UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

DEPARTAMENTO DE BIOQUÍMICA PROF. TUISKON DICK

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COFILINA-1: POTENCIAL PREDITIVO EM CÂNCER DE PULMÃO DE NÃO-PEQUENAS CÉLULAS

CAROLINA BEATRIZ MÜLLER

PORTO ALEGRE, 2017

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Lista de Abreviaturas

ADF- *actin-depolymerization factor*

ADP- *adenosine diphosphate*

ALK- anaplastic lymphoma kinase

BSA- *bovine serum albumin*

CDDP- *cis-diamminedichloridoplatinum(II)*

CFL1- cofilin-1

CFL2- cofilin-2

CIN- cronophin

CPNPC- câncer de pulmão de não-pequenas células

CPPC- câncer de pulmão de pequenas células

DAB- diaminobenzidina

DMSO- dimetilsulfóxido

DNA- *deoxyribonucleic acid*

DO- densidade óptica

EGF- *epidermal growth factor*

EGFR- *epidermal growth factor receptor*

FDR- *false discovery rate*

GEO- *gene expression omnibus*

GI50- *growth inhibition 50%*

GSH- glutationa reduzida

GSSG- glutationa dissulfeto

GST- glutationa s transferase

IHQ- imuno-histoquímica

KRAS- kirsten rat sarcoma viral oncogene homolog

LIMK- lim domain kinase

MAPK- mitogen activated protein kinases

MMR- *mismatch repair*

MRP- *multidrug resistance proteins*

NER- *nuclear excision repair*

NESK- skeletal muscle-specific kinases

NIH- *National Institutes of Health*

NLS- *nuclear localization signal*

PDB- *protein data bank*

PIP2- fosfatidilinositol 4,5-bifosfato

PLC-γ- fosfolipase C gama

PS- *performance status*

RMSD- *root-mean-square deviation*

RNA- *ribonucleic acid*

SQ-IHQ- semiquantificação imuno-histoquímica

SRB- sulforodamina-B

SSH- slingshot protein phosphatases

TESK- testicular protein kinase

TKI- *tirosine kinase inhibitor*

TNM- tumor-nódulo-metástase

TP53- tumor protein p53

Resumo

O câncer de pulmão é um dos tipos de câncer mais comumente diagnosticados. Além da alta incidência, o câncer de pulmão também é a causa mais frequente de mortes, contabilizando quase 20% do total relacionado ao câncer. Destes, cerca de 85% são do tipo CPNPC. O atual desafio da oncologia consiste em estabelecer o conceito de medicina personalizada na prática clínica, proposta que tem permeado diversos estudos na área. Recentes avanços na elucidação da biologia do câncer de pulmão levaram a identificação de potenciais biomarcadores com grande relevância clínica para pacientes com CPNPC. Nos últimos 10 anos, nosso grupo de pesquisa identificou e validou a cofilina-1 como biomarcador prognóstico em CPNPC e evidenciou seu potencial preditivo na resistência ao tratamento com agentes alquilantes. Com relação a esse último aspecto, faz-se necessário o desenvolvimento de novos estudos que visem melhor elucidar a relação entre a expressão da proteína cofilina-1 e a resistência/sensibilidade aos tratamentos disponíveis para pacientes com CPNPC. Assim, o nosso objetivo foi dar seguimento aos estudos já realizados pelo grupo na área e melhor explorar o papel biológico e clínico da cofilina-1 como biomarcador preditivo para CPNPC. Primeiramente nós realizamos 3 diferentes estratégias para avaliar a associação com resistência. Tanto a avaliação *in silico*, através da análise da rede gênica de *CFL1*, quanto dos métodos de seleção de resistência intrínseca/adquirida (protocolos de tratamento diferenciados com cisplatina) e transfecção demonstraram que a modulação da expressão gênica e a alteração no imunoconteúdo tem impacto direto na sensibilidade das células A549 ao tratamento com cisplatina. Em seguida, avaliamos diferentes mecanismos de resistência nas células com resistência inata e adquirida por meio de avaliação do efluxo da droga e por ensaios de dosagem de grupamentos tióis totais livres, GSH e atividade de glutationa-S-transferase (GST). Os resultados sugerem que a mesma possa ser devida ao mecanismo de reparo ao DNA. Baseados nesses dados e em extensa revisão da literatura, fundamentamos uma hipótese de colocalização nuclear e interação de cofilina-1 com receptor EGF (*epidermal growth factor*) no prognóstico e predição de resistência à agentes alquilantes. Por fim, tendo em vista a hipótese desenvolvida, avaliamos as possibilidades de interação dos marcadores por meio de cálculos de atracamento molecular (*docking*), análises estruturais e energia de interação entre os marcadores. Essa análise apontou que a interação mais provável é a do complexo cofilina-1/actina-EGFR intracelular, em que a ligação à porção intracelular de EGFR se dá por actina; sugerindo, assim, a possibilidade de uma função principal na translocação nuclear de moléculas que possam interagir com o receptor. Além disso, a análise da expressão dos marcadores em amostras tumorais evidenciou uma maior frequência de cofilina-1 nuclear em adenocarcinomas, subtipo histológico em que ocorre a maior incidência de mutações em EGFR. Esses dados sugerem que a interação entre os marcadores é plausível, embora não sejam suficientes para a compreensão integral do seu envolvimento nos mecanismos de resistência. Por fim, a explanação contribui com a organização e compilação de evidências que permitirão guiar estudos futuros no sentido de melhor avaliar a relação entre EGFR e cofilina-1 mediante estímulos comuns e, também, mensurar a contribuição dos marcadores na escolha de tratamentos mais adequados.

Abstract

Lung cancer is one of the most commonly diagnosed type of cancer. In addition to the high incidence, lung cancer is also the most frequent cause of death, accounting for almost 20% of all cancer-related deaths. NSCLC type corresponds to 85% of all cases diagnosed. The current challenge of oncology is to establish the concept of personalized medicine in clinical practice, a proposal that has permeated several studies in the field. Recent advances in the elucidation of lung cancer biology have led to the identification of potential biomarkers of great clinical relevance for patients with NSCLC. In the last 10 years, our group has identified and validated cofilin-1 as a prognostic biomarker in NSCLC and evidenced its predictive potential in resistance to treatment with alkylating agents. Regarding this last aspect, it is necessary to develop new studies to better elucidate the relationship between the expression of the cofilin-1 protein and the resistance/sensitivity to treatments available for NSCLC patients. our aim was to perform follow up studies to better explore the biological and clinical role of cofilin-1 as a predictive biomarker for NSCLC. First, we performed 3 different strategies to evaluate the association with resistance. Both the *in silico* assay, by analyzing differential gene expression levels of the *CFL1* gene network, and the intrinsic/acquired resistance (different cisplatin treatment protocols) and transfection selection methods have demonstrated that modulation of gene expression and immunocontent alteration have a direct impact on the sensitivity of A549 cells to cisplatin treatment. We then evaluated different mechanisms of resistance in cells with innate and acquired resistance, through efflux drug evaluation and dosing of free total thiol groups, GSH and glutathione-S-transferase activity (GST) assays. The results suggest that it may be due to the mechanism of DNA repair. Based on these data and an extensive review of the literature, we established a hypothesis of nuclear colocalization and interaction of cofilin-1 with EGF (epidermal growth factor) receptor on the prognosis and prediction of resistance to alkylating agents. Finally, considering the hypothesis developed, we evaluated the possibility of interaction of the markers by calculations of molecular docking, structural analysis and interaction energy between markers. This analysis indicated that the most likely interaction is the intracellular cofilin-1/actin-EGFR complex, in which the binding to the intracellular portion of EGFR is given by actin; this suggests the possibility of a major function in the nuclear translocation of molecules that may interact with the receptor. Furthermore, the analysis of the expression of the markers in tumor samples evidenced a higher frequency of nuclear cofilin-1 in adenocarcinomas, a histological subtype in which the highest incidence of EGFR mutations occurs. These data suggest that the interaction between markers is plausible, although not sufficient for an integral understanding of their involvement in resistance mechanisms. Finally, the explanation contributes to the organization and compilation of evidence that will guide future studies in order to better evaluate the relationship between EGFR and cofilin-1 by common stimuli and also to measure the contribution of the markers in choosing the most appropriate treatments

SUMÁRIO

PARTE I

1 INTRODUÇÃO

1.1 CÂNCER DE PULMÃO

O câncer de pulmão é considerado o tipo de câncer mais comumente diagnosticado. Um estudo mundial recente - GLOBOCAN 2012 - estimou 1,8 milhões de novos casos para o ano de 2012, sendo quase 60% desses casos são provenientes das regiões menos desenvolvidas do planeta. Além da alta incidência, o câncer de pulmão também é a causa mais frequente de mortes, contabilizando quase 20% do total relacionado ao câncer (Ferlay, Soerjomataram et al. 2015). Os dados brasileiros assemelham-se aos dados mundiais, as estimativas para o ano de 2016 apontaram aproximadamente 28 mil novos casos (INCA 2015).

A alta letalidade da doença, evidenciada por estudos locais e mundiais, está diretamente relacionada a sua sintomatologia tardia, o que dificulta a detecção precoce. Ou seja, 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 (Zamboni 2002). Essas características impactam diretamente na sobrevida dos pacientes. A taxa de sobrevida relativa em 5 anos é de 17% para pessoas diagnosticas com câncer de pulmão primário e ela cai para menos de 4% para aqueles que apresentam doença metastática (Horner, Ries et al. 2009)

O tabagismo é responsável por aproximadamente 90% dos casos, constituindo o principal fator de risco. Fumar aumenta de 5 a 10 vezes o risco de desenvolver câncer de pulmão, apresentando clara relação dose-resposta (Health and Services 1986). Embora a abrangência das campanhas antitabagistas tenha se ampliado e intensificado nos últimos anos, o real impacto dessas campanhas somente se dará em questão de décadas, uma vez que o desenvolvimento do câncer de pulmão ocorre após longo período de exposição ao tabaco, (de Sá, Coelho et al. 2016). Outros fatores de risco estão relacionados à exposição ocupacional a

agentes químicos, fatores dietéticos, doença pulmonar obstrutiva crônica e alguns fatores genéticos predisponentes ao câncer (Proctor 2001).

1.2 HISTOPATOLOGIA DO CÂNCER DE PULMÃO

O câncer de pulmão é subclassificado em dois principais tipos: câncer de pulmão de pequenas células (CPPC), composto por células malignas com componente neuroendócrino, e câncer de pulmão de não pequenas células (CPNPC), composto por uma grande variedade de padrões histopatológicos. 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, Brambilla et al. 2005).

A incidência dos adenocarcinomas aumentou nas últimas décadas, atualmente correspondendo a 60% dos CPNPC. O adenocarcinoma é, por definição, uma neoplasia maligna epitelial com diferenciação glandular e que localiza-se perifericamente. Podem apresentar ainda uma grande variedade de subclassificações como lepídica, papilar e acinar. Já os tumores escamosos correspondem a 20% das neoplasias pulmonares, e sua incidência tem diminuído em função de mudanças nos hábitos tabágicos. Ocorrem frequentemente nas porções centrais do pulmão e, a nível microscópico, apresentam regiões de intensa queratinização. Os carcinomas de grande células correspondem ao subtipo menos incidente, normalmente localizam-se perifericamente e apresentam aparência necrótica e indiferenciados quando analisados a nível celular (Mukhopadhyay and Katzenstein 2011, Lewis, Check et al. 2014, Zheng 2016).

Com os recentes avanços em biologia molecular e reconhecimento de características genéticas do câncer de pulmão, essa divisão em tumores de pequena células e não-pequenas células não é mais suficientemente acurada. A distinção dos pacientes conforme a presença de alterações biomoleculares ou genéticas é de extrema importância para o correto tratamento dos pacientes nessa era de medicina personalizada (Pao and Girard 2011).

1.3 DIAGNÓSTICO E ESTADIAMENTO DO CÂNCER DE PULMÃO

A investigação diagnóstica da doença é feita com base nos sintomas do paciente, histórico de exposição a fatores de risco, achados de exame físico e exames de imagem (Raio-x e tomografia computadorizada), porém a confirmação diagnóstica só pode ser firmada a partir dos exames patológicos, como citologia ou biópsias. (Rivera, Mehta et al. 2013).

Um vez diagnosticada, é preciso determinar a extensão anatômica da doença, o que é feito através do método de estadiamento. Os critérios anatômicos para o estadiamento do CPNPC são baseados no sistema TNM (tumor-nódulo-metástase), 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. Assim, de acordo com classificação dada a cada um desses descritores é possível indicar qual o estágio da neoplasia, podendo variar de I a IV (Sihoe and Yim 2004, Detterbeck, Boffa et al. 2009). Esse sistema já está na sua oitava atualização (Tabela 1), realizada pelo *American Joint Committee on Cancer* (AJCC) , e tem previsão de implantação na prática clínica em 1º de janeiro de 2018 (https://cancerstaging.org/About/news/Pages/Implementation-of-AJCC-8th-Edition-Cancer-Staging-System.aspx).

Tabela 1. Proposta de agrupamento da oitava edição do sistema de estadiamento TNM.

Carcinoma Oculto	TX	N0	M0
Estágio 0	Tis	N0	M0
Estágio IA1	**T1a(mi)**	**N0**	**M0**
	T1a	**N0**	**M0**
Estágio IA2	**T1b**	**N0**	**M0**
Estágio IA3	**T1c**	**N0**	**M0**
Estágio IB	T2a	N0	M0
Estágio IIA	T2b	N0	M0
Estágio IIB	**T1a-c**	**N1**	**M0**
	T2a	**N1**	**M0**
	T2b	N1	M0
	T3	N0	M0
Estágio IIIA	**T1a-c**	**N2**	**M0**
	T2a-b	N2	M0
	T3	N1	M0
	T4	N0	M0
	T4	N1	M0
Estágio IIIB	**T1a-c**	**N3**	**M0**
	T2a-b	N3	M0
	T3	**N2**	**M0**
	T4	N2	M0
Estágio IIIC	**T3**	**N3**	**M0**
	T4	**N3**	**M0**
Estágio IVA	**Qualquer T**	**Qualquer N**	**M1a**
	Qualquer T	**Qualquer N**	**M1b**
Estágio IVB	**Qualquer T**	**Qualquer N**	**M1c**

As modificações em relação à sétima edição estão destacadas em negrito. Adaptado de (Goldstraw, Chansky et al. 2016)

O estadiamento, associado a outros fatores prognósticos como *performance status* (PS), escala que indica a capacidade funcional do paciente (tabela 2), idade e tipo histológico, refletirá o prognóstico e guiará a terapêutica (Fernandez, Jatene et al. 2002).

Tabela 2. Performance Status: Escalas de Zubrod e Karnofsky

Escala de Zubrod (ECOG)	Escala de Karnofsky (%)
PS 0 - Atividade normal	**100** - nenhuma queixa: ausência de evidência da doença
	90 - capaz de levar vida normal; sinais menores ou sintoma da doença
PS 1 - Sintomas da doença, mas deambula e leva seu dia a dia normal	**80** - alguns sinais ou sintomas da doença com o esforço
	70 - capaz de cuidar de si mesmo; incapaz de levar suas atividades normais ou exercer trabalho ativo
PS 2 - Fora do leito mais de 50% do tempo	**60** - necessita de assistência ocasional, mas ainda é capaz de prover a maioria de suas atividades
	50 - requer assistência considerável e cuidados médicos freqüentes
PS 3 - No leito mais de 50% do tempo, carente de cuidados mais intensivos	**40** - incapaz; requer cuidados especiais e assistência
	30 - muito incapaz; indicada hospitalização, apesar da morte não ser iminente
PS 4 - Preso ao leito	**20** - muito debilitado; hospitalização necessária; necessitando de tratamento de apoio ativo
	10 - moribundo, processos letais progredindo rapidamente

Fonte: (PSICOMOTORA 2002)

1.4 TRATAMENTO

Como mencionado anteriormente, a maior parte dos diagnósticos para câncer de pulmão se dá quando a doença já está localmente avançada ou disseminada, uma vez que tumores iniciais geralmente não produzem sintomas. Entretanto, o diagnóstico nos estágios iniciais da doença é de fundamental importância, pois permite a ressecção cirúrgica do tumor, abordagem terapêutica que apresenta maior potencial de cura ao paciente (Barros, Valladares et al. 2006).

O tratamento cirúrgico é geralmente indicado nos estágios I, II e IIIA, em que o tumor ainda se encontra localizado. Nesse estágios, a sobrevida média varia de 70 a 40%. A remoção cirúrgica em estágio IV não é indicada; assim, a sobrevida média esperada para a maioria dos pacientes diagnosticados em estágio IV é muito baixa, em torno de 1% (Novaes, Cataneo et al. 2008).

Os pacientes submetidos à ressecção cirúrgica usualmente realizam terapia adjuvante, que envolve a realização de radioterapia e/ou quimioterapia no período pós-cirúrgico, a fim de eliminar células cancerosas remanescente, podendo assim contribuir para o aumento da

sobrevida (Alberti, Anderson et al. 1996). A quimioterapia neoadjuvante (pré-operatória) pode ser empregada em pacientes diagnosticados em estágio III e complementada no pós-operatório; no entanto, a morbidade pós-operatória dos pacientes submetidos a esse esquema se mostra aumentada (Novaes, Cataneo et al. 2008).

Aproximadamente 40% dos diagnósticos para câncer de pulmão são realizados em estágio IV. Para esses pacientes, o objetivo do tratamento é melhorar a sobrevida e reduzir eventos adversos relacionados à doença (Ramalingam and Belani 2008).

Conforme revisado por Zappa e Mousa, a Sociedade Americana de Clínica Oncológica estabelece que o tratamento de pacientes com PS de 0 ou 1 deve ser a combinação de cisplatina ou carboplatina e paclitaxel, gemcitabina, docetaxel, vinorelbina, irinotecam ou pemetrexed. Evidencias sugerem que pacientes com PS de 2 devam utilizar somente uma droga, geralmente não platina. Já pacientes com PS de 3 ou 4 não devem receber quimioterapia citotóxica, em função dos efeitos adversos, recebendo somente cuidados paliativos. Além disso, o tratamento deve ser interrompido caso o tumor cresça ou, após quatro ciclos, o tratamento não tenha diminuído o tumor (Zappa and Mousa 2016).

Embora a escolha do tratamento leve em consideração a extensão anatômica da doença e as condições gerais do paciente, o câncer de pulmão é uma desordem bastante heterogênea, seu desenvolvimento e manifestação variam muito de caso a caso. Cada subtipo histológico apresenta associações clinico-patológicas e moleculares únicas, características essas que não são consideradas nos esquemas de estadiamento (Sholl 2016).

A discriminação desses subtipos histológicos do CPNPC tornou-se um fator determinante na terapia. Recentemente, a identificação de anomalias moleculares em uma grande proporção de pacientes com câncer de pulmão permitiu o desenvolvimento de terapias-alvo personalizadas. O uso de biomarcadores preditivos para identificar tumores que tenham

melhor resposta a essas terapias significou uma mudança de paradigma no diagnóstico do câncer de pulmão e criou, em última análise, melhores expectativas para esses pacientes (Kerr, Bubendorf et al. 2014).

1.4.1 TERAPIAS ALVO EM CÂNCER DE PULMÃO

O atual desafio da oncologia consiste em estabelecer o conceito de medicina personalizada na prática clínica, proposta que tem permeado diversos estudos na área. Recentes avanços na elucidação da biologia do câncer de pulmão levaram a identificação de potenciais biomarcadores com grande relevância clínica para pacientes com CPNPC (Villalobos and Wistuba 2017).

A última classificação histológica do câncer de pulmão publicada pela Organização Mundial de Saúde (OMS) em 2015, por exemplo, já incluiu aspectos genéticos e imuno-histoquímicos dos diferentes subtipos tumorais (Travis, Brambilla et al. 2015).

O câncer de pulmão de pequenas células (CPPC), o carcinoma epidermóide e o carcinoma de grande células são tipos tumorais que tendem a ocorrer em fumantes pesados. Embora apresentem uma alta frequência geral de mutações relacionadas ao tabagismo e altas taxas de mutações em *TP53*, carecem de um "*driver*" oncogênico claro (Network 2012, Peifer, Fernández-Cuesta et al. 2012). Já os adenocarcinomas apresentam uma variedade de alterações oncogênicas que permitiram o uso de inibidores alvos na prática clínica. As mais comuns ocorrem nos genes "*Kirsten rat sarcoma viral oncogene homolog"* (*KRAS*)*, "epidermal growth factor receptor"* (*EGFR*)*,* e *"anaplastic lymphoma kinase"* (*ALK*), sendo as duas últimas mais frequentes em não-fumantes (Lindeman, Cagle et al. 2013).

Mutações espontâneas em *EGFR*, por exemplo, são geralmente oncogênicas, ou seja, elas ativam a via de sinalização *EGFR* na ausência de ligantes e promovem proliferação celular, sinais anti-apoptóticos e de sobrevivência. Essas vias de sinalização funcionam fazendo com

que as células *EGFR* mutadas tornem-se dependentes do *EGFR* ativo para sua sobrevivência. Inibidores de *EGFR* levam à "*up-regulation*" de moléculas pró-apoptóticas e resultam, por fim, na morte da célula através da ativação da via apoptótica mitocondrial intrínseca (Costa, Halmos et al. 2007, Sharma, Bell et al. 2007). Com base em estudos que demonstraram que o uso de inibidores tirosina-cinase resulta em maior sobrevida quando comparados ao o uso de cisplatina e carboplatina, drogas como gefitinib e erlotinib passaram a ser indicadas como tratamento de primeira linha aos pacientes de CPNPC que apresentem mutação em *EGFR* (L858R e E746-A750del) (Mok, Wu et al. 2009, Maemondo, Inoue et al. 2010).

Por muito tempo, o paradigma central da oncologia de precisão foi "o tratamento certo ao paciente certo, no momento certo"; a prática clínica, entretanto, demonstrou que o futuro dos estudos na área deve centrar-se em combinar as alterações genômicas mais críticas com as melhores drogas disponíveis (Warner 2017). A utilização dos inibidores tirosina-cinase (*TKI's*) no tratamento de pacientes que apresentem o diagnóstico de mutações específicas e responsivas a esse tratamento, é um exemplo da aplicação desse conceito.

Contudo, a população que se beneficia desse tratamento ainda é bastante restrita, caracterizada em sua maioria por mulheres não-fumantes com adenocarcinoma de pulmão (Boch, Kollmeier et al. 2013). Compreender a associação de outros biomarcadores preditivos ao *EGFR* e à resistência às terapias convencionais baseadas em agentes alquilantes pode auxiliar a discrimar e, potencialmente, aumentar a população responsiva ao tratamento com os *TKI's*.

1.5 COFILINA-1

A dinâmica e a reorganização dos filamentos de actina são "espaço-temporalmente" reguladas por proteínas ligantes de actina que controlam, de maneira cooperativa, a construção

e desconstrução das estruturas supramoleculares do citoesqueleto baseadas em filamentos de actina, tais como filopódios, lamelipódios, invadopódios, fibras de estresse e redes corticais de actina. Dentre essas proteínas está a família das ADF/cofilina-1, que compreende duas isoformas de cofilina, a cofilina-1 (*CFL1*, cofilina não-muscular ou cofilina-1) e a cofilina-2 (*CFL2*, cofilina muscular ou cofilina-m) e a ADF (*actin-depolymerization factor*), também conhecida como destrina (Moon and Drubin 1995, Bamburg 1999).

A cofilina-1, é uma proteína citosólica de 19 kDa ubiquamente presente nas células eucarióticas (Moon, Janmey et al. 1993, Gurniak and Witke 2005), sua estrutura está representada na figura 1. Essa proteína está diretamente relacionada com a regulação da polimerização e despolimerização de actina durante a migração celular (Maciver and Hussey 2002) e também com apoptose induzida por oxidantes (Zdanov, Klamt et al. 2010).

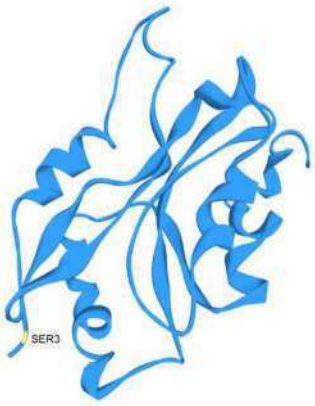

Figura 1. Representação tridimensional da estrutura da proteína cofilina-1. PDB 1Q8G, com destaque para o resíduo serina 3 (em amarelo), alvo de fosforilação por ação de cinases como a LIMK-1. Retirado de (Pope, Zierler-Gould et al. 2004)

Conforme revisado por Mizuno, a cofilina-1 prende-se aos filamentos de actina (F-actina) ligados a ADP e participa do seu "desmonte", através de atividades de quebra e despolimerização, aumentando assim a concentração de actina monomérica (G-actina). Dessa forma, a cofilina-1 também contribui com a polimerização de novos filamentos, uma vez que aumenta a disponibilidade de G-actina; além disso, a atividade de quebra cria novas

extremidades livres (*free barbed ends*) para polimerização, aumentando, consequentemente, o *turnover* dos filamentos de actina na célula (Mizuno 2013).

Existem cinco principais mecanismos regulatórios da atividade da cofilina-1. Primeiro, a fosforilação de sua serina 3, por *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 defosforilação da serina 3 por fosfatases, como a família das *SSH's* (*slingshot family proteins*) e a cronofina (*CIN*), resultando em sua ativação (figura 2). Terceiro, sua atividade pode ser inibida pela ligação a fosfatidilinositol-4,5-bifosfato (PIP_2). Quarto, a mudança de pH fora dos padrões fisiológicos pode aumentar a atividade da cofilina-1 quando em estado defosforilado (Wang, Eddy et al. 2007). Por último, a cofilina-1 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, Zdanov et al. 2009).

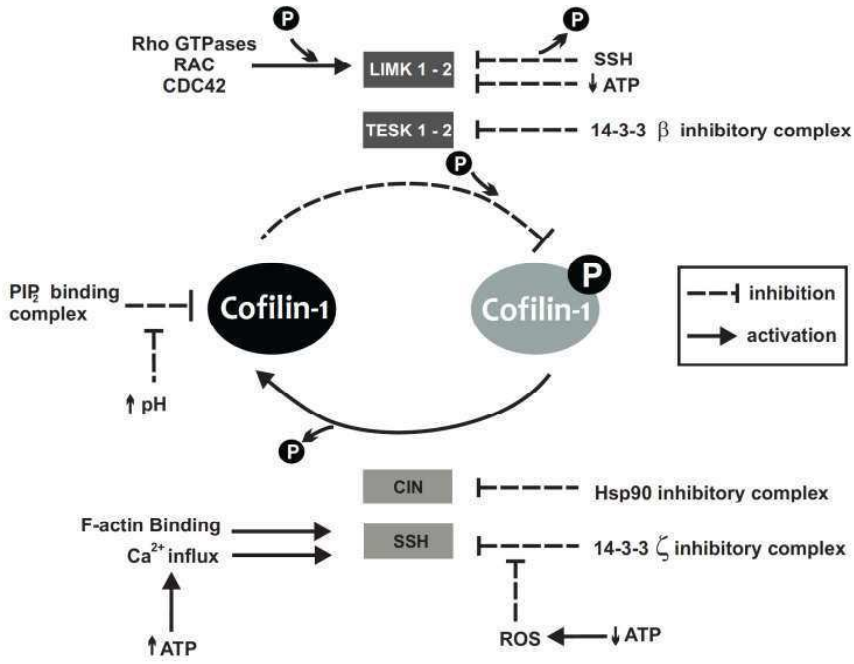

Figura 2. Controle da dinâmica dos filamentos de actina via fosfo-regulação de cofilina-1. A cofilina-1 é inativada pela fosforilação da serina 3 por ação das cinases *LIMK* e *TESK*; é

reativada por fostatases, como a família das *SSH*'s. Adaptado de (Schonhofen, de Medeiros et al. 2014)

A localização da cofilina-1 depende do tipo celular, estado de diferenciação e de seu estado de ativação (Hao, Wang et al. 2008). Estudos imuno-histoquímicos revelaram que a cofilina-1 pode, em resposta a agentes químicos e físicos, translocar-se para o núcleo e que essa importação depende do seu estado de fosforilação. A translocação nuclear de cofilina-1, mediante estímulos, requer defosforilação do domínio serina 3 para expor seu sinal de localização nuclear (NLS). Estudos demonstraram que o tratamento de fibroblastos em cultura com dimetilsulfóxido (DMSO) ou aquecimento resultou em diminuição das fibras de estresse no citoplasma e uma maior localização nuclear de actina, colocalizados com cofilina-1 (Abe, Nagaoka et al. 1993). Mais recentemente verificou-se que a actina é constantemente transportada para dentro e para fora do núcleo, e que o seu importe nuclear é dependente de cofilina-1 (Dopie, Skarp et al. 2012).

1.5.2 COFILINA-1 E CÂNCER

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. Sob estímulo de EGF (Epidermal Growth Factor), a defosforilação e ativação de cofilina-1 leva à remodelação do citoesqueleto, resultando nas protrusões celulares críticas para a migração e invasão de células. Essas alterações na morfologia da célula são, assim, diretamente dirigidas pela reestruturação da rede de actina do citoesqueleto celular, regulada pela cofilina-1 (Van Rheenen, Song et al. 2007).

Embora os mecanismos moleculares do envolvimento da cofilina-1 na agressividade das células tumorais ainda não tenham sido completamente elucidados, alterações na expressão de cofilina-1 tem sido relacionadas com o fenótipo maligno de diversos tipos tumorais. Shishkin

e colaboradores realizaram extensa revisão sobre esse tópico e citam relação da expressão de mRNA e da proteína como marcador prognóstico e/ou preditivo em tumores de mama, pulmão, próstata, bexiga e ovário (Shishkin, Eremina et al. 2016).

Nosso grupo tem realizado diversos estudos buscando melhor compreender a relação da proteína cofilina-1 com a agressividade do CPNPC. Em 2010, publicamos um trabalho em que testamos a capacidade prognóstica do gene da cofilina-1 (*CFL1*) em duas coortes independentes. Nesse mesmo estudo, 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*. Os resultados demonstraram 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 (figura 3). Um maior imunoconteúdo de cofilina-1 em linhagens celulares está relacionado a um maior índice de invasão e também resistência a agentes alquilantes (figura 4). Ambos os dados são importantes características de agressividade que tem direta relação com as quantidades de cofilina-1 (Castro, Dal-Pizzol et al. 2010). Entretanto, a maior parte dos indicativos preditivo e prognóstico do marcador foram baseados em dados de micro-arranjo, sendo, por essa razão, a principal limitação desse estudo.

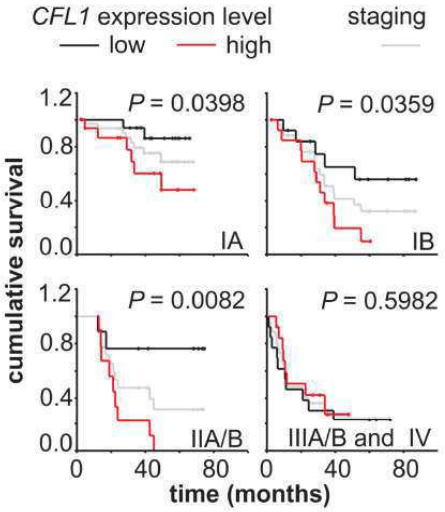

Figura 3. Valor prognóstico dos níveis de mRNA de CFL1 em pacientes com CPNPC. As curvas de mortalidade Kaplan-Meier demonstram que os níveis de CFL1 discriminam bom e mal prognóstico. Retirado de Castro, Dal-Pizzol et al, 2010

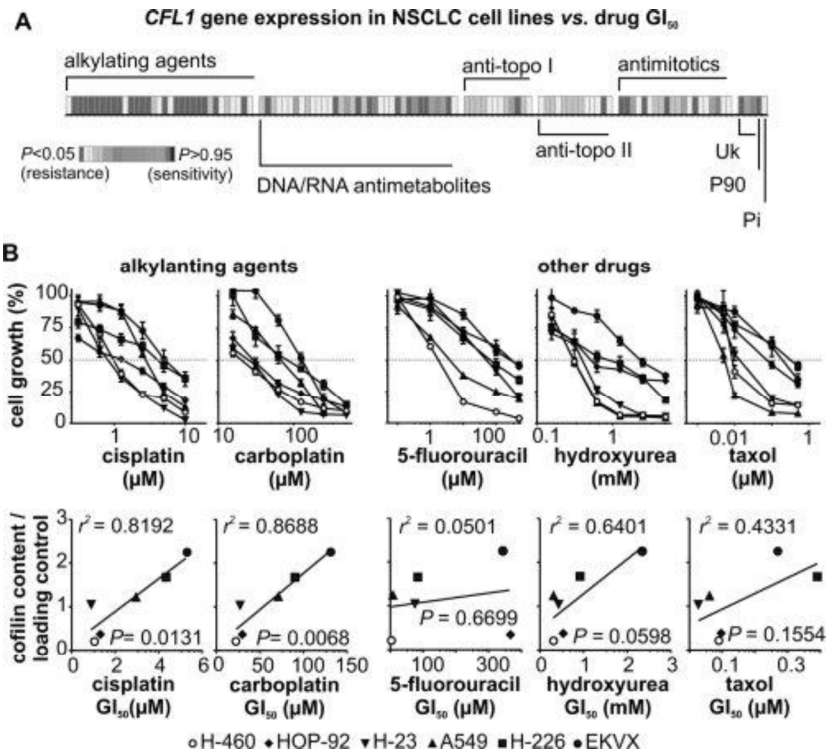

Figura 4. (A) Dados de microarranjo das linhagens celulares cruzados contra o valor de GI50 de 118 agentes quimioterápicos (do *NCI-60 drug discovery pipeline*); P<0,05 indica correlação negativa (resistência), enquanto que P>0,95 indica correlação positiva (sensibilidade). Cada coluna na matriz representa a correlação de Spearman entre a expressão gênica e a toxicidade

individual de cada droga. (B) Avaliação *in vitro* da citotoxicidade a drogas pelo método de sulfarodamina B (SRB). Linhagens com maior imunoconteúdo de cofilina apresentaram maior valor de GI50, indicando maior resistência ao tratamento. Retirado de Castro, Dal-Pizzol et al, 2010 (ANEXO 1)

Para que pudéssemos quantificar a proteína em amostras tumorais e superar essa limitação, em 2011, nós otimizamos um protocolo de Semi-quantificação Imuno-histoquímica (SQ-IHQ) para cofilina-1 em amostras de CPNPC. Foi o estabelecimento de um método de análise computadorizada que fez uso de uma técnica amplamente estabelecida em serviços de saúde, a imuno-histoquímica (IHQ). O software utilizado atribui valores de densidade óptica (DO) às diferentes intensidades da imunorreação. A aplicação deste método em uma coorte retrospectiva 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 (figura 5), o que reforça o valor da cofilina-1 como biomarcador prognóstico. (Müller, de Barros et al. 2011).

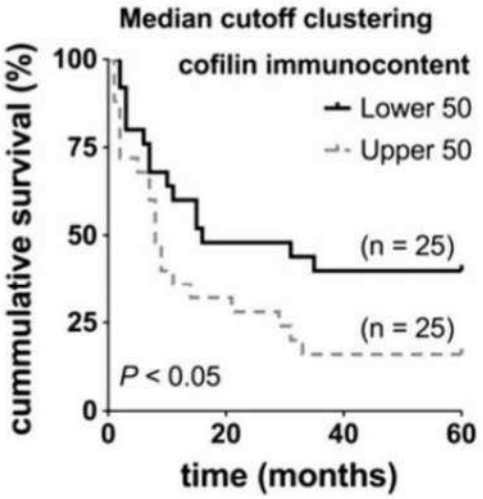

Figura 5. Curva de mortalidade Kaplan-Meier. O agrupamento de pacientes de acordo com o imunoconteúdo de cofilina demonstrou que a maior expressão do marcador está relacionada com menor sobrevida. Retirado de Müller, de Barros et al., 2011

No que diz respeito ao aspecto preditivo evidenciado anteriormente, realizamos em 2012 um estudo em que avaliamos a rede de interação gênica da cofilina-1 com relação aos principais mecanismos de resistência descritos pela literatura (*Nuclear Excision Repair* (NER), *Mismatch Repair* (MMR), inativação/efluxo e importação nuclear) em um modelo *in silico*, utilizando dados de expressão gênica de microarranjos. Os dados extraídos do *Gene Expression Omnibus* (GEO) foram analisados pelo software ViaComplex (Castro, Filho et al. 2009). Os resultados demonstraram que o grupo de genes relacionados à cofilina-1 tem comportamento similar ao grupo de genes relacionados ao reparo por NER e inativação/efluxo, indicando que o mecanismo de resistência a agentes alquilantes possa compreender uma possível interação dessas vias (figura 6) (De Bastiani 2012).

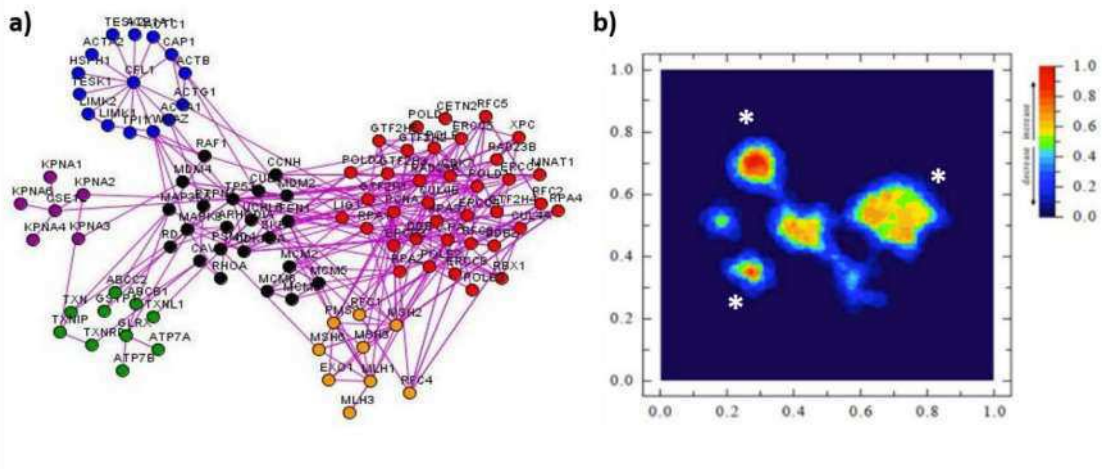

Figura 6. (a) Rede de resistência a drogas alquilantes. Em azul, os genes associados a cofilina; em vermelho, NER; em laranja MMR, em verde, inativação e efluxo; em roxo, importação nuclear; (b) Análise topográfica das redes de interação gene/proteína das células resistentes X células sensíveis ao tratamento com cisplatina. O gradiente de cor representa o estado funcional relativo. Os asteriscos sinalizam maior atividade dos genes da rede de cofilina, NER e inativação/efluxo. Adaptado de De Bastiani 2012

2 JUSTIFICATIVA

Ao longo da última década, nosso grupo de pesquisa validou a cofilina-1 como marcador prognóstico em CPNPC e evidenciou seu potencial preditivo na resistência ao tratamento com agentes alquilantes. Com relação a esse último aspecto, faz-se necessário o desenvolvimento de novos estudos que visem melhor elucidar a relação entre a expressão da proteína cofilina-1 e a resistência/sensibilidade aos tratamentos disponíveis para pacientes com CPNPC.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Explorar o papel biológico e clínico da cofilina-1 como biomarcador preditivo para CPNPC.

3.2 OBJETIVOS ESPECÍFICOS

i) *In vitro*:

- Avaliar mecanismos de resistência ao tratamento com agentes alquilantes utilizando linhagens que apresentam resistência intrínseca e adquirida ao tratamento com cisplatina

ii) Revisão da literatura científica disponível:

- Compilar e avaliar dados sobre o papel fisiológico da cofilina-1 no funcionamento celular, bem como sobre as flutuações na expressão e alterações de seu funcionamento nas células tumorais

- Avaliar as interações entre cofilina-1 e EGFR e suas possíveis implicações no fenótipo tumoral mais agressivo e de resistência à terapia com cisplatina.

iii) *In silico*:

- Testar as probabilidades de interação da cofilina-1 e do complexo cofilina-1/actina com diferentes porções do receptor EGF, baseados na hipótese de colocalização quando da translocação nuclear.

iv) Em estudos clínicos:

- Quantificar cofilina-1 e EGFR e avaliar a presença de marcação/colocalização nuclear desses marcadores em amostras de câncer de pulmão.

- Realizar correlação desses achados com os dados clinicopatológicos disponíveis.

PARTE II

4. RESULTADOS

Os resultados serão apresentados em dois capítulos, na forma de artigos publicados e na forma de metodologia/resultado nos resultados suplementares.

Capítulo 1
Artigo publicado na revista Tumor Biology.

"**High cofilin-1 levels correlate with cisplatin resistance in lung adenocarcinomas**"

Tumor Biol.
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RESEARCH ARTICLE

High cofilin-1 levels correlate with cisplatin resistance in lung adenocarcinomas

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Abstract High cofilin-1 levels have been shown to be an accurate prognostic biomarker in non-small cell lung cancer (NSCLC) and a predictive factor in drug resistance. Herein we explore the role of cofilin-1 in *cis*-diamminedichloroplatinum(II) (cisplatin) resistance. We evaluated cofilin-1 levels in intrinsically cisplatin-resistant A549 (ICR-A549) cells and determined the cisplatin toxicity in A549 cells transiently transfected and overexpressing *CFL1* plasmid. Moreover, expression levels (activity) of the *CFL1* gene network were analyzed in a cisplatin-resistant human lung adenocarcinoma cell panel. ICR-A549 cells, selected by challenging parental cells with 10-fold drug GI_{50} value, presented a sixfold increase in cisplatin GI_{50} value and an increased cofilin-1 immunocontent ($P<0.01$). In addition, cells transfected with cofilin-1 became more resistant to cisplatin ($P<0.01$). High activity of the *CFL1* gene network was found in a cisplatin-resistant adenocarcinoma cell panel ($P<0.01$). In vitro evidences suggest that cofilin-1 is a biological predictor of cisplatin resistance, supporting new treatment initiatives based on cofilin-1 levels to guide chemotherapeutic interventions in NSCLC patients.

Keywords Non-small cell lung cancer · Cisplatin resistance · Cofilin-1 · *CFL1* · Predictive biomarker

Introduction

Non-small cell lung cancer (NSCLC) remains the leading cause of cancer-related mortality worldwide, being responsible for almost 1.1 million deaths a year [1]. Unfortunately, since most of the cases are diagnosed with advanced pathologic (p)-stages of disease, curative pulmonary resection is no longer a therapeutic option and multimodality treatment became the indicative management of disease [2]. However, the effect of current therapies in improving the survival of NSCLC patients remains far from satisfactory, reflecting that the prognosis of NSCLC is still poor, with a 5-year survival probability of 49 % for early stages and less than 1 % for advanced stages [3].

Advances in molecular pathology led 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 treatment [4]. For example, the treatment of NSCLC had been revolutionized by the development of targeted agents (e.g., the FDA-approved drugs *erlotinib* and *gefitinib* for patients harboring specific *EGFR* mutations) [5], but decision in NSCLC management is still mainly based on the anatomic extent of the disease. Other factors, such as the molecular characterization of the tumor, are rarely included in decision-driven therapeutics [6].

Matheus Becker and Marco Antônio De Bastiani contributed equally to this work.

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Despite the large number of studies involving biomarkers for NSCLC, poor individual performance precludes their inclusion in the clinical practice [7]. Then, the identification of biomarkers that could add value to the TNM system is an important step in an individualized therapy and, ultimately, improves patient survival.

In this context, we have previously established the role of cofilin-1 as a prognostic biomarker for NSCLC patients [8, 9]. Using three independent clinical cohorts, we found that cofilin-1 levels are highly sensitive and specific in discriminating between good and bad NSCLC patient outcomes, especially in the early disease stage [8, 9]. In these studies, we also found an association between cofilin-1 and lung tumor migration and invasion.

Cofilin-1 (*CFL1* gene product; non-muscle isoform; 1072 Gene ID) is one of the major proteins responsible for cell migration processes, playing a key role in actin filament dynamics [10], and apoptosis induced by oxidants [11]. Cofilin-1 is overexpressed in several highly invasive cancer cell lines [12–14], as well as in biopsies of oral, renal, and ovarian carcinomas [15]. More importantly, cofilin-1 levels (protein and mRNA) were found to be correlated with resistance to 22 of 33 alkylating drugs tested [8]. These findings led us to propose the use of cofilin-1 levels as a prognostic and predictive NSCLC biomarker.

Herein we aimed to strengthen the association of cofilin-1 with cisplatin resistance in human NSCLC, based on three different experimental strategies: (1) evaluation of cofilin-1 immunocontent in the intrinsically cisplatin-resistant A549 (ICR-A549) NSCLC cell line, (2) determination of cisplatin toxicity in A549 cells transiently transfected and overexpressing *CFL1* plasmid, and (3) evaluation of the differential gene expression level (activity) of the cofilin-1 gene network in response to acute cisplatin treatment and in cisplatin-resistant human NSCLC cell panel.

Materials and methods

Cell line maintenance, treatments, and cisplatin resistance protocol

Exponentially growing human A549 NSCLC adenocarcinoma cells (obtained from NCI-Frederick cell line repository) were maintained in RPMI 1640 medium (Invitrogen) containing 10 % fetal bovine serum, 1 μg/mL of amphotericin B, and 50 μg/L of garamycin at 37 °C in a humidified atmosphere of 5 % CO_2. Cisplatin cytotoxicity (GI_{50} value) was determined with the sulforhodamine B (SRB) assay as a dose–response curve, following the NCI-60 drug screening protocol. Briefly, cells were seeded in a 96-well plate and treated for 72 h after overnight adherence. Cells were fixed with 10 % TCA, washed, and stained with 0.2 % SRB in 1 % acetic acid at room temperature. Bound dye was solubilized with 10 mM Tris buffer (pH 10.5), and a plate reader (Spectra Max GEMINI XPS, Molecular Devices, USA) was used to measure the optical densities of SRB at 490 nm. Once the cisplatin GI_{50} value was obtained, sub-confluent A549 cells plated in 75-cm^2 flasks were treated with 10-fold GI_{50} value for 24 h. The ICR-A549 cells were left to grow until semi-confluence, harvested, sub-cultured to re-evaluate the cisplatin GI_{50} value as previously described, or collected for *Western blot* immunoassay. We used rabbit cofilin-1 polyclonal antibody (Abcam; 1:2,000) in combination with horseradish peroxidase-linked secondary antibodies (1:10,000) from DakoCytomation. Bands were visualized by chemiluminescence (PIERCE) using X-ray film. Quantification was with ImageJ software. Data analyses were performed using GraphPad Prism 5.0 software.

Transient transfection and overexpression of wild-type cofilin-1 or mock

Transient transfections were performed with Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instructions. Briefly, A549 cells were seeded in 96-well plates overnight before transfection with 0.2 μg of cofilin-1 plasmid (pCMV-XL5) or empty plasmid (mock). DNA was mixed with the liposome reagent at a ratio of 1:2 before addition to cells. At 6 h after transfection, the medium was removed and fresh medium was added. Transfection efficiency was determined using a pGFP-N1 vector (Clontech) and evaluated by flow cytometry to be ~80 % after 48 h. Cofilin-1 levels in transfected cells were determined by *dot blot* immunoassay, where serial dilutions of samples (1, 2, 4, and 8 μL) were applied to a nitrocellulose membrane and cofilin-1 immunocontent were determined as described for Western blot.

Differential gene expression and enrichment analysis

We analyzed differential gene expression levels of the *CFL1* (human cofilin-1) gene network, as previously described [8], using microarray data from the GSE4127 dataset available at the Gene Expression Omnibus repository (http://www.ncbi.nlm.nih.gov/geo/). The GSE4127 dataset provides the transcriptional profiling of a set of 10 human lung adenocarcinomas (RERF LC-KJ, ABC-1, PC14, LU65, PC9, PC7, A549, LC2/ad, RERF LC-MS, and PC3 cells), with cytotoxicity data of several chemotherapeutic drugs, including cisplatin and carboplatin [16]. Differential gene expression (activity) and enrichment analysis were obtained using ViaComplex software version 1.0 [17], which estimates the relative expression level of groups of functionally associated genes (GFAG). Briefly, to obtain a quantitative parameter that characterizes the functional state of GFAG in the sample,

ViaComplex measures the information content using Shannon's entropy.

Statistical analysis

Data are expressed as means±standard deviation of at least three independent experiments performed in triplicate. Data were analyzed for significance by Student's *t* test or by one-way ANOVA, with Tukey's multiple comparison post hoc test. Differences were considered statistically significant when $P < 0.05$ (GraphPad® Software Inc., 5.0, San Diego, CA, USA).

Results

In a previous work, exploring data from the NCI-60 cell panel, we found a strong correlation between cofilin-1 (protein and mRNA levels) and increased GI_{50} value for several clinically relevant alkylating agents (including cisplatin and carboplatin). These findings allowed us to propose that cofilin-1 could be used as a new biological predictor of response to this class of anticancer drugs [8].

Trying to strengthen this observation, we first evaluated the expression levels of the *CFL1* gene network in an alternative cisplatin-resistant human NSCLC cell panel (GSE4127 dataset). The *CFL1* gene network consists of 19 genes (*LIMK1*, *LIMK2*, *YWHAG*, *YWHAZ*, *TPI1*, *HSPH1*, *NRK*, *ATP1A1*, *ACTA1*, *ACTA2*, *ACTB*, *ACTC1*, *ACTG1*, *TESK1*, *TESK2*, *SSH1*, *SSH2*, *SSH3*, *CAP1*) identified by the network-based model of *CFL1* interaction partners [8]. Bootstrap analysis showed a significant increase in gene expression level (activity) of the *CFL1* gene network in cisplatin-resistant adenocarcinomas ($P < 0.01$; Fig. 1a). To do so, the human lung adenocarcinoma cell lines were first clustered on the basis of cisplatin cytotoxic activity. LC2/ad, RERF-LC-MS, and PC-3 were considered cisplatin-resistant cells while RERF-LC-KJ, ABC-1, and PC14 the cisplatin-sensitive cells (means of the cisplatin GI_{50} value for each group were 12.10±7.97 vs. 2.45±0.43 μM, respectively, $P < 0.01$; Fig. 1b).

Moreover, we then explored the cofilin-1 immunocontent in ICR-A549 human adenocarcinoma cells. ICR-A549 cells were selected by challenging parental A549 cells with 10-fold the GI_{50} value of cisplatin for 24 h. Approximately 2 weeks after treatment, the GI_{50} value for cisplatin was found to be sixfold higher in ICR-A549 cells as compared to parental A549 cells (20.81±8.70 vs. 3.50±0.86 μM, respectively, $P < 0.0001$; Fig. 2a), and ICR-A549 cells presented a significant higher cofilin-1 immunocontent ($P < 0.01$; Fig. 2b). More interestingly, A549 cells transiently transfected and overexpressing a plasmid containing *CFL1* (human cofilin-1 gene) exhibit an increase in cisplatin resistance (increase in drug GI_{50} value), as compared to the mock (empty plasmid) group ($P < 0.01$; Fig. 3). Representative images of the dot blot immunoassay confirmed the transfection efficacy and showed a significant increase in cofilin-1 immunocontent after 48 h ($P < 0.05$).

All in all, the cumulative experimental data obtained with these in vitro studies support that a high cofilin-1 level is correlated with an increased resistance to cisplatin in human NSCLC cell lines.

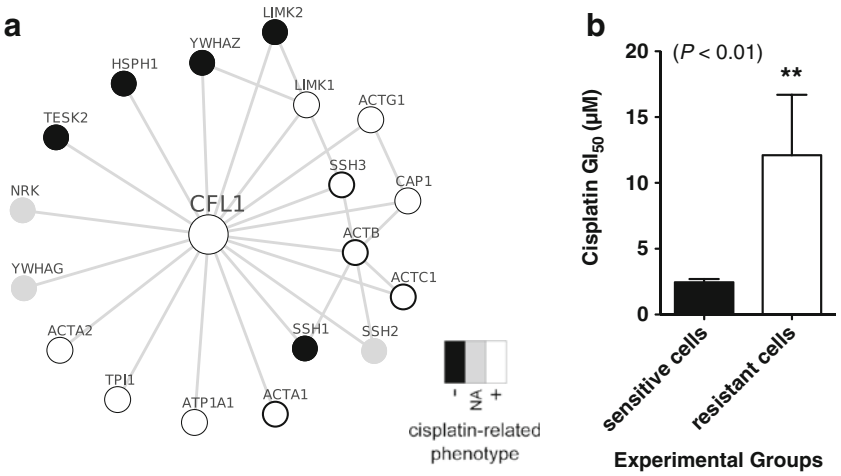

Fig. 1 Differential gene expression levels of the *CFL1* gene network in cisplatin-resistant lung adenocarcinoma cell panel. **a** STRING gene interactions representation of the *CFL1* gene network. A graphic model represents the *CFL1* functional gene network vs. cisplatin drug resistance profiles. Gene expression data of cisplatin-resistant cells were crossed against cisplatin-sensitive cells. *White nodes* are genes up-regulated in resistant phenotype, and *black nodes* are genes down-regulated in resistant phenotype (*gray nodes* are genes not represented in the microarray platform). *Connecting lines* indicate physical and/or functional associations according to experimental data (http://string.embl.de/) as described in Castro et al. [8]. The network drawn was built using a spring model algorithm. Further details are given in the "Materials and methods" section. This network is significantly enriched with up-regulated genes and compared to a bootstrap null distribution estimated in the software ViaComplex ($P < 0.01$). **b** Cisplatin-resistant cells were selected as described in the "Materials and methods" section. $^{**}P < 0.01$ (different from respective control group; Student's *t* test)

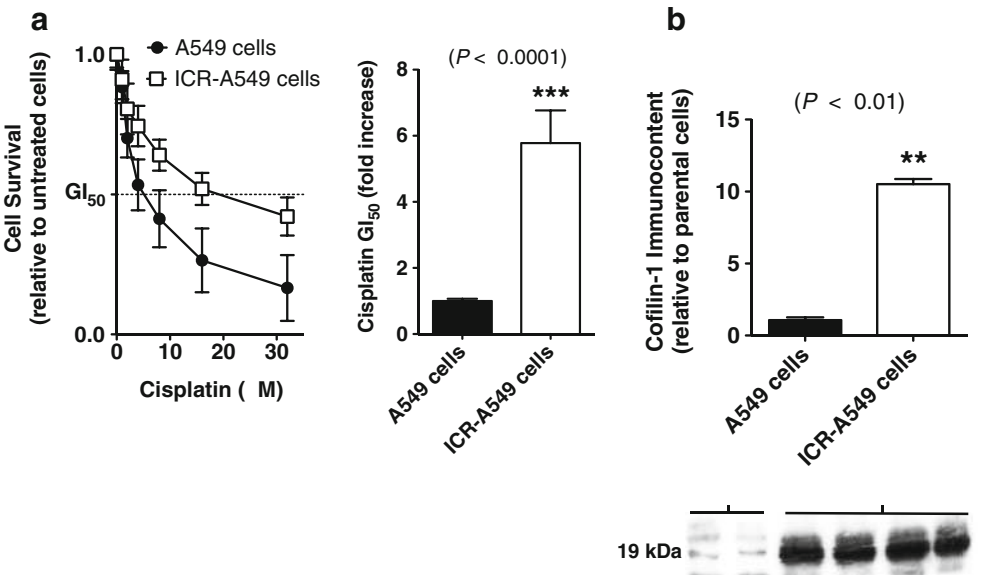

Fig. 2 Increased cofilin-1 immunocontent in intrinsically cisplatin-resistant A549 (*ICR-A549*) NSCLC cells. ICR-A549 cells were selected as described in the "Materials and methods" section and presented a sixfold increase in drug GI_{50} value (**a**) and an increase in cofilin-1 immunocontent (**b**) as compared to parental A549 cells. Data represent mean±S.D. of at least three independent experiments ($n=3$) performed in triplicate. $^{**}P<0.01$ (different from respective control group); $^{***}P<0.0001$ (Student's t test)

Discussion

Most of the NSCLC patients are diagnosed at advanced stage of disease, and some of them are refractory to platinum-based chemotherapy [18]. The primary cause of cancer treatment failure can be found in the biological properties of the malignant system. In that way, the responsibility of current chemotherapy inefficiency can be directly linked to cancer phenotype [19].

Several studies have consistently correlated cofilin-1 levels with a more aggressive phenotype in different tumor tissues [8, 20–22]. These observations were attributed to the critical role played by cofilin-1 in the regulation of cellular migration and invasion capacity [15, 23, 24]. In recent years, however, other functions have been attributed to cofilin-1, such as oxidant-induced apoptosis [11]. More importantly, cofilin-1 has been correlated with multidrug resistance in pancreatic cancers [12] and yeast [25] and with platinum resistance in ovarian cancer cells [26] and in human lung adenocarcinoma cell lines and tumor biopsies [27]. These results are in agreement with the findings presented here. Our data also support further clinical studies to validate the use of cofilin-1 protein as a new predictive biomarker in non-small cell lung cancer, being able to direct decisions in the management of patients with this disease. Therefore, patients with high cofilin-1 immunocontent may not respond adequately for a chemotherapy treatment based on alkylating agents.

Cisplatin constitutes the major therapeutic option in some clinical settings and often leads to an initial therapeutic success. Still, many patients (in particular, in the context of

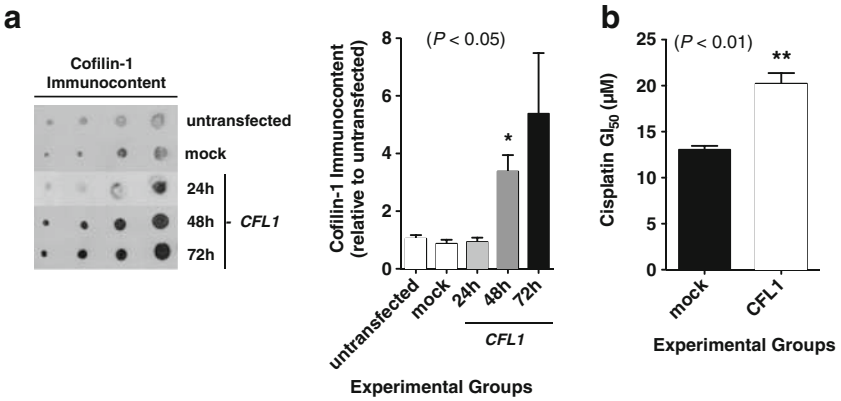

Fig. 3 Transient transfection and overexpression of cofilin-1 lead to an increase in cisplatin resistance in A549 cells. **a** Cells were transiently transfected with the plasmid containing the cDNA sequence of cofilin-1 (*CFL1*) or empty plasmid (mock) as described in the "Materials and methods" section, and cofilin-1 immunocontent was determined by dot blot analysis in different incubation times. **b** At 48 h after transfection with *CFL1* plasmid or mock, A549 cells were treated with different concentrations of cisplatin and drug GI_{50} were determined. Data represent mean±S.D. of at least three independent experiments ($n=3$) performed in triplicate. $^{*}P<0.05$ (different from respective control group; ANOVA, using Tukey's multiple comparison post hoc test); $^{**}P<0.01$ (Student's t test)

colorectal, lung, and prostate cancers) are intrinsically resistant to cisplatin-based therapies. Thus, the development of biomarkers that predict tumor resistance constitutes a goal with important clinical implications. Several mechanisms account for the cisplatin-resistant phenotype of tumor cells. Most described are drug reduced uptake/increased efflux (mediated mainly by the plasma membrane copper transporter *CTR1*, copper-extruding P-type ATPases *ATP7A*/*ATP7B*, and the member of the ABC family of transporters *MRP2*), increased inactivation (by GSH/γ-GCS/GST and metallothioneins), and increased repair capacity of DNA lesions (mediated by members of the nucleotide excision repair pathway such as *ERCC1* or by the machinery for homologous recombination *BRCA1*/*BRCA2*) (see review by Galluzzi et al. [28]). In this scenario, the role of cofilin-1 in tumoral cisplatin resistance is not evident. Cofilin-1 presents a nuclear localization signal in its primary structure and can, under a specific chemical or physical stimulus, translocate into the nucleus. However, the role of this protein in the nuclear compartment is still unclear [29–31].

Platinum-based chemotherapy is the therapeutic foundation of treatment both in the metastatic and adjuvant setting of NSCLC patients. Because cofilin-1 levels appear to be a marker of resistance to platinum agents, patients whose tumors harbor high levels of this protein would benefit from a different treatment modality. This discovery indicates that many individuals may be assigned to a therapy that has little chance of success in their particular case, something that will hopefully change as a result of this research. Thus, our findings could clearly impact cancer therapy. Ultimately, the refinement of patient stratification with the use of cofilin-1 levels, as all promising predictive biomarker, requires prospective validation in carefully designed randomized, large-scale clinical trials.

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Conflicts of interest None

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Resultados Suplementares ao Capítulo 1

Os resultados apresentados na revista *Tumor Biology* reforçam a relação entre variação na expressão do gene/proteína cofilina-1 e sensibilidade/resistência à cisplatina. A partir desses resultados, realizamos ensaios *in vitro* para avaliar os possíveis mecanismos de resistência à cisplatina nos modelos de resistência intrínseca e adquirida.

METODOLOGIA

- Cultivo Celular e Modelo Celular de Resistência:

A linhagem humana de CPNPC A549, obtida da ATCC, foi mantida em meio RPMI, suplementado com 10% de soro fetal bovino (FBS), Streptomicina e Penicilina, em uma atmosfera umidificada de CO_2 5% em 37°C. Conforme descrito no artigo publicado na revista *Tumor Biology* (figura 1B), para o modelo de resistência intrínseca, as células foram tratadas com dez vezes o valor de GI50 de cisplatina encontrado no ensaio de viabilidade. Para o modelo de resistência adquirida, as células foram tratadas com doses graduais e crescentes de cisplatina até a concentração de três micromolar. Após os tratamentos, a presença de resistência foi avaliada pela alteração do valor de GI50, utilizando a técnica de sulforodamina B (SRB) (Skehan, Storeng et al. 1990). Os tratamentos foram realizados com as células em ~ 75% de confluência. A quantidade de proteínas das amostras, para correção de dados dos ensaios bioquímicos, foi mensurada pela técnica de Bradford (Bradford 1976).

- Parâmetros de Resistência:

A avaliação do efluxo de droga foi feita utilizando o efluxo celular de rodamina 123 (Altenberg, Young et al. 1993) por citometria de fluxo. Nesse protocolo, as células são incubadas por 30 minutos com rodamina 123; em seguida, são lavadas com PBS a 0ºC. Depois, é feita uma nova incubação de 180 minutos para expulsão da droga e nova lavagem com PBS a 0ºC. Por fim, as células são tripsinizadas e é feita a avaliação por citometria.

Para abordar o mecanismo de inativação intracelular da droga, realizamos ensaios de dosagem de grupamentos tióis totais livres, GSH e atividade de glutationa-*S*-transferase (GST). Na técnica de dosagem de grupamentos tióis totais livres (Sedlak and Lindsay 1968), os grupos SH livres das proteínas reagem com DTNB *(5,5'-Dithiobis(2-nitrobenzoic acid)),* resultando na

formação da proteína tionitrofenilada e um ânion – 2-nitro-5-tiobenzoato –, amarelado. Após incubação de 1 hora com DTNB, é feita a leitura espectrofotométrica em 420 nm. Para o cálculo, tem-se que SH (mol)= (leitura final-branco) X (volume final do poço em litros)/(14,15 X 0,6), em que 14,15 $M^{-1}cm^{-1}$ é o fator de extinção molar e 0,6 é o fator de conversão de cuveta para placa.

Com relação à quantificação de GSH (Akerboom and Sies 1981), sabe-se que a reciclagem de GSH total (GSH + GSSG, em equivalents de GSH) é um procedimento sensível e específico. Como indicado na reação abaixo, GSH é oxidado por *5,5' -dithiobis(2-nitrobenzoic acid*) (DTNB) para gerar GSSG e *5-thio-2-nitrobenzoic acid* (TNB). GSSG é reduzido à GSH pela ação de glutationaredutase (GR), com a utilização de NADPH como cofactor. A taxa de formação de TNB é monitorada espectrofotometricamente a 412 nm e é proporcional à soma de GSH e GSSG presente. O ensaio pode ser também avaliado pelo decaimento de NADPH a 340 nm.

2GSH + DTNB → GSSG + TNB

A atividade de Glutationa-*S*-Transferase (GST) (Pabst, Habig et al. 1974) baseia-se na formação do conjugado de *1-chloro-2,4-dinitrobenzene* (CDNB) com glutationa reduzida, reação catalisada pela GST. A taxa de formação desse produto é acompanhada a 340 nm e é proporcional à atividade da enzima.

GSH + CDNB → GS-DNB + HCl

RESULTADOS

Tendo em vista os dados apresentados que evidenciam a correlação da expressão de cofilina-1 com resistência ao tratamento com ciplatina, nós realizamos testes in vitro para avaliação dos principais mecanismos de resitência. Para avaliação da inativação intracelular de drogas, quantificamos a quantidade de tióis reduzidos total, GSH e a atividade de glutationa-S-transferase (GST) em células controle e resistentes à cisplatina. A quantidade de tióis reduzidos total não mostrou diferença significativa. Porém a quantidade de GSH disponível mostrou diminuição significativa em relação ao controle nos grupos de resistência adquirida. Com relação à atividade de GST, observamos aumento significativo com relação ao controle nos grupos resistência adquirida 3.0 μM e resistência intrínseca. Esses dados encontram-se representados na figura 7.

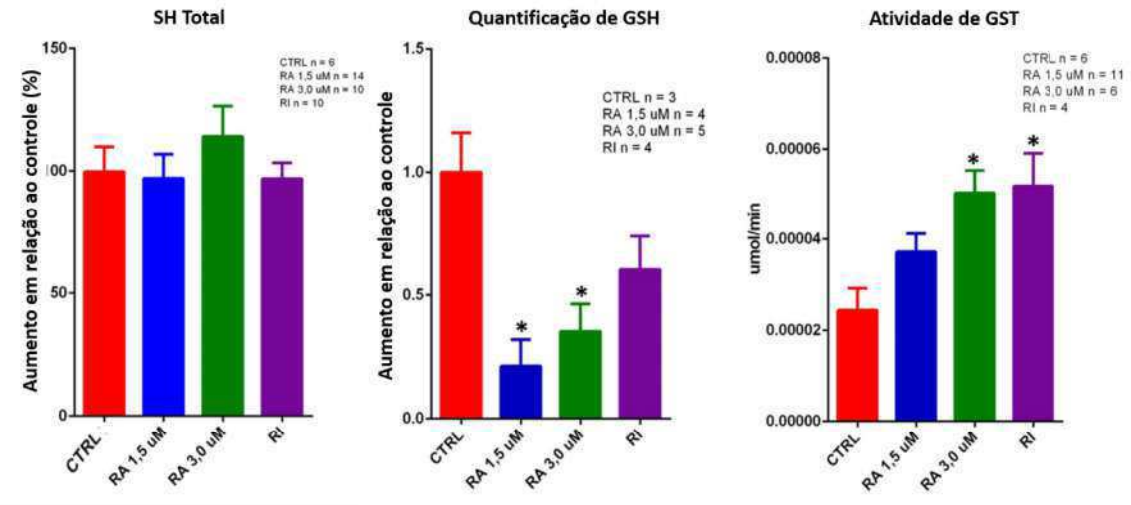

Figura 7. Avaliação *in vitro* dos parâmetros de resistência em células A549 com resistência intriseca e adquirida à cisplatina. A análise da quantidade de tióis não mostrou diferença significativa entre os grupos. A quantificação de GSH nos dois grupos de resistência adquirida (RA 1,5 e RA 3,0) foi significativamente menor quando comparado aos grupos CTRL (controle). A atividade de GST mostrou-se aumentado nos grupos RA 3,0 e RI quando comparados ao CTRL. Os resultados foram avaliados por Anova de uma via e pos teste de Tukey; foi considerado estatisticamente significativo $P < 0,05$.

Para a avaliação do efluxo extracelular de drogas, utilizamos citometria de fluxo. Conforme demonstrado na figura 8, o efluxo de rodamina 123 não mostrou diferenças significativas no perfil de dinâmica de exclusão entre os grupos experimentais testados.

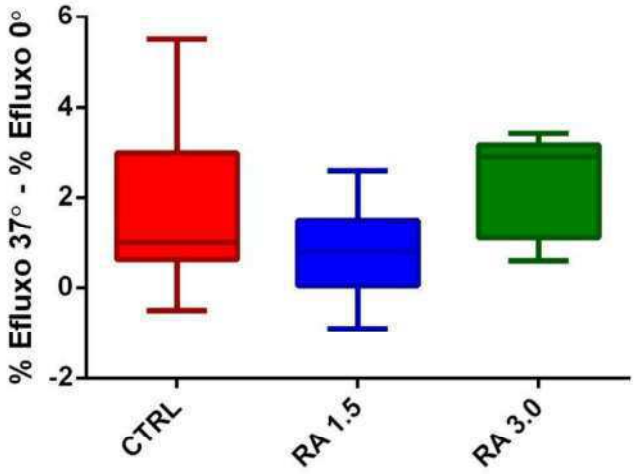

Figura 8. Efluxo extracelular de drogas por citometria de fluxo. A avaliação do efluxo de rodamina 123 não apresentou diferença estatística entre os grupos CRTL (controle) e resistência adquirida (RA 1,5 e RA 3,0). Para análise estatística foi realizada ANOVA de uma via; foi considerado estatisticamente significativo $P < 0,05$

Capítulo 2
Artigo publicado na revista Oncotarget.

"**Potential crosstalk between cofilin-1 and EGFR pathways in cisplatin resistance of non-small-cell lung cancer**"

Potential crosstalk between cofilin-1 and EGFR pathways in cisplatin resistance of non-small-cell lung cancer

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Keywords: NSCLC, EGFR, cofilin-1, chemotherapy resistance, personalized medicine

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ABSTRACT

Current challenge in oncology is to establish the concept of personalized medicine in clinical practice. In this context, non-small-cell lung cancer (NSCLC) presents clinical, histological and molecular heterogeneity, being one of the most genomically diverse of all cancers. Recent advances added Epidermal Growth Factor Receptor (EGFR) as a predictive biomarker for patients with advanced NSCLC. In tumors with activating EGFR mutations, tyrosine kinase inhibitors (TKI) are indicated as first-line treatment, although restricted to a very small target population. In this context, cofilin-1 (a cytosolic protein involved with actin dynamics) has been widely studied as a biomarker of an aggressive phenotype in tumors, and overexpression of cofilin-1 is associated with cisplatin resistance and poor prognosis in NSCLC. Here, we gather information about the predictive potential of cofilin-1 and reviewed the crosstalk between cofilin-1/EGFR pathways. We aimed to highlight new perspectives of how these interactions might affect cisplatin resistance in NSCLC. We propose that cofilin-1 quantification in clinical samples in combination with presence/absence of EGFR mutation could be used to select patients that would benefit from TKI's treatment. This information is of paramount importance and could result in a possibility of guiding more effective treatments to NSCLC patients.

INTRODUCTION

The current challenge in oncology is to establish the concept of personalized medicine in clinical practice [1]. Classification into subpopulations differed by their susceptibility to a particular disease and response to a specific treatment allows therapeutic intervention to be focused on patients who will greatly benefit from it, sparing those who will not [2].

For cancer therapeutics, the use of specific characteristics of mutational status and deregulated pathways of tumor itself might help to prevent, diagnose and treat the disease [3]. The central hypothesis is that treatment decisions based on tumor genotype and genomic profile, correlated with clinical factors, would improve clinical outcomes, as measured by response rate, survival and safety [4]. Furthermore, to guarantee that patients can access personalized medicine, a new paradigm has evolved, the "P4" (standing for *p*redictive, *p*reventive, *p*ersonalized and *p*articipatory medicine), based on scientific, organizational and wellness strategies. Thus, to achieve that, oncology will have to move from a reactive to a proactive discipline [5].

This approach has good application to heterogeneous disorders, such as lung cancer whose development and manifestation vary greatly from patient to patient. Lung cancer is a disease with clinical, histological and molecular heterogeneity, remaining one

of the leading causes of cancer mortality worldwide [6]. The lethality of this disease can be attributed to late diagnosis (hindering the possibility for surgical treatment), resistance to chemotherapy treatments and emerging of complications in advanced stages [7]. Additionally, traditional lung cancer chemotherapy is not curative and provides limited benefits, with average survival of less than one year. Nevertheless, we faced a decade of significant advances in the identification of key driver events in lung carcinogenesis and target lung cancer therapies [8]. The most prevalent type of lung cancer is non-small-cell lung cancer (NSCLC). It is also described as one of the most genomically diverse of all cancers [9]. This feature imposes a great challenge for prevention and treatment strategies, but at the same time provides a number of opportunities for intervention by ungrouping NSCLC into a variety of molecularly defined subsets [4, 6]. In view of such challenges, finding biomarkers that could overcome these obstacles and group patients according to optimal responsiveness and efficacy, would lead to a better treatment and management.

Recent advances added EGFR (Epidermal Growth Factor Receptor) and ALK (Anaplastic Lymphoma Kinase) as biomarkers that should be tested for in patients with advanced lung cancer. For tumors with activating EGFR mutations (e.g.: L858R and E746-A750del), EGFR tyrosine kinase inhibitors (EGFR-TKI) (such as gefitinib, erlotinib, and afatinib) are indicated as first-line treatment [10]. Although this treatment is already in clinical practice, there is still controversy about its effect on patients overall survival (OS); in addition, it seems to be very restricted to a target population composed primarily of non-smoking women with adenocarcinoma [11].

In this context, cofilin-1 – a small protein of 18 kDa – has been widely studied as a biomarker of a more aggressive phenotype of different types of cancer such as breast, gastrointestinal and NSCLC [12-14]. The comprehension of its association with EGFR and relation with conventional alkylating agent-based therapy resistance, could help to discriminate and increase the suitable population to TKI's treatment. Here, we gather information about cofilin-1 therapeutic prediction potential and review the crosstalk between cofilin-1 and EGFR pathways, highlighting new perspectives of how these interactions might affect cisplatin resistance in NSCLC.

Cofilin-1 and its predictive role in cancer chemotherapy

Cofilin-1 (*CFL1*; non-muscle isoform; Gene ID: 1072) is a conserved and ubiquous protein in mammals, classically involved with actin polymerization/depolymerization dynamics [15]. In the last decade, however, new and unexpected roles of this protein have been described in other pathological and physiological cellular situations, such as apoptosis induced by oxidants [16] and intracellular rods formation in neurodegenerative diseases [17-19].

Over the last 20 years, several studies have pointed cofilin-1 as an important protein in aggressive cancer cell behavior, due to its involvement in the coordination of tumor cell migration and invasion [12, 20-24]. There are four important mechanisms that regulate the activation status of cofilin-1: (1) its dephosphorylation at Ser3; (2) its release from phosphatidylinositol-4,5bisphosphate (PtdIns(4,5)P2); (3) its release from cortactin; and (4) regulation by oxidation/reduction of one of its four cysteins residues [16]. Dephosphorylation of cofilin-1 at Ser3 was the first activation mechanism to be well characterized. Slingshot (SSH) was shown to be a major phosphatase responsible for dephosphorylating cofilin-1 at Ser3, and chronophin (CIN) was recently identified as a cofilin-1 specific phosphatase. In addition, the serine-phosphatases PP1 and PP2A can also dephosphorylate cofilin-1 at Ser3. On the other hand, LIMK1 and LIMK2 as well as TES kinase 1 (TESK1) and TESK2 phosphorylate cofilin-1 at Ser3 *in vivo*. LIMK1/2 are the most well studied kinases and have been proposed to be the dominant kinase in the regulation of actin dynamics by mediating cofilin-1 inactivation. Cofilin-1 can still be inactivated by its interaction with PtdIns(4,5)P2 at the plasma membrane. This follows a general mechanism whereby membrane lipids have been shown to bind various actin regulatory proteins. In migrating cells, the hydrolysis of PtdIns(4,5)P2 can release cofilin-1 from its inhibitory interaction with the membrane lipids, resulting in the local activation of F-actin filament severing, protrusion and cell polarity. Finally, the binding of cofilin-1 to the actin regulatory protein cortactin also negatively regulates cofilin-1 activity, and this mechanism seems to be specific to invadopodia formation [12, 25, 26]. Deregulations of such pathways, favoring tumorigenesis, have been described in some extension for different types of carcinomas, like breast, oral, ovarian, prostate, melanoma and gastrointestinal cancer, indicating a strong prognostic correlation [12, 13, 27-31].

Regarding NSCLC, a series of correlational studies using meta-analysis of microarray data showed that mRNA level of *CFL1* in NSCLC can discriminate between good and bad prognosis, in which tumors with high expression of *CFL1* are associated with low overall survival (OS) [14, 32]. This microarray data was validated in a retrospective NSCLC cohort by a semi-quantitative immunohistochemistry method [33]. Meta-analysis of other independent cohorts microarray data also corroborates that cofilin-1 has a prognostic capability, indicating that patients with higher levels of this protein are more likely to be at the poorer outcome group (Figure 1). In these works, however, the relation of cofilin-1's expression with a more aggressive phenotype of tumors was attributed to its classical activity upon actin

cytoskeleton modulation, related to improved migration and invasion capacity in cancer cells, as reviewed recently [26]. Moreover, NSCLC cell lines with high cofilin-1 immunocontent have high invasive potential and were found to be resistant to cisplatin and carboplatin treatment (compounds that are gold-standard drugs used in NSCLC patient management), indicating that cofilin-1 might also present a predictive aspect to be explored [14].

Hints of a possible role of cofilin-1 in the cellular resistance against alkylating agents have been described in cisplatin/carboplatin resistant ovarian cell lines almost 10 years ago [34]. Regarding NSCLC, available data from pre-clinical studies point to the same direction [14, 35, 36]. Analysis of microarray data in a drug screening cell panel (NCI60 cell panel) of 118 chemotherapeutic compounds showed that *CFL1* mRNA level is correlated with resistance against 21 of 30 alkylating agents (such as cisplatin and carboplatin) tested [14]. High levels of cofilin-1 were found in cisplatin-resistant A549 NCSLC cells and A549 cells transiently overexpressing *CFL1* plasmid present an increased in GI_{50} value for cisplatin [36]. Wei and collaborators also found high levels of cofilin-1 in cisplatin-resistant NSCLC cell lines using proteomics studies [35]. These studies support the idea that high level of cofilin-1 correlates with cisplatin resistance.

Several mechanisms account for the cisplatin-resistant phenotype of tumor cells. Most described are drug reduced uptake/increased efflux (mediated mainly by the plasma membrane copper transporter CTR1, copper-extruding P-type ATPases ATP7A/ATP7B, and members of the ABC family of transporters MRP and MDR), increased inactivation (by GSH/γ-GCS/GST and metallothioneins), and increased repair capacity of DNA lesions (mediated by members of the nucleotide excision repair pathway such as ERCC1 or by the machinery for homologous recombination BRCA1/BRCA2) [37]. Cisplatin cytotoxic is described by its interaction with nucleophilic sites in N7 position of purines in DNA, forming DNA-protein interactions, inter and intra-strands crosslinks and DNA adducts [38], which are the main lesions responsible for cell death [39]. More than 90% of cisplatin-DNA adducts result in crosslinks 1.2 d (GpG) intra-strands, which modifies the three dimensional structure of the DNA molecule, enabling this site for several proteins recognition. These proteins include damage recognition components of the mismatch repair (MMR) complex, such as group 1 and 2 proteins of non-histone high mobility group of proteins (HMG1 and HMG2), proteins related to nucleotide excision repair (NER), among others [38, 40]. In this scenario, the precise mechanism that leads to cisplatin resistance is not well established. Cofilin-1 presents a nuclear localization signal in its primary structure and can translocate into the nucleus under specific chemical or physical stimuli (Figure 1) [41, 42]. These information hints the possibility that cofilin-1 could have a nuclear role in supporting the DNA repair system.

Although these data could potentially impact an

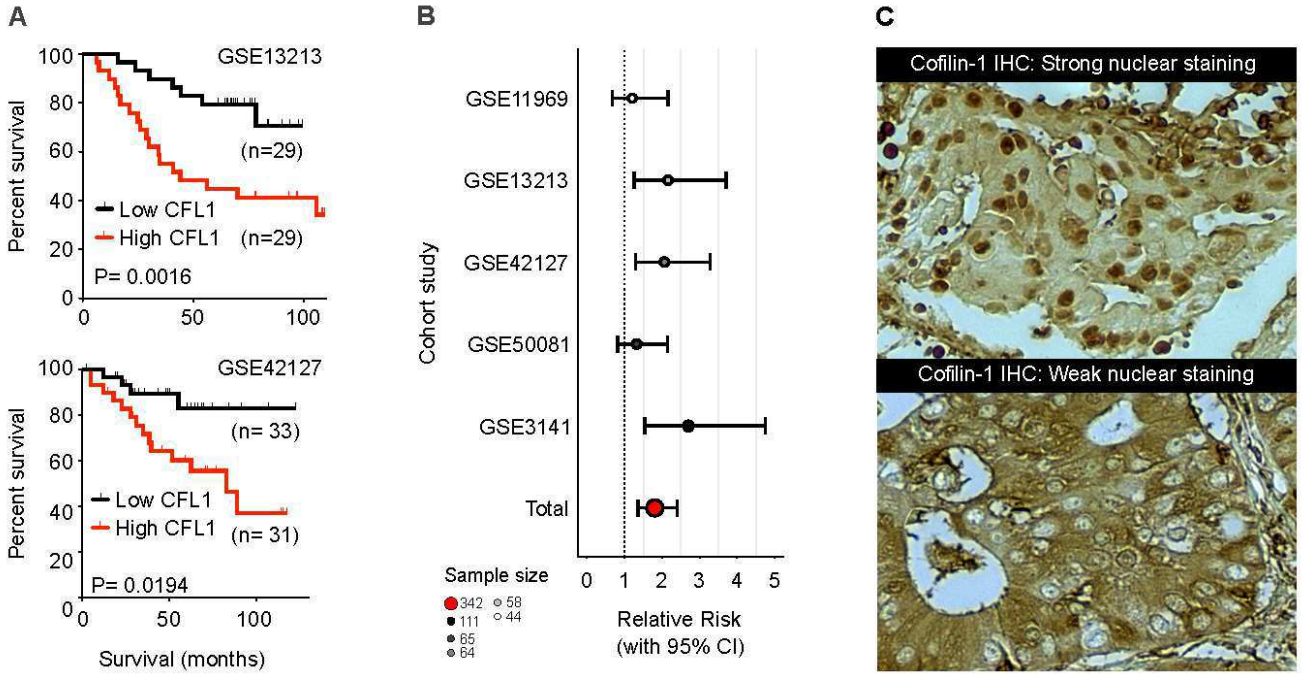

Figure 1: Meta-analysis results of cofilin-1 prognostic potential (A) Kaplan-Meier mortality curves indicating *CFL1* strength in predicting patient survival. (B) Forest plot of five different studies showing relative risk of death in high expressing *CFL1* mRNA patients. Microarray data were obtained from Gene Expression Omnibus (GEO) online repository (http://www.ncbi.nlm.nih.gov/geo/). (C) Immunohistochemistry for cofilin-1 in two different NSCLC slides, presenting presence/absence of nuclear staining.

appropriate treatment prediction, many questions related to these events remain to be answered. A *sine qua non* condition to use this information in patient benefit is to visualize cofilin-1 pathway interactions and how this might affect cellular resistance machinery.

EGFR: a biological marker in clinical practice

The EGF receptor (EGFR) belongs to the ErbB family of receptor tyrosine kinase (RTK) greatly known for its involvement with pro-tumorigenic pathways [43]. EGFR, or HER1, is one of a family of epidermal growth factor (EGF) receptors that also includes ErbB2/HER-2, ErbB3/HER-3, and ErbB4/HER-4. Binding of its ligands result in conformational change of EGFR, homodimerization or heterodimerization with other members of the receptor family, and autophosphorylation of the cytoplasmic tyrosine kinase domain. EGFR signaling network has an interactive nature, being one of the most deregulated molecular pathways found in human cancer. The major pathways downstream EGFR activation are Ras/Raf/MEK, PI3K/AKT/mTOR, JAK2/STAT3 and PLC-gamma/PKC [43-45]. All these pathways are important for tumor growth, progression and survival.

Besides that, EGFR at different subcellular location has different functions and overlapping signals [45]. Therefore, various strategies of targeting EGFR or its family members have been developed and are in different phases of clinical trials [46]. However, feedback and crosstalk circuits between signaling pathways could limit the selection of one driven gene mutation for treatment with a matching drug. This underlines the difficulty of using a single marker to predict patient susceptibility to a particular disease and response to a specific treatment. Another important factor of tumor aggressiveness is

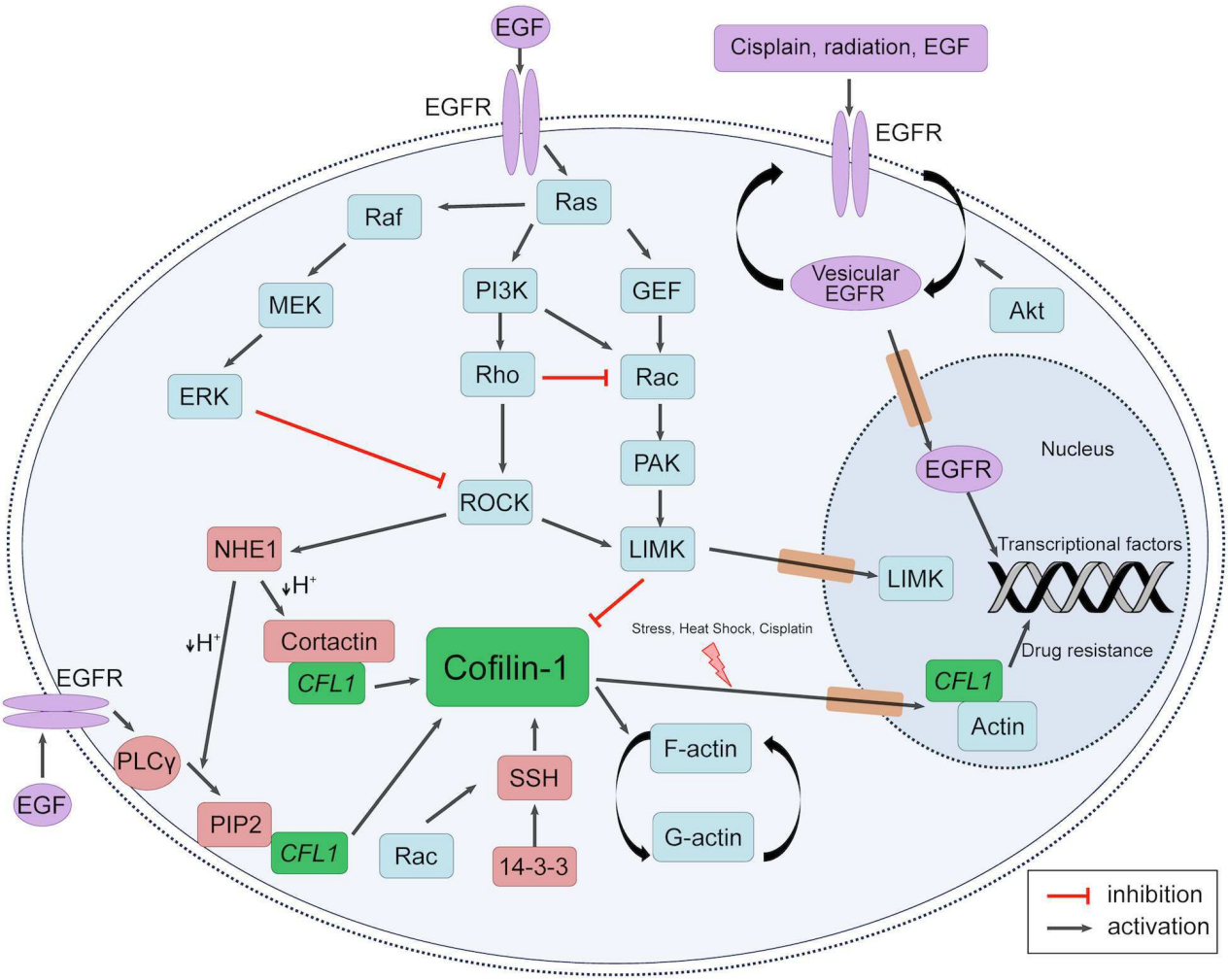

Figure 2: EGFR and Cofilin-1 cytosolic and nuclear crosstalk. Schematic representation of EGFR and cofilin-1 pathways intersections. Different local stimuli may result in cofilin-1 modulation through EGFR activation. Downstream EGFR pathways may activate cofilin-1 through dephosphorylation by SSH1 and release of cortactin and PIP_2 bounds by intracellular pH alteration; also, it may result in cofilin-1 inactivation by LIMK activity. Cofilin-1 and EGFR may also translocate into nucleus in response to external stimuli, indicating a possibility of related mechanisms of drug resistance.

the potential cell migration and ability to leave primary tumor sites. In this aspect, EGF has been shown to be an important chemotactic molecule both in physiological and in pathological situations [47]. In fact, in MDA-MB-231 breast cancer cells, PI3K and PLC-gamma pathways indeed promote migration [48]. Thus, research to identify active pathways downstream EGFR activation could lead the rationale for the development of multidrug combination therapies striking several critical points important to tumor development [49].

Cytosolic and nuclear crosstalks between Cofilin-1 and EGFR pathways

There is an intense crosstalk between EGFR and cofilin-1 pathways, as summarized in Figure 2. Indeed, EGFR downstream routes indirectly regulate all of the described cofilin-1 activation/inactivation mechanisms. Cofilin-1's major kinase, LIMK1, is modulated via EGFR-PI3K route. PI3K activates small Rho GTPases such as Rac and CDC42, which mediate activation of p21-activated kinase 1 (PAK1) and Rho-dependent protein kinase 1 (ROCK1). Afterwards, these kinases phosphorylate, and activate LIMK [50-53]. On the other hand, cofilin-1 dephosphorylation by SSH1 may also be modulated downstream EGFR [54]. As Kligys and collaborators have demonstrated, SSH1 activation occurs via Rac1 in keratinocytes [55]. Moreover, it is well established that EGFR signaling activates Rac1 [56]. Therefore, EGFR pathway can modulate the phosphorylation (and so the activation) state of cofilin-1.

Another intersection between EGFR and cofilin-1 pathways is via PLC gamma activation followed by PtdIns(4,5)P2 hydrolysis, an important mechanism of local cofilin-1 mobilization [25, 57]. Lastly, *tyr* phosphorylation of cortactin by Arg kinase, which is activated downstream of EGFR, regulates the interaction between the Na^+-H^+ exchanger 1 (NHE1) and cortactin. NHE1 increases the intracellular pH, which induces the release of cortactin-bound cofilin-1 [58, 59]. Therefore, EGFR pathway plays a pivotal role over cofilin-1 activity states in response to different cellular stimuli, leading to several ways to modulate cell adaptation either in pathological as well as physiological situations.

Nuclear localization of EGFR was first observed more than two decades ago in hepatocytes [60]. Only recently, however, the nuclear translocation of this protein was shown to be induced by several stimuli, such as EGF, ionizing radiation and cisplatin treatment [61]. Activation of EGFR results in its endocytosis and interaction with importin β1 via its tripartite nuclear localization sequence [62]. Moreover, EGFR undergoes to nucleus via a retrograde trafficking from Golgi apparatus to ER. Once embedded into the ER membrane, EGFR and importin β1 interface with nucleoporins in the nuclear pore complex (NPC) to shuttle EGFR from the outer nuclear membrane (ONM) to the inner nuclear membrane (INM) [63, 64]. Once EGFR is inside the nucleus, it may display four major functions: *i*) promote gene regulation (an independent kinase activity of EGFR), acting as a co-factor and increasing expression of target genes, like iNOS, COX-2, c-Myc, cyclins and others, contributing to several malignant phenotypes of human cancers; *ii*) phosphorylates proliferating cell nuclear antigen (PCNA), promoting its stability and contributing to cell proliferation and DNA repair (an activity dependent of its kinase activity); *iii*) interacts with DNA-dependent protein kinase (DNA-PK) and enhances the DNA repair machinery; *iv*) co-localizes with γH_2AX complex, enabling chromatin relaxation for DNA repair process [65, 66]. Therefore, it is not surprising that a growing body of evidence has demonstrated a strong association between nuclear EGFR and resistance to chemotherapy/radiotherapy in tumors.

It has been reported that cisplatin stimuli can induce EGFR activity and downstream events and this process is ligand-independent [67]. Regarding cisplatin resistance, murine NIH-3T3 fibroblasts cells treated with cisplatin had an increasing in nuclear EGFR associated with DNA-PKs, which contributed to cisplatin resistance [61]. This involvement of nuclear EGFR and DNA-PK enhancing DNA repair and cisplatin resistance was also demonstrated in human tumor cell lines [68]. Moreover, nuclear EGFR was correlated with shorter progression-free survival in early NSCLC stage [69]. This association with poor prognosis is in accordance with the fact that nuclear EGFR activity was related to tumor radio and chemoresistance. However, it is not yet clear how nuclear EGFR affects TKI and antibodies target therapies.

On the other hand, nuclear translocation of cofilin-1 was first described in 1987 by Nishida and collaborators in mouse fibroblast cell line C3H-2K stimulationed with 10% of dimethyl sulfoxide (DMSO) or heat shock at 42-43°C for 60 minutes [70]. Afterward, studies have shown that cofilin-1 nuclear translocation upon such stimuli requires dephosphorylation at serine-3 domain to expose its nuclear localization signal (NLS). Moreover, cofilin-1 seems to play an important role in cellular stress contexts by leading monomeric actin (G-actin) inside the nucleus, since G-actin does not have NLS [41, 71-73]. For example, Sotiropoulos and colleagues showed that monomeric actin is able to inhibit SRF (serum response factor)-dependent gene transcription activation inside the nucleus [74]. However, cofilin-1 appears to have functions besides actin translocation when inside the nucleus. Indeed, studies have pointed a direct role of cofilin-1 in modulation of transcription independently of actin [73, 75]. Additionally, the regulation of cofilin-1 inside the nucleus may also contribute to phenotype changes, since nuclear LIMK enhances human breast cancer progression [76]. Hence, the roles cofilin-1 may play inside the nucleus are still a prospect for further studies. Likewise, there

are no studies trying to associate nuclear cofilin-1 with patient's outcome/prognosis in lung cancer.

CONCLUSIONS AND FUTURE DIRECTIONS

Considering the information gathered here, it seems clear that cofilin-1 regulation and functions are closely related to EGFR activity. However, some evidences allow the assumption of a greater extent of these interactions. EGFR functions inside the nucleus have been subject of intense study, leading to many possible roles of its translocation upon several stimuli [77]. As presented in figure 2, cisplatin is one of these stimuli, which may lead to nuclear EGFR translocation in tumor cells and resistance to treatment, as result of an enhanced DNA repair [62]. In this same scenario, we have described a positive correlation between cofilin-1 expression and cisplatin resistance in NSCLC cell lines [14, 36]. Considering these facts, could cofilin-1 be affecting EGFR translocation to the nucleus? Indeed, cofilin-1 signaling plays a pivotal role in the regulation of efficient EGFR vesicular trafficking in invasive tumor cell [78, 79].

Since cofilin-1 has a nuclear location signal (NLS) and may enter into nucleus, as presented in figure 1C, would its activity be restricted to EGFR vesicular trafficking? Could nuclear cofilin-1 also play a direct role in the resistance mechanism to platinum compound? Dopie and colleagues have shown that actin constantly shuttles between cytoplasm and nucleus and they assign to cofilin-1 the role of regulating this continuous *steady-state* actin flow [73]. Based on this, cofilin-1 could be necessary to maintain a *pool* of actin inside the nucleus thus maintaining a "nuclearskeleton" of actin. This could contribute to the transcriptional action of EGFR within the nucleus. On the other hand, cofilin-1, as well as EGFR, can act directly on transcription. According to Obrdlik and Percipalle, cofilin-1 is a key regulator of pol II transcription and its interaction with actin would facilitate the association of transcription machinery with actively transcribed genes [75].

Therefore, seems that cofilin-1 and EGFR pathways are closely related in driving the resistance machinery to cisplatin. Further studies that could evaluate co-localization and activity of cofilin-1 and EGFR in cancer cells would help to elucidate how exactly they are working together towards resistance behavior against cisplatin treatment. Given that increased expression of cofilin-1 is directly related to cisplatin resistance, we propose that its quantification could be used in association with presence/absence of EGFR mutation to guide which patients would benefit better from TKI's treatment. Moreover, studies associating both variables with patient´s outcome could better elucidate this relationship. This information is of paramount importance and may, ultimately, result in a possibility of guiding more effective treatments to NSCLC patients, potentially expanding the target population.

CONFLICT OF INTEREST DECLARATION

The authors of this manuscript have no conflicts of interest to declare.

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Resultados Suplementares ao Capítulo 2

Considerando a hipótese formulada no artigo publicado na revista *Oncotarget*, nós realizamos ensaios *in silico* para avaliar as probabilidade de interação entre cofilina-1 e EGFR na hipótese de colocalização nuclear e ensaio clínico preliminar com base em *lung cancer tissue array* para testar a hipótese de colocalização e interação dos marcadores cofilina-1 e EGFR

- Ensaio *in silico* -

METODOLOGIA

- Cálculos de Atracamento (*docking)* Molecular, Análises Estruturais e Energia de Interação

As estruturas proteicas utilizadas neste trabalho correspondem aos arquivos com identificação no banco de dados *Protein Data Bank* – PDB: 1Q8G para cofilina-1 humana (Pope, Zierler-Gould et al. 2004); a porção extracelular do receptor EGFR foi representada pelas entradas 4UV7 (resíduos 25-645) (Lim, Yoo et al. 2016), 1MOX (resíduos 1-501) (Garrett, McKern et al. 2002) e 5SX4 (resíduos 335-525) (Sickmier, Kurzeja et al. 2016). Já a porção intracelular do receptor foi representada pelas estruturas 5HG8 (resíduos 695-1022) (Cheng, Nair et al. 2016), 3LZB (resíduos 696-1022) (Fidanze, Erickson et al. 2010) e 5JEB (resíduos 696-1022) (Novotny, Pollari et al. 2016). O arquivo 5L6W representa o complexo cofilina-1-LIMK1 (10.2210/pdb5l6w/pdb). O complexo actina-cofilina-1 foi obtido através da seleção do complexo resultante de menor energia entre os arquivos 1Q8G (cofilina-1) e 1J6Z (Otterbein, Graceffa et al. 2001). O *software* CluPro 2.0 (Comeau, Gatchell et al. 2004) foi utilizado para os cálculos de atracamento molecular, cujo algoritmo seleciona as conformações com propriedades eletrostáticas e de dessolvatação favoráveis, agrupa as estruturas resultantes através do RMSD (raiz do desvio quadrático médio) e seleciona os centros dos clusters mais populosos. Todos os cálculos foram realizados sem a imposição de restrições físicas, isso é, o ligante ficou livre para procurar pela melhor forma de ligação com o receptor. Após a conclusão dessa etapa, os dez melhores resultados selecionados pelo sistema de ranqueamento do programa foram normalizados por um método externo. Esse método consiste na avaliação da energia de interação entre ligante e receptor através do software FoldX (Schymkowitz, Borg et al. 2005). O *software* FoldX consiste em um conjunto de campo de forças empírico

desenvolvido para avaliar rapidamente o efeito de mutações na estabilidade, dobramento e dinâmica de proteínas e ácidos nucléicos, possibilitando o cálculo da energia livre de uma determinada molécula baseado em sua estrutura tridimensional. Primeiramente, a função *Repair*PDB foi aplicada para eliminar colisões provenientes de ângulos e torções inadequados e otimizar a estrutura. Após, a energia de interação, dada em kcal/mol, entre as proteínas dos complexos formados foi calculada e utilizada como parâmetro para definir a interação putativa de maior probabilidade ou mais favorecida de ocorrer. Os modelos selecionados e que apresentaram menor energia de interação foram visualizados e analisados através do *software Biovia Discovery Studio*.

RESULTADOS

Em um primeiro momento, foram realizados cálculos de *docking* entre a molécula de cofilina-1 (representada pela estrutura 1Q8G) e as porções extracelular e intracelular do EGFR. Para aumentar a diversidade dos complexos resultantes, optamos por utilizar três estruturas representativas da porção extracelular de EGFR (PDB 4UV7, 1MOX, 5SX4) e três estruturas representativas da porção intracelular de EGFR (PDB 5HG8, 3LZB, 5JEB). Os resultados obtidos compõem as tabelas 4 e 5 e sugerem que a interação é favorecida no complexo 4UV7-1Q8G, com energia de interação de -21.42 kcal/mol, e no complexo 5JEB-1Q8G, com energia de interação de -16.04 kcal/mol. Os complexos resultantes estão representados na figura 9.

Tabela 4. Energia de interação dos complexos cofilina-1 - EGFR (porção extracelular)..

Energia de interação dos complexos cofilina-1 - EGFR (porção extracelular)					
4UV7-1Q8G	ΔG	1MOX-1Q8G	ΔG	5SX4-1Q8G	ΔG
complexo 0	-12,19	complexo 0	-2,48	complexo 0	-12,64
complexo 1	**-21,42**	complexo 1	-5,42	complexo 1	-7,24
complexo 2	-6,55	complexo 2	-10,86	complexo 2	-12,43
complexo 3	-10,58	complexo 3	-7,08	complexo 3	-2,28
complexo 4	-5,55	complexo 4	-3,07	complexo 4	-3,81
complexo 5	-6,55	complexo 5	-13	complexo 5	-8,49
complexo 6	-13,09	complexo 6	-4,27	complexo 6	-13,42

complexo 7	-6,94	complexo 7	0,55	complexo 7	-8,24
complexo 8	-6,88	complexo 8	-6,42	complexo 8	-12,28
complexo 9	-6,55	complexo 9	-7,27	complexo 9	-10,46

Em azul, o complexo resultante de menor energia de interação. Energia de interação em kcal/mol

Tabela 5. Energia de interação dos complexos cofilina-1 - EGFR (porção intracelular).

Energia de interação dos complexos cofilina-1 - EGFR (porção intracelular)					
5HG8-1Q8G	ΔG	3LZB-1Q8G	ΔG	**5JEB-1Q8G**	ΔG
complexo 0	-12,53	complexo 0	-15,46	complexo 0	-4
complexo 1	-4,03	complexo 1	-17,13	complexo 1	-6,37
complexo 2	-6,47	complexo 2	-7,32	complexo 2	-8,89
complexo 3	-5,76	complexo 3	-6,89	complexo 3	-11,75
complexo 4	-6,5	complexo 4	-13,57	complexo 4	-11,84
complexo 5	-9,64	complexo 5	-8,95	complexo 5	-9,64
complexo 6	-5,02	complexo 6	-3,93	complexo 6	-12,15
complexo 7	-10,35	complexo 7	-12,36	**complexo 7**	**-16,04**
complexo 8	-6,24	complexo 8	-7,99	complexo 8	-11,45
complexo 9	-10,75	complexo 9	-13,88	complexo 9	-7,99

Em azul, o complexo resultante de menor energia de interação. Energia de interação em kcal/mol.

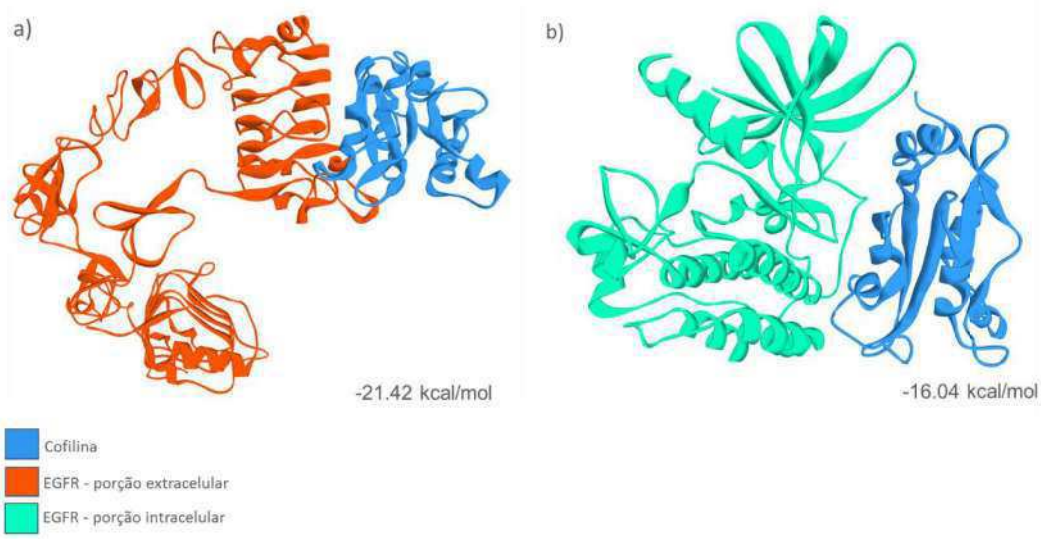

Figura 9. Complexos Cofilina-1-EGFR. Representação tridimensional das interações entre as estruturas de cofilina-1 e EGFR-porção extracelular (a) e cofilina-1 e EGFR-porção intracelular (b). Os resultados são representativos dos complexos que apresentaram menor energia de interação calculado em kcal/mol, através do software FoldX.

Estes resultados permitiram selecionar uma estrutura representativa da porção extracelular de EGFR e uma da porção citoplasmática do receptor, as quais foram submetidas

a novos cálculos de *docking* entre estruturas complexadas de cofilina-1 e LIMK1 e de cofilina-1 e actina, cujos resultados se encontram nas tabelas 6 e 7, respectivamente. Os complexos formados sugerem que a molécula de cofilina-1 complexada a uma molécula de LIMK1 apresenta menor energia de interação quando o *docking* é realizado com a porção extracelular de EGFR (-20.19 kcal/mol) em comparação com a porção citoplasmática de EGFR (-14.15 kcal/mol). Em contrapartida, o complexo cofilina-1-actina apresentou menor energia quando a interação é realizada com a porção intracelular de EGFR (-33.89 kcal/mol) em comparação com a porção extracelular (-23.13 kcal/mol). Estes complexos estão representados nas figuras 10 e 11, respectivamente.

Tabela 6. Energia de Interação dos complexos cofilina-1/LIMK1 e as porções extracelular (esquerda) e intracelular (direita) de EGFR..

Energia de interação dos complexos cofilina-1/LIMK1 - EGFR (porção extracelular)		**Energia de interação dos complexos cofilina-1/LIMK1 - EGFR (porção intracelular)**	
4UV7-5L6W COMPLEXO	ΔG	5JEB-5L6W COMPLEXO	ΔG
complexo 0	-13,92	complexo 0	-11,45
complexo 1	-18,28	complexo 1	-12,91
complexo 2	-16,15	complexo 2	-13,55
complexo 3	-18,46	complexo 3	-12,41
complexo 4	-14,24	complexo 4	-11,66
complexo 5	-19,24	complexo 5	-11,37
complexo 6	**-20,19**	complexo 6	-12,45
complexo 7	-18,93	complexo 7	-10,99
complexo 8	-8,66	**complexo 8**	**-14,15**
complexo 9	-10,85	complexo 9	-13,62
MÉDIA	**-15,89**	MÉDIA	**12,45**

Em azul, o complexo resultante de menor energia de interação. Energia de interação em kcal/mol

Tabela 7. Energia de Interação dos complexos cofilina-1/actina e as porções extracelular (esquerda) e intracelular (direita) de EGFR.

Energia de interação dos complexos cofilina-1/actina - EGFR (porção extracelular)		**Energia de interação dos complexos cofilina-1/actina - EGFR (porção intracelular)**	
4UV7-ACTCOF COMPLEXO	ΔG	5JEB-ACTCOF COMPLEXO	ΔG
complexo 0	-17,82	complexo 0	-19,52
complexo 1	-10,61	complexo 1	-9,96
complexo 2	-18,15	**complexo 2**	**-33,89**
complexo 3	-6,47	complexo 3	-13,47
complexo 4	-18,57	complexo 4	-18,36
complexo 5	-10,75	complexo 5	-12,14
complexo 6	-12,9	complexo 6	-11,16
complexo 7	-7,47	complexo 7	-6,85
complexo 8	**-23,13**	complexo 8	-15,07
complexo 9	-6,18	complexo 9	-18,66
MÉDIA	**-13,20**	MÉDIA	**-15,90**

Em azul, o complexo resultante de menor energia de interação. Energia de interação em kcal/mol.

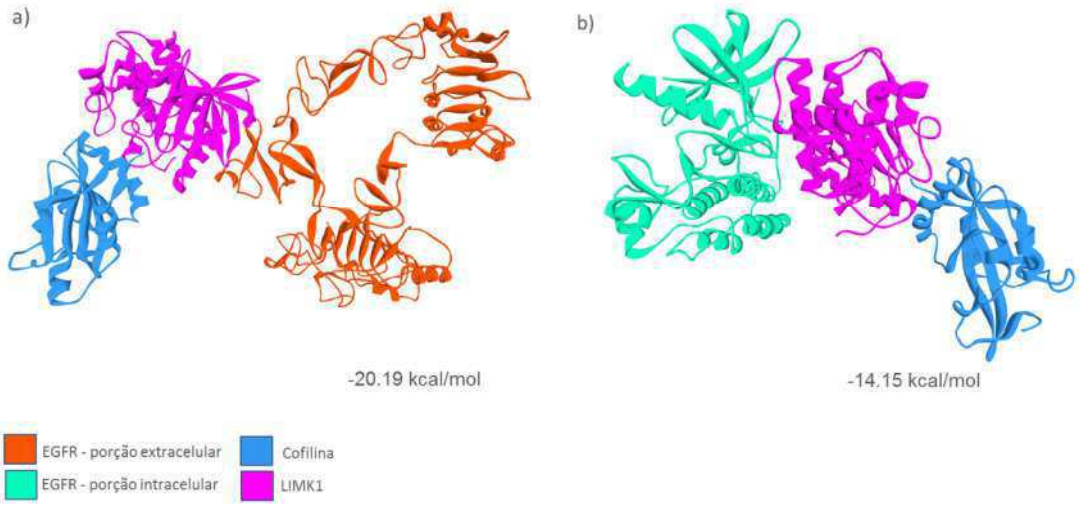

Figura 10. Complexos Cofilina-1/LIMK1-EGFR. Representação tridimensional das interações entre as estruturas de cofilina-1/LIMK1 e EGFR-porção extracelular (a) e cofilina-1 e EGFR-porção intracelular (b). Os resultados são representativos dos complexos que apresentaram menor energia de interação calculado em kcal/mol, através do software FoldX.

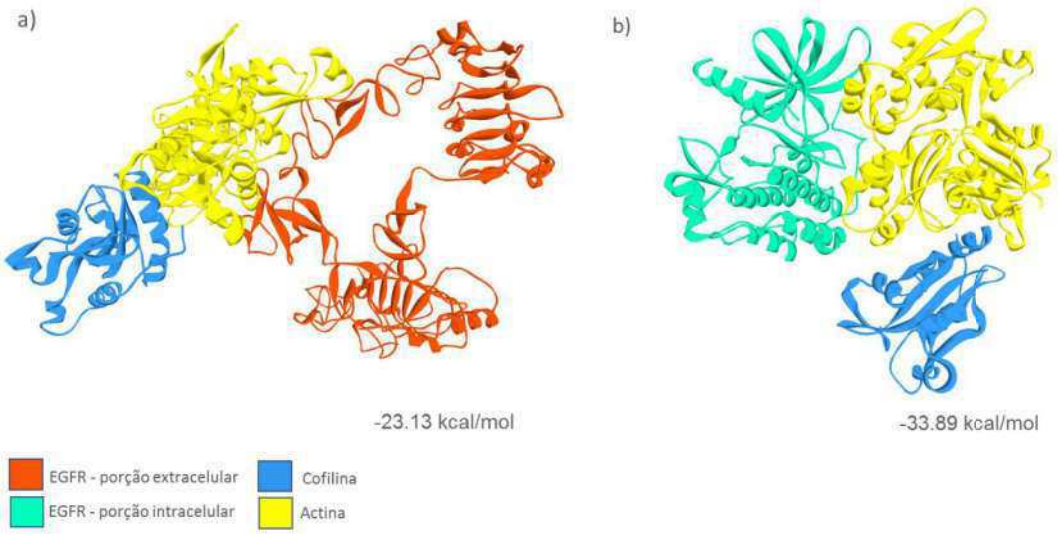

Figura 11. Complexos Cofilina-1/actina-EGFR. Representação tridimensional das interações entre as estruturas de cofilina-1/actina e EGFR-porção extracelular (a) e cofilina-1 e EGFR-porção intracelular (b). Os resultados são representativos dos complexos que apresentaram menor energia de interação calculado em kcal/mol, através do software FoldX.

- Ensaio clínico em *Tissue Array* -

METODOLOGIA

- *Lung Cancer Tissue Array:*

As lâminas de *tissue array* de câncer de pulmão foram obtidas da Abcam® (ab178190). Cada uma contém um total de 228 amostras teciduais e as respectivas informações de sexo, idade, patologia (subtipo histológico), grau de diferenciação e estadiamento. Todos os tecidos fornecidos foram fixados em formalina 10% tamponada, cortados em 4 µm de espessura e 1.1mm de tamanho e aplicados em lâminas adesivas, conforme informações do fabricante.

- Imuno-histoquímica:

As reações imuno-histoquímicas foram realizadas para detectar e quantificar dois marcadores, cofilina-1 e EGFR. Para tanto, utilizamos duas lâminas de *tissue array*, as quais foram igualmente submetidas à recuperação antigênica em banho-maria à 60ºC (em tampão citrato de sódio (pH 6,0) para reação de cofilina-1 e em tampão Tris/EDTA (pH 9,0) para reação de EGFR). Na sequência foi feito o bloqueio da peroxidase endógena com solução 5% de peróxido de hidrogênio em metanol. Para evitar marcações inespecíficas, as lâminas foram incubadas em solução 1% de albumina sérica bovina (BSA) (Sigma-Aldrich®) por uma hora. Em seguida, uma lâmina foi incubada com anticorpo primário policlonal de coelho anti-cofilina-1 (Abcam®; ab42475) na titulação 1:400 em BSA 1%; a outra lâmina foi incubada com anticorpo primário monoclonal de coelho anti-EGFR (D38B1) (Cell Signaling®; #4267) na titulação 1:100, *overnight* à 4ºC. Depois da incubação, ambas as lâminas foram lavadas em PBS e foi adicionado *HRP-labeled polymer conjugated* (Invitrogen®) e incubado por 45 minutos. Após a incubação com o polímero conjugado, as lâminas foram lavadas e expostas à solução de diaminobenzidina (DAB) 0,06% por 5 minutos e, então, lavadas em água corrente. Por fim, elas foram contracoradas com hematoxilina (Sigma-Aldrich®), desidratadas com álcool, clareadas com xileno e montadas. O controle negativo da reação foi obtido através do mesmo protocolo, porém com omissão do passo de incubação com anticorpo primário.

- Digitalização das Imagens:

As imagens digitais das reações imuno-histoquímicas foram obtidas utilizando o sistema *Evos XL Cell Imaging System* (Thermo Fisher Scientific®) em aumento de 20X. Para evitar erros sistemáticos, as imagens coradas das 228 amostras de cada lâmina foram digitalizadas em um mesmo dia, por um único observador e utilizando-se os mesmos parâmetros de captura e iluminação.

- Quantificação Imuno-histoquímica:

Primeiramente, foram selecionadas as áreas de parênquima tumoral de cada imagem. Para a análise das imagens, o *freeware* ImageJ v33 foi obtido do *website* do *National Intitutes of Health-NIH* (http://rsb.info.nih.gov/ij). No primeiro passo, estabeleceu-se um limiar para determinação do *background*; baseado nesse limiar, os pixels em primeiro plano foram convertidos em pixels de cor branca e os pixels de fundo (*background*) foram convertidos em pixels de cor preta. Assim, a imagem binária formada representou a reação de DAB analisada. A área da reação positiva para DAB foi estimada através do número de pixels/área (densidade integrada). Assim, os valores de densidade integrada atribuiram um valor a intensidade da reação, representando indiretamente o nível de expressão do marcador. As imagens também foram utilizadas para avaliar a presença de marcação nuclear nas reações de cofilina-1 e EGFR. Sempre que a contagem de núcleos marcados foi superior a 5%, a imagem era considerada positiva para marcação nuclear (Traynor, Weigel et al. 2013).

- Análise dos Dados:

Primeiramente, os resultados da quantificação imuno-histoquímica foram transformados em valores logarítmicos e os "*outliers*" foram removidos pelo método de Tukey. Em seguida, as informações de estadiamento dos paciente foram compiladas em 3 grupos "I-IIA", "IIB" e "III". Por fim, somente os grupos discriminados por patologia com mais de 15 casos foram selecionados. Para testar as correlações entre os valores de quantificação de cofilina-1 e EGFR e os dados clínico-patológicos, utilizamos regressões lineares. Os dados de marcação nuclear de cofilina-1 foram associados com as informações clínico-patológicas utilizando teste exato de Fisher, com ajuste FDR para comparações múltiplas pareadas. Essas análises foram realizadas usando o ambiente estatístico R (Team 2015).

RESULTADOS

Dos 228 casos iniciais, após aplicação dos critérios descritos anteriormente, obtivemos uma coorte resultante constituída de 198 pacientes. Desse total de pacientes, mais de 90% tinha mais de 40 anos no momento do diagnóstico; mais de 80% dos pacientes apresentava estágios iniciais da doença (IA-IIB). O tipo histológico mais frequente foi o carcinoma escamoso (51.5%), seguido de adenocarcinoma (33%); a maior parte dos tumores, 25%, tinha grau de diferenciação III (mais indiferenciado em relação a I e II). O dados completos da coorte, organizados por subgrupos, estão sumarizados na tabela 8.

Tabela 8. Distribuição dos pacientes por grupos organizados de acordo com as informações clinicopatológicas.

Características Clínicas e Patológicas	Total: 198 (100%)
Sexo	
Homens	46 (23,2%)
Mulheres	152 (76,8%)
Idade (anos)	
≤40	12 (6%)
41-59	93 (47%)
≥60	93 (47%)
Classificação Patológica	
Adenocarcinoma	65 (33%)
Adenoescamoso	15 (7.5%)
Carcinoma escamoso	102 (51.5%)
Carcinoma de pequenas células	16 (8%)
Grau	
I	12 (6%)
I-II	10 (5%)
II	40 (20.2%)
II-III	24 (12.1%)
III	51 (25.7%)
Não especificado	61 (31%)
Estadiamento	
IA-IIA	110 (55.5%)
IIB	59 (29.8%)
IIIA-IIIC	29 (14.7%)
IV	0 (0%)

A densitometria da reação de cofilina-1 variou entre os valores logarítmicos de 6,25 a 8.92, com média de 7,68. Já os valores densitométricos de EGFR variaram entre 0 e 1,09, com média de 0,53. A figura 12 apresenta imagens representativas da variação da intensidade da imunorreação para cofilina-1 e EGFR. As análises realizadas revelaram não haver diferença na

comparação independente das informações de sexo, idade, classificação patológica, grau de diferenciação e estadiamento com as quantificações das expressões de cofilina-1 e EGFR.

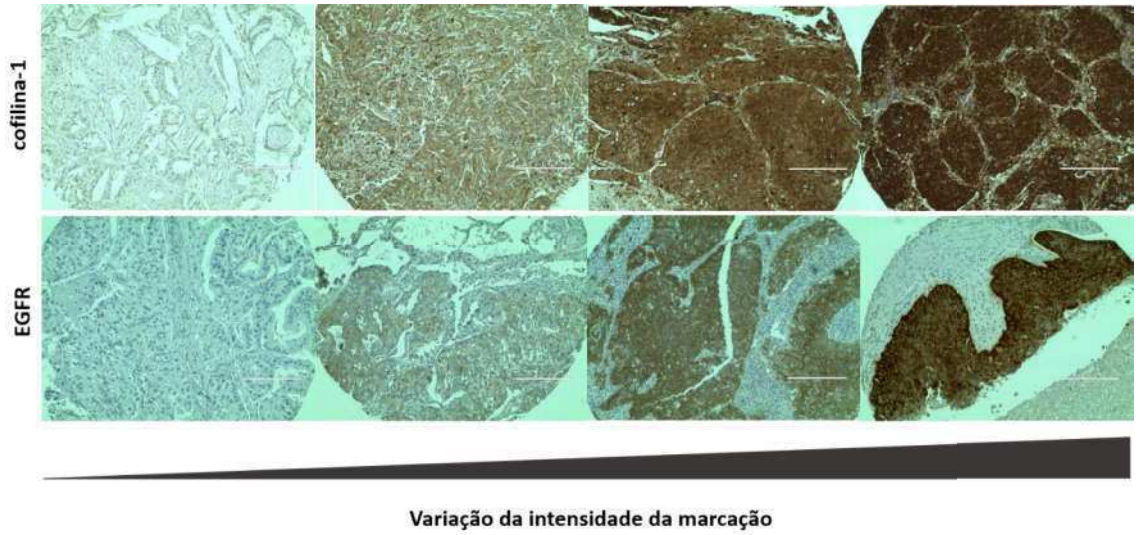

Figura 12. Variação de intensidade das imunorreações. A primeira e a segunda linha apresentam imagens de imunorreações de diferentes casos para marcação de cofilina-1 e EGFR, respectivamente. Da esquerda para direita, a intensidade da marcação varia de maneira crescente, de negativa a alta.

A análise de marcação nuclear foi positiva em 35 casos na reação de cofilina-1. Dentre esses casos, houve diferença na análise por subgrupo patologia, em que a análise das tabelas de contingência demonstrou diferença entre os dois subgrupos histológicos mais frequentes, escamoso e adenocarcinoma. A distribuição dos casos positivos para cofilina-1 nuclear é maior no grupo dos adenocarcinomas do que no grupo dos escamosos (figura 13).

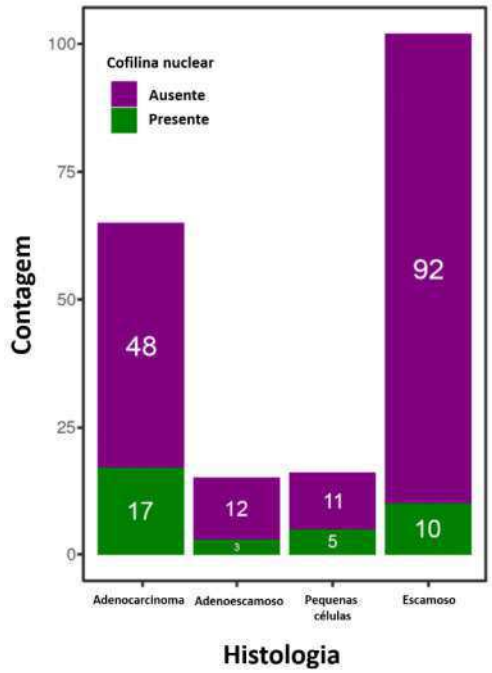

Figura 13. Comparação da presença/ausência de marcação nuclear em diferentes grupos histológicos. A análise por tabela de contingência permite a comparação entre as características de ausência (roxo) e presença (verde) nos diferentes subgrupos histológicos, evidenciando-se diferença estatisticamente diferente entre os grupos adenocarcinoma e escamoso.

PARTE III

5. DISCUSSÃO

Cerca de 85% dos pacientes diagnosticados com câncer de pulmão apresentam a forma predominante da doença, que é o CPNPC. O tratamento desses pacientes requer uma abordagem multidisciplinar que pode envolver a combinação de cirurgia, radioterapia e/ou quimioterapia, dependendo das condições de ressecabilidade, estágio e *status performance* (Ettinger, Wood et al. 2015).

A quimioterapia é um componente importante do tratamento em todos os estágios da doença, incluindo pacientes diagnosticados em estágios iniciais que realizaram tratamento cirúrgico e que podem se beneficiar ao receberem quimioterapia adjuvante. Para os pacientes diagnosticados em estágio avançado da doença (cerca de 77%), a quimioterapia caracteriza-se como a base do tratamento e é crítica para aumento da sobrevida e qualidade de vida (Chang 2011). As opções de tratamento para esse subgrupo envolvem regimes baseados em platina em/ou combinação com outras drogas (Ramalingam and Belani 2008).

O mecanismo de ação da cisplatina (e também da carboplatina) envolve a formação de ligações covalentes com as bases púricas de DNA. A consequente formação de adutos impede os processos de replicação e transcrição e pode levar a ativação de várias vias de transdução de sinal, como por exemplo vias relacionadas ao reconhecimento e reparo de dano ao DNA, à parada de ciclo e à apoptose (Kelland 2007).

Um impedimento significativo no sucesso da quimioterapia, e que pode resultar em um pior prognóstico para o paciente, está na habilidade das células tumorais em tornarem-se resistentes à cisplatina (Gurubhagavatula and Lynch 2005). Em estudo anterior, conforme descrito na Parte I, demonstramos que linhagens tumorais de CPNPC como maior imunoconteúdo de cofilina-1 apresentam resistência ao tratamento com cisplatina (Castro, Dal-Pizzol et al. 2010).

No C**apítulo 1** da presente tese, nós apresentamos diferentes ensaios com o objetivo de corroborar e melhor compreender a associação de cofilina-1 com resistência à cisplatina em CPNPC. Para confirmar os dados preliminares de associação com a quimioresistência, nós realizamos três diferentes estratégias experimentais.

Na primeira, comparamos, *in silico*, a expressão da rede do gene *CFL1* em um painel de linhagens de CPNPC humanas resistentes à cisplatina e verificamos uma maior atividade da rede no subtipo histológico adenocarcinoma. Com base nesse resultado, utilizamos a linhagem humana A549 (adenocarcinoma de pulmão) para selecionarmos células intrinsicamente resistentes ao tratamento com cisplatina.

O que nos leva à segunda abordagem, em que, a partir de nossas análises, verificamos que as células intrinsicamente resistentes, quando comparadas às células parentais, tinham a expressão de cofilina-1 aumentada. Além de corroborar dados prévios em câncer de pulmão, esse dado reforça relações semelhantes encontrados em outros tipos tumorais, como no câncer pancreático, em que a maior expressão de cofilina-1 está relacionada com resistência a multidrogas, e no câncer de ovário, em que também há correlação entre a expressão de cofilina e resistência à cisplatina (Sinha, Hütter et al. 1999, Yan, Pan et al. 2007).

A terceira estratégia objetivou verificar se a modulação da expressão do gene *CFL1* teria influência na resposta ao tratamento com cisplatina, o que se confirmou. A superexpressão do gene da cofilina-1 em células A549 resultou em resistência ao tratamento com cisplatina aumentada (maior valor de GI50) quando comparadas à células que foram transfectadas com o plasmídeo vazio.

Assim, demonstramos que tanto a modulação da expressão gênica quanto a alteração no imunoconteúdo tem impacto direto na sensibilidade das células A549 ao tratamento com cisplatina (Becker, De Bastiani et al. 2014). Embora tenhamos evidenciado essa associação,

faz-se necessário compreender os mecanismos envolvidos na resistência das células tumorais que apresentam um maior imunoconteúdo desta proteína.

A quimioresistência, comum em CPNPC, pode ser inata ou adquirida e pode envolver mais de um mecanismo celular. Dentre os principais mecanismos celulares de resistência à cisplatina temos o aumento do reparo ao dano do DNA, maior inativação intracelular da droga e redução do acúmulo intracelular da droga, por efluxo e/ou por inibição da captação (Chang 2011).

Ainda no **Capítulo 1**, avaliamos dois mecanismos de resistência à cisplatina em células tumorais. A cisplatina pode ser inativada por alguns constituintes citoplasmáticos, dentre eles a glutationa (GSH) e metalotioneínas ricas em cisteínas. A exposição crônica à cisplatina pode levar ao aumento da concentração de moléculas contendo tiol, resultando na diminuição do agente antitumoral disponível. Estudos em modelos tumorais e clínicos evidenciaram que o aumento na quantidade de GSH está diretamente relacionado com resistência (Siddik 2003). A conjugação de GSH com a cisplatina é catalisada pela Glutationa-S-transferase (GST), envolvida nas reações de detoxificação de xenobióticos, levando à inativação enzimática da cisplatina. Células tumorais resistentes à cisplatina apresentam aumento na expressão de GST acompanhado de aumento nos níveis de GSH (Sakamoto, Kondo et al. 2001). Por isso, quantificamos tióis reduzidos, GSH e atividade de GST em células.

Nossos resultados evidenciaram um aumento na atividade de GST nos grupos de resistência adquirida e intrínseca, que, entretanto, não foram acompanhados de aumento nos níveis de GSH. Além disso, não verificamos diferença nas quantidades de tióis reduzidos quando comparamos controles aos grupos de resistência.

Embora pareçam contraditórios, esses resultados não são suficientes para descartarmos o mecanismo de inativação intracelular da droga, uma vez que isso possa ser devido ao

constante efluxo do complexo cisplatina-GSH para fora da célula, conforme ilustrado na Figura 14, haja visto nosso ensaio tenha sido realizado em extrato celular. Esse efluxo é mediado por transportadores de membrana da família ABC conhecidos como MRP's (*Multidrug Resistance Proteins*) (Helen HW and Macus Tien 2010).

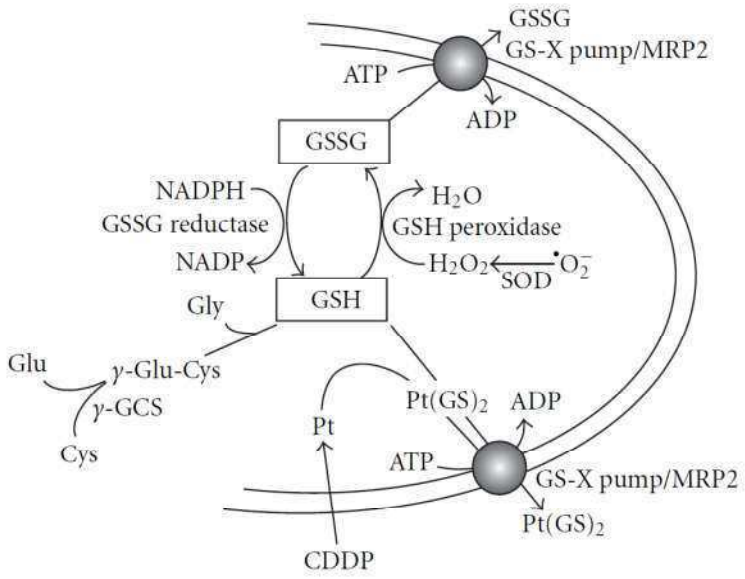

Figura 14. O papel da GSH no efluxo de Cisplatina mediado por MRP (*multidrug resistance protein*). A GSH pode ser oxidada por ação da GSH peroxidase em GSSG, que por sua vez pode ser reduzida novamente a GSH por ação da GSSG redutase. A GSSG é substrato para MRP2 enquanto a a GSH funciona como um cofator para o transporte de cisplatina (CDDP) mediado por MRP2. Adaptado de (Helen HW and Macus Tien 2010).

Considerando a relação existente entre inativação e efluxo, o segundo mecanismo avaliado foi a redução do acúmulo intracelular da droga por meio de efluxo. Trata-se de um mecanismo importante no desenvolvimento de resistência à cisplatina (Andrews, Velury et al. 1988). Os transportadores da família ABC funcionam como bombas de efluxo, eliminando do meio intracelular xenobióticos, agentes tumorais e constituintes endógenos aniônicos. Os transportadores, como já mencionado, são conhecidos como MRP's e realizam a eliminação dos substratos com gasto energético. A família de genes associada a resistência multidroga (MRP) é composta por pelo menos 7 membros, e o MRP2 parece estar consistentemente

associado ao efluxo de cisplatina em células resistentes e requer GSH como substrato (Kuo 2009).

Em nossa avaliação, não verificamos diferenças no perfil de exclusão da rodamina 123 entre os grupos controle e resistência. Cabe ressaltar, entretanto, que a redução intracelular dessa droga pode ser tanto devido a uma inibição da captação quanto ao aumento do seu efluxo e que é possível que a resistência seja resultado de um defeito no processo de captação, controlado por transportadores de cobre (Howell, Safaei et al. 2010). Cabe ressaltar também que a rodamina 123 é uma sonda não específica para MRP. Ela é exportada por membros da família ABC , especialmente ABCB1 (*MDR1*) (a principal proteína de resistência a múltiplas drogas), e bastante utilizada para investigação de resistência (Forster, 2012).

Embora, em conjunto, nossos resultados sugiram que a resistência não se deva ao acúmulo intracelular da droga, mais estudos necessitam ser realizados para descartarmos completamente a possibilidade de resistência relacionada a esses mecanismos.

Ainda assim, e sabendo que as células podem contar com um ou mais dos mecanismos descritos para o desenvolvimento de resistência, os resultados aqui apresentados sugerem a possibilidade de que a resistência verificada em nossas células, acompanhada de aumento na expressão de cofilina-1, seja resultado de um aumento na eficácia nos mecanismos de reparo ao DNA. Um aumento no reparo dos adutos formados por cisplatina no DNA pode atenuar a indução ao processo apoptótico. Esse mecanismo não é apresentado por todas as linhagens resistentes, mas, quando presente, resulta em resistência. A principal via é o reparo por excisão de nucleotídeos, NER (*nuclear excision repair*) (Furuta, Ueda et al. 2002). Além de NER, há o sistema de reparo por mal pareamento, MMR (*mismacht repair*), responsável por garantir estabilidade genômica (Martin, Hamilton et al. 2008). Essas vias não foram verificadas nesse trabalho e surgem como forte perspectiva para avaliação futura.

Esses indicativos com relação aos mecanismos de reparo ao DNA, somados aos resultados *in silico* previamente apresentados por nosso grupo, nos levaram a uma pesquisa aprofundada na literatura a respeito das flutuações na expressão e alterações na localização celular da cofilina-1 frente a diferentes estímulos. Essa pesquisa identificou uma série de intersecções entre as vias de EGFR e cofilina-1, incluindo a translocação nuclear.

O levantamento e análise desses dados da literatura nos permitiram formular uma hipótese de "responsabilidade integrada" dos dois marcadores nos mecanismos de resistência a cisplatina. Essas novas perspectivas foram publicadas na revista "*Oncotarget*", sob o título de: "*Potencial crosstalk between cofilin-1 and EGFR pathways in cisplatin resistance of non-small cell lung cancer*" (Müller, De Bastiani et al. 2015) e estão apresentadas no **Capítulo 2**.

Nesse artigo, nós revisamos a ação da proteína cofilina-1 na célula e os principais mecanismos modulatórios de sua atividade. Além disso, destacamos as alterações relacionadas com potencial prognóstico e preditivo desse marcador em diferentes tipos tumorais, cuja descrição foi detalhada na parte introdutória dessa tese. Entretanto, cabe aqui ressaltar que a estrutura primária da cofilina-1 contém uma sequência NLS (*nuclear localization signal*) (Iida, Matsumoto et al. 1992). Diferentes estímulos, como choque térmico (*heat shock*) e tratamento com dimetil sulfóxido (DMSO), induzem a translocação da proteína para o compartimento nuclear (Ohta, Nishida et al. 1989). Também é verificado que em linhagens tumorais em crescimento, há translocação nuclear espontânea (Samstag, Dreizler et al. 1996). Ainda, dados sugerem que a cofilina-1 nuclear tenha papel na transcrição da RNA polimerase I, através do direcionamento de actina (Percipalle 2013). Somado a isso, temos que essa translocação ocorre mediante modulação do *status* de fosforilação de sua serina 3, sendo necessária para a translocação, a ação de fosfatases para defosforilação da proteína (Nebl, Meuer et al. 1996). Embora ainda não haja dados que avaliem a translocação da proteína para núcleo mediante tratamento com agentes alquilantes, fica implícita a possibilidade de que a cofilina-1 possa ter

um papel de suporte ao sistema de reparo ao DNA, uma vez que verificamos que a resistência é acompanhada de aumento no imunoconteúdo de cofilina-1, e que esta proteína pode, em condições específicas, translocar-se para o núcleo.

O EGFR, por sua vez, é nodo de uma importante rede de sinalização envolvida com crescimento, progressão e sobrevivência das células tumorais (Goffin and Zbuk 2013). Por essa razão, mutações associadas à ativação contínua do receptor são alvo para terapias personalizadas com a utilização de inibidores tirosina cinase (Thunnissen, van der Oord et al. 2014). As diversas vias jusantes à ativação de EGFR possuem intensa interação com cofilina-1, uma vez que, indiretamente, regulam os mecanismos de ativação/inativação da proteína. Além disso, EGFR, assim como a cofilina-1, pode sofrer translocação para o compartimento nuclear. Essa translocação pode ocorrer mediante radiação ionizante e tratamento com cisplatina (Marti, Burwen et al. 1991). Uma vez dentro do núcleo, ele pode agir promovendo regulação gênica e auxiliando no reparo ao dano do DNA (Dittmann, Mayer et al. 2011, Brand, Iida et al. 2013). Suas funções relacionadas ao reparo do DNA, por associação com a DNA-PK, já foram relacionadas com o desenvolvimento de resistência à cisplatina (Liccardi, Hartley et al. 2011). Além disso, a presença de EGFR nuclear também foi associado a pior prognóstico em paciente com CPNPC em estágio inicial da doença (Traynor, Weigel et al. 2013). As ações individuais, modulações e suas intersecções foram compilados em um esquema apresentado na figura 15.

Esses dados reforçam a possibilidade de que a interação de EGFR com cofilina-1 vá além da modulação de sua atividade citoplasmática, uma vez que já evidenciamos aumento da expressão de cofilina-1 relacionado com pior do prognóstico (Castro, Dal-Pizzol et al. 2010, Müller, de Barros et al. 2011) e com resistência ao tratamento a cisplatina (Becker, De Bastiani et al. 2014). Mais recentemente, também verificamos que pacientes que possuem aumento da expressão de cofilina-1, tem pior resposta ao tratamento com radioterapia (Leal 2016).

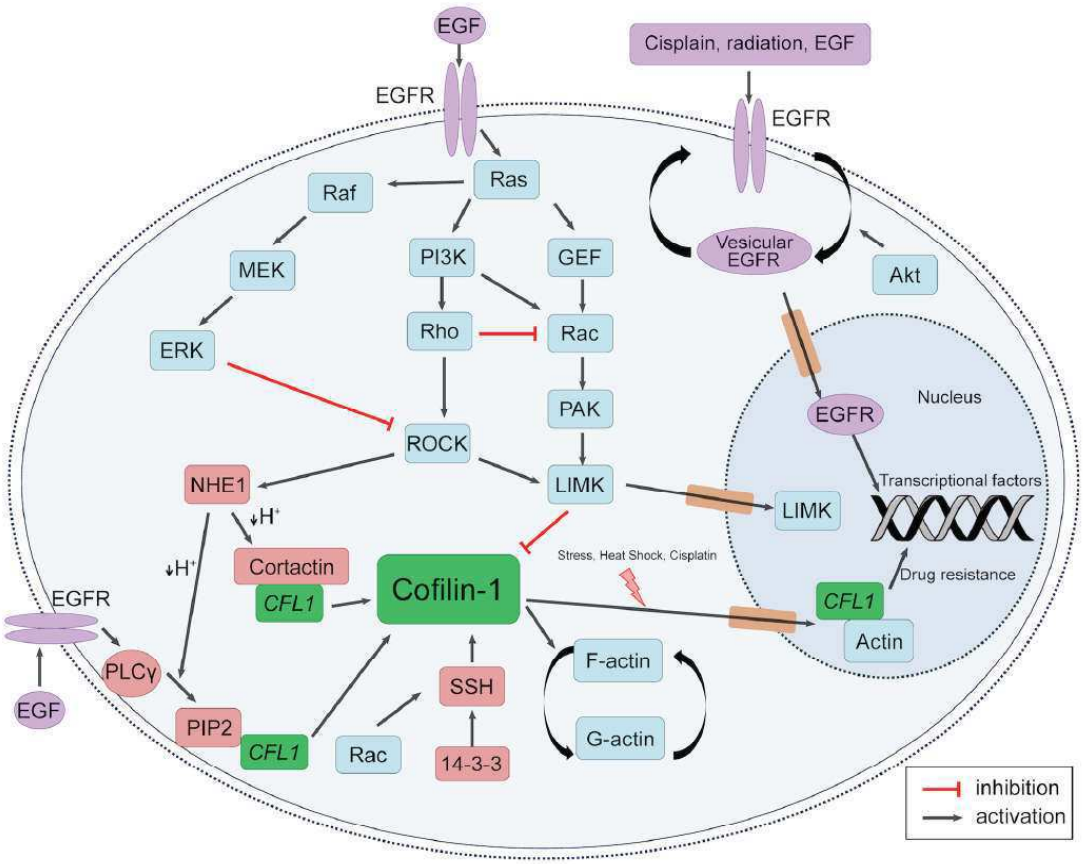

Figura 15. Interações citosólicas e nucleares de EGFR e cofilina-1. Representação esquemática das intersecções entre as vias de EGFR e cofilina-1. Diferentes estímulos locais podem resultar na modulação de cofilina através da ativação de EGFR. As vias jusantes a EGFR podem ativar cofilina-1 por defosforilação por ação da SSH1 (*slingshot 1*) e através da liberação da ligação à cortactina e PIP2, por alterações no pH; além disso, podem inativar cofilina-1 pela atividade de LIMK. EGFR e cofilina-1 podem translocar para o núcleo em resposta à estímulos externos, indicando a possibilidade de mecanismos relacionados a resistência a drogas. (Retirado de Müller, De Bastiani et al. 2015)

Evidências moleculares demonstram que cofilina-1 tem papel importante na regulação do tráfico vesicular de EGFR (Nishimura, Yoshioka et al. 2006) e que tem ação direta no constante transporte de actina para o núcleo, podendo ter participação na manutenção do nucleoesqueleto de actina (Dopie, Skarp et al. 2012). Além disso, é possível que exerça papel direto na transcrição gênica (Obrdlik and Percipalle 2011).

A riqueza das associações culminou com a proposição de que cofilina-1 e EGFR ajam em conjunto na regulação da maquinaria de resistência à cisplatina, o que nos levaria, na

sequência, a realizar uma série de experimentos *in silico* para a avaliar as possibilidades de interação entre a proteína e o receptor e guiar futuros experimentos *in vitro*. Assim, para melhor discutirmos as possíveis interações, fazemos aqui uma breve descrição as características estruturais e funcionais de EGFR e cofilina-1.

Estruturalmente, EGFR apresenta-se como uma proteína de 170kDa, 1186 resíduos de aminoácidos distribuídos em porções intra e extracelular de proporções semelhantes, intercalados por um domínio transmembranar (Ullrich, Coussens et al. 1984). A porção extracelular possui 4 segmentos, dos quais há dois domínios para interação com ligantes e dois domínios ricos em cisteína. A porção intracelular, por sua vez, apresenta domínio tirosina cinase, servindo como sítio de fosforilação e ancoragem para proteínas de sinalização *downstream* (Mayes and Waterfield 1984). A representação esquemática do receptor está ilustrada na figura 16.

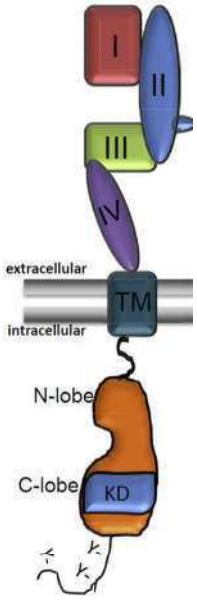

Figura 16. Representação esquemática de EGFR. A imagem apresenta as porções extracelular, transmembrana e intracelular do receptor; I: domínio para interação com ligantes 1; II: domínio rico em cisteína 1; III: domínio para interação com ligantes 2; IV: domínio rico em cisteína 2; TM: domínio transmembrana; KD: domínio tirosina cinase. Adaptado de Ceresa e Peterson, 2014.

A interação de um ligante à porção extracelular do receptor induz sua dimerização, levando a uma mudança conformacional, que aumenta a atividade catalítica de suas tirosinas cinases, resultando em autofosforilação. A cinase ativada fosforila resíduos de tirosina de um grande número de substratos celulares, incluindo PLC-γ (fosfolipase C-γ) e MAPK's (*mitogen-activated protein kinase*) (Soler, Beguinot et al. 1994). O complexo receptor/ligante ativado é endocitado e pode ou ser degradado por via lisossomal, ou reciclado pela membrana plasmática. Assim, o receptor move-se continuamente da superfície celular para endossomos, via endocitose mediada por clatrinas (Voldborg, Damstrup et al. 1997).

Entretanto, apesar de os receptores tirosina cinase residirem e atuarem na bicamada lipídica e em vesículas citosólicas, diversos estudos demonstraram que membros da família EGFR podem também ser detectados, em fragmentos e em seu comprimento total, no núcleo (Marti, Burwen et al. 1991, Lin, Makino et al. 2001, Schlessinger and Lemmon 2006). Conforme revisado por Wang e Hung, um dos mecanismos propostos para translocação nuclear, é o modelo de retrotranslocação, que envolve a fusão da vesícula endossomal com o Retículo Endoplasmático (Wang and Hung 2009).

Em comparação ao EGFR, a cofilina-1 é uma proteína estruturalmente simples e pequena, com cerca de 19 KDa e 166 resíduos de aminoácidos (Klejnot, Gabrielsen et al. 2013). Sua atividade regulatória da dinâmica do citoesqueleto de actina pode ser modulada mediante fosforilação/defosforilação de sua serina 3, alterações no pH e ligação ao PIP_2, conforme descrito em mais detalhe na parte introdutória dessa tese. Além desses mecanismos, há a possibilidade de oxidação de cisteína, cuja consequência é a formação de dímeros e oligômeros de cofilina, podendo contribuir para a formação de "*rods*" nucleares de actina-cofilina, particularmente em neurônios (Minamide, Maiti et al. 2010).

Tendo em vista o tamanho e complexidade estrutural do receptor EGF e a possibilidade de ambas as proteínas translocarem-se em seu comprimento total para o núcleo, nós testamos as possíveis interações entre cofilina-1 e as diferentes porções de EGFR, intracelular e extracelular.

Além disso, testamos também as possibilidades de interação entre os complexos cofilina-1/LIMK e cofilina-1/actina com as porções intra e extracelular do receptor, uma vez que tanto LIMK quanto actina podem também translocar-se para o núcleo e possuem íntima associação com regulação e função da proteína, respectivamente.

Comparando a energia de ligação do complexo cofilina-1-EGFR intra e extracelular, verificamos uma maior probabilidade de que, caso a interação ocorra, a mesma se dê com a porção extracelular. O mesmo também ocorreu na interação com o complexo cofilina-1/LIMK. Entretanto, quando avaliamos o complexo cofilina/actina, a maior probabilidade de interação se dá com a porção intracelular do receptor. Se compararmos a energia de ligação dos complexos mais favoráveis formados, a interação mais provável é a do complexo cofilina-1/actina-EGFR intracelular, em que a ligação à porção intracelular de EGFR se dá por actina; sugerindo, assim, a possibilidade de uma função principal na translocação nuclear de moléculas que possam interagir com o receptor.

Biologicamente, esse dado é bastante lógico, pois a cofilina-1 apresenta-se como um regulador crucial do fluxo contínuo de actina para o núcleo. Dopie e colegas demonstraram ainda que o silenciamento de cofilina-1 e a manipulação dos níveis de actina nuclear tem efeito sobre a atividade transcricional da célula (Dopie, Skarp et al. 2012), o que reforça ainda mais a real possibilidade de interação desse complexo com EGFR no núcleo, haja visto que o receptor pode estar envolvido com regulação gênica (Dittmann, Mayer et al. 2011).

A partir da possibilidade de interação entre cofilina-1, direta ou indiretamente, com EGFR, realizamos um estudo clínico preliminar para avaliarmos, através de imuno-histoquímica, a expressão e a possibilidade de co-localização nuclear de cofilina-1 e EGFR em amostras tumorais de pacientes com CPNPC.

Em nossa avaliação, não verificamos qualquer correlação dos diferentes níveis de expressão de cofilina-1 e EGFR com nenhum dos dados clinico-patológicos disponibilizados. A análise por subgrupos também não revelou diferenças significativas. Entretanto, salientamos aqui as limitações impostas pela coorte utilizada; os casos reunidos no *tissue array* são bastante heterogêneos, apresentando um grande número de subgrupos contando, cada um, com poucos casos, o que dificulta a análise.

Além disso, dados como tratamento e sobrevida não são disponibilizados pela empresa fornecedora do material, limitando as inferências sobre potencial preditivo e prognóstico. A predominância de carcinomas escamosos também não reflete os dados epidemiológicos mundiais, que indicam adenocarcinoma como o subtipo histológico mais frequente (Travis, Brambilla et al. 2011).

Outra limitação é a predominância de casos em estágios iniciais da doença e a inexistência de pacientes em estágio IV. A análise de EGFR deteve-se à expressão do *wild type*, uma vez que também não possuíamos as informações de mutações do receptor nos casos avaliados que validassem a avaliação de marcadores imuno-hitoquímicos para L858R e E746-A750del.

Quanto à análise dos marcadores no compartimento nuclear, não verificamos co-localização de EGFR e cofilina-1. A hipótese de interação dessas proteínas no núcleo não se confirmou nesse estudo. Cabe avaliarmos, entretanto, as potenciais interferências nessa constatação.

Embora não tenhamos detectado marcação nuclear de EGFR, verificamos a presença de cofilina-1 no núcleo, e que essa expressão é mais frequente em adenocarcinoma do que em carcinoma escamoso. Conforme discutido anteriormente, sabemos que a cofilina-1 tem um papel importante no fluxo constante de actina para o núcleo (Dopie, Skarp et al. 2012) o que justificaria, portanto, a maior probabilidade de a encontrarmos nesse compartimento. Contudo, é possível que a presença temporalmente concomitante dos marcadores no núcleo seja resultado de um insulto comum, como, por exemplo, o tratamento com cisplatina, uma vez que a droga induz translocação nuclear de EGFR (Marti, Burwen et al. 1991) e que o desenvolvimento de resistência a ela em células de adenocarcima pulmonar é acompanhado de um aumento na expressão de cofilina-1 (Becker, De Bastiani et al. 2014). Além disso, conforme indicado pelos dados *in silico*, é possível que não haja uma interação direta e, sim, que o tratamento com cisplatina induza a translocação nuclear de EGFR e que a cofilina-1 funcione carreando outra(s) molécula(s) para o compartimento que, em algum nível, irão interagir com EGFR no desenvolvimento do perfil de resistência.

O fato de verificarmos um número maior de casos com marcação nuclear de cofilina-1 no subtipo histológico adenocarcinoma é bastante relevante, haja visto que a presença de mutação do receptor EGF é mais comum nesse tipo tumoral (Kosaka, Yatabe et al. 2004). Se realmente houver relação direta ou indireta entre cofilina-1 e EGFR no mecanismo de resistência à cisplatina, a quantificação e a subcompartimentalização de cofilina-1 pode também ser fator determinante na escolha do tratamento de primeira linha, indicando possivelmente uma melhor resposta do paciente a inibidores tirosina cinase.

7. CONCLUSÃO

Os resultados expostos nessa tese foram publicados em revistas científicas internacionais ou, em conjunto com processos experimentais em andamento, irão compor futura submissão (resultados suplementares).

Com base no corpo de resultados apresentados, podemos sumarizar:

(i) Demonstramos que tanto a modulação da expressão gênica quanto a alteração no imunoconteúdo tem impacto direto na sensibilidade das células A549 ao tratamento com cisplatina, reforçando o envolvimento da proteína cofilina-1 no desenvolvimento de resistência à cisplatina em CPNPC

(ii) Avaliamos diferentes mecanismos de resistência a cisplatina, e os resultados aqui apresentados sugerem a possibilidade de que a resistência verificada em nossas células, acompanhada de aumento na expressão de cofilina-1, seja resultado de um aumento na eficácia nos mecanismos de reparo ao DNA.

(iii) Com base em extensa revisão da literatura, formulamos uma hipótese de "responsabilidade integrada" de cofilina-1 e EGFR nos mecanismos de resistência a cisplatina, em que propomos a colocalização nuclear e interação de ambos nesse processo.

(iv) Avaliamos, *in silico*, as probabilidades de interação entre cofilina-1 e EGFR e identificamos que a interação mais provável seria a do complexo cofilina-1/actina-EGFR intracelular, em que a ligação à porção intracelular de EGFR se dá por actina; sugerindo, assim, a possibilidade de uma função principal na translocação nuclear de moléculas que possam interagir com o receptor.

(v) Observamos uma expressão mais frequente de cofilina-1 nuclear no subtipo adenocarcinoma, tipo histológico em mais é mais comum encontrarmos mutação do receptor EGF.

Embora esses dados não sejam suficientes para a compreensão integral do envolvimento de cofilina-1 e EGFR nos mecanismos de resistência, a tese contribui com a organização e compilação de evidências que permitirão guiar estudos futuros no sentido de melhor avaliar a relação entre EGFR e cofilina-1 mediante estímulos comuns e, também, mensurar a contribuição dos marcadores na escolha de tratamentos mais adequados.

8. PERSPECTIVAS

Com base nos resultados e hipóteses aqui apresentados, surgem como perspectivas futuras ao grupo:

(i) avaliar mecanismos de reparo ao dano com DNA induzido pelo tratamento com cisplatina e observar possíveis correlações com as flutuações na expressão e localização celular de cofilina-1;

(ii) Estabelecimento de coorte de CPNPC como dados clínicos completos para a realização de Estudo Clínico (Coorte Retrospectiva):

Pacientes de estágios IA a IIB

- Avaliar a correlação entre quantidade de cofilina e desfecho clínico;
- Em pacientes diagnosticados com metástase linfonodal, determinar e avaliar a correlação entre as quantidades e sublocalização de cofilina-1 e EGFR na peça tumoral e no linfonodo acometido com a evolução do paciente;
- Avaliar o efeito dessas diferenças no desfecho do paciente de acordo com o esquema quimioterápico de escolha (se recebido);

Pacientes de estágio IIIA a IV

- Quantificar e sublocalizar cofilina-1 e EGFR nas biópsias de pacientes diagnosticados em estágios avançados da doença e avaliar a possível relação entre expressão dos marcadores e a resposta ao esquema quimioterápico ao qual foi submetido o paciente.

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ANEXOS

ANEXO 1

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 (2.5×10^4 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.

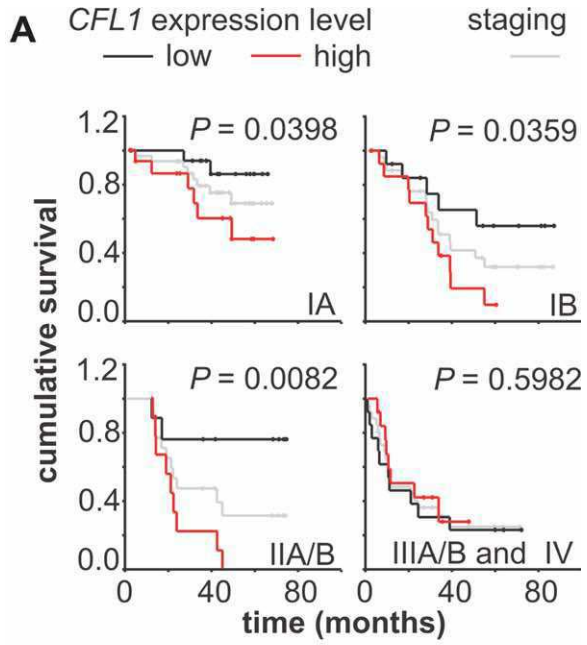
Bioinformatics Analysis

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

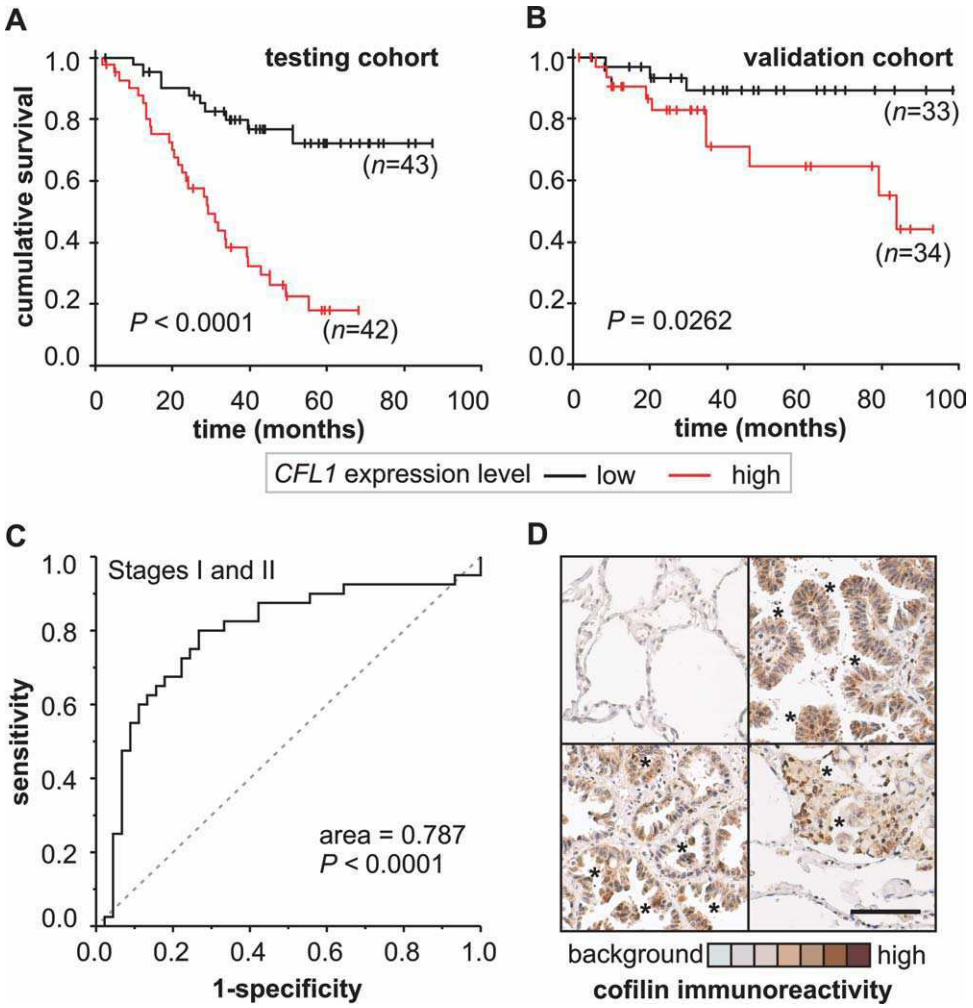


Figure 2. Biomarker performance in early stage nonsmall cell lung cancer (NSCLC) patients is shown. (A) Kaplan-Meier plots are shown for patients in stages I and II (n = 85) in the original cohort (testing cohort) stratified by *CFL1* expression level and (B) in an independent cohort (validation cohort) 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

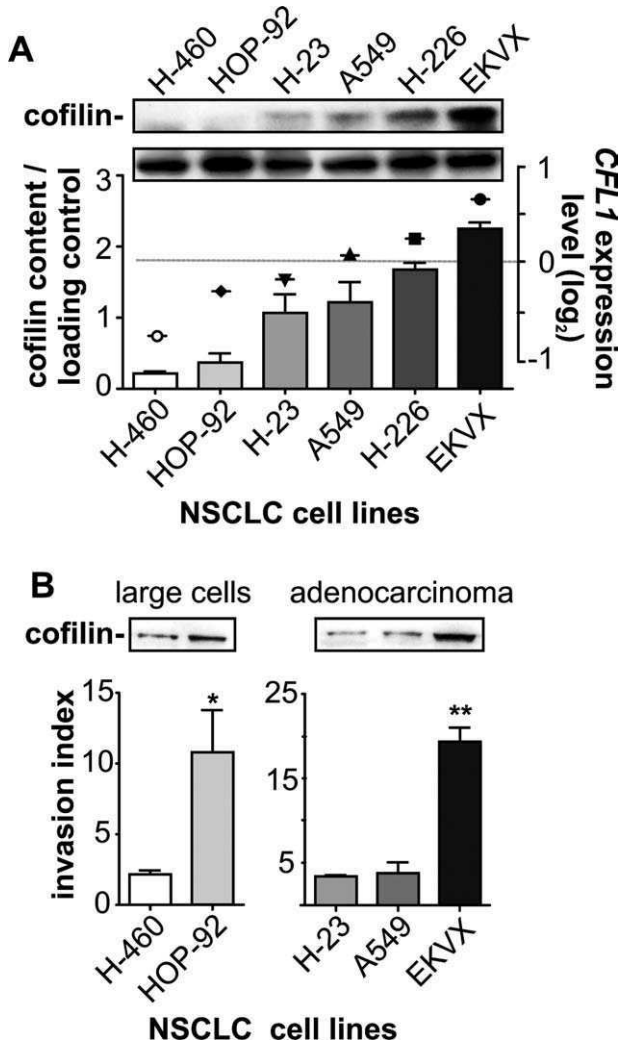


Figure 3. Cofilin immunocontent correlates with tumor invasiveness in vitro. Six human nonsmall cell lung cancer (NSCLC) cell lines composed of adenocarcinomas (H-23, A549, EKVX), large cells (H-460, HOP-92), and squamous-cell carcinomas (H-226) from the NCI-60 panel were selected based on different 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.

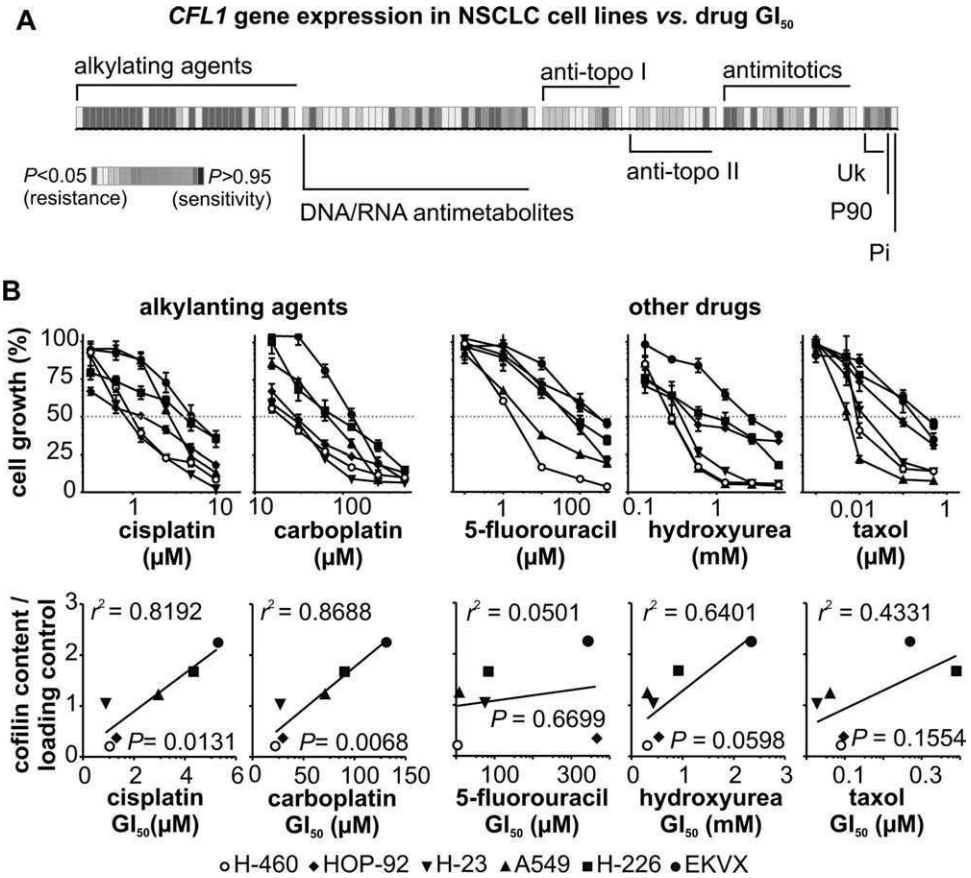


Figure 4. Colifin mRNA and protein levels correlate with drug resistance in vitro. (A) Microarray metadata of the cell lines are crossed against 50% growth inhibition dose (GI_{50}) values of 118 standard chemotherapy agents (from the NCI-60 drug discovery pipeline). *P* values have been color coded 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

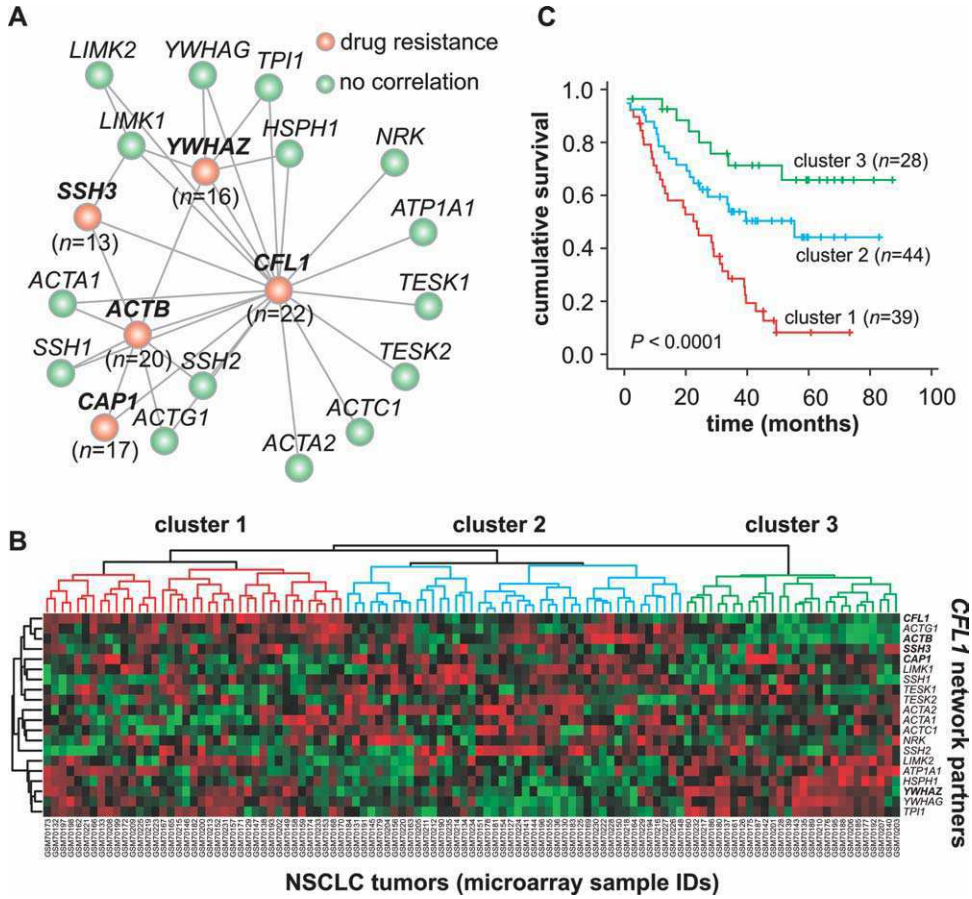


Figure 5. Prognostic and drug resistance markers of the *CFL1* functional gene network are shown. (A) A graphic model represents the *CFL1* functional gene network versus alkylating drug sensitivity/resistance profiles. Nodes represent gene products; connecting lines indicate physical and/or functional 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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ANEXO 2

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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

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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

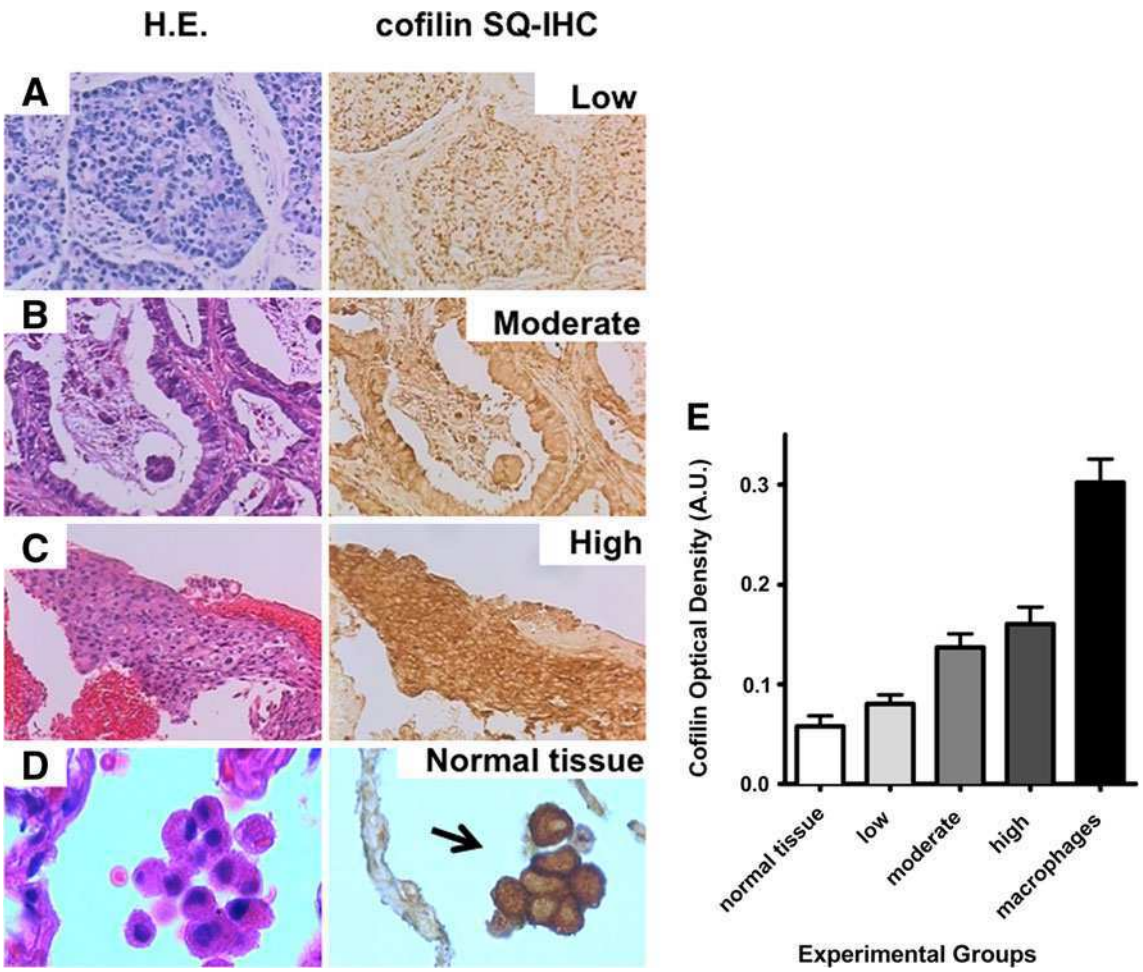

Fig. 1 Representative HE and cofilin-1 IHC staining of NSCLC cases are presented. IHC images correspond to **a** *low*, **b** moderate and **c** *high* cofilin-1 immunocontent (200× magnification). Healthy tissue **d** obtained from free resection margins of biopsies represents basal levels of cofilin-1,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

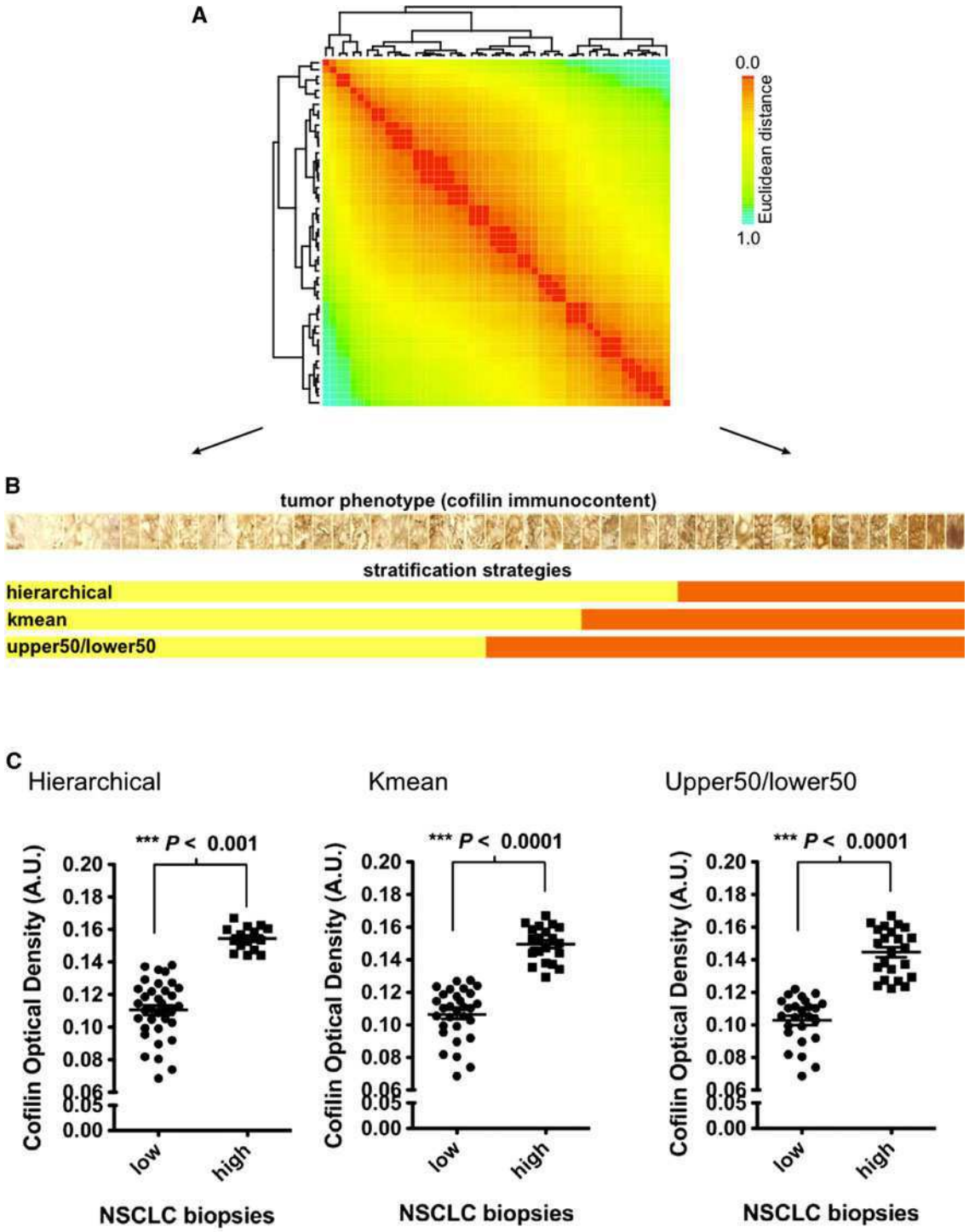

Fig. 2 Unsupervised clustering. **a** Hierarchical cluster analysis. The heat map shows the dissimilarities between clusters computed by the Euclidean distance (agglomeration method: "average"). The cluster tree is cut in $k = 2$ groups for subsequent analysis. **b** Tumor phenotype aliened with three 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

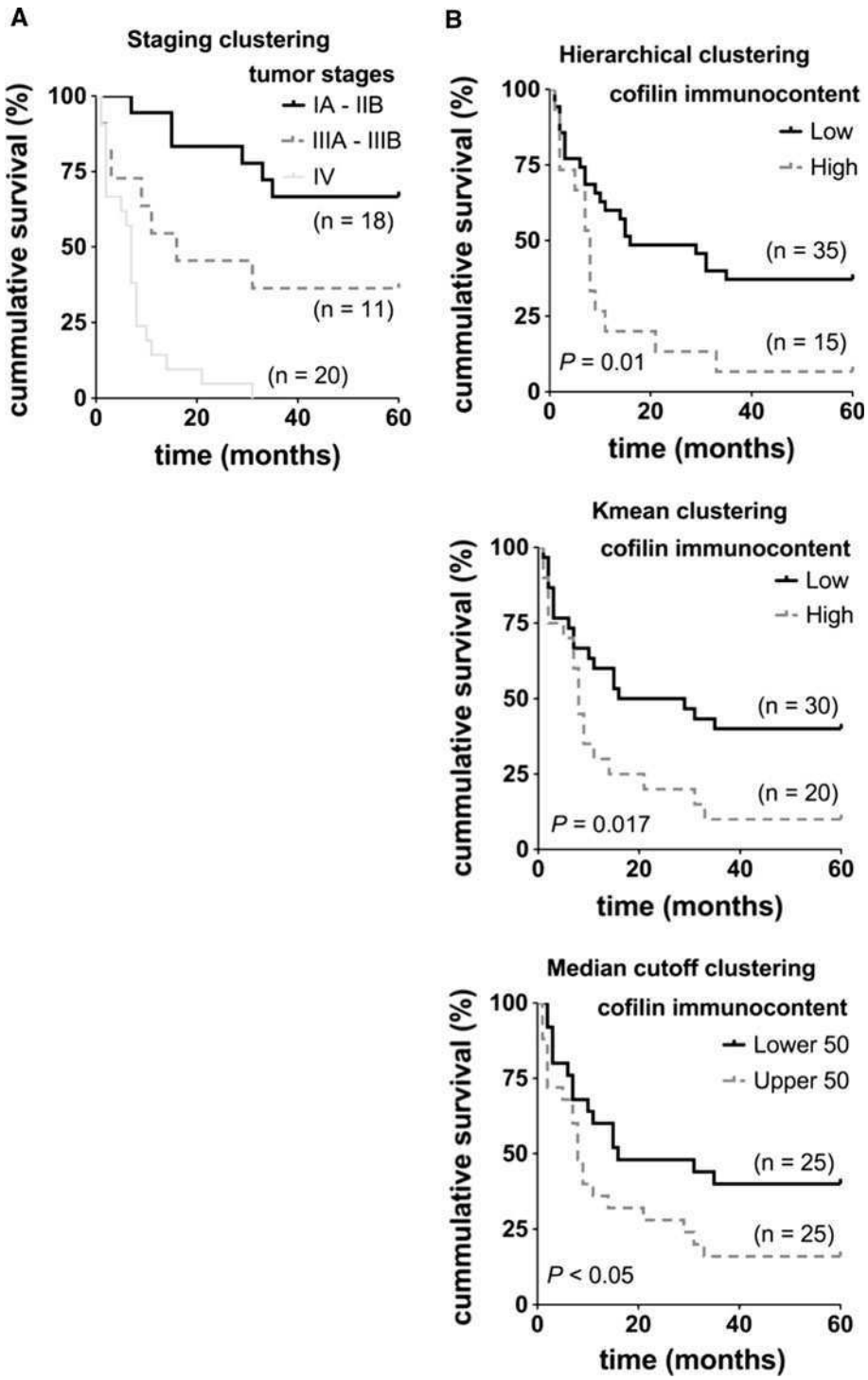

Fig. 3 Kaplan–Meier mortality curves were used to evaluate the cofilin-1 performance to discriminate between good and bad prognosis of NSCLC patients. These curves show the overall survival of patients in 5 years. The cumulative survival was also measured according the TNM staging system (**a**). Patients 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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