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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].

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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.

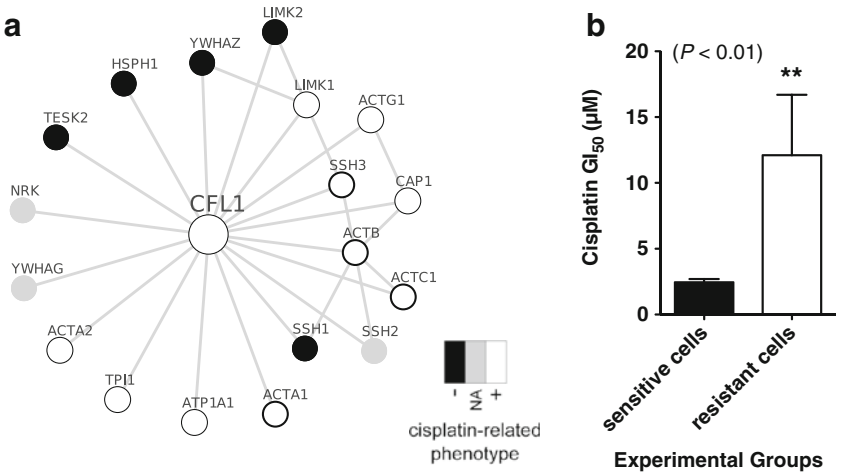

Fig. 1 Differential gene expression levels of the *CFL1* gene network in cisplatin-resistant lung adenocarcinoma cell panel. **a** STRING gene interactions representation of the *CFL1* gene network. A graphic model represents the *CFL1* functional gene network vs. cisplatin drug resistance profiles. 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)

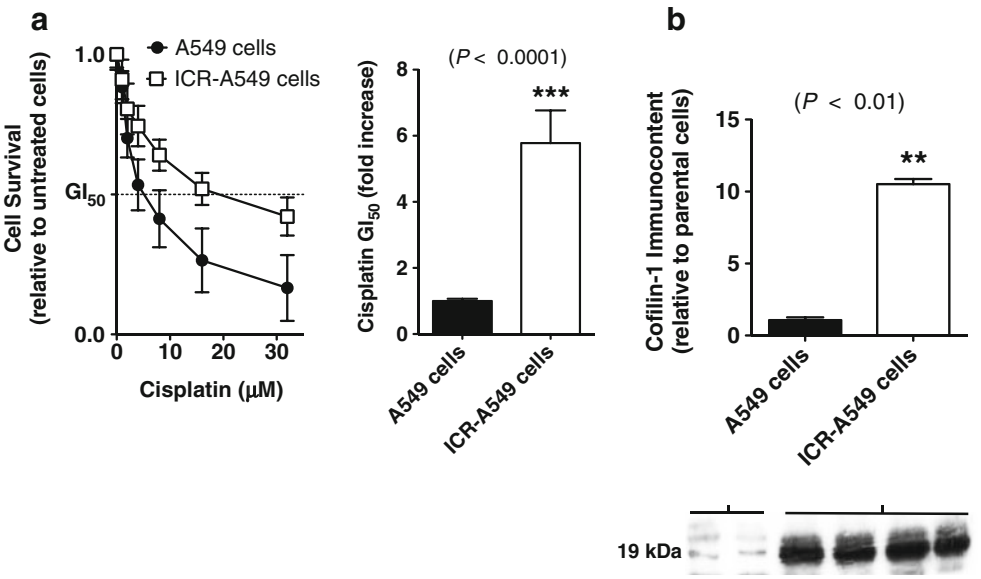

Fig. 2 Increased cofilin-1 immunocontent in intrinsically cisplatin-resistant A549 (*ICR-A549*) NSCLC cells. ICR-A549 cells were selected as described in the "Materials and methods" section and presented a sixfold increase in drug GI_{50} value (**a**) and an increase in cofilin-1 immunocontent (**b**) 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

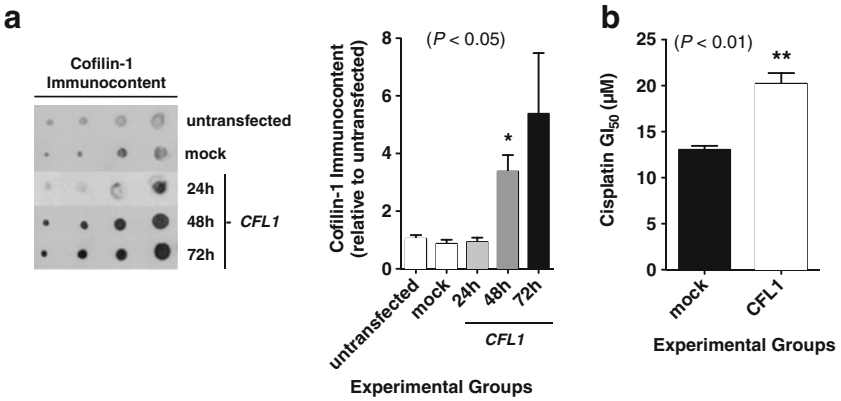

Fig. 3 Transient transfection and overexpression of cofilin-1 lead to an increase in cisplatin resistance in A549 cells. **a** Cells were transiently transfected with the plasmid containing the cDNA sequence of cofilin-1 (*CFL1*) or empty plasmid (mock) as described in the "Materials and methods" section, 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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