**UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL**

**INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE**

**DEPARTAMENTO DE BIOQUÍMICA PROF. TUISKON DICK**

**PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA**

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CAROLINA BEATRIZ MÜLLER

PORTO ALEGRE, JANEIRO DE 2012

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## O potencial prognóstico da Cofilina-1

ALUNA: CAROLINA BEATRIZ MÜLLER

ORIENTADOR: PROF. DR. FÁBIO KLAMT

Dissertação apresentada ao Programa de Pós-graduação em Ciências Biológicas: Bioquímica, como requisito para obtenção do grau de mestre em Ciências Biológicas: Bioquímica.

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**ÍNDICE**

# PARTE 1

## RESUMO

O câncer de pulmão é responsável por aproximadamente 13% do total de casos de neoplasias malignas e por cerca de 1.4 milhões de mortes por ano em todo mundo. Esta neoplasia apresenta-se sob dois principais subtipos: câncer de pulmão de pequenas células (CPPC) e câncer de pulmão de não-pequenas células (CPNPC). Cerca de 85% dos casos de câncer de pulmão são do tipo CPNPC. Os sinais e sintomas são secundários ao crescimento do tumor primário, ao comprometimento lobo-regional, à disseminação à distância, ou são secundários às síndromes paraneoplásicas. Essas características refletem diretamente sobre as taxas de mortalidade; de cada 100 novos casos, 80 são inoperáveis e a maioria morre dentro de 3 anos. Isso significa que, apesar dos diversos avanços no diagnóstico e tratamento, o prognóstico do câncer de pulmão permanece sendo extremamente ruim, com sobrevida média de 10 meses, e cumulativa total em 5 anos de aproximadamente 12%.

Atualmente, o prognóstico e a decisão terapêutica de pacientes com câncer de pulmão é baseada no TNM, Embora esse seja o procedimento considerado padrão-ouro entre os profissionais de saúde, ele não leva em consideração características biológicas do tumor. Nesse contexto, a identificação de biomarcadores para câncer pode agregar importantes informações ao já estabelecido sistema TNM e resultar em tratamentos mais eficientes e em menores taxas de mortalidade. Existem 5 fases distintas que conceitualizam o desenvolvimento de um biomarcador tumoral. Através dessas fases consecutivas, é possível que se desenvolvam ferramentas úteis para triagem populacional, capazes de serem implementadas na rotina clínica para predição de desfecho do paciente, resposta terapêutica e monitoramento da doença.

O presente projeto avaliou o valor prognóstico dos principais genes citados na literatura como potenciais biomarcadores para CPNPC, e verificou-se que nenhum deles apresentou significância na correlação estatística que indica poder prognóstico. Além disso, identificamos e validamos o papel prognóstico da cofilina-1 por meio de dados de microarranjo e quantificação de seu imunoconteúdo em biópsias de CPNPC. Para tanto, fizemos uso de meta-análise de bancos de dados e análise densitométrica das reações imuno-histoquímicas, seguida de correlação com dados de grau de diferenciação tumoral, classificação histológica, sexo, idade e desfecho relativo a cada caso. Além disso, desenvolvemos um método de baixo custo, fácil execução e ampla aplicação e reprodutibilidade, capaz de quantificar a proteína em amostras biológicas, com potencial para ser implementado na rotina clínica e aplicamos esse método em uma coorte restrospectiva de CPNPC. Confirmamos assim o papel prognóstico da cofilina-1. Estes achados seguem a lógica das fases de desenvolvimento de um biomarcador e representam um grande passo no seu processo de validação.



*Palavras-chave*: Imuno-histoquímica; Câncer de Pulmão Não de Pequenas Células; Cofilina-1; Prognóstico; Biomarcador

## ABSTRACT

Lung cancer accounts for approximately 13% of all malignant tumor cases and for about 1.4 million deaths per year worldwide. This cancer has two main subtypes: Small Cell Lung Cancer (SCLC) and Non-Small Cell Lung Cancer (NSCLC). About 85% of cases of lung cancer are NSCLC type. The signs and symptoms are secondary to the primary tumor growth, to regional lobe commitment and distant spread, or are secondary to paraneoplastic syndromes. These features reflect directly on mortality rates; 80 in every 100 new cases are inoperable and most die within 3 years. This means that, despite many advances in diagnosis treatment, the prognosis of lung cancer remains extremely poor, with median survival of 10 months, and total cumulative survival in 5-year of approximately 12%.

Currently, prognosis and therapeutic decisions in patients with lung cancer is based on TNM. Although this procedure is considered gold standard among health professionals, it does not take into account the biological characteristics of the tumor. In this context, the identification of cancer biomarkers may add important information to the already established TNM system and result in better treatments and lower mortality rates. There are five distinct phases that conceptualize a tumor biomarker development of. Through these successive phases, it is possible to develop useful tools for population screening, capable of implementation in clinical practice for prediction of patient outcome, therapeutic response and disease monitoring.

This project evaluated the prognostic value of major genes mentioned in literature as potential biomarkers for NSCLC and found that none of them showed statistical significance in the correlation that indicates prognostic power. It also identified and validated the prognostic role of cofilin-1 by microarray data and quantification of their immunocontent in biopsies of NSCLC. For this purpose, we used data meta-analysis and immunohistochemical reactions densitometric analysis, followed by correlation with data from tumor grade, histological classification, sex, age and outcome for each case. In addition, we developed a low-cost protocol, of easy implementation and wide application and reproducibility, able to quantify the protein in biological samples, with the potential to be implemented in clinical practice. We applied this method in a retrospective cohort of NSCLC and confirm the prognostic role of cofilin-1. These findings follow the logical phases of biomarker development and represent a major step in its validation process.



*Keywords:* Immunohistochemistry; Non-small cell lung cancer; Cofilin-1; Prognosis; Biomarker

## LISTA DE ABREVIATURAS

Actina-F – Actina Filamentosa;

Actina-G – Actina Globular;

ADF – Fator de Despolimerização de Actina;

AJCC –Comissão Mista Americana sobre Câncer;

CPNPC – Câncer de Pulmão Não de Pequenas Células;

CPPC – Câncer de Pulmão de Pequenas Células;

*CFL1* – Cofilina-1;

*CFL2* – Cofilina-2;

DMSO – Dimetilsulfóxido;

EGF – Fator de Crescimento Epidérmico;

EGFR – Receptor do Fator de Crescimento Epidérmico;

INCA – Instituto Nacional do Câncer;

LIMK1 – LIM cinase 1;

NESK –Cinase Músculo Esquelético Específica;

PIP2 – Fosfatidilinositol-4,5-bifosfato;

TESK 1 – Proteína cinase Testicular 1;

UICC – União Internacional Contra o Câncer

# 1. INTRODUÇÃO

## *1.1 Câncer de Pulmão*

O câncer de pulmão é responsável por aproximadamente 13% do total de casos de neoplasias malignas e por cerca de 1,4 milhões de mortes por ano em todo mundo (Jemal, 2011). No Brasil estima-se que haja cerca de 28 mil novos casos por ano. É, portanto, uma doença altamente letal, cuja sintomalogia não é comum nos estágios iniciais da doença, o que dificulta a detecção precoce. O tabagismo é responsável por aproximadamente 90% dos casos, constituindo o principal fator de risco. Outros fatores de risco estão relacionados à exposição ocupacional a agentes químicos, fatores dietéticos, doença pulmonar obstrutiva crônica e fatores genéticos predisponentes ao câncer (INCA, 2010).

Os sinais e sintomas são secundários ao crescimento do tumor primário, ao comprometimento lobo-regional, à disseminação à distância, ou são secundários às síndromes paraneoplásicas. Assim, mais de 90% dos pacientes são sintomáticos no momento do diagnóstico. Essas características refletem diretamente sobre as taxas de mortalidade; de cada 100 novos casos, 80 são inoperáveis e a maioria morre dentro de 3 anos. Isso significa que, apesar dos diversos avanços no diagnóstico e tratamento, o prognóstico do câncer de pulmão permanece sendo extremamente ruim, com sobrevida média de 10 meses, e cumulativa total em 5 anos de aproximadamente 12% (Zamboni, 2002).

Do ponto de vista anatomo-patológico, esta neoplasia apresenta dois principais subtipos: câncer de pulmão de pequenas células (CPPC) e câncer de pulmão de não-

pequenas células (CPNPC). O CPPC é tipicamente uma doença mais agressiva com elevada incidência de metástases precoces e distantes. A maior parte dos pacientes só é diagnosticada quando a doença já está em estágio avançado, e grande parte dos casos é irreversível (Garst, 2007). Cerca de 85% dos casos de câncer de pulmão são do tipo CPNPC, um tipo menos agressivo quando comparado ao CPPC. Esse tipo de tumor é composto por três principais tipos histológicos distintos: carcinoma epidermóide, adenocarcinoma e carcinoma de grandes células (Beadsmoore and Screaton, 2003; Beasley *et al.,* 2005) (Fig.1).

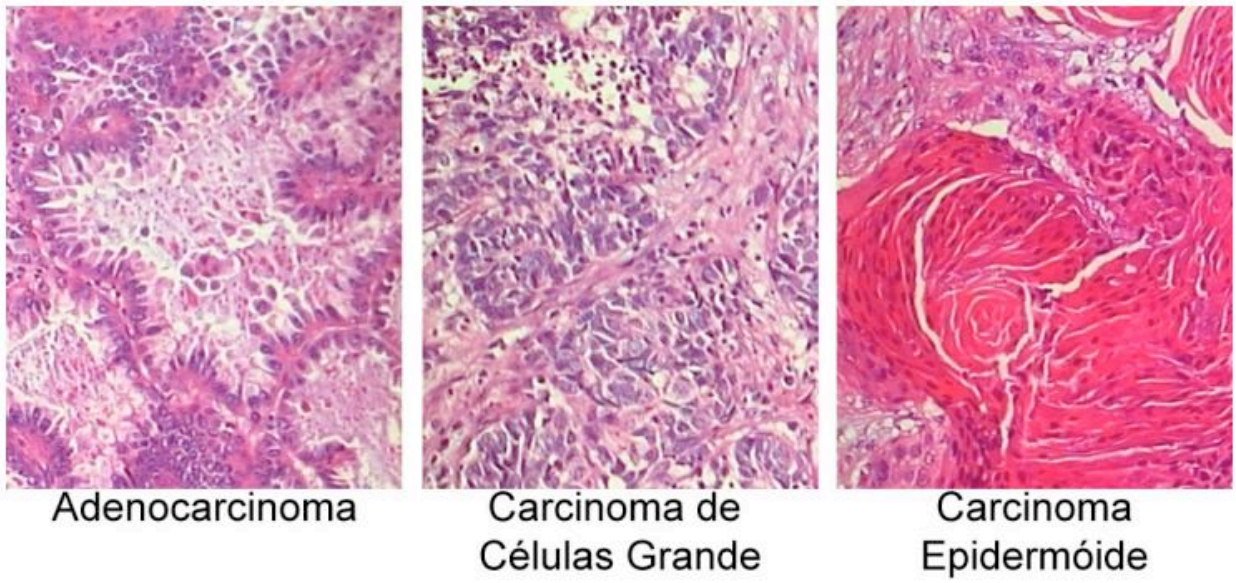


**Figura 1. Tipos histológicos de CPNPC.** Microfotografia representativa dos três principais subtipos histológicos de CPNPC: adenocarcinoma, carcinoma de células grande e carcinoma epidermóide. Imagens de lâminas histológicas de biópsias coradas com hematoxilina-eosina (HE) em aumento de 200x.

Trata-se, portanto, de uma doença que apresenta diversos desafios biológicos e clínicos que dificultam o progresso das iniciativas de saúde em prevenção, detecção precoce e direcionamento do tratamento. Nesse aspecto, quaisquer esforços que

sejam empregados no sentido de agregar informações e/ou aumentar a sobrevida dos pacientes representam importante avanço.

### *1.2 Biomarcadores*

Atualmente, o prognóstico e a decisão terapêutica de pacientes com câncer de pulmão é baseada no TNM, sistema de estadiamento em que“T” seguido de sufixo (1-4) indica o tamanho e a extensão do tumor primário, “N” seguido de sufixo (0-3) indica acometimento de linfonodos e “M” (0 ou 1) indica se há presença de metástase. A avaliação desses parâmetros proporciona uma descrição da extensão anatômica e do avanço da doença. Essa classificação é recomendada pela AJCC (American Joint Committee on Cancer) e pela UICC (Union Internationale Contre Cancer) (Mountain, 1986; Mountain, 1997; Mountain 2002).

Embora esse seja o procedimento considerado padrão-ouro entre os profissionais de saúde, ele não leva em consideração características biológicas do tumor (Detterbeck *et al.,* 2009). Nesse contexto, a identificação de biomarcadores para câncer pode agregar importantes informações ao já estabelecido sistema TNM e resultar em tratamentos mais eficientes e em menores taxas de mortalidade (Issaq *et al.,* 2011).

Biomarcadores são substâncias encontradas em fluidos biológicos ou tecidos, cuja detecção ou quantificação podem ser correlacionadas com características e comportamentos específicos da doença. Assim, métodos laboratoriais que quantifiquem os níveis de proteínas e metabólitos em amostras biológicas podem revelar importantes informações quanto a biologia de tumores. (MacNeil, 2004). Existem algumas divergências entre autores quanto à descrição e classificação de

biomarcadores. Em regra geral, os marcadores podem ser diagnósticos, prognósticos e preditivos. Sawyers (2008) caracterizou 3 tipos de biomarcadores (prognósticos, preditivos e farmacodinâmicos), ressaltando a contribuição que cada um dos quais pode prestar no desenvolvimento de drogas anticâncer (Fig. 2).

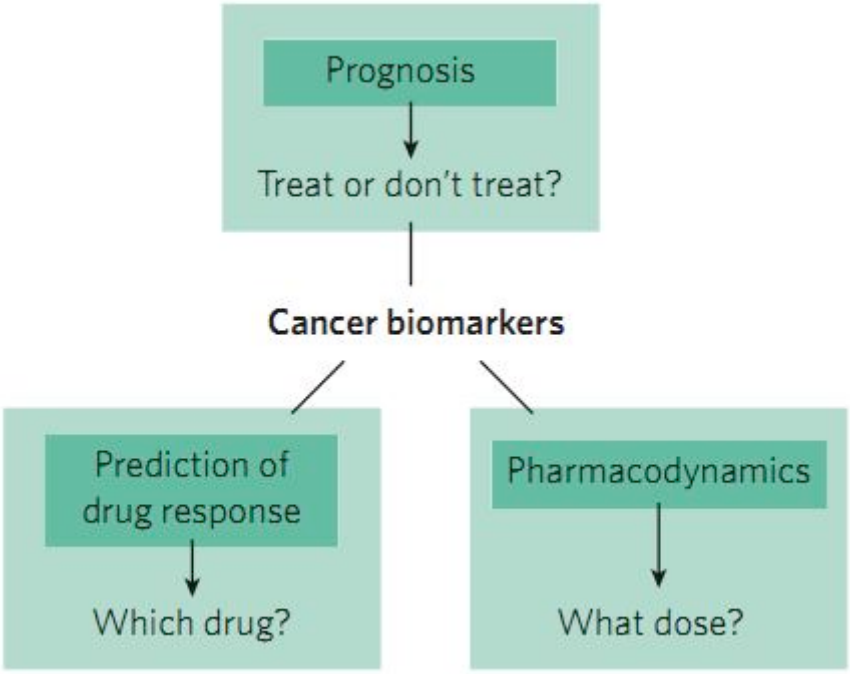


**Figura 2. Tipos de Biomarcadores.** Os biomarcadores podem ser utilizados para prognóstico: predizer o curso natural da doença, indicando um melhor ou pior desfecho do paciente. Eles também podem auxiliar o médico a decidir qual paciente responderá melhor a uma determinada droga (predição) e em qual dose essa droga seria mais efetiva (farmacodinâmica).(Retirado de Sawyers, 2008)

Embora os benefícios de um biomarcador para câncer de pulmão sejam indiscutíveis, seu estabelecimento e implementação na rotina clínica não são tarefas fáceis. Existem 5 fases distintas que conceitualizam o desenvolvimento de um biomarcador tumoral. São elas: **Fase 1**: Estudos pré-clínicos exploratórios com objetivo

de identificar potenciais biomarcadores; **Fase 2**: Desenvolvimento de ensaio capaz de identificar/quantificar o biomarcador (genes, proteínas) em amostras clínicas, testando sua especificidade e reprodutibilidade inter-laboratorial; **Fase 3**: Estudo retrospectivo longitudinal (avaliar capacidade de correlação); **Fase 4**: Estudos prospectivos de triagem; **Fase 5**: Estudos de Controle (avaliar impacto clínico) (Pepe *et al*., 2001; Issaq *et al.*; 2011).

Através dessas fases consecutivas, é possível que se desenvolvam ferramentas úteis para triagem populacional, capazes de serem implementadas na rotina clínica para predição de desfecho do paciente, resposta terapêutica e monitoramento da doença (Cho, 2007).

Biomarcadores tumorais para câncer de pulmão podem ser categoricamente classificados em biomarcadores do soro, teciduais e de escarro. Diversos estudos propõem biomarcadores para CPNPC; entretanto, os resultados reportados na literatura ainda são conflitantes e grande parte deles não consegue progredir do laboratório para clínica, estagnando na primeira fase de desenvolvimento. Muito disso deve-se a limitações como dificuldades na validação dos métodos (baixa sensibilidade; impossibilidade de aplicação por todos os estabelecimentos participantes do estudo) e a viabilidade da obtenção de amostras (dificuldades em coletar amostras de todos os pacientes; volume amostral inadequado). Essa situação evidencia a necessidade de mais investigações, novos ensaios moleculares e do desenvolvimento de um painel apropriado de biomarcadores (Strauss & Skarin, 1994; Niklinski *et al.,* 2001; Saijo, 2011).

Entender os processos envolvidos no desenvolvimento tumoral e no aparecimento de metástases é condição importante para que se possa verificar possíveis marcadores biológicos que estejam relacionados à agressividade do carcinoma. Sabe-se, por exemplo, que a motilidade da célula tumoral é condição indispensável para que ela tenha capacidade de migrar, invadir e realizar metástase. Assim, a identificação de vias moleculares da invasão de células tumorais constitui uma possibilidade fértil de estudo de novos marcadores capazes de indicar prognóstico e/ou melhorar estratégias terapêuticas (Wang *et al.*, 2007).

### *1.3 Cofilina*

A cofilina é uma proteína citosólica de 19 kDa integrante da família das ADF/cofilina *(ADF;actin-depolymerisation factor)* ubiquamente presente nas células eucarióticas *(*Moon *et al.,* 1993; Gurniak *et al.,* 2005*)* e que está diretamente relacionada com a regulação da polimerização e despolimerização de actina durante a migração celular (Maciver & Hussey, 2002) e também com apoptose induzida por oxidantes (Zdanov et al.,2010). Existem duas isoformas de cofilina, a cofilina-1 (*CFL1*, cofilina não-muscular ou cofilina-n) e a cofilina-2 (*CFL2*, cofilina muscular ou cofilina-m).

O remodelamento dos filamentos de actina é essencial durante a formação e retração das estruturas usadas na quimiotaxia, na migração celular e invasão de células tumorais. Existem cinco principais mecanismos regulatórios da atividade da cofilina. Primeiro, a fosforilação de sua serina 3 pela *LIMK1* e suas cinases relacionadas (*LIMK2, NESK (skeletal muscle-specific kinases), TESK1(testicular protein kinase 1) e TESK2 (testicular protein kinase2))* que a regulam inibindo sua atividade de

ligante de actina. Segundo, a desfosforilação da serina 3 por fosfatases, resultando em sua ativação. Terceiro, sua atividade pode ser inibida pela ligação a fosfatidilinositol-4,5-bifosfato (*PIP2*). Quarto, a mudança de pH fora dos padrões fisiológicos pode aumentar a atividade da cofilina quando em estado desfosforilado (Fig. 3) (Wang *et al.*, 2007). Por último, a cofilina quando oxidada, perde afinidade pela actina e transloca para mitocôndria, onde induz swelling e liberação do citocromo *c* mediante abertura do poro de transição de permeabilidade (Klamt *et al*,. 2009).

A localização da cofilina depende do tipo celular, estado de diferenciação e de seu estado de ativação (Hao *et al.*, 2008). Estudos imunoistoquímicos revelaram que a cofilina pode, em resposta a agentes químicos e físicos, se translocar para o núcleo; entretanto, esse mecanismo de transporte nuclear ainda não está bem esclarecido. É descrito que a importação de cofilina para o núcleo depende do seu estado de fosforilação, em que a desfosforilação de cofilina através de tratamento com dimetilsulfóxido (DMSO) ou aquecimento demonstrou maior localização nuclear da proteína. Além disso, a cofilina possui uma sequência sinal de localização nuclear (*NLS*), a quem infere-se a responsabilidade de sua translocação nuclear (Abe *et al.,* 1993).

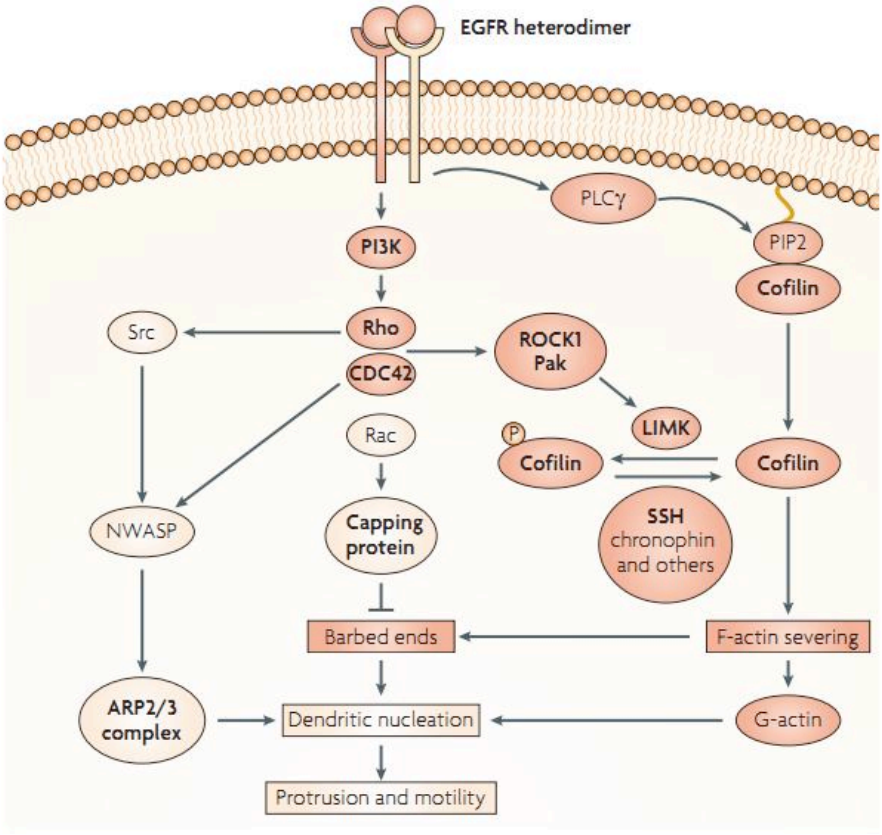


**Figura 3. Via de regulação da cofilina**. A via da cofilina é ativada em células tumorais através de estímulo do microambiente, como a sinalização de EGF detectado por EGFR. (Adaptado de Wang et al., 2007)

A cofilina atua ligando-se à actina filamentosa (actina-F) e quebrando-a, liberando actina globular (actina-G) como substrato para a formação de novos filamentos. Além disso, a quebra de actina-F resulta na formação de *free barbed ends,* que se caracterizam como extremidades livres, que podem ser alongadas e polimerizadas em novos filamentos. A ativação local de cofilina causa polimerização local de actina e, conseqüentemente, a protrusão da membrana celular. Sob estímulo de EGF (*Epidermal Growth Factor*), células cancerosas formam protrusões celulares críticas

para a quimiotaxia, como lamelipódios e invadopódios. Essas alterações na morfologia da célula são, assim, diretamente dirigidas pela reestruturação da rede de actina do citoesqueleto celular, regulada pela cofilina (van Rheenen *et al.,* 2007). O entendimento da regulação da cofilina e de suas funções celulares leva a conclusão de que a cofilina corresponde a um nodo funcional na biologia celular. Sua atividade é modulada por quase qualquer perturbação ou flutuação na fisiologia celular normal e ela, por sua vez, tem o potencial de corrigir ou atenuar essas flutuações (Bernstein & Bamburg, 2010). Por outro lado, estudos em câncer mama demonstraram que a cofilina-1 é coordenadamente regulada em células tumorais invasivas durante o processo de migração celular *in vivo* e que tem papel direto na intensidade das características invasivas e mestastáticas (Wang *et al*., 2007)

## 2. OBJETIVOS

### *2.1 Objetivo Geral*

O presente projeto avaliou o valor prognóstico dos principais genes citados na literatura como potenciais biomarcadores para CPNPC. Além disso, identificou e validou o papel prognóstico da cofilina-1 por meio de dados de microarranjo e quantificação de seu imunoconteúdo total e nos compartimentos citosólico e nuclear em biópsias de CPNPC. Para tanto, fizemos uso de meta-análise de dados e análise densitométrica das reações imuno-histoquímicas, seguida de correlação com dados de grau de diferenciação tumoral, classificação histológica, sexo, idade e desfecho relativo a cada caso.

### *2.2 Objetivos Específicos*

- Realizar meta-análise de dados da literatura médica, através de busca no pubmed dos principais genes citados como potenciais biomarcadores para CPNPC;

- Testar o valor prognóstico desses genes através de curvas de mortalidade Kaplan-Meier que correlacionem expressão gênica com a sobrevida de pacientes de uma coorte de CPNPC cujas informações estão disponíveis em um banco de dados público (GEO database (http://www.ncbi.nlm.nih.gov/projects/geo/; Series GSE3141));

- Testar o valor prognóstico do gene *CFL1* através de curvas de mortalidade Kaplan-Meier que correlacionem expressão gênica com a sobrevida de pacientes de uma coorte de CPNPC cujas informações estão disponíveis em um banco de dados público (GEO database (http://www.ncbi.nlm.nih.gov/projects/geo/; Series GSE3141));

- Desenvolver e padronizar método de quantificação imuno-histoquímica (SQ-IHQ) de cofilina-1 em espécimes de CPNPC emblocados em parafina;

- Reunir coorte retrospectiva de casos CPNPC e respectivas informações específicas para que se possa realizar o estudo de correlação destas com o imunoconteúdo de cofilina-1;

- Processar as biópsias embebidas em parafina em coloração hematoxilina–eosina (HE) para confirmação histológica e classificação de acordo com grau de diferenciação tumoral;

- Realizar técnica de imunoistoquímica para análise de imunorreatividade ao anticorpo anti-cofilina-1;

- Verificar a compartimentalização de cofilina-1 nas células tumorais, bem como quantificar, através de mensuração da densidade óptica (OD), o imunoconteúdo total e dos compartimentos citosólico e nuclear de cofilina-1 para cada caso;

- Correlacionar as medidas de OD total e dos compartimentos citosólico e nuclear de cada caso com as respectivas informações de grau de diferenciação tumoral, classificação histológica e desfecho do paciente adquiridas na montagem da coorte, com o objetivo de buscar possíveis correspondências ao imunoconteúdo de cofilina que possam indicar maior ou menor agressividade tumoral.

# PARTE 2

## 3. RESULTADOS

Os resultados da dissertação estão apresentados na forma de artigos publicados, dispostos por capítulos.

**Capítulo 1**

Artigo publicado na Revista "*Frontiers in Bioscience*"

**"*Bioinformatics approach for the validation of non-small cell lung cancer biomarkers*"**

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# Bioinformatics approach for the validation of non-small cell lung cancer biomarkers

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## TABLE OF CONTENTS



## 1. ABSTRACT

Non-small cell lung cancer (NSCLC) accounts for nearly 1 million deaths annually, worldwide. Conventional treatments offer limited benefits and patients have a survival rate of approximately 1 year. A biomarker for NSCLC could provide the potential benefits of early diagnosis, prognosis and could lead to important applications such as drug targeting. In a search for a biomarker with prognostic value, we reviewed the literature and tested potential biomarkers by performing a meta-data analysis using public databank of NSCLC biopsies containing gene expression data and clinical and pathologic information from 111 patients. We generated standard Kaplan–Meier mortality curves by clustering patients according to either biomarker expression levels or NSCLC stage grouping. Our statistical analyses show that all 60 potential biomarkers analyzed here have no prognostic value for NSCLC patient outcome.

## 2. INTRODUCTION

Lung cancer is the leading cause of cancer deaths worldwide, accounting for 1.3 million deaths annually (data from World Health Organization, 2008). The high mortality associated with this disease is primarily due to the fact that the majority of lung cancers are not detected until they have progressed to an advanced stage (1). Non-small cell lung cancer (NSCLC) represents nearly 85% of lung cancer cases. Compared with other major types of cancer such as colon, prostate, and breast cancers, the clinical outcome of conventional therapies for NSCLC remains poor, with a median survival of 9-11 months (2).

Therefore, there is an urgent need for more effective therapies, drugs, or treatments that could help decrease the incidence of NSCLC. Alternatively, cancer biomarker gives good guidance on many areas of cancer biology. Unlike uniformity of long-established TNM

system, the international standard protocol that allows the staging of carcinoma according to the extent of disease in the patient, cancer biomarkers are considered to be more suitable to the heterogeneous nature of cancer (3). In 2008, Sawyers discussed the three types of cancer biomarkers. These can be used for prognosis, to predict the natural course of a tumor, indicating whether the outcome for the patient is likely to be good or poor. They can also be used in prediction, helping to decide which patients are likely to respond to a given drug and at what dose it might be most effective (4). A biomarker for NSCLC could provide the potential benefits of early diagnosis, considering that the disease is usually detected in late stages when surgical removal of the tumor is no longer an option, and additionally, could lead to other important applications such as prognosis and drug targeting (5). An impressive number of molecular markers have been implicated in the prognosis of NSCLC; however, the results reported in literature are conflicting and none are in use in clinics. Thus, further investigation, newer molecular assays and the development of appropriate panel of molecular markers are still required (6).

Systematic analysis of gene expression using high-throughput screening of cDNA microarray libraries has been considered as an effective approach for identifying and validating potentials biomarkers for NSCLC (7). However, final validation should be done by testing a collection of well-defined clinical samples. Herein, we describe a bioinformatics-based approach to test and validate the prognostic value of potential NSCLC biomarkers. Our research group have been studying many aspects of tumor biology by different bioinformatics approaches (8-9). Here our approach is to correlate data collected from the literature with data on gene expression of a large and well-defined collection of NSCLC biopsies containing information on patients' clinical status and pathology to clinically evaluate the efficacy of potential biomarkers to predict patients outcome. Validation by clinical trials in large cohorts of patients is necessary before cancer-related phenotypes can be translated into the clinic as reliable biomarkers.

## 3. SEARCHING FOR NSCLC POTENTIAL BIOMARKER

### 3.1. Literature search

The list of genes presented in (Table 1) was compiled by searching the PubMed database for articles published in English between January 1985 and December 2009. Search criteria included subject heading terms for "biomarker", "prognosis", "gene expression" and "lung cancer". Genes reported in two or more articles during the period of our search, or in one article at least during the last 3 years were selected. Those articles describing pooled biomarkers into the same analysis were excluded from the list (*i.e.* combined performance for multiple genes). A total of 60 NSCLC was tested.

### 3.2. Tumor samples and microarray data

For the clinical validation of potential NSCLC prognostic biomarkers, we used a large, homogeneous, well-defined collection of samples from lung cancer biopsies, along with respective gene expression data and relevant clinical and pathologic information -such as age, sex, cancer histological type, and NSCLC staging in a cohort follow-up period of 72 months- on 111 patients (10). Data were obtained from the GEO database (http://www.ncbi.nlm.nih.gov/projects/geo/; Series GSE3141) and from the Duke Institute for Genome Sciences & Policy website (http://data.cgt.duke.edu/oncogene.php). All gene array data was on Affymetrix U133A GeneChip, from core biopsies of patients' tumor. A *sine qua non* condition for select a given gene was the presence of two or more microarray probes in NSCLC cohort used.

### 3.3. Survival data analysis

We used the SPSS software (SPSS for Windows, release 14.0.0, SPSS Inc., Chicago, IL) to generate Standard Kaplan–Meier mortality curves with their significance levels, for patient clusters. Survival curves were compared using the log-rank test; patients were clustered according to biomarker expression level or NCSLC stage grouping (*i.e.* initial and advanced staging).

## 4. DISCUSSION

Through a systematic MEDLINE literature inspection, we selected 60 genes as potential biomarkers to be validated using a clinical databank. These biomarkers constitute a diverse group of genes involved in different cellular functions. They code proteins such as transcription factors (*TFAP2B*, *MYC*), protein kinases (*PIK3CA*, *STAT1*, *KRAS*, *STYK1*, *LCK*), protein phosphatases (*DUSP6*), receptors (*AGER*, *EGFR*, *AMFR*), and several DNA repair systems (*ALKBH5*, *ALKBH3*, *FGFR1OP*, *ERCC1*) (See table 1 for complete list of potential prognostic biomarkers). These potential biomarkers are related in the most distinct ways with lung cancer; for example, mutations in the genetic region encoding the kinase domain of the epidermal growth-factor receptor (*EGFR*) predict the sensitivity of lung tumors to erlotinib or gefitinib (11), as well different mutation in *KRAS* predict that patients with lung cancer will fail to respond to these inhibitors (12).

Some genes of DNA repair system are also considered to be potential biomarkers to NSCLC. They have been constantly described as being related with sensitivity to chemotherapeutic drugs (13-14), specially alkylating agents, which are the most largely used anti-cancer drug for NSCLC treatment (15). These drugs are mutagenic, genotoxic and have the ability to damage DNA (16). Cisplatin is an alkylating agent widely used in NSCLC treatment; however, this type of cancer can presents inherently resistance against it. Like many DNA alkylators, cisplatin acts inhibiting DNA replication, which is the critical target in cancer treatment. In this case, the resistance against cisplatin is mainly determinate by the expression levels of nucleotide excision repair genes (NER). ERCC, a component of the NER complex, is a potential marker involved in prediction of resistance to cisplatin, which has been described to be related with its mRNA expression (17-19).

**Table 1.** Potential NSCLC biomarkers previously described

| Symbol[1] | Gene Name[1] | Gene ID[1] | *P-value*[2] | Ref. |
|---|---|---|---|---|
| *ABCC1* | ATP-binding cassette, sub-family C (CFTR/MRP), member 1 | 4363 | 0.6239 | [27] |
| *AGER* | advanced glycosylation end product-specific receptor | 177 | 0.0894 | [28] |
| *ALKBH1* | alkB. alkylation repair homolog 1 | 8846 | 0.5685 | [29] |
| *ALKBH5* | alkB. alkylation repair homolog 5 | 54890 | 0.1704 | [29] |
| *ALKBH7* | alkB. alkylation repair homolog 7 | 84266 | 0.6970 | [29] |
| *ALKBH8* | alkB. alkylation repair homolog 8 | 91801 | 0.5752 | [29] |
| *AMFR* | autocrine motility factor receptor | 267 | 0.6829 | [30,31] |
| *BCL2L1* | BCL2-like 1 | 598 | 0.0661 | [22] |
| *BIRC5* | baculoviral IAP repeat-containing 5 | 332 | 0.3584 | [32,33] |
| *BRCA1* | breast cancer 1, early onset | 672 | 0.5103 | [34] |
| *CALB1* | calbindin 1, 28kDa | 793 | 0.3541 | [35] |
| *CAV1* | caveolin 1, caveolae protein | 857 | 0.0808 | [36] |
| *CBLC* | Cas-Br-M (murine) ecotropic retroviral transforming sequence c | 23624 | 0.0608 | [7] |
| *CCNB2* | cyclin B2 | 9133 | 0.8282 | [28] |
| *CCND1* | cyclin D1 | 595 | 0.1177 | [29] |
| *CD9* | CD9 molecule | 928 | 0.7940 | [20,26] |
| *CDK8* | cyclin-dependent kinase 8 | 1024 | 0.4337 | [38] |
| *CRABP1* | cellular retinoic acid binding protein 1 | 1381 | 0.5485 | [27] |
| *CTSB* | cathepsin B | 1508 | 0.6717 | [39] |
| *DNAJB4* | DnaJ (Hsp40) homolog, subfamily B, member 4 | 11080 | 0.2820 | [40] |
| *DUSP6* | dual specificity phosphatase 6 | 1848 | 0.2842 | [41] |
| *EGFR* | epidermal growth factor receptor | 1956 | 0.6074 | [42,43] |
| *ERBB2* | v-erb-b2 erythroblastic leukemia viral oncogene homolog 2. | 2064 | 0.2379 | [42,10] |
| *ERBB3* | v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian) | 2065 | 0.5191 | [44] |
| *ERCC1* | excision repair cross-complementing rodent repair deficiency. | 2067 | 0.4269 | [43,45,46] |
| *FGFR1OP* | FGFR1 oncogene partner | 11116 | 0.1871 | [47] |
| *FOLR1* | folate receptor 1 (adult) | 2348 | 0.3262 | [48] |
| *GSTA1* | glutathione S-transferase alpha 1 | 2938 | 0.6353 | [35] |
| *HMGA2* | high mobility group AT-hook 2 | 8091 | 0.7014 | [49] |
| *HSP90AA1* | heat shock protein 90kDa alpha (cytosolic), class A member 1 | 3320 | 0.4367 | [50] |
| *IFI44* | interferon-induced protein 44 | 10561 | 0.9626 | [51] |
| *IGF2BP1* | insulin-like growth factor 2 mRNA binding protein 1 | 10642 | 0.8041 | [52] |
| *IL1A* | interleukin 1. alpha | 3552 | 0.4672 | [29] |
| *ILF3* | interleukin enhancer binding factor 3, 90kDa | 3609 | 0.7057 | [35] |
| *KIF14* | kinesin family member 14 | 9928 | 0.9346 | [53] |
| *KRAS* | v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog | 3845 | 0.4472 | [54] |
| *LARS2* | leucyl-tRNA synthetase 2, mitochondrial | 23395 | 0.8316 | [27] |
| *LCK* | lymphocyte-specific protein tyrosine kinase | 3932 | 0.5878 | [44] |
| *LST1* | leukocyte specific transcript 1 | 7940 | 0.2105 | [27] |
| *MBD2* | methyl-CpG binding domain protein 2 | 8932 | 0.4473 | [55] |
| *MMD* | monocyte to macrophage differentiation-associated | 23531 | 0.1662 | [41] |
| *MYC* | v-myc myelocytomatosis viral oncogene homolog (avian) | 4609 | 0.4586 | [56,57] |
| *P53AIP1* | p53-regulated apoptosis-inducing protein 1 | 63970 | 0.8171 | [58] |
| *PIK3CA* | phosphoinositide-3-kinase. | 5290 | 0.1111 | [59] |
| *PLAU* | plasminogen activator. urokinase | 5328 | 0.1314 | [60] |
| *PRDX2* | peroxiredoxin 2 | 7001 | 0.9166 | [61] |
| *PRSS3* | protease, serine, 3 | 5646 | 0.5814 | [62] |
| *RRM1* | ribonucleotide reductase M1 polypeptide | 6240 | 0.8498 | [43,45] |
| *SLC1A7* | solute carrier family 1, member 7 | 6512 | 0.4883 | [35] |
| *SLC2A1* | solute carrier family 2 (facilitated glucose transporter), member 1 | 6513 | 0.7739 | [27] |
| *SP100* | SP100 nuclear antigen | 6672 | 0.9490 | [7] |
| *STAT1* | signal transducer and activator of transcription 1. 91kDa | 6772 | 0.9245 | [41] |
| *STC1* | stanniocalcin 1 | 6781 | 0.1508 | [27] |
| *STYK1* | serine/threonine/tyrosine kinase 1 | 55359 | 0.6834 | [63] |
| *TAL2* | T-cell acute lymphocytic leukemia 2 | 6887 | 0.4593 | [35] |
| *TERF2* | telomeric repeat binding factor 2 | 7014 | 0.1028 | [64] |
| *TERT* | telomerase reverse transcriptase | 7015 | 0.7542 | [65] |
| *TFAP2B* | transcription factor AP-2 beta | 7021 | 0.1814 | [9] |
| *TOP2A* | topoisomerase (DNA) II alpha | 7153 | 0.1998 | [66] |
| *TYMS* | thymidylate synthetase | 7298 | 0.1742 | [67,68] |

[1]Gene symbols and names according to HUGO Gene Nomenclature Committee, HGNC database (http://www.genenames.org).
[2]Differences in survival were assessed with the log-rank test using SPSS version 14.0. In all statistical analyses, *P*-value less than 0.05

Despite the large literature about markers in NSCLC, our study shows that none of the genes we tested have their mRNA levels directly correlated with patient outcome. It is important to state that our analysis was based on gene expression status, not taking into account other relevant parameters, like gene mutation pattern, methylation, or cohort subgroups. As an example, *ERBB2* gene has prognostic value in patients with NSCLC when considering specific cohort gender (20). Likewise, *ERCC1* is effective in stage IIB-IIIA-IIIB of NSCLC (21) and *CD9* has prognostic value given the mutation status of *KRAS* gene (22).

Different experimental approaches have been used to establish each of the genes listed in table 1 as potentials biomarkers for predicting patient outcome. This

approach shows that biomarker candidates should be carefully tested in clinical samples and exemplifies a rational use of public high-throughput clinical data. In theory, it could be applied to validate any possible biomarker, optimizing the use of the information available in public databanks and serving as standard tool to guide future clinical trials. Thus, we would be maximizing the use of information already generated and increasing its applicability.

The panel generated by this tool must be further analyzed. Microarrays are well described as capable of determining the expression levels of thousands of genes simultaneously (23) and the ability to define cancer subtypes, recurrence of disease and response to specific therapies using DNA microarray-based gene expression signature has been demonstrated in multiple studies (24). Bild & Col. described the activation status of several oncogenic pathway based on the statistical combination of gene expression signatures (8). Nevertheless, we believe that the gene signatures should be obtained based on biological (not statistical) combination of high-throughput screening of cDNA microarray probes. In this scenario, fluctuation of gene expression within biological networks can be evaluated by landscape analysis, which can represent different functional states of the same gene network (25)

In summary, our research describes a reliable tool able to discriminate biomarkers performance, revealing that none of the 60 genes individually tested had shown sufficient statistical power to be safely included in clinical use, when compared to TNM system, which is considered gold standard by physicians. Therefore, these approach may strengthens the development of new biomarkers, since up-to-date there is still no prognostic biomarker (based on gene expression) available for NSCLC. As stated by Dr. Goldstraw in the last World Conference on Lung Cancer (26), it is still uncertain how to integrate the predictive information from biomarkers with the anatomical extent of disease described by the TNM system, which rises the possibility that T, N, and M could be joined by a B (biological) factor. Biomarkers will probably be the next major development in NSCLC staging.

## 5. ACKNOWLEDGEMENTS

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**Key Words:** Bioinformatic approach, Non-Small Cell Lung Cancer, prognostic biomarkers

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## **CAPÍTULO 1 -** RESULTADOS SUPLEMENTARES:

Avaliação potencial prognóstico – Curvas de mortalidade Kaplan-Meier

**Bioinformatics Approach for the Clinical Validation of Potential Non-Small Cell Lung Cancer Prognostic Biomarkers**

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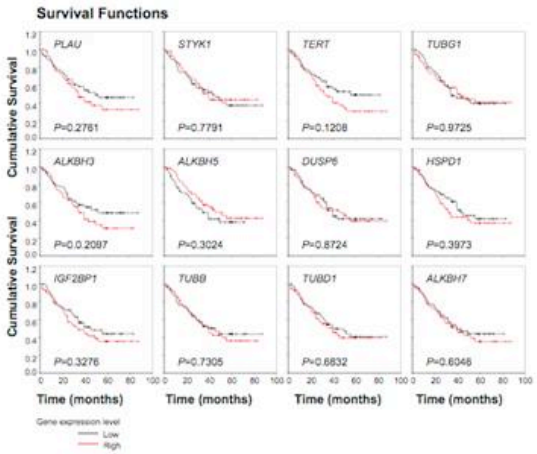


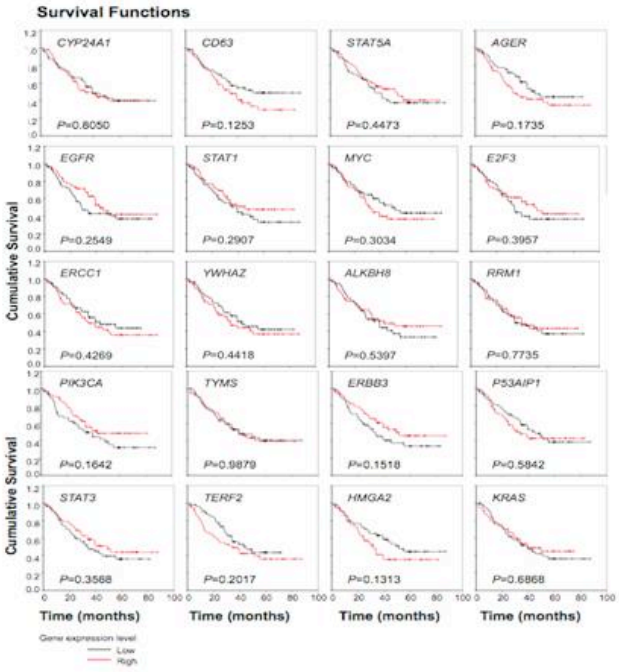

**Capítulo 2**

Artigo Publicado na Revista "*Cancer*"

**"*CFL1 expression levels as a prognostic and drug resistance marker in non-small-cell lung cancer*"**

Original Article

# *CFL1* Expression Levels as a Prognostic and Drug Resistance Marker in Nonsmall Cell Lung Cancer

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**BACKGROUND:** Nonsmall cell lung cancer (NSCLC) is the major determinant of overall cancer mortality worldwide. Despite progress in molecular research, current treatments offer limited benefits. Because NSCLC generates early metastasis, and this behavior requires great cell motility, herein the authors assessed the potential value of *CFL1* gene (main member of the invasion/metastasis pathway) as a prognostic and predictive NSCLC biomarker. **METHODS:** Metadata analysis of tumor tissue microarray was applied to examine expression of *CFL1* in archival lung cancer samples from 111 patients, and its clinicopathologic significance was investigated. The robustness of the finding was validated using another independent data set. Finally, the authors assayed in vitro the role of *CFL1* levels in tumor invasiveness and drug resistance using 6 human NSCLC cell lines with different basal degrees of *CFL1* gene expression. **RESULTS:** *CFL1* levels in biopsies discriminate between good and bad prognosis at early tumor stages (IA, IB, and IIA/B), where high *CFL1* levels are correlated with lower overall survival rate ($P < .0001$). Biomarker performance was further analyzed by immunohistochemistry, hazard ratio ($P < .001$), and receiver-operating characteristic curve (area = 0.787; $P < .001$). High *CFL1* mRNA levels and protein content are positively correlated with cellular invasiveness (determined by Matrigel Invasion Chamber System) and resistance (2-fold increase in drug 50% growth inhibition dose) against a list of 22 alkylating agents. Hierarchical clustering analysis of the *CFL1* gene network had the same robustness for stratified NSCLC patients. **CONCLUSIONS:** This study indicates that the *CFL1* gene and its functional gene network can be used as prognostic biomarkers for NSCLC and could also guide chemotherapeutic interventions. ***Cancer* 2010;116;3645–55.** © *2010 American Cancer Society.*

**KEYWORDS:** prognosis, biomarker, lung cancer, nonsmall cell lung cancer, cofilin, *CFL1* expression, drug resistance.

**Lung** cancer accounts for 1.3 million deaths annually (World Health Organization), of which 85% are of nonsmall cell lung cancer (NSCLC) patients. These patients present an average survival rate of 10 months, and only 15% survive for 5 years.[1] Currently, prognosis of NSCLC patients is done by considering patient performance status and tumor staging.[2,3] However, accumulating data[4] has shown that these have unsatisfactory power in predicting patient outcome or in guiding physicians on the best course of action for each patient. A novel prognostic method for early stage NSCLC patients can potentially increase survival rates by indicating those in need of more aggressive treatment.[5]

Lung cancers in particular show poor prognosis because of their ability to generate early metastasis within the lungs and then in distant organs. This behavior requires great cell motility, which is performed by several proteins that act on the actin cytoskeleton by regulating cycles of polymerization and depolymerization of actin filaments, which in turn generates cell motion.

One of the main proteins in charge of cell motility is cofilin (*CFL1*, cofilin-1; nonmuscle isoform; gene ID, 1072),[6] which is regulated by factors such as phosphorylation, pH, binding of phosphoinositides, and subcellular compartmentalization. In a recent study, we have found that cofilin mediates apoptosis in response to oxidative stress, which is a novel

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regulatory activity described for this protein.[7] The role of the cofilin pathway in cell mobility has been shown extensively.[8] Its activation occurs locally and in response to epidermal growth factor receptor (EGFR) signaling in chemotaxis.[9] High cofilin activity has been correlated with breast cancer invasion and metastasis,[10,11] where it is essential for directional sensing,[12] and with epithelial-mesenchymal transition, a process that is involved in the regulation of cell migration, adhesion, and invasion, suggesting the acquisition of a invasive phenotype.[13] Thus, we raised the hypothesis that cofilin amount in NSCLC could provide relevant information about a tumor's aggressiveness and therefore be used as a prognostic marker.

Herein, we assessed the potential prognostic value of *CFL1* as a NSCLC biomarker. To assay that, we used 3 different experimental approaches: the first based on the correlation of gene expression levels and patient outcome using meta-analysis of clinical data from a large, homogeneous, well-defined collection of samples from NSCLC cohorts; the second based on the analysis of in vitro data obtained with 6 different human NSCLC cell lines; and the third in which we constructed a network-based model of *CFL1* gene and analyzed the role of each network component on the cellular resistance profile to different chemotherapeutic drugs.

## MATERIALS AND METHODS

### *Cohort Studies and Data Analysis*

#### Patients, tumor samples, and microarray datasets

For NSCLC cohort analysis, we accessed a large well-defined collection of lung cancer samples with expression data and relevant clinical and pathologic information on 111 patients (testing cohort), from core biopsies of patients' tumors. The data were obtained from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/projects/geo/; Series GSE3141) and the Duke Institute for Genome Sciences and Policy website (http://data.cgt.duke.edu/oncogene.php). Gene array data are available on the Affymetrix (Santa Clara, Calif) U133 Plus 2.0 GeneChip.[14] To test the reproducibility of the data, we assessed a second, independent microarray data set (validation cohort), which is available on a different microarray platform (Affymetrix HG_U95Av2 GeneChip).[15] The validation cohort comprises microarray data from 86 tumor biopsies obtained from sequential patients seen at the University of Michigan Hospital for stage I or stage III lung adenocarcinomas. All gene array data of the validation cohort are available at http://dot.ped.med.umich.edu:2000/ourimage/pub/Lung/index.html

#### Survival data analysis

Standard Kaplan-Meier mortality curves and their significance levels were generated for clusters of patients using SPSS software (SPSS for Windows, release 14.0.0, SPSS Inc., Chicago, Ill). The survival curves are compared using the log-rank test, and patients are clustered according to either biomarker expression level or NCSLC stage grouping.[4,16]

#### Cox multivariable regression analysis

Multivariate Cox proportional hazards regression models were used to test the independent contribution of each variable on mortality. Graphical assessment was used to assess the Cox model's proportional hazard assumption. Results of multivariate analysis were summarized by calculating hazard ratios (HRs) and corresponding 95% confidence intervals (CIs).

#### Biomarker accuracy

The area under the receiver operating characteristic (ROC) curve was used to evaluate the biomarker's ability in discriminating patients who survived and those who died. An optimal cutoff value was obtained considering the combination of highest sensitivity and specificity.

### In Vitro *Assays*

#### Immunohistochemical staining

Paraffin-embedded sections of lung samples from 20 patients with NSCLC (classified according to World Health Organization criteria) were obtained as archival specimens from the Department of Pathology at the São João Batista Hospital in Criciúma, Brazil. Hematoxylin and eosin-stained slides of lung tissue were examined by a national board-certified pathologist. Selected areas of lung cancer and corresponding benign samples were sectioned into 3-μm slices, and immunohistochemical staining was performed according to the standard avidin-biotin immunoperoxidase complex technique. Rabbit polyclonal anti-human cofilin-1 antibody (Abcam, Cambridge, Mass) (1 μg/mL) was used as the primary antibody. The brownish color was considered to be evidence of a positive expression of cofilin-1 in the tumor cells. Unstained red blood cells and labeled macrophages were considered, respectively, as negative and positive internal controls. The

Helsinki Declaration of Human Rights was strictly observed when performing these experiments.

### *Cell culture and Western blot immunoassay*

The human NSCLC cell lines were obtained from the National Cancer Institute-Frederick Cancer Division of Cancer Treatment and Diagnosis tumor/cell line repository, and grown in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine at 37°C in 5% $CO_2$ in air. Exponentially growing cells were washed twice with phosphate-buffered saline and resuspended in lysis buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid, 1% Triton, 1 mM $Na_3VO_4$, and protease inhibitors. After sonication, 30 μg of protein was electrophoresed on 4% to 12% Bis-Tris NuPage gels (Invitrogen, Carlsbad, Calif), transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore, Billerica, Mass), and blocked with 5% milk. The following antibodies were used for Western blot immunoassay: rabbit polyclonal anticofilin (1:1000), rabbit polyclonal antiactin (1:2000) (Cytoskeleton, Denver, Colo). Horseradish peroxidase-linked secondary antibody (1:10,000) was from Dako-Cytomation (Carpinteria, Calif). Bands were observed by chemiluminescence using the ECL Detection kit from Amersham Biosciences (Fairfield, Conn) and exposure of x-ray film. Quantification of band was done with ImageJ 1.36b software (National Institutes of Health).

#### Drug cytotoxicity

Drug 50% growth inhibition dose ($GI_{50}$) was determined as described elsewhere. Briefly, exponentially growing NSCLC cell lines were treated with different concentrations of drugs (cisplatin, carboplatin, 5-fluorouracil, hydroxyurea, and paclitaxel [Taxol]) (Sigma, St. Louis, Mo). After 72 hours, the medium was removed, and cells were fixed with cold 10% trichloracetic acid (TCA) for 1 hour at 4°C. Plates were washed 5× with distilled water and left to dry at room temperature. Cells were stained with 0.4% of sulforhodamine B (Sigma) (w/v) in 1% acetic acid (v/v) at room temperature for 20 minutes. Sulforhodamine B was removed, and the plates were washed 5× with 1% acetic acid before air-drying. Bound dye was solubilized with 10 mM unbuffered Tris-base solution, and plates were left on a plate shaker for at least 10 minutes. Absorbance was measured in a 96-well plate reader (VERSA*max*, Molecular Devices, Sunnyvale, Calif) at 492 nm. $GI_{50}$ was calculated according to the concentration-response curve. The mean of 3 independent experiments for each condition run in triplicates was plotted.

#### Cell migration and invasion assays

In vitro migration and invasion assays were performed using the BioCoat Matrigel Invasion Chamber System (BD Biosciences, San Jose, Calif). Briefly, Matrigel inserts were rehydrated in RPMI medium, and cells ($2.5 \times 10^4$ cells) were seeded at each 24-well chamber. The chemoattractant (medium RPMI with 10% of FBS) was added to the lower wells, and the movement of cells through the 8.0-μm pore size Tran-swell cell culture inserts, either uncoated (migration) or Matrigel coated (invasion), was determined after 22 hours of incubation at 37°C in a humidified incubator with 5% $CO_2$ atmosphere. At the end of the assay, cells were removed from the top side of the insert using a cotton swab. Cells that penetrated to the underside surfaces of the inserts were fixed and stained with a HEMA 3 staining kit (Fisher Scientific, Waltham, Mass) and counted under the microscope. Data are expressed as the percentage invasion through the Matrigel relative to the migration through the uncoated membrane, and expressed as invasion index. The mean of 3 high power fields for each condition run in triplicates is plotted.

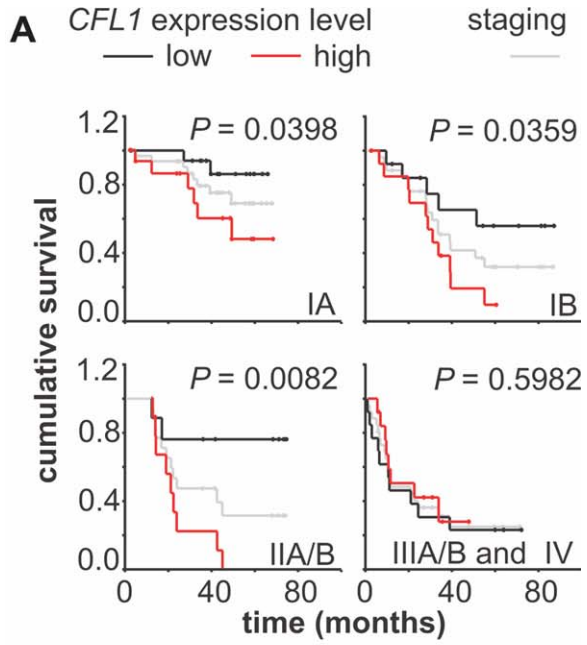
### *Bioinformatics Analysis*

#### Microarray data from the NCI-60 cancer cell panel

Transcript expression profiles of the 6 human NSCLC cell lines were obtained from the NCI-60 human tumor cell line anticancer drug screen (http://discover.nci.nih.gov/datasetsNature2000.jsp). To test the reproducibility of the data, we assessed a second, independent microarray data set available at http://discover.nci.nih.gov/cellminer/home.do (Robust Multi-array Average [RMA] normalized Affymetrix HG-U133A/B data set). This second microarray platform comprises the human transcriptome and consistently identifies gene probes (eg, it follows approved gene IDs from the HUGO Gene Nomenclature Committee, http://www. genenames.org/), allowing the proper identification of *CFL1* partners in the biological network analysis.

#### The drug database

For drug panel activity analysis, we considered those compounds listed in the Mechanism of Action drug activity database of the National Cancer Institute Developmental Therapeutics Program (http://discover.nci.nih.gov/datasetsNature2000.jsp). This panel consists of 118




B

Hazard Ratio for patient's survival*

| Variables | overall survival HR (95% CI) | *P*-value |
|---|---|---|
| Age (Years) | 1.02 (0.99-1.05) | 0.085 |
| Gender | | |
| Female | 1.00 | |
| Male | 1.06 (0.61-1.83) | 0.845 |
| *CFL1* level | | |
| Low | 1.00 | |
| High | 2.70 (1.54-4.75) | 0.001 |
| Type of cancer | | |
| Squamous-cell | 1.00 | |
| Adenocarcinoma | 1.32 (0.77-2.29) | 0.316 |
| Tumour stage | | |
| IA | 1.00 | |
| IB | 2.35 (1.09-5.07) | 0.030 |
| IIA/B | 2.76 (1.20-6.37) | 0.017 |
| IIIA/B-IV | 4.01 (1.90-8.46) | <0.001 |

*Cohort description in Table 1 (*n* = 111)

**Figure 1.** Prognostic value of *CFL1* mRNA levels in nonsmall cell lung cancer patients is shown. (A) A meta-analysis of cohort data grouped according to the International Staging System for Lung Cancer and *CFL1* gene expression level (ie, upper fifth vs lower fifth), and plotted as survival probabilities using the Kaplan-Meier method is shown. Black lines represent patients with low *CFL1* expression; red lines indicate high *CFL1* expression. Differences in survival rates were assessed with the log-rank test. Gray lines represent all patients according to tumor staging. *P* values <.05 were considered significant. (B) Cox multivariate regression analysis was used to estimate hazard ratios (HRs) for cohort clinical covariates and *CFL1* expression. HRs indicate that patients with high *CFL1* expression level presented poor outcome. CI, indicates confidence interval.

**Table 1.** Clinical Characteristics of the Original and Validation Cohorts

| Characteristic | *CFL1* Expression | | *P* |
|---|---|---|---|
| | High | Low | |
| Testing cohort, n=111 | 55 (49%) | 56 (51%) | |
| Age, y | 64.6 ± 9.6 | 64.9 ± 9.7 | .842 |
| **Sex** | | | |
| Men | 30 (54%) | 33 (59%) | .784 |
| Women | 25 (46%) | 23 (41%) | |
| **Tumor type** | | | |
| Adenocarcinoma | 28 (51%) | 30 (54%) | .928 |
| Squamous cell | 27 (49%) | 26 (46%) | |
| **Tumor TNM stage** | | | |
| Ia | 20 (36%) | 20 (36%) | .999 |
| Ib | 13 (24%) | 14 (25%) | |
| II | 9 (16%) | 9 (16%) | |
| III-IV | 13 (24%) | 13 (23%) | |
| Validation cohort, n=86 | 43 (50%) | 43 (50%) | |
| Age, y | 62.3 ± 8.8 | 65.1 ± 10.7 | .187 |
| **Sex** | | | |
| Men | 21 (49%) | 14 (33%) | .198 |
| Women | 22 (51%) | 29 (67%) | |
| **Tumor type/differentiation** | | | |
| Adenocarcinoma/well | 12 (28%) | 12 (28%) | .964 |
| Adenocarcinoma/moderate | 21 (49%) | 20 (47%) | |
| Adenocarcinoma/poor | 10 (23%) | 11 (26%) | |
| **Tumor TNM stage** | | | |
| I | 34 (79%) | 33 (77%) | .999 |
| III | 9 (21%) | 10 (23%) | |

compounds whose mechanisms of action are classified: 1) alkylating agents; 2) topoisomerase I inhibitor; 3) topoisomerase II inhibitor; 4) DNA/RNA antimetabolites (DNA binder, DNA incorporation, antifols, ribonucleotide reductase inhibitor, DNA synthesis inhibitor, RNA synthesis inhibitor); 5) antimitotics; and 6) others (protein synthesis inhibitor, HSP90 binder, or unknown). Drug activity against the NSCLC cell lines is expressed by $GI_{50}$ (also known as $IC_{50}$), and the entire $GI_{50}$ data set is available at http://dtp.nci.nih.gov/dtpstandard/cancerscreeningdata/index.jsp

### *CFL1* chemotherapeutic drug resistance/ sensitivity data analysis

The relation between the activity of the drug dataset (ie, 118 standard chemotherapy agents) and *CFL1* expression levels was estimated by Spearman correlation analysis with SPSS software (SPSS for Windows, release 14.0.0). Positive correlations occurred when relatively high levels of gene expression were found in relatively sensitive cell

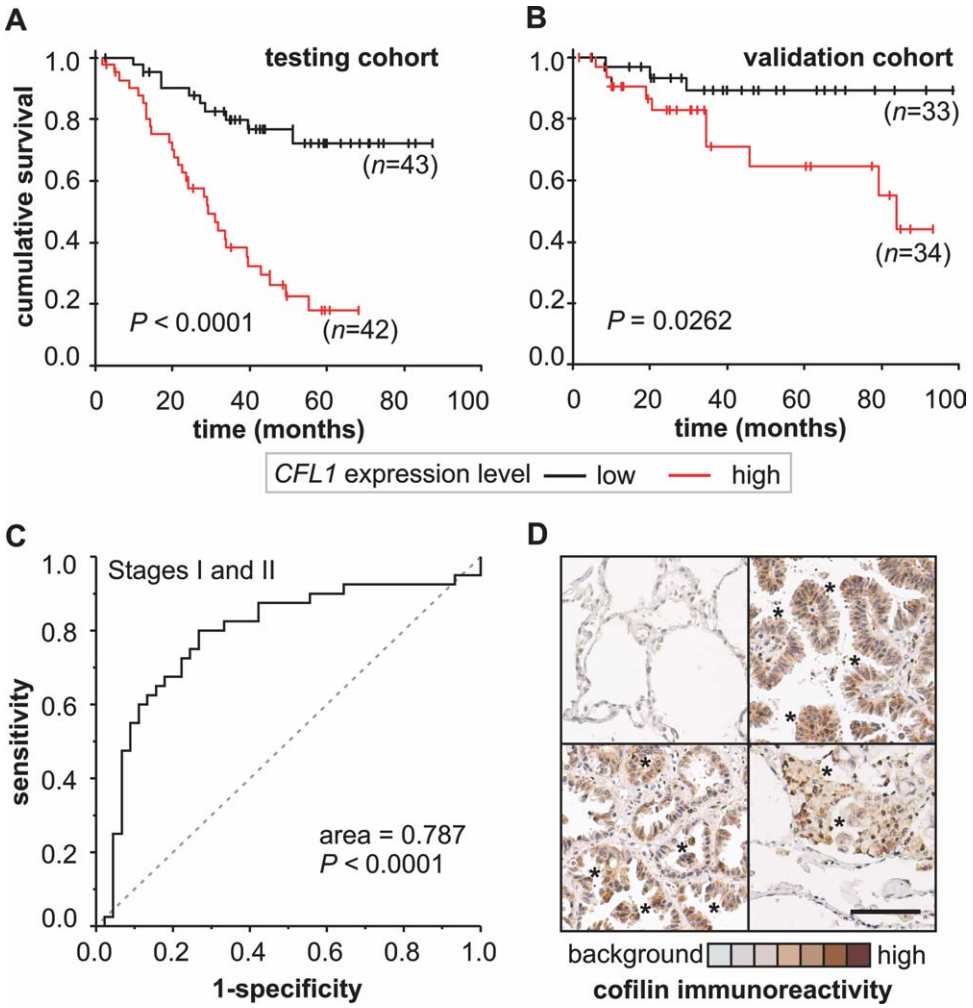



**Figure 2.** Biomarker performance in early stage nonsmall cell lung cancer (NSCLC) patients is shown. (A) Kaplan-Meier plots are shown for patients in stages I and II (n = 85) in the original cohort (testing cohort) stratified by *CFL1* expression level and (B) in an independent cohort (validation cohort) obtained from a different set of published NSCLC microarray data (n = 67). (C) Biomarker performance estimated by receiver operating characteristic analysis is shown. (D) Representative immunohistochemical (IHC) analysis of cofilin immunocontent in tumor biopsies is shown. Healthy human alveolar tissue obtained from tumor margins is mostly negative to cofilin IHC staining (upper left). High staining for cofilin is found within the neoplastic lung cells (asterisks). Original magnification, ×200; scale bar = 100 μM.

lines. Negative correlations occurred when relatively high levels of gene expression were found in resistant cell lines. Therefore, $P$ values $<.05$ indicate a significant negative correlation (resistance), and $P > 0.95$ indicates a significant positive correlation (sensitivity). Because of multiple comparisons, only drug categories showing reproducible results were considered for further analysis (ie, consistent results among the drugs of a given class).

### Construction of the network-based model of *CFL1* interaction partners

Experimental evidence of protein-protein interactions was obtained from the STRING database (http://string.embl.de/).[17] STRING integrates different curated, public databases containing information on direct and indirect functional protein-protein associations. We retrieved all proteins described in that database inferred by experimental evidence and that directly interact with *CFL1* (cofilin-1; nonmuscle isoform; Ensembl peptide ID, ENSP00000309629). The final network was drawn using a spring model algorithm and then handled in Medusa software (Candego, Stockholm, Sweden).[18]

### *CFL1* gene partner analysis

Microarray data of NSCLC cell lines were crossed against $GI_{50}$ values of 118 standard chemotherapy agents to estimate drug sensitivity/resistance profile according to the

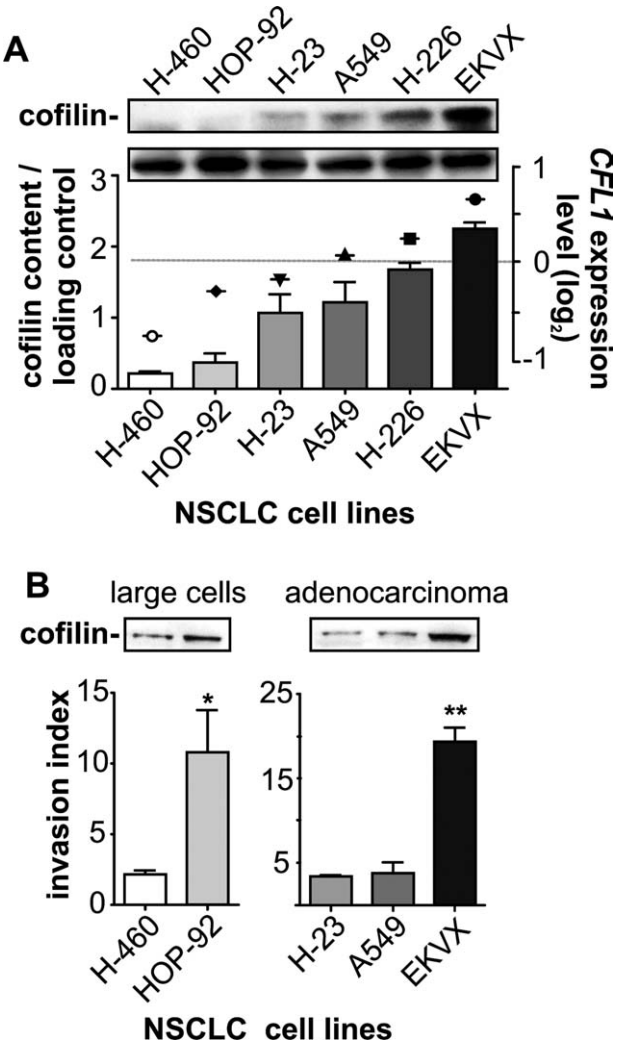



**Figure 3.** Cofilin immunocontent correlates with tumor invasiveness in vitro. Six human nonsmall cell lung cancer (NSCLC) cell lines composed of adenocarcinomas (H-23, A549, EKVX), large cells (H-460, HOP-92), and squamous-cell carcinomas (H-226) from the NCI-60 panel were selected based on different levels of *CFL1* gene expression (http://discover.nci.nih.gov/datasetsNature 2000.jsp) to establish the role of *CFL1* in tumor aggressiveness, evaluated by assays of cell invasion and drug resistance. (A) Western blot analysis shows that the pattern of *CFL1* mRNA (symbols) matches the level of cofilin immunocontent (bars). (B) Invasion index was obtained by determining the movement of cells through an 8.0-μm pore size, either uncoated (migration) or Matrigel-coated (invasion), attracted by a chemotactic gradient of serum. The mean of 4 fields for each condition in quadruplicates is plotted. *$P < .02$ (Mann-Whitney test); **$P < .0001$ (1-way analysis of variance).

expression levels of *CFL1* gene partners (ie, all genes identified in the network-based model of *CFL1* interaction partners). The statistical analysis follows the original method described in the National Cancer Institute's drug discovery program.[19]

### Clustering analysis and expression profile of *CFL1* gene network

The strategy to assess the functional status of tumor samples based on gene expression network profiles has been previously described.[20,21] Two-way hierarchical clustering analysis was performed with the Cluster 3.0 software package using the complete linkage clustering option.[22] For visualization purposes, the gene expression values were median-centered and normalized. The results were processed and observed in TreeView software.[23] The color intensity was set to the log2 ratio of the microarray signal. Probes of all genes listed in the *CFL1* gene network could be retrieved from the microarray platform (ie, the cohort study—its corresponding gene expression database—is provided on the Human Genome U133-Plus 2.0 Array).

## RESULTS

Kaplan-Meier estimates of patient cumulative survival by time (months) according to the expression level of *CFL1* showed that when patients are grouped by *CFL1* gene expression (upper fifth vs lower fifth of transcript abundance levels), the expression levels can be used to discriminate patients in early disease stages (IA, IB, IIA, and IIB) between good or bad outcome (Fig. 1A; based on metadata analysis). Data on microarray gene expression and patient information such as age, sex, cancer histological type, and NSCLC staging were considered (cohort description can be found in Table 1). Cox multivariate regression revealed that lower *CFL1* expression was significantly associated with high overall survival (HR for high risk vs low risk, 2.7; 95% CI, 1.5-4.7; $P = .001$) (Fig. 1B).

Analysis of 85 patients with disease stages I or II (the testing cohort), revealed that patients with high *CFL1* expression (n = 42) had an overall survival rate shorter than those with low *CFL1* expression (n = 43) (Fig. 2A). To test the robustness of this finding, we analyzed a second, independent data set of 67 patients in early stages (the validation cohort) (Fig. 2B). Our meta-analysis showed that high *CFL1* levels are associated with shorter overall survival in both cohorts. ROC curve analysis showed that *CFL1* sensitivity/specificity is high enough to indicate the outcome of patients with early disease stages (area under ROC curve = 0.787) (Fig. 2C). Immunohistochemical stains revealed an increased cofilin immunocontent within the neoplastic tissue (Fig. 2D). The data presented in Figures 1 and 2 suggest that *CFL1* levels can be used to indicate patient outcome.

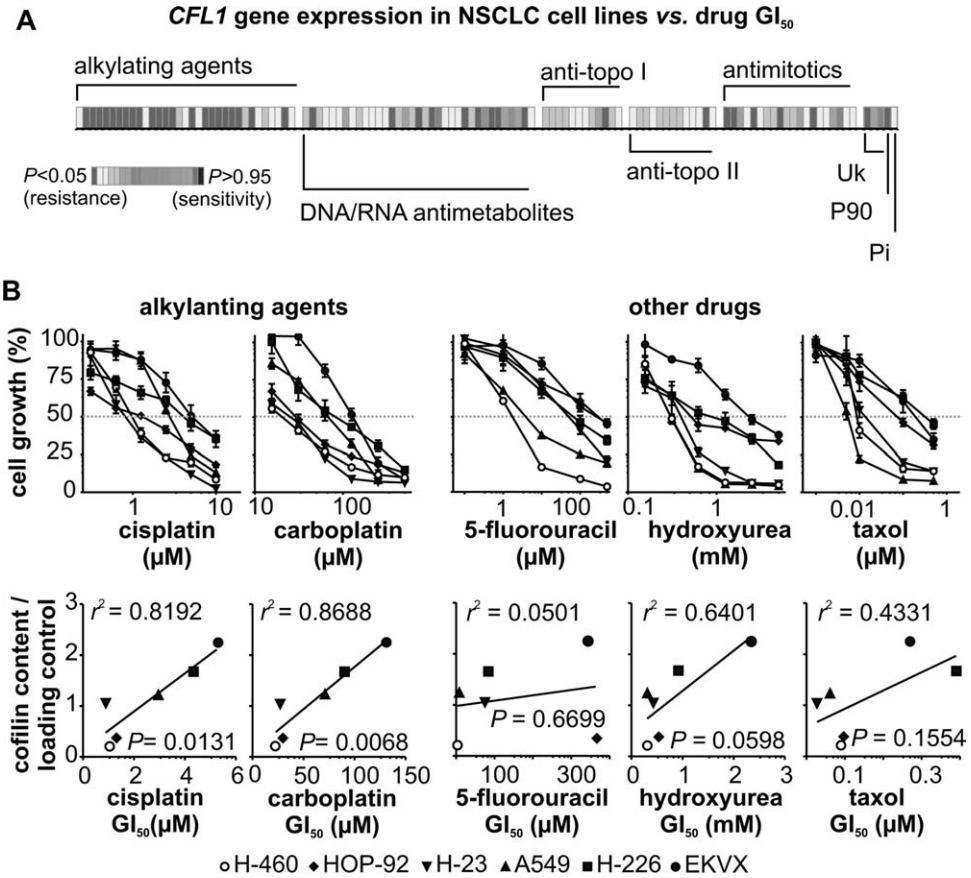



**Figure 4.** Colifin mRNA and protein levels correlate with drug resistance in vitro. (A) Microarray metadata of the cell lines are crossed against 50% growth inhibition dose ($GI_{50}$) values of 118 standard chemotherapy agents (from the NCI-60 drug discovery pipeline). *P* values have been color coded according to the scale shown; $P < .05$ indicates a significant negative correlation (resistance), whereas $P > .95$ indicates a significant positive correlation (sensitivity). The major mechanism of drug action is shown (the term "alkylating agents" is used broadly to include platinating agents; Uk indicates unknown; P90, hsp90 binder; Pi, protein synthesis inhibitor). Each column within the matrix represents the Spearman correlation between gene expression and toxicity of an individual drug. (B) In vitro validation of the cytotoxicity for selected drugs assayed by the sulforhodamine B method is shown (upper plots). The obtained drug $GI_{50}$ values were correlated with cofilin immunocontent (lower plots). NSCLC indicates nonsmall cell lung cancer.

We also investigated whether *CFL1* levels could provide additional insights into the pathophysiology of NSCLC, predicting tumor aggressiveness and/or chemotherapy response. To do that, we used NSCLC data from the US National Cancer Institute in vitro anticancer drug screen (NCI-60 cancer panel).[19] Six human cell lines of the 3 major histological types of NSCLC, namely adenocarcinomas cells (H-23, A549, EKVX), squamous cells carcinomas (H-226), and large cells carcinomas (H-460, HOP-92), were analyzed. Relative levels of *CFL1* gene expression obtained by microarray are presented in Figure 3A (symbols) and match the amount of cofilin protein evaluated here (Fig. 3A; bars). Then, using the BD BioCoat Matrigel Invasion System (to assess the tumor's metastatic potential) (BD Biosciences), we found that different histological types expressing higher *CFL1* levels presented higher invasion indexes, which indicates a more aggressive invasiveness behavior (Fig. 3B).

In addition to this higher invasiveness potential, analysis of microarray data of the 6 cell lines and respective $GI_{50}$ values of 118 standard chemotherapy agents (from the NCI-60 drug discovery pipeline) revealed that high levels of *CFL1* mRNA are also correlated with resistance against different anticancer drugs—mainly alkylating agents (Fig. 4A; meta-analysis) (for a list of all correlated alkylating drugs see Table 2). Exposure of the cell lines to different concentrations of selected chemotherapy drugs (namely cisplatin, carboplatin, 5-fluorouracil, hydroxyurea, and paclitaxel) revealed significant correlations between cofilin immunocontent and

**Table 2.** List of Alkylating Agents for Which *CFL1* mRNA Levels Are a Biomarker[a] for Drug Resistance

| Class[b] | Drugs | $R_s$ | *P* |
|---|---|---|---|
| A2 | Porfiromycin | 0.771 | .036 |
| A6 | Carmustine (BCNU) | 1.000 | .000 |
| A6 | Chlorozotocin | 0.943 | .002 |
| A6 | Clomesone | 0.943 | .002 |
| A6 | Lomustine (CCNU) | 0.771 | .036 |
| A6 | Mitozolamide | 0.943 | .002 |
| A6 | PCNU | 0.943 | .002 |
| A6 | Semustine (MeCCNU) | 0.886 | .009 |
| A7 | Asaley | 0.771 | .036 |
| A7 | Carboplatin | 0.829 | .021 |
| A7 | Chlorambucil | 0.829 | .021 |
| A7 | Cisplatin | 0.829 | .021 |
| A7 | Cyclodisone | 0.943 | .002 |
| A7 | Hepsulfam | 0.771 | .036 |
| A7 | Iproplatin | 1.000 | .000 |
| A7 | Mechlorethamine | 0.943 | .002 |
| A7 | Melphalan | 0.771 | .036 |
| A7 | Piperazine mustard | 0.943 | .002 |
| A7 | Piperazinedione | 0.771 | .036 |
| A7 | Spiromustine | 0.886 | .009 |
| A7 | Uracil mustard | 0.829 | .021 |
| A7 | Yoshi-864 | 0.771 | .036 |

BCNU indicates bischloroethylnitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; MeCCNU, 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea.

[a] Meta-analysis data of chemotherapeutic drugs from a panel of 33 alkylating agents (from Fig. 4A) tested for positive correlation (resistance) between drug 50% growth inhibition dose (μM) and the pattern of *CFL1* gene expression in 6 human nonsmall cell lung cancer cell lines (A549, EKVX, HOP-92, NCI-H226, NCI-H23, NCI-H460) obtained from the NCI-60 cell panel.

[b] Mechanism of action codes: A2, alkylating at N-2 position of guanine; A6, alkylating at O-6 position of guanine; A7, alkylating at N-7 position of guanine.

resistance to cisplatin and carboplatin, the 2 alkylating agents tested (Fig. 4B; in vitro analysis).

By using the same approach on drug resistance, we evaluated the resistance profile against alkylating agents of each gene product that interacts directly with *CFL1*. Four of cofilin's partners (*CAP1, ACTB, SSH3*, and *YWHAZ* genes) show a resistance profile similar to cofilin, suggesting that a functional network is correlated with this tumor phenotype. These results are presented as network-based model of the cofilin biological pathway (Fig. 5A, red nodes), where nodes represent gene products, and connecting lines indicate physical and/or functional associations according to experimental data (http://string.embl.de/).

To further explore the role of this gene network in NCSLC patient outcome, a cluster analysis was carried out using the data bank from the testing cohort. As the microarray data set from this cohort study was produced on the Affymetrix U133 Plus 2.0 platform, all genes listed in our network could be retrieved. Complete linkage clustering of tumor samples is shown in TreeView format (Fig. 5B). From the Heat Map, we identified 3 large tumor clusters, which were then used to restratify the NSCLC patients according to the gene expression profile. Kaplan-Meier estimates based on this new stratification showed that the *CFL1* gene network can also be used to discriminate patients' outcomes (Fig. 5C).

## DISCUSSION

Although much progress has been made in reducing overall mortality rates, cancer is a major public health problem worldwide, accounting for more deaths than heart disease. Most recent epidemiological data show a notable trend in stabilization of incidence rates for all cancer and a continued decrease in the cancer death rate.[1] Whereas the decrease in death rates for colorectal, breast, and prostate cancer largely reflects improvements in early detection and treatment, the decrease in lung cancer death rates reflects manly the reduction in tobacco use.[24,25]

In this scenario, NSCLC is the leading cause of deaths annually. Currently, prognosis of NSCLC patients is still based almost exclusively on the anatomical extent of disease, which may have reached its limit of usefulness for predicting outcomes.[4] Advances in molecular pathology have led to the development of many candidate biomarkers with potential clinical value. However, according to the TNM tumor staging system, few tumors are formally staged with the addition of molecular biomarker information (eg, TNM + S; where S = serum levels of the selected biomarker), which does not include lung cancers.[3]

Herein we are proposing the use of *CFL1* gene expression levels as a prognostic and predictive NSCLC biomarker based on the following findings: 1) *CFL1* mRNA levels are highly sensitive and specific in discriminating between good and bad patient outcome in 2 independent cohorts—specially in early stage disease—where tumors with low expression of the *CFL1* gene are associated with high overall survival; 2) an association exists between cofilin immunocontent and tumor invasion; 3) cells with high cofilin mRNA and protein levels are resistant to alkylating drug treatment; and 4) 4 other genes that interact in the *CFL1* pathway (named *SSH3*, *YWHZ*, *CAP1*, and *ACTB*) also demonstrate the same resistance profile.

As previously shown, to be able to generate early metastasis, tumor cells require the activity of cofilin to

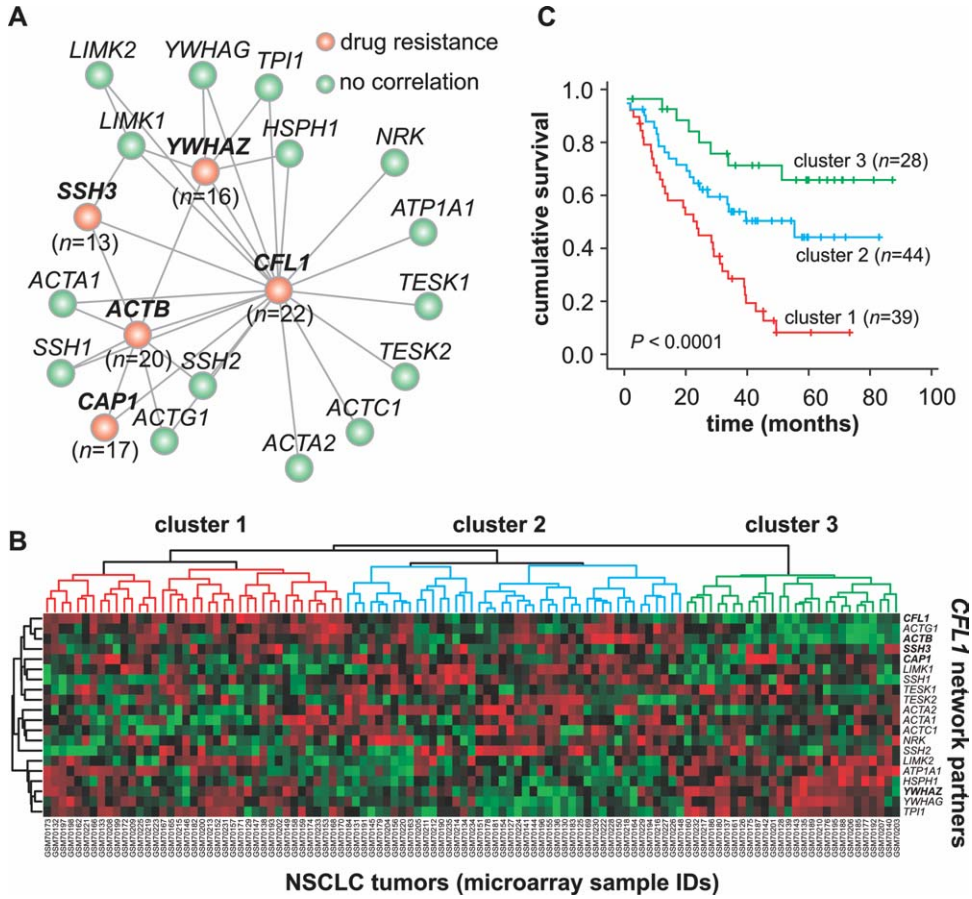



**Figure 5.** Prognostic and drug resistance markers of the *CFL1* functional gene network are shown. (A) A graphic model represents the *CFL1* functional gene network versus alkylating drug sensitivity/resistance profiles. Nodes represent gene products; connecting lines indicate physical and/or functional associations according to experimental data (http://string.embl.de/). Gene expression data (http://discover.nci.nih.gov/cellminer/home.do) were crossed against 50% growth inhibition dose values of all alkylating agents identified in the resistance panel (Fig. 4A). Four *CFL1* network partners follow the same resistance profile (red nodes; n = number of drugs for which gene expression showed correlation). The network drawn was built using a spring model algorithm. Further detains are given in Materials Methods. (B) Two-way hierarchical clustering analysis of nonsmall cell lung cancer (NSCLC) tumors is shown. This panel presents the NSCLC cohort data (referred to as Testing cohort in Table 1) arranged according to the gene expression profile of all *CFL1* network components. Complete linkage clustering of tumor samples is shown in TreeView format. The color intensity is relative to the log2 ratio of the microarray signal (red, positive values; green, negative values). For visualization purposes, the gene expression values were median centered and normalized using Cluster 3.0 software. (C) A Kaplan-Meier plot of the entire NSCLC cohort data (n = 111) is shown, where patients are stratified according to the hierarchical clustering analysis of the *CFL1* functional gene network.

modulate actin cytoskeleton, generating cell mobility.[9,10] Therefore, as cofilin is associated with epithelial-mesenchymal transition and tumor invasion, it stands to reason that NSCLC patients with high tumor *CFL1* expression levels present low overall survival rates, even in early stage disease. Our data obtained by in vitro experiments suggest that cofilin levels also could be used to predict tumor resistance to alkylating agents. The correlation between high levels of cofilin and alkylating drug resistance probably is the most important finding of this study, because this class of drugs is among the most effective cytotoxic agents for advanced cancer treatments and has long been the cornerstone of NSCLC management.[26,27] Although this treatment improves patient survival, the benefit is stage-dependent. Unfortunately, intrinsic or acquired resistance to alkylating agents is frequently encountered and severely limits its therapeutic potential.[28] Our findings may have great impact on survival rates, as currently there is no way to predict and identify potential responders.

Although we focused our analysis on the role of *CFL1* gene in alkylating drugs resistance, we also expanded the potential biological relevance of our findings by testing the role of other cofilin partners on tumor resistance. Doing so, we obtained a signature based on 5

biological related genes (members of the cofilin pathway). These genes can be used in combination to characterize the tumor resistance phenotype. This approach is consistent with other studies that have proposed the use of gene combination to enhance biomarker robustness, which may potentially deal better with intrinsic intra- and inter-sample heterogeneity.[29-31] For instance, Chen et al[32] have described a biomarker cluster comprising the combination of *DUSP6*, *MMD*, *STAT1*, *ERBB3*, and *LCK* gene expression to predict the clinical outcome of NSCLC patients. This signature was obtained based on the statistical (not biological) combination of high-throughput screening of cDNA microarray probes. Likewise, other authors have used the same strategy to identify low/high NSCLC risk phenotypes.[14,15]

In this sense, our 5-gene signature emerges from a functional gene network comprising all described cofilin partners. To further explore this finding using the NSCLC cohort data, we assigned subsets of tumors (clusters) based on related expression patterns, represented in the tree structure, or dendrograms. By using all *CFL1* gene network components, the hierarchical clustering analysis put together the similar network datasets, stratifying NSCLC patients in 3 large subgroups, whose outcomes differ to the same extent as observed for *CFL1* gene alone. The effect of this strategy of assessing tumors is the distribution of biomarker task among related genes, as opposed to focus on 1 or several nonrelated ones, which can potentially reduce the effect of random fluctuations on biomarker performance. Further investigation of the molecular properties of this network should be helpful to validate these genes as prognostic and predictive markers in NSCLC, or even in other cancer types, given that the *CFL1* gene is widely expressed[33] and more specifically in some subtypes (eg, colorectal adenocarcinomas[33,34]).

Our findings have clear implications for NSCLC management and therapy, as *CFL1* expression levels can be used to indicate which patients should receive a more aggressive therapy in an attempt to reverse the poor prognosis. Because *CFL1* expression levels also correlate with drug resistance, our findings can also be used to decide the best course of action for each patient, representing a contribution to translational medicine for treating NSCLC. In the adjuvant setting, for example, cisplatin-based chemotherapy constitutes the standard first-line treatment for patients with early stage and good performance status.[27] Because *CFL1* expression appears to be a marker of resistance to platinum agents, patients whose tumors harbor high levels of *CFL1* would benefit from a different treatment modality. In these cases, possible trials to test alternative adjuvant regimens would be based on the combination of other drugs commonly used in NSCLC (eg, gemcitabine, docetaxel, and vinorelbine) or EGFR-targeted monoclonal antibodies. The combination of EGFR inhibitors with first-line chemotherapy is currently under evaluation, and efforts have been made to identify subgroups of NSCLC patients who respond to these agents.[35-37] The refinement of patient stratification with the use of *CFL1* gene signature provides the opportunity to design a prospective, large-scale, randomized clinical trial that would evaluate these ideas.

## CONFLICT OF INTEREST DISCLOSURES

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**Capítulo 3**

Artigo Publicado na Revista "*Journal of Cancer Research and Clinical Oncology*"

**"*Validation of cofilin-1 as a biomarker in non-small cell lung cancer: application of quantitative method in a retrospective cohort*"**

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ORIGINAL PAPER

# Validation of cofilin-1 as a biomarker in non-small cell lung cancer: application of quantitative method in a retrospective cohort

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**Abstract**
*Purpose* Cofilin is a cytoskeletal protein whose overexpression has been associated with aggressiveness in several types of malignancies. Here, we established and optimized a simple semi-quantitative immunohistochemistry (SQ-IHC) method for cofilin quantification in tumor biopsies, and applied it in a retrospective cohort of NSCLC patients aiming at validating the use of cofilin-1 as a prognostic biomarker.
*Methods* The SQ-IHC method for cofilin-1 quantification was established and applied in a NSCLC cohort. An archival collection of biopsies from 50 patients with clinicopathological information and 5 years follow-up was accessed. Association between cofilin-1 immunocontent and clinical outcome was assessed using standard Kaplan–Meier mortality curves and the log-rank test. To evaluate the robustness of our findings, three different partitional clustering strategies were used to stratify patients into two groups according to the biomarker expression level (hierarchical clustering, Kmeans and median cutoff).
*Results* In all the three different partitional clustering we used, survival analysis showed that patient with high cofilin-1 immunocontent had a lower overall survival rate ($P < 0.05$), and could be used to discriminate between good and bad prognosis. No other correlation was found when the variables age, sex or histological type were tested in association with patients outcome or with cofilin immunocontent.
*Conclusions* Our method showed good sensitivity/specificity to indicate the outcome of patients according to their cofilin immunocontent in biological samples. Its application in a retrospective cohort and the results presented here are an important step toward the validation process of cofilin-1 as a prognostic biomarker.

**Keywords** Immunohistochemistry · Non-small cell lung cancer · Cofilin-1 · Prognosis · Biomarker

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## Introduction

Lung cancer is the most frequently diagnosed cancer and the most common cause of cancer mortality worldwide, being responsible for almost 1.3 million deaths a year (Jemal et al. 2010). Nearly 85% of lung cancer cases are represented by non-small cell lung cancer (NSCLC) (Molina et al. 2008). Although significant advances have

been achieved in conventional therapies, poor prognosis and short survival time of patients, as well as the limited value of any sort of conventional therapy, are factors all demanding novel and more effective therapy (Yuan et al. 2009).

Decision in NSCLC patient management is still based solely on the anatomic extent of the disease. Other factors, such as the molecular biological characterization of the tumor, are not included (Detterbeck et al. 2009). However, advances in molecular pathology underwent to the development of an impressive number of biomarkers that could provide information about cancer heterogeneity and could have important applications such as prediction and planning of the treatment (Cho 2007). Despite the large number of studies involving biomarkers for NSCLC, poor individual performance precludes their inclusion in the clinical practice (Muller et al. 2011). The identification of biomarkers that could add value to TNM system is an important step in individualized therapy and, ultimately, an improving in patient survival (Pérez–Soler 2009).

Cofilin-1 (*CFL1* gene product; non-muscle isoform; Gene ID: 1072) is one of the major proteins responsible for cell migration processes, playing a key role in actin filaments dynamics (Wang et al. 2007), and apoptosis induced by oxidants (Klamt et al. 2009; Zdanov et al. 2010). Bernstein and Bamburg (2010) suggest that cofilin-1 plays a major role in cell biology, and that any interference with its normal activity is highly likely to have severe repercussions. Under EGF (Endothelial Growth Factor) stimulation, cancer cells use cofilin-1 to locally restructure the actin cytoskeleton network, leading cell migration and invasion (van Rheenen et al. 2007). Cofilin-1 is overexpressed in the highly invasive C6 rat glioblastoma cell line, A549 human lung cancer cells and human pancreatic cancer cells (Sinha et al. 1999; Gunnersen et al. 2000; Keshamouni et al. 2006). The spontaneous overexpression of cofilin-1 can also be detected in invasive sub-populations of breast tumor cells in rats, as well as in biopsies of oral, renal and ovarian carcinoma (Wang et al. 2007).

Based on this biological information, we recently described, using two independent clinical cohorts, that *CFL1* levels in NSCLC biopsies can discriminate good and bad prognosis, where high *CFL1* levels are correlated with lower overall survival rate and resistance to several alkylating drugs (Castro et al. 2010). Even though promising, these findings were obtained based on high throughput microarray meta-data analysis of tumor biopsies, which limits a more broad application in health services.

Immunohistochemistry, in the other hand, is a technique widely used and well established in hospital services. In surgical pathology, it is used as a diagnostic, prognostic and predictive tool. More than identifying the presence or absence of a biomarker, immunohistochemistry can be used to quantify its expression (Honig et al. 2005; De Matos et al. 2006).

Aiming at validating the use of cofilin-1 as a prognostic biomarker in NSCLC, here we establish and optimize the experimental conditions for a semi-quantitative immunohistochemistry (SQ-IHC) analysis of cofilin-1 in NSCLC biopsies and evaluated the correlation of cofilin-1 levels—as optical densities (OD)—with patient outcome.

## Materials and methods

### Patient cohort and clinicopathological review

Formalin-fixed paraffin-embedded NSCLC tumors from patients diagnosed between 2003 and 2005 were obtained from the Pathology Service at the Hospital de Clinicas de Porto Alegre (HCPA), Brazil. The pathological diagnoses were reviewed and classified by two independent pathologists at collaborating institute, according to World Health Organization criteria. Information such as sex, age, histological type, NSCLC staging and patient outcome were collected. Inclusion criteria were non-small cell lung primary tumor and clinical follow-up of at least 5 years available. The research program, including studies on archival and stored materials, was approved by the Research Ethics Committee of the HCPA (#08-216).

### Immunohistochemistry (IHC)

The corresponding archived paraffin-embedded specimens were sectioned into 4 μm slices, de-paraffinized and antigen retrieval was performed in a water bath for 30 min with sodium citrate buffer (pH 6.0). Endogenous peroxidases were blocked with 5% hydrogen peroxide in methanol. To avoid nonspecific background staining, slides were incubated for 1 h with 1% bovine serum albumin (BSA) (Sigma®) in PBS. Rabbit polyclonal anti-cofilin-1 primary antibody (Abcam®; catalog number AB42475) (diluted 1:200 in 1% BSA) was incubated overnight at 4°C. After incubation, HRP-labeled polymer conjugated (Invitrogen®) was added and incubated for 45 min, rinsed, exposed to a solution of diaminobenzidine (0.06%) for 5 min and then rinsed in running water. Next, they were dehydrated with alcohol, cleared in xylene and mounted. Negative controls were obtained performing the same protocol above described, with the omission of primary antibody, representing in optical density (OD) measurements the background staining value. The brownish-color was considered to be a positive expression of cofilin-1 in cells.

### SQ-IHC

The intensity of cofilin-1 IHC reaction was quantitatively measured using a Zeiss® Imager AI (200×) microscope coupled to Image Pro Plus® Software 6.1. For each case, 5 images were captured on the same day by a single observer. Images were converted to gray scale 8, and the OD generated by the immunoreaction was measured in equidistant areas of interest (AOI) (Xavier et al. 2005). Immunoreactivity was based in the Beer–Lambert Law and the OD was calculated using the following formula: $A(x,y) = -\log$ [(intensity($x$,$y$)-black)/(incident-black)], where: A is absorbance, intensity ($x$,$y$) is the intensity at pixel ($x$,$y$), black is the intensity generated when no light goes through the material, and incident is the intensity of the incident light. Additionally, to reduce the time required to perform OD measurements, some macros were created using Auto Pro Language.

### Survival data analysis

Standard Kaplan–Meier mortality curves and their significance levels were generated for clusters of patients using SPSS software (SPSS for Windows, release 14.0.0, SPSS Inc., Chicago, Ill). The survival curves are compared using the log-rank test, and patients are clustered according to either biomarker expression level or NSCLC stage grouping. Clustering analysis was carried out in R using *stats* Package (R Development Core Team 2009).

## Results

### Cohort characteristics

The patients' clinicopathological features are summarized in Table 1. The major part of the NSCLC cases analyzed, 31 (62%), are male. Adenocarcinoma was the histological subtype with highest incidence, accounting for 30 (60%) cases, followed by 15 (30%) cases diagnosed as squamous cells, and 3 cases (6%) as large cells (see Table 1). Patient distribution according to tumor staging showed that 20 (40%) cases were diagnosed at an advanced stage of disease (i.e., stage IV). Through Kaplan–Meier mortality curves, the relation between cumulative survival in 5 years and tumor staging (IA- IIB, IIIA-IIIB and IV) of each patient, showed that the more advanced the disease, the lower is the patient survival, suggesting that our cohort patient follows the natural course of the disease. No other correlation was found when the variables age, sex or histological types were tested in association with patients' outcome or with cofilin immunocontent.

### Cofilin-1 expression and patients overall survival

Applying our SQ-IHC protocol in our NSCLC samples, we obtained different numerical values of optical density (OD), which corresponds to cofilin-1 immunocontent. Figure 1 presents representative images of IHC reactions with low, moderate and high cofilin-1 amount that reflects

**Table 1** Epidemiological and clinical features of the NSCLC Cohort according to cofilin immunocontent

| Characteristics | Cofilin immunocontent | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Median cutoff | | | Kmean | | | Hierarchical | | |
| | Lower 50 | Upper 50 | *P* | Low | High | *P* | Low | High | *P* |
| Cohort, $n = 50$ | 25 (50%) | 25 (50%) | | 30 (60%) | 20 (40%) | | 35 (70%) | 15 (30%) | |
| Age, years | 61.4 ± 11 | 63.6 ± 9 | | 61.8 ± 11 | 65.0 ± 10 | | 62.4 ± 10 | 63.3 ± 10 | |
| Sex | | | 0.29 | | | 0.20 | | | 0.10 |
| Men | 14 (56%) | 17 (68%) | | 17 (57%) | 14 (70%) | | 20 (57%) | 11 (73%) | |
| Women | 11 (44%) | 8 (32%) | | 13 (43%) | 6 (30%) | | 15 (43%) | 4 (27%) | |
| Histological type | | | 0.16 | | | 0.10 | | | 0.13 |
| Adenocarcinoma | 11 (44%) | 19 (76%) | | 15 (50%) | 15 (75%) | | 20 (57%) | 10 (67%) | |
| Squamous cell | 12 (48%) | 3 (12%) | | 13 (44%) | 2 (10%) | | 13 (37%) | 2 (13%) | |
| Large cell | 1 (4%) | 2 (8%) | | 1 (3%) | 2 (10%) | | 1 (3%) | 2 (13%) | |
| Undetermined | 1 (4%) | 1 (4%) | | 1 (3%) | 1 (5%) | | 1 (3%) | 1 (7%) | |
| Tumor stage (TNM) | | | 0.57 | | | 0.64 | | | 0.47 |
| IA/IB–IIA/IIB | 11 (44%) | 5 (20%) | | 13 (44%) | 3 (15%) | | 14 (40%) | 2 (13%) | |
| IIIA/IIIB | 8 (32%) | 3 (12%) | | 10 (33%) | 1 (5%) | | 10 (28%) | 1 (7%) | |
| IV | 4 (16%) | 16 (64%) | | 5 (16%) | 15 (75%) | | 8 (23%) | 12 (80%) | |
| Undetermined | 2 (8%) | 1 (4%) | | 2 (7%) | 1 (5%) | | 3 (9%) | 0 (0%) | |

an increase in OD values. Sample OD values were distributed within a small range of variation, between 0.0578, the lowest value attributed to the healthy tissue derived from resection margins, and 0.302, the highest value derived from intense migratory cells as macrophages (Fig. 1). For sample identification, hematoxylin/eosin staining is also shown. Following this, we performed three different unsupervised clustering strategies to stratify patients into two groups according to the cofilin-1 levels (Fig. 2). In these three ways of partitioning, the Kaplan–Meier mortality curves indicated that those who have higher cofilin-1 levels present lower cumulative survival rate in 5 years ($P < 0.05$) (Fig. 3). Data on patient information such as age, sex, cancer histological type and NSCLC staging according to cofilin-1 levels were considered for each type of case grouping (see Table 1).

## Discussion

Biomarkers use could be a great advance in cancer treatment due to its potential role in early diagnosis, therapy guidance and prognosis monitoring of cancers. However, the currently available lung cancer biomarkers are not sensitive or specific enough to be used clinically in the diagnosis, patient stratification, prognosis or drug responses (Sung and Cho 2008).

When it comes to NSCLC, an impressive number of markers are related in the prognosis of this disease; however, the results reported in literature are conflicting and none are in use in clinics (Chi–Shing 2007). Thus, further investigation, newer assays and the development of an appropriate panel of molecular markers are still required (Niklinski et al. 2001). Sawyers (2008) emphasizes that this is an expensive and lengthy process. Besides the biomarker

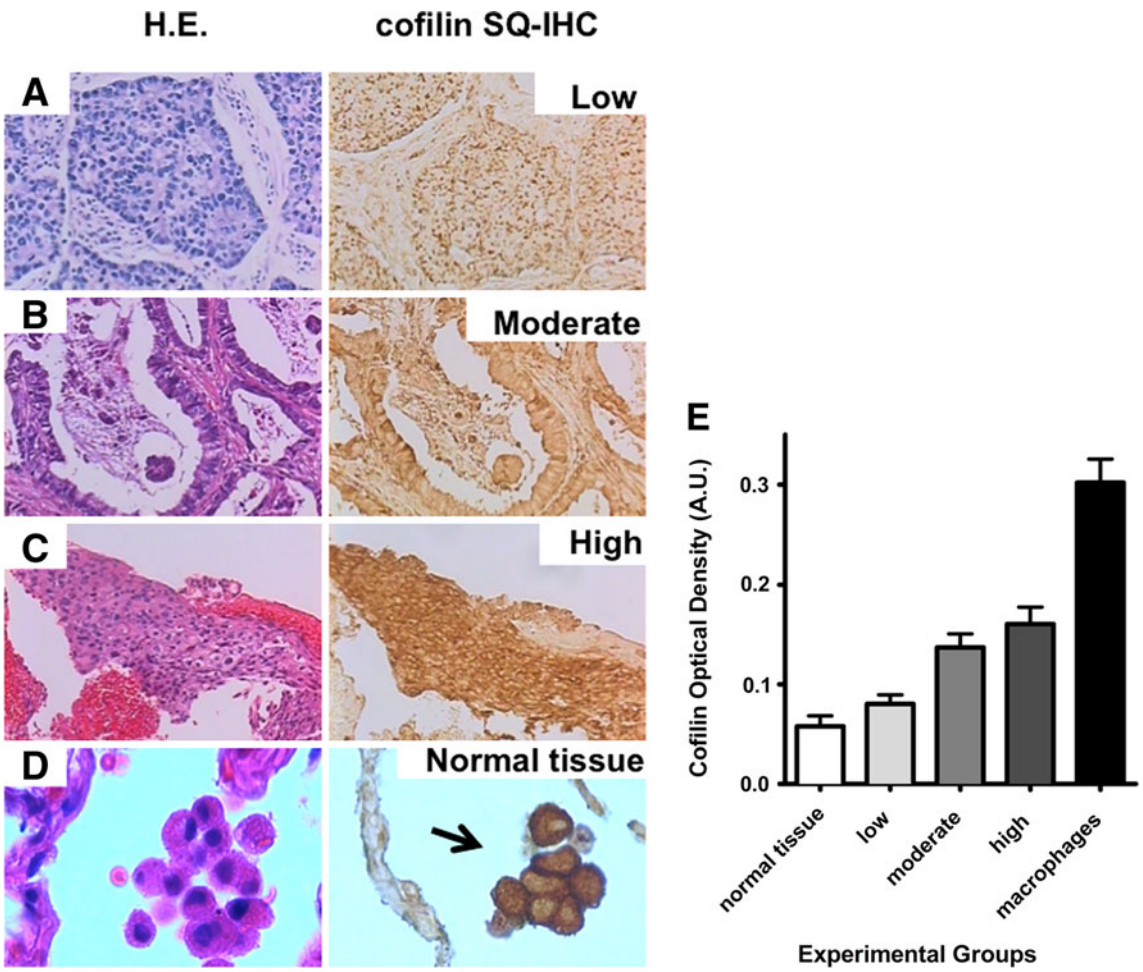


**Fig. 1** Representative HE and cofilin-1 IHC staining of NSCLC cases are presented. IHC images correspond to **a** *low*, **b** moderate and **c** *high* cofilin-1 immunocontent (200× magnification). Healthy tissue **d** obtained from free resection margins of biopsies represents basal levels of cofilin-1, and macrophages were considered positive staining (*arrow*) (1,000× magnification). **e** O.D. values obtained by SQ-IHC for each case

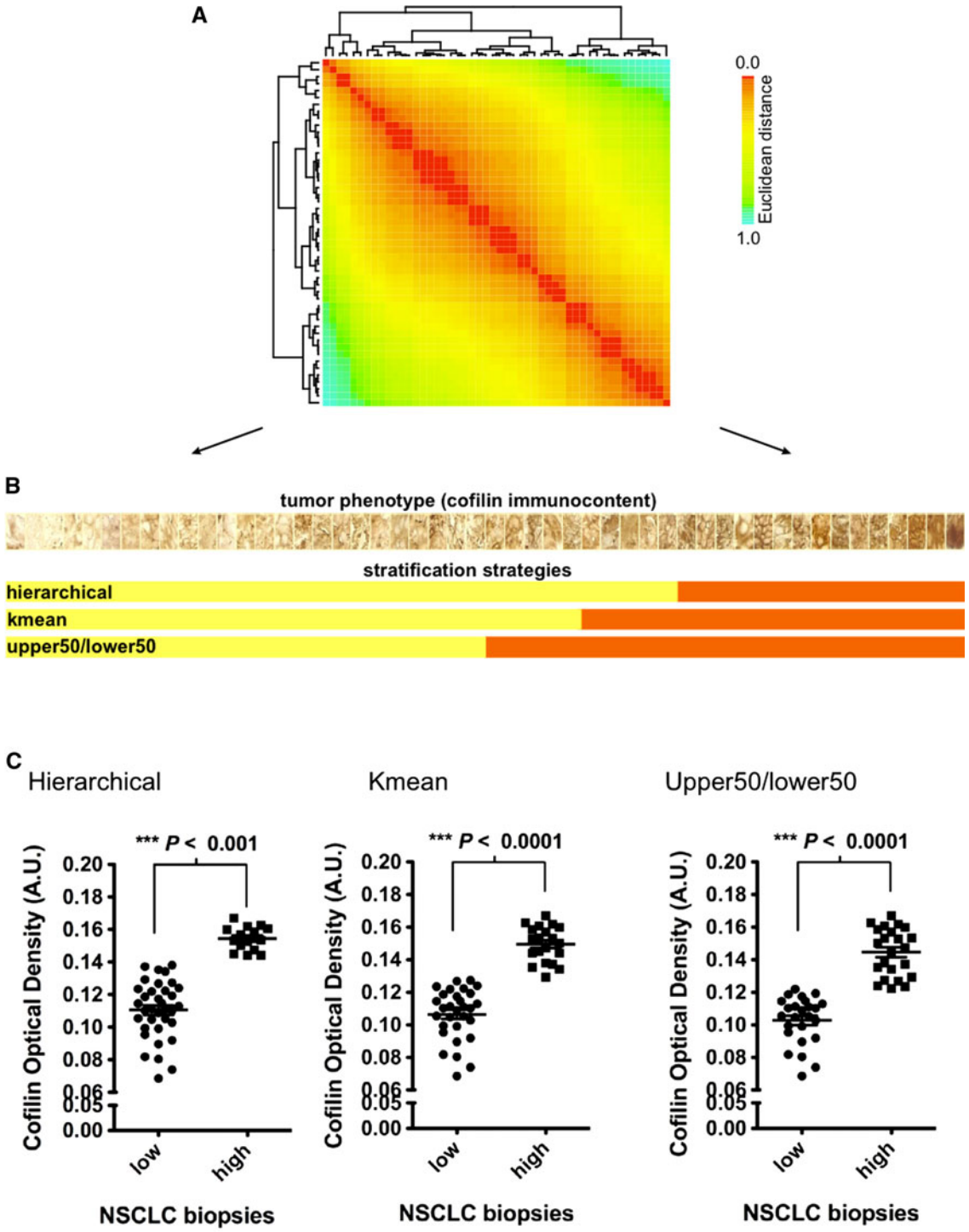


**Fig. 2** Unsupervised clustering. **a** Hierarchical cluster analysis. The heat map shows the dissimilarities between clusters computed by the Euclidean distance (agglomeration method: "average"). The cluster tree is cut in $k = 2$ groups for subsequent analysis. **b** Tumor phenotype aliened with three different partitions. *Hierarchical*: as specified in **a**; *kmeans*: clustering performed by Hartigan–Wong algorithm ($k = 2$); *upper50/lower50*: median cutoff partitioning. **c** original data grouped according to the cluster assignment. The plots present the same dataset where points represent the cofilin-1 optical density for each specific tumor

identification, it is necessary to develop a reliable assay to measure it in biological samples (validation) and that could also be able to perform clinical distinction (qualification).

In prior exploratory studies, we have identified *CFL1* gene as a potential prognostic and predictive biomarker in NSCLC. *CFL1* mRNA levels are highly sensitive and

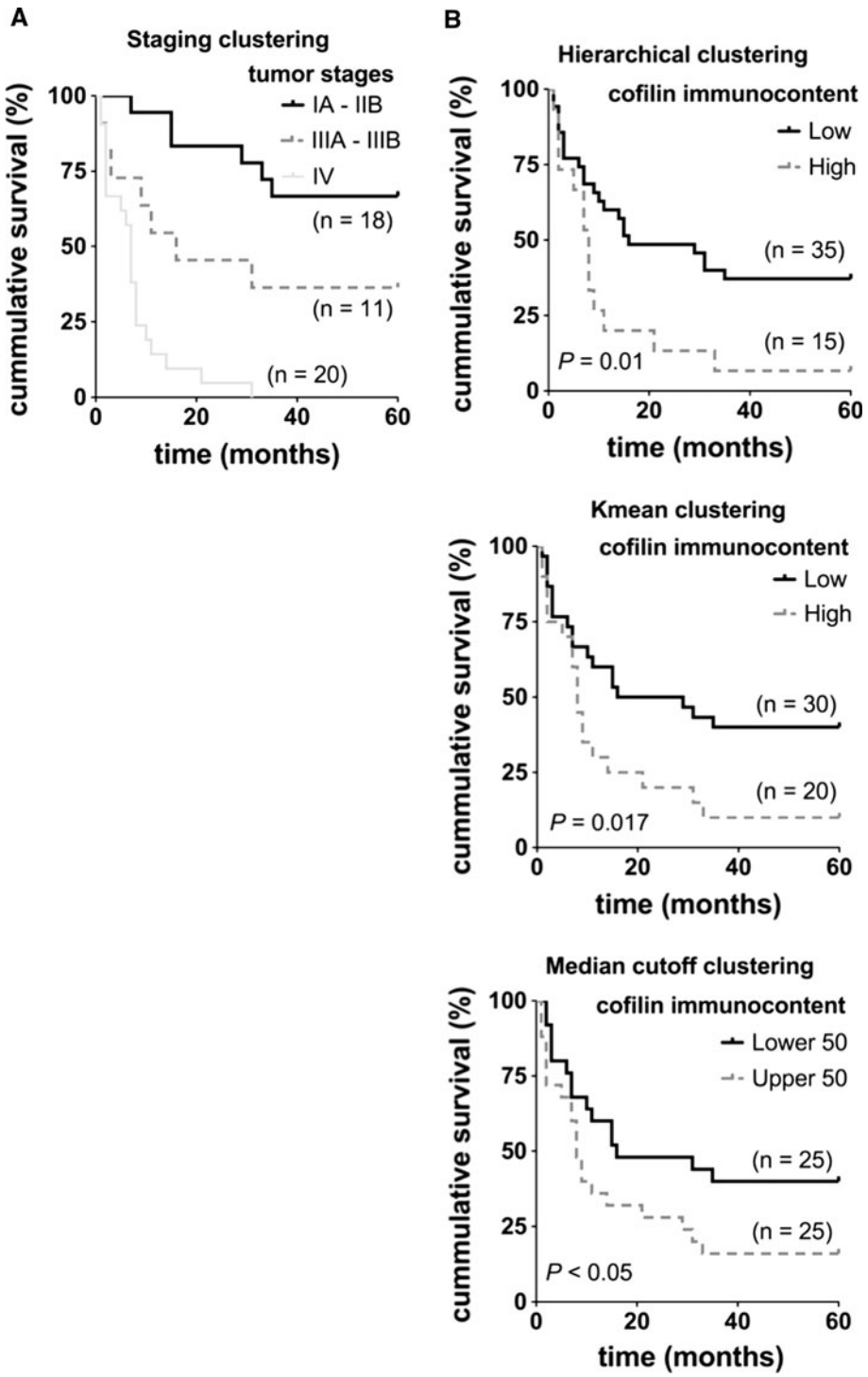


**Fig. 3** Kaplan–Meier mortality curves were used to evaluate the cofilin-1 performance to discriminate between good and bad prognosis of NSCLC patients. These curves show the overall survival of patients in 5 years. The cumulative survival was also measured according the TNM staging system (**a**). Patients were clustered according cofilin-1 content by three distinct approaches: Median cutoff, Kmeans and hierarchical (**b**)

specific in discriminating between good and bad prognosis. Also, using NSCLC cell lines, we found that cofilin-1 immunocontent is associated with tumor invasion and resistance to alkylating drug treatment (Castro et al. 2010). However, to be clinically acceptable, the biomarker should present not only sensitivity and specificity. Biomarker evaluation in tumor samples needs to be accurate, economical, easy to perform and reproducible by different technicians across different laboratories (Pepe et al. 2001; Issaq et al. 2011). Following the validation proposal, in this report we established a simple SQ-IHC method to detect and quantify cofilin-1 immunocontent in tumor biopsies. Further, we evaluated the potential prognostic role of cofilin-1 in NSCLC based on a standard procedure widely used for diagnosis and prognosis of cancer and other diseases in clinical practice.

The majority of the IHC analysis uses scoring systems to discriminate the immunostaining. This method, though widely used by pathologists, has many biases, for instance the differences of visual acuity and interpretation between different observers (Taylor and Levenson 2006). In search of more objective quantification methods, there is a gradual introduction of automated systems for IHC analysis (Cregger et al. 2006; Walker 2006). The protocol proposed here has good reproducibility and specificity and can avoid abovementioned biases.

Using this method, we measured the cofilin-1 immunocontent of each case. We also measure it in healthy lung tissue (to assume the basal level of cofilin-1) and in macrophages found in this tissue (highly mobile cells which are expected to have high expression of cofilin-1). These range of values lead us to infer that tumors have higher amounts of cofilin-1 than healthy tissue, and small variations of these OD values are associated with different outcomes for each patient. So, the correlation between cofilin-1 and survival rates indicates that levels of cofilin-1 can actually discriminate good and bad prognosis, confirming the same relation previously found by microarray data (Fig. 1). Even though we found some cases with nuclear and membranous staining, only the total amounts of cofilin-1 were actually able to discriminate prognosis. Also, two independent pathologists performed the tumor cell grading differentiation of our cohort. Using Kaplan–Meier mortality curves, we analyzed the possible correlation between this information and cofilin immunocontent with patient outcome. No statistical correlation was found (data not shown). Moreover, we can assume that, despite the simplicity and the innumerous intrinsic interferences of IHC, the SQ-IHC protocol established here is able to measure the differences in immunocontent of cofilin-1.

For comparative purposes, the performance of our SQ-IHC protocol was compared with a traditional scoring system of immunoreactions intensity (negative, low, moderate or strong) performed by two independent observers. Even though one observer obtained the same findings of our quantitative protocol (i.e., high cofilin-1 amount indicates bad prognosis), there was no inter-observer consistency in discriminating prognosis (data not shown). This reinforce that our protocol can actually avoid some of the major biases of traditional scoring system, as low reproducibility, different individual visual acuity and misinterpretation of data.

Another data that reinforce the relation between total cofilin-1 amounts and tumor aggressiveness, according the OD values, is the majority of high cofilin-1 cases observed are adenocarcinoma (Table 1). Adenocarcinomas grow more slowly than squamous cell carcinomas, but tend to metastasize widely and earlier (Mountzios et al. 2009), which important characteristics in cancer malignancy. The histologic pattern of lung cancer incidence has been changed in the past few decades in a number of countries; the frequency of adenocarcinoma has risen and that of squamous cell carcinoma has declined (Wingo et al. 1999), as observed in our cohort where 60% of the cases are adenocarcinoma, followed by squamous cell and large cell type. Far more men than women still die from lung cancer each year, as show our data, but the gender gap in lung cancer mortality is steadily narrowing and will eventually disappear (Alberg et al. 2007). Further, in our study population a high prevalence of patients was diagnosed in advanced stages, which can be explained by the late onset of lung cancer symptoms.

In summary, the optimization protocol for SQ-IHC of cofilin-1 in NSCLC biopsies described here enables its implementation to perform correlation studies (i.e.: cofilin-1 amount *vs* patient outcome; histological type and/or tumor differentiation status). Moreover, its application in a retrospective cohort and the results presented here are an important step toward the validation process of cofilin-1 as a prognostic biomarker. The refinement of patient stratification with the use of SQ-IHC of cofilin-1 provides the opportunity to design a prospective, large-scale, randomized clinical trial that would evaluate these ideas.

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**Conflict of interest** We declare that we have no conflict of interest.

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**PARTE 3**

## 4. DISCUSSÃO

O câncer de pulmão é uma doença heterogênea tanto por suas características biológicas quanto pela conduta clínica a ser adotada para cada indivíduo (Lehtiö & De Petris, 2010). É importante observar que suas altas taxas de mortalidade devem-se não só ao fato de a maioria dos casos serem diagnosticados em estágios avançados da doença, mas também à carência de terapias efetivas, sendo em boa parte ineficazes mesmo para pacientes de estágios iniciais, cuja sobrevida é surpreendentemente baixa (Granville & Dennis, 2005).

Ao contrário da uniformidade estabelecida pelo sistema TNM, utilizado para indicar prognóstico e delinear a terapia, os biomarcadores fornecem informações sobre a biologia do tumor, adequando-se melhor à heterogeneidade do câncer (Sung & Cho, 2008). Na literatura foram identificados alguns potenciais biomarcadores para câncer de pulmão; entretanto, poucos provaram-se úteis na clínica, principalmente devido à baixa sensibilidade, especificidade e reprodutibilidade que apresentaram. Há, portanto, uma necessidade de identificação de melhores biomarcadores que possam predizer a evolução da doença para cada paciente e abrir caminho para o desenvolvimento de novas estratégias terapêuticas (Tarro, Perna & Esposito, 2009).

Calcados na intenção de contribuir com o provimento de informações que pudessem estabelecer um ou mais marcadores capazes de auxiliar no diagnóstico, prognóstico e/ou predição da doença, nosso grupo iniciou um estudo com finalidade de prospectar potenciais biomarcadores para CPNPC através de meta-análise de banco de dados, conforme disposto no **capítulo 1** da seção anterior.

Nesse estudo, uma inspeção sistemática da literatura nos levou a 60 genes com potencial biomarcador. Entre eles, genes codificadores de proteínas cinases, fosfatases, fatores de transcrição e uma série de receptores, tendo relações diversas com o câncer de pulmão. Utilizando dados de microarranjo e informações clinicopatológicas, nós testamos a correlação direta entre os níveis de mRNA e a sobrevida total dos pacientes com CPNPC. As curvas de mortalidade Kaplan-Meier geradas a partir desses dados nos permitiram avaliar o valor prognóstico dos biomarcadores.

Em nossa análise, não houve correlação entre os níveis de mRNA e desfecho dos pacientes, indicando que nenhum dos genes que prospectamos da literatura foi capaz de, sozinho, discriminar bom ou mal prognóstico. Embora impactante, é importante ressaltar que nossa análise foi baseada unicamente na expressão gênica e na sobrevida dos pacientes. Não levamos em consideração padrões de mutação, metilação ou subgrupos da coorte, como sexo, idade, estadiamento ou grau de diferenciação. Dessa forma, sugerimos que os candidatos a biomarcadores sejam cuidadosamente avaliados, levando em consideração variáveis do grupo e do próprio marcador que possam ter influência na sobrevida dos indivíduos. Sugerimos também a utilização da ferramenta que descrevemos nessas avaliações a fim de auxiliar o delineamento dos estudos de potenciais biomarcadores tumorais.

Além disso, como foi dito, nós avaliamos o valor prognóstico dos genes individualmente. Muitos estudos têm proposto correlações com assinaturas gênicas baseadas em combinações estatísticas, como o de Bild e colaboradores (2006) que descreveu a ativação várias vias oncogênicas baseado nessas assinaturas. Nós

acreditamos na validade dessas avaliações, não é necessário que se saiba o exato mescanismo de ação do biomarcador proposto, todavia sugerimos que essas assinaturas sejam baseadas em combinações biológicas (interações entre os genes em vias relevantes para a biologia de tumores), e não somente estatísticas, e que as flutuações das expressões gênicas sejam avaliadas através das redes formadas através dessas interações.

Um outro aspecto importante que permeia a realização desse trabalho e que cabe ser valorizado, é a optimização das informações. A montagem de uma coorte é um processo muito dispendioso. A seleção de pacientes, a coleta de informações adequadas e de material biológico, são processos demorados, que requerem pessoal especializado e que, muitas vezes por razões éticas, podem inviabilizar a realização do estudo. Portanto, a utilização de bancos de dados públicos, que disponibilizam informações a respeito de coortes montadas por grandes grupos de pesquisa, têm vital valor no delineamento de futuros ensaios. É uma forma de maximizar a informação já gerada e aumentar sua aplicabilidade (Müller *et al.,* 2011a).

Em suma, nesse trabalho nós descrevemos uma ferramenta capaz de testar individualmente a capacidade prognóstica de biomarcadores e, além disso, visualizamos um cenário que confirma a carência de biomarcadores prognósticos para CPNPC na rotina clínica.

Tendo em vista a ineficiência em discriminar prognóstico dos candidatos testados, atentamo-nos a evidências reportadas na literatura a respeito de um marcador fortemente associado a agressividade de outros tipos tumorais. Sabe-se que em tumores malignos primários, as células cancerosas invadem os tecidos

circundantes e atingem os vasos sanguíneos e/ou linfáticos. Esse passo e os subseqüentes do processo metastático, que levam a formação de tumores secundários em locais distantes, requerem a migração ativa em um microambiente complexo. Como já descrito, a maquinaria de actina possui um papel central na regulação da migração celular, dessa forma, as proteínas ligantes de actina da família das ADF/cofilina desenvolvem função importante na migração e na invasão de células cancerosas (van Troys *et al.,* 2008). Wang, em 2007, confirma essa relação descrevendo que a super-expressão espontânea de cofilina-1 pode ser detectada em sub-populações invasivas de células tumorais mamárias de rato, assim como em biópsias de carcinoma oral, renal e ovariano.

Sidani, em 2007, demonstrou que a supressão de cofilina-1 resulta em inibição da polimerização de actina e, por conseguinte, à diminuição nas taxas de protrusão e aumento de actina-F e seus agregados dentro da célula. A cofilina-1 também aparece superexpressa na linhagem murina de glioblastoma C6, na linhagem humana de câncer de pulmão A549 e em células humanas de câncer pancreático (Sinha *et al.*, 1999; Gunnersen *et al.,* 2000; Keshamouni *et al.,* 2006).

Essas evidências nos levam ao **capítulo 2** da segunda parte dessa dissertação, em que propusemos o gene *CFL1* como candidato biomarcador para CPNPC.

Esse foi um estudo multidisciplinar, em que não só testamos a capacidade prognóstica do gene utilizando a ferramenta descrita no **capítulo 1** em duas coortes independentes, como também utilizamos ferramentas de bioinformática e realizamos testes *in vitro* para avaliar o valor preditivo do marcador. Além disso, construímos um modelo de rede de interação do gene *CFL1.*

A avaliação dos resultados nos mostrou que o nível de mRNA de *CFL1* é altamente sensível e específico para discriminar entre bom e mau prognóstico, principalmente nos estágios iniciais da doença, onde a alta expressão do gene está associada com menor sobrevida. Um maior imunoconteúdo de cofilina-1 em linhagens celulares está relacionado a um maior índice de invasão e também resistência a agentes alquilantes. Ambos os dados são importantes características de agressividade que tem direta relação com as quantidades de cofilina-1 (Castro *et al.,* 2010).

Devemos, entretanto, dedicar especial atenção aos dados que mostram a correlação negativa existente entre a expressão do gene *CFL1*, bem como do imunoconteúdo de cofilina em linhagens celulares, com os agentes alquilantes. O perfil de resistência foi encontrado não só em relação aos altos níveis de expressão de *CFL1,* mas também em relação a seus parceiros de interação gênica *(CAP1, ACTB, SSH3, YWHAZ)*, sugerindo uma rede funcional correlacionada com esse fenótipo tumoral, o que aumenta a robustez do biomarcador.

Os esquemas quimioterápicos baseados em agentes alquilantes são amplamente aceitos como tratamento de primeira linha para CPNPC ( Buzaid & Hoff, 2008). São compostos capazes de substituir um átomo de hidrogênio por um radical alquil, se ligando ao DNA de modo a impedir a separação dos filamentos na dupla hélice, o que é indispensável para replicação celular. As principais drogas dessa categoria incluem ciclofosfamida, cisplatina, carboplatina e ifosfamida (INCA, 2011).

O potencial preditivo da cofilina-1 em triar pacientes menos responsivos às combinações com alquilantes representa um grande impacto no que diz respeito à estratégia terapêutica e deve ser melhor explorado em estudos futuros (vide anexo 3).

Os potenciais preditivo e prognóstico do gene *CFL1* descritos nesse trabalho foram baseados, principalmente, em dados de micro-arranjo. Embora tenhamos realizado alguns testes aplicando a técnica de IHQ para cofilina-1 em espécimes tumorais emblocados em parafina, ainda seria necessário desenvolver um métoddo que nos permitisse quantificar a cofilina-1 em amostras biológicas.

Para compreendermos os passos que foram dados a partir desse ponto em direção à validação, devemos ter em mente as fases de desenvolvimento de um biomarcador. Os **capítulos 1 e 2** discutidos até aqui atenderam aos propósitos da fase 1, em que verificamos a carência de biomarcadores para CPNPC e identificamos o potencial prognóstico e preditivo da expressão do gene *CFL1* para essa doença.

Contudo, além da identificação do biomarcador, é necessário que se desenvolva um método confiável para mensurá-lo em amostras biológicas (validação) e que ele seja capaz de realizar distinção clínica (qualificação) (Sawyers, 2008). Mais além, não basta que esse biomarcador seja sensível e específico. Para que seja clinicamente aceitável, sua detecção e/ou quantificação precisa ser acurada, de baixo custo, fácil de ser realizada e reprodutível em diferentes laboratórios. Isso torna o estabelecimento de um biomarcador na prática clínica um processo lento e caro (Pepe *et al.,* 2001).

A técnica de imuno-histoquímica possui ampla aplicação na patologia, fisiologia e biologia celular, sendo uma valiosa ferramenta para identificação de proteínas, visualização de componentes celulares e para o estudo de vias de sinalização (Muñoz *et al.,* 2004). É uma técnica amplamente utilizada e bem estabelecida em serviços hospitalares (quando comparada às técnicas de biologia molecular, como PCR em

tempo real), que não apenas assegura a detecção de antígenos, mas também possibilita a determinação de sua localização morfológica (Honig *et al.*, 2005).

Na patologia cirúrgica, a IHQ é usada tanto como uma ferramenta diagnóstica para diferenciar tecidos malignos de benignos, quanto uma ferramenta prognóstica para estudar a presença de moléculas envolvidas na agressividade do tumor. A imunoistoquímica, mais do que identificar a presença ou ausência de um biomarcador, pode ser usada para quantificar sua expressão (De Matos *et al.,* 2006).

Apesar da ampla aplicabilidade da técnica de imuno-histoquímica, maior que muitos métodos bioquímicos e moleculares de análise, um grande número de estudos dedica esforços no desenvolvimento de métodos que maximizem a aplicação da técnica, permitindo formas de quantificação da imunomarcação. Essa busca deve-se a sérias limitações do olho humano em obter informações quantitativas das imagens e à baixa reprodutibilidade do processo (Bernardo *et al.,* 2009). Por essas razões, o que se observa é a progressiva introdução de métodos de quantificação mais objetivos, através do uso de sistemas automatizados para a análise imuno-histoquímica (Cregger *et al.*, 2006).

Baseados nessas credenciais, no **capítulo 3** nós otimizamos um protocolo de Semi-quantificação de Imuno-histoquímica (SQ-IHQ) para cofilina-1 em amostras de CPNPC. Trata-se de um método de análise computadorizada que faz uso de uma técnica amplamente estabelecida em serviços de saúde, a imuno-histoquímica (IHQ). O software utilizado atribui valores de densidade óptica (DO) ás diferentes intensidades da imunorreação. Além do baixo custo, o protocolo estabelecido apresenta boa reprodutibilidade e especificidade, além de evitar grande parte do viéses presentes no

tradicional sistema de escore utilizado para semiquantificar reações de IHQ, como diferenças de acuidade visual e interpretação inter-observadores.

Apesar da simplicidade da técnica e das suas inúmeras interferências intrínsecas, a aplicação deste método em uma coorte restrospectiva de 50 casos de CPNPC evidenciou diferenças no imunoconteúdo de cofilina-1. A análise, realizada através de curvas de mortalidade Kaplan-Meier, corroborou os resultados previamente encontrados através de dados de micro-arranjo. Correlacionando informações de sobrevida dos pacientes com os valores de DO, concluímos que pacientes com maior imunoconteúdo de cofilina-1 apresentavam menor sobrevida, o que confirma o valor da cofilina-1 como biomarcador prognóstico (Müller *et al.,* 2011b).

O **capítulo 3** abrange, dessa forma, as fases 2 e 3 do desenvolvimento do biomarcador, visto que desenvolvemos um ensaio capaz de quantificar o marcador em amostras biológicas e o aplicamos em um estudo retrospectivo, cujos resultados confirmaram o potencial indicado na fase 1.

Os resultados dispostos nessa dissertação foram publicados em revistas científicas internacionais (capítulos 1, 2 e 3) e receberam reconhecimento de mídias digitais (anexos 1 e 2) e impressa (anexo 3). A idealização de estudos futuros e desenho de um kit para quantificação de biomarcador prognóstico e preditivo para CPNPC, baseada nesses dados, foi reconhecida pelo Prêmio Santander Universidades, na categoria Ciência e Inovação – Saúde (anexo 4). A propriedade intelectual dos mesmos foi garantida à Universidade através do depósito de patente no INPI (PI0802917-2 A2) denominado “MÉTODO E KIT DE DIAGNÓSTICO E/OU PROGNÓSTICO DE CÂNCER DE PULMÃO (anexo 5).

## 5. CONCLUSÃO

O corpo dos resultados dessa dissertação, resumidamente, cumpre os seguintes passos para o estabelecimento de um novo biomarcador em câncer de pulmão:

- 1) por meio de meta-análise, análise de dados de micro-arranjo e testes *in vitro*, identificamos a cofilina-1 como biomarcador prognóstico e preditivo;
- 2) Desenvolvemos um método de baixo custo, fácil execução e de ampla aplicação e reprodutibilidade que é capaz de quantificar a proteína em amostras biológicas, com potencial para ser implementado na rotina clínica;
- 3) Aplicamos esse método em uma coorte restrospectiva de CPNPC e confirmamos o papel prognóstico da cofilina-1.

Estes achados representam grande passo no processo de validação da cofilina-1 como biomarcador prognóstico. Evidentemente, temos ciência de que é preciso ainda explorar o potencial preditivo, indicado no capítulo 2. A translação de informações provenientes de pesquisa básica irá gerar a possibilidade de futuros ensaios clínicos prospectivos que darão seguimento às fases 4 e 5.

Por fim, temos ciência dos esforços realizados e dos muitos que ainda deverão ser aplicados nesse longo caminho. Contudo, sentimo-nos satisfeitos em poder contribuir com informações que possam indicar caminhos alternativos no prognóstico e predição do tratamento do CPNPC.

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# ANEXOS

ANEXO 1- Artigo publicado no site “Ciência Diária”

http://cienciadiaria.com.br/2010/06/04/brasileiros-identificam-biomarcador-para-predicao-e-prognostico-de-cancer-de-pulmao/

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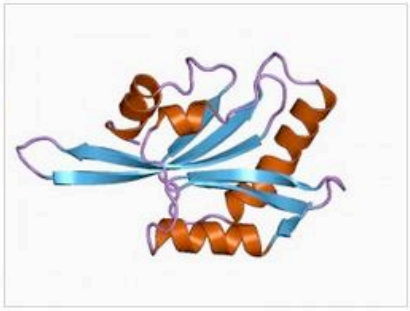
# Brasileiros identificam biomarcador para prognóstico de câncer de pulmão

**Submitted by Ciência Diária on Friday, 4 June 2010** — **Sem comentários**

A era da medicina "tamanho único" vai finalmente virar história; afinal, não é novidade que drogas e tratamentos não funcionam da mesma forma para todos. Para câncer de pulmão de células não pequenas (CPCNP) – um câncer que mata aproximadamente 1 milhão de pessoas por ano – o tratamento hoje disponível é basicamente o mesmo para todos os pacientes, apesar de alguns responderem ao tratamento e outros não. Novos marcadores biológicos e ferramentas de prognóstico são urgentemente necessários para ajudar os médicos a decidirem a melhor linha de ação para cada paciente com CPCNP. Parece que, agora, isso não está longe da realidade, pelo menos para o tratamento desta doença.



Estrutura de um representante de ADF/cofilina, proteína que pode ser usada como biomarcador no prognóstico de câncer de pulmão de células pequenas. Crédito: Wikipedia.

Atualmente, o prognóstico de CPCNP é feito com base no desempenho geral do paciente e na fase do tumor. Mesmo assim, os médicos não sabem quais pacientes sobreviverão por apenas dez meses ou quais conseguirão se manter por cinco anos. Agora, um estudo publicado na revista Cancer ("*CFL1 expression levels as a prognostic and drug resistance marker in nonsmall cell lung cancer*", DOI 10.1002/cncr.25125 – Níveis de expressão CFL1 como um marcador para prognóstico e de resistência à medicação em câncer de pulmão de células não-pequenas, DOI 10.1002/cncr.25125) apresenta fortes indícios de que uma proteína chamada cofilina (CFL1) pode ajudar os médicos a determinar o prognóstico do paciente e a identificar aqueles que necessitam de um tratamento mais agressivo. O estudo também indica que, com base nesta proteína, os médicos podem decidir quais medicamentos usar e quais evitar, ao tratar pacientes com CPCNP. A possibilidade dos médicos saberem antecipadamente quais drogas poderão atuar melhor em cada paciente, antes de prescrevê-las, representa um grande avanço no tratamento do câncer.

O grupo liderado pelo Dr. Fábio Klamt, pesquisador do Departamento de Bioquímica da Universidade Federal do Rio Grande do Sul, Brasil, descobriu que os níveis de cofilina podem ser usados para indicar quais pacientes, nos estágios iniciais da doença, tem um bom prognóstico e quais não têm. Enquanto estudavam um grande conjunto de amostras de células de CPCNP, os pesquisadores descobriram que as biópsias nas quais altos níveis de cofilina estavam presentes eram

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- Humanos preferem trabalhar em grupo; chimpanzés não

Anexo 2 – Artigo publicado no site “Science Dayle”

http://www.sciencedaily.com/releases/2010/06/100601205800.htm

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## Science News



# A Prognostic and Predictive Biomarker for Nonsmall Cell Lung Cancer

*ScienceDaily (June 1, 2010)* — The era of one-size-fits-all medicine will eventually be history; after all it is no news that drugs and treatments do not work the same for everyone. For patients with nonsmall cell lung cancer (NSCLC), a lung cancer that kills approximately 1 million people annually, the treatment currently available is basically the same for all patients, despite the fact that some patients may respond to treatment while others may not. New biological markers and prognostic tools are urgently needed to help doctors decide on the best course of action for each NSCLC patient. It now appears that this reality is not years away, at least for NSCLC treatment.

Currently, NSCL prognosis is made based on patient's general performance and tumor staging. Still, doctors do not know which patients with NSCLC may survive for only 10 months and which for 5 years. Now, a study published ahead of print in the journal *Cancer* presents strong evidence that a protein called cofilin (CFL1) may help doctors in determining patient prognosis and in identifying those in need of a more aggressive treatment. The study also indicates that based on this protein, doctors may decide which drugs to use, and which to avoid, when treating NSCL patients. The possibility of doctors knowing before prescription which drugs are likely to work best for each patient represents a great advance in cancer treatment.

The group led by Dr. Fábio Klamt, a researcher at the Department of Biochemistry at the Federal University of Rio Grande do Sul, Brazil found that cofilin levels can be used to indicate which patients in the early stages of the disease have a good prognosis and which do not. When studying a large collection of NSCL cancer samples, the researchers found that the biopsies in which high levels of cofilin were found were from patients who had survived for shorter periods than those from patients with lower expression of the protein.

**See Also:**

Health & Medicine
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**Cisplatin Is More Effective Than Carboplatin For Treating Nonsmall Cell Lung Cancer** (June 6, 2007) — Some patients with advanced nonsmall cell lung cancer have slightly higher survival rates when treated with the chemotherapy drug cisplatin than another platinum-based drug, carboplatin, according to ... > *read more*

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Anexo 3 – Artigo Publicado na Revista “Ciência Hoje”

Volume 47, 2010 – pg 56

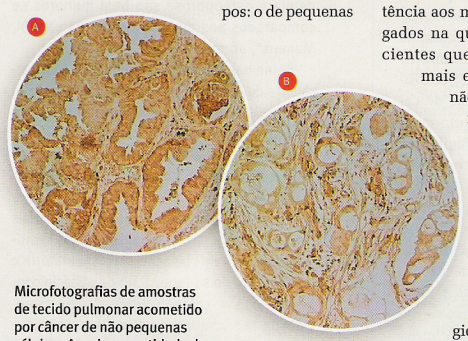


BIOQUÍMICA Teor de cofilina em tumores permite definir tratamento ideal para câncer de pulmão

# Cuidado sob medida

Uma proteína encontrada no organismo humano, a cofilina, é a nova arma dos pesquisadores para combater um tipo de tumor pulmonar maligno conhecido como câncer de pulmão de não pequenas células. De acordo com estudo realizado no Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul (UFRGS), tratamentos personalizados podem ser definidos a partir da identificação da quantidade da proteína cofilina em amostras de tumor. “Essa proteína funciona como um biomarcador na busca do tratamento ideal para o paciente”, afirma o bioquímico Fábio Klamt, coordenador do estudo.

O câncer de pulmão, segundo a Organização Mundial da Saúde, classifica-se em dois tipos: o de pequenas células e o de não pequenas células. Este último é o tipo mais frequente e abrange os adenocarcinomas, os carcinomas epidermoides e os carcinomas de grandes células.




Microfotografias de amostras de tecido pulmonar acometido por câncer de não pequenas células. A maior quantidade da proteína cofilina em A (indicada pelo tom mais amarronzado da imagem) revela um tumor agressivo e com pior prognóstico. A baixa expressão da proteína em B (indicada pelos tons mais claros de marrom) sugere um tumor menos agressivo. As duas amostras (em aumento de 200 vezes) foram obtidas pela técnica de coloração denominada imuno-histoquímica, muito empregada em serviços de patologia

FOTOS CAROLINA BEATRIZ MULLER

O trabalho de pesquisa desenvolvido na UFRGS revelou que o câncer de não pequenas células evolui rapidamente para uma forma mais agressiva em indivíduos com maior quantidade de cofilina no tumor. A substância é fundamental no processo de migração celular, estando assim diretamente associada à malignidade do câncer. “A cofilina tem influência na agressividade da doença, uma vez que facilita sua disseminação por metástases”, explica Klamt.

A equipe constatou ainda que a quantidade de cofilina no tumor tem relação também com a resistência aos medicamentos empregados na quimioterapia. Os pacientes que apresentam níveis mais elevados da proteína não respondem ao tratamento com os quimioterápicos tradicionais. “A partir da avaliação da quantidade de cofilina presente nas amostras tumorais, podemos compreender melhor o processo biológico de cada paciente e estabelecer o tratamento mais adequado”, ressalta Klamt.

Iniciada em 2004, a pesquisa foi feita com base na análise de pacientes, tumores e bancos de dados clínicos de diversas partes do mundo. O desafio atual da equipe do bioquímico da UFRGS é verificar se há relação entre a cofilina e outros tipos de câncer.

## Para poucos, por enquanto

A determinação dos níveis de cofilina em tumores é um processo sofisticado. Embora envolva um procedimento cirúrgico padrão – realização de uma biópsia para retirada de amostras de tecido ou células do paciente –, a quantificação da substância requer o emprego de aparato tecnológico de alto custo e que demanda treinamento específico. Por enquanto, o exame é restrito a poucas pessoas.

“Isso é natural, pois estamos no limiar de um avanço científico que deverá ter impacto direto na medicina”, explica Klamt. Mas a intenção de sua equipe é que, em breve, o exame possa ser feito de modo amplo. “Estamos trabalhando para que a medição do teor de cofilina se torne mais acessível, e ela possa ser introduzida já no serviço de patologia, para diagnosticar a doença em seu estágio inicial”, diz Klamt.

Segundo o Instituto Nacional de Câncer (Inca), o câncer de pulmão matou 20.485 no Brasil em 2008. Três quartos dessas mortes foram causados pelo câncer de pulmão de não pequenas células. Para 2010, o Inca estima a descoberta de aproximadamente 27 mil novos casos da doença, a grande maioria (quase 18 mil casos) em homens.

**Bruno Baggio**
Especial para *Ciência Hoje*/PR

Anexo 4 – Prêmio Santander

Categoria Ciência e Inovação – Saúde

# PRÊMIOS SANTANDER UNIVERSIDADES
## EDIÇÃO 2011

CONECTANDO GRANDES IDEIAS, VALORIZANDO GRANDES PROJETOS

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| Direcionamento in vivo de antígenos do HIV para células dendríticas | | |
| **SAÚDE** | **Fábio Klamt** | **UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL** |
| KIT DE QUANTIFICAÇÃO DE BIOMARCADOR PROGNÓSTICO E PREDITIVO DE CÂNCER DE PULMÃO | | |
| **INDÚSTRIA** | **Ricardo Tansheit** | **Pontifícia Universidade Católica do Rio de Janeiro** |
| SISTEMA DE CARACTERIZAÇÃO DA CONFIABILIDADE HUMANA NAS ATIVIDADES INDUSTRIAIS DE OPERAÇÃO, MANUTENÇÃO E INSPEÇÃO | | |

Anexo 5 – Propriedade Intelectual

INPI (PI0802917-2 A2)

2008.06.24 2008-002917(+2008BR-002917) *(2010.03.09)* G01N 33/574, C12Q 1/68

**Diagnosing lung cancer using diagnostic kit for use in clinical oncology, involves identifying and/or extracting biological material related to cofilin 1 gene from sample of lung cells (Por)**

**C2010D18384**

Addnl. Data: CASTRO M A A, DAL-PIZZOL F, KLAMT F, MOREIRA J C F, SHACTER E
2008.06.24 2008BR-002917

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NOVELTY

Lung cancer diagnosing involves identifying and/or extracting a biological material related to cofilin 1 (CFL1) gene from sample of lung cells by using PCR and/or microarray. A modulation index of CFL1 gene activity is estimated for sample of lung cells. The biological material related to CFL1 gene is quantified and compared with pre-established value.

DETAILED DESCRIPTION

The modulation index is obtained by using equation (I). $C_1$ =((Exp $C_1$ )/Exp $C_1$ + k((1/M)?(Exp $C_1$ ))) (I).

B(4-E1, 4-E2E, 4-E3E, 4-E5, 4-N4, 11-C8E, 12-K4A1, 12-K4F) C(4-E1, 4-E2E, 4-E3E, 4-E5, 4-N4, 11-C8E, 12-K4A1, 12-K4F) D(5-H9, 5-H18B) J(4-B3, 4-F)

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Exp $C_1$ = level of gene expression CFL1;
M = total number of genes used as control gene expression; and
~k = calibration of Exp C.

USE

Method for diagnosing lung cancer using diagnostic kit for use in clinical oncology.

ADVANTAGE

The lung cancer diagnosing method uses a kit for diagnosing especially lung cancer based on gene expression of CFL1, which is used as biomarker of lung cancer in non-small cell lung carcinoma (NSCLC), and thus it can be advantageously applied in clinical oncology.

TECHNOLOGY FOCUS

Biology - Preferred Components: The lung cancer is non-small cell lung carcinoma (NSCLC). The biological material is selected from DNA, RNA and/or proteins.
(20ppDwgNo.0/0)

BR 200802917-A+