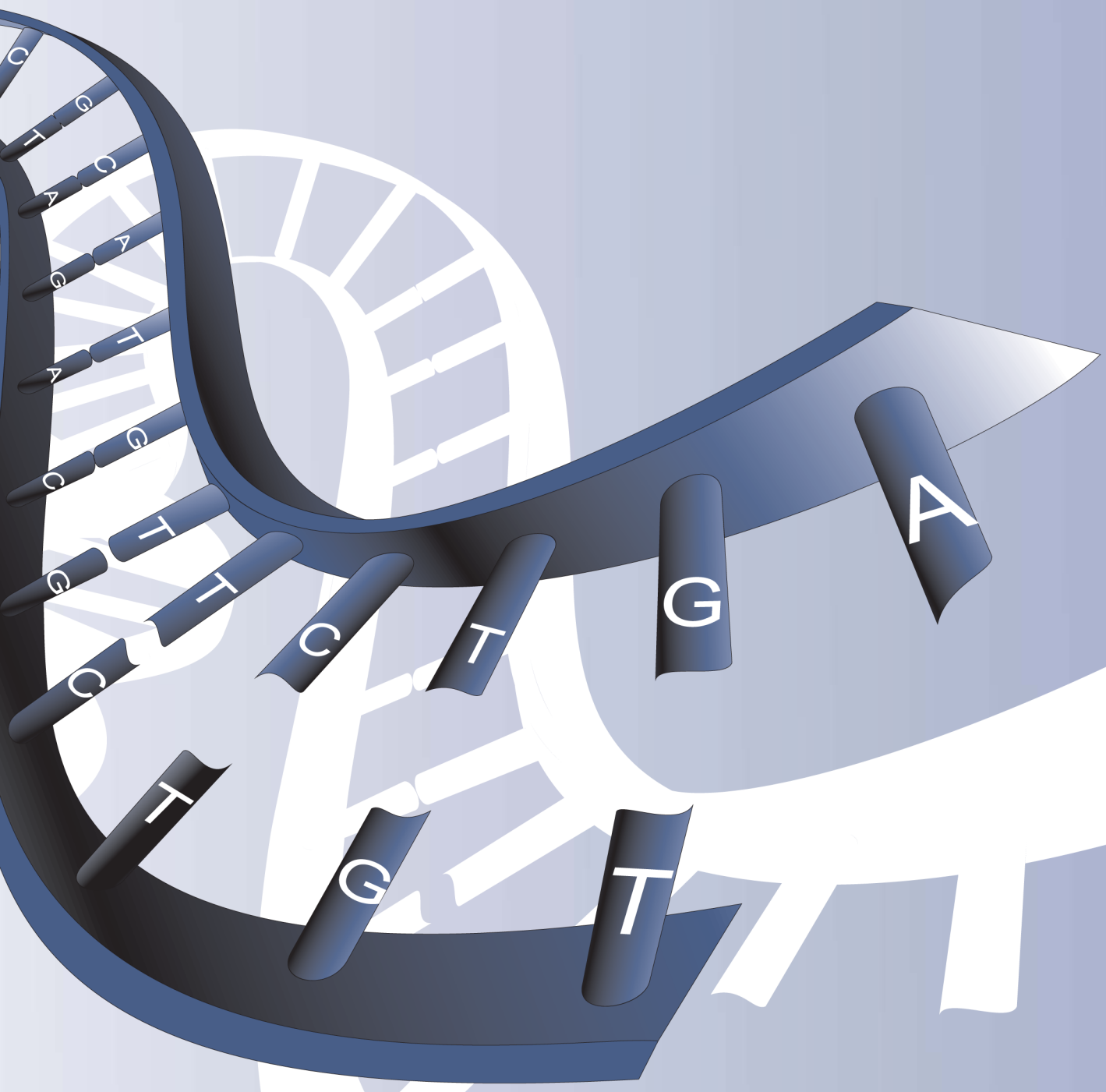
Catarino R, Araújo A, Coelho A, Gomes M, Nogueira A, Lopes C, Medeiros RM. "Prognostic significance of telomerase polymorphism in non-small cell lung cancer." Clin Cancer Res. 2010 Jul 15;16(14):3706-12.

Catarino R, Pereira D, Coelho A, Matos A, Breda E, Lopes C, Medeiros R."Oncogenic virus-associated neoplasia: a role for cyclin D1 genotypes influencing the age of onset of disease?" Biochem Biophys Res Commun. 2008 May 23;370(1):118-22.

Catarino R, Ferreira MM, Rodrigues H, Coelho A, Nogal A, Sousa A, Medeiros R. "Quantification of free circulating tumor DNA as a diagnostic marker for breast cancer." DNA Cell Biol. 2008 Aug;27(8):415-21.

Catarino R, Breda E, Coelho V, Pinto D, Sousa H, Lopes C, Medeiros R. "Association of the A870G CCND1 polymorphism with genetic susceptibility to nasopharyngeal carcinoma." Head and Neck. 2006 Jul;28(7):603-8.

I, hereby declare that I have actively participated in the gathering and study of the material included in each of the publications presented and had written the manuscripts in collaboration with the other authors.



ABSTRACT

ABSTRACT

Introduction: Cancer is today considered a public health problem, despite the decreases observed in cancer death rates in high-resource countries (1). There is a critical need for new research approaches aimed at improving cancer management. Identification and characterization of the genetic changes that drive human cancer development and progression may provide us with a variety of molecular markers that can redefine the criteria for cancer diagnosis and provide new tools for prognosis evaluation.

The existence of extracellular nucleic acids (DNA and RNA) in the blood was first described by Mandel and Metais. These authors demonstrated that DNA and RNA can be found in serum of both healthy and ill individuals (2). Various studies showed that the same genetic alterations in the primitive tumors were found in the free circulating DNA (fcDNA) of cancer patients, thus confirming the origin of the circulating DNA from the native tumor (reviewed in (3)).

Telomerase reactivation and cell cycle control bypass are key mechanisms for cancer cells to avoid senescence. Telomerase has reverse transcriptase activity, ability to prolong telomeres and its main components are the catalytic subunit (hTERT) and the RNA template (hTR). Matsubara and colleagues (4) screened the promoter region of *hTERT* for functional polymorphisms and a frequent T to C transition was found 1327 bp upstream the transcription starting site (*-1327T/C*). This study demonstrated that the *hTERT -1327* polymorphism is correlated with telomere length, indicating that telomere dysfunction may be modulated by these genetic variants. Cyclin D1 (CCND1) is a key regulatory protein at the G1/S checkpoint of the cell cycle. It forms complexes with CDK4 or CDK6 and is responsible for the phosphorylation of the retinoblastoma tumor suppressor protein, resulting in the release of E2F transcription factors that allow cells to enter into S-phase (5-7). Betticher and colleagues (8) identified a single base pair polymorphism (*A870G*) in *CCND1* and the genotypes have been significantly associated with carcinogenesis and clinical outcome in a variety of cancers (reviewed in (9)). Functional analyses have revealed that the polymorphism seems to modulate alternate splicing of *CCND1* mRNA, producing two different transcripts. The protein product of an alternately spliced transcript, cyclin D1b, harbors distinct functions as compared to full length cyclin D (cyclin D1a) (10).

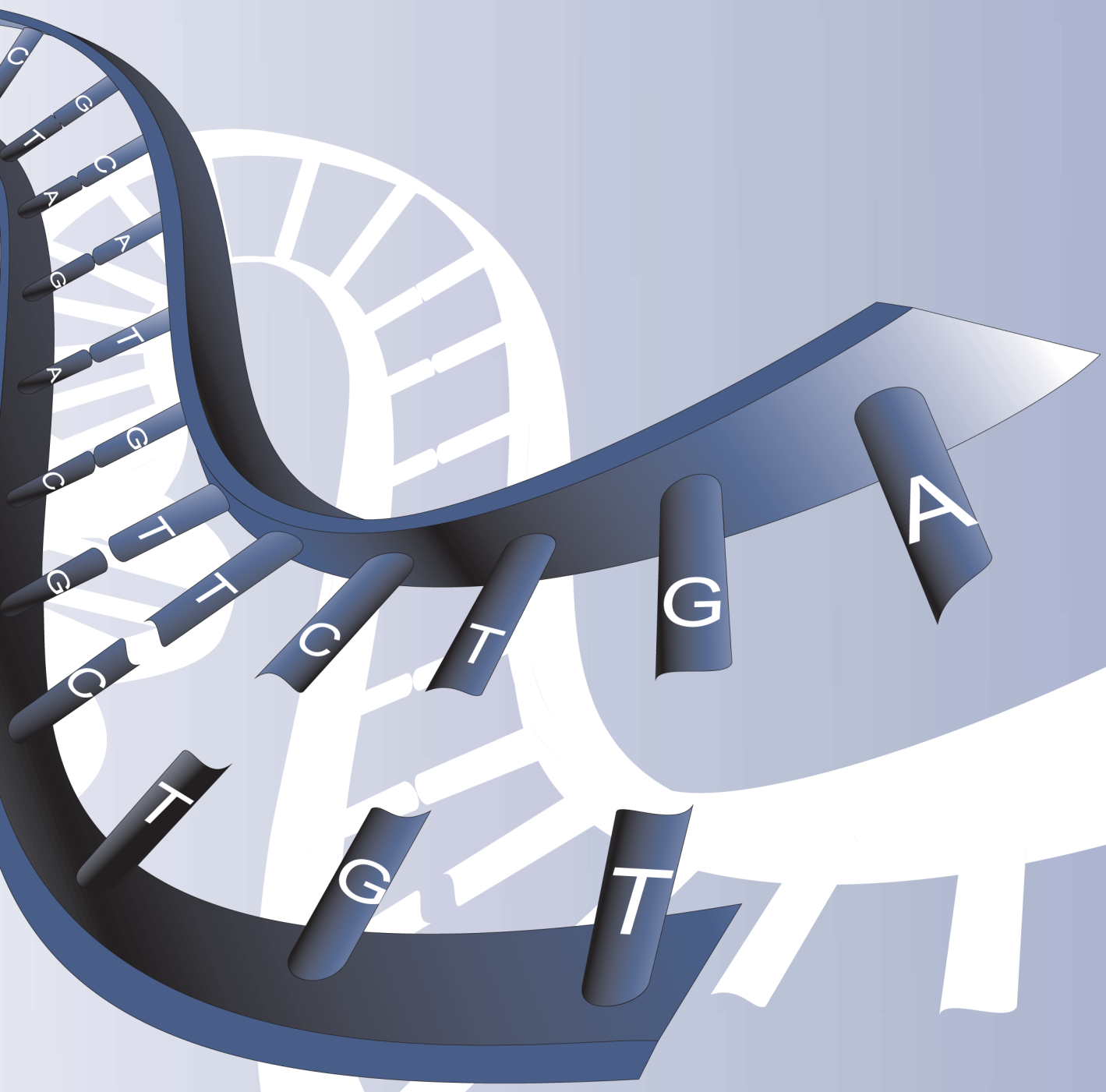
Aims: The aims of this study include the evaluation of the implications of circulating DNA quantification in cancer diagnosis and prognostic assessment

and the study of genetic variants in telomerase and cyclin D1 genes involved in tumor development and treatment response of cancer patients.

Material and Methods: Cancer cases admitted at Portuguese Institute of Oncology were recruited to the study. Control individuals were also recruited, from the same geographical area as case subjects. A peripheral blood sample was taken from each individual and DNA extraction was performed from plasma and blood mononuclear cells. fcDNA was measured through a real-time PCR method and the genetic variants of telomerase and cyclin D1 genes were evaluated using real-time PCR and PCR-RFLP methodologies, respectively.

Results: We used a real-time PCR based approach to quantify the amount of cell free circulating DNA. Our results demonstrate increased levels of circulating DNA in lung and breast cancer patients compared to control individuals. Regarding the evaluation of genetic variability of telomerase and cyclin D1 in cancer, our results indicate that lung cancer patients carrying the telomerase T-allele variant present a better treatment response of first-line chemotherapy, with a higher overall survival. Concerning cyclin D1 polymorphism, out results demonstrate an increased risk of lung, cervical and nasopharyngeal cancer development in individuals carrying the *CCND1 GG* genotype. Moreover, this genetic variant seems to be correlated with an earlier onset of these diseases.

Conclusions: This study suggests a non-invasive diagnostic tool that can be further investigated for cancer detection and assessment of its potential applicability as a complementary diagnostic tool in clinical practice. The assessment of telomerase and cyclin D1 genetic variants could help in the definition of susceptibility profiles for human cancer development, supplement prognosis of survival and may be promising molecular markers of treatment response in these patients. In the attempt of optimizing responses, minimizing toxicities, and reducing the elevated costs associated with chemotherapy failure, this analysis may contribute to the development of a pharmacogenomic profile, indicating the more appropriate chemotherapeutic protocol for each individual patient.



RESUMO

RESUMO

Introdução: O cancro é actualmente considerado um problema de saúde pública, apesar da diminuição das taxas de mortalidade em países desenvolvidos (1). Novas abordagens de investigação são necessárias com o objectivo de melhorar o diagnóstico e o tratamento dos doentes oncológicos. A identificação e caracterização de alterações genéticas que levam ao desenvolvimento de cancro e à sua progressão podem fornecer marcadores moleculares úteis na definição de critérios de diagnóstico de cancro e proporcionar novas ferramentas para a avaliação do prognóstico dos doentes oncológicos.

A presença de ácidos nucleicos no sangue periférico foi primeiramente descrita por Mandel e Metais. Estes autores demonstraram que pode ser encontrado DNA e RNA no soro de indivíduos saudáveis e de doentes (2). Vários estudos demonstraram que as alterações genéticas dos tumores podem ser encontradas no DNA livre circulante no plasma (fcDNA) dos indivíduos com cancro, confirmando que a origem do DNA livre em circulação no plasma deriva do tumor primário do doente (revisto em (3)).

A reactivação da enzima telomerase e a desregulação do ciclo celular são mecanismos chave para as células tumorais evitarem a senescência. Neste estudo, foram analisadas variações genéticas nos genes da telomerase e da ciclina D1 e as suas possíveis correlações com o desenvolvimento de cancro e resposta a tratamento.

A telomerase apresenta actividade de transcriptase reversa e capacidade para prolongar os telómeros, sendo os seus componentes principais a subunidade catalítica (hTERT) e o *template* de RNA (hTR). Um estudo realizado por Matsubara e colaboradores demonstrou a presença de variantes genéticas no gene *hTERT*, especificamente o polimorfismo *hTERT -1327 C/T*, localizado no promotor do gene (4). Este estudo demonstra que o polimorfismo está correlacionado com o tamanho dos telómeros, indicando que a disfunção dos telómeros pode ser modulada pela presença destas variantes genéticas. A ciclina D1 (CCND1) é uma proteína chave na regulação do ponto de controlo G1/S do ciclo celular, formando complexos com a CDK-4 ou CDK-6. Estes complexos são responsáveis pela fosforilação da proteína retinoblastoma, resultando na libertação de factores de transcrição E2F, que permitem a progressão através da fase S do ciclo celular (5-7). Betticher e colaboradores (8) identificaram um polimorfismo no gene da ciclina D1 (*CCND1 A870G*) e os diferentes genótipos têm sido associados à

carcinogénese e prognóstico de várias neoplasias (revisto em (9)). O polimorfismo parece modular o *splicing* alternativo do RNAm e produzir dois transcriptos diferentes (*a* e *b*), que estão simultaneamente presentes em vários tecidos. Estudos funcionais revelam que o produto proteico de um transcripto derivado de *splicing* alternativo, a isoforma ciclina D1b, apresenta funções distintas da isoforma completa (ciclina D1a) (10).

Objectivos: Os objectivos deste estudo incluem a avaliação das implicações da quantificação de DNA circulante no diagnóstico de cancro e avaliação de prognóstico e o estudo de variantes genéticas nos genes da telomerase e ciclina D1 envolvidas no desenvolvimento tumoral e resposta ao tratamento de doentes oncológicos.

Material e Métodos: Neste estudo foram admitidos doentes oncológicos que deram entrada no Instituto Português do Oncologia FG do Porto, EPE. Indivíduos controlo, sem doença oncológica conhecida e provenientes da mesma área geográfica que o grupo de casos, foram também admitidos no estudo. Foram recolhidas amostras de sangue periférico de todos os indivíduos e a extracção de DNA foi efectuada a partir de plasma e células mononucleadas. A quantificação de fcDNA foi efectuada recorrendo à técnica de PCR em tempo-real e a análise dos polimorfismos nos genes da telomerase e ciclina D1 foi realizada através das metodologias PCR em tempo-real e PCR-RFLP, respectivamente.

Resultados: Os resultados deste estudo demonstram uma quantidade de DNA circulante superior em doentes com cancro do pulmão e cancro da mama, quando comparados com indivíduos saudáveis. Relativamente à avaliação das variantes genéticas nos genes da telomerase e ciclina D1, os resultados indicam que doentes com cancro do pulmão com a variante T do polimorfismo da telomerase apresentam uma melhor resposta ao tratamento de quimioterapia de primeira linha, com uma sobrevivência mais longa. No que concerne ao estudo do polimorfismo no gene da ciclina D1, a análise dos resultados demonstra que indivíduos com o genótipo *CCND1 GG* apresentam um risco acrescido para o desenvolvimento de cancro do pulmão, do colo do útero e da nasofaringe. Mais ainda, esta variante parece estar associada a uma idade de diagnóstico mais jovem, sendo o desenvolvimento destas neoplasias mais precoce em indivíduos com este genótipo.

Conclusões: Este estudo sugere uma ferramenta de diagnóstico não invasiva que pode ser investigada em estudos futuros para a detecção de cancro e avaliação da sua possível aplicabilidade como ferramenta de diagnóstico

complementar na prática clínica. A análise das variantes genéticas da telomerase e ciclina D1 pode ajudar na definição de perfis de susceptibilidade para cancro, no estabelecimento do prognóstico e auxiliar na investigação de marcadores moleculares de resposta ao tratamento nestes doentes. Assim, na tentativa de optimizar respostas, minimizar toxicidades e reduzir os custos elevados associados à falência da quimioterapia, esta análise pode contribuir para o desenvolvimento de um perfil farmacogenómico, indicando o protocolo mais adequado e individualizado para cada doente.



INTRODUCTION

1. INTRODUCTION

Cancer constitutes one of the most important medico-biological problems, remaining generally unsolved. In Europe, cancer is an important public health problem. The most common incident forms of cancer in 2004 were lung cancer (13.3% of all incident cases), followed by colorectal cancer (13.2%) and breast cancer (13%). Lung cancer was also the most common cause of cancer death (341 800 deaths), followed by colorectal (203 700), stomach (137 900) and breast cancers (129 900) (1, 11).

Although the involvement of numerous genetic factors in the pathogenesis of neoplasia is well proven, the understanding of the complex molecular mechanisms underlying neoplastic growth is still incomplete. Intense investigation of cancer-related genetic alterations at the somatic cell level has improved the knowledge about the sequence of molecular events leading to malignant transformation of somatic cells and clonal neoplastic growth. It is now believed that most types of cancer share a relatively small number of molecular, biochemical and cellular traits called acquired capabilities (12).

Hanahan and Weinberg suggested the following six alterations relevant for malignancy: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. Neoplastic growth should not be regarded as a cell-autonomous process intrinsic to the cancer cell. Cancer development strongly depends upon changes in interactions between malignant cells and their normal neighbors (12). Furthermore, even at the level of cancer cell, acquired genetic changes affect only a small fraction of its genome. Tumor tissue grows as an abnormal analogue of the tissue of its origin, being influenced and often governed by the interplay of multiple basically normal physiologic processes and corresponding regulatory mechanisms. In neoplasia, these mechanisms are not necessarily damaged, but deregulated or taken out of their physiological context, so that their otherwise beneficial role can be totally distorted. It is well known that both cancer initiation risk and later neoplastic events (tumor growth, invasion, metastatic spread, response to therapeutic interventions and survival) are strongly affected by factors predetermined by individual's genetic background. The presence of polymorphic gene variants in the human genome provides an extensive genetic variation, affecting both normal physiological mechanisms and cancer pathogenesis (13).

Tumor development goes through several consecutive stages involving complex sequences of interactions between cancer-specific phenomena (initiating mutagenesis by genotoxic carcinogens, cell transformation followed by expansion of transformed clones and eventually tumor growth, progression, invasion and metastasis) and respective protective and modulating mechanisms. Understanding the role of genetic variation affecting these diverse protective/modulating systems is crucial for unraveling the differences in 'tumor behavior', prognosis and response to therapeutic interventions.

Uncontrolled cell proliferation is generally accepted as the hallmark of cancer (6, 14). Genetic damage, which causes somatic mutations and leads to malignant transformation implying cell multiplication and loss of death control, is believed to be the main triggering mechanism of neoplasia (6). Nevertheless, most growth-controlling pathways in cancer cells are, at least initially, largely intact, their complex biomachinery being a subject of physiological diversity depending on the presence of intrinsic polymorphic gene variants (15).

Alterations in genes encoding regulatory proteins of two major interconnected signaling pathways controlled by RB and TP53 are reported for many tumors (6), however, there is little understanding of the role of individual genetic background as a factor in the deregulation of cell proliferation, differentiation and death control.

The increasing amount of information regarding the role of genetic variation in human diseases has lead to putative new biomarkers and the development of new fields, as pharmacogenomics (PGx). Pharmacogenomics is a rapidly developing field, especially in oncology. In the most ideal situation pharmacogenomics will allow oncologists to individualize therapy based on patients' individual germline genetic test results. This can help to improve efficacy, reduce toxicity and predict non-responders in a way that alternative therapy can be chosen or individual dose adjustments can be made (16). It has been observed that the interpatient variability in response to medications is associated with a spectrum of outcomes, ranging from failure to demonstrate an expected therapeutic effect, to an adverse reaction resulting in significant patient morbidity and mortality, as well as increasing healthcare costs (17).

The goal of the emerging disciplines of pharmacogenomics is to personalize therapy based on an individual's genotype. To date, the success of PGx has spread across all fields of medicine. Genetic information has been used in the identification of disease risk, choice of treatment agents and guiding drug

dosing. This is particularly important for chemotherapeutic agents, which in general affect both tumor and non-tumor cells and thus have a narrow therapeutic index, with the potential for life-threatening toxicity (17).

1.1 Circulating DNA levels in cancer diagnosis and prognosis assessment

Increased knowledge of molecular pathogenesis of human tumors may offer a basis for the use of molecular markers in biologic fluids for early detection and prognostic assessment. Researchers are trying to develop methods that use biological materials that derive from non-invasive procedures, like plasma and expectoration, in order to locate possible biomarkers for early diagnosis and to follow-up high-risk individuals. Among these biomarkers, nucleic acids and their alterations are of greatest interest (18).

DNA from tumor cells shows alterations that cannot be found in normal cells and such anomalies are also detectable in the circulating DNA of cancer patients (19). This DNA seems to be independent of the stage of disease and seems to be correlated with tumor size (20). The existence of extracellular nucleic acids (DNA and RNA) in the blood is not a recent concept. Mandel and Metais demonstrated in 1948 that DNA and RNA can be found in serum of both healthy and ill individuals (2). Various studies showed that the same genetic alterations in the primitive tumors were found in the circulating DNA of cancer patients, thus confirming the origin of the circulating DNA from the native tumor (reviewed in (3)).

The mechanism of circulating DNA release is still not fully understood. The hypotheses that explain the presence of circulating native DNA include the active release of DNA from the tumor, the lysis of neoplastic and metastatic cells and necrosis or apoptosis of neoplastic cells (21).

Recently, various tests that are responsive of the quantification of circulating plasma DNA were published, namely methodologies based on quantitative Real-Time PCR. The quantification of circulating DNA through a sophisticated molecular method can be a useful diagnostic tool to differentiate patients with tumors from healthy individuals and can identify those with increased risk of cancer.

In 2003, Sozzi and co-workers showed that the average concentration of circulating DNA in lung cancer patients was eight times greater than healthy controls (19). There are several studies that have valued the different concentration of free plasma DNA among patients with lung tumors and healthy

individuals, and have used different data analysis methods and laboratory procedures (21). So far, the different methods employed for sample processing and storage, for the extraction and quantification of plasma DNA and the choice of different target genes, have not allowed the use of circulating DNA in the clinical practice. The evaluation of the data reproducibility obtained from other groups is necessary for any eventual future application of this test.

Previous studies found elevated plasma DNA levels in cancer patients comparing to control individuals, independently of tumor stage, indicating that plasma DNA is an early event during carcinogenesis (22).

There are multiple potential uses for free circulating DNA (fcDNA) quantification in cancer diagnosis and prognosis. However, direct comparison of the available data is often prevented by differences in the parameters analysed and by lack of standardized methodology and analysis procedures.

1.2 Genetic variability in cancer

A malignant tumor is the result of a series of DNA alterations in a single cell, or clones of that cell, which lead to loss of normal function, aberrant or uncontrolled cell growth and often metastasis. Several of the frequently altered genes have been identified, including proto-oncogenes and tumor suppressor genes. Other genetic alterations are also necessary, including in genes involved in DNA repair, cell cycle control, angiogenesis and telomerase production (12).

With the exception of rare familial cancers, primarily caused by a germline inheritance of a specific mutation, a sporadic cancer may acquire mutations as a result of genotoxic exposure to external or internal agents, such as tobacco carcinogens, dietary factors, infectious agents, sex hormones and consequent DNA adduct formation. The likelihood of a mutation occurring and persisting in subsequent clones may be heavily dependent on the efficiency with which potentially toxic exposures are metabolized and excreted, and also the efficiency with which small mistakes in DNA replication are rectified. It is this caretaker role of carcinogenesis that is likely to vary strongly between individuals, because of the population variability in polymorphic genes that regulate these processes. Many studies have tried to identify these low risk, but highly prevalent variations (23).

Telomerase reactivation and cell cycle control bypass are key mechanisms for cancer cells to avoid senescence. We studied genetic variations in telomerase and

cyclin D1 genes and assessed their role in cancer development and treatment response in cancer patients.

1.2.1 Telomerase genetic variants

Cellular senescence is considered a stress response triggered by a number of "counting mechanisms," such as telomere shortening. Importantly, the mechanisms underlying cellular senescence are involved in protection against cancer and may also be involved in the aging process. Even though senescent cells in vitro may remain viable indefinitely, although incapable of proliferation, the situation in vivo may be more complex (24).

Telomeres consist of repetitive DNA elements at the end of linear chromosomes that protect the DNA ends from degradation and recombination (25, 26). Telomeres are TTAGGG repeat complexes bound by specialized nucleoproteins at the ends of chromosomes in all eukaryotic cells. By capping the ends of chromosomes, telomeres prevent nucleolytic degradation, end-to-end fusion, irregular recombination and other events that are normally lethal to a cell (26).

Due to the intrinsic inability of the replication machinery to copy the ends of linear molecules, telomeres become progressively shorter with every round of cell division. Eventually, telomeres reach a critically short length, behaving as double-stranded DNA breaks that activate the TP53 tumor suppressor protein resulting in telomere-initiated senescence or apoptosis (27). Telomerase is a ribonucleoprotein with DNA polymerase activity that elongates telomeres (28), but its level of activity in most adult tissues is not sufficient to compensate for the progressive telomere attrition that occurs with aging (29). The generation of telomerase-deficient mice demonstrated that telomerase is the main cellular activity responsible for maintaining telomere length. Importantly, in the case of in vitro cultured cells, ectopic expression of telomerase was sufficient to prevent telomere shortening and this resulted in immortalization of human fibroblasts, thus formally demonstrating that telomere exhaustion is a critical factor leading to cellular senescence (27).

The telomere clock limits not only the proliferation of normal non-tumor cells, but also the proliferation of those cells that are already on the road to neoplastic transformation. This is best illustrated by the fact that essentially all human cancers have acquired mechanisms to maintain telomeres, generally through the expression of high levels of telomerase (30, 31). Exceptionally, immortal human

cell lines and tumors may maintain their telomeres in the absence of telomerase, through a mechanism known as alternative lengthening of telomeres (ALT), which involves homologous recombination between telomeres (32). These observations in human cancer strongly support the concept that telomerase is a tumorigenic factor that enables tumor progression. In agreement with this, mice deficient in telomerase activity are significantly resistant to cancer induced by a variety of genetic defects or carcinogenic treatments (27).

The final cellular outcome responsible for telomere mediated protection against cancer involves senescence and apoptosis. Whether a cell senesces or undergoes apoptosis likely depends on both cell type and context. Moreover, preneoplastic cells are exposed not only to dysfunctional short telomeres but also to other potential triggers of senescence or apoptosis, such as oncogenic signaling (27). Telomere-induced senescence has been shown to act as a tumor suppressor mechanism in telomerase-deficient mice expressing the oncogene Myc in B cells (33).

Telomerase main components are the catalytic subunit (hTERT) and the RNA template (hTR). The enzyme is active during embryonic development but silenced in most somatic cells after birth (29). The genetic integrity of the cell is maintained, in part, by the architecture of telomeres. This genetic integrity, however, is gradually lost as telomeres progressively shorten with each cell replication cycle (34, 35). Although telomere shortening is inversely associated with age, telomere length has been found to vary considerably in human peripheral blood lymphocytes from individuals of the same age (36, 37). Little is known about the impact of hereditary *hTERT* gene variations. Matsubara and colleagues (4) screened the promoter region of *hTERT* for functional polymorphisms and a frequent T to C transition was found 1327 bp upstream the transcription starting site (*-1327T/C*). Individuals homozygous for the *-1327C/C* genotype showed shorter telomere length in their peripheral leucocytes compared to the *-1327T/T* and *-1327T/C* genotypes.

1.2.2 Cyclin D1 genetic variants

The cell cycle is a critical regulator of the processes of cell proliferation and growth as well as of cell division after DNA damage. It governs the transition from quiescence (G0) to cell proliferation, and through its checkpoints, ensures the fidelity of the genetic transcript.

A frequent target in carcinogenesis is the deregulation of G1–S phase progression of the cell cycle. The transition through G1 to S phase is regulated by cyclins, cyclin-dependent kinases (CDKs) and their inhibitors (38). Cyclin D1 (CCND1) is a key regulatory protein at the G1/S checkpoint of the cell cycle, as it forms complexes with CDK4 or CDK6 and is responsible for the phosphorylation of the retinoblastoma tumor suppressor protein, resulting in the release of E2F transcription factors that allow cells to enter into S-phase (5–7). The G1/S checkpoint is frequently altered in many epithelial tumors and may confer growth advantage and enhance tumorigenesis (39). Amplification of *CCND1* and altered expression of the protein have been reported in a variety of tumors (7, 40).

Cyclin D1 is a focal point for integrating mitogenic stimulation with cellular proliferation [1,2]. Mitogenic signals typically induce increases in cyclin D1 mRNA expression and translation, thereby increasing the cellular pool of the protein product. The pro-proliferative function of cyclin D1 is mediated through its ability to regulate the cell cycle machinery and excessive cyclin D1 expression and/or activity is a hallmark of several tumor types [3,4]. Given the importance of cyclin D1 in human disease, concerted effort has been directed at delineating the mechanisms by which cyclin D1 is dysregulated in cancer. Intragenic somatic mutations of cyclin D1 are rare, but a polymorphism of cyclin D1 that occurs in a splice donor site has been epidemiologically linked to altered cancer risk or prognosis in a number of tumor types (reviewed in (9)).

It has been reported that CCND1 mRNA is alternatively spliced to produce two transcripts (*a* and *b*), which are present simultaneously in a variety of normal tissues and cancer cells (8). Betticher and colleagues (8) identified a single base pair polymorphism (*A870G*) in *CCND1*, and *CCND1* genotypes have been significantly associated with carcinogenesis and clinical outcome in a variety of cancers (reviewed in (9)). Recent functional analyses have revealed that the protein product of an alternately spliced transcript, cyclin D1b, harbors distinct functions as compared to full length cyclin D (cyclin D1a).

The aims of this study include the evaluation of the implications of circulating DNA quantification in cancer diagnosis and prognostic assessment and the study of genetic variants involved in tumor development and treatment response of cancer patients. This study was designed to outline a simple blood test, based on circulating DNA quantification (through amplification of *hTERT*) which might be used as a non-invasive complementary diagnostic tool, assess its specificity and sensibility to identify lung and breast cancer cases and evaluate its potential value

to monitor these diseases. Furthermore, this study also includes the evaluation of genetic variations involved in cancer development and treatment response of cancer patients, namely single nucleotide polymorphisms in cyclin D1 and telomerase genes. In this context, inter-individual genetic variations may constitute an important factor in the definition of susceptibility profiles for cancer development and variability of treatment response in cancer patients (41-44). In the attempt of optimizing responses, minimizing toxicities, and reducing the elevated costs associated with chemotherapy failure, this analysis may contribute to the development of a pharmacogenomic profile, indicating the more appropriate chemotherapeutic protocol for each individual patient.


OBJECTIVES

2. OBJECTIVES

2.1 General objectives

- Evaluation of the implications of circulating DNA quantification in cancer diagnosis and prognostic assessment.
- Study of genetic variants involved in tumor development and treatment response of cancer patients.

2.2 Specific objectives

The aims of this study include:

- To outline a quantitative circulating plasma DNA test, based in real-time PCR methodology, through the amplification of *hTERT* (human telomerase reverse transcriptase)
- Evaluation of its potential value as a diagnostic cancer marker
- Investigation of the importance of circulating DNA levels in monitoring the disease of cancer patients
- Assessment of the influence of telomerase genetic variability (*hTERT* polymorphism) and cyclin D1 genetic variants (*CCND1* polymorphism) in the susceptibility to human tumors
- Evaluation of the role of cyclin D1 and telomerase polymorphisms as predictive molecular markers of treatment response in cancer patients.



RESULTS / PUBLICATIONS

3.1 CIRCULATING DNA LEVELS IN CANCER DIAGNOSIS AND PROGNOSIS

Circulating DNA: diagnostic tool and predictive marker for overall survival of NSCLC patients

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ABSTRACT

Background: The purpose of our study was to determine whether the amounts of circulating DNA (cDNA) could discriminate between NSCLC patients and healthy individuals and assess the value of cDNA levels as a prognostic marker of this disease.

This study was designed to validate the quantification of circulating DNA in order to design a useful test for the early identification of NSCLC patients and the monitoring of lung cancer progression.

Methods: We conducted a hospital-based case-control study of 309 individuals, including 205 controls and 104 cases. The circulating DNA levels were assessed through real-time PCR methodology. The performance of the test was evaluated with a ROC curve.

Results: We found increased levels of circulating DNA in NSCLC patients compared to control individuals (269.95 vs 122.67 ng/ml, p<0.0001). Increased plasma cell free DNA concentration was a strong risk factor for lung cancer presence, conferring an increased risk for the presence of this disease (aOR, 12.69; 95% CI, 4.95-32.52; p<0.0001). We also found a decreased overall survival time in patients presenting high cDNA levels (above 20.0 ng/ml), when compared to lower cDNA concentrations (hazard ratio, HR=3.77; 95% CI: 1.16-12.28; *P*=0.028). The area under the ROC curve was 0.88 (95% CI, 0.84-0.92; P<0.0001). The concentration of circulating DNA proved to be an important risk factor for the presence of NSCLC and a prognostic index in the follow-up of these patients.

Conclusions: Quantification of circulating DNA by real-time PCR may be a good and simple tool for lung cancer detection with potential to clinical applicability together with other current methods used for monitoring the disease. These

results could have practical implications such as the use in screening programs and a possible prognostic significance in the follow-up.

INTRODUCTION

Lung cancer is a worldwide problem. At the time of diagnosis, 50% of patients have advanced incurable disease. Different chemotherapy combinations — with or without targeted therapies — yield similar results despite the continuous efforts of clinicians (1).

Non-small cell lung cancer (NSCLC) accounts for about 80% of lung cancers. No more than 10% of these can be treated with surgery because of the lack of an early diagnosis or because of a high frequency of metastasis at diagnosis (2). The prognosis is poor with only 10—15% of patients surviving 5 years after diagnosis. This dismal prognosis is attributed to the lack of efficient diagnostic methods for early detection and lack of successful treatment for metastatic disease. Within the last decade, rapid advances in molecular biology and radiology have provided a rational basis for improving early detection and patients' outcome. The aim for early detection is to identify lung cancer at a stage early enough to be curable by surgery. Prognostic factors predict survival independent of the treatment applied and can classify patients as high or low risk. The most important prognostic factor is stage according to the TNM system (3, 4). Other prognostic factors include clinical aspects, as gender, age, weight loss and cardiovascular disease, elevated lactate dehydrogenase levels, FDG-PET scan and pathological aspects (5, 6). A major hurdle in the attempts to improve the survival of these patients has been the lack of a simple, non-invasive and effective test for early prediction of therapeutic efficacy.

The finding that tumors are capable of shedding DNA into the blood stream, which can be recovered from both serum and plasma and used as surrogate source of tumor DNA, has opened new areas in cancer diagnosis and prognosis (7). A number of studies have examined the mechanism behind origin

and release of free DNA in the circulation and its clinical implications for lung cancer diagnosis, prognosis, and monitoring the effect of cytotoxic therapies (7).

The presence of extracellular nucleic acids in the human bloodstream was first described in 1948 by Mandel and Métais (8). In recent years, many studies identified genetic alterations in cell-free circulating DNA (cDNA) from plasma or serum and tumor DNA in many tumor types, including lung cancer (9).

Previous studies indicate that circulating nucleic acids in peripheral blood are originated from tumors, through apoptosis, necrosis or cell lysis of tumor cells (10, 11). The presence of tumor DNA in blood is probably the result, in variable proportions, of these different mechanisms, such as apoptosis, necrosis, cell lysis and circulating tumor cells lysis, which produce DNA leakage or excretion.

There are multiple potential uses for cDNA quantification in cancer diagnosis and prognosis. It may represent a valuable source of tumor DNA when the exact position of a suspected primary lesion is not clearly defined, or when biopsies are not available (12). However, direct comparison of the available data is often prevented by differences in the parameters analysed and by lack of standardized methodology and analysis procedures. Any future application of plasma or serum DNA analysis for diagnostic purposes will depend on the reproducibility and reliability of results, both of which require optimization and equivalent procedures. For this study, we selected an assay designed for the human telomerase reverse transcriptase (*hTERT*) genomic sequence that was consistently developed in a previous study (13). The quantification of circulating DNA through a sophisticated molecular method can be a useful diagnostic tool to differentiate patients with tumors from healthy individuals and can identify those with increased risk of cancer. There are several studies that have valued the different concentration of free plasma DNA among patients with lung tumors and

healthy individuals, and have used different data analysis methods and laboratory procedures (14). So far, the different methods utilized for sample processing and storage, for the extraction and quantification of plasma DNA and the choice of different target genes, have not allowed the use of circulating DNA in the clinical practice (15). The evaluation of the reproducibility of data obtained from other groups is necessary for any eventual future application of this test. For this purpose, we decided to apply a real-time PCR methodology previously described (16), in order to confirm the reproducibility of the results. We processed our samples using the same analytic and pre-analytic methods that have been reported.

The present study was designed to analyze the efficacy of circulating plasma DNA levels in discriminating lung cancer patients from healthy individuals. Additionally, the utility of circulating plasma DNA as a prognostic marker of survival and in predicting response to therapy and disease progression was also assessed.

MATERIALS AND METHODS

Study population

We evaluated 104 newly diagnosed and untreated patients with non-small lung cancer (NSCLC) and 205 control individuals. Since 2002, 104 consecutive Caucasian patients admitted to the Portuguese Institute of Oncology of Porto (IPO-Porto), Portugal, with cytological or histological confirmed NSCLC, have been prospectively recruited to the study (median age 64.0 years; mean age 63.2 years; sd 10.0).

Considering the patients´ gender, 20 (18.9%) were female and 86 (81.1%) male individuals and regarding smoking habits, 24 (23.1%) were non-smokers and 80 (76.9%) were smoker or former-smoker individuals.

The control group consisted of 205 healthy individuals, 78 (38.0%) male and 127 (62.0%) female individuals, with a median age of 50.0 years (mean age 47.3; standard deviation 11.6), without clinical history of cancer, from the same geographic area as the case group.

The patients were evaluated according to the TNM staging system, and the assessment of tumour response to chemotherapy was based on RECIST (Response Evaluation Criteria In Solid Tumors). The first line chemotherapeutic protocol consisted of platin-based doublet chemotherapy in combination with a third-generation cytotoxic compound such as paclitaxel, gemcitabine or docetaxel. The chemotherapeutic protocols were as follows: cisplatin (80 mg/m2 on day 1) + paclitaxel (175 mg/m2 on day 1 every 3 weeks); cisplatin (100 mg/m2 on day 1) + gemcitabine (1250 mg/m2 on days 1 and 8 every 3 weeks); carboplatin (AUC 6 on day 1) + paclitaxel (175 mg/m2 on day 1 every 3 weeks); carboplatin (AUC 6 on day 1) + gemcitabine (1000 mg/m2 on days 1 and 8 every 3 weeks).

The median follow-up time was 9 months (range 1–35 months). Patients' distribution according to the stage at the time of diagnosis was 4 patients (3.8%) presenting localized disease (stages I and II) and 100 (96.2%) with advanced disease (stages III and IV). From all patients, the histological type distribution was as follows: 38 patients (36.6%) with epidermoid NSCLC, 54 (51.9%) with NSCLC adenocarcinoma and 12 (11.5%) with other NSCLC histological types.

The study was approved by the institute's ethics committee. Informed written consent was obtained from all the subjects, according to Helsinki Declaration.

Sample Collection and DNA Isolation

A 5 ml sample of peripheral blood extracted from each patient was collected in tubes containing EDTA and plasma was immediately separated from the cellular fraction by centrifugation at 2500 rpm for 10 min at 4°C. The resulting supernatant (plasma) was frozen at -80°C. DNA was extracted from plasma by using QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The purified DNA from 200µL of plasma was eluted in a final volume of 70µL of buffer AE used for the elution and stored at -20°C.

DNA Quantification in Plasma

The quantification of cDNA was performed by a real-time quantitative PCR method, based on a continuous monitoring of fluorescence by an optical system (17). The probe is labelled by two fluorescent dyes, one serves as a reporter on 5' end (VIC dye; Applied Biosystems, Foster City, CA). The emission spectrum of the dye is quenched by a second fluorescent dye at the 3' end (TAMRA; Applied Biosystems). Primers and probes were designed previously by Sozzi and co-workers to amplify the gene of interest, the *hTERT* single copy gene mapped on

5p15.33 (13). The primers and probe's sequences were the following: primer forward, 5'-GGC ACA CGT GGC TTT TCG- 3'; primer reverse, 5'- GGT GAA CCT CGT AAG TTT ATG CAA- 3'; probe, VIC5'- TCA GGA CGT CGA GTG GAC ACG GTG-3' TAMRA.

Fluorogenic PCRs were carried out in a reaction volume of 25μL in a 7300 ABIPRISM System (Applied Biosystems). Each PCR reaction consisted of TaqMan Universal Mastermix (Applied Biosystem), probe (7.5mmol/L), primer forward (5 mmol/L), primer reverse (5mmol/L) and sterile water. DNA solution (2.5μL) was used in each real-time PCR reaction. Thermal cycling was initiated with a first denaturation step of 50ºC for 2 minutes and then 95ºC for 10 minutes followed by 50 cycles of denaturation at 95ºC for 15 seconds and annealing and extension at 60ºC for 1 minute. Each plate consisted of patient samples in duplicates and triplicate blanks of water as negative control. The calibration curve of each plate was calculated based on a dilution series of the TaqMan Control Human Genomic DNA Standard (Applied Biosystems) at 10ng/μL: 1, 0.1, 0.01, 0.001 and 0.0001 ng/μL. All the data were analyzed using the 7300 System – SDS Software (version 1.2.3) Sequence Detection Software (Applied Biosystems).

Statistical Analysis

Data analysis was performed using the computer software SPSS for Windows (version 18.0) (SPSS Inc, Chicago, IL).

Linear amplification down to the last dilution was obtained in each experiment (Pearson correlation coefficient, 0.999 to 0.995).

DNA concentrations between healthy controls and patients were compared using the Mann-Whitney U- test. The Mann-Whitney test was also used to compare DNA levels and patients´ clinical-pathological characteristics. $P \leq 0.05$

was considered statistically significant. For the diagnostic discrimination of DNA concentrations between cancer patients and controls the area under the curve of the receiver operating characteristic curve (AUC-ROC) was assessed non-parametrically. A *p*-value of ≤0.05 was regarded as significant. An AUC-ROC equal to 1 denotes perfect discrimination between patients with cancer and patients without cancer, a value equal to 0.5 denotes the lack of discrimination, and values in between indicate a degree of discrimination between strong and poor.

Chi-square analysis was used to compare categorical variables and a 5% level of significance was used in the analysis. The odds ratio (OR) and its 95% confidence interval (CI) were calculated as a measurement of the association between the amount of cDNA and probability of lung cancer presence. Logistic regression analysis was used to calculate the adjusted OR (aOR) and 95% CI, with adjustment for age, gender and smoking status.

The probabilities of survival were calculated, and the means and life tables were computed using the product-limit estimate of Kaplan and Meier. The curves were examined by the Log rank test, a statistical test for equality of survival distributions. Survival duration was defined as the time between diagnosis and either death or the last clinical evaluation of the patient. Clinical-pathological data and cause of death was determined from the patient's medical records.

RESULTS

Distributing the cDNA values between healthy and NSCLC patients

Our study was developed to investigate cell free circulating DNA (cDNA) by a real time PCR quantitative approach, performing all the technical procedures necessary for accurate and reproducible results. The quantification of cDNA was performed as previously described, by a real-time quantitative PCR assay (13). This quantitative assay shows a strong discrimination power and a high sensitivity and specificity using the *hTERT* gene as a target sequence for quantification of cDNA in plasma. For patients with two or more samples collected after diagnosis and before treatment, the mean cDNA concentration was calculated.

Plasma DNA was not associated with age and smoking history in both case and control groups. However, considering the control group, cDNA levels were increased in men individuals (Table 1). There were no statistically significant differences in cDNA concentrations between probands in different histological groups and tumor stages (Table 2).

The mean cDNA plasma concentration in the control group (mean rank 122.7 ng/ml), was statistically different from the mean cDNA plasma concentration of the patient group (mean rank 270.0 ng/ml) (Mann-Whitney test, $p<0.0001$) (Figure 1).

Sensitivity and specificity for circulating plasma DNA levels as a diagnostic marker

The ROC curve was made to evaluate the diagnostic power of the circulating DNA concentration. The ROC analysis is performed through the study of the function that links the sensibility with 1-specificity. The subtended area by

the ROC curve (Figure 2) shows a synthetic index of the overall capacity of the test in differentiating between healthy and ill individuals. The closer the area gets to the unit, the greater its discriminating ability. According to our data, the area under the ROC curve is 0.88 (95% CI, 0.84–0.92; p<0.0001). We found that lower cut-off values increased the sensitivity of the assay but at the cost of specificity and vice versa. Using the results of the ROC curve, an analysis was made on the test performance with respect to the different threshold values (Table 3). The results showed that, with a threshold of 20 ng/mL, there is a probability of illness of 71% when the test is positive (PPV). A DNA cut-off level of >20 ng/ml differentiated between lung cancer patients and controls with a specificity of 83% and sensitivity of 79%.

cDNA Concentration and risk of NSCLC

An elevated concentration of plasma cDNA was associated with a higher chance for the presence of NSCLC. Our results indicate that high cDNA concentrations are more prevalent among cancer cases, comparing to control individuals (Table 4). Data analysis demonstrates that individuals with high cDNA concentrations present an increased chance for the presence of NSCLC.

Keeping the cut-off value as the reference value equal to 6 ng/mL, the relative risks of illness by DNA classes are shown in Table 4. The risk of pathology increases as the DNA concentrations increase: with concentrations between 6.0 and 20.0 ng/mL the risk for illness is over 3 times higher, when compared with cDNA concentrations lower than 6 ng/mL (OR, 3.33; 95% CI, 1.29–8.58; p=0.010); high concentrations greater than 20.0 ng/mL are associated with a risk of illness of over 36 times higher (OR, 36.71; 95% CI, 15.49–86.99; p<0.0001). For the cut-off value of 20 ng/ml, logistic regression

analysis adjusted by age, gender and smoking status confirmed these associations (aOR, 12.69; 95% CI, 4.95-32.52; p<0.0001).

cDNA as a prognostic marker of NSCLC

We looked at correlation between pre-treatment circulating plasma DNA levels and survival time in 73 NSCLC patients, who received a first line chemotherapeutic protocol consisting of platin-based doublet chemotherapy in combination with a third-generation cytotoxic compound such as paclitaxel, gemcitabine or docetaxel and for which survival data was available. Survival analysis was performed according to plasma DNA levels distribution using the Kaplan-Meier method and Cox-regression analysis.

The mean survival rates were statistically different according to the patients' cDNA concentrations, indicating that patients with high cDNA levels presented a lower mean survival time than the other patients (16.8 vs 22.4 months; Log Rank test, p = 0.024). Using a multivariate Cox regression model, we found a decreased overall survival time in patients presenting high cDNA levels (above 20.0 ng/ml), when compared lower cDNA concentrations, with stage (P=0.171) as covariate (hazard ratio, HR=3.77; 95% CI: 1.16-12.28; *P*=0.028) (Figure 3).

DISCUSSION

Diagnostic assays based on blood sample analysis are becoming an area of study with growing interest, mainly because of the simplicity of sampling and the future potential of automation of the technical methods for clinical applicability. The presence of circulating tumor DNA in plasma of patients with lung cancer arouse great interest since, with a simple blood test, a valid marker could be set out for a possible screening, diagnosis, prognosis, progression of disease and the monitoring of treatment response. Unfortunately, the standardization of the test is difficult because of the lack of a suitable standard test and the use of different extraction and quantification methods (14, 18).

The presence of abnormally high levels of free circulating DNA in plasma/serum of cancer patients was first demonstrated in 1977 (19). The possibility of recovering tumor-derived DNA from the patient's blood has provoked great expectation. This method offers a non-invasive means to obtain tumor surrogate material, which could represent a unique source for diagnostic and prognostic applications. However, only recently cell free DNA is becoming an issue of growing interest and its possible use as a marker for cancer diagnosis or prognosis has been investigated. Recent studies demonstrated higher cDNA levels in lung cancer patients comparing to control individuals, however, the laboratorial methods have not been consistently evaluated, namely the procedures for DNA isolation and quantification (20). Moreover, there are some contradictory results regarding the analyses of the clinical-pathological features, such as clinical staging and the prognostic and predictive value of quantification of cell free DNA and relapse-free evaluation that should be studied in more detail (7, 11, 21). A number of methods have been described to assess the amount of DNA present in plasma, including competitive binding radioimmunoassay, SYBER green, PicoGreen and others, but most lack the

sensitivity and specificity required. In the present work, we used real-time PCR for DNA quantification, which can be regarded as the standard method currently available (22).

In our study, we found no statistically significant differences in cDNA concentrations between probands in different histological groups and tumor stages. However, there is some controversy in the relationship between DNA levels and the clinical-pathological features (23). Conflicting data have also been reported about circulating DNA as a prognostic factor in lung cancer patients. Some studies showed a correlation between an elevated plasma DNA concentration and poor survival (11, 24), whereas other studies did not report such a relationship (16, 22). This might be explained by differences in patient selection, covering both non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), and both treated and untreated patients. Moreover, techniques for sample collection and DNA quantification differed between these studies. Thus, at present, the prognostic value of circulating DNA for survival has not been established yet. Also, the relationship of circulating DNA with age, gender, histology, stage and pulmonary inflammatory conditions is not clear. Our results are in agreement with the observations found in other studies, regarding the lack of correlation between plasma DNA levels and clinical-pathological parameters (11). A correlation with age was reported in some (1, 13), but not in all studies (16, 24). Similarly, no correlation has been established with histologic subtypes (16, 25-27). Disparity also exists with regard to variables such as clinical staging. Some studies report that plasma DNA was highest in patients with stage IV disease (26), whereas in other studies there was no such association (11, 13, 16, 19, 25, 27). As already discussed, these discrepancies in results across the studies might be due to technical differences prevalent in various studies. However, these differences provide an insight into the present status of

circulating DNA research and of the need for larger controlled studies with standardized procedures.

We found increased levels of circulating DNA in NSCLC patients compared to control individuals. Similar results were also found in other studies, with higher DNA plasma concentrations found in cancer patients when compared to healthy individuals (13, 16, 22, 28-30). According to the majority of the quantitative studies performed until the present time, cell free circulating DNA is observed in healthy subjects at concentrations between 0 and 100 ng/ml of blood with an average of 30 ng/ml, whereas in cancer patients the concentration in plasma or in serum varies between 0 and 1000 ng/ml, with an average of 180 ng/ml (12). However, these values constitute just a reference to evaluate cell free DNA concentrations, as the percentage of circulating DNA originated from tumor cells can vary from one cancer model to another (12). The plasma DNA concentrations observed in this study were similar to the cell free DNA level averages described in the literature (1, 11, 16, 31).

The presence of free circulating DNA can be found in patients with malignant pathologies, but also in healthy individuals and in patients with non-malignant diseases, namely erythematic lupus, rheumatoid arthritis, lung embolism, myocardium infarction, traumas or invasive therapeutics practices (32-34). However, DNA concentrations seem to be much higher in patients with malignant pathologies (23, 34). There are various advanced hypotheses about the release mechanisms of DNA in circulation. For healthy individuals, it is presumed that circulating DNA originates from the death of lymphocytes or other nucleated cells, whereas for patients with neoplastic pathologies, there were several hypothesis, namely lysis of tumor cells (12).

The purpose of our study was to confirm the results of the previous researches about the free circulating DNA's capacity of differentiating healthy

individuals from patients with NSCLC cancer and to validate the method used for future clinical application.

Increased plasma cDNA concentration was associated with a higher probability of lung cancer presence. To our knowledge, similar OR values were never reported previously for any biologic marker and could be of substantial benefit in clinical practice. Our study has confirmed the literature data, indicating that higher DNA concentrations can be correlated with the condition of the disease.

High plasma DNA levels were also associated with decreased overall survival. Although there are conflicting results (28), some studies also found a correlation between an elevated plasma DNA concentration and poor survival (11, 22, 24, 29).

A recent study has demonstrated that the levels of cell free DNA present in cancer patients constitute a stable parameter over time and its variations may be due to real clinical alterations in the patient, as long as all the technical and methodological steps are controlled (35). Therefore, it becomes important to carefully monitor the methods for blood sampling, plasma DNA isolation and DNA quantification. Some quantification methods, such as colorimetric kits, are not based on the amount of amplifiable DNA but in the total amount of nucleic acids, which can include double-stranded and single-stranded DNA and RNA (13). The differences of the results observed in literature may also reflect biological causes (histology, tumor origin, stage or tumor size) or technical issues (blood processing, cell free DNA isolation and quantification). There is insufficient data for the comparison of all these parameters between different types of cancer, as it is still unclear, whether all types of cancer can release altered cell free DNA and at the same rate (12). It has been demonstrated that clearance of cell free DNA from the bloodstream seems to be a rapid process,

and one possible explanation is based on the cell free DNA sensibility to plasma nucleases, such as DNAse1 (36). Moreover, a study has demonstrated that, for a patient with a tumor load of 100g in size, up to 3.3% of the tumor DNA entered the circulation every day (37). In another study, mutations in *TP53* or *KRAS2* were detected in whole blood at least one year before diagnosis in 67% of patients, which indicates that tumor DNA is released in blood before conventional diagnosis and enhances the importance of this marker in cancer prevention (38).

We think that the results are promising enough to encourage further research in the area of circulating DNA as a tool for monitoring therapeutic efficacy in lung cancer patients. Identification of additional, more specific, and more sensitive plasma-based biomarkers, which can be used in combination with circulating DNA, may further improve the diagnostic power of current imaging tools for indicating therapeutic efficacy.

Conclusions

This study demonstrates that higher levels of free circulating DNA can be detected in patients with lung cancer compared to healthy individuals by a quantitative PCR assay. We have shown that the analytic procedure reported by Sozzi et al (13) is useful to discriminate between healthy subjects and NSCLC patients. The reproducibility of this method could represent a first step for its future application in the clinical practice.

Although some concerns regarding the sample size and age of case and control groups may be evaluated, this study suggests a non-invasive diagnostic tool that can be further investigated in future prospective studies for detection of lung cancer and assessment of its potential applicability as a complementary diagnostic and prognostic tool in clinical practice. We believe that our results may help to improve discussion between researchers in the field and that

different methodological approaches must be considered regarding the real meaning of cDNA in lung cancer patients.

Levels of circulating DNA could also identify higher-risk individuals for this disease screening and chemoprevention trials. Furthermore, plasma DNA could also be used to detect cancer specific molecular markers, such as mutations and amplifications, and individualize and monitor drug treatment, namely resistance to targeted therapies without the need for repeated tumor biopsies in metastatic disease.

We found a significant decrease in the overall survival for higher concentrations of cell free circulating DNA and a very significant increase in the patients' risk for the presence of lung cancer in individuals with high cDNA levels. Cell free circulating DNA may represent an important source of biomarkers at several steps of carcinogenesis, including early detection of preneoplastic lesions and monitoring of cancer. Moreover, levels of plasma DNA could be tested as a potential intermediate biomarker of the efficacy of intervention.

Large-scale prospective studies are necessary for population-based studies and molecular epidemiologic studies, in order to implement a clinical application in lung cancer detection, diagnosis, prognosis and prediction of treatment response.

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Tables

Table 1 – Correlation between circulating plasma DNA levels and demographic characteristics of the subjects

Characteristics	Mean rank of plasma DNA level (ng/ml)			
	Cases (n=104)	P* value	Controls (n=205)	P* value
Age (years)				
< 64	54.70	0.614	101.48	0.342
≥ 64	51.68		69.33	
Gender				
Female	43.95	0.123	91.57	<0.001
Male	55.72		121.62	
Smoking history				
No	50.33	0.688	58.72	0.217
Yes	53.15		66.79	

* Mann-Whitney test

Table 2 – Correlation between circulating plasma DNA levels and clinic-pathological parameters of NSCLC patients

Characteristics	Mean rank of plasma DNA level (ng/ml)	
	Cases (n=104)	P* value
Histological type		
Epidermoid	51.91	0.981
Non-Epidermoid	52.05	
Tumor stage		
I/II	64.25	0.427
III/IV	52.03	

* Mann-Whitney test

Table 3 – Test performance

Cut-off value (ng/ml)	Sensitivity	Specificity	PPV	NPV
3	100.0	25.85	41.08	100.0
6	93.40	50.73	49.50	93.69
9	92.45	63.90	56.98	94.24
12	89.62	68.78	59.75	92.76
15	87.74	74.63	64.14	92.17
20	79.24	83.41	71.19	88.60

Table 4 – Frequency and *odds ratio* of plasma DNA concentrations among the control group and NSCLC patients

cDNA levels (ng/ml)	**Controls (n=205)** N (%)	**Patients (n=104)** N (%)	**OR***	**95% CI***	**P***
< 6	104 (93.7)	7 (76.3)	1.00	Reference	–
[6-20[	67 (81.7)	15 (18.3)	3.33	1.29 – 8.58	0.010
≥ 20	34 (28.8)	84 (71.2)	36.71	15.49 – 86.99	< 0.0001
< 20	171 (88.6)	22 (11.4)	1.00	Reference	–
≥ 20	34 (28.8)	84 (71.2)	19.20	10.58 – 34.87	< 0.0001

*For 20 ng/ml cut-off value, P<0.0001, OR=12.69 and 95% CI: 4.95-32.52, using logistic regression analysis adjusted by age, gender and smoking status

Figures

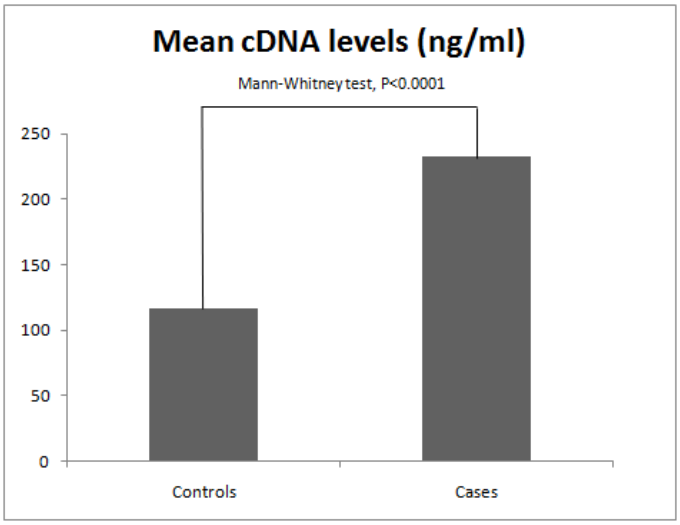


Figure 1 - Mean rank of cDNA plasma concentrations in pre-treatment NCLC patients and control individuals. *Mann-Whitney U-test.

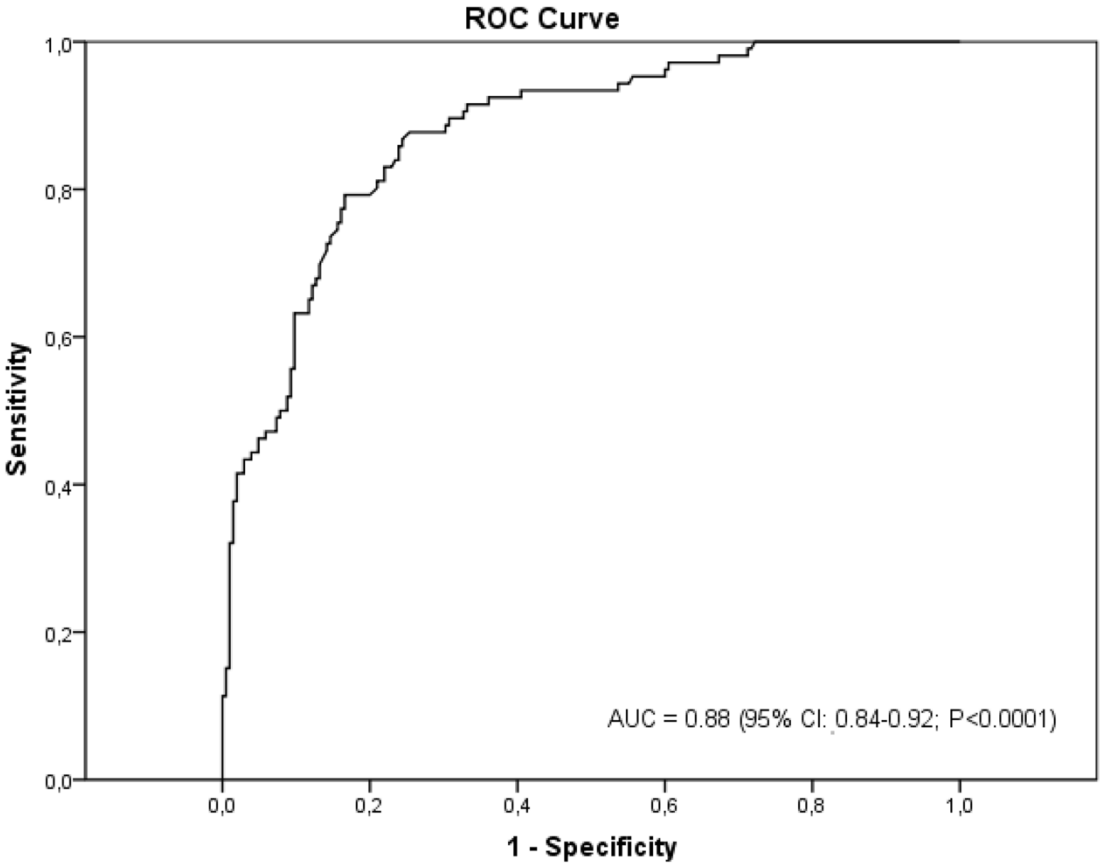


Figure 2 – Receiver-operating characteristics (ROC) curve to calculate sensitivity and specificity of circulating plasma DNA as a tumor marker of NSCLC.

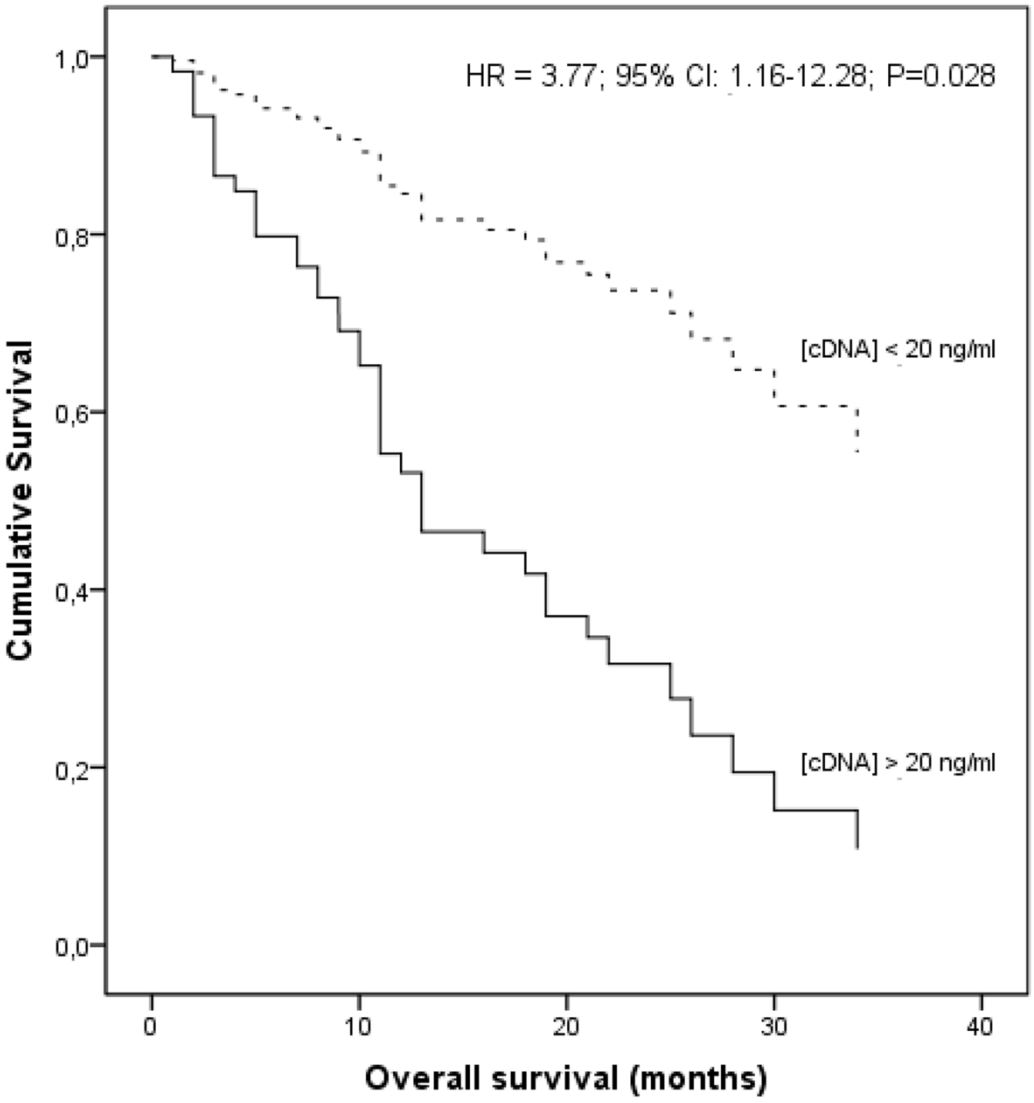


Figure 3 – Cox regression analysis of overall survival according to cDNA levels, with tumor stage as covariate.

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Quantification of Free Circulating Tumor DNA as a Diagnostic Marker for Breast Cancer

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Aim: To determine whether the amounts of circulating DNA could discriminate between breast cancer patients and healthy individuals by using real-time PCR quantification methodology. *Methods:* Our standard protocol for quantification of cell-free plasma DNA involved 175 consecutive patients with breast cancer and 80 healthy controls. *Results:* We found increased levels of circulating DNA in breast cancer patients compared to control individuals (105.2 vs. 77.06 ng/mL, $p < 0.001$). We also found statistically significant differences in circulating DNA amounts in patients before and after breast surgery (105.2 vs. 59.0 ng/mL, $p = 0.001$). Increased plasma cell-free DNA concentration was a strong risk factor for breast cancer, conferring an increased risk for the presence of this disease (OR, 12.32; 95% CI, 2.09–52.28; $p < 0.001$). *Conclusions:* Quantification of circulating DNA by real-time PCR may be a good and simple tool for detection of breast cancer with a potential to clinical applicability together with other current methods used for monitoring the disease.

Introduction

Breast cancer (BC) is the most common cancer in the Western female population with more than 1,152,161 of new cases each year and 411,093 cancer deaths per year (Kamangar *et al.*, 2006). The world age-standardized incident rate in the year 2000 was 35.7 per 100,000 individuals (Baselga and Norton, 2002). This disease represents the second leading cause of death by cancer. Although its incidence rate has been clearly increasing, due to population aging, the mortality rate has not increased in the same proportion. Estimates from randomized clinical trials and population-based models suggest that early detection and improved treatment might reduce breast cancer mortality rates by 25–30% (Kamangar *et al.*, 2006). For this reason, there is a need to develop new approaches that may facilitate earlier diagnosis and more effective treatments.

Increased knowledge of molecular pathogenesis of breast cancer offers a basis for the use of molecular markers in biologic fluids for early detection, as well as identification of higher-risk individuals.

The presence of extracellular nucleic acids in the human bloodstream was first described in 1948 by Mandel and Métais (Mandel and Métais, 1948). In recent years, many studies identified genetic alterations in cell-free circulating DNA (fcDNA) from plasma or serum and tumor DNA in many tumor types, such as lung cancer (Esteller *et al.*, 1999), breast cancer (Silva *et al.*, 1999), colon cancer (Anker *et al.*, 1997; Kopreski *et al.*, 1997; Hibi *et al.*, 1998), and liver cancer (Wong *et al.*, 1999). The genetic alterations include *K-ras*, *N-ras*, and *p53* gene mutations, aberrant promoter hypermethylation of tumor suppressor genes, and changes in microsatellites detected by polymorphic markers. Other types of circulating nucleic acids, such as viral DNA, mitochondrial DNA, and mRNA, constitute promising tumor biomarkers, as well as free circulating DNA.

Previous studies indicate that circulating nucleic acids in peripheral blood are originated from tumors, through apoptosis, necrosis, or cell lysis of tumor cells (Anker *et al.*, 1999; Gautschi *et al.*, 2004). Some authors indicate that fcDNA shows the typical ladder profile of apoptotic cells (Jahr *et al.*, 2001). However, it is common to detect large, quasi-genome-size DNA fragments, which can be explained by the lysis of circulating tumor cells (Gautschi *et al.*, 2004; Gormally *et al.*, 2007). The presence of tumor DNA in blood is probably the result, in variable proportions, of these different mechanisms, such as apoptosis, necrosis, cell lysis, and circulating tumor cells lysis, which produce DNA leakage or excretion.

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There are multiple potential uses for fcDNA quantification in cancer diagnosis and prognosis. For instance, it may represent a valuable source of tumor DNA when the exact position of a suspected primary lesion is not clearly defined, or when biopsies are not available (Gormally *et al.*, 2007). However, direct comparison of the available data is often prevented by differences in the parameters analyzed, and by lack of standardized methodology and analysis procedures. Any future application of plasma or serum DNA analysis for diagnostic purposes will depend on the reproducibility and reliability of results, both of which require optimization and equivalent procedures. For this study, we selected an assay designed for the human telomerase reverse transcriptase (*hTERT*) genomic sequence that was performed consistently in a previous study (Sozzi *et al.*, 2003). Amplification of *hTERT* was therefore used as a marker of the total amount of DNA present in plasma samples. Our working hypothesis was based on the use of a single-copy gene, such as *hTERT*, as an indicator of the global amount of circulating DNA.

The quantitative analysis fcDNA in plasma may constitute an important noninvasive diagnostic tool, requiring only a limited blood sample.

The purpose of our study was to determine whether the amounts of circulating DNA could discriminate between breast cancer patients and healthy individuals by using real-time PCR-based DNA quantification methodology and determine the kinetics of circulating plasma DNA in surgically treated patients.

Materials and Methods

Patients and controls series

We evaluated the amount of fcDNA in plasma samples of 255 women, including 175 consecutive patients with breast cancer, subjected to breast surgery and 80 healthy controls, admitted at the Portuguese Institute of Oncology, Porto. Patients with a confirmed diagnosis of breast cancer and healthy individuals gave their informed consent to be included in the study.

Sample collection and DNA isolation

A 5 mL sample of peripheral blood extracted from each patient was collected in tubes containing EDTA, and plasma was immediately separated from the cellular fraction by centrifugation at 2500 rpm for 10 min at 4°C. The resulting supernatant (plasma) was frozen at −80°C. DNA was extracted from plasma by using QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The purified DNA from 200 μL of plasma was eluted in a final volume of 70 μL of buffer AE used for the elution and stored at −20°C.

DNA quantification in plasma

The quantification of fcDNA was performed by a real-time quantitative PCR method, based on a continuous monitoring of fluorescence by an optical system (Heid *et al.*, 1996). The probe is labeled by two fluorescent dyes, one serves as a reporter on 5′ end (VIC dye; Applied Biosystems, Foster City, CA). The emission spectrum of the dye is quenched by a second fluorescent dye at the 3′ end (TAMRA; Applied Biosystems). Primers and probes were designed previously by Sozzi *et al.* to amplify the gene of interest, the *hTERT* single-copy gene mapped on 5p15.33 (Sozzi *et al.*, 2003). The primers and probe's sequences were the following: primer forward, 5′-GGC ACA CGT GGC TTT TCG-3′; primer reverse, 5′-GGT GAA CCT CGT AAG TTT ATG CAA-3′; probe, VIC5′-TCA GGA CGT CGA GTG GAC ACG GTG-3′ TAMRA.

Fluorogenic PCRs were carried out in a reaction volume of 25 μL in a 7300 ABIPRISM System (Applied Biosystems). Each PCR reaction consisted of TaqMan Universal Mastermix (Applied Biosystems), probe (7.5 mmol/L), primer forward (5 mmol/L), primer reverse (5 mmol/L), and sterile water. DNA solution (2.5 μL) was used in each real-time PCR reaction. Thermal cycling was initiated with a first denaturation step of 50°C for 2 min and then 95°C for 10 min followed by 50 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Each plate consisted of patient samples in duplicates and triplicate blanks of water as negative control. The calibration curve of each plate was calculated based on a dilution series of the TaqMan Control Human Genomic DNA Standard (Applied Biosystems) at 10 ng/μL: 1, 0.1, 0.01, 0.001 and 0.0001 ng/μL. All the data were analyzed using the 7300 System–SDS Software (version 1.2.3) Sequence Detection Software (Applied Biosystems).

Statistical analysis

Data analysis was performed using the computer software SPSS for Windows (version 12.0) (SPSS, Chicago, IL).

Linear amplification down to the last dilution was obtained in each experiment (Pearson correlation coefficient, 0.999 to 0.995).

DNA concentrations between healthy controls and patients were compared using the Mann–Whitney *U*-test. The Kruskal–Wallis test was used to compare DNA levels and patients' clinical-pathological characteristics. $p \leq 0.05$ was considered statistically significant.

Chi-square analysis was used to compare categorical variables, and a 5% level of significance was used in the analysis. The odds ratio (OR) and its 95% confidence interval (CI) were calculated as a measurement of the association between the amount of fcDNA and probability of breast cancer presence. Logistic regression analysis was used to calculate the adjusted OR (aOR) and 95% CI, with adjustment for age. We established fcDNA concentration cut-off values, according to the patients' distribution percentiles. For p50 (median fcDNA concentration—high fcDNA concentration), the DNA concentration was 106.0 ng/mL, and for p75 (very high fcDNA concentration), the DNA concentration was 171.6 ng/mL.

The probabilities of survival were calculated, and the means and life tables were computed using the product-limit estimate of Kaplan and Meier. The curves were examined by the Breslow (generalized Wilcoxon) test, a statistical test for equality of survival distributions. Survival duration was defined as the time between diagnosis and either death or the last clinical evaluation of the patient. Clinical-pathological data and cause of death were determined from the patient's medical records.

Results

Patients' clinical-pathological parameters and circulating DNA levels

Our study was developed to investigate fcDNA by a real-time PCR quantitative approach, performing all the technical

Table 1. Patients' Clinical-Pathological Characteristics

Characteristics	*Patients* (n = *175*) *N* (%)	*Controls* (n = *80*) *N* (%)
Age, years		
Age ± standard deviation	49 ± 14.8	38 ± 11.9
Histology		
Ductal invasive carcinoma	125 (71.4)	
Lobular invasive carcinoma	7 (4.0)	
Medular carcinoma	2 (1.1)	
Papillary carcinoma	2 (1.1)	
Ductal *in situ*	5 (2.9)	
Other	34 (19.5)	
Tumor stage		
I	34 (19.4)	
II	69 (39.4)	
III	39 (22.2)	
IV	7 (4.0)	
Data not available	26 (15.0)	
Alive	135 (77.1)	
Dead	17 (9.7)	
Data not available	23 (13.2)	

procedures necessary for accurate and reproducible results. The clinical-pathological parameters of breast cancer patients and age distribution among case and control groups are shown in Table 1. There were no statistically significant differences in fcDNA concentrations between probands in different histological groups and tumor stages (Table 2).

fcDNA levels in breast cancer patients and controls

The quantification of fcDNA was performed as previously described, by a real-time quantitative PCR assay with a diagnostic performance accessed in a previous study (Sozzi *et al.*, 2003). This quantitative assay shows a strong discrimination power and a high sensitivity and specificity using the *hTERT* gene as a target sequence for quantification of fcDNA in plasma. For patients with two or more samples collected after or before surgery, the mean fcDNA concentration was calculated. The mean fcDNA plasma concentration in the control group, 77.1 ng/mL, was statistically different from the mean fcDNA plasma concentration of the patient group before surgery (105.2 ng/mL) ($p = 0.0004$). Patients subjected to preoperative chemotherapy were not considered in the analysis. Our results demonstrate that the mean DNA quantity is significantly lower in patients whose blood was collected after surgery comparing with the group before surgery (105.2 vs. 59.0 ng/mL) ($p = 0.001$) (Fig. 1).

Table 2. Plasma DNA Levels and Patients' Characteristics

Characteristics	*Mean rank of plasma DNA level (ng/mL)*	p[a] *value*
Histology		
Ductal invasive carcinoma	77.98	0.644
Lobular invasive carcinoma	82.14	
Medular carcinoma	39.00	
Papillary carcinoma	65.00	
Ductal *in situ*	85.00	
Other	61.18	
Tumor stage		
I	73.07	0.637
II	76.95	
III	70.27	
IV	91.50	

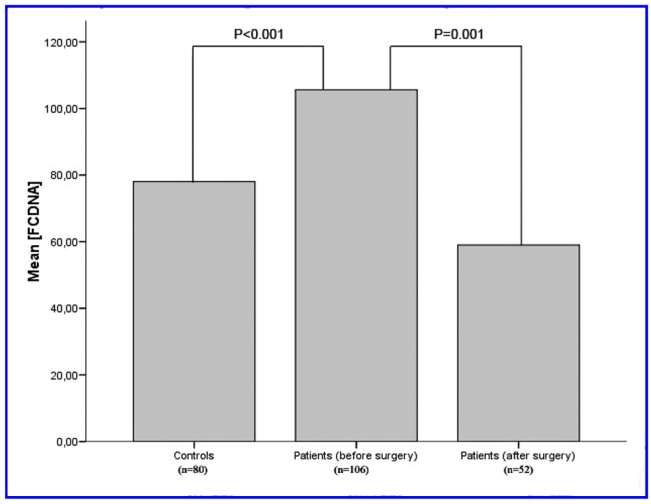


FIG. 1. Mean rank of fcDNA plasma concentration in controls, patients before surgery, and patients after surgery. *Mann–Whitney *U*-test.

fcDNA concentration as a risk factor for breast cancer

An elevated concentration of plasma fcDNA was associated with a higher chance for the presence of breast cancer. Our results indicate that high fcDNA and very high fcDNA concentrations are more prevalent among cancer cases, comparing to control individuals (Table 3). Data analysis demonstrates that women with high and very high fcDNA concentrations present an increased chance for the presence of breast cancer, of 8- and 12-fold, respectively (OR, 8.01; 95% CI, 3.49–18.38; $p < 0.001$ for high [fcDNA], and OR, 12.32; 95% CI, 2.09–52.28; $p < 0.001$, for very high [fcDNA]). Logistic regression analysis adjusted by age confirmed these associations.

Circulating plasma DNA and global survival

Regarding the overall survival rates using Kaplan–Meier methodology (Fig. 2), we observed the presence of very high fcDNA concentrations in the influence of overall survival of breast cancer patients.

The mean survival rates were statistically different according to the patients' fcDNA concentrations, indicating that patients with very high fcDNA concentrations presented a lower mean survival time than the other patients (79.3 vs. 106.2 months; $p = 0.05$). The overall follow-up time was 156 months, and the number of patients censured in the analysis was 91 patients with very high DNA concentrations and 24 patients with lower DNA levels. The estimated 5-year

Table 3. Frequency and Odds Ratio of Plasma DNA Concentrations among the Control Group and Breast Cancer Patients

		Patients (n = *175*) *N* (%)	*Controls* (n = *80*) *N* (%)	*OR*[a]	*95% CI*[a]	p[a] *value*
High [fcDNA]	No	99 (56.6)	73 (91.2)	8.01	3.49–18.38	<0.001
	Yes	76 (43.4)	7 (8.8)			
Very high [fcDNA]	No	133 (76.0)	78 (97.5)	12.32	2.90–52.28	<0.001
	Yes	42 (24.0)	2 (2.5)			

[a]For high [fcDNA], $p < 0.001$, OR = 6.48, and 95% CI: 2.76–15.20; for very high [fcDNA], $p = 0.003$, OR = 9.30, and 95% CI: 2.14–40.35, using logistic regression analysis adjusted by age.

survival rate was 79.3% for women presenting very high fcDNA concentrations, compared with 89.60% for the other patients who did not present these fcDNA values.

Discussion

Diagnostic assays based on blood sample analysis are becoming an area of study with growing interest, mainly because of the simplicity of sampling and the future potential of automation of the technical methods for clinical applicability. The presence of abnormally high levels of free circulating DNA in plasma/serum of cancer patients was first demonstrated in 1977 (Leon *et al.*, 1977). The possibility of recovering tumor-derived DNA from the patient's blood has provoked great expectation. This method offers a noninvasive means to obtain tumor surrogate material, which could represent a unique source for diagnostic and prognostic applications. However, only recently cell-free DNA is becoming an issue of growing interest and its possible use as a marker for cancer diagnosis or prognosis has been investigated. There are some reports in breast cancer models with interesting results about this subject, but the majority consists in qualitative analysis of plasma and serum DNA (Chen *et al.*, 1999; Shao *et al.*, 2001; Silva *et al.*, 2002). Some quantitative (Shapiro *et al.*, 1983; Lee *et al.*, 2001; Wu *et al.*, 2002; Fleischhacker and Schmidt, 2007) studies reported increased concentrations of circulating plasma DNA of cancer patients, in comparison with healthy individuals. However, the laboratorial methods have not been consistently evaluated, namely, the procedures for DNA isolation and quantification (Sozzi *et al.*, 2005). Moreover, there are some contradictory results regarding the analyses of the clinical-pathological features, such as clinical staging and the prognostic and predictive value of quantification of cell-free DNA and relapse-free evaluation that should be stud-

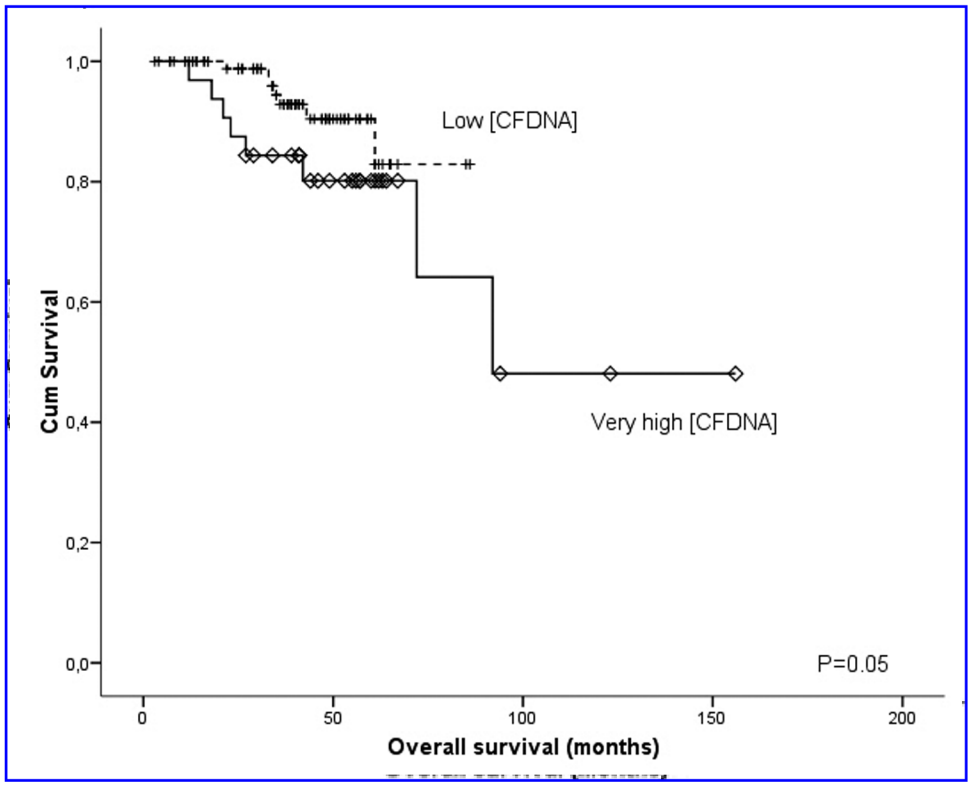


FIG. 2. Association of fcDNA concentration with overall survival by Kaplan–Meier curves and Breslow test.

ied in more detail (Silva *et al.*, 2002; Gautschi *et al.*, 2004; Fleischhacker and Schmidt, 2007). A number of methods have been described to assess the amount of DNA present in plasma, including competitive binding radioimmunoassay, SYBER green, PicoGreen, and others, but most lack the sensitivity and specificity required.

To measure with greater accuracy the fcDNA amount, a quantification approach based on real-time PCR was developed. A single-copy gene, the amplification of which is specific and robust, represents the ideal target for DNA-based quantitative real-time PCR assay. This assay is a specific and sensible method, providing an accurate quantification of low plasma DNA concentrations and minimizes the risk of carryover contamination of the post-PCR processing from conventional PCR (Sozzi *et al.*, 2003). There are promising results in recent studies performed in lung cancer models, using a more accurate method to measure the amount of free circulating DNA by real-time PCR (Sozzi *et al.*, 2003; Gautschi *et al.*, 2004). In the present work, we used real-time PCR for DNA quantification, which can be regarded as the standard method currently available.

In our study, there were no statistically significant differences in fcDNA concentrations between probands in different histological groups and tumor stages (Table 2), and these results could be due to insufficient study power. However, there is some controversy in the relationship between DNA levels and the clinical-pathological features (Ziegler *et al.*, 2002). Our results are in agreement with the observations found in other studies regarding the lack of correlation between plasma DNA levels and clinical-pathological parameters (Gautschi *et al.*, 2004).

We found increased levels of circulating DNA in breast cancer patients compared to control individuals. Similar results were also found in other studies, with higher DNA plasma concentrations found in breast cancer patients when compared to healthy individuals (Gal *et al.*, 2004; Huang *et al.*, 2006; Skvortsova *et al.*, 2006). Although the DNA concentration values measured in these studies were lower, this difference could be due to the different experimental procedures used. We also found statistically significant differences in circulating DNA amounts in patients before and after breast surgery (105.2 vs. 59.0 ng/mL, $p = 0.001$). Our results are in agreement with the findings of a previous study (Sozzi *et al.*, 2001). Sozzi *et al.* demonstrate that a relevant drop in DNA levels is already visible at 1–6 months after tumor removal and reaches the level observed in normal healthy subjects. These data suggest that in tumor-free patients, plasma DNA either is released at lower rates or is rapidly degraded.

The difference in plasma DNA levels between patients and controls is concordant with the results of other quantitative studies in other cancer models (Sozzi *et al.*, 2003; Gautschi *et al.*, 2004). According to the majority of the quantitative studies performed until present, fcDNA is observed in healthy subjects at concentrations between 0 and 100 ng/mL of blood with an average of 30 ng/mL, whereas in cancer patients the concentration in plasma or in serum varies between 0 and 1000 ng/mL, with an average of 180 ng/mL (Gormally *et al.*, 2007). However, these values constitute just a reference to evaluate cell-free DNA concentrations, as the percentage of circulating DNA originated from tumor cells can vary from one cancer model to another (Gormally *et al.*, 2007). The plasma DNA concentrations observed in this study were similar to the cell-free DNA level averages described in the literature.

Increased plasma fcDNA concentration was associated with a higher probability of breast cancer presence. To our knowledge, similar OR values were never reported previously for any biologic marker and could be of substantial benefit in clinical practice.

High plasma DNA levels were also associated with decreased overall survival ($p = 0.043$). Although there are conflicting results (Gal *et al.*, 2004), similar results were found with significant associations between high plasma DNA levels and decreased patients' survival (Gautschi *et al.*, 2004; Huang *et al.*, 2006).

The purpose of our study was to determine whether the amounts of circulating DNA could discriminate between breast cancer patients and healthy individuals by using real-time PCR-based DNA quantification methodology and determine the kinetics of circulating plasma DNA in surgically treated patients.

A recent study has demonstrated that the levels of cell-free DNA present in cancer patients constitute a stable parameter over time and its variations may be due to real clinical alterations in the patient, as long as all the technical and methodological steps are controlled (Frattini *et al.*, 2005). Therefore, it becomes important to carefully monitor the methods for blood sampling, plasma DNA isolation, and DNA quantification. Some quantification methods, such as colorimetric kits, are not based on the amount of amplifiable DNA but in the total amount of nucleic acids, which can include double-stranded and single-stranded DNA and RNA. Therefore, the results can vary significantly, and the best option is, in our opinion, the quantification by real-time PCR (Sozzi *et al.*, 2003). The differences of the results observed in literature may also reflect biological causes (histology, tumor origin, stage, or tumor size) or technical issues (blood processing, cell-free DNA isolation, and quantification). There are insufficient data for the comparison of all these parameters between different types of cancer, as it is still unclear whether all types of cancer can release altered cell-free DNA and at the same rate (Gormally *et al.*, 2007). It has been demonstrated that clearance of cell-free DNA from the bloodstream seems to be a rapid process, and one possible explanation is based on the cell-free DNA sensibility to plasma nucleases, such as DNAse1 (Lo *et al.*, 1999). Moreover, a recent study has demonstrated that for a patient with a tumor load of 100 g in size, up to 3.3% of the tumor DNA entered the circulation every day (Diehl *et al.*, 2005). In another study, mutations in *TP53* or *KRAS2* were detected in whole blood at least 1 year before diagnosis in 67% of patients, which indicates that tumor DNA is released in blood before conventional diagnosis and enhances the importance of this marker in cancer prevention (Mao *et al.*, 1994).

Conclusions

This study demonstrates that higher levels of free circulating DNA can be detected in patients with breast cancer compared to healthy individuals by a quantitative PCR assay.

Although some concerns regarding the sample size and age of case and control groups need to be evaluated, this study suggests a noninvasive diagnostic tool that can be further investigated in future prospective studies for detection of breast cancer and assessment of its potential applicability as a

complementary diagnostic tool in clinical practice. We believe that our results may help to improve discussion between researchers in the field and that different methodological approaches must be considered regarding the real meaning of fcDNA in breast cancer patients.

Levels of circulating DNA could also identify higher-risk individuals for this disease screening and chemoprevention trials. Further, plasma DNA could also be used to detect cancer specific molecular markers, such as mutations and amplifications, and individualize and monitor drug treatment, namely, resistance to targeted therapies without the need for repeated tumor biopsies in metastatic disease.

We found a significant decrease in the overall survival for higher concentrations of fcDNA and a very significant increase in the patients' risk for the presence of breast cancer in women with high fcDNA levels. fcDNA may represent an important source of biomarkers at several steps of carcinogenesis, including early detection of preneoplastic lesions and monitoring of cancer. Moreover, levels of plasma DNA could be tested as a potential intermediate biomarker of the efficacy of intervention.

Large-scale prospective studies are necessary for population-based studies and molecular epidemiologic studies to implement a clinical application in breast cancer detection, diagnosis, prognosis, and prediction of treatment response.

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3.2 GENETIC VARIABILITY IN CANCER

Clinical Cancer Research

Prognostic Significance of Telomerase Polymorphism in Non–Small Cell Lung Cancer

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Abstract

Purpose: Lung cancer is the leading cause of death in oncologic patients of western countries, with very low survival rates. Telomerase main components are the catalytic subunit (hTERT) and the RNA template (hTR). A functional polymorphism in the *hTERT* gene was found in the promoter region (−1327T/C), and individuals homozygous for the −1327C/C genotype present shorter telomere length compared with T-carrier genotypes. Our purpose was to investigate the potential prognostic role of the *hTERT* functional genetic variant in non–small cell lung cancer (NSCLC) patients.

Experimental Design: We prospectively conducted a study involving 226 patients with NSCLC treated with a first-line chemotherapeutic standard protocol. A follow-up study was undertaken (median follow-up time, 26 months) to evaluate treatment response and overall survival of NSCLC patients. The *hTERT* −1327T/C genetic variants were analyzed by allelic discrimination with real-time PCR.

Results: Our results indicate an influence of the telomerase genetic variants in the overall survival of NSCLC patients. Cox regression analysis showed a significantly higher median estimated cumulative survival of 26.5 months in T-carrier patients, compared with that of 19.3 months in CC patients (hazard ratio, 0.52; 95% confidence interval, 0.35-0.77; $P = 0.001$).

Conclusions: Telomerase functional polymorphism in the *hTERT* gene may contribute as a prognostic factor in NSCLC patients. Our findings indicate that *hTERT* genetic variants, by modulating telomere length, may confer an advantage in chemotherapy response. The assessment of telomerase genetic variants could supplement prognosis of survival in the course of NSCLC and may be a promising molecular marker of treatment response in these patients. *Clin Cancer Res; 16(14); 3706–12. ©2010 AACR.*

Lung cancer is the leading cause of death in oncologic patients in western countries (1). Approximately 85% of lung cancer patients have a histologic diagnosis of non–small cell lung cancer (NSCLC). Due to the delay in clinical diagnosis, only a relatively small proportion of patients (20-25%) have a resectable disease at presentation and the 5-year estimated survival rates are disappointing (2–4). Therapeutic failure and the side effects of anticancer therapy remain very important issues for future research (5). Therefore, the study of tumor and patient genetic profiles, relative to drug-related genes, may offer new opportunities for tailoring treatments.

Telomeres are repetitive, noncoding DNA [TTAGGG] elements at the ends of chromosomes. Telomerase is a multimeric ribonucleoprotein complex consisting of at least a functional RNA (hTR) that contains the template region complementary to the telomeric sequence and a reverse transcriptase protein component (hTERT) that catalyzes the addition of telomeric repeats to the ends of chromosomes (6–9). In most human somatic cells, telomeres become shorter with each cell division due to incomplete lagging DNA strand synthesis and oxidative damage (10, 11). Telomere lengths are affected by their starting set points, the cellular activity of telomerase, the cell's history of cell division, and environmental effects. Among the different molecular factors implicated in carcinogenesis, telomere dysfunction emerges as an early event associated with genetic instability. Genetic integrity is gradually lost as telomeres progressively become shorter with each cell replication cycle (12). This telomere shortening is a result of end-replication problems caused by DNA polymerase having difficulty in replicating the very ends of linear DNA. Telomere shortening may induce cells to undergo apoptosis or may induce chromosomal instability (10). In fact, telomere dysfunction (short telomeres) has been associated with the initiation and progression of human neoplasia (13).

Activations of the hTERT or alternate lengthening of telomeres (ALT) pathways are obligate for senescence bypass and for neoplastic transformation of normal cells (14).

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Translational Relevance

One of the main aims of clinical or translational research in cancer is the search for genetic factors that could foresee treatment outcomes, in biological activity and toxic effects. The assessment of telomerase genetic variants could supplement prognosis of survival in the course of non–small cell lung cancer and may be a promising molecular marker of treatment response in these patients. This genetic analysis might allow the selection of patients who would have the greatest benefit from chemotherapy. Furthermore, a better knowledge of the underlying molecular profile of the host and the tumor will facilitate screening for lung cancer susceptibility and tailoring of chemotherapy in individual patients, choosing those most likely to respond, adjusting doses more precisely to reduce less adverse effects, and establishing safety profiles based on individual genetic analyses.

Telomerase activity or hTERT expression or both are increased in cancers and both are prognostic factors in various cancer types (15–22). Furthermore, several studies using animal models and human NSCLC tissues have reported that TERT mRNA and TERT protein are overexpressed in lung cancer biopsies compared with normal lung tissues (23–25).

Although telomere shortening is inversely associated with age, telomere length has been found to vary considerably in human peripheral blood lymphocytes from individuals of the same age (26, 27). Matsubara and colleagues (28) screened the promoter region of *hTERT* for functional polymorphisms, and a frequent T to C transition was found 1,327 bp upstream the transcription starting site (−1327T/C). Individuals homozygous for the −1327C/C genotype showed lower telomerase activity and shorter telomere length in their peripheral leucocytes compared with the −1327T/T and −1327T/C genotypes.

The purpose of our study was to determine whether *hTERT* genetic variants are of prognostic and/or predictive value in NSCLC patients who have undergone a platin-based doublet chemotherapy in combination with a third-generation cytotoxic compound.

There are few studies regarding the *hTERT* −327T/C polymorphism. So far, this genetic variant has been associated with telomerase activity, telomere length, and coronary artery disease development (28–30). To the best of our knowledge this is the first study reporting a role of these telomerase genetic variants in cancer, specifically in NSCLC patients.

Materials and Methods

Study population

Starting in 1997, 226 consecutive Caucasian patients admitted to the Portuguese Institute of Oncology of Porto (IPO-Porto), Portugal, with cytologically or histologically confirmed NSCLC, were prospectively recruited to the study (median age, 63.5 years; mean age, 62.5 years; SD, 10.2). The recruited NSCLC patients were divided in two groups, according to tumor stage and treatment of the disease: stage I and II patients with surgical resection done at IPO-Porto, and stages III and IV patients treated with platinum-based chemotherapy between 1997 and 2009, which had follow-up data. The patients were evaluated according to the tumor-node-metastasis staging system, and the assessment of tumor response to chemotherapy was based on the Response Evaluation Criteria in Solid Tumors. The first-line chemotherapeutic protocol consisted of platin-based doublet chemotherapy in combination with a third-generation cytotoxic compound such as paclitaxel, gemcitabine, or docetaxel. The chemotherapeutic protocols were as follows: cisplatin (80 mg/m^2 on day 1) plus paclitaxel (175 mg/m^2 on day 1 every 3 weeks); cisplatin (100 mg/ m^2 on day 1) plus gemcitabine (1,250 mg/m^2 on days 1 and 8 every 3 weeks); carboplatin [area under curve (AUC) 6 on day 1] plus paclitaxel (175 mg/m^2 on day 1 every 3 weeks); carboplatin (AUC 6 on day 1) plus gemcitabine (1,000 mg/m^2 on days 1 and 8 every 3 weeks).

The median follow-up time was 26 months (range, 1-135 months). Patients' distribution according to the stage at the time of diagnosis was 29 patients (12.5%) presenting localized disease (stages I and II) and 203 (87.5%) with advanced disease (stages III and IV). Considering the patients' gender, 49 (21.1%) were female and 183 (78.9%) were male individuals. Regarding smoking habits, 63 (27.2%) were nonsmokers and 169 (72.8%) were smokers or former smokers. For all patients, the histologic type distribution was as follows: 87 patients (37.5%) with epidermoid NSCLC, 112 (48.3%) with NSCLC adenocarcinoma, and 33 (14.2%) with other NSCLC histologic types.

This study was conducted according to Helsinki Declaration principles. Antecubital peripheral venous blood sample was collected from each subject at the time of recruitment. DNA was extracted from peripheral blood samples using the QIAamp DNA Mini Kit (Qiagen), according to the manufacturer's protocol.

hTERT −1327 T/C genotyping

The *hTERT* −1327 T/C polymorphism (rs 2735940) was analyzed by allelic discrimination with real-time PCR, through the 5′-nuclease assay (TaqMan) using the ABI Prism 7300HT Sequence Detection System (Applied Biosystems). Assay and PCR conditions were according to the included protocol except that PCR was run on 10 ng DNA in a 10 μL reaction volume. PCR plates were read and data were analyzed using Allelic Discrimination Program (SDS v2.1 software, Applied Biosystems).

Quality control procedures implemented for genotyping included double sampling in about 10% of the samples to assess reliability and the use of negative controls to prevent false positives. Two authors independently

reviewed the genotyping results, data entry, and statistical analyses.

Statistical analysis

Genotype proportions among groups were compared with Pearson χ^2 test. Overall survival was the end point of this analysis and was calculated from the date of diagnosis to the patient's date of death. Data were collected from patients' medical records. The associations between *hTERT* polymorphism and survival were estimated by Cox regression analysis. Cox regression models were used to adjust for potential confounders with *hTERT* genotypes fitted as indicator variables. Analysis of data was done using the computer software SPSS for windows (version 13.0).

Results

Table 1 describes the genotype distributions of the *hTERT* −1327C/T functional polymorphism among NSCLC cases. The frequencies of CC, CT, and TT genotypes were 0.25, 0.43, and 0.32, respectively. Using the recessive model, we found the frequency of the CT and TT genotypes (T-carrier genotypes) to be 0.75. Observed versus expected genotype frequencies were calculated, and no deviation from Hardy-Weinberg equilibrium was observed ($P = 0.33$).

We found no statistically significant differences in genotype distributions according to the patients' clinicopathologic characteristics, namely, histologic type (epidermoid and nonepidermoid cases; $P = 0.870$) and tumor stage (stages I/II and III/IV; $P = 0.837$). Moreover, the *hTERT* genotype frequencies did not differ significantly among NSCLC cases considering smoking habits (smoker/former smoker and nonsmoker cases; $P = 0.061$), gender (male and female; $P = 0.174$), and age (age <64 years and >64 years; $P = 0.246$).

Regarding survival analysis, Fig. 1 presents the Cox regression analysis of survival curves of patients with III and IV tumor stages considering *hTERT* genotypes. Using a multivariate Cox regression model, we found an increased overall survival time for T-carrier patients, when compared with CC genotype, with histologic type ($P = 0.523$), gender ($P = 0.691$), smoking status ($P = 0.707$), and age ($P = 0.459$) as covariates [hazard ratio (HR), 0.52; 95% confidence interval (95% CI), 0.35-0.77; $P = 0.001$; Table 2). The median estimated cumulative survival in T-carrier patients was significantly higher at 26.5 months, compared with that of CC patients at 19.3 months (Fig. 1).

This difference was more evident when considering nonepidermoid tumor histologic type, with a statistically significant increase in overall survival of patients with T-carrier genotypes, compared with CC genotype, with an estimated median overall survival of 29.8 months for T-carrier patients and 19.3 months for CC patients (HR, 0.46; 95% CI, 0.27-0.78; $P = 0.004$), adjusted by gender ($P = 0.689$), smoking status ($P = 0.675$), and age ($P = 0.872$; Fig. 2).

Regarding epidermoid histologic type, we found no association between *hTERT* genotypes and overall survival (HR, 0.53; 95% CI, 0.27-1.05; $P = 0.068$). We found no

Table 1. *hTERT* −1327C/T genotype frequencies among NSCLC patients, according to the patients' characteristics

	hTERT genotypes, *n* (%)			*P**	hTERT genotypes, *n* (%)		*P**
	CC	CT	TT		CC	T carrier	
Histology							
Epidermoid	22 (26.2)	35 (41.7)	27 (32.1)	0.870	22 (26.2)	62 (73.8)	0.617
Nonepidemoid	33 (23.2)	63 (44.4)	46 (32.4)		33 (23.2)	109 (76.8)	
Stage							
I/II	8 (27.6)	13 (44.8)	8 (27.6)	0.837	8 (27.6)	21 (72.4)	0.731
III/IV	50 (24.6)	86 (42.4)	67 (33.0)		50 (24.6)	153 (75.4)	
Smoking habits							
Yes	48 (28.4)	65 (38.5)	56 (33.1)	0.061	48 (28.4)	121 (71.6)	0.050
No	10 (15.9)	34 (54.0)	19 (30.1)		10 (15.9)	53 (84.1)	
Gender							
Male	8 (16.3)	26 (53.1)	15 (30.6)	0.174	8 (16.3)	41 (83.7)	0.114
Female	50 (27.3)	60 (32,8)	73 (39.9)		50 (27.3)	133 (72.7)	
Age							
<64 years	25 (21.6)	48 (41.4)	43 (37.1)	0.246	25 (21.6)	91 (78.4)	0.225
>64 years	33 (28.4)	51 (44.0)	32 (27.6)		33 (28.4)	83 (71.6)	
All patients	58 (25.0)	99 (42.7)	75 (32.3)		58 (25.0)	174 (75.0)	

*χ^2 test.

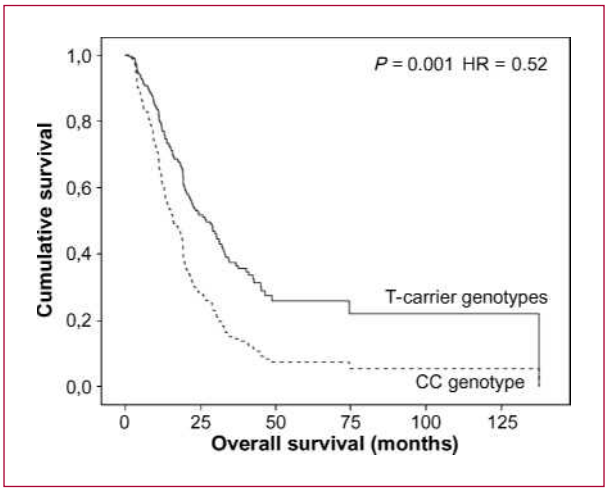


Fig. 1. Cox regression analysis of overall survival according to the *hTERT* −1327 C/T polymorphism in stage III and IV NSCLC patients.

association between *hTERT* genotypes and time to progression in stages III and IV NSCLC patients (P = 0.436; data not shown).

Discussion

Telomerase has reverse transcriptase activity and the ability to prolong telomeres; its main components are the catalytic subunit (hTERT) and the RNA template (hTR). The enzyme is active during embryonic development but silenced in most somatic cells after birth (7). Many malignant tumors show telomerase activity and thereby telomere lengthening capacity (31).

Telomeres stabilize chromosome ends and prevent them from undergoing degradation and recombination. With increasing cell divisions, telomeres become shorter due to incomplete replication of the lagging strand during DNA synthesis. Under normal physiologic conditions, cell cycle checkpoints such as pRB and TP53 control further cellular activities by activating genetic programs of cell cycle arrest, differentiation, or senescence. Moreover, when telomeres become critically shortened and compromise genomic stability, chromosome ends activate DNA damage response pathways that can induce apoptosis (32). In cancer, these mechanisms are often inactivated, and telomeres can become dysfunctional by several mechanisms, such as loss or alterations of telomere-binding proteins involved in telomere maintenance, and DNA damage due, for example, to oxidative stress. Total loss of telomeric DNA can promote the formation of end-to-end chromosome fusions that lack TTAGGG repeats at the fusion point. Telomere loss may be compensated by the recombination-based ALT pathway or, as seen in the majority of human cancers, by the enzyme telomerase (8, 33).

Because of the general requirement for telomerase in cancers, it is potentially a universal anticancer drug target. Cisplatin selectively reduces telomerase activity in a specific manner in tumor cells so that telomerase inhibition might be a component of the efficacy of cisplatin in the treatment of cancer. Interestingly, cisplatin-adduct formation in the telomere unit may block the telomerase reaction, and DNA adducts of cisplatin may inhibit the conventional replication of the telomere repeats (34).

hTERT mRNA expression seems to be most important for telomerase activity, but alternative splicing, posttranslational alterations, and hTERT localization in the cell contribute as well (35). Less is known about the impact of hereditary *hTERT* gene variations. Matsubara et al. (28) screened the promoter region of *hTERT* for functional polymorphisms in a Japanese healthy population, and a frequent T to C transition was found 1,327 bp upstream the transcription starting site (−1327T/C). Individuals homozygous for the −1327C/C genotype showed shorter telomere length in their peripheral leucocytes compared with the −1327T/T and −1327T/C genotypes.

In this study we report for the first time a role of 1327 T/C *hTERT* genetic variants in cancer prognosis and clinical outcome of NSCLC patients. So far, this genetic variant has been associated with telomerase activity, telomere length, and coronary artery disease development (28–30).

In this study, our results indicate an influence of the telomerase genetic variants in overall survival of NSCLC patients. Multivariate Cox regression analysis indicated an increased overall survival for T-carrier patients, when compared with CC genotype, after adjustment for tumor histologic type, stage, smoking status, age, and gender (HR, 0.52; 95% CI, 0.35-0.77; P = 0.001). The median estimated cumulative survival in T-carrier patients of 26.5 months was significantly higher compared with that of CC patients of 19.3 months.

This difference was more evident regarding nonepidermoid NSCLC histologic type, with the median estimated cumulative survival of 29.8 months in T-carrier patients being significantly higher compared with the 19.3 months of CC patients (HR, 0.46; 95% CI, 0.27-0.78; P = 0.004).

Matsubara and coworkers (28, 29) showed that the −1327T/C polymorphism within the *hTERT* promoter region has functional roles: the −1327T sequence is associated with higher transcriptional activity, lack of age-dependent telomere shortening, longer telomere length, and telomerase activity. The relationship of the −1327T/C polymorphism to telomere shortening, telomere length, and telomerase activity was found in normal peripheral leukocytes. Transcriptional regulation of hTERT has a key role in telomerase activity and telomere shortening. Approximately 25% higher promoter activity in the −1327T-sequence was found compared with the −1327C-sequence, and the T allele was strongly associated with longer telomere length. Thus, the *hTERT* T allele with higher hTERT transcriptional activity is associated with more effective extension of the telomeric end during cell division. Another study found an overrepresentation of the −1327C/C genotype in patients with coronary artery disease compared with controls, presenting shorter telomeres compared with other patients with alternative genotypes, indicating that

Table 2. Cox multivariate regression analysis of *hTERT* polymorphism and other potential factors of overall survival in stage III and IV NSCLC patients

	P	HR (95% CI)
hTERT polymorphism		
CC genotype	—	1.00 (reference)
T carrier	0.001	0.52 (0.35-0.77)
Age		
< 64 years	—	1.00 (reference)
> 64 years	0.459	0.88 (0.62-1.25)
Histologic type		
Epidermoid	—	1.00 (reference)
Nonepidermoid	0.523	0.88 (0.60-1.30)
Gender		
Female	—	1.00 (reference)
Male	0.691	0.89 (0.50-1.60)
Tobacco smoke		
Nonsmoker	—	1.00 (reference)
Smoker	0.707	0.90 (0.51-1.59)

a subgroup of patients is more prone to telomere shortening (29).

Tumors with excessive telomere alterations are therefore likely to possess the most extensive phenotypic variability and have the greatest probability of containing cells capable of invasion, extravasation, and metastasis, i.e., an aggressive tumor phenotype. Numerous groups have hypothesized that altered telomere length could predispose cells to gain the necessary properties to metastasize and cause recurrent disease, and thereby be a predictor of clinical outcome (36). Although not entirely consistent on the underlying mechanisms, several studies have indicated that telomere alterations are associated with parameters of clinical outcome in patients with lung cancer (37–39). A recent study (39) indicated a significant poor clinical outcome in NSCLC patients presenting telomere shortening, a finding that emerged as an independent prognostic marker in multivariate analysis. Therefore, considering that most NSCLCs display telomerase activity, the capacity to identify patients who have tumors with telomere shortening may have clinical relevance. According to telomere hypothesis, telomere shortening prevents somatic cells from dividing and states senescence. However, cells may escape from the senescence barrier if key tumor suppressor genes, especially *TP53* and/or *p16/Rb*, lose their function. In lung cancer, these pathways are frequently inactivated and when telomeres become critically shortened genomic stability becomes compromised.

In other tumor types, such as prostate cancer, it has been suggested that reduced telomere content is associated with poor clinical outcome and markers of disease progression. Thus, reduced telomere content values conferred a relative hazard of 5.02 compared with tumors with greater telomere content (40). Moreover, studies in breast cancer have shown that telomere attrition is associated with parameters of increased risk and poor outcome, with low telomere content conferring an adjusted relative hazard of 4.43 (36, 41).

Because hTERT reactivation is a mechanism for cancer cells to avoid senescence (42) and the latter could be induced by chemotherapy, the predictive value of *hTERT* genetic variations for benefit to first-line chemotherapy needs evaluation. Because short telomeres have been associated with poor prognosis in NSCLC patients, and the *hTERT* T allele correlated with telomere elongation, this genetic variant may confer an advantage in treatment response in these patients. Furthermore, because cisplatin mechanism of action involves aduct formation in the telomere unit, the modulation of long telomeres through *hTERT* T variant may originate a more available and easier target for the cytotoxic activity of this compound, strengthening cisplatin activity and conferring an improved response of NSCLC patients presenting the long telomere variant.

Recently, telomerase has been intensively studied as a target for novel cancer gene therapy and therapeutics (43). Our findings that *hTERT* genetic variants, by modulating telomere length, may confer an advantage in chemotherapy response, according to different types of NSCLC, suggest that patients with long telomeres could have better responses to telomerase-based therapies.

The assessment of telomerase genetic variants could supplement prognosis of survival in the course of NSCLC and may be a promising molecular marker of treatment response in these patients. Platinum and taxol compounds play a central role in cancer chemotherapy, and although treatment is limited by side effects, they continue to have widespread application. One of the main aims of clinical or

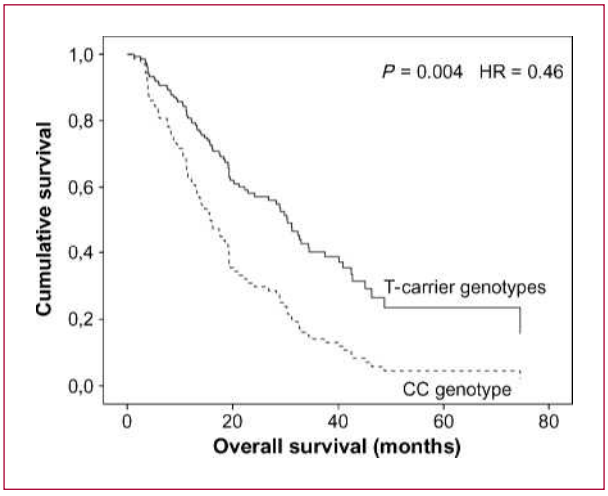


Fig. 2. Cox regression analysis of overall survival in stage III and IV NSCLC patients with nonepidermoid tumors according to the *hTERT* –1327 C/T polymorphism.

translational research in cancer is the search for genetic factors that could foresee treatment outcomes, in biological activity and toxic effects. This genetic analysis might allow the selection of patients who would have the greatest benefit from chemotherapy. Furthermore, a better knowledge of the underlying molecular profile of the host and the tumor will facilitate screening for lung cancer susceptibility and tailoring of chemotherapy in individual patients, choosing those most likely to respond, adjusting doses more precisely in order to reduce less adverse effects, and establishing safety profiles based on individual genetic analyses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Cyclin D1 polymorphism in Non-Small Cell Lung Cancer

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ABSTRACT

Cyclin D1 (CCND1) is a key regulatory protein at the G1/S checkpoint of the cell cycle. The purpose of our study was to assess the role of *CCND1* genetic variants influencing the genetic susceptibility and age of onset of non-small cell lung cancer (NSCLC). We conducted a hospital-based case-control study of 1234 individuals, including 892 controls and 342 cases. The polymorphism analysis was performed in blood samples by PCR-RFLP methodology. Logistic regression analysis adjusted by age, gender and smoking status indicates that individuals carrying two G-alleles have an increased genetic susceptibility for the development of NSCLC (aOR=2.70, 95% CI 1.60-4.56, *P*<0.0001). Moreover, our results indicate that the waiting time for onset of NSCLC in patients homozygous (GG) for *CCND1* genotypes (65 years) was 2 years earlier in comparison with patients carrying AG or AA genotypes (63 years) (Breslow test: P=0.0003). Our results may be important in contributing to a more extensive knowledge of the mechanisms involved in lung carcinogenesis, as CCND1 may be an important target for the development of new strategies for cancer treatment and prevention.

Key Words: Cyclin D1, Genetic Polymorphism, NSCLC

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths worldwide (1). Despite the fact that 80—90% of the cases are related to cigarette smoking, only around 15% of all smokers develop lung cancer during their lifetime. The cumulative smoking dose is clearly the most relevant parameter affecting individual lung cancer risk, but experimental data suggest that genetic factors may play an additional role. These factors include polymorphisms of genes involved in carcinogen-detoxification, DNA repair, immune system and cell cycle control (2-5). Because not all of the data are consistent, the modulation of risk or prognosis conferred by these and other gene polymorphisms in lung carcinogenesis needs further evaluation.

A consensus is emerging that gene-gene and gene-environment interactions in multiple cancer-related pathways may play a crucial role in this multi-step disease. The intricate and versatile cell cycle regulatory network is essential for mammalian cells to undergo an orderly series of critical cellular events including replication, division, proliferation, and differentiation. It has been proposed that a stepwise accumulation of multiple genetic alterations in the cell cycle control network may precede the initiation of malignant transformation (6).

Mammalian cell cycle progression is primarily regulated by interactions between cyclins, a family of periodically expressed proteins, and their binding partners, cyclin-dependent kinases (CDKs). Among the eight well studied cyclins, CCND1 (cyclin D1) is the most important cyclin that promotes cell cycle transition from the G1 to the S phase through interacting with either CDK4 or CDK6 (7).

Anomalies of cell cycle regulation genes have been frequently observed in a variety of human malignancies, including lung cancer (8). Dysfunctions of proto-oncogenes, such as CCND1, are commonly associated with increased cell

proliferation, defective apoptosis, elevated cancer risk, and poor survival (9, 10). The cyclin D1 (*CCND1*) gene encodes the cell cycle protein cyclin D1, which is essential for passage through the G1 restriction point, leading to the progression towards S-phase (11). Cyclin D1 represents a physiologic link between mitogens and the cell cycle machinery, as its expression in normal cells is induced by growth signals involving Ras, Raf, and the mitogen-activated protein kinase (MAPK) pathway (12). Cyclin D1 has been found to be over-expressed in several cancers. In non-small cell lung cancer, over-expression of cyclin D1 has been detected in 47% of lesions (13). Further investigations of the *CCND1* gene led to the detection of alternative splicing of the cyclin D1 mRNA, producing the two variant transcripts *CCND1a* and *CCND1b*, which are present simultaneously in a variety of normal tissues and cancer cells (14). Splicing of the primary *CCND1* transcript is modulated by a frequent A/G single nucleotide polymorphism located at nucleotide 870 in the splice donor region of exon 4 of the gene. Betticher and colleagues (14) identified a single base pair polymorphism (A870G) in *CCND1*, and *CCND1* genotypes have been significantly associated with carcinogenesis and clinical outcome in a variety of cancers (15).

The purpose of our study was to assess the role of *CCND1* genotypes influencing the genetic susceptibility for NSCLC and age of onset of this disease. The objective was to expand our current understanding of the role of cell cycle control genetic variations in lung cancer susceptibility.

PATIENTS AND METHODS

We conducted a hospital-based case-control study of 1234 individuals. Since 1997, 342 consecutive Caucasian patients admitted to the Portuguese Institute of Oncology of Porto (IPO-Porto), Portugal, with NSCLC, have been prospectively recruited to the study (median age 64.0 years; mean age 63.0 years; sd 9.9).

The control group consisted of 892 healthy individuals, 631 (51.9%) male and 585 (48.1%) female individuals, with a median age of 43.0 years (mean age 41.5; standard deviation 12.2), without clinical history of cancer, from the same geographic area as the case group. Regarding smoking habits, 481 (57.1%) were non-smokers and 362 (42.9%) were smoker or former-smoker individuals.

The NSCLC recruited patients were divided in two groups, according to tumour stage and treatment of the disease: stage I and II patients with surgical resection performed at the IPO-Porto and stages III and IV patients treated with platinum-based chemotherapy, between 1997 and 2009 which had follow-up data. The patients were evaluated according to the TNM staging system, and the assessment of tumour response to chemotherapy was based on RECIST. The first line chemotherapeutic protocol consisted of platin-based doublet chemotherapy in combination with a third-generation cytotoxic compound such as paclitaxel, gemcitabine or docetaxel. The chemotherapeutic protocols were as follows: cisplatin (80 mg/m2 on day 1) + paclitaxel (175 mg/m2 on day 1 every 3 weeks); cisplatin (100 mg/m2 on day 1) + gemcitabine (1250 mg/m2 on days 1 and 8 every 3 weeks); carboplatin (AUC 6 on day 1) + paclitaxel (175 mg/m2 on day 1 every 3 weeks); carboplatin (AUC 6 on day 1) + gemcitabine (1000 mg/m2 on days 1 and 8 every 3 weeks).

The median follow-up time was 26 months (range 1-135 months). Patients' distribution according to the stage at the time of diagnosis was 71

patients (18.3%) presenting localized disease (stages I and II), and 317 (81.7%) with advanced disease (stages III and IV). Considering the patients´ gender, 79 (20.3%) were females and 311 (79.7%) male individuals and regarding smoking habits, 101 (26.0%) were non-smokers and 287 (74.0%) were smoker or former-smoker individuals. From all patients, the histological type distribution was as follows: 151 patients (38.8%) with epidermoid NSCLC and 238 (61.2%) with non-epidermoid NSCLC.

This study was conducted according to Helsinki Declaration principles.

POLYMERASE CHAIN REACTION/RESTRICTION FRAGMENT LENGTH POLYMORPHISM (PCR-RFLP) ANALYSIS

Antecubital peripheral venous blood sample was collected from each subject at the time of recruitment. DNA was extracted from peripheral-blood samples using the QIAamp DNA Mini Kit (Qiagen, Hilden,Germany), according to the manufacturer's protocol. The detection of the A870G polymorphism of *CCND1* was carried out essentially as previously described (14). The PCR reactions consisted of nearly 0.2 µg of genomic DNA, 30 pmol of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_{2,}$ 1 × *Taq* Buffer, and 1 U of *Taq* DNA polymerase to a final volume of 50 µl. Primers used in the analysis were CY26 (5´GTG AAG TTC ATT TCC AAT CCG C 3´) and CY27 (5´ GGG ACA TCA CCC TCA CCC TCA CTT AC 3´). Thirty five cycles were performed, consisting of an initial heating at 95°C for 10 min to activate the enzyme, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, with a final extension step at 72°C for 2 min.

PCR products (15 µl) were digested with 1 U *ScrF1* at 37°C for 4 hr, and visualized by electrophoresis on 3% agarose gels containing 0.5 µg/ml ethidium bromide. The 167 bp PCR product generated is not cut by *ScrF1* if the A allele is

present, whereas the product from the G allele is cut to produce fragments of 145 and 22 bp.

STATISTICAL ANALYSIS

Analysis of data was performed using the computer software SPSS for Windows (Version 17.0) and Epi Info (version 6.04). Chi-square analysis was used to compare categorical variables with a 5% level of significance. The odds ratio (OR) and its 95% confidence interval (CI) were calculated as a measurement of the association between *CCND1* genotypes NSCLC risk. Logistic regression analysis was used to calculate the adjusted OR (aOR) and 95% CI for the influence of the *CCND1* genotypes in the risk for cancer with adjustment for age, gender and smoking history. The Hardy-Weinberg equilibrium was tested by a Pearson goodness of fit test to compare the observed versus the expected genotype frequencies. Overall survival (OS) was the endpoint of this analysis and was calculated from the date of diagnosis to the patient's date of death. Data were collected from patients' medical records. The associations between *CCND1* polymorphism and survival were estimated through Cox regression analysis. Cox regression models were used to adjust for potential confounders with *CCND1* genotypes fitted as indicator variables.

Finally, we considered a basic question: "For a newborn individual, what is the probability that he will experience onset of advanced disease before the age of X, supposing he survives that long?", assuming that all cases were at an identical risk of NSCLC at birth (16). To address this question, we hypothesized that *CCND1* genotypes may alter the onset of these tumors. To analyse the data, we defined age of onset for cancer as the outcome and *CCND1* genotype as an independent variable. We tested the association between age of onset and the A870G *CCND1* polymorphism by comparing Kaplan-Meier survival curves

according to *CCND1* genotype. We therefore considered the waiting time for onset of disease (WTOD) as the interval between the time of initial exposure to the risk factor (CCND1) and the time of onset of disease. We estimated the cumulative probabilities for having NSCLC by the Kaplan-Meier methodology (17). The primary analysis of time-to-event for WTOD was performed with the use of two-sided log-rank test at the 5% level of significance.

RESULTS

***CCND1* genotyping**: The frequencies of *CCND1* AA, AG and GG genotypes were 24.2, 57.4 and 18.4%, respectively, in normal controls, and 22.5, 51.2 and 26.3%, respectively, in the NSCLC case group. The G allele presented a higher frequency among NSCLC cases, indicating an increased risk of 1.6-fold for individuals carrying the GG genotype (OR=1.58, 95% CI 1.18-2.13, *P*=0.002). Logistic regression analysis adjusted by age, gender and smoking status, indicates that individuals carrying two G-alleles have a 2.70-fold increase in the risk for the development NSCLC (aOR=2.70, 95% CI 1.60-4.56, *P*<0.0001) (Table 1).

The genotype distributions of A870G *CCND1* functional polymorphism among NSCLC cases are described in Table 2. The frequencies of AA, AG and GG genotypes were 0.23, 0.51 and 0.26, respectively. We found no statistically significant differences in genotype distributions according to the patients´ clinico-pathological characteristics, namely, histological type (epidermoid and non-epidermoid cases; *P*=0.789) and tumor stage (stages I/II and III/IV; *P*=0.482). Moreover, the *CCND1* genotype frequencies did not differ significantly among NSCLC cases considering smoking habits (smoker/former-smoker and non-smoker cases; *P*=0.520), gender (male and female; *P*=0.315) and age (age under 64 years and above 64 years; *P*=0.606).

Regarding survival analysis, Cox regression analysis showed no statistically significant differences of survival curves considering *CCND1* genotypes (P=0.643) (Figure 1).

WTOD and *CCND1* genotype: The waiting time for onset of disease, i.e., the cumulative probabilities of developing disease according to *CCND1* genotypes for the development of NSCLC are shown in Figure 2.

The mean WTOD was 67 years for individuals with *CCND1* AA genotype, 65 years for AG genotype and 63 years for GG genotype (Breslow test: P=0.006). Considering the recessive model, Kaplan-Meier analysis (Figure 2) showed that the WTOD in patients homozygous (GG) for *CCND1* genotypes (63 years) was 2 years earlier in comparison with patients carrying AG or GG genotypes (65 years) (Breslow test: P=0.004).

Cox regression analysis of hazard curves confirmed this association (Figure 3). Using a multivariate Cox regression model, we found an increased WTOD for patients carrying GG genotype, when compared with AA/AG genotypes, with gender (*P*=0.137) and smoking status (*P*=0.033) as covariates (hazard ratio, HR=1.28; 95% CI: 1.00-1.63; *P*=0.047).

DISCUSSION

Several genetic polymorphisms contributing to individual's susceptibility to cancer have been studied regarding their association with cancer risk (18, 19). In this study, a single-nucleotide polymorphism in *CCND1* was analysed in order to evaluate its importance in the development and disease onset of NSCLC.

Over-expression of cyclin D1 in NSCLC (20, 21) and in apparently normal bronchial epithelia of lung cancer patients (22) has been correlated with smoking and with poor prognosis, suggesting that altered regulation of *CCND1* is an early event in lung carcinogenesis. Altogether, there is strong evidence that de-regulation of *CCND1* is an important step in lung carcinogenesis and that A870G gene polymorphism has biological and phenotypic significance.

The A870G polymorphism at codon 242 within the conserved splice donor site of exon 4 of the gene appears to modulate the splicing of CCND1 mRNA, originating two transcripts (*a* and *b*), which are present in a variety of tissues (14). The transcript *a* is identical to the reported CCND1 cDNA. However, transcript *b* fails to splice at the exon 4/intron 4 boundary, does not contain exon 5, and terminates downstream of exon 4. The main difference in the cyclin D1 proteins encoded by the two transcripts (*a* and *b*) is in the C-terminal PEST-rich region (destruction box) encoded by exon 5 which is responsible for rapid intracellular degradation and turnover of the G1 cyclins (23).

Our results indicate that individuals carrying the *CCND1* GG genotype have increased genetic susceptibility for the development of NSCLC. In this study, we also demonstrate that the GG genotype is associated with an earlier age of onset of lung cancer, compared to *CCND1* AG and AA genotypes. Although there are conflicting results, these findings are consistent with previous findings suggesting that *CCND1* GG genotype is associated with cancer development (15, 24-33).

It has been suggested that the variant A allele is a major source of variant transcript *b* in several types of cancer cells. The AA genotype increases the products of transcript *b* in tumor tissue cells, resulting in an increase of an altered protein that lacks the PEST-region with increased half-life (14, 23). The presence of the A allele has been reported to be positively associated with increased risk for several cancers (15). However, a study reported, surprisingly, that cyclin D1b protein does not inappropriately accumulate in cells and exhibits stability comparable to cyclin D1a. This study also suggests that cyclin D1a is a better catalyst of RB (retinoblastoma protein) phosphorylation/inactivation (34). These data support the emerging view that CCND1 alternate transcripts encode proteins with differing independent biological functions. Furthermore, it has been reported that cyclin D1 is over-expressed in bronchial epithelia, and the protein is mainly localized to the cytoplasm, with nuclear localization of cyclin D1 being associated with premalignant transformation (22).

The direction of the biological impact of cyclin D1 expression depends on the state of the cell in accordance with its checkpoint function. While cyclin D1 is best known for its proliferating effect (35, 36), experimental evidence suggests that under conditions such as oxidative stress, or senescence, cyclin D1 can exhibit S-phase entry and DNA replication and promote growth arrest, as well as apoptosis (37, 38). The context-dependent dual role of cyclin D1 in cell proliferation and growth arrest may explain the inconsistent associations observed between *CCND1* genotype and cancer risk.

Carcinogenic and mutagenic agents contained in tobacco smoke and air pollution induce the formation of aromatic DNA adducts, which have been indicated as playing an important role in the etiology of lung cancer. Moreover, tobacco smoke is also a major source of oxidative stress and may induce the production of endogenous DNA lesions, through oxidative DNA damage and lipid

peroxidation of cell membranes (39). Nitric oxide (NO) and reactive oxygen species (ROS) play important physiologic roles as mediators of signaling processes. However, high concentrations of NO and ROS result in damage to cellular and extracellular components. Excessive production of endogenous and/or exogenous ROS and NO is implicated in the pathogenesis of lung cancer. NO and its metabolites interact with ROS to generate potent nitrating agents leading to protein nitration, which is one of the several chemical modifications that occur during oxidative/nitrosative stress (39). *In vitro* experiments of breast tumor and other tumor cells exposed to oxidative stressors demonstrate that cyclin D1 activation and overexpression is also able to activate molecular pathways resulting in cell-cycle arrest and apoptosis (38). Turner and co-workers (40) provided *in vivo* evidence of cyclin D1 as a caretaker gene offering downstream protection against oxidative damage. Our findings extend this evidence to situations of more moderate oxidative burden and suggest that modulation of the biological function of cyclin D1 by oxidative damage may lead to differential impact of the *CCND1* polymorphism in lung carcinogenesis.

It is possible that these conflicting results in part reflect the many different mechanisms through which deregulated expression of CCND1 can occur in cancer, and the direction and magnitude of the CCND1 effect in cancer development. Functional studies in the future may help to elucidate the conflicting experimental findings and influence of *CCND1* genotypes on tumor behaviour in different cell types.

In conclusion, our results may be important in contributing to a more extensive knowledge of the mechanisms involved in lung carcinogenesis, as CCND1 may be an important target for the development of chemoprevention or therapeutic strategies and raises the possibility of using cyclin D1 as a molecular

marker to identify high-risk individuals, allowing the development of effective surveillance and early intervention strategies.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST STATEMENT

None declared.

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Table 1 – Prevalence and odds ratio of *CCND1* genotypes among control group and NSCLC patients

	Patients (n=342)		**Controls** (n=892)		**OR**	**95%CI**	***P***
	n	%	n	%			
Genotypes							
AA/AG	252	73.7	728	81.6	1.00	reference	
GG	90	26.3	164	18.4	1.58*	1.18–2.13*	0.002*

**P*<0.0001, aOR=2.70 and 95% CI=1.60–4.56 using logistic regression analysis adjusted by age, gender and smoking history.

Table 2 – *CCND1* genotype frequencies among NSCLC patients, according to the patients´ characteristics

	***CCND1* genotypes**				***CCND1* genotypes**		
	AA	AG	GG	*P**	AA/AG	GG	*P**
	N (%)	N (%)	N (%)		N (%)	N (%)	
Histology							
Epidermoid	27 (20.5)	69 (52.3)	36 (27.3)	0.744	96 (72.7)	36 (27.3)	0.789
Non-epidemoid	50 (24.0)	104 (50.0)	54 (26.0)		154 (74.0)	54 (26.0)	
Stage							
I/II	17 (27.9)	30 (49.2)	14 (23.0)	0.530	47 (77.0)	14 (23.0)	0.482
III/IV	60 (21.6)	142 (51.1)	76 (27.3)		202 (72.7)	76 (27.3)	
Smoking habits							
Yes	135 (25.6)	285 (54.0)	108 (20.5)	0.060	48 (28.4)	121 (71.6)	0.520
No	92 (20.1)	279 (61.1)	86 (18.8)		420 (79.5)	108 (20.5)	
Gender							
Male	151 (23.2)	374 (57.5)	125 (19.2)	0.315	525 (80.8)	125 (19.2)	0.209
Female	142 (24.4)	312 (53.5)	129 (22.1)		454 (77.9)	129 (22.1)	
Age							
<64 years	242 (23.1)	592 (56.5)	214 (20.4)	0.270	834 (79.6)	214 (20.4)	0.606
>64 years	50 (27.6)	91 (50.3)	40 (22.1)		141 (77.9)	40 (22.1)	
All patients	77 (22.5)	175 (51.2)	90 (26.3)		252 (73.7)	90 (26.3)	

*Qui-square test

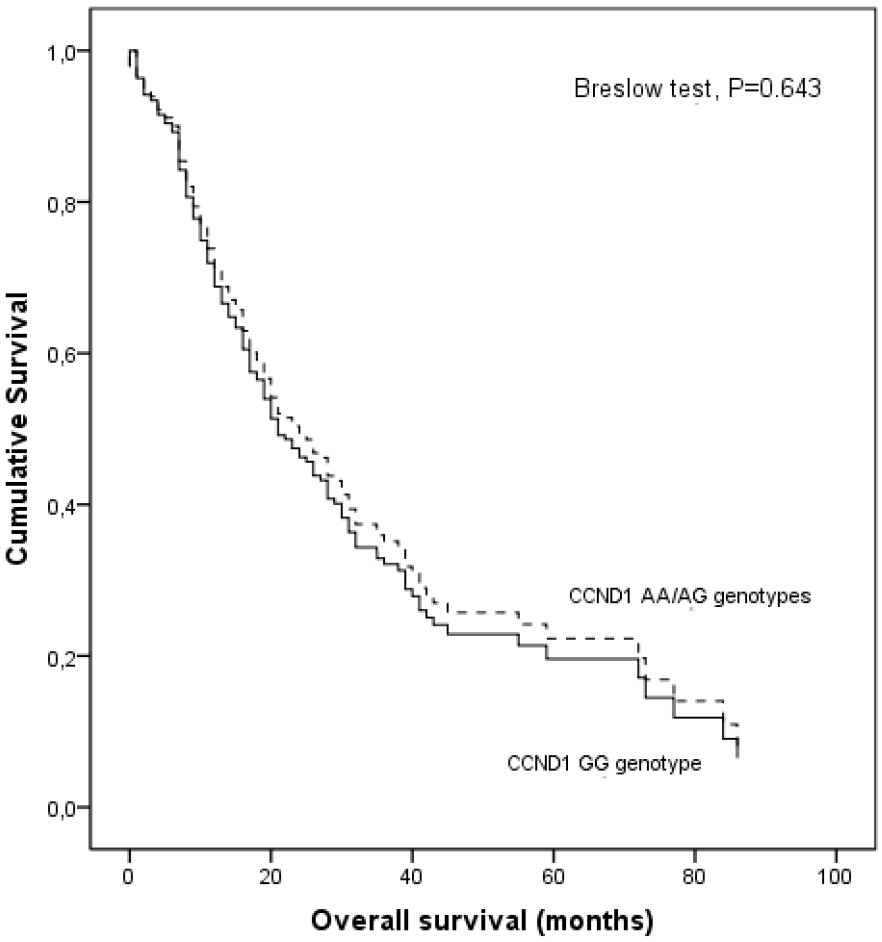


Figure 1 – Cox regression analysis of overall survival according to *A870G CCND1* polymorphism in NSCLC patients.

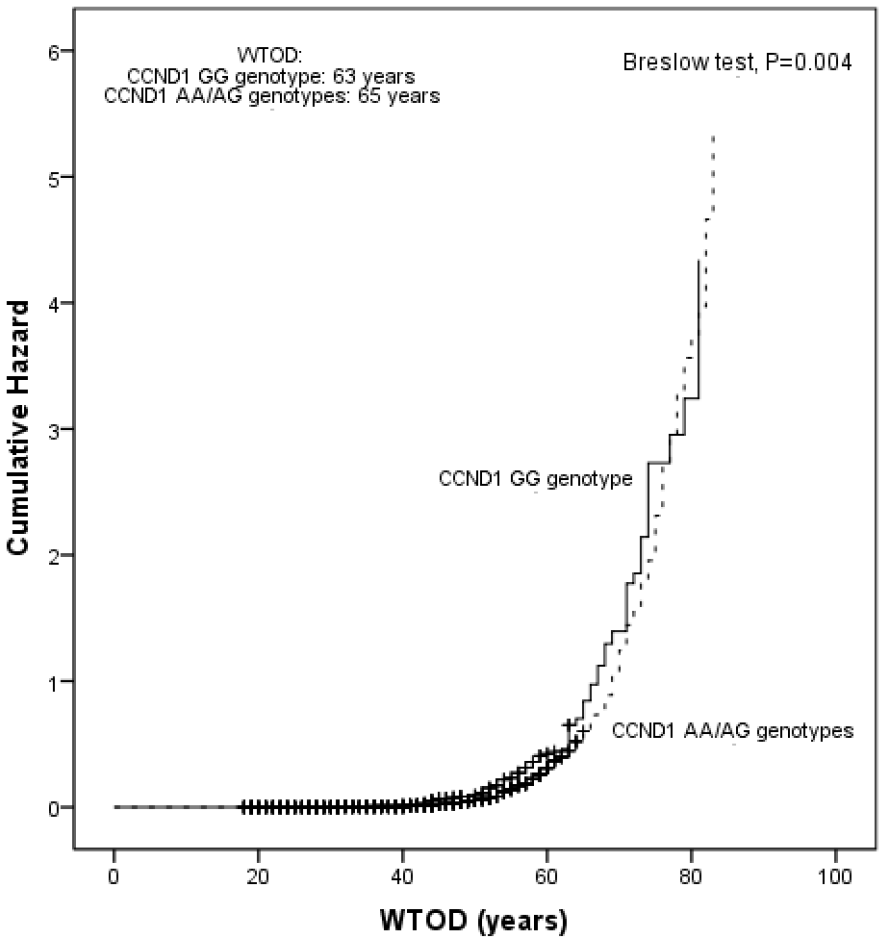


Figure 2 – Association between GG C*CND1* genotype and the waiting time to onset of NSCLC. Cumulative hazard function plots by the Kaplan-Meier methodology and Breslow test. WTOD – waiting time to onset of disease.

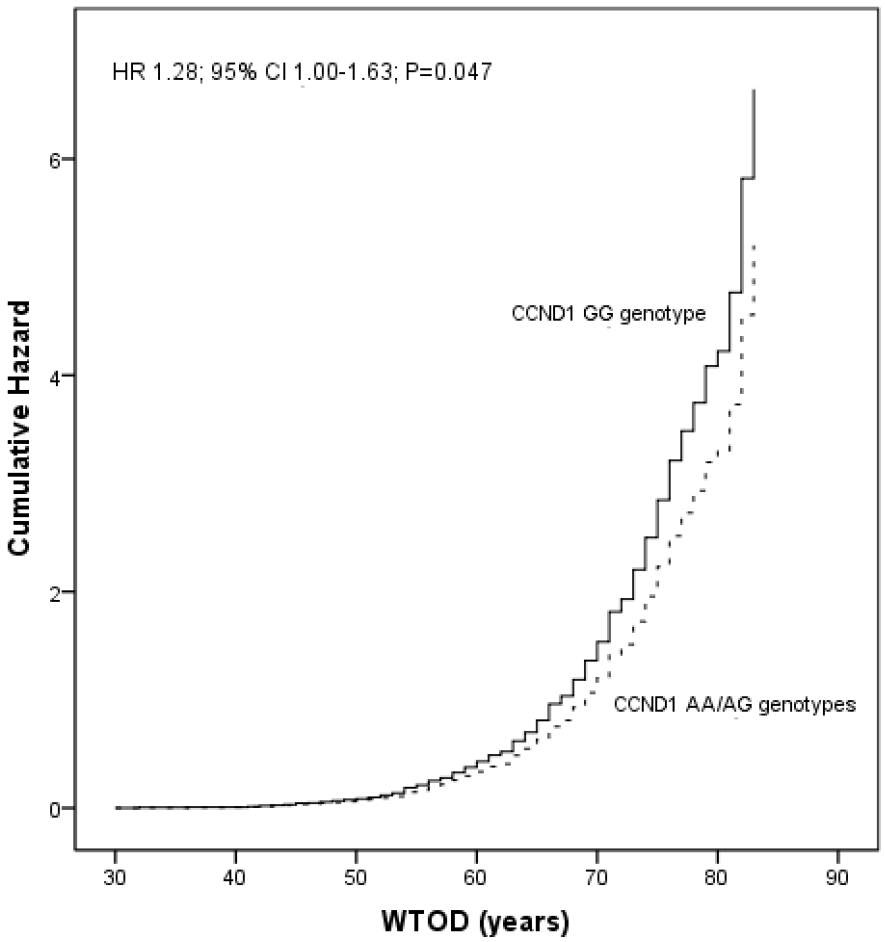


Figure 3 – Cox regression analysis hazard curves according to *A870G CCND1* polymorphism in NSCLC patients. WTOD – waiting time to onset of disease.

ORIGINAL ARTICLE

ASSOCIATION OF THE A870G CYCLIN D1 GENE POLYMORPHISM WITH GENETIC SUSCEPTIBILITY TO NASOPHARYNGEAL CARCINOMA

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Abstract: *Background.* Nasopharyngeal cancer (NPC) is multifactorial, and the genetic background may be a crucial etiologic factor. Cyclin D1 (CCND1) is a key regulator of the cell cycle, and its altered activity is associated with the development of cancer.

Methods. We analyzed the A870G *CCND1* polymorphism by polymerase chain reaction/restriction fragment length polymorphism (PCR-RFLP) in 281 individuals, including 94 patients with NPC and 187 healthy individuals.

Results. Our results indicate that individuals carrying two G alleles have a 2.17-fold increase in the risk for the development of NPC (odds ratio [OR], 2.17; 95% confidence interval [CI], 1.19–3.98; $p = .016$). Age-adjusted logistic regression analysis confirmed this association (adjusted odds ratio [aOR], 2.14; 95% CI, 1.14–4.04; $p = .018$). Multivariate analysis demonstrates an independent association between GG *CCND1* genotype (aOR, 2.06), male sex (aOR, 2.66), and age at diagnosis (aOR, 2.02) regarding the development of undifferentiated NPC. The proportion of NPC cases attributable to the GG *CCND1* genotype was 14.76%.

Conclusions. Our results may be important in the definition of a biologic predictive profile for the development of NPC within our population. © 2006 Wiley Periodicals, Inc. *Head Neck* **28:** 603–608, 2006

Keywords: cyclin D1; genetic polymorphism; nasopharyngeal cancer; head and neck; cancer risk

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Nasopharyngeal cancer (NPC) has a remarkably distinctive geographic and ethnic distribution with particularly high rates among Southern China and Southeast Asia. NPC is a rare malignant disease, with an incidence less than 1 per 100,000 persons per year in Caucasians from North America and other Western countries. Independent of race/ethnicity, men are twofold to threefold more frequently affected than women. The dramatic difference in the incidence among populations and geographic areas suggests a strong association of NPC with genetic and environmental factors.[1] In contrast to other head and neck cancers, a unique feature of NPC is its strong association with Epstein-Barr virus (EBV).[2] However, NPC oncogenesis is not simply a conse-

quence of EBV infection. It probably results from a viral reactivation in combination with other events, such as cellular genetic lesions caused by environmental carcinogens and/or some immune defects. The contribution of other pathogenic factors, especially genetic and environmental factors, should be considered.[2] In addition to EBV, numerous other environmental factors have been shown to be associated with the development of NPC. In particular, long-term cigarette smoking, consumption of salted fish and foods containing nitrosamine or nitrosamine precursors at an early age, and occupational exposure to wood dust have been shown to be consistently associated with this disease.[1] Host factors previously shown to be associated with NPC development include human leukocyte antigen (*HLA*) class I and II alleles[3] and polymorphisms of genes responsible for carcinogen metabolism (*CYP2E1*), detoxification (*GSTM1*), and DNA repair (*XRCC1* and *hOGG1*).[4–6]

A frequent target in carcinogenesis is the deregulation of G1 to S phase progression of the cell cycle. The transition through G1 to S phase is regulated by cyclins, cyclin-dependent kinases, and their inhibitors.[7] Cyclin D1 (CCND1) is a key regulatory protein at the G1/S checkpoint of the cell cycle. It forms complexes with CDK4 or CDK6 and is responsible for the phosphorylation of the retinoblastoma tumor suppressor protein, resulting in the release of E2F transcription factors that allow cells to enter into S-phase.[8–10] The G1/S checkpoint is frequently altered in many epithelial tumors and may confer growth advantage and enhance tumorigenesis. Amplification of *CCND1* and altered expression of the protein have been reported in a variety of tumors, including esophagus, colon, breast, head and neck, and lung.[11–16]

Recently, it has been reported that CCND1 mRNA is alternatively spliced to produce two transcripts (*a* and *b*), which are present simultaneously in a variety of normal tissues and cancer cells.[17,18] Betticher and colleagues[18] identified a single base pair polymorphism (A870G) in *CCND1,* and *CCND1* genotypes have been significantly associated with carcinogenesis and clinical outcome in a variety of cancers.[19–23]

The aim of our study was to investigate the influence of *CCND1* genotypes on the genetic susceptibility to NPC.

PATIENTS AND METHODS

Patients. We tested the association between the *CCND1* A870G polymorphism and risk of NPC with a case-control study. Ninety-four patients with histologically confirmed NPC were selected for this study. Patients were admitted to the Portuguese Oncology Institute-Porto, Portugal, during the period from 1997 to 2003. The median age at diagnosis was 50 years (SD, 14.5). The control group consisted of 187 healthy individuals, with a median age of 55 years (SD, 16.8), without clinical history of cancer, from the same geographic area as the case group. All samples were taken after informed consent according to the declaration of Helsinki.

Polymerase Chain Reaction/Restriction Fragment Length Polymorphism Analysis. DNA was extracted from peripheral blood leukocytes from each study subject using a salting out protocol.[24] The detection of the A870G polymorphism of *CCND1* was carried out essentially as previously described.[18] The polymerase chain reactions consisted of nearly 0.2 μg of genomic DNA, 30 pmol of each primer, 0.2 m*M* of each dNTP, 1.5 m*M* MgCl2, 1 × *Taq* buffer, and 1 U of *Taq* DNA polymerase to a final volume of 50 μL. Primers used in the analysis were CY26 (5′-GTG AAG TTC ATT TCC AAT CCG C-3′) and CY27 (5′-GGG ACA TCA CCC TCA CCC TCA CTT AC-3′). Thirty-five cycles were performed, consisting of an initial heating at 95°C for 10 minutes to activate the enzyme, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute, with a final extension step at 72°C for 2 minutes.

PCR products (15 μL) were digested with 1 U *ScrF1* at 37°C for 4 hours and visualized by electrophoresis on 3% agarose gels containing 0.5 μg/mL ethidium bromide. The 167-bp PCR product generated is not cut by *ScrF1* if the A allele is present, whereas the product from the G allele is cut to produce fragments of 145 and 22 bp (Figure 1).

Statistical Analysis. Analysis of data was performed using the computer software SPSS for Windows (version 12.0) and Epi Info (version 6.04). Chi-square analysis was used to compare categorical variables, and a 5% level of significance was used in the analysis. The odds ratio (OR) and its 95% confidence interval (CI) were calculated as a measurement of the association between *CCND1* genotypes and NPC risk. Multivariate logistic regression analysis was used to calculate the adjusted OR (aOR) and 95% CI for the influence of the *CCND1* genotypes in the risk for NPC with adjustment for age and sex. The

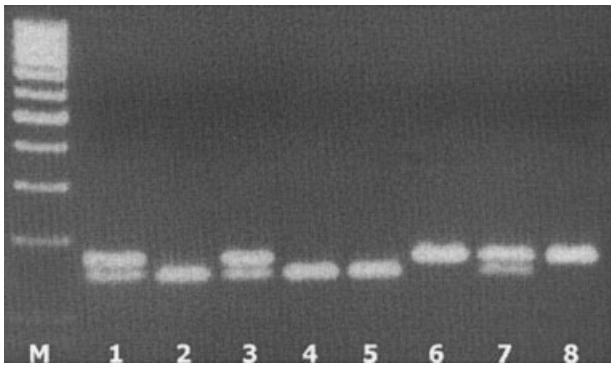


FIGURE 1. Analysis of the *CCND1* genotypes—restriction fragment length polymorphism of the polymerase chain reaction products. M, 100-bp ladder; cases 1, 3, and 7, *CCND1* heterozygous (G/A); cases 2, 4, and 5, homozygous GG; cases 6 and 8, homozygous AA.

Hardy–Weinberg equilibrium was tested by a Pearson goodness of fit test to compare the observed versus the expected genotype frequencies. We calculated the attributable proportion (AP) using the following formula: AP = PRF × (1-1/OR), where AP is the fraction of disease attributable to the risk factor and PRF is the percentage of the risk factor in case subjects and OR is the odds ratio.

RESULTS

The distribution of *CCND1* genotypes among patients and controls and the risk of NPC caused by the influence of *CCND1* polymorphism are shown in Table 1.

The frequencies of AA, AG, and GG genotypes were 28.9%, 56.1%, and 15.0%, respectively, in normal controls, and 27.7%, 44.6%, and 27.7%, respectively, in the patient group. The genotype distribution of both groups was in the Hardy-Weinberg equilibrium ($p = .80$ in the patient group and .36 in the control group). The analysis of the frequencies of *CCND1* genotypes indicates that individuals carrying two G-alleles have a 2.17-fold increase in the risk for the development of NPC (OR, 2.17; 95% CI, 1.19–3.98; $p = .016$). Age-adjusted logistic regression analysis confirmed the association between the presence of the GG *CCND1* genotype and increased genetic susceptibility for the development of NPC (aOR, 2.14; 95% CI, 1.14–4.04; $p = .018$).

The characteristics of patients with NPC and controls are shown in Table 2.

Multivariate logistic regression analysis indicated the association of the GG *CCND1* genotype (aOR, 2.06; 95% CI, 1.04–4.09; $p = .039$), male sex (aOR, 2.66; 95% CI, 1.45–4.86; $p = .002$), and age at diagnosis older than 50 years (aOR, 2.02; 95% CI, 1.12–3.62; $p = .019$) with undifferentiated histologic type of NPC (undifferentiated carcinoma of nasopharyngeal type, UCNT) risk, demonstrating the independent association between the *CCND1*

Table 1. Prevalence and odds ratio of *CCND1* genotypes among control group and patients with nasopharyngeal carcinoma.

	Patients ($n = 94$)		Controls ($n = 187$)				
	n	%	*n*	%	aOR	95%CI	*p* value
Genotypes							
AA	26	27.7	54	28.9			
AG	42	44.6	105	56.1			
GG	26	27.7	28	15.0			
Recessive model							
AA/AG	68	72.3	159	85.0	1.00	Reference	
GG	26	27.7	28	15.0	2.17*	1.19–3.98*	.016*

**p = .016, aOR = 2.14 and 95% confidence interval (CI) = 1.14–4.04 using logistic regression analysis adjusted by age.*

Table 2. Characteristics of patients with nasopharyngeal carcinoma and controls.

	No. (%)	
Characteristic	Patients ($n = 94$)	Controls ($n = 187$)
Sex		
Female	25 (26.6)	103 (55.1)
Male	69 (73.4)	84 (44.9)
Total	94 (100.0)	187 (100.0)
Age, y		
Median ± SD	50 ± 14.5	55 ± 16.8
Histology		
Well differentiated	1 (1.1)	
Moderately differentiated	11 (11.7)	
Undifferentiated	69 (73.4)	
Not stated	13 (13.8)	
Total	94 (100.0)	
Staging		
I	2 (2.1)	
II	11 (11.7)	
III	21 (22.4)	
IV	41 (43.6)	
Not stated	19 (20.2)	
Total	94 (100.0)	
Nodular metastasis		
Yes	58 (61.7)	
No	24 (25.5)	
Not stated	12 (12.8)	
Total	94 (100.0)	
Distant metastasis		
Yes	2 (2.1)	
No	80 (85.1)	
Not stated	12 (12.8)	
Total	94 (100.0)	

Table 3. Multivariate analysis of the *GG CCND1* genotype, sex, and age at diagnosis regarding the susceptibility to undifferentiated histologic type of nasopharyngeal carcinoma.

	p value*	aOR*	95% CI*
GG genotype	.039	2.06	1.04–4.09
Age ≥50 y	.019	2.02	1.12–3.62
Male sex	.002	2.66	1.45–4.86

Abbreviations: aOR, ; CI, confidence interval.
**P, aOR, and 95% CI using logistic regression analysis.*

GG genotype and the development of UCNT, as shown in Table 3. For patients with NPC, the proportion of NPC cases attributable to the GG CCND1 genotype was 14.76%.

DISCUSSION

NPC is a human malignancy consistently associated with EBV. However, the etiology of NPC is complex and multifactorial, and exposure to nonviral carcinogens and genetic predisposition are other crucial etiologic factors.

Several genetic polymorphisms contributing to an individual's susceptibility to cancer have been studied regarding their association with NPC risk.[3–6] In this study, a single-nucleotide polymorphism in *CCND1* was analyzed to evaluate its importance in the development of NPC. The A870G polymorphism at codon 242 within the conserved splice donor site of exon 4 of the gene seems to modulate the splicing of CCND1 mRNA, originating in two transcripts (*a* and *b*), which are present in a variety of tissues.[18,25,26] The transcript *a* is identical to the reported CCND1 cDNA. However, transcript *b* fails to splice at the exon 4/intron 4 boundary, does not contain exon 5, and terminates downstream of exon 4. The main difference in the cyclin D1 proteins encoded by the two transcripts (*a* and *b*) is in the C-terminal PEST-rich region (destruction box) encoded by exon 5, which is responsible for rapid intracellular degradation and turnover of the G1 cyclins.[18,25,27]

It has been suggested that the variant A allele is a major source of variant transcript *b* in several types of cancer cells. The AA genotype increases the products of transcript *b* in tumor tissue cells, resulting in an increase of an altered protein that lacks the PEST-region with increased half-life.[18,26,28] However, a recent study reported, surprisingly, that cyclin D1b protein does not inappropriately accumulate in cells and exhibits stability comparable to cyclin D1a. This study also suggests that cyclin D1a is a better catalyst of RB phosphorylation/inactivation.[29]

Our results indicate that individuals carrying the *CCND1* GG genotype have increased risk for the development of NPC (aOR, 2.14) and suggest a predictive biologic profile for the development of UCNT, indicating that individuals older than 50 years (aOR, 2.02), of male sex (aOR, 2.66), and carriers of the GG *CCND1* genotype (aOR, 2.06) have an increased susceptibility for the development of UCNT. These results are consistent with previous findings suggesting that *CCND1* GG genotype is associated with cancer development.[19,20,23,30–33] Matthias et al[19,20] reported that the *CCND1* GG genotype was associated with poorly differentiated tumors of the head and neck and reduced the disease-free interval in laryngeal and pharyngeal carcinomas, independently from tumor differentiation, providing a link between *CCND1* A870G alleles with CCND1 expression and clinical outcome in squamous cell carcinoma of the head and neck. Monteiro et al[23] reported a correlation between GG genotype and increased susceptibility for laryngeal tumor development. Another recently published study demonstrated that EBV-LMP1 could regulate cell growth by activation of cyclin D1 expression by means of the nuclear factor kappa B (NF-κB) pathway in NPC and suggested that A870G polymorphism was associated with susceptibility to NPC.[31] However, controversial results have been reported regarding the role of *CCND1* genotypes in cancer development.[22,28,34,35] The mechanism for this association is unknown, although because the G allele splices less of transcript *b* than the A allele, individuals with *CCND1* GG may have different cellular levels of CCND1 than individuals with *CCND1* AA.

Furthermore, these results suggest that the effect of genotype on tumor behavior may exhibit some degree of tissue specificity.[19] It is possible that these conflicting results in part reflect the many different mechanisms through which deregulated expression of CCND1 can occur in cancer. Functional studies in the future may help to explain the conflicting experimental findings and influence of *CCND1* genotypes on tumor behavior in different cell types.

The contribution of genetic polymorphisms to the risk for cancer may be dependent on the studied population, as well as on several environmental and other factors that influence that population. Geographic or ethnic differences have been reported regarding the genotype frequency of several polymorphisms. Our results within the Por-

tuguese population are consistent with a recently published study in our population.[23] Further studies may include the analysis of other genetic polymorphisms that have been already associated with cancer risk to characterize the genetic profile of NPC susceptibility.[36–42]

In conclusion, our results may be important in the definition of a biologic predictive profile for the development of NPC within our population. Furthermore, the knowledge of the mechanisms involved in NPC carcinogenesis may help to identify targets for the development of chemoprevention or therapeutic strategies.

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Oncogenic virus-associated neoplasia: A role for cyclin D1 genotypes influencing the age of onset of disease?

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ABSTRACT

Cyclin D1 (CCND1) is a key regulatory protein at the G1/S checkpoint of the cell cycle. The purpose of our study was to assess the role of *CCND1* genotypes influencing the age of onset of oncogenic virus-associated neoplasia. We conducted a hospital-based case-control study of 581 individuals, including 247 controls and 334 cases (108 nasopharyngeal and 226 cervical cancer cases). The polymorphism analysis was performed in blood samples by PCR–RFLP methodology. Age-adjusted logistic regression analysis indicates that individuals carrying two G-alleles have an increased genetic susceptibility for the development of oncogenic virus-associated cancers (aOR = 2.02, 95% CI 1.30–3.14, $P = 0.002$). Moreover, our results indicate that the waiting time for onset of oncogenic virus-associated neoplasia in patients homozygous (GG) for *CCND1* genotypes (52 years) was 12 years earlier in comparison with patients carrying AG or AA genotypes (60 years) (log-rank test: $P = 0.0003$). Our results may be important in contributing to a more extensive knowledge of the mechanisms involved in oncogenic virus-associated carcinogenesis, as CCND1 may be an important target for the development of new strategies for cancer treatment and prevention.

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Oncogenic viruses are currently considered the second most important cause of cancer in humans and contribute to 15–20% of all cancers in the world, some of them being very common, like cervical carcinomas. Human recognized cancer viruses include Human Papillomavirus (HPV) and Epstein–Barr virus (EBV) and although each virus has unique features, it is becoming clearer that all these oncogenic agents target multiple cellular pathways to support malignant transformation and tumor development [1].

Oncogenes encoded by tumor viruses play integral roles in the viral conquest of the host cell by subverting crucial and relatively no-redundant regulatory circuits that regulate cellular proliferation, differentiation, apoptosis, and life span. Some viral oncoproteins subvert cellular safeguard mechanisms intended to eliminate cells that have acquired abnormalities that interfere with normal cell division. Viruses that encode such activities can contribute to initiation as well as progression of human cancers [2].

HPV and EBV are common infectious agents that persist after primary infection in a latent state with occasional shedding of virus. Therefore, one of the fundamental questions in the etiology of cervical cancer (associated with high risk HPV infection) and nasopharyngeal cancer (associated with EBV infection) that are linked to infection with such ubiquitous viruses is why cancer develops in a few people when many are infected. These tumors share a DNA viral etiology and present similar histopathological findings. Moreover, there are several similar aspects of infection with HPV and EBV [3].

Although it is well known that HPV and EBV are directly involved in cervical and nasopharyngeal cancer development, respectively, some researchers acknowledge that viral infection is a necessary but not sufficient factor for cancer development. The progression from infection to cancer involves other environmental and host factors. Single-nucleotide polymorphism (SNP) markers should be considered in the determination of the combinations of genetic factors involved in precancerous changes to cancer development [4–8].

A frequent target in carcinogenesis is the deregulation of G1–S phase progression of the cell cycle. The transition through G1 to S phase is regulated by cyclins, cyclin-dependent kinases and their inhibitors [9]. Cyclin D1 (CCND1) is a key regulatory protein at the G1/S checkpoint of the cell cycle. It forms complexes with CDK4 or CDK6, and is responsible for the phosphorylation of the

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retinoblastoma tumor suppressor protein, resulting in the release of E2F transcription factors that allow cells to enter into S-phase [10–12]. The G1/S checkpoint is frequently altered in many epithelial tumors and may confer growth advantage and enhance tumorigenesis [13]. Amplification of *CCND1* and altered expression of the protein have been reported in a variety of tumors [12,14].

It has been reported that CCND1 mRNA is alternatively spliced to produce two transcripts (*a* and *b*), which are present simultaneously in a variety of normal tissues and cancer cells [15]. Bettticher and colleagues [15] identified a single base pair polymorphism (A870G) in *CCND1*, and *CCND1* genotypes have been significantly associated with carcinogenesis and clinical outcome in a variety of cancers [16].

The purpose of our study was to assess the role of *CCND1* genotypes influencing the age of onset of oncogenic virus-associated neoplasia.

Patients and methods

We conducted a hospital-based case-control study of 581 individuals, including 247 controls and 334 cases (108 nasopharyngeal and 226 cervical cases) diagnosed at the Portuguese Oncology Institute-Porto, Portugal, during the period from 2000 to 2004. The median age at diagnosis of nasopharyngeal cancer patients was 49.0 years (mean age 47.5; standard deviation 14.5). Considering the cervical cases, the median age at diagnosis was 47.0 years (mean age 47.2; standard deviation 12.3). The control group consisted of 247 healthy individuals, with a median age of 55.5 years (mean age 51.1; standard deviation 16.8), without clinical history of cancer, from the same geographic area as the case group. All samples were taken after informed consent according to the declaration of Helsinki. For clinical and statistical purposes, we defined two groups of malignant cases, with a strong association with oncogenic virus: oncogenic virus-associated nasopharyngeal cases (OVNC), and oncogenic virus cervical cases (OVCC). In OVNC group, 84 undifferentiated nasopharyngeal cancers were included (24 were differentiated nasopharyngeal cancers, and thereby not associated with EBV). The OVCC group included 202 cervical malignant cases (50 High-Grade Squamous Intraepithelial Cervical Lesions of the cervix—HSIL—and 154 invasive epidermoid cervical cancers).

Polymerase chain reaction/restriction fragment length polymorphism (PCR–RFLP) analysis. DNA was extracted from peripheral blood leukocytes from each study subject using a salting out protocol [17]. The detection of the A870G polymorphism of *CCND1* was carried out essentially as previously described [15]. The PCR consisted of nearly 0.2 μg of genomic DNA, 30 ρmol of each primer, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1× Taq Buffer, and 1 U of Taq DNA polymerase to a final volume of 50 μl. Primers used in the analysis were CY26 (5′GTG AAG TTC ATT TCC AAT CCG C 3′) and CY27 (5′ GGG ACA TCA CCC TCA CCC TCA CTT AC 3′). Thirty five cycles were performed, consisting of an initial heating at 95 °C for 10 min to activate the enzyme, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, with a final extension step at 72 °C for 2 min.

PCR products (15 μl) were digested with 1 U *ScrF1* at 37 °C for 4 h, and visualized by electrophoresis on 3% agarose gels containing 0.5 μg/ml ethidium bromide. The 167 bp PCR product generated is not cut by *ScrF1* if the A allele is present, whereas the product from the G allele is cut to produce fragments of 145 and 22 bp.

Statistical analysis. Analysis of data was performed using the computer software SPSS for Windows (Version 12.0) and Epi Info (version 6.04). Chi-square analysis was used to compare categorical variables and a 5% level of significance was used in the analysis. The odds ratio (OR) and its 95% confidence interval (CI) were calculated as a measurement of the association between *CCND1* genotypes and ICC and NPC risk. Logistic regression analysis was used to calculate the adjusted OR (aOR) and 95% CI for the influence of the *CCND1* genotypes in the risk for cancer with adjustment for age. The Hardy–Weinberg equilibrium was tested by a Pearson goodness of fit test to compare the observed versus the expected genotype frequencies.

Finally, we considered a basic question: "For a newborn individual, what is the probability that he will experience onset of advanced disease before the age of X, supposing he survives that long?", assuming that all cases were at an identical risk of cervical and nasopharyngeal cancers at birth [18]. To address this question, we hypothesized that CCND1 genotypes may alter the onset of these cancers. To analyse the data, we defined age of onset for cancer as the outcome and *CCND1* genotype as an independent variable. We tested the association between age of onset and the A870G *CCND1* polymorphism by comparing Kaplan–Meier survival curves according to *CCND1* genotype. We therefore considered the waiting time for onset of disease (WTOD) as the interval between the time of initial exposure to the risk factor (CCND1) and the time of onset of disease. We estimated the cumulative probabilities for having cervical and nasopharyngeal cancers by the Kaplan–Meier methodology [19]. The primary analysis of time-to-event for WTOD was performed with the use of two-sided log-rank test at the 5% level of significance.

Results

CCND1 genotyping

The frequencies of *CCND1* AA, AG and GG genotypes were 27.9, 55.9 and 16.2%, respectively, in normal controls, and 31.1, 54.9 and 14.0%, respectively, in the female only control group. The frequencies of the polymorphism in case groups were: 27.7% for the AA genotype, 45.5% for AG genotype and 26.7% for GG genotype in oncogenic virus-associated cervical cases (OVCC) group and 25.0, 47.6 and 27.4% for AA, AG and GG genotypes, respectively, in the oncogenic virus-associated nasopharyngeal cases (OVNC) group. The genotype distribution of both groups was in the Hardy–Weinberg equilibrium ($P = 0.679$ in the case group and $P = 0.341$ in the control group).

Age-adjusted logistic regression analysis indicates that individuals carrying two G-alleles have a 2.44-fold increase in the risk for the development OVCC (aOR = 2.44, 95% CI 1.38–4.30, $P = 0.002$) and a 2.09-fold increased risk for the development of OVNC (aOR = 2.09, 95% CI 1.15–3.79, $P = 0.016$). For all cases, the presence of the GG *CCND1* genotype was also associated with an increased genetic susceptibility for the development of oncogenic virus-associated cancers (aOR = 2.02, 95% CI 1.30–3.14, $P = 0.002$) (Table 1).

WTOD and CCND1 genotype

The waiting time for onset of disease, the cumulative probabilities of having disease, according to *CCND1* genotypes for the OVCC group are shown in Fig. 1. The mean WTOD was 60 years for carri-

Table 1
Age-adjusted logistic regression analysis of the GG *CCND1* genotype regarding the genetic susceptibility to oncogenic virus-associated cancers

	P	aOR	95% CI
Oncogenic virus-associated cases			
Cervical	0.002	2.44	1.38–4.30
Nasopharyngeal	0.016	2.09	1.15–3.79
All cases	0.002	2.02	1.30–3.14

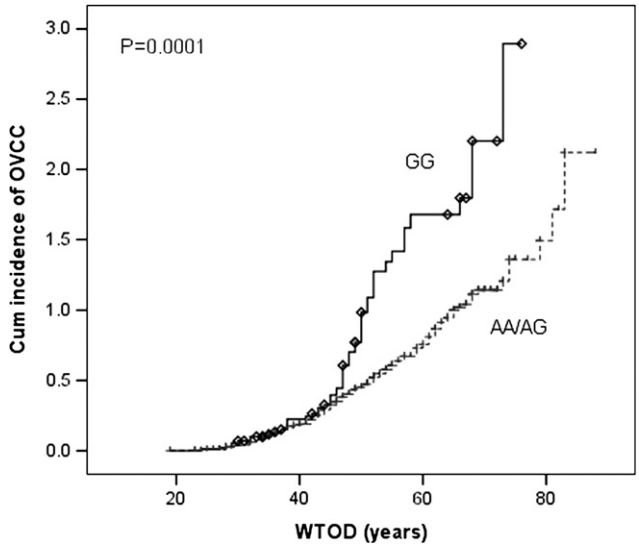


Fig. 1. Association between GG *CCND1* genotype and the waiting time to onset of disease (WTOD) for the OVCC (oncogenic virus-associated cervical cases) group. Cumulative hazard function plots by the Kaplan–Meier methodology and log-rank test ($P = 0.0001$).

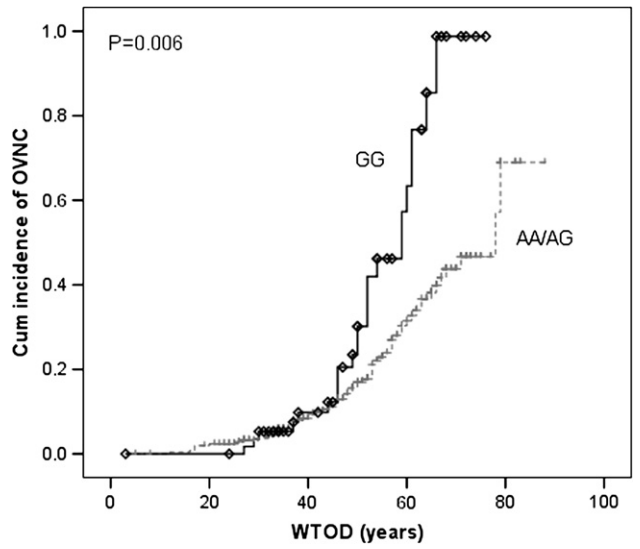


Fig. 2. Association between GG *CCND1* genotype and the waiting time to onset of disease (WTOD) for the OVNC (oncogenic virus-associated nasopharyngeal cases) group. Cumulative hazard function plots by the Kaplan–Meier methodology and log-rank test ($P = 0.006$).

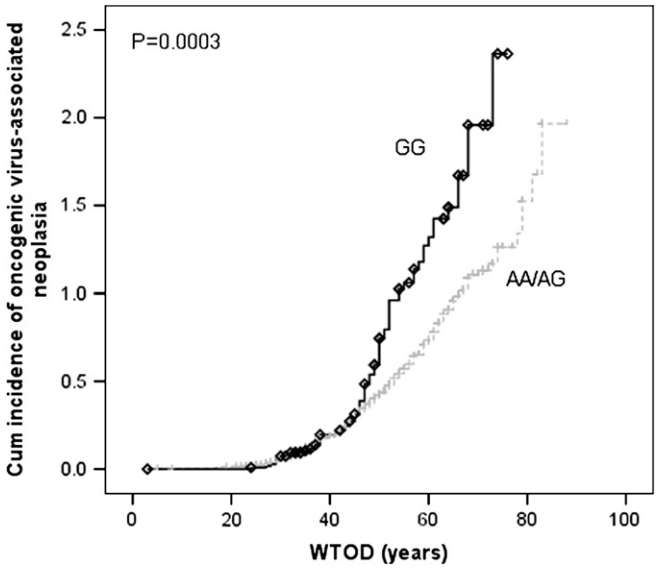


Fig. 3. Association between GG *CCND1* genotype and the waiting time to onset of oncogenic virus-associated cancers. Cumulative hazard function plots by the Kaplan–Meier methodology and log-rank test ($P = 0.0003$). WTOD, waiting time to onset of disease.

ers of the AA or AG genotypes and 50 years for carriers of the GG *CCND1* genotype (log-rank test: $P = 0.0001$). Regarding the OVNC group, the mean WTOD for patients carrying AA or AG genotypes was 73 years and 61 years for patients carrying the GG genotype (log-rank test: $P = 0.006$) (Fig. 2). Considering all oncogenic virus cases, Kaplan–Meier analysis (Fig. 3) showed that the WTOD in patients homozygous (GG) for *CCND1* genotypes (52 years) was 12 years earlier in comparison with patients carrying AG or GG genotypes (60 years) (log-rank test: $P = 0.0003$).

Discussion

Oncogenic viruses trigger persistent infections, which can stimulate uncontrolled cell growth by inducing cell transformation. Most oncogenic DNA viruses integrate transforming sets of genes into the host chromosome and encode proteins that bind and inactivate cell growth regulatory proteins. Tumor viruses play an important role for the development of a substantial fraction of human malignancies, including common cancers, such as carcinomas of the cervix uteri, nasopharyngeal cancers, hepatocellular carcinomas, or lymphomas [20]. Malignant transformation typically requires additional genetic alterations of the host cell, to which tumor viruses can contribute by destabilizating the cellular genome [21].

Several genetic polymorphisms contributing to individual's susceptibility to cancer have been studied regarding their association with cancer risk [22–24]. In this study, a single-nucleotide polymorphism in *CCND1* was analysed in order to evaluate its importance in the development and disease onset of cervical and nasopharyngeal cancers. The A870G polymorphism at codon 242 within the conserved splice donor site of exon 4 of the gene appears to modulate the splicing of CCND1 mRNA, originating two transcripts (*a* and *b*), which are present in a variety of tissues [15,25]. The transcript *a* is identical to the reported CCND1 cDNA. However, transcript *b* fails to splice at the exon 4/intron 4 boundary, does not contain exon 5, and terminates downstream of exon 4. The main difference in the cyclin D1 proteins encoded by the two transcripts (*a* and *b*) is in the C-terminal PEST-rich region (destruction box) encoded by exon 5 which is responsible for rapid intracellular degradation and turnover of the G1 cyclins [25,26].

Our results indicate that individuals carrying the *CCND1* GG genotype have increased genetic susceptibility for the development oncogenic virus-associated neoplasia (aOR = 2.02). In this study, we also demonstrate that the GG genotype is associated with an earlier age of onset of oncogenic virus-associated cancers, compared to *CCND1* AG and AA genotypes. These results are consistent with previous findings suggesting that *CCND1* GG genotype is associated with cancer development [16,27–36].

It has been suggested that the variant A allele is a major source of variant transcript *b* in several types of cancer cells. The AA genotype increases the products of transcript *b* in tumor tissue cells, resulting in an increase of an altered protein that lacks the PEST-region with increased half-life [15,25]. The presence of the A allele has been reported to be positively associated with increased risk for several cancers [12,37,38]. However, a recent study reported, surprisingly, that cyclin D1b protein does not inappropriately accumulate in cells and exhibits stability comparable to cyclin D1a. This study also suggests that cyclin D1a is a better catalyst of RB (retinoblastoma protein) phosphorylation/inactivation [39]. These data support the emerging view that CCND1 alternate transcripts encode proteins with differing independent biological functions.

The direction of the biological impact of cyclin D1 expression depends on the state of the cell in accordance with its checkpoint function. While cyclin D1 is best known for its proliferating effect [40,41], experimental evidence suggests that under conditions such as oxidative stress [42–45] or senescence [46,47], cyclin D1 can exhibit S-phase entry and DNA replication and promote growth arrest, as well as apoptosis. The context-dependent dual role of cyclin D1 in cell proliferation and growth arrest may explain the inconsistent associations observed between *CCND1* genotype and cancer risk.

Oxidative stress, primarily due to increased generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), is a feature of many viral infections, including HPV and EBV infections [48–51]. ROS and RNS modulate the permissiveness of cells to viral replication, regulate host inflammatory and immune responses, and cause oxidative damage to both host tissue and progeny virus [48]. *In vitro* experiments of breast tumor and other tumor cells exposed to oxidative stressors demonstrate that cyclin D1 activation and overexpression is also able to activate molecular pathways

resulting in cell-cycle arrest and apoptosis [45,52]. Turner and coworkers [44] provided *in vivo* evidence of cyclin D1 as a caretaker gene offering downstream protection against oxidative damage. Our findings extend this evidence to situations of more moderate oxidative burden and suggest that modulation of the biological function of cyclin D1 by tumor viruses may lead to differential impact of the *CCND1* polymorphism on oncogenic virus-associated malignant lesions.

It is possible that these conflicting results in part reflect the many different mechanisms through which deregulated expression of CCND1 can occur in cancer, and the direction and magnitude of the CCND1 effect in cancer development. Functional studies in the future may help to elucidate the conflicting experimental findings and influence of *CCND1* genotypes on tumor behaviour in different cell types.

In conclusion, our results may be important in contributing to a more extensive knowledge of the mechanisms involved in oncogenic virus-associated carcinogenesis, as CCND1 may be an important target for the development of chemoprevention or therapeutic strategies.

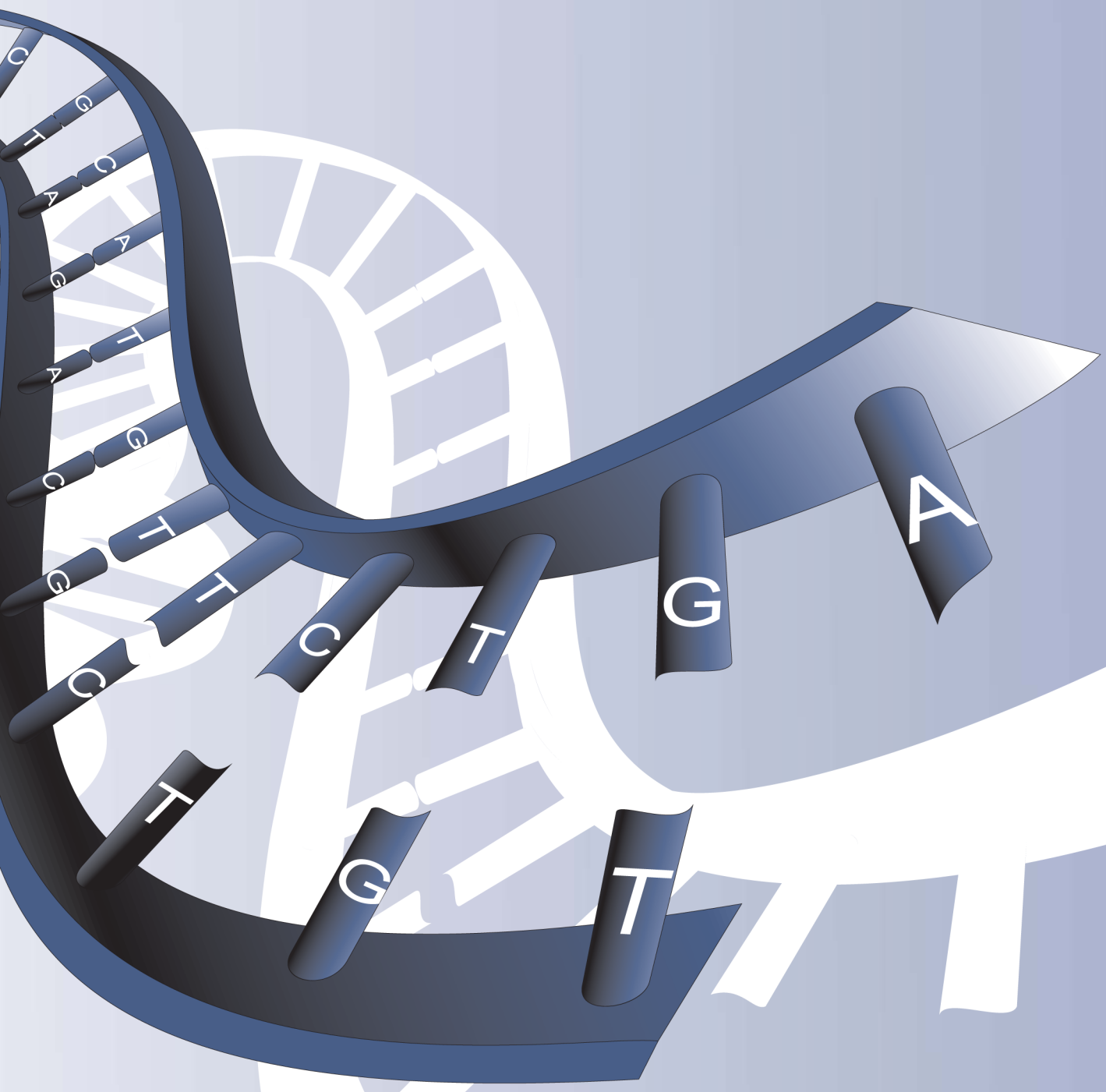
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GENERAL DISCUSSION AND CONCLUSIONS

4. GENERAL DISCUSSION AND CONCLUSIONS

Despite decreases in the cancer death rates in high-resource countries, the number of cancer cases and deaths is projected to more than double worldwide over the next 20–40 years. Cancer is now the third leading cause of death, with approximately 12 million new cases and 7.6 million cancer deaths estimated to have occurred globally in 2007. By 2030, it is projected that there will be approximately 26 million new cancer cases and 17 million cancer deaths per year (2). Given the magnitude of the projected demographic trends and their disproportionate impact in countries that can least afford increased health care expenditures, preventive measures offer the most reasonable approach (1). For these reasons, there is a critical need for new research approaches aimed at improving cancer management. Identification and characterization of the genetic changes that drive human cancer development and progression may provide us with a variety of molecular markers that can ultimately redefine the criteria for cancer diagnosis and provide new tools for early detection through the development of sensitive procedures aimed at detecting these alterations in easily accessible samples, such as blood and sputum (20).

Cancer is a disease of the genome, which is invariably altered at multiple sites in cancer cells. The goal of cancer research is to define these molecular defects and turn these discoveries into effective treatment and prevention regimens (45). Pharmacogenomics (PGx) is expected to improve patients' treatment outcomes by increasing efficacy and decreasing toxicity, and eventually reducing overall healthcare costs. Medical oncologists are striving to individualize cancer treatment in an effort to maximize efficacy and minimize toxicity in these patients. Identifying host genetic variations that contribute to drug efficacy and/or the risk of toxicity will provide means for tailoring cancer therapy. Genetic variation could explain variations in pharmacokinetics, alterations in activity or expression of the target, or proteins involved in the drugs' mechanism of action (17).

4.1 Circulating DNA levels in cancer diagnosis and prognosis assessment

Diagnostic assays based on blood sample analysis are becoming an area of study with growing interest, mainly because of the simplicity of sampling and the future potential of automation of the technical methods for clinical applicability (46). The possibility of recovering tumor-derived DNA from the patient's blood has opened new prospects. This method offers a non-invasive means to obtain

tumor surrogate material, which could represent a unique source for diagnostic and prognostic applications. However, only recently cell free DNA (fcDNA) is becoming an issue of growing interest and its possible use as a marker for cancer diagnosis or prognosis has been investigated. There are some reports in breast cancer models with interesting results about this subject, but the majority consists in qualitative analysis of plasma and serum DNA (47-49). Some quantitative studies reported increased concentrations of circulating plasma DNA of cancer patients, in comparison with healthy individuals (21, 50-52). However, the laboratorial methods have not been consistently evaluated, namely the procedures for DNA isolation and quantification (53). Moreover, there are some contradictory results regarding the analyses of the clinical-pathological features, such as clinical staging and the prognostic and predictive value of cell free DNA quantification and relapse-free evaluation that should be studied in more detail. A number of methods have been described to assess the amount of DNA present in plasma, including competitive binding radioimmunoassay, SYBER green, PicoGreen and others, but most lack the sensitivity and specificity required (21, 49, 54).

To measure with greater accuracy the fcDNA amount, a quantification approach based on real-time quantitative polymerase chain reaction (real-time PCR) was developed. The amplification of a single copy gene is specific and robust and represents the ideal target for DNA-based quantitative real-time PCR assay. This assay is a specific and sensitive method, providing an accurate quantification of low plasma DNA concentrations and minimizes the risk of carryover contamination of the post-PCR processing from conventional PCR (19). There are promising results in recent studies performed in lung cancer models, using a more accurate method to measure the amount of free circulating DNA by real-time PCR (19, 54). In the present work, we used real-time PCR for DNA quantification, which can be regarded as the standard method currently available.

We found increased levels of circulating DNA in lung and breast cancer patients compared to control individuals. Similar results were also found in other studies, with higher plasma DNA concentrations found in breast cancer patients when compared to healthy individuals (20, 55-57). Sozzi and co-workers demonstrated that a relevant drop in DNA levels is already visible at 1-6 months after tumor removal and reaches the level observed in normal healthy subjects (19). These data suggest that in tumor-free patients, plasma DNA either is released at lower rates or is rapidly degraded.

The differences in plasma DNA levels between patients and controls are concordant with the results of other quantitative studies in other cancer models (19, 54). According to the majority of the quantitative studies performed until present, cell free circulating DNA is observed in healthy subjects at concentrations between 0 and 100 ng/ml of blood with an average of 30 ng/ml, whereas in cancer patients the concentration in plasma or in serum varies between 0 and 1000 ng/ml, with an average of 180 ng/ml (22). However, these values constitute just a reference to evaluate cell free DNA concentrations, as the percentage of circulating DNA originated from tumor cells can vary from one cancer model to another (22). The plasma DNA concentrations observed in this study were similar to the cell free DNA level averages described in the literature (reviewed in (22)).

In a healthy person, it is believed that fcDNA enters circulation via apoptosis of lymphocytes and other nucleated cells. The notion that apoptosis as the primary source of fcDNA has been supported by the fact that normal plasma DNA on electrophoresis exhibits band sizes equivalent to a whole number multiple (1-5x) of nucleosomal DNA (185–200 bps). In cancer as well, apoptosis has been advanced as the possible origin of fcDNA on the basis of the fact that circulating DNA often represents ladder like electrophoretic pattern (as seen in pancreatic and lung cancer) which is similar to that shown by apoptotic cells (58).

One of the hypotheses for the origin of fcDNA in cancer is based on ‘‘micrometastases’’ of tumor origin which are shed into circulation. Some studies reported that the amount of DNA isolated from plasma of cancer patients was very high and did not correspond to the number of cancer cells present in the circulation (47). Thus, the number of cancer cells as per the amount of circulating DNA should have been 10000 cells per ml, while the authors detected much lower number of cells in plasma. Therefore, the above hypothesis of micrometastases does not seem accurate. High amounts of DNA found in plasma of patients with large or advanced/metastatic tumors are thought to arise from tumor necrosis. However, it was noted that after radiation therapy, which is presumed to induce cell death/necrosis, circulating DNA levels surprisingly decreased in 90% of patients. Therefore, the hypothesis based on necrosis mediated release of fcDNA in plasma became controversial. It was suggested by Leon et al. that the decrease in free DNA levels following radiotherapy may be due to arrest of cellular proliferation by radiation (46). Cell-free nucleic acids can also contain small amounts of DNA of T-cell and mitochondrial origin (58).

Enzymes DNase I and II present in circulation degrade DNA and, therefore, minimal levels of plasma DNA are detected in healthy people. However, low activity of DNase I and II is seen in malignant diseases. This is supported by the fact that inhibitors of DNase have been detected in tumors, which explains why the elevated DNA levels in circulation are noticed (58). Spontaneous and active release of DNA by proliferating cancer cells is another possibility that cannot be ignored, as the activated lymphocytes are shown to release DNA in vitro (59).

A published study has demonstrated that the levels of cell free DNA present in cancer patients constitute a stable parameter over time and its variations may be due to real clinical alterations in the patient, as long as all the technical and methodological steps are controlled (60). Therefore, it becomes important to carefully monitor the methods for blood sampling, plasma DNA isolation and DNA quantification. Some quantification methods, such as colorimetric kits, are not based on the amount of amplifiable DNA, but in the total amount of nucleic acids, which can include double-stranded and single-stranded DNA and RNA. Therefore, the results can vary significantly and the best option is, in our opinion, the quantification by real-time PCR (19). The differences between the results observed in literature may also reflect biological causes (histology, tumor origin, stage or tumor size) or technical issues (blood processing, cell free DNA isolation and quantification). There is insufficient data for the comparison of all these parameters between different types of cancer, as it is still unclear whether all types of cancer can release altered cell free DNA and at the same rate (22). A recent study has demonstrated that, for a patient with a tumor load of 100g in size, up to 3.3% of the tumor DNA entered the circulation every day (61). In another study, mutations in *TP53* or *KRAS2* were detected in whole blood at least one year before diagnosis in 67% of patients, which indicates that tumor DNA is released in blood before conventional diagnosis and enhances the importance of this marker in cancer prevention (62).

Although some concerns regarding the sample size and age of case and control groups may be evaluated, this study suggests a non-invasive diagnostic tool that can be further investigated in future prospective studies for cancer detection and assessment of its potential applicability as a complementary diagnostic tool in clinical practice. We believe that our results may help to improve discussion between researchers in the field and that different methodological approaches must be considered regarding the real meaning of fcDNA in cancer patients.

Levels of circulating DNA could also identify higher-risk individuals for this disease screening and chemoprevention trials. Furthermore, plasma DNA could also be used to detect cancer specific molecular markers, such as mutations and amplifications, and individualize and monitor drug treatment, namely resistance to targeted therapies without the need for repeated tumor biopsies in metastatic disease.

Cell free circulating DNA may represent an important source of biomarkers at several steps of carcinogenesis, including early detection of preneoplastic lesions and monitoring of cancer. Moreover, plasma DNA levels could be tested as a potential intermediate biomarker of the efficacy of intervention.

We think that the results are promising enough to encourage further research in the area of circulating DNA as a tool for monitoring therapeutic efficacy in lung cancer patients. Identification of additional, more specific, and more sensitive plasma-based biomarkers, which can be used in combination with circulating DNA, may further improve the diagnostic power of current imaging tools for indicating therapeutic efficacy. Large-scale prospective studies are necessary for population-based studies and molecular epidemiologic studies, in order to implement a clinical application in cancer detection, diagnosis, prognosis and prediction of treatment response.

4.2 Genetic variability in cancer

DNA-repair and cell-cycle checkpoint pathways allow cells to deal with both endogenous and exogenous sources of DNA damage. How much an individual is exposed to these agents and how their cells respond to DNA damage are critical determinants of whether or not that individual will develop cancer. These cellular responses are also important for determining toxicities and responses to current cancer therapies, most of which target the DNA (63).

Several genetic polymorphisms contributing to individual's susceptibility to cancer have been studied regarding their association with cancer risk (43, 64-67). Because telomerase reactivation and cell cycle control bypass are key mechanisms for cancer cells to avoid senescence (68) and the latter could be induced by chemotherapy, the predictive value of *hTERT* and *CCND1* genetic variations in treatment response of cancer patients subjected to chemotherapeutic regimens needs evaluation.

4.2.1 Telomerase genetic variants

Telomerase has reverse transcriptase activity and ability to prolong telomeres, and its main components are the catalytic subunit (hTERT) and the RNA template (hTR). Telomeres stabilize chromosome ends and prevent them from undergoing degradation and recombination. With increasing cell divisions, telomeres shorten due to incomplete replication of the lagging strand during DNA synthesis. When telomeres become critically shortened and compromise genomic stability, chromosome ends activate DNA damage response pathways that can induce apoptosis (69). In cancer, these pathways are often inactivated and telomeres can become dysfunctional by several mechanisms, such as loss or alterations of telomere-binding proteins involved in telomere maintenance and DNA damage. Total loss of telomeric DNA can promote the formation of end-to-end chromosome fusions which lack TTAGGG repeats at the fusion point. Telomere loss may be compensated by the recombination based alternative lengthening of telomeres (ALT) pathway or, as seen in the majority of human cancers, by the enzyme telomerase (70, 71).

hTERT mRNA expression seems to be most important for telomerase activity, but also alternative splicing, posttranslational alterations and hTERT localization in the cell contribute to this activity (72). Less is known about the impact of hereditary *hTERT* gene variations. Matsubara et al. (73) found a frequent T to C transition in *hTERT* gene, 1327 bp upstream the transcription starting site (*-1327T/C*). The *-1327T/C* polymorphism within the *hTERT* promoter region has functional roles: the *-1327T* sequence is associated with higher transcriptional activity, lack of age-dependent telomere shortening, longer telomere length, and telomerase activity. Approximately 25% higher promoter activity in the *-1327T* sequence was found, compared to the *-1327C* sequence and the T allele was strongly associated with longer telomere length. Thus, *hTERT T* allele with higher hTERT transcriptional activity is associated with more effective extension of the telomeric end during cell division. Another study found an overrepresentation of the -1327C/C genotype in patients with coronary artery disease compared to control individuals, presenting shorter telomeres compared to other patients with alternative genotypes, indicating that a subgroup of patients is more prone to telomere shortening (4).

Tumors with excessive telomere alterations are therefore likely to possess the most extensive phenotypic variability and have the greatest probability of containing cells capable of invasion, extravasation and metastasis, i.e., an

aggressive tumor phenotype. Numerous groups have hypothesized that altered telomere length could predispose cells to gain the necessary properties to metastasize and cause recurrent disease, and thereby be a predictor of clinical outcome (74). Although not entirely consistent on the underlying mechanisms, several studies indicated that telomere alterations are associated with parameters of clinical outcome in lung cancer patients (75-77). A recent study (77) indicated a significant poor clinical outcome in NSCLC patients presenting telomere shortening, a finding that emerged as an independent prognostic marker in a multivariate analysis. Therefore, considering that most NSCLCs display telomerase activity, the capacity to identify patients who have tumors with telomere shortening may have clinical relevance. According to telomere hypothesis, telomere shortening prevents somatic cells from dividing and states senescence. However, cells may escape from the senescence barrier if key tumor suppressor genes, especially *TP53* and/or *p16/Rb,* lose their function. In lung cancer, these pathways are frequently inactivated and when telomeres become critically shortened, genomic stability results compromised.

In other tumor types, such as prostate cancer, it has been suggested that reduced telomere content is associated with poor clinical outcome and disease progression. Reduced telomere content values conferred a relative hazard of 5.02 compared with tumors with greater telomere content (78). Also, studies in breast cancer have shown that telomere attrition is associated with parameters of increased risk and poor outcome, with low telomere content conferring an adjusted relative hazard of 4.43 (74, 79).

As hTERT reactivation is a mechanism for cancer cells to avoid senescence (68) and the latter could be induced by chemotherapy, we evaluated the predictive value of *hTERT* genetic variations in first-line chemotherapy of non-small cell lung cancer (NSCLC) patients. Cisplatin selectively reduces telomerase activity in tumor cells through a specific manner, so that telomerase inhibition might be a component of the drugs' efficacy in the treatment of cancer. Interestingly, cisplatin-adduct formation in telomere unit may block the telomerase reaction and DNA adducts of cisplatin may inhibit the conventional replication of the telomere repeats (80). Our results demonstrate an increased survival time in NSCLC presenting the telomerase T-allele variant. Since short telomeres have been associated with poor prognosis in NSCLC patients and the *hTERT* T allele correlated with telomere elongation, this genetic variant may confer an advantage in treatment response in these patients. Furthermore, since

cisplatin mechanism of action involves aduct formation in the telomere unit, the modulation of long telomeres through *hTERT* T variant may originate a more available and easier target for the cytotoxic activity of this compound, strengthening cisplatin activity and conferring an improved response of NSCLC patients presenting the long telomere variant.

Recently, telomerase has been intensively studied as a target for novel cancer gene therapy and therapeutics (81). Our findings that *hTERT* genetic variants, through modulating telomere length, may confer an advantage in chemotherapy response, according to different types of NSCLC, suggest that patients with long telomeres could have better responses to telomerase-based therapies.

Platinum and taxol compounds play a central role in cancer chemotherapy and, although treatment is limited by side effects, they continue to have widespread application. The assessment of telomerase genetic variants could supplement prognosis of survival in the course of NSCLC and may be promising molecular markers of treatment response in these patients.

4.2.2 Cyclin D1 genetic variants

Several findings are consistent with a model in which cyclin D1 serves as a key sensor and integrator of extracellular signals of cells in early to mid G1 phase, mediating its function through binding the CDKs (82). Cyclin D1 (CCND1) is the regulatory subunit of the holoenzymes that phosphorylate and, together with sequential phosphorylation by cyclin E/CDK2, inactivate the cell-cycle inhibiting function of the retinoblastoma protein (pRb). pRb serves as a gate keeper of the G1 phase and passage through the restriction point leads to DNA synthesis (83). Over-expression of cyclin D1 is known to correlate with the early onset of cancer and risk of tumor progression and metastasis (84, 85). pRb silences specific genes that are active in the S phase of the cell cycle through repression of E2F transcriptional activity. This activity is thought to be derepressed by cyclin D1 (82).

The *CCND1 A870G* polymorphism at codon 242 within the conserved splice donor site of exon 4 of the gene appears to modulate the splicing of CCND1 mRNA, originating two transcripts (*a* and *b*), which are present in a variety of tissues (8). The transcript *a* is identical to the reported CCND1 cDNA. However, transcript *b* fails to splice at the exon 4/intron 4 boundary, does not contain exon 5, and terminates downstream of exon 4. The main difference in the cyclin D1 proteins encoded by the two transcripts is in the C-terminal PEST-rich region

(destruction box) encoded by exon 5 which is responsible for rapid intracellular degradation and turnover of the G1 cyclins (86). Both genetic variants (A and G alleles) have been reported to be positively associated with increased risk for several cancers (9, 87-90). It has been suggested that the variant A allele may result in increased expression of *CCND1b* and G allele is associated with both cyclin D1 isoforms. *CCND1b* lacks the PEST-region, presents increased nuclear half-life and is a more potent oncogene, whereas *CCND1a* is a better catalyst of pRB phosphorylation/inactivation (10). These data support the emerging view that CCND1 alternate transcripts encode proteins with differing independent biological functions in tumour development. Thus, both isoforms encoded by G allele, seem to render a more aggressive phenotype, with increased transforming capacity and ability to promote cell cycle progression and proliferation. Our results indicate that the G-allele is associated with a higher risk of lung, cervical and nasopharyngeal cancers, as well as with a lower age at diagnosis in these patients. Similar results were found by other studies (88-91). The direction of the biological impact of cyclin D1 expression depends on the state of the cell in accordance with its checkpoint function. While cyclin D1 is best known for its proliferating effect, experimental evidence suggests that under conditions such as oxidative stress or senescence, cyclin D1 can exhibit S-phase entry and DNA replication and promote growth arrest, as well as apoptosis (92, 93). The context-dependent dual role of cyclin D1 in cell proliferation and growth arrest may explain the inconsistent associations observed between *CCND1* genotype and cancer risk.

One of the main aims of clinical or translational research in cancer is the search for genetic factors that could foresee treatment outcomes, in biologic activity and toxic effects. The assessment of telomerase and cyclin D1 genetic variants could supplement prognosis of survival in the course of cancer and may be promising molecular markers of treatment response in these patients. This genetic analysis may allow selection of patients who will have the greatest benefit from chemotherapy. Furthermore, a better knowledge of the underlying molecular profile of the host and the tumor will facilitate screening for cancer susceptibility and tailoring of chemotherapy in individual patients, choosing those most likely to respond, adjusting doses more precisely in order to reduce less adverse effects and establishing safety profiles based on individual genetic analyses.



FUTURE PERSPECTIVES

5. FUTURE PERSPECTIVES

Molecular markers within easily accessible samples from cancer patients may be of great interest in oncology. Late diagnosis constitutes a frequent problem in some tumors, with undesirable treatment responses and low overall survival of these patients. This study suggests a non-invasive diagnostic tool that can be further investigated in future prospective and larger scale studies for cancer detection and assessment of its potential applicability as a complementary diagnostic tool in clinical practice. Upcoming works should include a wider sample size, including cancer cases and control individuals.

This analysis of plasma DNA could also be performed in other tumors, especially those that lack programmed screenings, namely colorectal and gastric cancers, and other less incident neoplasia, like pancreatic and bladder cancers.

Moreover, in addition to quantitative analysis of plasma DNA, qualitative works studying specific genetic alterations in fcDNA and its possible correlation with those found in solid tumors should provide valuable information.

Finally, an analysis of fcDNA levels as possible predictive markers of treatment response could also be carried out in oncologic patients undergoing chemotherapeutic schemes.

Numerous gene variants frequently found in human populations may influence different stages of the neoplastic growth. These variants act through their products involved in various regulatory systems and metabolic chains at different levels of biological organization. Proliferation, differentiation and death of transformed and even malignant cells can be affected by pre-existing polymorphisms in genes exerting regulation of these basic processes. Likewise, genetic background emerges as a key factor in setting the rules of cell interactions defining tumor growth and spread.

Impressive recent progress in decoding the human genome has provided information about hundreds of thousands of potentially important gene polymorphisms. In this situation, the selection of polymorphisms to be analyzed in relation to a certain pathogenetic component becomes a critical element defining success of a research approach. For this reason, combined investigation of groups of functionally important gene variants within regulatory or metabolic cascades appears to be of the most importance in these studies. An analysis of interaction between polymorphisms should be included in different genes in determining drug response. It will be important to consider groups of potentially

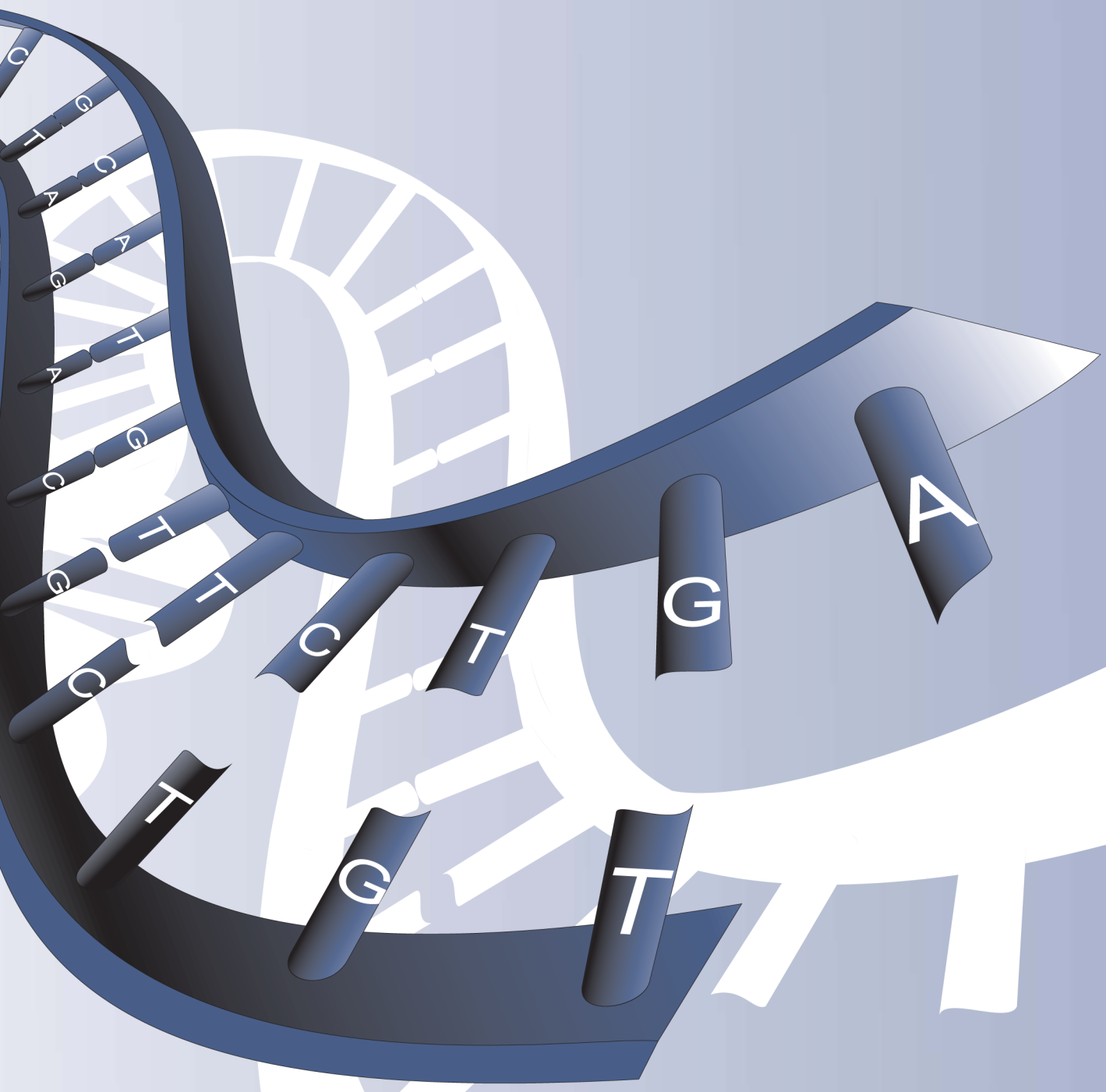
interacting genes as a set, such as those that act in common pathways, to identify interactions between polymorphisms in different genes. Furthermore, the behavior of most drugs will be influenced by a wide range of gene products (transporters, metabolizing enzymes, targets and others) and in many cases the importance of polymorphisms in one of the relevant genes might depend on polymorphisms in other genes. Thus, the study of other genetic variants involved in drug metabolic pathways should be included in future works.

To detect even relatively strong associations between genetic variants at a specific locus and variation in drug response, many cases and controls are needed. Future studies should include a wider population of cancer cases and control individuals.

The cyclin D1 and telomerase genetic polymorphisms should also be studied in other tumor models, namely, prostate and breast cancers. Functional studies should also be performed, in order to confirm the associations between the genetic variants and its possible phenotypes.

Most pharmacogenomic studies are retrospective and the genetic work has been carried out after the responses have already been observed. Few gene variants have been studied prospectively to assess how knowledge of the relevant genotype might improve clinical outcomes, for example, by adjusting doses or selecting the most appropriate drug as a function of the genotype. Translating pharmacogenomics research into improved therapies will require an expansion of prospective studies of how variants influence drug response.

To the extent that gene variants could offer significant diagnostic value on how patients respond to medicine, it seems likely that health-care providers will benefit economically by paying for pharmacogenomic diagnostics, as well as providing better clinical care for their patients both from savings on expensive medicines that do not work in many patients and by savings on costs that are associated with acquired drug resistances. Future prospective studies should focus on the effect of pharmacogenomics in patient outcome and combine this with cost effectiveness evaluations, developing predictive models that may help in deciding when pretreatment genetic screening is useful.



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