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Loss of C/EBP-β LIP drives cisplatin resistance in malignant pleural mesothelioma

Joanna Kopecka[a,1], Iris C. Salaroglio[a,1], Luisella Righi[b], Roberta Libener[c], Sara Orecchia[c], Federica Grosso[d], Vladan Milosevic[a], Preeta Ananthanarayanan[a], Luisa Ricci[a], Enrica Capelletto[e], Monica Pradotto[e], Francesca Napoli[b], Massimo Di Maio[f], Silvia Novello[e], Menachem Rubinstein[g], Giorgio V. Scagliotti[e,1], Chiara Riganti[a,1,*]

[a] Department of Oncology, University of Torino, via Santena 5/bis, 10126, Torino, Italy
[b] Pathology Unit, Department of Oncology at San Luigi Hospital, University of Torino, Regione Gonzole 10, 10043, Orbassano, Italy
[c] Pathology Division, S. Antonio and Biagio Hospital, Spalto Marengo, 15121, Alessandria, Italy
[d] Oncology Division, S. Antonio and Biagio Hospital, Spalto Marengo, 15121, Alessandria, Italy
[e] Thoracic Unit and Medical Oncology Division, Department of Oncology at San Luigi Hospital, Regione Gonzole 10, University of Torino, Orbassano, Italy
[f] Medical Oncology Division, Department of Oncology at Mauriziano Hospital, Largo Filippo Turati 62, 10128, University of Torino, Italy
[g] Department of Molecular Genetics, The Weizmann Institute of Science, Herzl Street 234, 76100, Rehovot, Israel

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ABSTRACT

Objectives: Cisplatin-based chemotherapy is moderately active in malignant pleural mesothelioma (MPM) due to intrinsic drug resistance and to low immunogenicity of MPM cells. CAAT/enhancer binding protein (C/EBP)-β LIP is a pro-apoptotic and chemosensitizing transcription factor activated in response to endoplasmic reticulum (ER) stress.

Materials and methods: We investigated if LIP levels can predict the clinical response to cisplatin and survival of MPM patients receiving cisplatin-based chemotherapy. We studied the LIP-dependent mechanisms determining cisplatin-resistance and we identified pharmacological approaches targeting LIP, able to restore cisplatin sensitiveness, in patient-derived MPM cells and animal models. Results were analyzed by a one-way analysis of variance test.

Results: We found that LIP was degraded by constitutive ubiquitination in primary MPM cells derived from patients poorly responsive to cisplatin. LIP ubiquitination was directly correlated with cisplatin chemosensitivity and was associated with patients' survival after chemotherapy. Overexpression of LIP restored cisplatin's pro-apoptotic effect by activating CHOP/TRB3/caspase 3 axis and up-regulating calreticulin, that triggered MPM cell phagocytosis by dendritic cells and expanded autologous anti-tumor $CD8^+CD107^+$ T-cytotoxic lymphocytes.

Proteasome inhibitor carfilzomib and lysosome inhibitor chloroquine prevented LIP degradation. The triple combination of carfilzomib, chloroquine and cisplatin increased ER stress-triggered apoptosis and immunogenic cell death in patients' samples, and reduced tumor growth in cisplatin-resistant MPM preclinical models.

Conclusion: The loss of LIP mediates cisplatin resistance, rendering LIP a possible predictor of cisplatin response in MPM patients. The association of proteasome and lysosome inhibitors reverses cisplatin resistance by restoring LIP levels and may represent a new adjuvant strategy in MPM treatment.

Abbreviations: ANOVA, analysis of variance; ATF6, activating transcription factor 6; C/EBP, CAAT/enhancer binding protein; IRE1, inositol-requiring enzyme 1; CHOP/GADD153, C/EBP homologous protein/growth arrest/DNA damage inducible 153; CEA, carcino-embrionic antigen; ChIP, chromatin immunoprecipitation; DC, dendritic cells; ER, endoplasmic reticulum; EMA, epithelial membrane antigen; EIF2AK3/PERK, eukaryotic translation initiation factor-2α kinase 3; ICD, immunogenic cell death; HMC, human mesothelial cells; MPM, malignant pleural mesothelioma; OS, overall survival; qRT-PCR, quantitative Real Time-PCR; PFS, progression free survival; SPSS, Statistical Package for Social Science; TBS, Tris-buffered saline; TRB3, *tribbles*-related protein 3; UPN, Unknown Patient Number; WT1, Wilms tumor-1 antigen

* Corresponding author.

E-mail addresses: joanna.kopecka@unito.it (J. Kopecka), irischiara.salaroglio@unito.it (I.C. Salaroglio), luisella.righi@unito.it (L. Righi), rlibener@ospedale.al.it (R. Libener), sorecchia@ospedale.al.it (S. Orecchia), fgrosso@ospedale.al.it (F. Grosso), vladan.milosevic@unito.it (V. Milosevic), preeta.ananthanarayanan@unito.it (P. Ananthanarayanan), luisa.ricci@gmail.com (L. Ricci), enrica.capelletto@gmail.com (E. Capelletto), monica.pradotto@unito.it (M. Pradotto), francesca.napoli@unito.it (F. Napoli), massimo.dimaio@unito.it (M. Di Maio), silvia.novello@unito.it (S. Novello), menachem.rubinstein@weizmann.ac.il (M. Rubinstein), giorgio.scagliotti@unito.it (G.V. Scagliotti), chiara.riganti@unito.it (C. Riganti).

[1] These authors equally contributed to the work.

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1. Introduction

Malignant pleural mesothelioma (MPM), an asbestos-related cancer, is usually diagnosed at advanced stage, when chemotherapy – based on cisplatin associated with pemetrexed or raltitrexed – is the only available therapeutic option [1,2]. The success of chemotherapy is limited by the intrinsic chemoresistance [1] and immune-evasive nature of MPM. Such immune-evasive microenvironment in MPM relies on the presence of immune-suppressive/immune-tolerant cells in the MPM [3,4], on the high levels of immune-suppressive immune-checkpoints on both MPM cells and surrounding T-lymphocytes [5,6], on the low amount of tumor-associated antigens of MPM cells [7], due to its low mutational burden [8].

In sensitive cells, cisplatin induces DNA damage, hampers DNA repair [9] and elicits nuclear-independent effects, such as dispersal of Golgi apparatus [10] and apoptosis induced by endoplasmic reticulum (ER) stress [11]. Upon ER stress, cancer cells expose on their surface the “eat-me” signal calreticulin, leading to dendritic cells (DC)-mediated phagocytosis and activation of autologous anti-tumor cytotoxic $CD8^+$ T-lymphocytes [12]. This process is known as immunogenic cell death (ICD) [12]. MPM cells however do not translocate calreticulin from ER to surface [13,14], resulting ICD-refractory. This is an additional mechanism explaining the low immunogenicity of MPM cell.

Solid tumors respond to chemotherapy-induced ER stress by activating adaptation and survival pathways if the stress is limited, or pro-apoptotic pathways if the stress persists [15,16]. The ER stress-induced transcription factor CAAT/enhancer binding protein (C/EBP)-β is involved in both responses. At the early ER stress phase, the pro-survival isoform C/EBP-β LAP is produced. Upon prolonged ER stress, the isoform C/EBP-β LIP (LIP) is formed and activates C/EBP homologous protein/growth arrest/DNA damage inducible 153 (CHOP/GADD153) protein, which promotes apoptosis by activating *tribbles*-related protein 3 (TRB3) and caspase 3 [17–20]. At the present there are no data available about gene alterations (mutation, amplification or deletion) in the 87 MPM evaluated by the Tissue Cancer Genome Atlas (https://cancergenome.nih.gov), nor about the expression of C/EBP-β LAP/LIP isoforms in MPM, according to Protein Tissue Atlas (http://www.proteinatlas.org).

We recently reported that chemoresistant tumors lack LIP, because of its constitutive ubiquitination. LIP loss mediates chemoresistance by increasing the expression of the drug efflux transporter P-glycoprotein and by preventing the ER stress-dependent pro-apoptotic response [12].

Here, we investigated if LIP mediates the resistance to cisplatin in MPM. We identified clinically feasible pharmacological strategies that restore the sensitivity of MPM to cisplatin by preventing LIP degradation.

2. Materials and methods

2.1. Chemicals

Cell culture plastic ware were obtained from Falcon (Becton Dickinson, Franklin Lakes, NJ). Electrophoresis reagents were obtained from Bio-Rad Laboratories (Hercules, CA). Carfilzomib was purchased from Biorbyt Ltd. (Cambridge, UK) and torin 1 was from Selleckchem (Munich, Germany). The protease inhibitor cocktail set III was obtained from Millipore (Billerica, MA). Unless specified otherwise, all reagents were purchased from Sigma Chemicals Co (St. Louis, MO).

2.2. Cells

Primary human mesothelial cells (HMC) were isolated from three patients with pleural fluid secondary to congestive heart failure, with no history of a malignant disease. Nine primary human MPM samples (3 epithelioid MPM, 3 biphasic MPM, 3 sarcomatous MPM) were obtained from diagnostic thoracoscopies. Histological and clinical features are shown in Supplementary Tables S1–S2. All patients, identified with Unknown Patient Numbers (UPN), received 5 cycles of cisplatin 75 mg/m^2 every 21 days. Tissue was digested in medium containing 1 mg/ml collagenase and 0.2 mg/ml hyaluronidase for 1 h at 37 °C. Cells were seeded in culture and used within passage 6. The Ethical Committee of Biological Bank of Mesothelioma, S. Antonio e Biagio Hospital, Alessandria, Italy, and San Luigi Gonzaga Hospital, Orbassano, Italy, approved the study (#9/11/2011; #126/2016). Murine AB1 cells were purchased from Sigma Chemicals Co. Cells were grown in Ham’s F10 nutrient mixture medium (primary HMC/MPM cells) or DMEM (AB1 cells), supplemented with 10% v/v fetal bovine serum (FBS), 1% v/v penicillin-streptomycin.

2.3. Quantitative real time-PCR (qRT-PCR)

RNA was extracted and reverse-transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories). qRT-PCR was performed using IQ™ SYBR Green Supermix (Bio-Rad Laboratories). The same cDNA preparation was used for measuring genes of interest and the housekeeping gene *S14*. Primer sequences were designed using qPrimerDepot software (http://primerdepot.nci.nih.gov/). Relative gene expression levels were calculated using Gene Expression Quantitation software (Bio-Rad Laboratories).

2.4. Immunoblotting

Protein or tumor extracts (20 μg) were subjected to SDS-PAGE and probed with the following antibodies: C/EBP-β (directed against the common C-terminus of LIP and LAP, Santa Cruz Biotechnology Inc., Santa Cruz, CA), CHOP/GADD153 (Abcam, Cambridge, UK), TRB3 (Proteintech, Chicago, IL), caspase-3 (GeneTex, Hsinhu City, Taiwan), β-tubulin (Santa Cruz Biotechnology Inc.). To detect ubiquitinated C/EBP-β, 100 μg protein extracts were immuno-precipitated overnight with the anti-C/EBP-β antibody, using 25 μl of PureProteome Magnetic Beads (Millipore). Immunoprecipitated samples were then probed with an anti-mono/polyubiquitin antibody (Axxora, Lasuane, Switzerland). Blotting was followed by the peroxidase-conjugated secondary antibody. The membranes were washed with Tris-buffered saline/Tween 0.01% v/v and proteins were detected by enhanced chemiluminescence. Band density was calculated using ImageJ software (http://www.rsb.info.nih.gov/ij/).

2.5. Cell viability and growth

Cell viability with neutral red staining and crystal violet staining were performed as reported [20]. IC_{50} was calculated with the CompuSyn software (http://www.combosyn.com). Quantitation of crystal violet staining was performed by dissolving crystal violet with 1% v/v acetic acid and reading the absorbance of each well at 570 nm (HT Synergy 96-well microplate reader, Bio-Tek Instruments, Winoosky, VT). The mean absorbance of untreated cells was considered 100%; the absorbance units of the other experimental conditions were expressed as percentage towards untreated cells.

2.6. Cell cycle analysis

1×10^4 cells were harvested, washed with PBS, treated with 0.25 mg/ml RNAse and stained for 15 min with 50 μg/ml propidium iodide. Cell cycle distribution was analyzed by Guava® easyCyte flow cytometer (Millipore, Billerica, MA), using the InCyte software (Millipore).

2.7. Over-expression of C/EBP-β LAP and LIP

The pcDNA4/TO expression vectors (Invitrogen Life Technologies, Milan, Italy) for LAP and LIP, produced as reported previously [17],

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