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Targeting nicotinamide N-methyltransferase decreased aggressiveness of osteosarcoma cells

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Abstract

Background: Osteosarcoma (OS) is a primary bone malignancy that mostly affects young people, characterized by high metastatic potential, and a marked chemoresistance that is responsible for disease relapse in most patients. Therefore, it is necessary to identify novel molecules to setup targeted strategies to improve the clinical outcome. The enzyme nicotinamide N-methyltransferase (NNMT) catalyses the N-methylation of nicotinamide and other analogs, playing a crucial role in the biotransformation of drugs and xenobiotics. NNMT overexpression was reported in a wide variety of cancers, and several studies demonstrated that is able to promote cell proliferation, migration and resistance to chemotherapy. The aim of this study was to explore the potential involvement of NNMT in OS.

Methods: Immunohistochemical analyses have been performed to evaluate NNMT expression in selected OS and healthy bone tissue samples. Subsequently, OS cell lines have been transfected with vectors targeting NNMT mRNA (shRNAs) and the impact of this downregulation on migration, cell proliferation, and response to chemotherapeutic treatment was also analysed by wound healing, MTT, SRB and Trypan blue assays, respectively.

Results: Results showed that OS samples display a significantly higher NNMT expression compared with healthy tissue. Preliminary results suggest that NNMT silencing in OS cell lines is associated to a decrease of cell proliferation and migration, as well as to enhanced sensitivity to chemotherapy. Data obtained showed that NNMT may represent an interesting marker for OS detection and a promising target for effective anti-cancer therapy.

KEYWORDS

anti-cancer therapy, cell migration, cell proliferation, molecular biomarker, nicotinamide N-methyltransferase, osteosarcoma

E.N. Serritelli and D. Sartini were contributed equally to this work.

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1 | INTRODUCTION

Osteosarcoma (OS) represents a rare and aggressive form of bone cancer that occurs at a rate of 3.4 individuals per million per year worldwide, with a higher prevalence in males than females. It primarily affects children and young patients, but it can be diagnosed in individuals of all ages. OS arises from primitive bone-forming cells, leading to the development of malignant tumours within bone tissue.¹ The most common sites for OS onset are long bones, particularly around the knee, but it can also occur in the pelvis and shoulders. Patients often present with local pain, swelling and limited joint mobility in the affected bone.² Unfortunately, these nonspecific symptoms often lead to delayed diagnosis, resulting in advanced and metastatic-stage disease. The pathogenesis of OS is complex and still unclear, risk factors such as intense linear bone growth during adolescence, hereditary cancer syndromes, disruption in critical signalling pathways and genetic mutations could contribute to the aetiology of this disease.³ Moreover, the exposure to several environmental agents like radium, beryllium, chromium has been implicated in its development. OS is a high-grade neoplasm recognized for its pronounced chemo- and radioresistance and tendency to metastasize to the lung.

Diagnosis of OS typically involves a combination of clinical evaluation, imaging studies and histopathological examination. X-rays, computer tomography (CT), magnetic resonance imaging (MRI) and bone scans are commonly used to visualize the tumour and assess its extent. Treatment of OS usually involves a multimodal approach, combining chemotherapy, surgical resection of the tumour and radiation therapy.¹ The current standard management involves surgical excision (limb salvage or amputation) combined with adjuvant chemotherapy by using of multiple cytotoxic agents like cisplatin, doxorubicin and high-dose methotrexate/ifosfamide. Radiotherapy is frequently used in combination with chemotherapy in tumours localized in inaccessible locations. Preoperative radiotherapy may be used to improve the success of limb salvage techniques and to reduce the risk of cancer recurrence. Limb salvage procedures along with neoadjuvant chemotherapy have revolutionized the treatment strategies, offering improved functional outcomes for patients. However, the presence of metastatic disease remains a significant negative prognostic factor, with long-term survival rates remaining sub-optimal.⁴ Results obtained by several 'omics' studies led to the discovery of many chromosomal abnormalities and mutations, as well as dysregulated genes, reasonably allowing OS to potentially respond to immunotherapy, by using check-point inhibitors and

targeted therapy.³ In this context, there is a pressing need to discover new target molecules for implementation of effective anti-cancer therapies.

Nicotinamide N-methyltransferase (NNMT) is a cytosolic enzyme involved in the second phase of metabolism, that catalyses the N-methylation of nicotinamide (NAM) and other related compounds, using S-adenosyl-L-methionine (SAM) as methyl donor and yielding N1-methylnicotinamide (MNAM) and S-adenosyl-L-homocysteine (SAH) as byproduct. MNAM may be excreted through urine or may be subsequently oxidized to N1-methyl-2-pyridone-5-carboxamide or N1-methyl-4-pyridone-3-carboxamide, by aldehyde oxidase enzyme and undergo urinary excretion. NNMT activity can regulate the intracellular levels of NAM, that is a precursor of nicotinamide adenine dinucleotide (NAD⁺), a coenzyme involved in redox reactions and other processes, such as mechanisms of DNA repair after structural damage and gene expression regulation at transcriptional level. After N-methylation, NAM can no longer be used as a NAD⁺ precursor substrate, thus NNMT activity plays a fundamental role in the regulation of NAD⁺ homeostasis, indirectly affecting a wide number of NAD-dependent enzymes.⁵ Based on research findings documented in various studies, NNMT has been found to be overexpressed in a wide range of solid tumours.^{5–11} The impact of this dysregulation on the phenotype of cancer cells is gradually being elucidated. Specifically, the collective evidence from these studies clearly demonstrates that NNMT can enhance cell proliferation, migration and resistance to chemo- and radiotherapy.

The focus of this research was to explore the role of NNMT in OS, by performing preliminary immunohistochemical analysis on healthy and tumour bone tissue samples. Subsequent analyses, carried out on OS cell lines transfected with vectors encoding short hairpin RNAs targeting NNMT mRNA, have been focused on evaluating the role played by enzyme dysregulation in tumour cell phenotype, concerning aspects related with viability, invasiveness and chemosensitivity.

2 | MATERIALS AND METHODS

2.1 | Tissue samples

A retrospective study was conducted in accordance with the principles of the Declaration of Helsinki on a total of 20 formalin-fixed and paraffin embedded (FFPE) tissue samples obtained from the archives of Section of Pathology, Department of Biomedical Sciences and Public Health of Polytechnic University of Marche. The

specimens included 15 OSs and five healthy bone tissues as control group.

2.2 | Immunohistochemistry

The expression of NNMT enzyme in tumour and healthy bone tissues was evaluated by immunohistochemical analysis using a rabbit polyclonal antibody against human NNMT (1:1500 dilution) (Sigma-Aldrich) as previously reported.¹² Briefly, sections were cut from FFPE blocks and, following installation on poly-L-lysine-coated glass slides, were deparaffinized in xylene and rehydrated through a graded series of alcohols. Samples were then treated with EnVision FLEX Target Retrieval Solution Low pH (Dako, Carpinteria, California, USA) to achieve antigen retrieval, and incubated with 3% H₂O₂ for 7 min to neutralize the endogenous peroxidase. Following a washing with EnVision FLEX Wash Buffer for 5 min, sections were incubated for 1 h in a humidified atmosphere at room temperature with antibody against human NNMT and then washed and incubated with EnVision FLEX/HRP (Dako) for 20 min. After a further washing, samples were incubated with EnVision FLEX DAB+ Chromogen (Dako) for 10 min. Sections were then counterstained with Mayer's haematoxylin (Bio-Optica, Milano, Italy) and permanently mounted on glass slides. Clear renal cell carcinoma was used as a positive control, whereas negative control slides were obtained by replacing primary antibody with rabbit IgG isotype. Stained cells were counted in at least 10 fields per sample (field area: 0.07 mm²; magnification: x 400), using a Nikon Eclipse E600 light microscope equipped with a Nikon DS-Vi1 digital camera, and quantified as a percentage of the total counted cells. Analyses were independently performed by two investigators blinded to the patient group (E.S. and G.G.), using a light microscope equipped with a digital camera. Stained cells were counted in at least 10 fields per sample (field area: 0.07 mm², magnification: x 400) and quantified as a percentage of the total counted cells. Agreement between observers was always >95%. Discrepancies were resolved by simultaneous re-examination of the slides, using a double-headed microscope.

2.3 | Cell lines and culture conditions

Human OS cell lines U-2 OS and Saos-2, purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA), were cultured in DMEM High Glucose media supplemented with 10% foetal bovine serum (FBS) and 50 µg/mL gentamicin, at 37°C in a humidified 5% CO₂ incubator.

2.4 | shRNA-mediated gene silencing of NNMT enzyme

To induce NNMT knockdown, OS cell lines were seeded in 24-well plates (4 × 10⁴ cells/well) the day before transfection. Plasmids (0.5 µg/well) encoding shRNAs targeting different regions of NNMT transcript (pLKO.1-164, pLKO.1-330, pLKO.1-448 and, pLKO.1-711) or an empty vector (pLKO.1-puro) were used to transfect 80% confluent cells, whereas control cells were treated with transfection reagent only (mock). Transfection protocol was performed using FuGENE HD Transfection Reagent (Promega, Madison, WI, USA), according to the manufacturer's instructions. 48 h following transfection, the medium was removed and replaced with a new one containing 1 µg/mL puromycin, in order to select, for each sample, those cellular clones harbouring vectors containing DNA element conferring resistance to puromycin. At the end of puromycin selection, Real-Time PCR and Western blot analysis were used to estimate the efficiency of NNMT gene silencing in U-2 OS and Saos-2.

2.5 | Real-time PCR

For Real-Time PCR analysis, total RNA was isolated from cell pellets (1 × 10⁶), according to manufacturer's protocol, reverse transcribed (2 µg) with M-MLV Reverse and used as template. The following primers were used: 5'-GAA TCA GGC TTC ACC TCC AA-3' (forward) and 5'-TCA CAC CGT CTA GGC AGA AT-3' (reverse) for NNMT, and 5'-TCC TTC CTG GGC ATG GAG T-3' (forward) and 5'-AGC ACT GTG TTG GCG TAC AG-3' (reverse) for β-actin. Genes were run in duplicate for 40 cycles at 94°C for 30 s and 58°C for 30 s, using SsoFast EvaGreen Supermix (Bio-Rad Laboratories, USA). Fold changes in relative gene expression were calculated by $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct(NNMT) - Ct(\beta\text{-actin})$, and $\Delta(\Delta Ct) = \Delta Ct(NNMT \text{ silencing plasmid}) - \Delta Ct(\text{empty plasmid})$.

2.6 | Western blot analysis

A Western blot analysis was set up to evaluate NNMT protein levels. Cell pellets (2 × 10⁶ cells) were suspended in 200 µL lysis buffer and homogenized using a syringe. Lysates were centrifuged at 16,000 × g at 4°C for 10 min, obtaining protein extract. 50 µg samples were subjected to 15% SDS-PAGE and transferred to polyvinylidene fluoride membranes (PVDF). After blocking and washing procedures, blots were probed with rabbit polyclonal antibody against NNMT (Sigma-Aldrich) (1:1000 dilution) or with mouse monoclonal antibody against

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz) (1:250), for 1 h, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma-Aldrich) (1:150,000 dilution) or with HRP-conjugated goat anti-mouse IgG (Biorad) (1:5000) for 2 h. NNMT was observed using enhanced SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA). Chemiluminescent bands were then acquired using a ChemiDoc XRS+ System (Bio-Rad Laboratories, Hercules, CA, USA), and signal intensity was quantified using Image Lab Software (Bio-Rad Laboratories, Hercules, CA, USA).

2.7 | MTT and Trypan blue exclusion assays

Cell viability of transfected OS cells was evaluated by MTT assay at different time points. U-2 OS and Saos-2 cells were seeded in a 96-well plate (1×10^4 cell/well) and allowed to attach to the plate overnight. At fixed time points (0, 24, 48 and 72 h), 10 μ L of MTT reagent (5 mg/mL in phosphate buffered saline) were added to the cells in complete medium (100 μ L/well). After incubation of 3 h at 37°C, the medium was discarded, and 200 μ L of isopropanol per well were added to dissolve formazan crystals, formed following MTT conversion by mitochondrial dehydrogenases. Reaction product was quantified by measuring the absorbance at 550 nm using an ELISA plate reader. Results were expressed as percentage of the control, that equals 100% and corresponds to the absorbance value of each sample at 0 h, and were presented as mean values $\pm$ standard deviation of three independent analyses.

Proliferation ability of OS cells downregulating NNMT, as well as of controls, was estimated by trypan blue exclusion assay at different timepoints (0, 24, 48 and 72 h). Cells were seeded on 6-well plates at a density of 3×10^5 cells/well in serum-free medium. The day after, representing the 0 h timepoint, the medium was replaced with a complete one, and for each time point, cells were harvested by using 500 μ L trypsin and centrifuged at 300 x g for 3 min. Cell pellet was resuspended in 500 μ L complete medium, trypan blue was added, and cells were counted using Burker's chamber. The number of viable cells (negative to staining) was expressed as percentage of the control (control equals 100% and corresponds to the number of viable counted cells of each sample at 0 h) and showed as mean values $\pm$ standard deviation. Each experiment was run in triplicate and independently repeated three times.

2.8 | Monolayer wound healing assay

In order to verify whether NNMT silencing influenced cell migration capability, U-2 OS and Saos-2 cells were seeded into 6-well plates (5×10^5 cells/well) and allowed to attach until 100% confluency. Cells monolayers were scratched using sterile 200 μ L pipette tip to create a vertical wound. After scratching, wounded monolayers were washed three times and incubated in DMEM High Glucose media supplemented with L-glutamine 2 mM and 0.5% FBS. Wounded monolayers were photographed at 0, 4, 8, 24 and 28 h after scratching. Images were analysed using software ImageJ (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, <https://imagej.nih.gov/ij/>, 1997–2018), and each experiment was performed in triplicate and independently repeated three times.

2.9 | Treatment with chemotherapeutic drugs

Cisplatin (CDDP) and doxorubicin (DOXO) were selected since representing two of the most used chemotherapeutic drugs for OS treatment, in order to evaluate cell sensitivity to chemotherapy after NNMT silencing. OS cells, both those downregulating NNMT and controls, were seeded in 96-well plates (3×10^3 cell/well). The day after, cells started to be treated with CDDP or DOXO at different concentrations (0.01, 0.1 and 1 μ M). For each sample, sensitivity to drugs was evaluated through MTT assay at different time points (24, 48 and 72 h after starting treatment with drug) and represented as percentage of decrease in cell viability. For each sample, sensitivity to CDDP or DOXO was also evaluated through Sulforhodamine B (SRB) assay that is based on the ability of the SRB dye to bind basic protein amino acid residues. The amount of dye incorporated by the cells indicates the cell number. Cells (5×10^3 cell/well) were plated in 96-well plates and the day after, cells started to be treated with CDDP or DOXO at different concentrations (0.01, 0.1 and 1 μ M). For each sample, sensitivity to drugs was evaluated through SRB assay at different time points (24, 48 and 72 h) after starting treatment with drug as previously described.¹³

2.10 | Statistical analysis

GraphPad Prism software (GraphPad Software, San Diego, CA, USA) was used to statistically analyse the data obtained. One-way analysis of variance (ANOVA) was

adopted to evaluate differences among examined samples. A $p < 0.05$ was accepted as statistically significant.

3 | RESULTS

3.1 | NNMT expression in bone tissue samples

NNMT immunohistochemical expression was evaluated both in healthy and pathological tissues. The cytoplasmic expression of the enzyme was significantly higher in OS bone tissue samples than in controls. In particular, OS cells exhibited a significantly ($p < 0.05$) good/strong immunostaining (percentage of stained cells: 81.88 ± 23.29) compared with that of healthy bone specimens (percentage of stained cells: $20.00 \pm 20\%$) (Figure 1).

3.2 | Efficiency of NNMT knockdown in U-2 OS and Saos-2 cells

NNMT silencing was confirmed by Real Time PCR and Western Blot analysis. Real Time PCR results showed a significant ($p < 0.05$) reduction of NNMT mRNA expression in U-2 OS cells transfected with pLKO.1-330 (0.2 ± 0.018), pLKO.1-448 (0.065 ± 0.002) and pLKO.1-711 (0.005 ± 0.00048) compared to cells treated with an empty vector (1.00 ± 0.58) (Figure 2A). Analogously, in Saos-2 cell line, treatment with pLKO.1-164 (0.48 ± 0.079), and pLKO.1-711 (0.14 ± 0.052) led to a significant reduction of NNMT expression respect to control (1.00 ± 0.1 ;

pLKO.1-puro) (Figure 2B). Western Blot analysis, coupled with densitometric analysis of immunoreactive bands, corroborated Real-Time PCR data showing a decreased NNMT expression in U-2 OS cells transfected with pLKO.1-330 (0.7 ± 0.055), pLKO.1-448 (0.51 ± 0.040) and pLKO.1-711 (0.2 ± 0.011) compared with those treated with empty vector (1.00 ± 0.089 ; pLKO.1-puro) (Figure 2C,E). Regarding Saos-2 cells, efficiency of NNMT knockdown was demonstrated only for cells transfected with pLKO.1-164 (0.47 ± 0.043) and pLKO.1-711 (0.22 ± 0.015) compared to control cells (1.00 ± 0.098) (Figure 2D,F). Therefore, cells transfected with the most efficient plasmids were selected for subsequent experiments.

3.3 | Impact of NNMT silencing on viability, proliferation and migration capability of OS cells

MTT assay, used to assess cell viability after cells transfection, showed a significant ($p < 0.05$) reduction of cell growth in U-2 OS cell line transfected with the three different plasmids respect to cells transfected with pLKO.1-puro at 48 and 72 h timepoints (Figure 3A). Saos-2 treated with pLKO.1-164 showed a decrease of cell viability only at 24 h, whereas cells transfected with pLKO.1-711 showed a significant ($p < 0.05$) reduction of cell viability at all timepoints respect to control cells (Figure 3C). Cell proliferation, measured by Trypan Blue staining, was decreased in U-2 OS cells treated with pLKO.1-711 at 24, 48 and 72 h, and at 72 h only in cells transfected with pLKO.1-330 and pLKO.1-448 (Figure 3B). Moreover, this assay clearly revealed that enzyme silencing

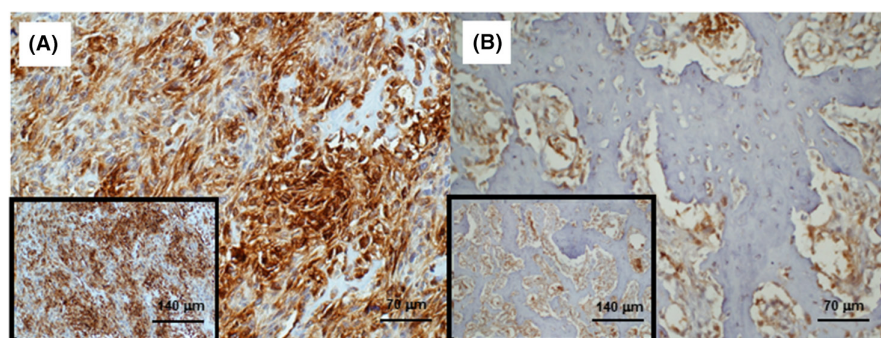
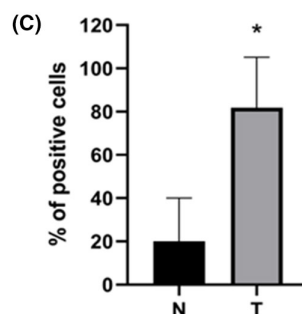


FIGURE 1 Immunohistochemical expression of NNMT in osteosarcoma and healthy bone tissue sections. Representative images showing NNMT cytoplasmic immunopositivity in osteosarcomas (A) and control tissues (B) (scale bar 70 μm ; inserts, scale bar 140 μm). Comparison between enzyme levels in osteosarcomas (T) and healthy bone tissues (N), (C). Values represent the mean percentage of positive cells $\pm$ standard deviation (* $p < 0.05$).



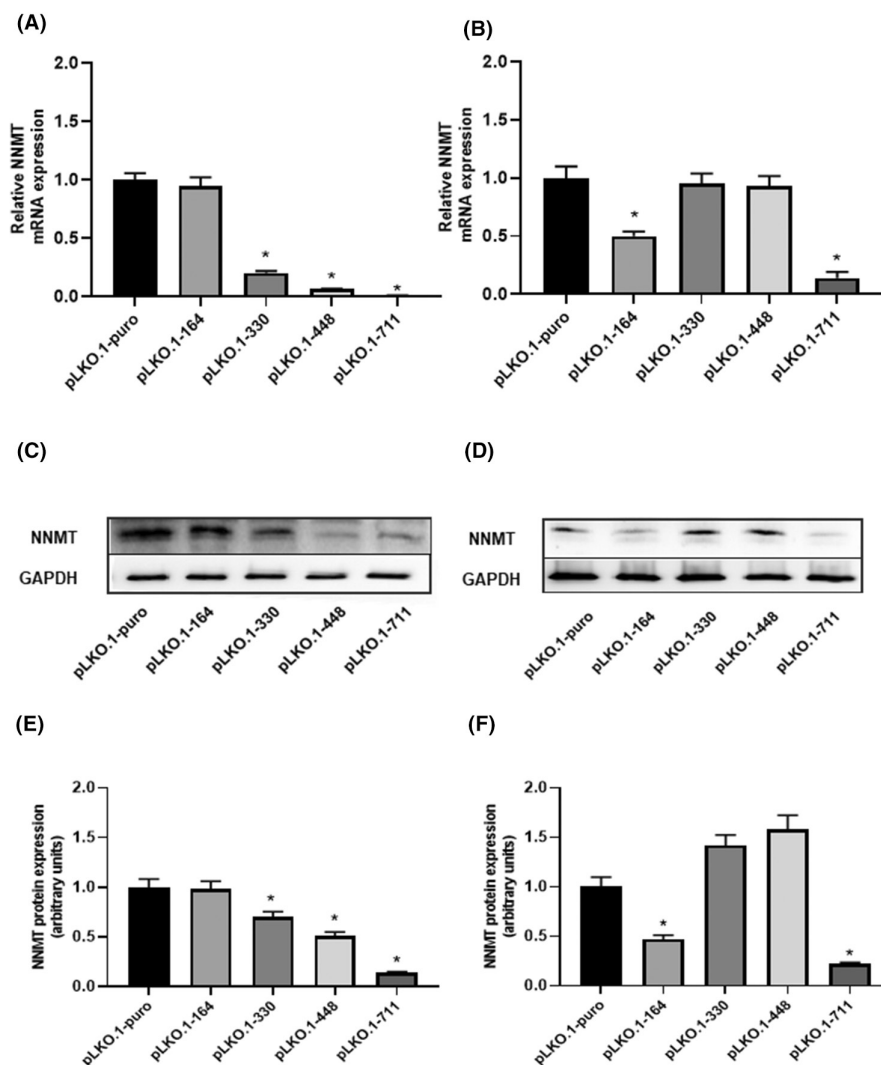


FIGURE 2 Efficiency of NNMT knockdown in OS cell lines. U-2 OS (A,C,E) and Saos-2 (B,D,F) cells were treated with plasmids targeting against NNMT (pLKO.1-164, 1-330, 1-448, 1-711), or with an empty vector used as control (pLKO.1-puro). NNMT mRNA expression was analysed by Real-Time PCR in transfected compared to control cells (A,B). Protein lysates, obtained from NNMT silenced and control cells, were analysed for NNMT expression by Western blot (C,D), followed by densitometry (E,F) ($n = 3$). Values are expressed as mean $\pm$ standard deviation (* $p < 0.05$).

led a marked reduction ($p < 0.05$) of cell proliferation at all tested timepoints in Saos-2 cells transfected with pLKO.1-711 compared to control cells, and at 48 and 72 h in those cells treated with pLKO.1-164 (Figure 3D). Monolayer wound healing assay was used to analyse the effect of NNMT silencing on the migration capability of OS cells, that in U-2 OS cells was reduced following knockdown with plasmids pLKO.1-448 and pLKO.1-711 at 24 and 28 h, and at 28 h only with plasmid pLKO.1-330, in comparison with control cells (Figure 4A, C), while the invasiveness of Saos-2 cells downregulating NNMT, was significantly decreased even earlier, starting from 4 h respect to control cells (Figure 4B, D).

3.4 | Sensitivity of U-2 OS and Saos-2 cells to the treatment with chemotherapeutic drugs

In order to evaluate the effect of NNMT silencing on cell chemoresistance, OS transfected cells were treated with

CDDP and DOXO at fixed concentrations (0.01 0.1 and 1 μ M) and then cell viability was assessed by using MTT assay at 24, 48 and 72 h from the treatment.

In U-2 OS cells, NNMT knockdown sensitized cells to the treatment with DOXO at 0.01, 0.1 μ M concentrations, showing a significant reduction of cell viability at 72 h timepoint compared to the controls, as well as to the treatment with CDDP at 0.01 μ M, while a reduction of cell viability was detected at 48 and 72 h timepoints respect to cells treated with the empty vector (pLKO.1-puro) after treatment with CDDP at 0.1 and 1 μ M (Figure 5A-F).

In Saos-2 cells transfected with plasmids vector coding shRNA against NNMT mRNA, enzyme knockdown led to a reduced proliferative capacity at 48 and 72 h after treatment with DOXO at 0.01 and 0.1 μ M and with CDDP 0.01 μ M respect to control, and at 72 h only in cells treated with CDDP 0.1 μ M. Interestingly, Saos-2 transfected with plasmid pLKO.1-164 showed a significant reduction of cell viability after treatment with CDDP 0.1 μ M in all tested timepoints (Figure 5G-L). SRB assay confirmed the MTT data showing in NNMT-downregulating U-2

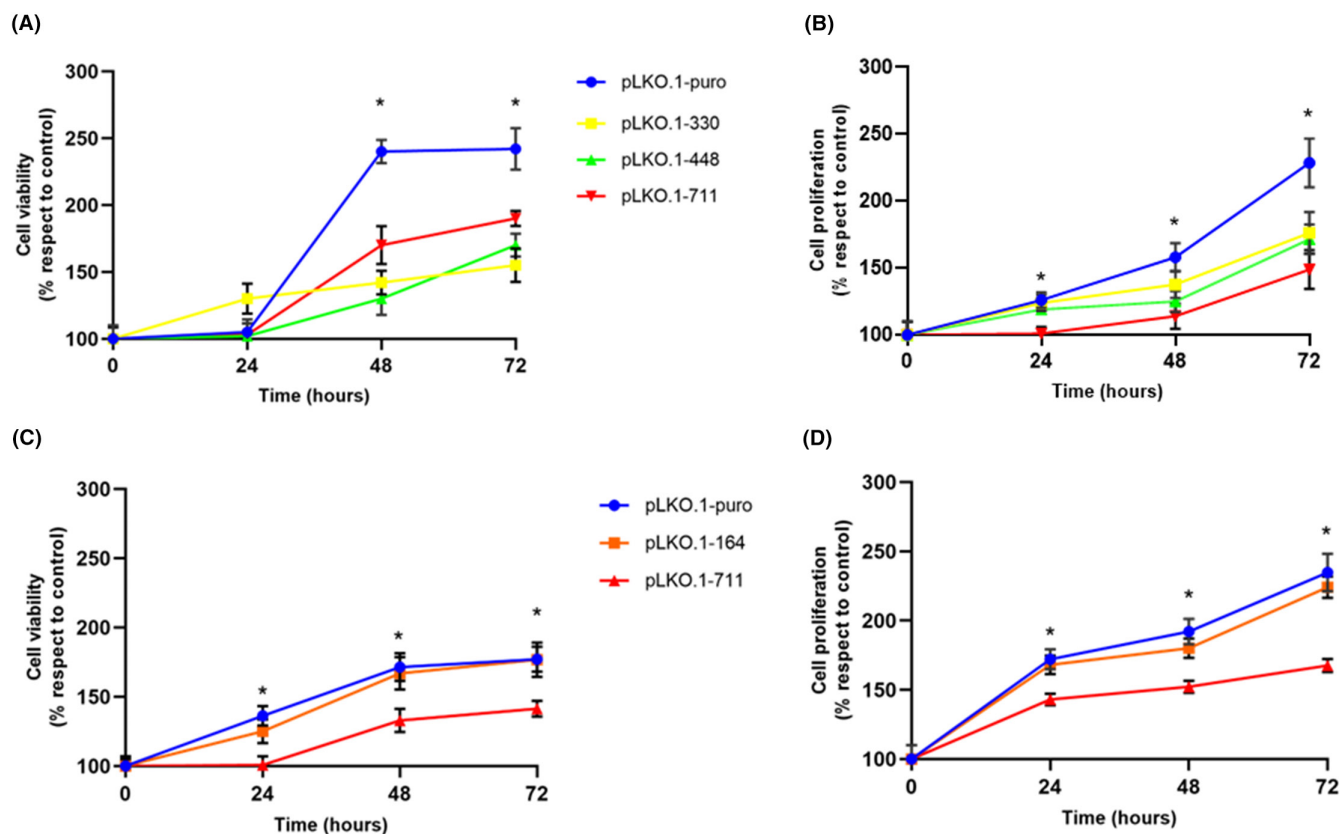


FIGURE 3 Impact of NNMT knockdown on OS cells viability and proliferation. Cell viability was analysed by MTT (A,C) and Trypan blue exclusion (B,D) assays, in U-2 OS (A,B) and Saos-2 (C,D) cell lines ($n=3$). Values are expressed as mean $\pm$ standard deviation (* $p < 0.05$).

OS cells a significant reduction of cell viability at 48 and 72h timepoints after treatment CDDP at 0.01, 0.1 and 1 μ M, and after treatment with DOXO at 0.01 and 0.1 μ M respect to control cells. In Saos-2 cells, NNMT silencing led to a reduced proliferative capacity at 24, 48 and 72h after treatment with DOXO at 0.01 and 0.1 μ M and with CDDP at 0.01, 0.1 and 1 μ M. Finally, in all samples analysed, exposure to DOXO 1 μ M resulted in an excessive cell death, thus preventing to appreciate potential differences in cell viability. Taken together, these data demonstrated that NNMT silencing enhances the sensitivity of OS cells to chemotherapeutic drugs (Figure 6).

4 | DISCUSSION

OS represents a primary bone neoplasm, mostly affecting young people. Due to its high metastatic potential, it is often associated with rapid progression and poor prognosis.² The presence of undetectable micrometastases at the time of diagnosis demonstrates the malignant nature of this cancer, thus leading OS patients to be treated with surgery coupled with systemic chemotherapy.^{4,14} Since OS is notoriously radioresistant,^{15,16} conventional treatment prior to (neoadjuvant) and following (adjuvant) surgery is

based on systemic chemotherapy. A combination called MAP, that includes high-dose of cisplatin, methotrexate and doxorubicin, is considered the first-line treatment of both neoadjuvant and adjuvant chemotherapy.^{17,18} However, OS is a particularly chemoresistant tumour that responds only to high dose drugs and rapidly acquires resistance, thus leading to poor survival rates.² Therefore, the elucidation of molecular mechanisms underlying resistance to chemotherapy, as well as the identification of new molecular targets for effective treatment of OS patients is fundamental.

The aim of this study was to analyse the involvement of the enzyme NNMT in OS, highlighting its contribution to cancer cell phenotype. NNMT is a phase II drug-metabolizing enzyme that catalyses the NAM and pyridines using the methyl group derived from SAM, thus playing a critical role in the biotransformation and detoxification of many drugs and xenobiotic molecules.⁵ No data are currently available about a potential association of NNMT with OS. Therefore, it seemed to be interesting to explore the role of the enzyme in this neoplasm, by performing immunohistochemical analysis in bone tissues and in vitro studies on OS cell lines. The expression level of NNMT was evaluated in tumour and healthy tissues of selected FFPE specimens by immunohistochemistry to

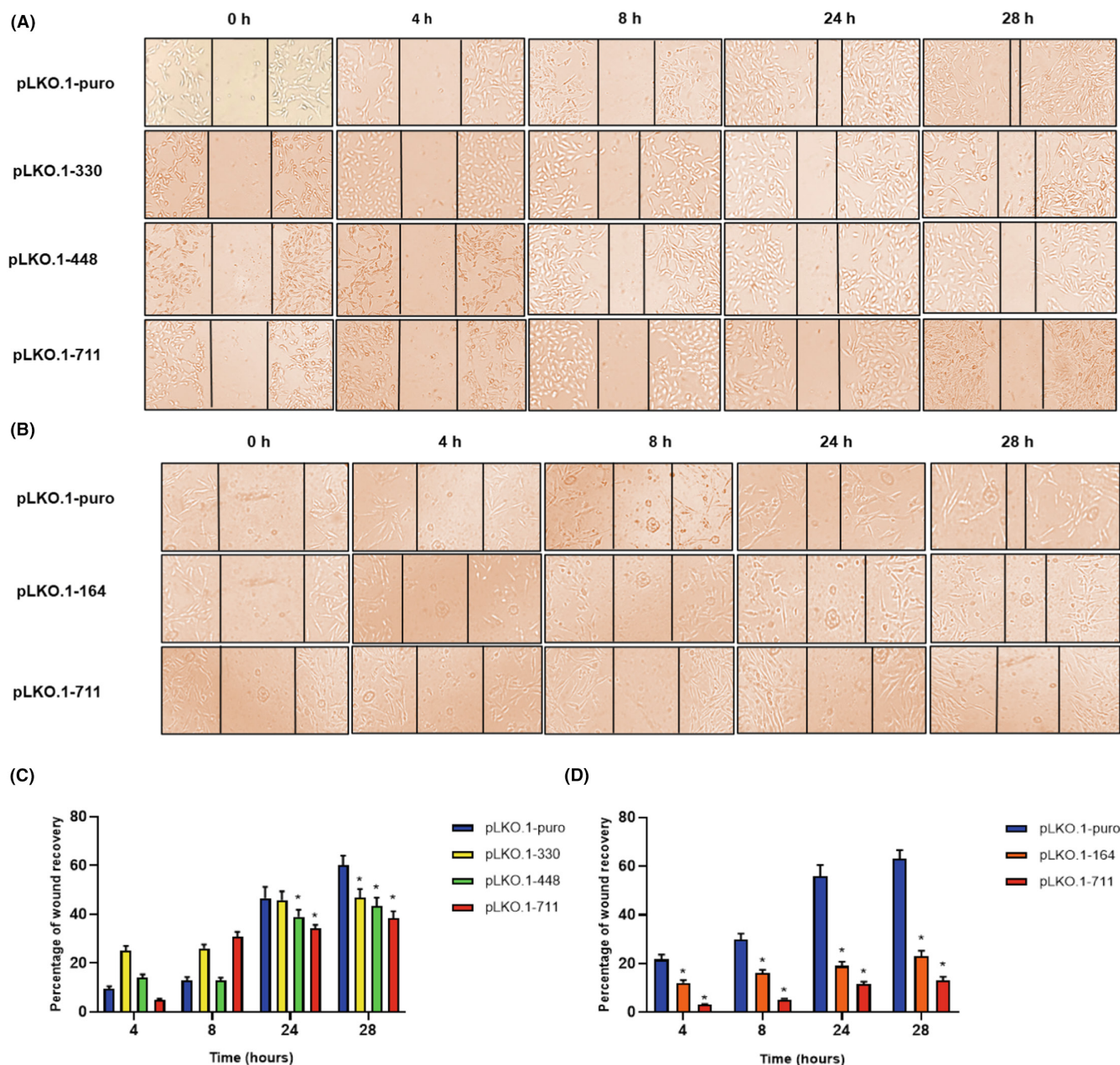


FIGURE 4 Effect of NNMT silencing on OS cells migration. OS cells were subjected to wound healing assay to analyse migration ability of U-2 OS (A) and Saos-2 (B) cells, by measuring percentage of wound recovery compared with 0 h, as reported in bar diagrams (C for U-2 OS and D for Saos-2) ($n = 3$). Values are expressed as mean $\pm$ standard deviation (* $p < 0.05$).

speculate whether NNMT was dysregulated in this neoplasm. Our results showed that the cytoplasmic expression of the enzyme was significantly higher in tumour samples compared to controls. Subsequently, shRNA-mediated knockdown of NNMT in U-2 OS and Saos-2 cell lines was carried out to investigate the effects of such dysregulation in OS cell metabolism and in cancer cell phenotype in terms of viability, proliferation, and invasiveness. Finally, it was assessed the impact of modulation of NNMT expression on chemoresistance of OS cell lines. MTT, SRB, trypan blue and wound healing assays revealed a significantly reduction of cell viability, proliferation and

migration of OS cell lines downregulating NNMT respect to control cells. Moreover, OS cells exhibiting significant enzyme knockdown as well as controls, were treated with different chemotherapeutic drugs (cisplatin and doxorubicin), at fixed concentrations and timepoints. Afterwards, the impact of NNMT expression modulation on cells subjected to chemotherapeutic treatment was assessed by the evaluation of cell viability that resulted significantly decreased in NNMT downregulating cells compared with controls.

In our previous studies, we found an enhanced expression of NNMT in several cancers, such as clear cell renal

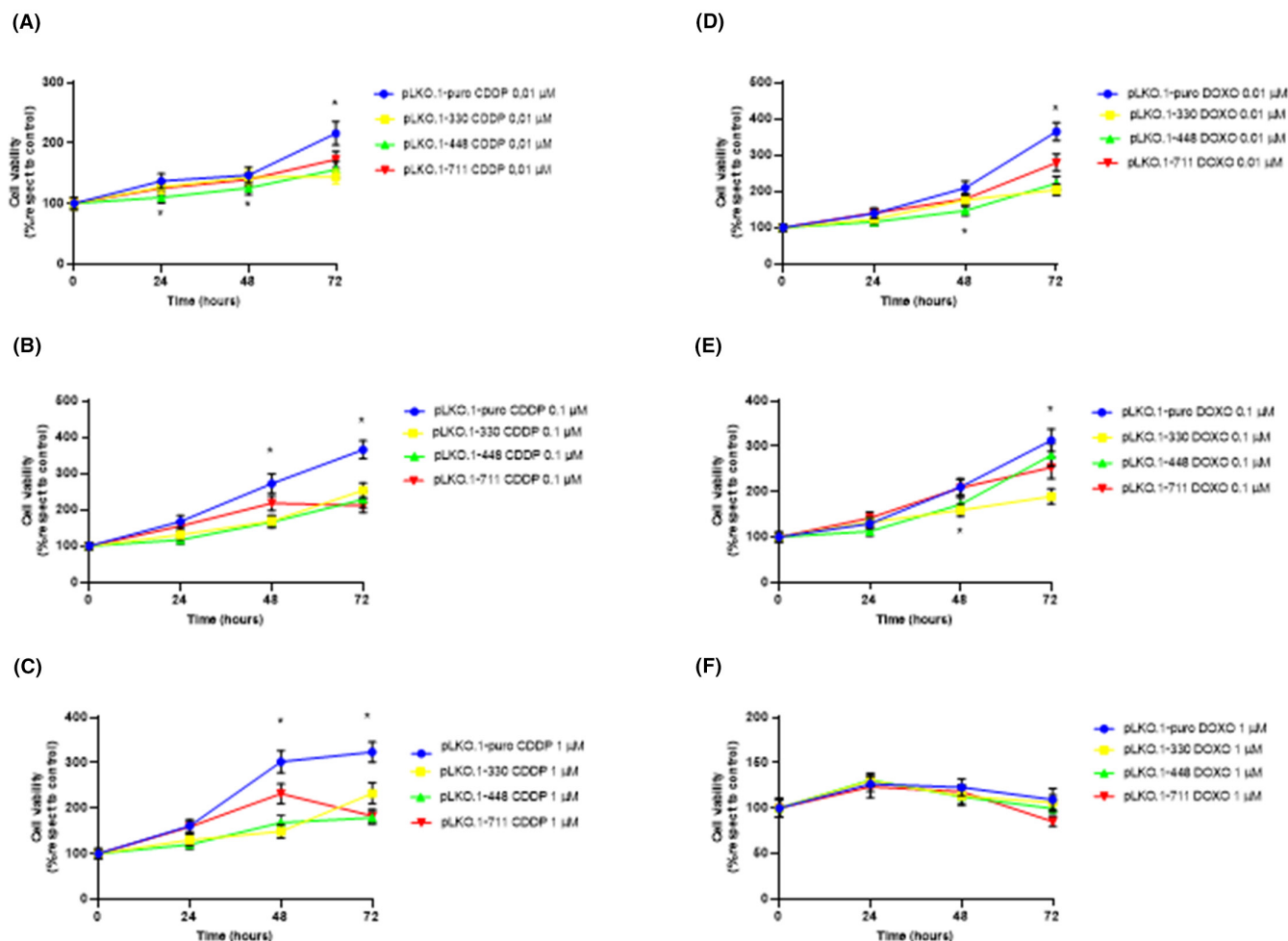


FIGURE 5 Impact of NNMT knockdown on chemosensitivity of OS cells. The effect of cisplatin (CDDP) and doxorubicin (DOXO) treatment at different concentration levels on viability of U-2 OS (A–F), and Saos-2 (G–L) cells was evaluated by using MTT assay. Measurements ($n=3$) were performed at 0, 24, 48, 72 h timepoints. All values are reported as mean $\pm$ standard deviation (* $p < 0.05$).

cell carcinoma,¹⁹ non-small cell lung cancer,²⁰ bladder urothelial carcinoma,⁸ oral squamous cell carcinoma¹⁰ and recently in skin malignancies.^{7,21} In these neoplasms, NNMT was shown to increase cell proliferation, viability, invasiveness, migration and metastatic potential.²² Analyses carried out on 786-O ccRCC cell line transfected with plasmids against NNMT mRNA showed that the enzyme efficiently inhibited tumour progression and metastasis in vivo and promoted cellular invasiveness in vitro by the activation of the PI3K/Akt/SP1/MMP-2 pathway.²³ In human 253J bladder urothelial cancer cell line, NNMT silencing led to a significant decrease of chemotaxis and chemokinesis related to cell migration, highlighting the crucial role of the enzyme in tumour metastasis and invasiveness.²⁴ Experiments of NNMT silencing in Bcap-37 and MDA-MB-231 human breast cancer cell lines showing endogenous high enzyme expression levels, led to apoptosis activation and significantly reduction of cell growth and tumorigenicity in vivo. On the contrary, NNMT overexpression in MCF-7 and SK-BR-3 breast cancer cell lines that

lacked constitutive enzyme expression has produced an opposite effect.²⁵ In the pancreatic cancer cell line PANC-1, NNMT silencing led to a reduction of cell proliferation and migration, as well as invasiveness, suggesting enzyme involvement in cell proliferation and metastatic capacity of these cancer cells.²⁶ In non-small cell lung cancer cell line, which displayed high endogenous NNMT levels, NNMT knockdown significantly inhibited colony formation.²⁷ In addition, treatment of two oesophageal squamous-cell carcinoma (ESCC) cell lines with plasmids targeted to NNMT mRNA significantly suppressed cell migration and proliferation, inhibited epithelial-mesenchymal transition (EMT) via the Wnt/ β -catenin pathway and promoted cell cycle and apoptosis.²⁸ Moreover, in the PC3 prostate cancer cells, the induction of the enzyme overexpression stimulated cell proliferation, invasive ability and migration capability, by increasing the histone deacetylase SIRT1 mRNA levels.²⁹ Our previous studies demonstrated that NNMT silencing led to decreased tumorigenicity and cell growth of PE/CA-PJ15, KB and A549 cancer cells.^{30–32} On

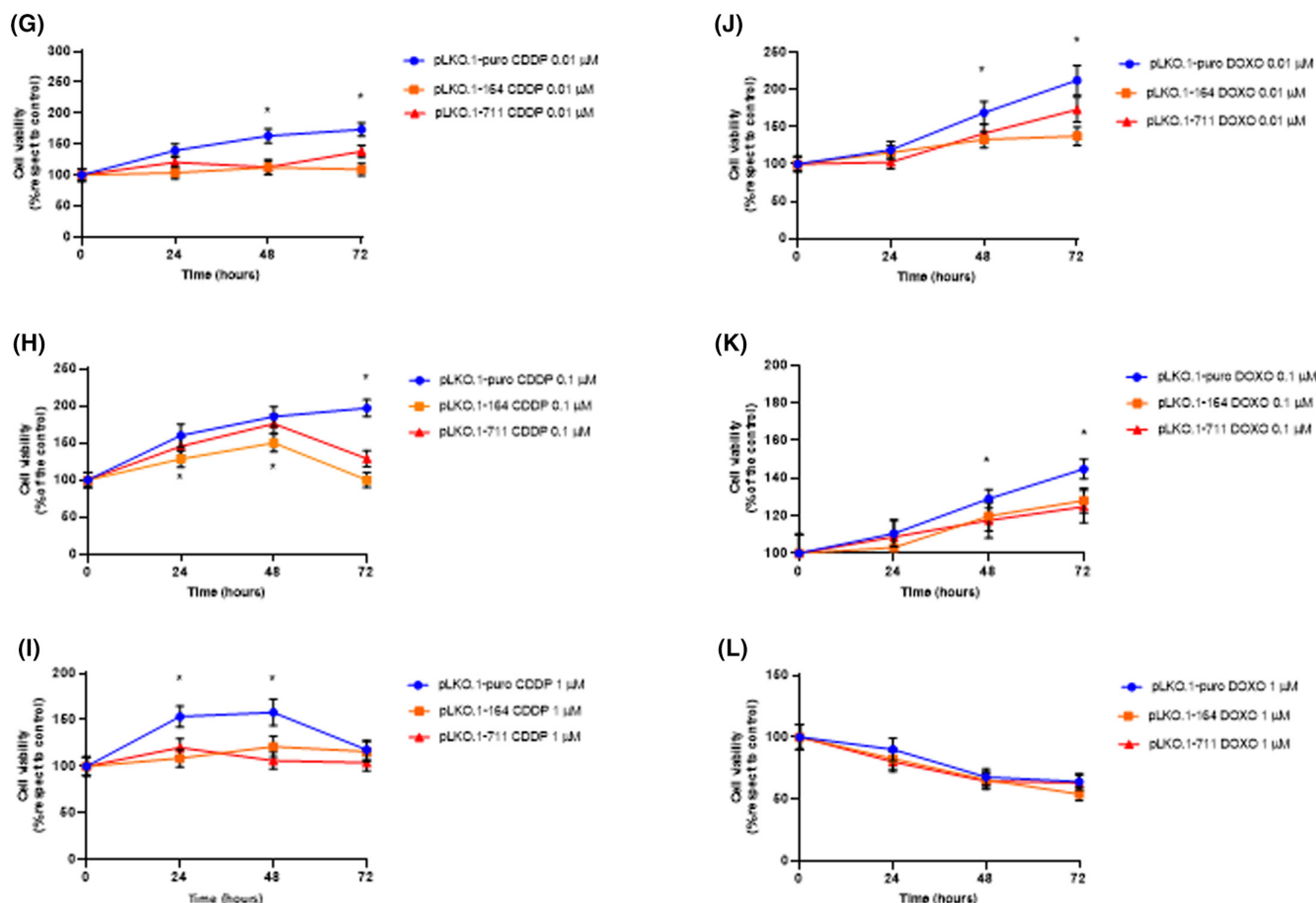


FIGURE 5 (Continued)

the contrary the induction of the enzyme overexpression in the HSC-2 OSCC cell line was significantly correlated with enhanced cell proliferation.³³ Moreover, we demonstrated that cancer stem cell (CSC) enrichment is associated with enhancement of NNMT expression in different cancer cell lines. Considering the implication of CSCs in carcinogenesis and in development of metastases, these results could suggest an important role of NNMT in the metabolism of tumour cell.³⁴ Up to date, it has not been completely clarified the specific mechanism by which NNMT enzyme can participate to carcinogenesis and tumour progression. NNMT catalytic activity influences the regulation the intracellular levels of nicotinamide, that is a specific precursor of the NAD⁺, available for the energy metabolism, thus modulating multiple pathways linked to cellular viability or apoptosis.³⁵ Some of these pathways include Poly(ADP-ribose) polymerases (PARPs) enzymes, that are important for the correct DNA repair after structural damage which prevents the malignant transformation.³⁶

Several studies clearly evidenced a correlation between NNMT expression and resistance to chemotherapy. Analyses performed on colorectal cancer cell lines showed that, the induction of NNMT upregulation in

SW480 cell line lacking endogenous enzyme expression promoted colony formation, cell proliferation, cell cycle progression, increased ATP level, and inhibited apoptosis.³⁷ NNMT downregulation in HT-29 cells, displaying high enzyme levels, decreased resistance to 5-fluorouracile (5-FU) by limiting the production of reactive oxygen species consequent to 5-FU treatment.³⁸ Moreover, in CRC cell lines characterized by high NNMT expression levels it was found that curcumin administration can attenuate NNMT-induced cell proliferation and 5-FU resistance by enhancing ROS production and triggering cell cycle arrest.³⁹ Experiments leading to NNMT overexpression or downregulation demonstrated that the enzyme decreased 5-FU sensitivity in human ESCC cells through the promotion of the Warburg effect, suggesting that the enzyme could contribute to predict the treatment effects of the therapeutic activity of 5-FU in this malignancy.⁴⁰ Wang et al. reported that breast cancer patients with high NNMT expression had shorter survival and lower chemotherapy efficacy compared to patients with low NNMT expression, suggesting that NNMT expression promotes chemoresistance in breast cancer patients. Moreover,

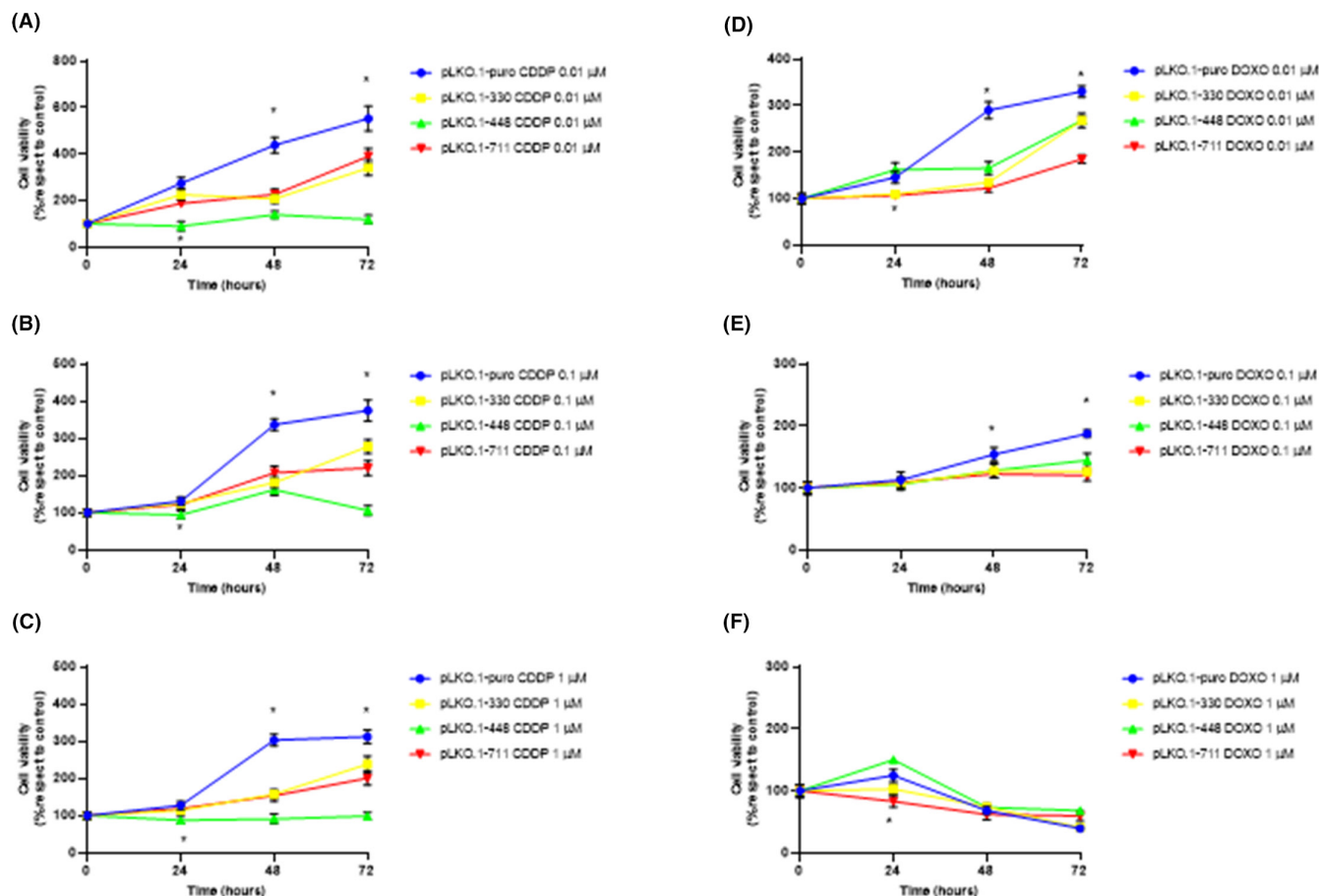


FIGURE 6 Effect of NNMT silencing on chemosensitivity of U-2 OS and Saos-2 cells. The effect of cisplatin (CDDP) and doxorubicin (DOXO) treatment at different concentration levels on viability of U-2 OS (A–F), and Saos-2 (G–L) cells was evaluated by SRB assay. Measurements ($n=3$) were performed at 0, 24, 48, 72 h timepoints. All values are reported as mean $\pm$ standard deviation (* $p < 0.05$).

utilizing in vitro cell lines either expressing endogenous high levels of NNMT, or NNMT-overexpressing cell lines stably transfected with pcDNA3.1-NNMT vector, it was demonstrated that higher levels of NNMT are associated with an increased resistance to paclitaxel and doxorubicin, while the opposite effect was observed in breast cancer cell lines transfected with plasmids encoding for shRNAs targeted to NNMT. Taken together these results confirmed that NNMT expression exerts a positive effect on the chemoresistance of breast cancer cells, and this mechanism has been associated to SIRT1 stabilization and activity, since NNMT-related resistance in breast cancer cells treated with SIRT1-specific siRNA was reverted.⁴¹ Moreover, we recently observed in A375 and WM-115 melanomas cell lines a significant reduction of cell proliferation and migration capability of both cell lines after NNMT silencing and an increase of the chemosensitivity to the treatment with dacarbazine, demonstrating that NNMT may play an important role in chemotherapy resistance.⁴² Taken together, all these evidences strongly support the hypothesis to target

NNMT, in order to set up effective therapeutic strategies against OS.

Since the beginning, the interest of scientific community towards the elucidation of NNMT involvement in cancer has been focused on solid tumours of epithelial origin. However, the recent gain of knowledge in the context of the EMT process featuring oncogenesis, as well as of the discovery of the crucial role played by tumour microenvironment, acted as drivers to shift the attention to other research fields, including not only the speculation of enzyme involvement in mesenchymal neoplasms but also the clarification of the NNMT contribution in tumour-surrounding stromal cells. In the light of these considerations, our study could be considered both pivotal and pioneering, since focused on analysing for the first time the expression and phenotypic impact of the enzyme in OS, the latter representing a rare but aggressive mesenchymal neoplastic disorder. Concerning the state-of-the-art of studies investigating the expression of NNMT in tumour stromal cells, a study by Eckert et al. reported that stromal NNMT expression was fundamental

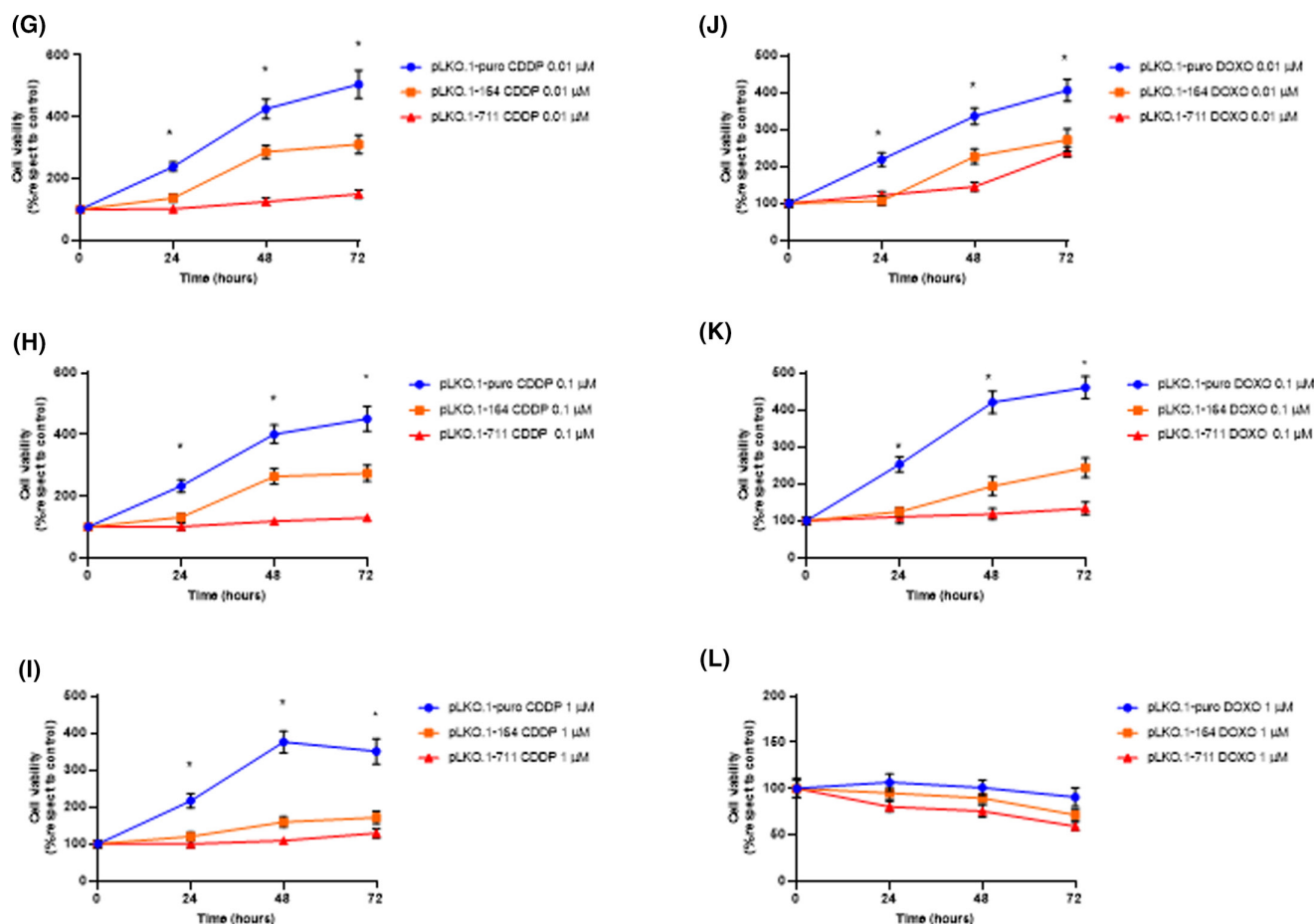


FIGURE 6 (Continued)

for functional activity of the cancer-associated fibroblasts (CAFs) and stromal NNMT expression promoted ovarian cancer proliferation and metastasis also in vivo. Furthermore, high NNMT expression level in tumour-stroma was responsible of SAM depletion and histones hypomethylation with a consequent aberrant gene expression.⁴³ The overexpression of NNMT in ovarian cancer tumour-stroma was also reported by Harmankaya et al. where NNMT has been observed to progressively increase expression in the stroma of ovarian neoplasms from benign cysts to high grade serous carcinomas, thus being correlated to poor survival.⁴⁴ An upregulation of NNMT has been described also in the tumour-stroma of colorectal cancer. NNMT expression levels in malignant cells and stromal cells were associated with several unfavourable clinicopathological features, such as advanced TNM stage, lymph node metastasis and distant metastasis. Furthermore, the release of MNAM by tumour-stroma cells was found to promote the migration and invasion of colorectal cancer cells.⁴⁵ Finally, a recent study performed by Zhao et al. reported high levels of NNMT expression in the stroma of oral squamous cell carcinoma. This overexpression was found to

be associated in the enrichment of H3K27me3 at the promoter of the lysyl oxidase (LOX) gene, a crucial enzyme for collagen I cross-linking, thus authors suggested that NNMT expression by tumour-stroma cells might favour tumour invasion by promoting extracellular matrix remodelling.⁴⁶ The sum of these data greatly highlighted the sophisticated role played by NNMT in regulating the crosstalk between tumour associated fibroblasts surrounding tumour bulk and cancer cells, thus conferring tumour phenotype a severe aggressive behaviour, with important potential consequences in clinical practice.

The present study is in line with other ones available in literature, describing a tumour-promoting role for NNMT. Our results demonstrated that NNMT is upregulated in OS and it could represent a promising marker for the detection of this malignancy. Moreover, our findings clearly demonstrated that NNMT promotes tumour cell proliferation, migration and chemoresistance of OS cells. Nonetheless, we are aware of the limitations of our study. Firstly, our immunohistochemical analyses involved a limited number of patients, due to the rarity of this malignancy. Moreover, our promising in vitro findings require a further validation in in vivo models. However, in the

light of our results, assessing NNMT expression may be a promising approach for histological diagnosis of OS. In addition, due to its involvement in cancer aggressiveness, suppressing the NNMT activity represents an interesting strategy for counteracting cancer growth and chemoresistance, providing the rationale for an effective anti-cancer therapy based on the use of specific inhibitors of the enzyme.

AUTHOR CONTRIBUTIONS

ME and DS designed the study. CR and ES selected tissue samples and performed immunohistochemistry. VP, ENS, MC and RC performed cell-based assays. DS, MJVH and MC performed statistical analyses. ENS and DS wrote the original draft of the manuscript. VP, RC, NIM, and ME reviewed and edited the manuscript.

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CONFLICT OF INTEREST STATEMENT

All authors declare that they have no conflict of interest.

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