

CDKN1A upregulation and cisplatin-pemetrexed resistance in non-small cell lung cancer cells

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Received September 17, 2019; Accepted February 27, 2020

DOI: 10.3892/ijo.2020.5024

Abstract. Cisplatin-pemetrexed is a frequently adopted first-line treatment for patients with advanced non-small cell lung cancer (NSCLC) ineligible for biological therapy, notwithstanding its limited efficacy. In the present study, the RAL cell line, an epidermal growth factor receptor (*EGFR*)-wild-type, *p53*- and *KRAS*-mutated model of NSCLC, was used to investigate novel biomarkers of resistance to this treatment. Cells were analyzed 96 h (96 h-post wo) and 21 days (21 days-post wo) after the combined treatment washout. Following an initial moderate sensitivity to the treatment, the cell growth proliferative capability had fully recovered. Gene expression analysis of the resistant surviving cells revealed a significant upregulation of *CDKN1A* expression in the cells at 96-h post-wo and, although to a lesser extent, in the cells at 21 days-post wo, accompanied by an enrichment of acetylated histone H3 in its promoter region. *CDKN1A* was also upregulated at the protein level, being mainly detected in the cytoplasm of the cells at 96 h-post wo. A marked increase in the number of apoptotic cells, together with a significant G1 phase block, were observed at 96-h post wo in the cells in which *CDKN1A* was knocked down, suggesting its involvement in the modulation of the response of RAL cells to the drug combination. On the whole, these data suggest that *CDKN1A* plays a role in the

response to the cisplatin-pemetrexed combination in advanced *KRAS*-mutated NSCLC, thus suggesting that it may be used as a promising predictive marker.

Introduction

Lung cancer is the leading cause of cancer-associated mortality worldwide (1-3). More than 80% of patients with lung cancer suffer from non-small cell lung cancer (NSCLC), and the majority have advanced disease at diagnosis with a poor prognosis (4).

Adenocarcinoma is the most common subtype of NSCLC. In addition to epidermal growth factor receptor (*EGFR*) mutations that occur in approximately 50% of patients, the oncogene *KRAS*, responsible for tumor formation, and the tumor suppressor gene *TP53*, implicated in cell cycle regulation, cell proliferation and apoptosis, are also frequently mutated in this type of tumor, with *EGFR* and *KRAS* mutations generally mutually exclusive (1). The role of such mutations in the selection of the anticancer treatment is still under debate, even though it appears that they may be associated with differential sensitivity patterns to currently available therapies (5,6).

Specific targeted therapies are available for patients with advanced disease harboring *EGFR* mutations or anaplastic lymphoma kinase (*ALK*) translocation, and immunotherapy has also been increasingly adopted on the basis of the programmed death protein 1 (PD-1) level. It is still not clear how the remaining mutations could drive the selection of anticancer treatment. The American Society of Clinical Oncology and National Comprehensive Cancer Network (NCCN) recommends a combination of two cytotoxic drugs for the first-line therapy of patients who are not eligible for biological treatment (7). The antifolate drug, pemetrexed, is frequently used in combination with cisplatin, since an improved overall survival has been observed in patients treated with this combination compared to those undergoing the cisplatin-gemcitabine regimen (8). However, acquired drug resistance mechanisms

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Key words: non-small cell lung cancer, *CDKN1A*, platinum-based chemotherapy, drug resistance

are common and compromise the efficacy of chemotherapy for lung cancer (8-11). The mechanism of action of cisplatin relies on the induction of DNA damage (12), resulting in cell cycle arrest and subsequent apoptosis. Neoplastic cells activate several genes involved in DNA damage repair to withstand platinum-based chemotherapy. Among these, cyclin-dependent kinase inhibitor 1A (*CDKN1A*) plays a central role in cell cycle progression, regulating both G1/S and G2/M checkpoints. Upon DNA damage, *CDKN1A* is transiently recruited to target sites to facilitate their repair. However, *CDKN1A* activity is dependent on the *p53* status. In fact, upon *p53* loss-of-function mutation, *CDKN1A* overexpression drives cells to acquire a more aggressive phenotype that is capable of escaping cell block, senescence and apoptosis (13).

The aim of the present study was to identify novel potential biomarkers involved in the onset of resistance to the cisplatin-pemetrexed combination in an *EGFR*-wild type (wt) NSCLC cell line (RAL) harboring *KRAS* and *TP53* mutations.

Materials and methods

Cells and cell culture. The NSCLC cell line, RAL, is derived from a metastatic lesion of lung adenocarcinoma of a 52-year-old female previously treated with cisplatin (14). The identity of the patient was irreversibly anonymized prior to specimen processing. The cell line is characterized by the following: *EGFR*-wt, *KRAS* mutation at exon 1 (p.G12C, missense, not functional, deleterious), *TP53* mutation at exon 7 (p.G244C, missense, not functional, deleterious) and no *ALK* rearrangement. The cells were grown in Dulbecco's modified Eagle's medium/HAM F12 (1:1) supplemented with 10% fetal bovine serum, 2 mM of L-glutamine (EuroClone) and 10 µg/ml of insulin (Sigma-Aldrich; Merck KGaA) in a humidified atmosphere with 5% CO₂ at 37°C. The population doubling time was 65 h, as previously described (14).

Chemosensitivity assay. Cisplatin (Ebewe Pharma-Sandoz) and pemetrexed (Eli Lilly Italia Spa) were tested at plasma peak concentrations (10 and 320.5 µM, respectively). Cell viability was assessed by MTS assay according to the manufacturer's protocol (CellTiter 96[®] Aqueous One Solution Cell Proliferation assay, Promega Corp.). Experiments were run in octuplicate and each experiment was repeated 3 times. To ensure that the exposure time was compatible with the half-life of the drugs administered as in a clinical setting, the cells were treated with pemetrexed or cisplatin alone and washed out with drug-free medium after 3 and 6 h of exposure, respectively, followed by up to 96 h of recovery, as previously described (15). For the combination treatment, the cells were exposed to cisplatin and pemetrexed for 6 h and washed out with drug-free medium, followed by 96 h of recovery (96 h-post wo).

Flow cytometry. Untreated cells, and those at 96-h post wo and 21 days post-treatment washout (21 days-post wo) were collected and fixed with ice-cold 70% ethanol. The cells at 21 days-post wo were allowed to recover by replacement of the fresh medium 3 times/week. No split was carried out, in order to permit the RAL cells to form resistant clones and repopulate the flask. Flow cytometric analysis was performed using

a FACSCanto flow cytometer (BD Biosciences). Data acquisition and analysis were performed using FACSDiva software (Version 6.1.3, BD Biosciences). Samples were run in triplicate (10,000 events for each replica). Data report the mean value of 3 experiments with standard deviations <5%.

TUNEL assay. Cells were fixed in 1% paraformaldehyde in phosphate-buffered saline (PBS) on ice for 15 min, suspended in cold ethanol (70%) and stored overnight at -20°C. The cells were then washed twice in PBS, resuspended in PBS containing 0.1% Triton X-100 for 5 min at 4°C, incubated in 50 µl of solution containing TdT and FITC-conjugated dUTP deoxynucleotides 1:1 (Roche Diagnostics GmbH) in a humidified atmosphere for 90 min at 37°C in the dark, washed in PBS, counterstained with propidium iodide (PI, 200 µg/ml, MP Biomedicals) and RNase (10,000 U/ml, Sigma-Aldrich; Merck KGaA) for 30 min at 4°C in the dark, and analyzed by flow cytometry.

Cell cycle distribution analysis. The cells were fixed in ethanol (70%) and stained in a solution containing 100 µg/ml of PI (Sigma-Aldrich; Merck KGaA), 10,000 U/ml of RNase (Sigma-Aldrich; Merck KGaA) and 0.01% NP40 (Sigma-Aldrich; Merck KGaA). After 24 h, the samples were analyzed by flow cytometry. Data were elaborated using ModFit (DNA Modelling System) software (Version 4.1.7, Verity Software House Inc.), and expressed as fractions of cells in the different cell cycle phases.

Annexin V assay. The cells were washed once in PBS, incubated with 250 µl of Annexin V-FITC in binding buffer (eBioscience) for 15 min at 37°C in a humidified atmosphere in the dark, and then washed in PBS and suspended in binding buffer. Immediately before flow cytometric analysis, PI was added to a final concentration of 5 µg/ml to distinguish between total apoptotic cells (Annexin V⁺ and PI⁻ or PI⁺) and necrotic cells (Annexin V⁻ and PI⁺).

siRNA transfection. *CDKN1A* Silencer Select Validated siRNA (Thermo Fisher Scientific, Inc.) and a validated Negative Universal Control[™] (Invitrogen; Thermo Fisher Scientific, Inc.) were used. The cells were seeded in 25 cm²-flasks at a density of 2.5x10⁵ cells. The transfections were carried out using the TransIT-X2 Dynamic Delivery System (Mirus Bio LCC) and Opti-MEM GlutaMax medium (Invitrogen; Thermo Fisher Scientific, Inc.) without antibiotics. siRNA/TransIT-X2 was incubated with the cells for 25 min. The total incubation time before the combinational treatment was 24 h.

Cellular fractionation, protein extraction and western blot analysis. Cells (7x10⁶) that had either been treated with cisplatin and pemetrexed or under the control condition were washed in hypotonic buffer (10 mM Tris, 5 mM MgCl₂). Triton X-100 (10% stock) was added to a 0.3% final concentration. The suspension was passed twice through a 22-gauge needle with a syringe and centrifuged at 800 x g for 10 min at 4°C to remove debris. The supernatant was collected. Protease/phosphatase inhibitor cocktail (Sigma-Aldrich; Merck KGaA) was added and protein lysate was saved as the cytosolic fraction. Nuclei were isolated following 2 subsequent centrifugations at

800 x g for 10 min at 4°C and lysed in RIPA buffer containing 1 µg/ml of DNase (Voden Medical Instruments). Western blot analysis was performed as previously described (16). The list of the specific antibodies and conditions used is available upon request.

RNA extraction, purification and reverse transcription. Total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and purified with the RNeasy MinElute Cleanup kit (Qiagen GmbH) according to the manufacturer's instructions. DNase enzyme digestion (Qiagen GmbH), was performed to exclude genomic DNA contamination. Purified RNA was eluted with 14 µl of RNase-free water (Qiagen GmbH). A total of 500 ng of purified RNA were reverse transcribed into cDNA using the RT² First Strand kit (SABiosciences Corp.) in a volume of 20 µl reaction and diluted with 91 µl of nuclease-free water, following the manufacturer's instructions.

RT² Profiler™ PCR array and quantitative (real-time) PCR (qPCR). The Human Oncogenes and Tumor Suppressor genes RT² Profiler™ PCR Array (complete list of genes is presented in Table SI) was used to profile the expression of 84 different key genes that promote oncogenesis. A mix containing 102 µl of cDNA, 1,248 µl of water and 1,350 µl of 2X RT² SYBR Green Master Mix (SABiosciences Corp.) was prepared and 25 µl were added to each well. qPCR was performed using the Real-Time Cycler 7500 (Applied Biosystems) with the following thermal profile: 95°C for 10 min; 40 cycles: 95°C for 15 sec and 60°C for 1 min; dissociation curve at 95°C for 1 min, 55°C 30 sec and 95°C for 30 sec. Raw data from qPCR were exported and analyzed using online software at the GeneGlobe Data Analysis Center website (Qiagen GmbH). Fold changes in gene expression were calculated using the 2^{-ΔΔC_q} method (17).

Differentially expressed genes (with >2-fold changes in expression) were validated with 3 biological independent replicates by RT-qPCR (Table SIIA), using 1 µl of cDNA, 0.4 µM of specific primers, 12.5 µl of RT² SYBR-Green Master Mix and water up to a final volume of 25 µl. The relative gene expression was calculated as previously mentioned.

A protein-protein interaction (PPI) network and functional module were established using the Search Tool for the Retrieval of Interacting Genes (STRING) (18). A confidence score of ≥0.4 and a maximum number of interactors of (=) 0 were set as the cut-off criteria.

Bisulfite sequencing. *CDKN1A* and *RASSF1* gene promoter methylation was analyzed by bisulfite sequencing (BS). A total of 1 µg of genomic DNA was bisulfite-treated using the EZ DNA Methylation Kit (Zymo Research), which induces the chemical conversion of unmethylated cytosine to uracil, and eluted in 20 µl of elution buffer. A total of 3 µl of bisulfite-treated DNA were amplified by PCR in a total volume of 20 µl, with 4 mM of MgCl₂, 0.5 mM of each dNTP, 0.2 µM of each primer, and 0.5 unit of Taq Hot Start Thermostable DNA polymerase (EuroClone). The primers were designed using the Methyl Primer Express® Software v1.0 (Applied Biosystems) and their sequences are presented in Table SIIIB. Amplifications were performed using the following thermal

profile: 95°C for 2 min followed by 38 cycles at 95°C for 30 sec, 59°C for 30 sec and 72°C for 30 sec. The PCR products were purified from agarose gel using the EuroGold Gel Extraction kit (EuroClone) and cloned using the pGEM-T Easy Vector System (Promega Corp.) following the manufacturer's instructions. In total, 10 white colonies for each time point were resuspended in 50 µl of water and tested by colony PCR using the same PCR conditions as before, adding an extra step at 95°C for 10 min to induce cell lysis. PCR products were then purified by QIAquick PCR Purification kit (Qiagen GmbH). Sequencing of PCR products was performed using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems) on the Applied Biosystems 3130 Avant Genetic Sequencer.

Chromatin immunoprecipitation (ChIP). ChIP assay was performed as previously described (19). Washes of immunoprecipitated (IP) samples were carried out for 1 min using the HulaMixer® Sample Mixer (Thermo Fisher Scientific, Inc.), setting the rotation as follows: Orbital (rpm) 58-1, Reciprocal (deg) 13°-01, Vibro (pause) 5° off. IP purified DNA and inputs were amplified using genomic primer sets specific for *RASSF1* and *CDKN1A* gene promoters designed to overlap the regions investigated by BS (Table SIIIB). RT-qPCR was performed in a total volume of 20 µl including 5 µl of IP-DNA, 0.1 µM of each primer and 14 µl of SsoAdvanced™ Universal SYBR®-Green Supermix (Bio-Rad Laboratories, Inc.). Amplification was carried out in the CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.) using a two-step protocol consisting of 95°C for 3 min, 95°C for 10 sec and 64°C for 30 sec, 40 cycles, followed by a melting curve step to confirm the specificity of the PCR products. ChIP data were analyzed using the Percent Input Method: input Ct values corresponding to 1% of initial chromatin were adjusted to 100% and used to normalized Ct values of IP-DNA samples.

Immunocytochemistry (ICC). Cell blocks were prepared by fixing untreated cells, and cells at 96-h post wo and 21 days- post wo in 10% neutral-buffered formalin for 24 h and then embedding them in paraffin using Bio Agar gel (Bio Optica). Sections 4-µm-thick were cut and placed on positive-charged slides (Bio Optica). Immunostaining was performed with the Ventana Benchmark XT system (Ventana Medical Systems, Tucson, AZ) and the Optiview DAB Detection kit (Ventana Medical Systems), an indirect, biotin-free system for mouse IgG, mouse IgM and rabbit primary antibodies detection. The kit aims to identify targets by immunohistochemistry (IHC) and ICC in formalin-fixed, paraffin-embedded sections that are stained on the Ventana automated slide stainers and visualized by light microscopy. Incubation time and temperature are set by the stainer automatically. Antibodies against CDKN1A (DCS-60.2, Cell Marque, Ventana Medical Systems), thymidylate synthase (TS; clone 4H4B1, Life Technologies; Thermo Fisher Scientific, Inc.) and the DNA excision repair cross-complementation group 1 (ERCC1; clone 8F1, Neomarkers) were used. The CDKN1A antibody was pre-diluted by the supplier and incubated at room temperature for 16 min. For TS and ERCC1 detection, antibodies diluted at 1:300 and 1:100 were used, respectively, and incubated at room temperature for 32 min. Sections were automatically counterstained at room temperature for 16 min with hematoxylin II (Ventana Medical Systems).

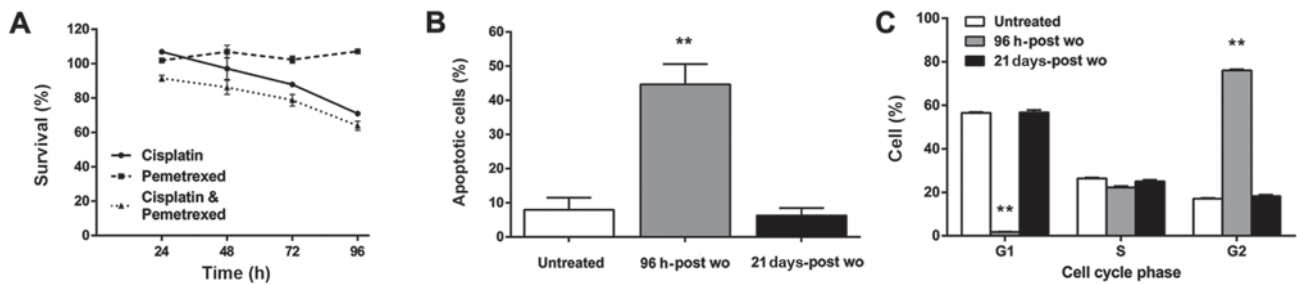


Figure 1. Cytotoxic activity of cisplatin and pemetrexed in RAL cells. (A) Effect of cisplatin and pemetrexed used alone and in combination on cell survival evaluated by MTS assay at 24, 48, 72 and 96 h after drug washout. Plasma peak concentrations were used for both drugs. Mean values and SEM of 3 independent experiments performed in octuplicate are reported. (B) Percentage of apoptotic cells evaluated by TUNEL assay in the cells at 96 h- and 21 days-post wo. Mean values and SEM of 3 independent experiments are reported. (C) Cell cycle analysis 96 h- and 21 days-post wo. Mean values and SEM of 3 independent experiments are reported. ** $P < 0.01$. post wo, post-treatment washout.

Hematoxylin and eosin staining, at room temperature for 16 and 1 min respectively, was also performed for each sample. Biomarker expression was semi-quantitatively analyzed as the percentage of immunopositive tumor cells out of the total number of tumor cells. All samples were evaluated by a trained pathologist and analyzed using an upright light microscope in bright field (Axioscope, Zeiss).

Statistical analysis. Three biological replicates for each time-point (untreated cells, and cells at 96 h-post wo and 21 days-post wo) were analyzed for each experiment and the results were reported as the mean values and standard deviation (SD) or standard error (SEM). Time points were compared using the non-parametric Friedman's test followed by the Bonferroni post-hoc test. Reported P -values were two-sided and $P < 0.05$ was used as the threshold for significance. Correction for multiple testing was not performed due to the explorative aim of this analysis. Statistical analyses were carried out using STATA/MP 15.0 for Windows (Stata Corp. LP).

Results

Cytotoxic effect analysis. The anti-proliferative effects of cisplatin and pemetrexed used as single agents or in combination were evaluated using the RAL cells by MTS assay at 24, 48, 72 and 96 h after drug washout. The RAL cells exhibited no or only slight sensitivity to pemetrexed and cisplatin, with 100 and 71% of cell survival at 96 h, respectively. Although a higher cytotoxic effect was observed at the end of the exposure time to both drugs, the half-maximal inhibitory concentration (IC_{50}) value was never reached (64% of cell survival at 96 h-post wo) (Fig. 1A).

TUNEL assay (Fig. 1B) revealed a percentage of apoptotic cells consistent with that of non-surviving cells detected by MTS assay when the two drugs were used in combination. In particular, a significant increase in the number of apoptotic cells was observed at 96 h-post wo (44.6%, $P = 0.002$). Notably, the cells had fully recovered 21 days after the end of treatment and began to grow and divide normally. Cell cycle distribution analysis (Fig. 1C) revealed a significant decrease in the number of cells in the G_0G_1 phase (1.7%) and a marked increase in the number of cells in the G_2M phase at 96 h-post wo (76%), whereas no differences with respect to the untreated samples were detected in the cells in the S phase. These cell cycle

perturbations had fully recovered at 21 days-post wo when the cell percentages in the G_0G_1 or G_2M phase were similar to those of the untreated cells (56.7% vs. 56.5%, and 18.2% vs. 17.1%, respectively).

Gene expression analysis. The expression of 84 oncogenes and tumor suppressor genes (TSGs) was analyzed in the untreated cells, and in the cells at 96 h-post wo and 21 days-post wo. In total, 11/84 genes were not expressed (*ESR1*, *FOXD3*, *HGF*, *KIT*, *MOS*, *MYCN*, *ROS1*, *RUNX3*, *TNF*, *TP73* and *WT1*) and 2/84 were unevaluable (*BCL2* and *WWOX*). Among the genes included in the panel, 8 (*CDKN1A*, *CDKN3*, *EGF*, *FHIT*, *JAK2*, *MYC*, *RASSF1* and *RBI*) and 3 (*CDKN1A*, *CDKN3* and *RASSF1*) were differentially expressed by >2 -fold in the cells at 96 h-post wo and 21 days-post wo, respectively (Fig. 2A and B). In particular, RT-qPCR analysis of the cells at 96 h-post wo confirmed that *CDKN1A* ($P = 0.002$), *EGF* ($P = 0.001$) and *JAK2* ($P = 0.009$) were significantly upregulated with a >2 fold change, whereas *FHIT* was downregulated ($P = 0.008$). A significant increase in *CDKN1A* mRNA expression was also maintained and confirmed in the cells at 21 days-post wo ($P = 0.011$) (Fig. 2C). The STRING database used to visualize protein-protein interaction (PPI) revealed a network with high degree of connectivity between the differentially expressed genes, *KRAS* and *AKT*, with a PPI enrichment P -value equal to 0.00106 (Fig. S1).

Epigenetic modifications associated with the *CDKN1A* promoter region. Subsequently, it was investigated whether epigenetic modifications (DNA methylation and histone marks) are associated with gene expression, by performing BS and ChIP on the RAL cells at 96 h and 21 days following the combined treatment. The results for the *CDKN1A* gene were compared with those obtained for *RASSF1*, a tumor suppressor gene often silenced and hypermethylated in RAL cells (15). The promoter regions of *CDKN1A* and *RASSF1* are shown in Fig. 3A and B, respectively.

DNA methylation analysis was performed by BS in 10 clones corresponding to the untreated cells, and cells at 96 h-post wo and 21 days-post wo. The methylation percentage of each cytosine was calculated by the average of the methylation status of the 10 clones. The promoter region of the *CDKN1A* gene was completely unmethylated (data not shown), whereas the *RASSF1* gene promoter exhibited a hypermethylated ($>40\%$)

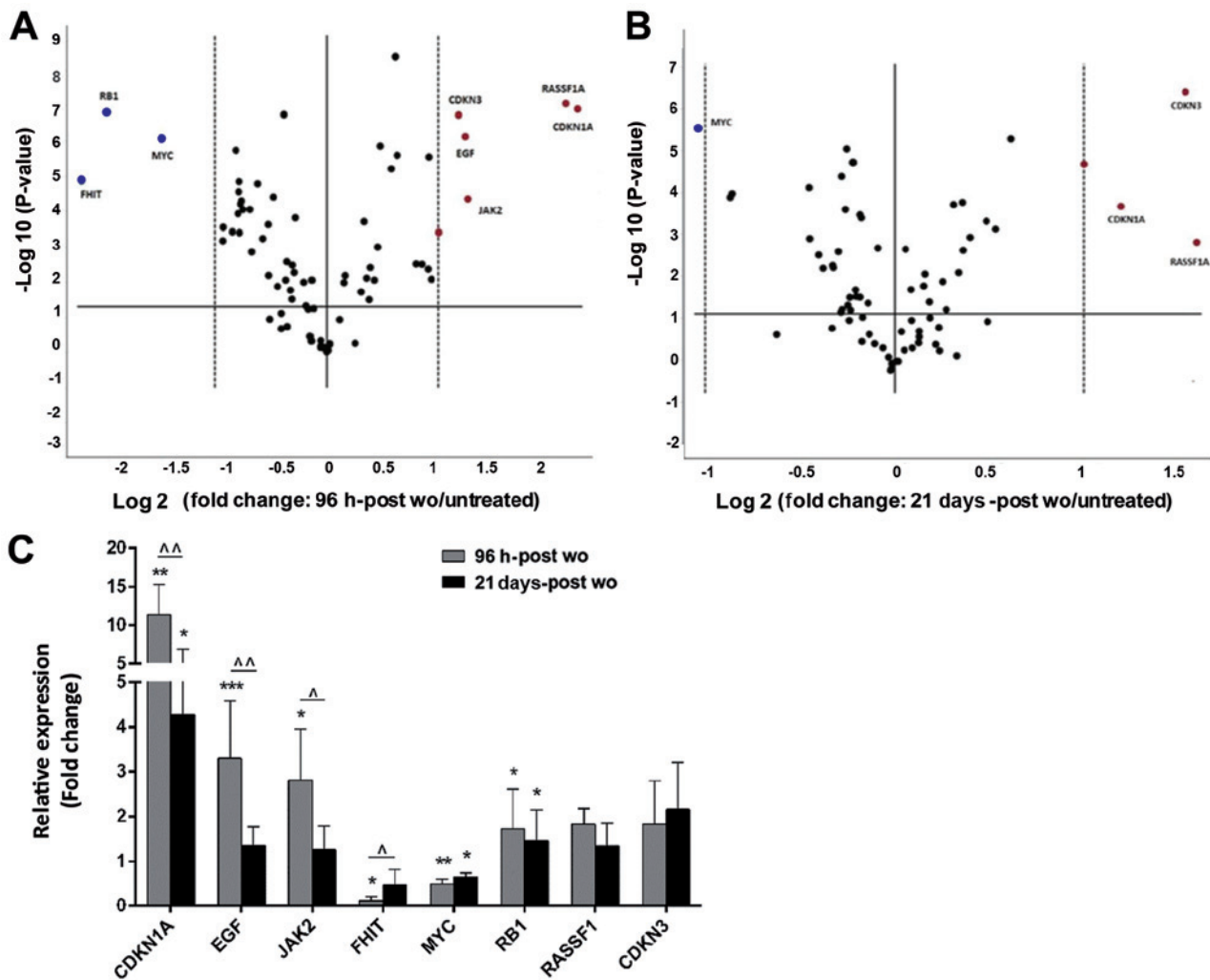


Figure 2. Effect of cisplatin and pemetrexed on the expression of oncogenes and tumor suppressor genes in RAL cells. (A and B) Volcano plots of PCR array results in cells at 96 h-post wo and 21 days-post wo. The solid black line indicates a 1.0-fold change in gene expression, while the dotted lines indicate the desired threshold of a 2.0-fold change in gene expression. The horizontal line in bold indicates the desired 0.05 threshold for the P-value of the ANOVA test. Blue, black and red spots indicate downregulated, unaltered and upregulated genes, respectively. (C) qPCR of significant genes resulted modulated by PCR array. The relative expression (fold change) of each gene was calculated using the $2^{-\Delta\Delta C_q}$ method, assuming the expression of each gene in untreated cells as 1. Mean values and SEM of 3 independent experiments are reported. * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$ indicates significant differences in the cells at 96 h-post wo and 21 days-post wo compared to the untreated control cells. Δ $P < 0.05$; $\Delta\Delta$ $P < 0.005$ indicates significant differences in the cells at 96 h-post wo compared those at 21 days-post wo. post wo, post-treatment washout.

CpG island (Fig. 3C). No significant differences were detected among the treated and untreated cells.

Three post-transcriptional histone modifications were investigated by ChIP assay: Two of these were associated with transcriptional active chromatin, i.e., the acetylated form of the H3 histone tail (acH3) and trimethyl-Lysine 4 of H3 histone tail (H3K4me3), and one enriched in transcriptional silenced chromatin domains, trimethyl-Lysine 27 of H3 histone tail (H3K27me3). The chromatin histone marks (acH3 and H3K4me3) corresponding to a transcriptionally open chromatin region were ≥ 7 -fold enriched in the promoter region of the *CDKN1A* gene compared to *RASSF1* (Fig. 3D and E). Conversely, the repressive histone mark H3K27me3 showed comparable results in the two genes (Fig. 3F). Isotype rabbit IgG was used as the background control (Fig. 3G). No substantial differences between the untreated cells and cells surviving the combination treatment were detected in histone modifications associated with the promoters of both genes,

with the exception of a significant 4-fold increase in acH3 enrichment in the *CDKN1A* promoter detected in the cells at 96 h-post wo with respect to the untreated cells, and cells at 21 days-post wo (Fig. 3D).

Effects of cisplatin and pemetrexed on *CDKN1A*, *TS* and *ERCC1* protein expression and subcellular localization.

An upregulation of *CDKN1A* protein expression was found in the cells at 96 h-post wo with respect to the controls (85 and 10% immunopositive cells, respectively), whereas *CDKN1A* expression in the cells at 21 days-post wo was equal to baseline (5% immunopositive cells) (Fig. 4A). In particular, it was observed that 10% of cells at 96 h-post wo exhibited a strong cytoplasmic expression (Fig. 5A) that was virtually absent in the untreated cells, and in cells at 21 days-post wo.

The results of western blot analysis of whole lysates (Fig. S2) were in line with the ICC results, confirming that

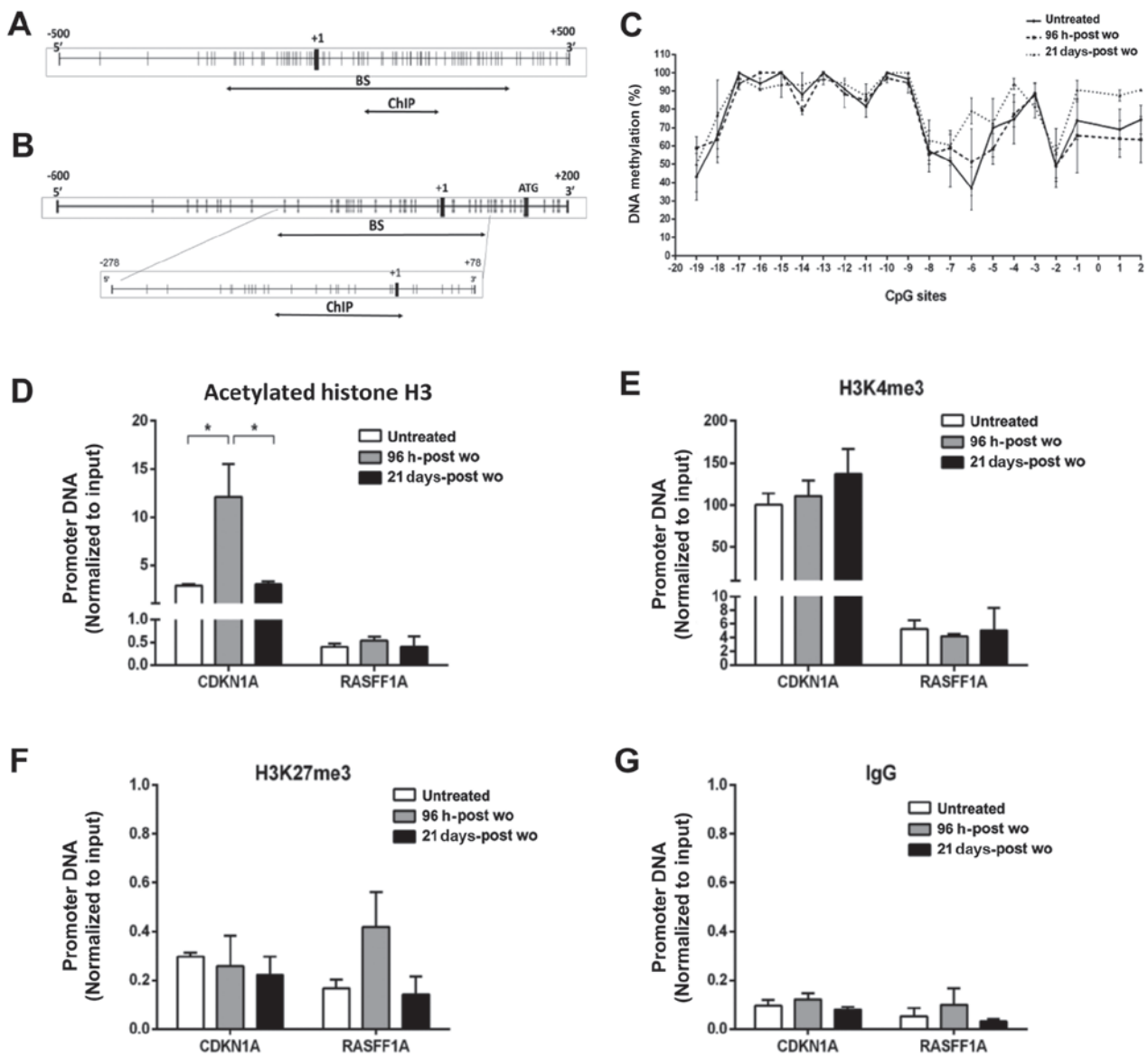


Figure 3. Effect of cisplatin and pemetrexed on epigenetic modifications associated with *CDKN1A* and *RASSF1* gene promoters. CpG island report of (A) *CDKN1A* and (B) *RASSF1* promoter regions. Each vertical bar represents a CpG site. The regions amplified by the primer sets are indicated by arrows. Bisulfite sequencing (BS) primers were designed to overlap the 5' region close to the transcription start site (+1). ChIP primers were designed to be included in the region analyzed by BS. (C) Percentage of DNA methylation of *RASSF1* promoter detected by BS analysis (*CDKN1A* gene promoter was completely unmethylated and thus not included). (D-G) ChIP analysis of histone modifications associated with *CDKN1A* and *RASSF1* promoter regions. Data are relative to immunoprecipitated DNA obtained with antibodies recognizing (D) acetylated lysines of H3 histone tail (H3K4me3) and (F) trimethylated-Lysine 27 of H3 histone tail (H3K27me3). (G) Rabbit IgG was used as background control. Chromatin from untreated RAL cells was compared with chromatin from cells at 96 h- and 21 days-post wo RAL cells. Ct values were normalized to inputs and reported as mean value and SEM of 3 independent experiments. * $P < 0.05$. post wo, post-treatment washout.

the cells at 96 h-post wo exhibited a marked upregulation of *CDKN1A* protein expression, while in the cells at 21 d-post wo, the protein level decreased with respect to the cells at 96 h-post wo.

Moreover, western blot analysis of protein lysates from subcellular compartments revealed that *CDKN1A* overexpression observed following treatment was mainly related to a protein increment in the cytoplasmic fraction. Again, the increased *CDKN1A* expression was more evident in the cytoplasm of the cells at 96 h-post wo (Fig. 5B).

We also characterized the RAL cells for the expression of TS and ERCC1, known to be potential markers of response to pemetrexed and cisplatin, respectively. The RAL cells

exhibited a high basal nuclear and cytoplasmic expression of TS, 80% of which were strongly immunopositive. Strong TS stain intensity was also observed in the cytoplasm (Fig. 4B). An additional upregulation of cytoplasmic TS was observed in the cells at 96 h-post wo, whereas only 40% of the cells were immunopositive at 21d-post wo. As regards ERCC1 protein expression, almost 100% of the untreated cells and cells at 96 h-post wo were strongly positive, whereas the cells at 21 days-post wo exhibited a somewhat lower positivity (75%) (Fig. 4C). The TS and ERCC1 protein expression levels were also confirmed by western blot analysis (Fig. S2). In all the immunocytochemical evaluations, the cells at 96 h-post wo appeared

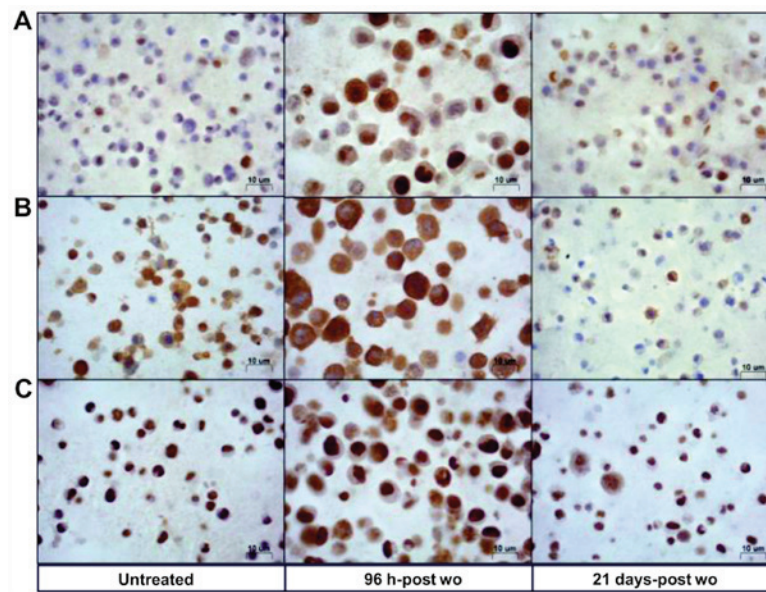


Figure 4. Effect of cisplatin and pemetrexed on protein expression of CDKN1A, TS and ERCC1 in RAL cells. Representative immunocytochemical staining for (A) CDKN1A, (B) TS and (C) ERCC1 in untreated cells, at 96 h-post wo and 21 days-post wo; x40 magnification. post wo, post-treatment washout; TS, thymidylate synthase; ERCC1, DNA excision repair cross-complementation group 1.

larger than their untreated counterparts, according to the well-known acute effects of cisplatin treatment (20,21).

Promotion of cell death by CDKN1A knockdown in the cells treated with the cisplatin-pemetrexed treatment combination. The role of *CDKN1A* gene in the cellular response to cisplatin and pemetrexed was investigated by transient silencing. The *CDKN1A* mRNA levels decreased significantly with respect to those observed in cells transfected with an appropriate negative control (scrambled oligonucleotide), confirming *CDKN1A* knockdown by siRNA. Moreover, its protein level was undetectable in cells transfected with *CDKN1A* siRNA (Fig. S3).

The RAL cells in which *CDKN1A* was silenced and which were exposed to the cisplatin-pemetrexed combination and evaluated 96 h after washout exhibited a consistently significant increase in cell death compared to the scramble control-transfected cells at 96 h-post wo. TUNEL assay revealed that there were 73.6% of apoptotic cells when *CDKN1A* was knocked down compared with 44% of apoptotic cells in the untreated cells transfected with *CDKN1A* siRNA (Figs. 6A and S4). As was expected, when scramble RNA was used instead of the *CDKN1A* siRNA probes, the cisplatin-pemetrexed combination induced a considerable increase in cell death compared to the untreated control conditions (40.1% vs. 22.1%, respectively) (Fig. 6A). The increase in cell death detected by TUNEL assay in the cells at 96 h-post wo with *CDKN1A* silencing was also confirmed by Annexin V assay, which identified both early and late apoptosis. In the 96 h-post wo condition, *CDKN1A* silencing was associated with 51.9% of cells in early apoptosis compared with 23.2% cells in the untreated siRNA counterparts and 9.4% in the cells at 96 h-post wo transfected with the scramble control (Figs. 6B and S4).

Finally, a cell cycle distribution analysis revealed that the cisplatin-pemetrexed combination led to a significant G2 phase block (Figs. 6C and S4) that was more evident in the

cells at 96 h-post wo than in the untreated cells transfected with either *CDKN1A* siRNA (58.6% in 96 h-post wo vs. 10.9% in untreated cells) or scramble RNA (56% in 96 h-post wo vs. 18.2% in untreated cells). Furthermore, as *CDKN1A* inhibits the activity of cyclin-CDK2, -CDK1 and -CDK4/6 complexes, functioning as a regulator of cell cycle progression during G1 and S phases, a significant increase in the cells in the G1-phase was also detectable in the cells transfected with siRNA at 96 h-post wo with respect to their scramble control counterparts (11.7 and 2.4%, respectively). No significant differences in percentages of S phase cells were detected in the untreated cells. Treatment with the cisplatin-pemetrexed combination caused an increase in the number of cells in the S phase (41.6% vs. 28.3% in the cells at 96 h-post wo and untreated cells, respectively). When *CDKN1A* was knocked down, the percentage of cells in the S phase decreased to the level of the untreated cells (29.7%).

Discussion

Platinum-based regimens used in combination with other chemotherapeutic compounds, such as pemetrexed, are still the optimal treatment selection for the adjuvant treatment of NSCLC and as the first-line therapy of advanced disease in patients whose tumor molecular signature limits the usefulness of biological therapies (22). However, the majority of patients are susceptible to disease progression due to acquired resistance.

The aim of the present study was to identify novel biomarkers of sensitivity/resistance to the cisplatin-pemetrexed treatment combination using the RAL cell line, an *in vitro* model representative of a subset of NSCLC patients who cannot benefit from biological therapies. The data of the present study revealed a significant increase in antitumor activity when both drugs were used in combination, rather than as single agents. For this reason, the present study aimed

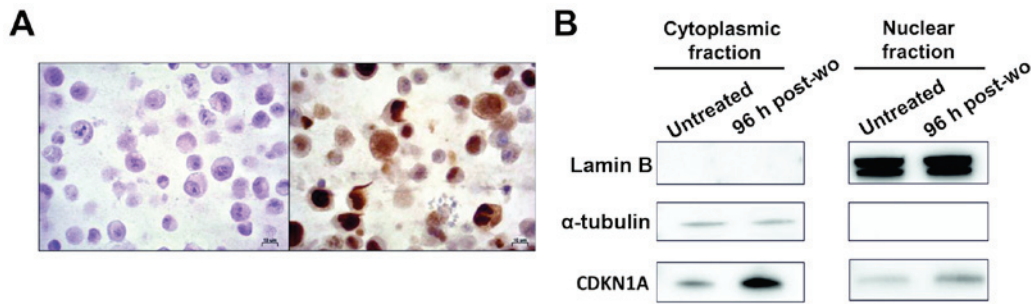


Figure 5. Effect of cisplatin and pemetrexed on *CDKN1A* expression and its cellular localization. (A) Representative hematoxylin and eosin staining (left panel) and *CDKN1A* staining (right panel) of cells at 96 h-post wo; x40 magnification. (B) Representative western blot of the *CDKN1A* expression level in cytoplasmic and nuclear protein fractions of untreated cells and cells at 96 h-post wo. Lamin B and α -tubulin were used as loading controls of the nuclear and cytoplasmic fractions, respectively. post wo, post-treatment washout.

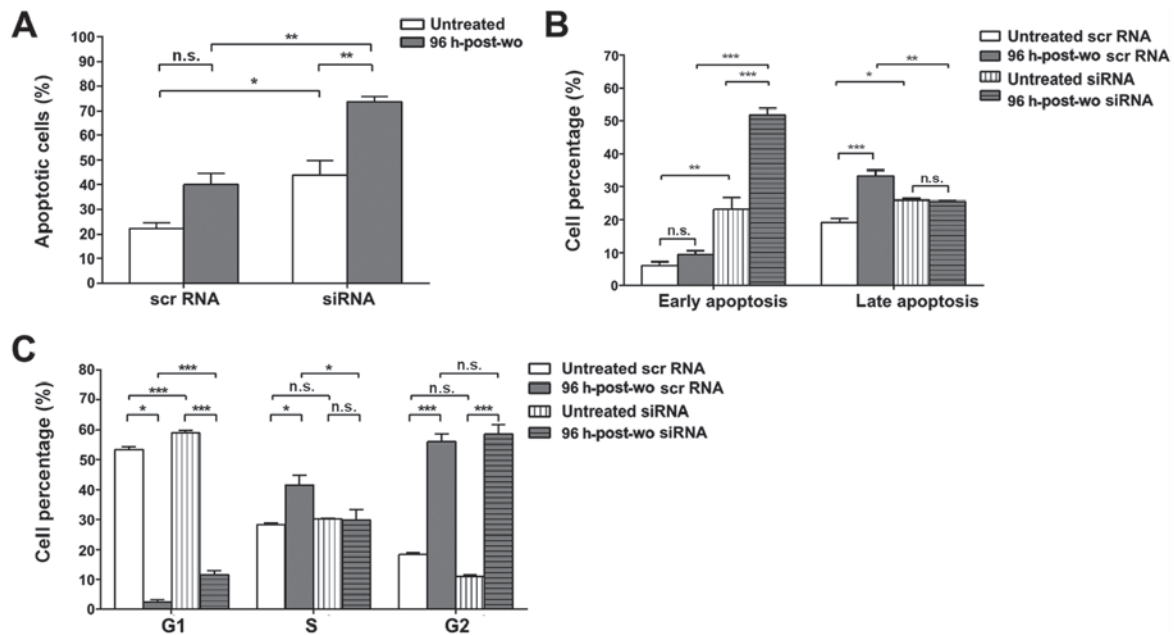


Figure 6. Effect of *CDKN1A* knockdown on cytotoxic activity of the cisplatin and pemetrexed combination in RAL cells. (A) Percentage of apoptotic cells evaluated by TUNEL assay in cells at 96 h-post wo with respect to untreated cells transfected with either scramble (scr) or *CDKN1A* siRNA. (B) Evaluation by Annexin-V assay of the percentage of apoptotic (early or late) cells in untreated and 96 h-post wo cells transfected with either scramble (scr) or *CDKN1A* siRNA. (C) Cell cycle analysis in untreated cells and cells at 96 h-post wo transfected with either scramble (scr) or *CDKN1A* siRNA. Mean values and SEM of 3 independent experiments are reported. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. n.s., not significant, post wo, post-treatment washout.

to perform a more in depth investigation of the combination of the two drugs, with no further investigations on single drugs. The observed antitumor activity was transient and 21 days after the treatment washout, the RAL cells had fully resumed their proliferation capacity.

Firstly, TS and ERCC1 protein expression was analyzed, since it is known that these markers may play a role in the response to pemetrexed and cisplatin respectively, even though contradictory results have been observed (23-27). In the present study, both markers were highly expressed either in the untreated cells and in the cells at 96 h-post wo, thus confirming their limited utility as response markers in this cell line.

Subsequently, the expression of genes involved in different biological pathways was analyzed to identify the genes implicated in the resistance of RAL cells to the cisplatin-pemetrexed treatment.

To this purpose, the gene profiling of 84 TSGs and oncogenes was performed followed by the validation of altered gene expression by qPCR. Among the genes confirmed as differentially expressed in the cells at 96 h-post wo, *FHIT* was the only one downregulated, perhaps contributing to platinum drug resistance via the serine threonine kinase AKT, particularly in *TP53*-mutated patients (28-30). The *CDKN1A*, *EGF* and *JAK2* mRNA expression levels were >2 -fold higher in the cells at 96 h-post wo compared to the untreated cells. Both *EGF* and *JAK2* are upstream regulators of STAT3 activity. STAT3 acts as a transcription factor, inducing the expression of genes involved in cancer progression, apoptosis resistance, cell proliferation and sustained angiogenesis (31). The significant overexpression of *EGF* and *JAK2* genes suggests that the activation of the JAK2/STAT3 signaling pathway may play a role in the resistance to chemotherapy observed in the model in the present study, as reported elsewhere (32).

Since it was the most upregulated gene in the cells at 96 h-post wo and the only gene still upregulated at 21 d-post wo, the role of *CDKN1A* was further investigated. The *CDKN1A* gene plays a crucial role in DNA repair, cell differentiation and apoptosis through the regulation of the cell cycle. It blocks DNA replication by binding to proliferating cell nuclear antigen and inhibits the activity of cyclin-CDK4 or -CDK2 complexes. As a result, the cell cycle is arrested in the G1/S or G2/M transitions (33,34). The role of *CDKN1A* in apoptosis is multifaceted and remains unclear (35-37). Some studies have suggested that it may mediate cell cycle arrest following DNA damage in either a p53-dependent or -independent manner, leading to an increase in apoptosis (38,39). Others have found that the inhibition of cell cycle progression, associated with elevated levels of *CDKN1A* gene expression, prevents apoptosis (34,40). Such a positive or a negative influence on cell growth and survival may result from its subcellular localization (34,41). Its cytoplasmic localization may favor cell proliferation as it acts as a chaperone for cyclin E, particularly when p53 is damaged or absent (42). Conversely, *CDKN1A* nuclear localization is associated with its better known function of modulator of cell cycle arrest and negative regulator of DNA repair pathways, resulting in unresolved DNA damage and growth inhibition (36).

Shoji *et al* reported that *CDKN1A* expression, in a large fraction of patients with completely resected pathologic stage I-IIIa NSCLC, was associated with a favorable prognosis (43). Moreover, a number of studies have demonstrated that *CDKN1A* modulation is involved in the response to different therapies in many cancer types (44-51). For example, cytoplasmic *CDKN1A* localization in testicular embryonal carcinoma leads to cisplatin resistance. In particular, high cytoplasmic *CDKN1A* levels exert a protective effect against cisplatin in embryonal carcinoma cell lines (52). The present study demonstrated that only 10% of the untreated RAL cells expressed *CDKN1A*, in line with the weak positive expression reported by Rosetti *et al* (46). The cytoplasmic expression revealed by western blot analysis (Fig. 5B) in untreated cells could be linked to its oncogenic activity in such an aggressive cell line, which is metastasis-derived.

In the present study, a significant *CDKN1A* upregulation was detected in the cells at 96 h-post wo, both at the mRNA and protein level, having a 10-fold higher mRNA expression and a marked cytosolic *CDKN1A* upregulation with respect to the untreated cells. In the cells at 21 d-post wo, *CDKN1A* remained upregulated, even though at a lower extent (Figs. 2C and S2).

Chromatin accessibility to transcriptional machinery is a crucial regulator of gene expression known to be involved in the clinical course of lung cancer (53). The analysis of epigenetic modifications associated with the *CDKN1A* gene promoter revealed that its gene expression regulation was not mediated by DNA methylation, but rather by chromatin histone marks H3K4me3 and acH3, representative of a transcriptionally open and active chromatin structure (Fig. 3). Although the increased level of association of acH3 in the promoter region of *CDKN1A* in 96 h-post wo cells compared to untreated cells correlated with increased gene expression, it was not sufficient to confirm epigenetics as the main molecular mechanism involved in the modulation of *CDKN1A* gene expression.

Taking into account the subcellular localization of *CDKN1A* in RAL cells surviving the combined chemotherapeutic action of cisplatin and pemetrexed, the high cytoplasmic expression levels of the protein appear consistent with the observed reduction in drug efficacy. It has been demonstrated that the phosphorylation of *CDKN1A* and its translocation to the cytosol, with the consequent progression of cell cycle through G1/S phase (54) and activation of anti-apoptotic pathways, may be AKT-mediated (55) and interfere with the cytotoxic effect of chemotherapeutic drugs (54-59). Preclinical and clinical data have also highlighted that PI3K/AKT/mTOR pathway inhibitors may be used in combination with other agents for the treatment of NSCLC (60).

In the present study, it was demonstrated that in the NSCLC cell line harboring *KRAS* G12C and *TP53* G244C mutations, *CDKN1A* silencing increased apoptosis and G1/S cell cycle arrest, thus increasing the efficacy of the cisplatin-pemetrexed combination. These findings support the hypothesis that *CDKN1A* functions as an oncogene, promoting cancer cell proliferation by inhibiting apoptosis, and highlight its potential role as a therapeutic response indicator of the pemetrexed-cisplatin combination in NSCLC.

A retrospective study to evaluate *CDKN1A* gene expression and localization in association with patient response to standard chemotherapy could also confirm *CDKN1A* role as a predictive biomarker of response to therapy in *KRAS*- and *TP53*-mutated NSCLC.

Acknowledgements

The authors would like to thank Mrs. Gráinne Tierney and Mr. Cristiano Verna of Istituto Scientifico Romagnolo per lo Studio e la Cura dei Tumori, for providing editorial assistance.

Funding

The present study was partially supported by Istituto Oncologico Romagnolo.

Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

Authors' contributions

AZ, AP, EG, DC, AT, PU, AD and CM conceived the idea for and designed the study. AZ, AP, CM, FP, SR and FFa were responsible for data acquisition and interpretation. FFa performed the statistical analysis. AZ, CM and AP drafted the manuscript. EG, GM, DC, AT, PU and AD critically revised the manuscript for important intellectual content. All authors read and approved the present version of the manuscript for submission and the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors confirm that they have no competing interests.

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