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Cadm1's expression levels in peripheral blood: A potential marker for diagnosis, disease progression and clinical outcome for NSCLC

Dissertação de Candidatura ao grau de Mestre em Oncologia – Especialização em Oncologia Molecular submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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Informação Técnica

TÍTULO:
Cadm1's expression levels in peripheral blood: a potential marker for diagnosis, disease progression and clinical outcome for NSCLC

Tese de Candidatura ao Grau de Mestre em Oncologia – Especialização em Oncologia Molecular submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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DATA: outubro 2015

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1ª EDIÇÃO: outubro 2015

“When nothing is sure, everything is possible.” Margaret Drabbler

Agradecimentos

Ao terminar mais uma etapa da minha vida não podia deixar de agradecer àqueles que foram essenciais para a realização deste projeto.

Em primeiro lugar gostava de agradecer à Professora Doutora Berta Silva, Diretora do Mestrado em Oncologia, pela oportunidade de ingressar neste mestrado.

Ao Professor Doutor Rui Medeiros, meu orientador, agradeço a oportunidade de fazer parte da sua equipa. Obrigada por toda a ajuda e disponibilidade que me ofereceu, pelo entusiasmo que demonstrou por este projeto, e por me ensinar a pensar além do óbvio.

Um grande agradecimento à Doutora Mónica Gomes. Não seria possível ter feito este trabalho sem a sua ajuda. Mostrou-se disponível para me ensinar e ajudar desde o primeiro momento. Motivou-me quando os resultados não foram os que eu esperava. Ajudou-me a ver e a pensar no que pode vir depois deste trabalho e isso foi essencial para que eu não desmotivasse. Obrigada por tudo o que me ensinou, pelos “brainstormings” e pelo exemplo que deu na maneira como trabalha. Foi essencial para a realização desde projeto.

A todos os colegas do piso -1, muito obrigada pela recetividade e disponibilidade. Todos, sem exceção, se mostraram disponíveis para me ajudar, ensinar e acompanhar, sempre que precisei.

Quero muito agradecer à Andreia, Liliana, Luísa, Joana e Marta por me fazerem sentir que os 300 quilómetros de distância que nos separam não mudam a nossa amizade. Conseguiram estar “presentes” sempre que precisei e foram um pilar essencial nesta jornada. São cinco pessoas fantásticas e cinco profissionais que admiro muito.

A toda a família e amigos que me fazem feliz por voltar a casa, e que não me fizeram sentir a distância nos últimos dois anos.

Obrigada mãe, por me mostrar todos os dias o que é empenharmo-nos num projeto e levá-lo até ao fim. “Quando acreditamos no que fazemos, queremos sempre fazer melhor” e eu espero um dia conseguir deixar a minha marca nos projetos em que me envolvo tal como a mãe faz. Obrigada pela mãe que é, por acreditar em mim e por me ter apoiado em tudo o que fiz até aqui.

Pai, desde pequena que me ensinou a questionar o que me dizem e a pensar por mim em vez de ir atrás dos outros. Esse espírito crítico e “cético” é essencial num investigador e esteve bem presente ao longo deste trabalho. Desde o primeiro dia que mostrou interesse no que eu faço, questionou as minhas escolhas e fez-me querer justificar cada passo do meu trabalho. Obrigada por tudo isto e pelo exemplo que é como pai, como homem e como profissional.

À Mariana e ao António, agradeço por me fazerem sentir orgulhosa por ser irmã

mais velha. Fazem-me querer ser melhor do que sou para dar o melhor exemplo possível.

Ao meu tio Carlos Maria, por me ter feito voltar às raízes, pelas gargalhadas e pelas histórias. Esses fins de semana foram essenciais para renovar energias e preparar uma boa semana.

Por último, e mais importante de todos, quero agradecer à tia Lena e à Mafalda. Deram-me muito mais que um teto. Deram-me uma casa, uma nova família. Cresci, com o exemplo que dão todos os dias e nada disto teria sido o mesmo sem as duas. Obrigada pelos miminhos, pela companhia, pelas histórias e por me fazerem sentir em casa e parte da família. Nunca esquecerei o quão importante foram nestes dois anos, especialmente durante a elaboração deste trabalho.

A todos, muito, muito obrigado!

Abbreviations

A	
A	Adenine
AJCC	American Joint Committee on Cancer
ATL	Adult T-cell leucemia
B	
BRCA2	Breast Cancer 2
C	
C	Cytosine
CADM1	Cell Adhesion Molecule 1
CASK	Calcium/Calmodulin-Dependent Serine Protein Kinase
cDNA	Complementary Deoxyribonucleic Acid
cfDNA	Circulating Cell free DNA
CI	Confidence Interval
CRTAM	class-I MHC-restricted T cell-associated molecule
Ct	Cycle threshold
CTC	Circulating Tumor Cells
D	
DNA	Deoxyribonucleic Acid
E	
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
EML4-ALK	Echinoderm Microtubule-associated protein-like 4 - Anaplastic Lymphome Kinase
G	
G	Guanine
GUSβ	glucuronidase, beta
H	
HER-2	Human Epidermal growth factor Receptor 2
I	
IARC	International Agency for Research on Cancer
IgCAM	immunoglobulin superfamily cell adhesion molecule
IGSF4	ImunoGlobulin SuperFamily 4
K	
K-RAS	Kirsten Rat Sarcoma
L	
LOH	Loss of Heterozigoty

M

miRNA	microRNA
MPP3	Membrane Protein, Palmitoylated 3
mRNA	messengerRNA

N

necl-2	nectin-like molecule 2
NK cells	Natural Killer Cells
nm	nanometers
NPV	Negative Predictive Value
NSCLC	Non-Small Cell Lung Cancer

O

OR	Odds Ratio

P

PPV	Positive Predictive Value

Q

qPCR	Real Time Polymerase Chain Reaction

S

SCLC	Small Cell Lung Cancer
SD	Standard Deviation
SgIGSF	Spermatogenic Immunoglobulin Superfamily
SynCAM	Synaptic Cell Adhesion Molecule

T

T	Thymine
TSG	Tumor Suppressor Genes
TSLC1	Tumor Suppressor of Lung Cancer 1

Y

YAC	Yeast Artificial Chromosome

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Resumo

Resumo

Cada vez mais, o cancro representa um crescente problema de saúde pública a nível global e estima-se que em 2025 o número de novos casos atinja os 19,3 milhões devido ao crescimento e envelhecimento da população mundial.

O cancro do pulmão é a principal causa de morte por doença oncológica no mundo. A maioria dos cancros de pulmão (80%) são cancros de pulmão de não-pequenas-células (CPNPC). Destes, mais de 65% dos pacientes apresenta doença localmente avançada ou metastática. É operável se diagnosticado em estadios precoces e a taxa de sobrevivência a cinco anos é aproximadamente 50%-70%. No entanto, a taxa de sobrevivência a cinco anos diminui para 2%-5% para os pacientes cuja doença já disseminou aquando do diagnóstico. Embora o diagnóstico precoce possa salvar vidas, os atuais métodos de rastreio para os indivíduos de alto-risco não demonstraram melhorias na sobrevivência. É, então, essencial que se desenvolva uma abordagem não invasiva de rastreio baseada em biomarcadores moleculares que permita uma melhoria na avaliação de risco e um diagnóstico precoce.

Um possível marcador é o CADM1 (*Cell Adhesion Molecule 1*), um gene supressor tumoral localizado na região 11q23.2 que codifica para uma glicoproteína da superfamília das imunoglobulinas cuja expressão se encontra diminuída em muitos tumores sólidos. Este gene está associado com a supressão tumoral e também com a diminuição da metastização, especialmente em CPNPC. Na grande maioria dos casos a diminuição da expressão do CADM1 parece resultar do silenciamento por metilação do promotor.

Com o presente trabalho propusemo-nos a avaliar a utilização de níveis de expressão de Cadm1 como biomarcador de diagnóstico e/ou prognóstico de CPNPC. Para validar o Cadm1 como um marcador de diagnóstico, elaborou-se um estudo de caso-controlo de base hospitalar, usando amostras de sangue provenientes de 89 doentes com CPNPC e 52 indivíduos saudáveis. Numa segunda fase, realizou-se um estudo de *follow-up* a 89 doentes com CPNPC para avaliar a associação entre os níveis de expressão de Cadm1 no sangue periférico, a progressão da doença e o *outcome* clínico.

Ao compararmos os perfis de expressão de Cadm1 entre os indivíduos saudáveis e os indivíduos com CPNPC, observamos claras diferenças entre os grupos. Indivíduos com CPNPC apresentam níveis mais baixos de expressão de Cadm1 comparativamente com indivíduos saudáveis que apresentam níveis mais elevados. (OR=2,147; IC95%=1,068-4,32; p=0,031), não estando estas diferenças relacionadas com o sexo do indivíduo (HR=0,401; IC95%= 0,181-0,887; p=0,024).

Não foram encontradas diferenças nos níveis de expressão de Cadm1 entre os vários estadios da doença (nem entre estadios iniciais e estadios avançados nem entre

não metastizados e estadios metastizados).

Os níveis de expressão de Cadm1 no sangue periférico parecem influenciar apenas a sobrevivência a um ano dos indivíduos não metastáticos e não dos indivíduos com tumores em estadio IV. Indivíduos com tumores não metastáticos e baixos níveis de expressão de Cadm1 em circulação apresentam uma pior taxa de sobrevivência do que aqueles que têm tumor não-metastático, mas elevados níveis de Cadm1 (p=0,016).

Apesar de os níveis de expressão de Cadm1 no sangue periférico aparentemente não representarem um bom marcador de prognóstico, os nossos resultados suportam o seu uso como biomarcador de diagnóstico apresentando uma sensibilidade de 57,3%, especificidade de 61,5%, VPP de 72% e VNP de 54%.

Não existindo à data um marcador ideal para diagnóstico de cancro do pulmão e visto que os métodos utilizados apresentam maiores custos, maiores riscos de indução de efeitos secundários e impossibilidade de aplicação em toda a população, concluímos, com base nos nossos resultados, que o Cadm1 poderá ser um potencial biomarcador de diagnóstico de CPNPC através de uma simples amostra de sangue.

Abstract

Abstract

More and more, cancer has become an emerging public health problem worldwide and it is estimated that there will be 19.3 million new cases per year by 2025 due to growth and ageing of the global population. Lung cancer is the leading cause of cancer-related death worldwide. Most lung cancers (80%) are non-small-cell lung cancers (NSCLC) and of these, more than 65% of the patients present with locally advanced or metastatic disease. When diagnosed in an early stage it's operable and the five-year survival rate is approximately 50%–70%. However, the five-year survival rate drops to 2%–5% for patients whose cancers are diagnosed after their tumors have spread distantly. Although early detection can save lives, the current screening tests for high-risk individuals have not resulted in improvements in disease-specific survival. It has become imperial to develop a non-invasive approach based on molecular biomarkers that may significantly improve the assessment of risk and early diagnosis.

One candidate marker is cell adhesion molecule 1 (CADM1), a putative tumor suppressor gene located in 11q23.2 region, which encodes a glycoprotein of the immunoglobulin superfamily and that is found to be downregulated in many solid tumors. It has been related both with suppression of tumorigenesis and decreased metastization, especially in non-small cell lung cancer (NSCLC) and in most cases its downregulation appears to be silencing by promoter methylation.

The aim of the present study was to evaluate if Cadm1 expression levels can be used as biomarker for diagnosis and/or prognosis for NSCLC. To evaluate the potential of using Cadm1 expression levels in peripheral blood as a diagnostic marker for NSCLC we conducted a hospital-based case-control study using blood samples from 89 NSCLC patients and 52 healthy individuals. In a second phase we performed a follow-up study with 89 NSCLC patients to evaluate the association between Cadm1's expression levels in peripheral blood and disease progression and clinical outcome.

When we compared the expression profiles of healthy individuals against NSCLC patients, we observed clear differences of expression between both groups. Cadm1 presents lower expression levels in NSCLC patients compared to the higher levels measured in healthy individuals (OR=2.147; CI95%=1.068-4.32; p=0.031). Also, we observed that this differences are independent of gender (HR=0.401; CI95%= 0.181-0.887; p=0.024).

When studying the association between Cadm1's expression levels and disease progression we didn't find any differences between early stages and more advanced stages, or between non metastatic and metastatic tumors.

Furthermore, Cadm1's expression levels in peripheral blood only appears to

influence one-year survival in non-metastatic patients and not in those who have stage IV tumors. Patients with non-metastatic tumors and low Cadm1's expression levels in circulation have lower survival rates that those who have non-metastatic tumor but high Cadm1's expression levels (p=0.016).

Even though it appears that Cadm1's expression levels in peripheral blood aren't a good prognostic marker, our results support the potential of using Cadm1 as a diagnostic biomarker with a sensibility of 57.3%, specificity 61.5%, PPV of 72% and NPV of 54%.

Since there isn't an ideal screening method for lung cancer and the methodologies used are either expensive, have side effects, or are not accessible or applicable to everybody we conclude, based on these results, that Cadm1 may become a potential biomarker for NSCLC through a simple blood test.

Introduction

1. Introduction

1.1. Cancer: Epidemiology and general concepts

More and more, cancer has become an emerging public health problem worldwide and it is estimated that there will be 19.3 million new cases per year by 2025 due to growth and ageing of the global population [1]. At present time, it represents the first and the third cause of death in the developed countries and in the less developed countries, respectively [2]. According to GLOBOCAN 2012 - the International Agency for Research on Cancer (IARC) online database - there were 14.1 million new cancer cases, 8.2 million cancer deaths in 2012, compared to 12.7 million and 7.6 million, respectively, registered in 2008 [1]. Estimates prevalence rates for 2012 show that there were 32.6 million people alive (over the age of 15 years) who had had a cancer diagnosed in the previous five years [3].

The term 'cancer' defines over one hundred different diseases that can arise from virtually any tissue or organ in the body. It is a heterogeneous disease, with different etiology and natural history that develops through the interactions between environmental and genetic factors, and involves deregulation of multiple pathways responsible for the fundamental cell processes, such as death, proliferation, differentiation and cell migration [4]. Hanahan and Weinberg proposed that cancer cells acquire six biological capabilities during the multistep development of human tumors that are shared by nearly all cancers and are considered to represent the hallmarks that govern the conversion of normal cells into cancer cells. Those include growth signal autonomy, insensitivity to anti-growth signals, apoptosis evasion, replicative immortality, angiogenesis induction and tissue invasion and metastasis. Conceptual progress in the last decade has added two emerging hallmarks of potential generality to this list - reprogramming of energy metabolism and evading immune destruction. In addition to cancer cells, tumors exhibit another dimension of complexity for they contain a repertoire of recruited, ostensibly normal cells that contribute to the acquisition of hallmark traits by creating the ‘‘tumor microenvironment’’ [5].

The process of carcinogenesis is very complex and involves a progressive accumulation of genetic alterations that lead to the transformation of a normal cell to a neoplastic cell [5]. Carcinogenesis can be divided in three main stages: Initiation, promotion and progression.

The initiation process consists on the acquisition of non-lethal genetic lesions by the somatic cells, caused by carcinogens (chemical, physical or biological), by epigenetic alterations or by inherited germline alterations [6]. If this cell is stimulated by promoting agents like chemicals or hormones and isn’t able to repair the damages, there is an

accumulation of genetic alterations that provides a selective advantage for these cells over normal cells. This is the promotion stage: there is a selective clonal expansion of initiated cells which results in a larger population of cells that are at risk of further genetic changes and malignant conversion [7]. Tumor progression comprises the expression of the malignant phenotype and the tendency of malignant cells to acquire more aggressive characteristics over time. During this process, and through interaction with environmental factors, there may occur genetic and epigenetic alterations that allow the activation of proto-oncogenes and inactivation of tumor suppressor genes (TSGs). Subsequently, multiple events occur that result in the disruption of processes such as cell proliferation and apoptosis, which in turn lead to the growth and development of malignancies. The mutations acquired during neoplastic development make the cells less sensitive to apoptotic signals, cell growth stop and destruction by the immune system. Cumulatively, the self-sufficiency of these cells in growth factors provides them with greater proliferative capacity, angiogenesis and invasive potential. These factors combined with increase in inflammation, genomic instability and cell energy deregulation, favor cancer development and systemic dissemination, enhancing the metastatic process [8].

Even though there has been new developments and achievements in the field of Oncology, either in the early detection of some neoplasia and in the development of new treatments more effective in disease management, the numbers are still increasing and the acquired resistance to these treatments has become a difficult obstacle to overcome.

1.2. Lung Cancer

Lung cancer has been the most common cancer in the world for several decades. There were estimated to be 1.8 million new cases in 2012 (12.9% of the total). The disease remains as the most common cancer in men worldwide (1.2 million, 16.7% of the total) with the highest estimated age-standardized incidence rates in Central and Eastern Europe (53.5 per 100,000) and Eastern Asia (50.4 per 100,000), in 2012. In women, the incidence rates are generally lower (approximately 0.6 million, 8.8% of the total) and the geographical pattern is a little different, mainly reflecting different historical exposure to tobacco smoking. Furthermore, lung cancer is the most common cause of death from cancer worldwide, estimated to be responsible for nearly one in five deaths (1.59 million deaths, 19.4% of the total) which is more than breast, colon and prostate cancer combined [1, 2].

In Portugal, lung cancer is the fourth most common neoplasia (4192 cases, 8.5% of total), but has the second highest mortality rate (3441 deaths, 14.3% of the total) following closely the 15.7% death rate caused by colorectal cancer [9].

According to American Cancer Society estimates, the 1- and 5-year relative survival

rates for lung cancer cases diagnosed during 2003-2009 were 43% and 17%, respectively [10]. The majority of lung cancer cases in men (~85%) and approximately 47% of lung cancer cases in women are estimated as being the consequence of tobacco smoking [11]. Primary prevention should continue to be a major focus of public health campaigns for there is overwhelming evidence that tobacco smoking is the major cause of lung cancer not only based on epidemiological studies. Lung tumors of smokers frequently contain a typical, though not specific, molecular footprint in the form of G:C>T:A mutation in TP53 gene which are probably caused by benzo[a]pyrene one of the many carcinogens in tobacco smoke [11].

However, there are other factors that may play a role in susceptibility to lung cancer or modulate the development of the neoplasia. Occupational exposure to carcinogens like arsenic, asbestos and other chemicals, pulmonary chronic disease, environmental pollution, pulmonary tuberculosis or even family history, viruses, and auto-immune diseases are considered to be possible risk factors, but further studies are necessary to clarify their association with lung cancer [11].

One of the biggest problems associated with poor survival in lung cancer cases is that most patients present with metastatic disease at time of diagnosis (~65%) [12, 13]. Metastasis is a multistep process in tumor development that culminates in a systemic spread of cancer cells. For reasons still unclear, some cells from the primary tumor suffer molecular alterations that causes them to separate from the tumor and enter circulation and invade another organ where a new tumor starts developing. Treatment against metastasis is still very poor because of the heterogeneity of the metastasis since the same type of tumor can develop metastasis in different sites, and even in the same tumor, different cells have a potential to metastasize.

A correct evaluation of the histologic subtype and staging at the time of diagnosis is necessary for treatment and patient's prognosis [14]. Staging for lung tumors is according to the TNM system, proposed by the American Joint Committee on Cancer (AJCC), that classifies the tumor according to its size and invasive potential (T), with the involvement of regional lymph nodes (N) and the absence or presence of long distance metastasis (M) [14-16].

Even though 50% of lung carcinomas exhibit more than one major histological type, two main categories are distinguished: small cell lung cancer (SCLC) accounting for about 20% of lung cancer cases, and non-small cell lung cancer (NSCLC) that comprises a number of histological subtypes representing near 80% of lung cancers [11].

1.3. Non-small cell lung cancer

NSCLC is representative of many different histological subtypes of lung cancer. The most frequent histological subtypes are: large cell/undifferentiated carcinomas; squamous cell carcinomas (SCC) and adenocarcinomas [11].

This neoplasia is often asymptomatic in early stages. As a result, of all the patients with NSCLC, more than 65% present with locally advanced or metastatic disease at diagnosis [12].

When diagnosed in an early stage it's operable and the five-year survival rate is approximately 50%–70%. However, the five-year survival rate drops to 2%–5% for patients whose cancers are diagnosed after their tumors have spread distantly [17]. Until the late 1990s, treatment of advanced lung cancer was based on platinum combination therapy, irrespective of histological subtype and without any option for further lines of treatment [18].

Since then, the introduction and development of cytotoxic drugs (such as gemcitabine, vinorelbine, docetaxel, and paclitaxel) and patient selection based on the recognition that different histological subtypes and driver mutations determine the biology of these malignancies and predict drug efficacy, changed the treatment of NSCLC. This resulted in improvement of overall survival up to 12 months and longer in clinical studies [18].

Even though the new approach to NSCLC treatment improved the overall survival there has been only a minor improvement in five-year survival from lung cancer in the last few decades. It's obvious that the sooner it's caught, the better the outcome.

According to the World Health Organization, early diagnosis and screening are the two main components for early detection. Early diagnosis - or the awareness of early signs and symptoms - is particularly relevant when there are no effective screening methods or no screening and treatment interventions implemented. Screening aims to identify individuals with abnormalities suggestive of a specific cancer or pre-cancer and refer them promptly for treatment or when feasible for diagnosis and treatment.

Although early detection can save lives, screening tests for high-risk individuals using sputum cytology or chest X-rays have not resulted in improvements in disease-specific survival [19, 20]. Annual screening with low-dose CT scanning was associated with a significant reduction in lung cancer mortality in one large study of high-risk individuals [21]. However, more data are needed on the cost effectiveness of screening that takes into account the frequency of screening and both the benefits and harms (such as false positives and over-diagnosis) before recommendations can be made for large-scale screening programs [21]. Microarray-based gene expression profiling has successfully been used in clinical cancer research to sub-classify cancer entities, to predict prognosis or response to

therapy, and to identify underlying mechanisms of tumor development but again, more data are needed [22].

Until the last decade, NSCLC was considered a single disease, and systemic treatment of metastatic NSCLC was limited to platinum-based chemotherapy doublets resulting in approximately 20% response rates and median survival of 8 months [23].

Since then, molecular diagnosis has been playing a major role in understanding the pathogenesis, diagnosis and treatment of lung cancer which has led to the identification of biomarkers in NSCLC [24].

The clinical challenge, therefore, is to develop a non-invasive approach based on molecular biomarkers that may significantly improve the assessment of risk and early diagnosis.

1.4. Biomarkers

According to the National (American) Cancer Institute, a biomarker is “a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease”. They typically differentiate an affected patient from a person without the disease and can be detected in the circulation (whole blood, serum or plasma), excretions or secretions (stool, urine, sputum), and thus easily assessed non-invasively and serially, or can be tissue-derived and require either biopsy or special imaging evaluation.

Biomarkers can be divided into: Diagnostic biomarkers; prognostic biomarkers; treatment biomarkers and prevention biomarkers.

Diagnostic biomarkers, also called predictive biomarkers (targets for diagnostic intervention), are identified by characterizing key mutations and molecular pathways involved in tumor development and proliferation. They help optimize therapy decisions by providing information about the likelihood of a response to a chemotherapeutic intervention [25]. Prognostic biomarkers are assigned to a specific tumor type by determining the occurring polymorphism, mutation or the change in DNA methylation or gene expression, or by detecting the presence of specific miRNA molecules or CTCs in the peripheral blood. They enable the monitoring of the advances in anti-cancer therapy, the assessment of the stage of the tumor and its potential malignancy, as well as the prognosis of disease remission in every case individually [25]. Treatment and prevention biomarkers are subgroups that require more accurate molecular analysis to guide individual therapy by identifying patients with different outcome risks (such as recurrence of the disease) [25].

Diagnostic and prognostic biomarkers particularly aid in identifying who is at risk, diagnose at an early stage, select the best treatment modality and monitor response to

treatment [26].

Over the last decade, the oncology field has witnessed significant advances in understanding these differences specific to a type of cancer or between patients, and instead of treating a particular histology, or organ system, it is now clear that a cancer with a specific histology represents a constellation of diseases with distinct molecular profiles and sensitivities to treatment (Figure 1). Personalized therapy based on patient's individual biologic and molecular profile is a promising approach to optimize efficacy in the available agents. This understanding has allowed us to deliver targeted therapy, which utilizes agents that affect a known aberrant pathway or molecular target in the cancer cell or tumor microenvironment. The treatment is no longer based on a “one drug fits all” approach but instead a more personalized strategy where treatment is driven by biomarker expression profiles [27-29].

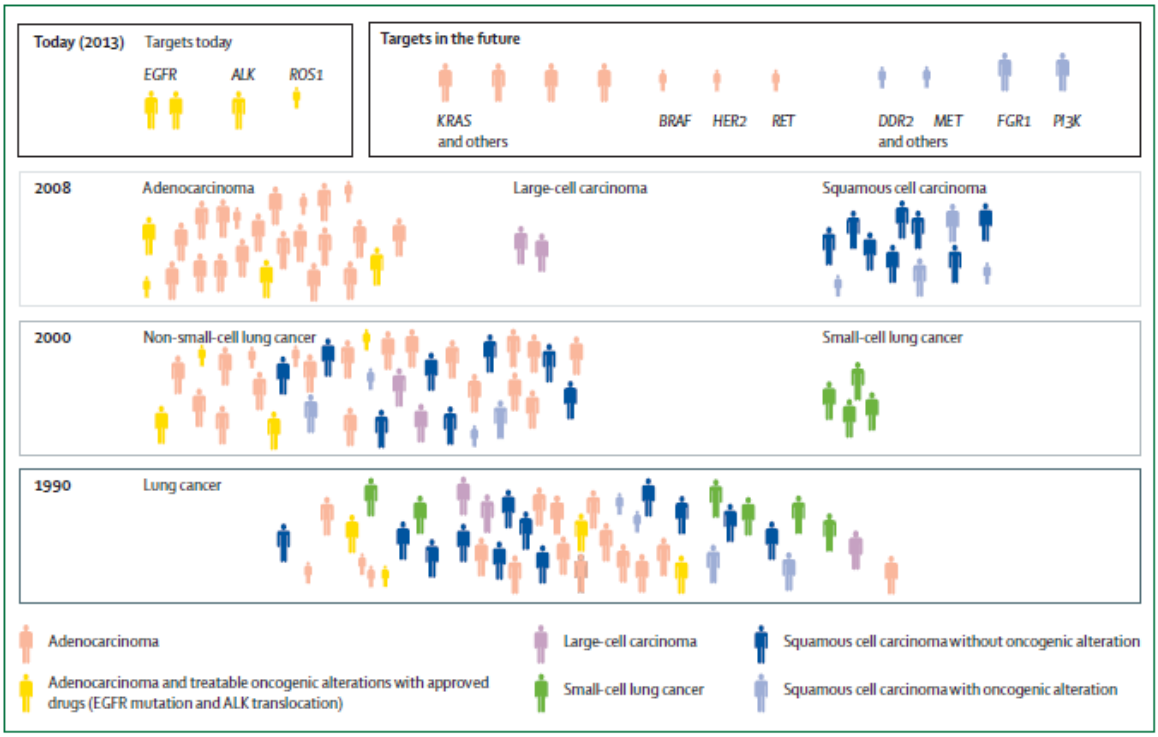

Figure 1 Evolution of lung cancer histology over time. The colors denote different histological subtypes [18]

Mutations in specific genes that affect DNA repair in germline cells may be inherited and sometimes a mutation in only one allele is enough to impair the whole repair system. This is the case, for example, of mutated BRCA2 gene in breast cancer. Patients identified with mutations in genes like these could, and should, undergo regular screening examinations to allow earlier detection of any tumor that might be growing asymptomatically [30].

TP53 mutations are the most frequent in all histological types of lung cancer. This

gene encodes the p53 protein that plays multi anti-proliferative roles in particular in response to genotoxic stress. Inactivating TP53 mutations (mostly missense mutations) are detected in up to 50% of all human tumors [31]. Although p53 is not a typical cancer-specific antigen, its central role in the control of cell growth and apoptosis and frequent mutations in tumors make p53 a unique target for cancer therapy.

Some of these biomarkers are the result of mutations in genes like EGFR, K-RAS, HER-2, BRAF, or translocations like the echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase (EML4-ALK) translocation and their frequencies are represented in (figure 2) [32].

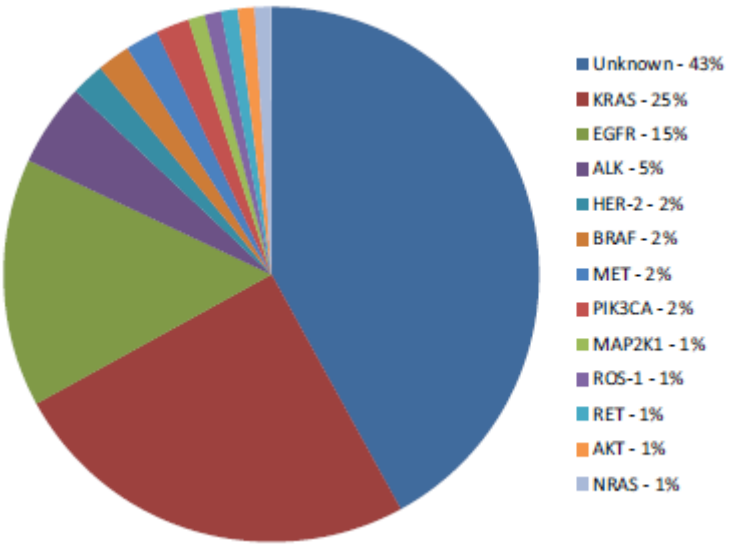

Figure 2 Molecular subsets of lung adenocarcinoma [32].

The following are three of the most promising molecular biomarkers studied in lung cancer. The Kirsten Rat Sarcoma (KRAS) gene was first described in human cancers in 1980s, where it was found to be active in tumor tissue but not in normal host tissues [33]. Mutations have been found in approximately 17% of all NSCLC, and are seen in 25%-34% of adenocarcinomas and non-squamous tumors, but are rarely seen in squamous cell carcinomas. However, a multivariate analysis looking at prognostic factors found that K-RAS mutational status was a weak prognostic factor (relative risk 0.641, P=0.066) [34, 35].

The Epidermal Growth Factor Receptor (EGFR) is one of a family of receptors that is overexpressed in about 10%-15% of cases. It has been proved that somatic mutations in exons 19 or 21 of the gene are related to tumor sensitivity to therapies with tyrosine kinase inhibitors (TKIs) such as gefitinib or erlotinib [36, 37].

Anaplastic lymphoma kinase (ALK) gene rearrangement was first reported in 2007 by Soda et al. In this translocation, a deletion and translocation in chromosome 2p brings

the EML4 gene in conjunction with ALK receptor tyrosine kinase, making a fusion protein in which the tyrosine kinase activity is permanently turned on. This translocation is detected in approximately 5% of patients with adenocarcinoma and are usually seen in non-smokers or very light smokers [38].

Unfortunately, the number of patients for whom targeted therapy is suitable is still very small (Figure 2). The access to tumor tissue for biomarker assessment and *de novo* molecular and genomic tumor heterogeneity (that may be further increased during the biomarker-driven therapy) remains a serious challenge. Ongoing research in detection of cell-free circulating tumor DNA (cfDNA) and circulating tumor cells (CTCs) may become clinically relevant alternatives for tumor biopsy that will provide measurements of the total tumor burden as well as identify mutations arising during therapy that may be responsible for development of acquired resistance [32].

1.5. CADM1

Cell Adhesion Molecule 1(CADM1) is also referred to as IGSF4, TSLC1, SgIGF, SynCAM, NECL2 according to the different functions in different tissues [39-43].

In 1998, Murakami et al observed that loss of heterozygosity (LOH) on chromosome 11q23 occurred in patients with non-small cell lung carcinomas (NSCLC) [44]. In 1999, a new member of ImunoGlobulin SuperFamily (IGSF) was isolated from that region and named as IGSF4 [39]. In 2001, by combinatorial analyses of YAC transfer into human NSCLC cells with a tumorigenicity assay in nude mice using A549 cell line, the IGSF4 gene was identified as a tumor suppressor in NSCLC and was renamed as Tumor Suppressor of Lung Cancer 1(TSLC1) [40, 41]. In the same year and the next, researchers of testis and brain found that this molecule played a significant role in spermatogenic-cell attachment and synapse formation. Accordingly, IGSF4/TSLC1 was renamed as Spermatogenic Immunoglobulin Superfamily (SgIGSF) and Synaptic Cell Adhesion Molecule (SynCAM), respectively [43, 45]. In 2003 Ito et al. found a role for the molecule in mast-cell adhesion in the course of mast cell research [46].

This region encodes an immunoglobulin superfamily cell adhesion molecule (IgCAM) homologous with the neural cell adhesion molecule and is involved in Ca^{2+}-independent cell-cell adhesion and cell signal transduction [47, 48].

Cadm1 is a type I transmembrane glycoprotein located in the chromosome 11q23.2, it has 442 aminoacids with a molecular weight of 75 kD in normal epithelium including the lung, 70 kD in the brain, and approximately 100 kD in the testis. This difference in molecular weight appears to be a result of post-translational modification, in addition to a variation in splicing [49, 50].

At the N-terminal there is a signal sequence followed by three Immunoglobulin-like domains that interact through a homo and heterophilic manner. There is a transmembrane domain and a small cytoplasmic domain as shown in figure 3 [40, 45, 46, 51].

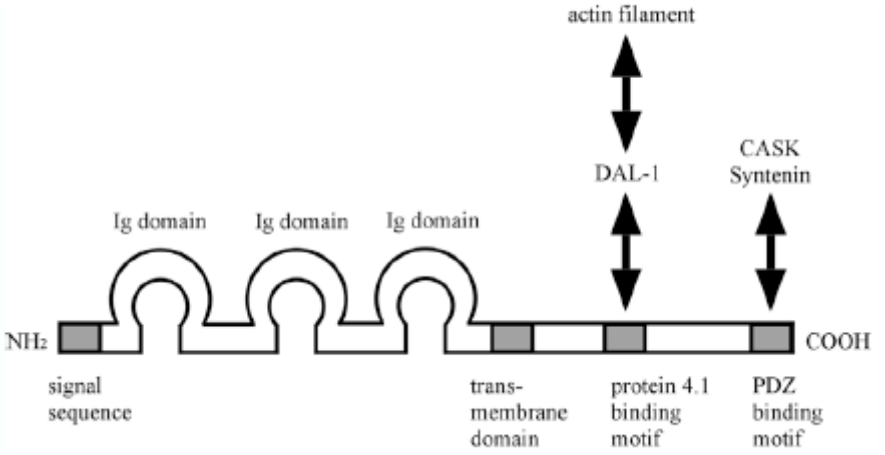

Figure 3 Schematic representation of the structure of CADM1. CADM1 contains from the NH2-terminal to the COOH-terminal signal peptide, three Ig domains, a transmembrane domain, protein 4.1 binding motif and PDZ binding motif [52].

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The overall structure of CADM1/TSLC1 is similar to that of another important family of IgCAM, namely the nectins, and so it is also called Nectin-like molecule 2 (Necl-2) [49].

The extracellular domain of CADM1 mediates the formation of homodimers or heterodimers with other CAM members, including Necl-1, CRTAM (class-I MHC-restricted T cell-associated molecule) and Nectin-3 to regulate cell adhesion [49, 50].

The cytoplasmic domain contains the protein 4.1 binding motif and directly associates with DAL-1, a gene product of another lung tumor suppressor belonging to the protein 4.1 family and interacts with the actin filament through an anchoring protein, DAL1 [53].

The cytoplasmic C-terminal sequence harbors a PDZ-domain protein-interaction sequence that is homologous to that of the synaptic cell-surface proteins neurexin and syndecan, which bind to the PDZ-domain protein CASK and syntenin [52]. Via the PDZ-binding domain CADM1 interacts with pals2, and MPP3 [52].

CADM1's role as a cell adhesion molecule has been well established. Molecules that are identical to human CADM1 or its murine orthologues have been found to play key roles in the adhesion of spermatogenic cells to Sertoli cells, mast cells to fibroblasts, presynaptic cells to postsynaptic cells, tumor cells to natural killer (NK) cells, and antigen-presenting cells to CD8+ T cells through homophilic or heterophilic interactions [42, 43, 46, 54, 55]. Its role as a tumor suppressor, has been shown in a variety of cancers of epithelial origin [40]. Also, Mao et al. reported that high CADM1/TSLC1 expression levels from a recombinant adenovirus vector (Ad-TSLC1) inhibited cell proliferation and induced

apoptosis in A549 cells [56].

Studies in nude mice have demonstrated that re-expression of CADM1 suppresses in vivo tumorigenicity of NSCLC and nasopharyngeal carcinoma cell lines [56, 57].

Apoptosis induction, as well as the formation or maintenance of an epithelial cell structure through homophilic interaction, is one of the possible molecular mechanisms of CADM1/TSLC1 in the tumor suppression of carcinomas.

In contrast, CADM1/TSLC1 has been found to be ectopically expressed in adult T-cell leukemia (ATL), and could be implicated in tumor invasion through interactions of the leukemia cells with the endothelial cells [58]. Another interesting study challenging CADM1's role as a tumor suppressor is one by Kikushi et al. They studied a splicing variant of CADM1 present in Small Cell Lung Cancer and their results suggest that CADM1 enhances the malignant features of SCLC, as is observed in ATL, and could provide a molecular marker specific to SCLC [59].

It is now known that CADM1/TSLC1 is expressed universally in human tissues and is frequently silenced in a variety of human carcinomas, such as lung, prostate, liver, stomach, pancreatic and breast carcinoma [48, 56].

Besides mutations and deletions, DNA methylation of CpG islands in the promoter region of cancer-related genes is a frequently acquired epigenetic event in the pathogenesis of many human malignancies leading to gene silencing. So far, aberrant methylation (referred to as methylation) has been described for several genes in various malignancies including lung cancer [60].

Promoter methylation of the CADM1/TSLC1 gene was demonstrated in NSCLC, pancreatic cancers, hepatocellular carcinoma, and prostate cancers, cancers from the esophagus, stomach, nasopharynx, breast, and uterine cervix, as well as meningioma, and seems to be the main mechanism underlying the expression silencing [41, 49, 60-62].

Furthermore, methylation or loss of CADM1 expression was observed preferentially in tumors at advanced pathological stages rather than in early stages and found to be associated with tobacco smoking [63, 64]. Fukami et al. demonstrated that CADM1 promoter methylation was significantly associated with tumor progression of NSCLC from pT1 to pT2 and from stage Ia to IIb [61]. And Heller et al. showed that promoter methylation also occurred more frequently in tumors at grade 3, compared with tumors at grade 1 or 2 in breast cancer [65].

In an immunohistochemical study using a specific antibody against CADM1/TSLC1, Uchino *et al*. demonstrated that CADM1/TSLC1 expression was inversely correlated with advanced disease stage, lymph node involvement, lymphatic permeation, and vascular invasion. 4-year survival and disease-free survival are significantly shorter in patients with lung adenocarcinoma lacking CADM1 expression [66].

For all that's been previously said, it seems clear that CADM1 plays an important role in cancer development, especially in NSCLC since it is downregulated in most cases and appears to be correlated with tumor progression, but can it be used as a biomarker?

Objectives

2. Objectives

2.1. Main Objective

The aim of the present study is to evaluate if Cadm1 expression levels can be used as biomarker for diagnosis and/or prognosis for NSCLC patients.

Thereby, the present study can be divided in two different parts:

2.2. Specific Objectives

1. Assess Cadm1's expression levels in peripheral blood as a possible diagnostic marker for NSCLC;
2. Evaluate the association between Cadm1's expression levels in peripheral blood and tumor progression, namely 1-year survival.

Material and Methods

3. Material and Methods

3.1. Patient selection and sample collection

3.1.1. The first part of this work consisted of a hospital-based case-control study involving 141 individuals (89 NSCLC patients and 52 healthy individuals) all Caucasian, from the north of Portugal. All participants signed an informed consent according to the Helsinki declaration.

The case group consisted of 69 males and 20 females all Caucasian with non-small cell lung cancer diagnosed and treated at the Portuguese Institute of Oncology of Porto (mean age = 61.2; SD=15.7). After patients signed an informed consent according to the Helsinki declaration a blood sample was collected before any medical intervention.

All the demographic and histopathological data were collected from the clinical files of each patient and the staging of each patient was made according to the AJCC TNM classification system 2010 (7th edition).

For the control group, 52 healthy Caucasian individuals with no history of cancer (mean age = 50.22; SD=17), were randomly recruited from the north of Portugal. All demographic data were collected through a written form, authorized by the ethics committee of the Portuguese Institute of Oncology of Porto, and signed by each individual, according to the Helsinki declaration.

All the clinicopathological and demographic data are described in table 1.

Approximately 8 ml of peripheral blood were collected from all individuals throught a standard method of intravenous collection with EDTA tubes. The blood tubes were centrifuged 5 minutes at 2800 rpm in order to separate the peripheral blood cells and then preserved in Tripure ® (Roche) at 80ºC.

3.1.2. The second part consisted of a follow-up study involving the 89 NSCLC patients from the case group of the first part. All the clinicopathological data are described in table 1.

Table 1 Clinicopathological and demographic characteristics of the participants

		Cases (n=89)		Controls (n=52)	
		n	%	n	%
Gender					
Masculin		69	77.5	17	32.7
Feminin		20	22.5	35	67.3
	Total	89	100.0	52	100.0
Age					
Mean ± SD		61,2 ± 15,7		50,22 ± 17	
Histology					
SCC		32	36.0		
Non-SCC		57	64.0		
	Total	89	100.0		
Stage					
I		3	3.4		
II		1	1.1		
III		36	40.4		
IV		49	55.1		
	Total	89	100.0		
Smoking					
Smokers and Ex-Smokers		66	74.2	10	19.2
Non Smokers		22	24.7	41	78.9
No information		1	1.1	1	1.9
	Total	89	100.0	52	100.0

3.2. RNA extraction and cDNA synthesis

After collecting the samples, the RNA was extracted from the peripheral blood cells (preserved in Tripure®) using an extraction kit (GeneJetRNAPurification KIT, Thermo Scientific®) and following the manufacturer's instructions. The concentration and purity of the isolated RNA were measured at 260 and 280 nm using the *NanoDrop® ND-1000* spectrophotometer.

The mRNA samples were then used as template for cDNA synthesis using a High capacity RNA-to-cDNA kit from Applied Biosystems®, according to manufacturer's instructions.

3.3. Cadm1 relative quantification

The Cadm1 mRNA expression was analyzed by quantitative real-time PCR. The reactions were carried out on a StepOne™ qPCR - Real-Time PCR machine, containing 1X Master mix (*Applied Biosystems®*), with 1X probes (*TaqMan® Gene Expression Assay – Hs00942509_m1 – Applied Biosystems®*), cDNA sample and GUSβ endogenous control (TaqMan® Pre-Developed Assay – NM-000181 – Applied Biosystems®) which was used to

normalize the results regarding the biomarkers, since it presents a constant expression level. Data analysis was made using StepOneTM Sofware v2.2 (*Applied Biosystems*®) with the same baseline and threshold set for each sample, in order to generate threshold cycle (Ct) values for all the mRNAs. The mRNAs quantification (Cadm1 and Gusβ) was performed in duplicate and negative control lacking cDNA was included in all reactions.

The results were confirmed by two independent investigators.

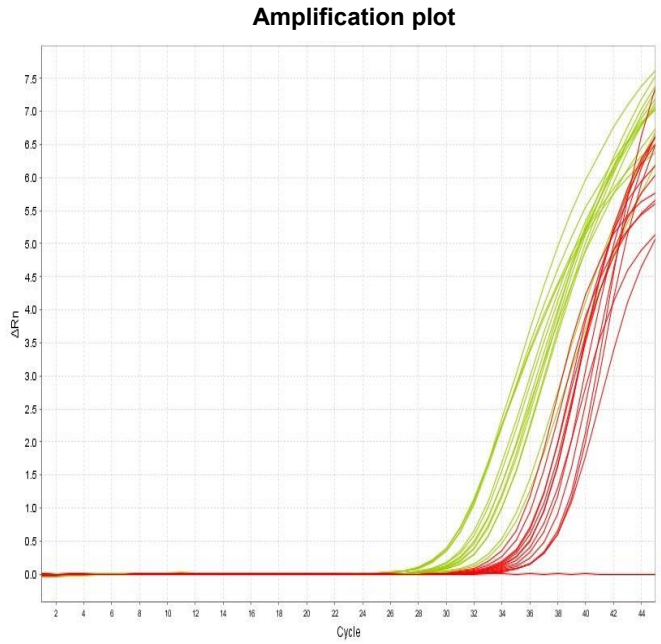

Figure 4 Amplification curves of Cadm1's relative expression

3.4. Statistical Analysis

For the statistical analysis we used IBM®SPSS®Statistics software for Windows (Version 22.0). The 2-ΔΔCt method (Livak method) and the t' Student test were used to evaluate the differences in the expression levels of the normalized mRNA.

Chi-square analysis (X^2) was used to compare the categorical variables. *p* values under 0,05 were considered statistically significant.

The probability associated of between our variables and NSCLC is indicated by the Odds Ratio (OR) using a 95% confidence interval (CI 95%).

We also analyzed a multivariate logistic regression to obtain the OR and respective CI 95% for the influence of the different variables in the risk of developing NSCLC metastasis adjusting for possible confounding variables.

The Kaplan-Meier method and Log-rank test were used to compare the mRNA expression profile influence in the patients' one-year survival.

The odds ratio (OR), specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV), as well as the receiver operating characteristic (ROC) curves were used to evaluate the potential of Cadm1 as a NSCLC biomarker.

Results

4. Results

4.1. Cadm1 as a diagnostic marker for NSCLC

4.1.1 Association between Cadm1 expression levels from peripheral blood and NSCLC.

In order to clarify and evaluate the effective potential of using Cadm1's expression levels in circulation as a NSCLC biomarker, a ROC curve was constructed and odd ratio (OR), specificity, sensitivity, positive predictive value (PPV) and negative predictive value (NPV) were determined. Using the results of the ROC curve, an analysis was made on the test performance with respect to the different threshold values, and a cut-off value was determined corresponding to $\Delta Ct=5.13$. Values under the cut-off point were determined Low Cadm1 and values above the cut off represented the High Cadm1 expression group.

The odd ratio (OR) represents the odds of an individual having NSCLC, given that they have Low Cadm1 expression levels compared to the odds of the individual being healthy, given that they have High Cadm1 expression [67]. The specificity reveals the probability of detecting High Cadm1 given that the individual tested is healthy [68]. On the other hand, the sensitivity reflects the proportion of the detection of Low Cadm1 in NSCLC samples [68]. The positive predictive value (PPV) reveals the probability of having NSCLC among those who have Low Cadm1 expression levels, whereas the negative predictive value (NPV) indicates the probability of being NSCLC-free among those who have High Cadm1 expression levels [68].

The frequencies of Cadm1 expression levels in the control (n=52) and case groups (n=89) are described in table 2. According to our results, the majority of participants with High Cadm1 were in the control group (61.5%) and most of the participants with Low Cadm1 expression belonged to the case group (57.3%).

There is a statistical significance between the groups (OR=2.147; CI 95%=1.068-4.32; *p*=0.031). Moreover, the specificity of Low Cadm1 expression levels is 61.5%, and the sensitivity is 57.3%. Additionally, the PPV value is 72% and NPV is 54%.

Table 2 Frequency of Cadm1 expression levels in the control and NSCLC groups.

	Cases (n=89)		Controls (n=52)		OR	CI95%	*p*
	n	%	n	%			
High Cadm1	38	42.7	32	61.5			
Low Cadm1	51	57.3	20	38.5	**2.147**	**1.068-4.32**	**0.031**

p- *p* value from X^2 test; OR- Odds Ratio; CI 95% - Confidence Interval at 95%

In order to better visualize these differences we created the graphic below where it is clear that the higher percentage of individuals with low Cadm1 expression levels belong to our case group and the higher percentage of individuals with high Cadm1 expression levels belong to the control group.

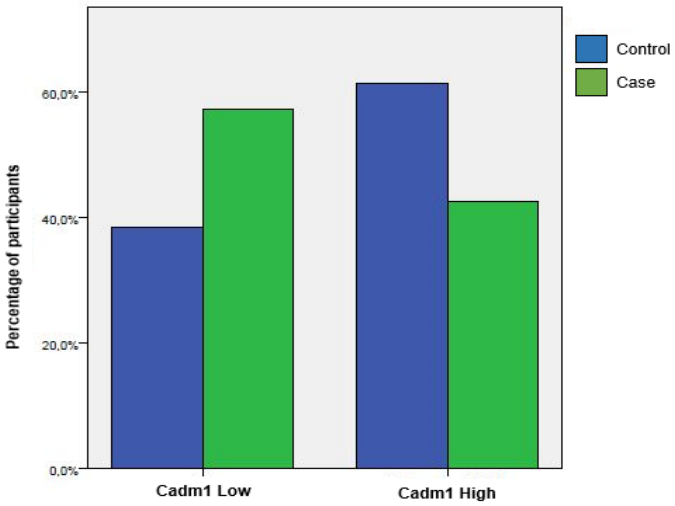

Figure 5 Percentage of participants (cases and controls) corresponding to the different Cadm1's expression profiles

To have a better understanding of our sensitivity and specificity values, we compared our study with other methods currently in use for diagnosing NSCLC.

Table 3 Comparison of specificity and sensitivity values for the currently used methods for diagnosing NSCLC

Screening method	Specificity (%)	Sensitivity (%)	References
Cadm1 expression levels	**61.5**	**57.3**	**Current work**
Low Dose CT	92.6 / 98.3	88.9 / 94.6	[69] / [70]
Chest x-ray	78.3	92.6	[69]
Panel of 6 antibodys agains 6 tumor related antigens	89-91	36-39	[70]

These results suggest that assessing Cadm1's expression levels in peripheral blood may be a potential diagnostic marker for NSCLC.

To further evaluate the potential of monitoring Cadm1 expression levels to diagnose NSCLC we studied the association between gender and the gene's expression levels (table 4) to eliminate possible confounding bias. According to our results, Cadm1's expression levels are independent of gender.

Table 4 Logistic Regression for Cadm1 expression

	HR	CI 95%	p
Cadm1 High/Low	0.401	**0.181-0.887**	**0.024**
Gender	7.639	3.459-16.871	<0.001

p- p value from X^2 test; HR- Hazard Ratio; CI 95% - Confidence Interval at 95%

4.2. Cadm1 influence in tumor progression and NSCLC advanced stages

4.2.1 Association between Cadm1 expression levels and NSCLC advanced stages

To study whether there was an association between Cadm1 expression levels and the evolution of the tumor we analyzed the expression in early stages (I and II) versus advanced stages (III and IV) as described in table 5. We couldn't find any differences with statistical significance between the groups (OR=2.30; CI 95%=0.231-23.147; *p*=0.464).

Table 5 Association between Cadm1 expression levels and tumor stages

	Stage I + II	Stage III + IV	Total	OR	CI 95%	*p*
Cadm1 mRNA	n (%)	n (%)	n (%)			
High	1 (25.0)	37 (43.5)	38 (42.7)	-	-	
Low	3 (75.0)	48 (56.5)	51 (57.3)	2.30	0.231-23.147	0.464

p- p value from X^2 test; OR- Odds Ratio; CI 95% - Confidence Interval at 95%

4.2.2. Association between Cadm1 expression levels and NSCLC long distance metastasis selecting for possible confounding variables

4.2.2.1. Selecting for Gender

In order to eliminate gender as a possible confounding variable we analyzed the differences in Cadm1's expression levels between non-metastatic stages and metastatic stages regarding gender. We couldn't find any differences with statistical significance between the groups in neither female (OR=2.667; CI 95%=0.237-30.066; *p*=0.417) or male (OR=1.200; CI 95%=0.466-3.093; *p*=0.706).

Table 6 Association between Cadm1 expression levels, metastization and gender

Gender	Cadm1	Stage I, II and III n (%)	Stage IV n (%)	OR	CI 95%	*p*
Female	High	1 (14.3)	4 (30.8)	-		
	Low	6 (85.7)	9 (69.2)	2.667	0.237-30.066	0.417
Male	High	15 (45.5)	18 (50.0)	-		
	Low	18 (54.5)	18 (50.0)	1.200	0.466-3.093	0.706

p- *p* value from X^2 test; OR- Odds Ratio; CI 95% - Confidence Interval at 95%

4.2.2.2. Selecting for Smoking status

In order to eliminate the smoking status as a possible confounding variable we analyzed the differences in Cadm1's expression levels between non-metastatic stages and metastatic stages regarding smoking status. We couldn't find any differences with statistical significance between the groups in neither Smoking (OR=1.466; CI 95%=0.554-3.882; *p*=0.441) or non-smoking (OR=1.200; CI 95%=0.166-8.659; *p*=0.856).

Table 7 Association between Cadm1 expression levels, metastization and smoking status

Smoking status	Cadm1	Stage I, II and III n (%)	Stage IV n (%)	OR	CI 95%	*p*
Smoking	High	13 (41.9)	18 (51.4)			
	Low	18 (58.1)	17 (48.6)	1.466	0.554-3.882	0.441
Non-smoking	High	2 (25.0)	4 (28.6)			
	Low	6 (75.0)	10 (71.4)	1.200	0.166-8.659	0.856

p- *p* value from X^2 test; OR- Odds Ratio; CI 95% - Confidence Interval at 95%

4.2.2.3. Selecting for Histology

In order to eliminate histology as a possible confounding variable we analyzed the differences in Cadm1's expression levels between non-metastatic stages and metastatic stages regarding the different histologic subtypes. We couldn't find any differences with statistical significance between the groups in neither Squamous Cell Carcinoma (OR=1.469; CI 95%=0.350-6.168; *p*=0.598) or Non-Squamous Cell Carcinoma (OR=1.067; CI 95%=0.360-3.159; *p*=0.907).

Table 8 Association between Cadm1 expression levels, metastization and histologic subtype

Histologic subtype	Cadm1	Stage I, II and III n (%)	Stage IV n (%)	OR	CI 95%	*p*
SCC	High	7 (36.8)	6 (46.2)			
	Low	12 (63.2)	7 (53.8)	1.469	0.350-6.168	0.598
Non-SCC	High	9 (42.9)	16 (44.4)			
	Low	12 (57.1)	20 (55.6)	1.067	0.360-3.159	0.907

p- *p* value from X^2 test; OR- Odds Ratio; CI 95% - Confidence Interval at 95%

4.3. The influence of Cadm1 expression levels in 1year survival rates

When analyzing the association between Cadm1's expression levels with one-year survival, distinguishing non-metastatic (stages I, II and III) and metastatic (stage IV) we observe different results. According to figure 6A (non-metastatic) there is a difference with statistical significance in 1-year survival rates of patients with Low Cadm1 versus patients with High Cadm1 (p=0.016). In figure 6B (metastatic) the difference between the two groups (High Cadm1 and Low Cadm1) doesn't have statistical significance (p=0.751).

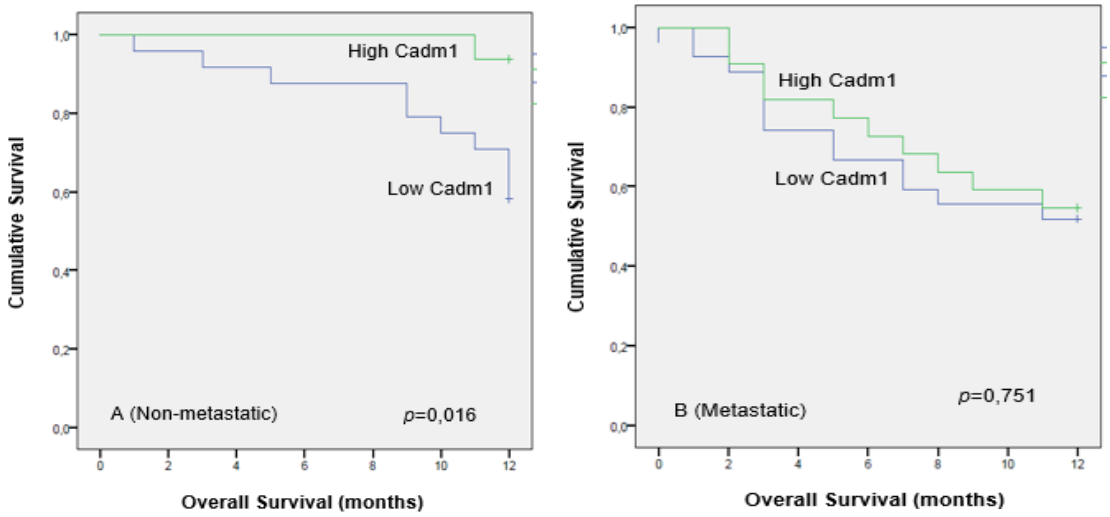

Figure 6 One-year survival by Kaplan-Meier for non-metastatic (A) and metastatic (B) NSCLC according to Cadm1's expression levels in the blood.

Discussion

5. Discussion

In the past years it has become clear that the key to lower cancer mortality rates is in early diagnosis and personalized therapy. Because cancer is a very complex disease, finding a specific marker for early diagnosis is complicated and finding a specific target for treatment has proven difficult for most tumors.

With the consistent increase in lung cancer incidence and low survival rates it has become imperial to find tools that allow early diagnosis. It is clear that people with tumors diagnosed in early stages have far better survival rates than those whose tumors were in more advanced stages. For lung cancer, one of the obstacles to early diagnosis is the fact that symptoms only appear in more advanced stages and 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 and for monitoring therapeutic efficacy [21, 29].

One potential marker for NSCLC development is CADM1. Even though Cadm1 is expressed in a variety of normal tissues, nowadays, CADM1 is well established as a tumor suppressor, not only in lung cancer, but in different tumors and appears to be downregulated in more advanced stages of the disease [48-53]. This downregulation is mostly due to promoter methylation but can also be the result of LOH. Because CADM1 is an adhesion molecule, it seems logic that its expression is lower when tumor has metastasized, since this process requires cell detachment from the primary tumor.

The use of a biomarker for early detection and for monitoring disease progression implies that with a simple test we can differentiate an affected patient from a person without the disease. This means diagnosing tumors in earlier stages that consequently are easier to treat and also, adjust therapy strategies according to each patient's response.

To the best of our knowledge, our study is the first to measure Cadm1 levels in peripheral blood. All the others report the use of tissue samples obtained directly from the tumor to quantify the molecule. When measuring Cadm1's levels in circulation we're referring to its expression in blood cells and not plasma or serum.

With this in mind, we established two main goals in order to evaluate the potential of using this method for early diagnosis, monitoring disease progression and evaluate clinical outcome: the first was to determine if there was an association between circulating mRNA levels of the CADM1 gene and non-small cell lung cancer in order to validate Cadm1 as a good diagnostic marker; the second was to study the association of circulating CADM1's expression levels and tumor progression and clinical outcome.

5.1. Association between Cadm1 expression levels from peripheral blood and NSCLC.

In the beginning of the process of carcinogenesis the cell must undergo a change, genetic and / or epigenetic, which may lead to changes in gene activity resulting in the rupture of the homeostatic balance [71]. Some of these alterations lead to inactivation of tumor suppressor genes (TSGs) and the effect of their inactivation plays a major role in cancer and has been described for several genes in various malignancies.

CADM1 is a TSG that has been reported to be functionally involved in pulmonary carcinogenesis and that is highly methylated in most NSCLC cases and inactivation of this gene by promoter methylation has been proposed as a candidate marker for early lung cancer detection [40, 72].

Even though the process through which Cadm1 acts to suppress tumorigenesis is still not clear, being a tumor suppressor it seems logic to find higher levels of Cadm1 in healthy individuals compared with patients with cancer.

According to our results, there is a difference with statistical significance between our case and control groups: individuals with Low Cadm1 expression levels, have a higher probability of being diagnosed with NSCLC (OR=2.147; CI95%=1.068-4.32; *p*=0.031) when compared with individuals with High Cadm1 expression levels. The majority of our control group had High Cadm1 expression levels (61.5%) while in NSCLC group, the majority presented with Low Cadm1 expression levels (57.3%).

If CADM1 is a TSG, healthy individuals will have High Cadm1 expression levels. Patients with NSCLC, however, will have lower Cadm1's expression levels since the disruption of the balance between oncogenes and tumor suppressor genes is a crucial step for carcinogenesis. With CADM1 downregulated, there is a reduction of the signals to stop cell growth and a decreasing in the level of cell adhesion which allows the tumor to develop [73].

The diminished levels of Cadm1 in the blood cells of patients with NSCLC compared to healthy individuals may be due to different factors. It has been proposed that Cadm1 interacts with the immune system [49, 74], so it is logical to suppose that low Cadm1's expression in the blood cells of NSCLC patients is the result of the interaction between tumor cells and blood cells. Tumor cells signal the cells in circulation to lower their Cadm1 expression levels. If these blood cells signal other cells to lower their Cadm1 expression, the result could be the establishment of a microenvironment with a reduced tumor suppressor activity consequently promoting tumor development, invasion and metastization. How this interaction works, and the pathways leading to Cadm1 decrease in the blood cells is still unknown.

On the other hand, we can also suggest that the difference in Cadm1 expression in circulating blood cells between healthy individuals and NSCLC patients is the cause for tumor formation instead of a consequence of the tumor. It is possible to assume that because individuals from our case group had a polymorphism that resulted in lower Cadm1 expression, they were more susceptible to develop NSCLC than our control group. If this was the case, it wouldn't be the tumor modulating Cadm1's expression levels in circulation. The fact that those people had less Cadm1 by default would be the reason to why they are more susceptible to develop NSCLC. Weyden et al. showed that Cadm1-null mice died significantly faster than their wildtype littermates due to the spontaneous development of tumors at an earlier age, which supports our suggestion [75].

Whatever the cause for the difference of expression levels, our results support the potential of using Cadm1 as a diagnostic biomarker with a sensibility of 57.3%, specificity 61.5%, PPV of 72% and NPV of 54%.

At the present time, there isn't an ideal screening method for lung cancer and the methodologies used are either expensive, or have side effects, or are not accessible or applicable to everybody. When compared to the current methods used, Cadm1 expression levels in peripheral blood seems to have low specificity and sensitivity (table 3). However, it is important to note that this is a non-invasive method, with no side effects (contrary to those of CT scans and X-Ray), it's less expensive, faster to obtain results and applicable to everyone. Having a PPV of 72% means that an individual who has Low Cadm1 expression levels in blood cells has a 72% chance of already having NSCLC and should therefore be recommended for further diagnostic tests. Based on these results Cadm1 has a potential to be a remarkable biomarker for NSCLC through a simple blood test.

5.2 Association between circulating Cadm1 expression levels and tumor progression and clinical outcome

5.2.1. Circulating levels of Cadm1 and tumor progression

There is still a lot to understand about the role CADM1 plays in tumor development and the pathways it is involved in, but many different studies have reported a correlation between loss of Cadm1 expression and advanced stages (poor prognosis) and metastasis [50, 63, 66]. Inhibition of Cadm1 in the tumor was proven to be important in metastasis formation in different studies and its re-expression found to prevent metastasis formation.

Our results, however, didn't show an association between Cadm1 circulating levels and tumor progression. We compared Cadm1's expression levels for early stages (stages I and II) against advanced stages (III and IV) and found no statistical association (p=0.464;

OR=2.30; CI95%=0.231-23.147). This means that there are no differences in Cadm1's circulating levels during disease progression, and so, evaluating Cadm1's expression in the blood doesn't appear to be a good method.

Since Cadm1 has been proposed to be associated with metastasis formation, and even though we obtained no association between Cadm1 and progression from early to advanced stages, we decided to check if there was any difference between Cadm1's expression levels in patients with non-metastasized tumors (stages I, II and III) versus metastasized (stage IV).

Again, we didn't find any association even when we eliminated possible bias due to confounding variables (gender, smoking status and histologic subtype).

Since CADM1 is a tumor suppressor gene that induces apoptosis and inhibits tumor growth, it makes sense that there is a decreased expression with tumor progression [56]. Less Cadm1 means less apoptotic signals and growth inhibition. Also, less Cadm1 means less cell adhesion, which is one of the initial steps to metastasis.

However, when measuring Cadm1's levels in circulation we're referring to its expression in blood cells that may not be representative of tumor behavior.

These results suggest that the interaction between tumor cells and blood cells, that decreases Cadm1 expression in the latter, happens in the beginning of the carcinogenic process since they don't show any difference with tumor progression or metastasis formation. Cadm1 seems to be more important for disease initiation rather than progression, and the reason why Cadm1's expression levels change in tumor cells and not in blood cells in more advanced stages is due to different factors involved with tumor development.

This explanation is still plausible if we consider the hypothesis of the existence of a putative polymorphism for this gene. If there is indeed a difference in the gene that causes lower expression levels in some individuals, the tumor progression won't affect Cadm1 levels in other cells beside tumor cells.

5.2.2. Circulating Cadm1 levels and clinical outcome

Lastly, we decided to evaluate the influence of Cadm1 in one-year survival rates. The prognosis of patients with stage IV NSCLC is still poor, most large-phase III trials have shown a median survival time from 8 to 10 months and 1-year survival rate of 30%–35%, that drops to 0%–1% for the 5-year survival rate [76, 77].

It is clear that patients with non-metastatic NSCLC and Low Cadm1's expression levels have a worst survival rate than those with High Cadm1 (p=0.016) Yet the same isn't true for stage IV. In this case, Cadm1 apparently doesn't affect one-year survival (p=0.751) (Figure 6).

Relative to non-metastatic tumors, our results show that NSCLC patients with Low Cadm1 have a worst prognosis than those who have High Cadm1 which is in accordance to what should be expected: individuals with low Cadm1 levels have less cell adhesion, less tumor suppressor activity and therefore are more likely develop into a more aggressive tumor culminating in worst prognosis [75].

For patients with tumors that have already metastasized, we don't see any differences in survival between patients with low circulating levels against high circulating levels. As stated previously, CADM1 is associated with metastasis formation since it codes for an adhesion molecule, and in order for a tumor to metastasize its cells need to loose adherence. But the fact that Cadm1's expression levels don't seem to be relevant for survival in stage IV patients suggests that after metastization, CADM1 stops being significant. In this stage there are other factors that have a bigger effect on survival.

To conclude, according to our study, the assessment of Cadm1's expression levels in the blood seems to be a good diagnostic marker for NSCLC. It's a simple method that allows to differentiate between healthy individuals and NSCLC patients with statistical significance.

On the other hand, we didn't find evidence that this is a good method to analyze tumor progression. But evaluating Cadm1's expression levels in the blood seems to possibly be a good prognostic factor for non-metastatic tumors since there are different expression profiles between metastatic and non-metastatic tumors.

Conclusion and future perspectives

6. Conclusion and future perspectives

The study of potential biomarkers has become one of the most invested areas in cancer research. In the recent years, knowledge about cancer biomarkers has increased tremendously providing great opportunities for improving the management of cancer patients by enhancing the efficiency of detection and efficacy of treatment.

The reality of the non-existence of a standard screening test for the early detection and follow-up in NSCLC has been haunting oncologists for the past years since lung cancer (specifically NSCLC) is one the most lethal neoplasia. The future of cancer therapy lies in the use of biomarkers that offer the potential to identify and treat cancer years before it is either visible or symptomatic. Exploring the presence of such markers that does not require the tumor tissue to detect them, but are obtained from a simple blood sample will not only facilitate easy detection without even minimal surgical procedure, but will also identify candidates for population based screening. This is a major improvement and believed to be the best way to lower incidence and mortality rates for lung cancer.

Being a cell adhesion molecule known to suppress tumor development that is silenced in most lung cancer patients, Cadm1 gene has been in the spotlight for a potential lung cancer biomarker for a couple of decades. Its role as a tumor suppressor is well established for various types of cancer even though the pathways involving this process aren't clear. Also, the correlation between lower Cadm1 expression and lung carcinogenesis has proven to be strong. A better and clearer understanding of how Cadm1 works, the pathways it is involved in, the molecules it interacts with etc. are a few of the subjects that need intensive research.

With this work we've demonstrated that there is a relevant difference of circulating Cadm1 levels between healthy individuals and cancer patients that could be used as a screening test for NSCLC. We proposed two scenarios for the diminished expression seen in cancer patients compared to healthy individuals: The tumor, with lower Cadm1, interacts with the cells in circulation signaling them to lower Cadm1's levels in order to promote a decrease in the tumor suppressor activity of the tumor surrounding cells; Or, there is a difference in Cadm1 expression levels due to a polymorphism in the gene that results in a lower Cadm1 expression by default, making that person more susceptible to develop NSCLC compared to those who don't have the polymorphism.

This opens many possible courses of investigation for the future.

Analyzing our sample for a possible polymorphism in the CADM1 gene and determine if there is an association between that polymorphism and risk for developing NSCLC and clinical outcome would be of interest.

To validate the hypothesis that our data is the result of tumor interaction with blood cells it would be interesting to analyze both tumor tissue and blood samples from each individual in order to evaluate a possible correlation between them.

Also, knowing more about what triggers Cadm1 silencing in tumor initiation may prove essential to understand it's role in tumor formation and development.

Another interesting idea would be testing Cadm1's expression levels in patients before and after treatment to understand treatment efficacy and the possibility of using Cadm1 as a marker for monitoring tumor development and treatment efficacy.

There is a lot we don't know about this molecule and its role in lung cancer development, but according to our study, the assessment of Cadm1's expression levels in the blood seems to potentially be a good diagnostic marker for NSCLC. It's a simple, fast and accessible method that allows to differentiate healthy individuals from NSCLC patients. Continuing this work is essential to understand Cadm1 and its role in NSCLC.

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7. References

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