Nicotinamide N-methyltransferase gene silencing enhances chemosensitivity of melanoma cell lines

PIGMENT CELL & MELANOMA Research

Nicotinamide N-Methyltransferase Gene Silencing Enhances Chemosensitivity of Melanoma Cell Lines.

Journal:	*Pigment Cell & Melanoma Research*
Manuscript ID	21-O-035.R2
Manuscript Type:	Original Article
Date Submitted by the Author:	11-May-2021
Complete List of Authors:	Campagna, Roberto; Università Politecnica delle Marche, Department of Clinical Sciences Salvolini, Eleonora; Università Politecnica delle Marche, Department of Clinical Sciences Pompei, Veronica; Università Politecnica delle Marche, Department of Clinical Sciences Pozzi, Valentina; Università Politecnica delle Marche, Department of Clinical Sciences Salvucci, Alessia; Università Politecnica delle Marche, Department of Clinical Sciences Molinelli, Elisa; Università Politecnica delle Marche, Department of Clinical and Molecular Sciences Brisigotti, Valerio; Università Politecnica delle Marche, Department of Clinical and Molecular Sciences Sartini, Davide; Università Politecnica delle Marche, Department of Clinical Sciences Campanati, Anna; Università Politecnica delle Marche, Department of Clinical and Molecular Sciences Offidani, Annamaria; Università Politecnica delle Marche, Department of Clinical and Molecular Sciences Emanuelli, Monica; Università Politecnica delle Marche, Department of Clinical Sciences; Università Politecnica delle Marche, New York-Marche Structural Biology Center (NY-MaSBiC)
Keywords:	Nicotinamide N-methyltransferase, Cutaneous melanoma, Cell proliferation, Cell migration, Chemosensitivity

TITLE

Nicotinamide N-Methyltransferase Gene Silencing Enhances Chemosensitivity of Melanoma Cell Lines

ABSTRACT

Melanoma accounts for less than 5% of all cutaneous neoplasms but is responsible for the greater part of skin cancer-related deaths. Therefore, the identification of molecules that could serve as therapeutic target is urgent. This study focused on the enzyme nicotinamide N-methyltransferase (NNMT). The effect of NNMT knockdown on cell proliferation and migration of A375 melanoma cells was evaluated by MTT and wound healing assays, respectively. Viability of A375 cells downregulating NNMT was also explored under treatment with dacarbazine, a chemotherapeutic drug approved for advanced melanoma treatment. The impact of enzyme knockdown on cell proliferation and chemosensitivity was also investigated in WM-115 melanoma cells. Results obtained demonstrated that NNMT silencing led to a significant reduction of cell proliferation and migration of A375 cells. Moreover, enzyme downregulation was associated with an increase of melanoma cells sensitivity to treatment with dacarbazine. Analogous effects induced by enzyme knockdown on cell proliferation and chemosensitivity were also found in WM-115 cell line. Our data seem to demonstrate that NNMT could represent a promising molecular target for the effective treatment of this form of skin cancer.

KEYWORDS

Nicotinamide N-methyltransferase; cutaneous melanoma; cell proliferation; cell migration; chemosensitivity.

1. INTRODUCTION

Melanoma arises from the transformation of cutaneous or mucous melanocytes. Cutaneous malignant melanoma represents less than 5% of all malignant skin neoplasms but, despite the low incidence, it is responsible for the majority of skin cancer-related deaths, due to its tendency to metastasize [1,2]. Its incidence varies greatly between countries and shows a steady increase [3]. Ultraviolet radiation exposure represents the main environmental risk factor, although host risks factors, such as genetic predisposition and the number of nevi, play an important role in tumor development [3].

If melanoma is diagnosed at an early stage, surgical excision is curative in the majority of cases [4]. Unfortunately, approximately 10% of neoplasms are detected at an advanced stage. These lesions are more difficult to treat although several therapeutic agents have been recently approved [3-5]. Despite progress in advanced malignant melanoma therapy, the patient prognosis remains poor [6]. The identification of reliable biomarkers represents therefore a primary goal.

In the present study, we focused on nicotinamide N-methyltransferase (NNMT), a cytosolic enzyme catalyzing the N-methylation of nicotinamide, pyridines, and other structural analogues [7], which was previously found to be upregulated in many solid tumors, among which cutaneous [8] and oral melanoma [9]. Moreover, a reduced proliferation and tumorigenicity of several cell lines, associated with NNMT downregulation, was also reported [10-12], while enzyme overexpression led to an increase of cell viability

[13]. Taken together, these results indicate that NNMT may be involved in tumor initiation and growth, thus supporting its potential as a novel diagnostic and prognostic biomarker, as well as an interesting therapeutic target.

Our aim was to analyze enzyme expression levels in human skin melanoma A375 cell line by Real-Time PCR and Western blot, and to evaluate the effect of NNMT gene silencing on cell proliferation and migration by means of MTT and wound healing assays, respectively. Subsequently, proliferation of NNMT downregulating A375 and control cells was evaluated after treatment with dacarbazine, in order to explore the potential involvement of the enzyme in sensitivity of skin melanoma cells to chemotherapy. The impact of NNMT knockdown on cell proliferation and chemosensitivity was further confirmed in WM-115 melanoma cell line.

2. MATERIALS AND METHODS

2.1 Cell lines and reagents

The human malignant melanoma cell line A375 was purchased from the American Type Culture Collection, while WM-115 melanoma cells were kindly provided by Dr. Claudia Matteucci, Department of Experimental Medicine and Surgery, University of Rome "Tor Vergata", Rome, Italy. Cells were cultured in Dulbecco's Modified Eagle's Medium (D-MEM) (Lonza, Basel, Switzerland) supplemented with L-glutamine 2mM, 10% fetal bovine serum (FBS) and 50µg/ml gentamicin, at 37°C in a humified 5% CO2 incubator.

2.2 shRNA-mediated gene silencing of NNMT

To achieve NNMT knockdown in A375 and WM-115 cells, a set of pLKO.1 vectors containing stem-loop cassettes encoding for different short hairpin RNAs (shRNAs) targeting different region of NNMT mRNA was used (Sigma-Aldrich, Saint Louis, MO), according to what previously reported [12].

The day before transfection, cells were seeded in 24-well plates ($4x10^4$ cells/well) with different plasmids (0.5µg for each well) coding each a specific shRNA against NNMT (pLKO.1-164, pLKO.1-330, pLKO.1-711), or with a combination of all three plasmids (0.5µg final DNA amount, named as pLKO.1-mix). Control cells were transfected with an empty vector (pLKO.1-puro) or treated with transfection reagent only (Mock).

Transfection was performed by using FuGENE HD Transfection reagent (Promega, Madison, WI, USA) according to manufacturer's instruction. Forty-eight hours from the beginning of transfection, cellular clones stably downregulating NNMT started to be selected by cultivating cells with complete medium containing 1ug/ml puromycin. For all subsequent experiments, puromycin resistant cells were maintained in complete selection medium

The efficiency of NNMT silencing in A375 and WM-115 cells was evaluated by Real-Time PCR and Western blot analysis.

2.3 Real-Time PCR

Total RNA isolation, reverse transcription and Real-Time PCR assay were performed according to the schemes described elsewhere [11].

Cell pellets ($1x10^6$ cells) were homogenized in a lysis buffer and total RNA was isolated with SV Total RNA Isolation System (Promega, Madison, WI, USA), according to manufacturer's protocol. Quantity and quality of RNA were spectrophotometrically evaluated at 260nm and 280nm. Total RNA (2ug) was reverse transcribed with M-MLV Reverse Trascriptase (Promega), using random primers in a total volume of 25µl for 60 min at 37°C.

The quantitative evaluation of NNMT mRNA expression was carried out using Real-Time PCR assay performed with CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). cDNA, generated as described above, was used as template. The primer oligonucleotides sequences were 5′-GAATCAGGCTTCACCTCCAA-3′ (forward) and 5′-TCACACCGTCTAGGCAGAAT-3′ (reverse) for NNMT, and 5′-TCACACCGTCTAGGCAGAAT-3′ (forward) and 5′-AGCACTGTGTTGGCGTACAG-3′ (reverse) for β-actin. Genes were run in duplicate using SsoFaste EvaGreen Supermix (Bio-Rad Laboratories) for 40 cycles at 95°C for 30 s and 58°C for 30s. All samples were tested in triplicate with the reference gene β-actin for data normalization to correct for variations in RNA quality and quantity. Direct detection of amplification products was monitored by measuring the fluorescence produced by EvaGreen. The measurements were then plotted against cycle numbers. Threshold cycle (Ct) was defined as the cycle number at which the first fluorescence increase above the threshold was detected. Changes in relative NNMT expression were calculated by $2^{-\Delta(\Delta Ct)}$, where ΔCt = Ct (NNMT)-Ct (β-actin) and Δ(ΔCt) = ΔCt (pLKO.1-164, pLKO.1-330, pLKO.1-711, pLKO.1-mix or pLKO.1-puro) - ΔCt (Mock).

2.4 Western blot

A Western blot assay was used to evaluate NNMT protein levels. Cells homogenization and Western blot procedure were performed as previously described [12].

Cell pellets ($2x10^6$ cells) were suspended in 200µl of lysis buffer (phosphate buffered saline containing 1% Nonidet P-40, 0.1% sodium dodecyl sulphate, 1mM phenylmethylsulphonyl fluoride and 2µg/ml aprotinin) and homogenized by passing (3–10 times) through a 30G needle attached to a 1ml syringe. Lysates were centrifuged at 16000xg at 4°C for 10 min and supernatants represented protein extract. 50µg of each sample were subjected to 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking and washing, membranes were incubated with rabbit polyclonal antibody against NNMT (Sigma-Aldrich) (1:1000 dilution) or with rabbit polyclonal antibody against β-actin (Sigma-Aldrich) (1:8000 dilution) for 1h, followed by an incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma-Aldrich) (1:150000 dilution) for 1h. An incubation (5 min) with Super-Signal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA) allowed the detection of the NNMT-related chemiluminescent signals.

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). Each experiment was performed in triplicate and independently repeated three times.

2.5 Protein concentration assay

Protein concentration was evaluated through the Bradford method using bovine serum albumin as standard.

2.6 MTT assay

Cell proliferation was evaluated using a colorimetric assay that quantifies the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to insoluble formazan by dehydrogenase enzymes of undamaged mitochondria of living cells. The MTT assay was performed in accordance with what previously described [12].

Briefly, cells downregulating NNMT (pLKO.1-164, pLKO.1-330, pLKO.1-711 and pLKO.1-mix for A375 cell line; pLKO.1-711 for WM-115 cells), as well as controls (pLKO.1-puro and Mock) cells, were seeded in 96-well plates ($1x10^3$ cells/well for A375 cells; $3.5x10^3$ cells/well for WM-115 cell line). The day after seeding, cell proliferation was evaluated (0h, 24h, 48h and 72h) by measuring the conversion of the tetrazolium salt MTT to formazan crystals. 100µl D-MEM containing 8.4µl MTT reagent (5mg/ml in phosphate buffered saline) were added to cells. After 4h of incubation at 37°C, medium was discarded, formazan crystals were dissolved by addition of 200µl of dimethyl sulfoxide and the reaction product was quantified by assessing the absorbance at 540nm.

Each experiment was repeated three times. Results were expressed as percentage of the control (control equals 100% and corresponds to the absorbance value of each sample at 0h) and presented as mean values ± standard deviation of three independent experiments performed in triplicate.

2.7 Monolayer wound healing assay

In order to evaluate migration capability, A375 cells were seeded into 6-well plates ($3x10^5$ cells/well) and allowed to grow up to 100% confluency. The wound on cells monolayers was made using sterile 200µl pipette tips. After scratching, cell debris were removed by washing cell monolayers three times and medium was replaced with D-MEM supplemented with L-glutamine 2mM and 0.5% FBS. Wounded monolayers were monitored under a microscope equipped with a camera and photographed at 0h, 4h, 8h, 24h and 28h after scratching. Images were analyzed by the software ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018). Each experiment was run in triplicate and independently replicated three times.

2.8 Chemotherapeutic treatment

Cells were seeded in 96-well plates ($1x10^3$ cells/well for A375 cell line; $3.5x10^3$ cells/well for WM-115 cells). The day after seeding, A375 and WM-115 cells started to be treated with complete medium, containing

(Sigma-Aldrich) 100 and 10µg/ml dacarbazine, respectively, previously dissolved in HCl 1M at 50mg/ml stock solution. Untreated cells were grown in complete medium containing HCl only, diluted at 2mM final concentration. This HCl concentration value (which did not affect the intracellular pH) corresponds to that obtained in samples subjected to treatment with chemotherapeutic drug. MTT assay was used to estimate the cell proliferation in untreated samples (0h) and at different time points (24h, 48h and 72h) after starting treatment with drug.

2.9 Statistical analysis

Data analysis was performed using GraphPad Prism software version 8.00 for Windows (GraphPad Software, San Diego, USA). Differences between groups were established using two-way analysis of variance (ANOVA). A p-value <0.05 was considered as statistically significant.

3 RESULTS

3.1 Efficiency of shRNA-mediated gene silencing of NNMT

In order to achieve enzyme knockdown, A375 and WM-115 cell lines were transfected with plasmids encoding shRNA targeting a different region of NNMT. Control cells were transfected with an empty vector (pLKO.1-puro) or treated with transfection reagent only (Mock). The effect of shRNA treatment on enzyme expression was determined by measuring the NNMT mRNA and protein levels, through Real-Time PCR and Western blot, respectively.

Results obtained showed that A375 cells transfected with plasmids encoding shRNA targeting NNMT mRNA displayed a significant ($p<0.05$) enzyme downregulation (0.48±0.02 for pLKO.1-164, 0.49±0.05 for pLKO.1-330, 0.37±0.03 for pLKO.1-711 and 0.56±0.03 for pLKO.1-mix) compared to Mock (1.00±0.07) or to cells treated with pLKO.1-puro empty vector (1.04±0.03) (Figure 1a). Western blot analysis confirmed a decreased NNMT expression in cells transfected with pLKO.1-164, pLKO.1-330, pLKO.1-711 or pLKO.1-mix compared with those treated with empty vector (pLKO.1-puro) or with transfection reagent only (Mock) (Figure 1b and c).

Analogously, WM-115 transfected with pLKO.1-711 exhibited significantly ($p<0.05$) lower NNMT mRNA levels (0.24±0.01) compared with controls (1.00±0.07 for mock; 1.04±0.03 for pLKO.1-puro) (Figure 1d).

3.2 Effect of shRNA-mediate gene silencing of NNMT on cell proliferation and migration

To explore the role of NNMT in cancer cell metabolism and evaluate the biological effect of enzyme downregulation, cell proliferation and migration were analyzed at different time points.

The influence of NNMT knockdown on cell proliferation was assessed by MTT assay, and results of colorimetric assay were expressed as relative cell proliferation referred to the control (absorbance at 0h and equal to 100%). As shown in Figure 2a, A375 cells treated with empty vector (pLKO.1-puro) showed no significant difference in cell proliferation with respect to Mock. Interestingly, enzyme downregulation

determined a significant ($p<0.05$) decrease of cell proliferation at 24h, 48h and 72h, for all cellular clones stably encoding shRNAs against NNMT mRNA (pLKO.1-164, pLKO.1-330, pLKO.1-711 and pLKO.1-mix) compared with controls (pLKO.1-puro and Mock). Moreover, cell proliferation was significantly ($p<0.05$) decreased in WM-115 cell line downregulating NNMT compared with controls (Figure 2b).

Monolayer wound healing assay was performed to monitor the migration ability of A375 cells. For each sample, results were reported as percentage of wound recovery with respect to 0h time point. Compared with Mock or cells treated with empty vector (pLKO.1-puro), the migration rate of A375 cells was significantly ($p<0.05$) decreased after NNMT silencing at all examined time points (4h, 8h, 12h, 24h and 28h) (Figure 3, panels a and b).

3.3 NNMT influence on A375 and WM-115 cells sensitivity to chemotherapeutic treatment

MTT assay was used to evaluate the effect of treatment with dacarbazine on cell proliferation. Cell treatment with medium containing 2mM HCl (Untreated) determined no significant difference in cell proliferation compared to cells treated with complete medium only (data not shown). At 48h and 72h time points, treatment with dacarbazine significantly ($p<0.05$) reduced proliferation of both A375 cells transfected with plasmids encoding shRNAs targeting NNMT mRNA (pLKO.1-164, pLKO.1-330, pLKO.1-711 and pLKO.1-mix) and Mock.

Interestingly, the decrease of cell proliferation upon treatment with dacarbazine was markedly enhanced in NNMT downregulating A375 cells compared with that detected in Mock (Figure 4a-b). Similarly, NNMT silenced WM-115 cells (pLKO.1-711) showed an increased sensitivity to treatment with dacarbazine with respect to control (Mock) (Figure 4e).

4 DISCUSSION

Cutaneous malignant melanoma (CMM) represents a widespread form of cancer associated with a high mortality rate. Excisional surgery is the first therapeutic option for early-stages CMM, followed by lymph node dissection, if appropriate [14]. However, about 9% of patients displays regional metastatic disease, while 4% of patients are diagnosed showing distant metastasis [15]. These patients display a poor prognosis, since their tumors are disseminated and unresectable. Moreover, unlike other solid tumors, advanced CMM typically responds weakly to traditional chemotherapy [14]. However, due to the lack of other effective therapeutic strategies, chemotherapy is still the best options for these patients, if affected with melanomas that do not harbor somatic mutations, such as those related with BRAF, NRAS, or cKIT. These subjects, in fact, cannot receive benefit from targeted immunotherapy based on specific inhibitors and without chemotherapy undergo disease progression [16].

The alkylating agent dacarbazine is used for treatment of advanced-stage CMM. This molecule gained FDA approval for the melanoma treatment in 1975 and therefore represents a longstanding chemotherapeutic drug. Several phase I and phase II clinical trials demonstrated partial and complete response rates in 15–28% and 3–5% treated patients, respectively, and durable responses were found in <2% subjects, only [17]. In order

to try to increase the efficacy of dacarbazine, a number of clinical studies have been carried out by using this compound combined with immunotherapies or other chemotherapeutic agents [18]. However, no improvement in overall survival was observed, but only an increase of side effects and the reduction of life quality [18]. Therefore, despite poor response rates, the overall outcome from these studies demonstrated that dacarbazine is still the standard chemotherapeutic option for patients with metastatic CMM [16,19-20]. In the light of these considerations, it is urgent to identify molecules, involved in melanoma initiation and progression, which are associated with resistance to chemotherapy, aiming to set up the basis for a targeted treatment of this neoplasm. The purpose of this research work was to explore the role played by NNMT in melanoma cell metabolism, focusing on aspects related to the possibility of the enzyme to affect cell sensitivity to chemotherapeutic treatment.

An overexpression of NNMT was found in numerous neoplasms, including clear cell renal cell carcinoma (ccRCC) [21,22], oral squamous cell carcinoma (OSCC) [23-25], non-small cell lung cancer (NSCLC) [26], bladder urothelial carcinoma (BUC) [27,28], and recently CMM [8] and basal cell carcinoma (BCC) [29].

NNMT upregulation in ccRCC displayed to be inversely related with the size of tumor, leading to hypothesize that the enzyme may be implicated in cancer progression [21]. In OSCC, NNMT overexpression was negatively correlated with pT, lymph node metastasis, pathological staging and histological grading, thus supporting its potential contribution in tumor growth and differentiation [23,24]. In pathological samples obtained from CMM patients, an inverse correlation was found between enzyme levels and clinicopathological parameters, such as Breslow thickness, Clark level, the presence/number of mitoses and ulceration [8]. The recent study, showing NNMT upregulation in BCC compared with healthy tissue margin, revealed that enzyme expression was significantly enhanced in nodular forms with respect to the infiltrative subtype, thus highlighting an interesting inverse correlation between NNMT levels and tumor aggressiveness [29]. The above results, taken together, seem to suggest that the enzyme could represent a candidate as molecular biomarker for both diagnosis and prognosis in the above-mentioned neoplasms.

NNMT expression was also found to be significantly higher in exfoliated cells from urines of patients with BUC compared with those of controls. Furthermore, statistical analyzes revealed an inverse correlation between tumor enzyme levels and histological grade, demonstrating the excellent diagnostic accuracy of a potential NNMT-based urine test [28]. Similarly, preliminary results indicated an upregulation of NNMT in saliva samples collected from OSCC patients compared with those of healthy subjects, strongly suggesting its potential application as a new salivary diagnostic biomarker for early and non-invasive detection of oral cancer [25].

The mRNA and protein levels of the NNMT, as well as its catalytic activity were also determined in several human tumor cell lines and corresponding cell populations enriched with cancer stem cells (CSCs). Data obtained revealed that enzyme expression was markedly increased in CSC-enriched populations compared with parental counterparts [30,31]. Given the role played by CSCs in tumor initiation, progression

and relapse, as well as in resistance to radio- and chemotherapy, these results strongly highlight the leading function exerted by NNMT in the metabolism of the cancer cells.

To date, it is not clear the exact mechanism by which NNMT may promote tumorigenesis and tumor progression. It has been proposed that NNMT can affect the methylation state of tumor cells not only by altering the ratio SAM:SAH ratio, but more likely by storing methylation units in tumor cells in form of MNA, thus fine-tuning the methylation state of cancer cells [32]. Furthermore, NNMT acts as a master regulator of the homeostasis of nicotinamide, a key precursor of the NAD^+. Therefore, the catalytic activity of the NNMT can control the amount of nicotinamide inside the cell available for the energy metabolism, thus influencing and modulating multiple pathways linked to both cellular survival and death [33]. Some of them, involving SIRT1 and the PARPs enzymes, are crucial for the correct DNA repair which prevents the neoplastic transformation [33, 34].

Results obtained in the present study clearly demonstrated that NNMT knockdown led to a significant reduction of cell proliferation and migration of melanoma cell lines. Moreover, the enzyme downregulation in melanoma cells was associated with an increased sensitivity to treatment with dacarbazine. In the context of studies carried out to explain the significance of NNMT upregulation in solid tumors and to clarify the impact of such dysregulation within cellular events promoting malignant transformation, a great contribution was made from our research group. Enzyme downregulation was significantly associated with a decrease of cell viability and anchorage-independent cell growth of HeLa-derived KB cancer cells [10], PE/CA PJ-15 OSCC cells [11] and NSCLC tumor cell line A549 [35]. Further-more, NNMT knockdown led to a considerable reduction of tumor formation capacity of PE/CA-PJ15 cells upon subcutaneous transplantation into athymic mice [11]. Conversely, the induction of enzyme overexpression in HSC-2 OSCC cell line was significantly associated with enhanced cell proliferation [13].

Treatment with short interfering RNAs against NNMT mRNA led to a marked reduction of chemotaxis and chemokinesis of the human BUC cell line 253J, thus highlighting the crucial role exerted by the enzyme in cellular events promoting cancer cell invasion and metastasis [36]. shRNA-mediated gene silencing of NNMT significantly reduced tumor formation and metastatic spread of 786O ccRCC cells subcutaneously transplanted into athymic mice, through the activation of the PI3K/Akt/SP1/MMP-2 pathway [37]. NNMT knockdown in Bcap-37 and MDA-MB-231 human breast cancer cells, originally exhibiting high enzyme levels, was associated with the induction of apoptosis via the mitochondria-mediated pathway and led to a significant reduction of cell growth in vitro, as well as tumor formation in vivo. Converse effects on cellular phenotype emerged after the induction of enzyme upregulation in MCF-7 and SK-BR-3 breast cancer cell lines displaying no endogenous NNMT expression [38]. By mimicking the above reported experimental procedure, NNMT role was explored in SW480 and HT-29 colorectal cancer (CRC) cell lines, lacking and showing high constitutive enzyme levels, respectively. Results obtained demonstrated that NNMT was able to enhance cell proliferation and colony formation ability, inhibit apoptotic pathway, promote the progression of cell cycle, and lead to an increase of ATP levels [39]. Furthermore, NNMT downregulation was significantly associated with reduction of cell growth and migration, as well as invasive capacity of the human pancreatic cancer cell

line PANC-1, thus highlighting enzyme involvement in cell proliferation and meta-static potential of cancer cell [40].

In the human NSCLC H1993 cells, which displayed high endogenous NNMT expression levels and were resistant to the treatment with the inhibitor of the tyrosine kinase domain of the EGFR gefitinib, enzyme knockdown significantly enhanced sensitivity to this molecule and suppressed colony formation ability [41]. The induction of NNMT overexpression in PC3 prostate cancer cell line enhanced cell proliferation, invasive capacity and migration ability, by increasing mRNA levels of histone deacetylase SIRT1. On the contrary, treatment with nicotinamide reversed the above reported effects, since suppressed the expression of SIRT1 and markedly decreased the invasive capacity of PC3 cells [42]. NNMT upregulation in BGC-823 gastric cancer cells led to the induction of transforming growth factor-β1 (TGF-β1) expression, with consequent activation of TGF-β1/Smad signaling, which in turn promotes epithelial-mesenchymal transition (EMT) as a key event in tumor invasion and metastasis [43]. Similarly, treatment of EC9706 and TE1 esophageal squamous cell carcinoma cells with short interfering RNAs targeted to NNMT mRNA significantly suppressed cell viability and migration, induced cell cycle arrest, promoted apoptosis and inhibited EMT via Wnt/β-catenin pathway [44]. Taken together, all these results seem to indicate that NNMT is a fundamental mediator of crucial events inducing malignant transformation, thus representing a potential molecular target for cancer therapy.

A few studies evaluated the ability of NNMT to influence the efficacy of drugs used for chemotherapeutic treatment. Enzyme downregulation diminished resistance to 5-fluorouracile (5-FU) in CRC HT-29 cells, while the opposite effect was induced in CRC cell line SW480 upon NNMT overexpression. The ability of NNMT to increase chemosensitivity was found to be mediated by the enzyme reaction product N1-methylnicotinamide, which reduces the production of reactive oxygen species induced by 5-FU treatment [45]. Furthermore, a recent study carried out in esophageal squamous cell carcinoma cell lines showed that NNMT knockdown increases 5-FU sensitivity of TE1 cells via suppressing Warburg effect, while overexpression of NNMT in EC1 and Eca109 cells increased the 5-FU sensitivity, thus suggesting that NNMT might be a potential therapeutic target to enhance the therapeutic efficacy of 5-FU [46]. Moreover, ectopic expression of NNMT in MDA-MB-231 breast cancer cells significantly inhibited apoptosis and suppression of colony formation induced by adriamycin and paclitaxel, through the induction SIRT1 expression [47].

Results obtained in this work, seems to suggest that NNMT is somehow involved in mechanisms promoting melanoma cell resistance to chemotherapy. In this light, further studies will clarify whether the combination of enzyme inhibitors with dacarbazine could enhance sensitivity of A375 cells to chemotherapeutic treatment. Indeed, cloning, expression and purification of human recombinant NNMT allowed to determine the crystal structure of the enzyme, as well as to identify the main amino acid residues involved in catalysis exerted by NNMT [7], thus leading to design and assay new and effective NNMT inhibitors [48].

To the best of our knowledge, this is the first study to evaluate the role played by NNMT in cellular events featuring melanoma progression and spread, as well as to explore its potential involvement in molecular processes affecting chemoresistance. Although further analyses are necessary to give a deeper insight into the

mechanisms by which the enzyme could participate to melanoma tumorigenesis, our results seem to demonstrate that NNMT could represent an interesting molecular target for effective treatment of this form of skin cancer.

ACKNOWLEDGMENTS

We would like to offer our sincere thanks to Dr. Claudia Matteucci (Department of Experimental Medicine and Surgery, University of Rome "Tor Vergata", Rome, Italy), for providing with WM-115 melanoma cell line.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Ossio, R., Roldán-Marín, R., Martínez-Said, H., Adams, D.J., Robles-Espinoza, C.D. (2017). Melanoma: a global perspective. *Nat Rev Cancer*, 17, 393-394.
2. Erdei, E., Torres, S.M. A new understanding in the epidemiology of melanoma. (2010). *Exp Rev Anticancer Ther*, 10, 1811–1823.
3. Leonardi, G., Falzone, L., Salemi, R., Zanghì, A., Spandidos, D.A., Mccubrey, J.A., Candido, S., Libra, M. (2018). Cutaneous melanoma: From pathogenesis to therapy. *Int J Oncol*, 52, 1071–1080.
4. Ross, M.I., Gershenwald, J.E. (2011). Evidence-based treatment of early-stage melanoma. *J Surg Oncol*, 104, 341–353.
5. Malas, S., Harrasser, M., Lacy, K.E., Karagiannis, S.N. (2014). Antibody therapies for melanoma: New and emerging opportunities to activate immunity. *Oncol Rep*, 32, 875–886.
6. Wiesner, T., Zbyszewski, A. (2018). Progress in the diagnosis and therapy of melanoma. *Br J Dermatol*, 178, 12-14.
7. Peng, Y., Sartini, D., Pozzi, V., Wilk, D., Emanuelli, M., Yee VC. (2011). Structural basis of substrate recognition in human nicotinamide N-methyltransferase. *Biochemistry*, 50, 7800-7808.
8. Ganzetti, G., Sartini, D., Campanati, A., Rubini, C., Molinelli, E., Brisigotti, V., Cecati, M., Pozzi, V., Campagna, R., Offidani, A., Emanuelli, M. (2018). Nicotinamide N-methyltransferase: potential involvement in cutaneous malignant melanoma. *Melanoma Res*, 28, 82-88.
9. Mascitti, M., Santarelli, A., Sartini, D., Rubini, C., Colella, G., Salvolini, E., Ganzetti, G., Offidani, A., Emanuelli, M. (2019). Analysis of nicotinamide N-methyltransferase in oral malignant melanoma and potential prognostic significance. *Melanoma Res*, 29, 151-156.
10. Pozzi, V., Mazzotta, M., Lo Muzio, L., Sartini, D., Santarelli, A., Renzi, E., Rocchetti, R., Tomasetti, M., Ciavarella, D., Emanuelli, M. (2011). Inhibiting proliferation in KB cancer cells by RNA interference-mediated knockdown of nicotinamide N-methyltransferase expression. *Int J Immunopathol Pharmacol*, 24, 69-77.

11. Pozzi, V., Sartini, D., Morganti, S., Giuliante, R., Di Ruscio, G., Santarelli, A., Rocchetti, R., Rubini, C., Tomasetti, M., Giannatempo, G., Orlando, F., Provinciali, M., Lo Muzio, L., Emanuelli, M. (2013). RNA-mediated gene silencing of nicotinamide N-methyltransferase is associated with decreased tumorigenicity in human oral carcinoma cells. *PLoS One*. 8, e71272.
12. Sartini, D., Seta, R., Pozzi, V., Morganti, S., Rubini, C., Zizzi, A., Tomasetti, M., Santarelli, L., Emanuelli, M. (2015). Role of nicotinamide N-methyltransferase in non-small cell lung cancer: in vitro effect of shRNA-mediated gene silencing on tumourigenicity. *Biol Chem*, 396, 225-234.
13. Seta, R., Mascitti, M., Campagna, R., Sartini, D., Fumarola, S., Santarelli, A., Giuliani, M., Cecati, M., Muzio, L.L., Emanuelli, M. (2019). Overexpression of nicotinamide N-methyltransferase in HSC-2 OSCC cell line: effect on apoptosis and cell proliferation. *Clin Oral Investig*, 23, 829-838.
14. Coit, D.G., Thompson, J.A., Algazi, A., Andtbacka, R., Bichakjian, C.K., Carson, W.E., Daniels, G.A., DiMaio, D., Ernstoff, M., Fields, R.C., et al. (2016). Melanoma, Version 2.2016, NCCN Clinical Practice Guidelines in Oncology. *J. Natl. Compr. Cancer Netw*. JNCCN, 14, 450–473.
15. Siegel, R.L., Miller, K.D., Jemal, A. (2015). Cancer statistics, 2015. CA. *Cancer J. Clin*, 65, 5–29.
16. Wilson, M.A., Schuchter, L.M. (2016). Chemotherapy for Melanoma. *Cancer Treat Res*, 167, 209-229.
17. Crosby, T., Fish R., Coles, B., Mason, M. D. (2000). Systemic treatments for metastatic cutaneous melanoma. *Cochrane Database Syst Rev* 2:CD001215.
18. Lui, P., Cashin, R., Machado, M., Hemels, M., Corey-Lisle, P.K., Einarson, T.R. (2007). Treatments for metastatic melanoma: Synthesis of evidence from randomized trials. *Cancer Treat Rev*, 33, 665–680.
19. Chapman, P.B., Hauschild, A., Robert, C., Haanen, J.B., Ascierto, P., Larkin, J., Dummer, R., Garbe, C., Testori, A., Maio, M., Hogg, D., Lorigan, P., Lebbe, C., Jouary, T., Schadendorf, D., Ribas, A., O'Day, S.J., Sosman, J.A., Kirkwood, J.M., Eggermont, A.M., Dreno, B., Nolop, K., Li, J., Nelson, B., Hou, J., Lee, R.J., Flaherty, K.T., McArthur, G.A., BRIM-3 Study Group. (2011). Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med*, 364, 2507–2516.
20. Eggermont, A.M., Kirkwood, J.M. (2004) Re-evaluating the role of dacarbazine in metastatic melanoma: what have we learned in 30 years? *Eur J Cancer*, 40, 1825–1836.
21. Sartini, D., Muzzonigro, G., Milanese, G., Pierella, F., Rossi, V., Emanuelli, M. (2006). Identification of nicotinamide N-methyltransferase as a novel tumor marker for renal clear cell carcinoma. *J Urol*, 176, 2248-2254.
22. Campagna, R., Cecati, M., Pozzi, V., Fumarola, S., Pompei, V., Milanese, G., Galosi, A.B., Sartini, D., Emanuelli, M. (2018). Involvement of transforming growth factor beta 1 in the transcriptional regulation of nicotinamide N-methyltransferase in clear cell renal cell carcinoma. *Cell Mol Biol (Noisy-le-grand)*, 64, 51-55.

23. Sartini, D., Santarelli, A., Rossi, V., Goteri, G., Rubini, C., Ciavarella, D., Lo Muzio, L., Emanuelli, M. (2007). Nicotinamide N-methyltransferase upregulation inversely correlates with lymph node metastasis in oral squamous cell carcinoma. *Mol Med*, 13, 415-421.
24. Emanuelli, M., Santarelli, A., Sartini, D., Ciavarella, D., Rossi, V., Rubini, C., Lo Muzio, L. (2010). Nicotinamide N-Methyltransferase upregulation correlates with tumour differentiation in oral squamous cell carcinoma. *Histol Histopathol*, 25, 15-20.
25. Sartini, D., Pozzi, V., Renzi, E., Morganti, S., Rocchetti, R., Rubini, C., Santarelli, A., Lo Muzio, L., Emanuelli, M. (2012). Analysis of tissue and salivary nicotinamide N-methyltransferase in oral squamous cell carcinoma: basis for the development of a noninvasive diagnostic test for early-stage disease. *Biol Chem*, 393, 505-511.
26. Sartini, D., Morganti, S., Guidi, E., Rubini, C., Zizzi, A., Giuliante, R., Pozzi, V., Emanuelli, M. (2013). Nicotinamide N-methyltransferase in non-small cell lung cancer: promising results for targeted anti-cancer therapy. *Cell Biochem Biophys*, 67, 865-873.
27. Sartini, D., Muzzonigro, G., Milanese, G., Pozzi, V., Vici, A., Morganti, S., Rossi, V., Mazzucchelli, R., Montironi, R., Emanuelli, M. (2013). Upregulation of tissue and urinary nicotinamide N-methyltransferase in bladder cancer: potential for the development of a urine-based diagnostic test. *Cell Biochem Biophys*, 65, 473-483.
28. Pozzi, V., Di Ruscio, G., Sartini, D., Campagna, R., Seta, R., Fulvi, P., Vic,i A., Milanese, G., Brandoni, G., Galosi, A.B., Montironi, R., Cecati, M., Emanuelli, M. (2018). Clinical performance and utility of a NNMT-based urine test for bladder cancer. *Int J Biol Markers*, 33, 94-101.
29. Pompei, V., Salvolini, E., Rubini, C., Lucarini, G., Molinelli, E., Brisigotti, V., Pozzi, V., Sartini, D., Campanati, A., Offidani, A., Emanuelli, M. (2019). Nicotinamide N-methyltransferase in nonmelanoma skin cancers. *Eur J Clin Invest*, 49, e13175.
30. Pozzi, V., Sartini, D., Rocchetti, R., Santarelli, A., Rubini, C., Morganti, S., Giuliante, R., Calabrese, S., Di Ruscio, G., Orlando, F., Provinciali, M., Saccucci, F., Lo Muzio, L., Emanuelli, M. (2015). Identification and characterization of cancer stem cells from head and neck squamous cell carcinoma cell lines. *Cell Physiol Biochem*, 36, 784-798.
31. Pozzi, V., Salvolini, E., Lucarini, G., Salvucci, A., Campagna, R., Rubini, C., Sartini, D., Emanuelli, M. (2020). Cancer stem cell en-richment is associated with enhancement of nicotinamide N-methyltransferase expression. *IUBMB Life*. 72, 1415-1425.
32. Ulanovskaya OA, Zuhl AM, Cravatt BF. (2013) NNMT promotes epigenetic remodeling in cancer by creating a metabolic methylation sink. *Nat Chem Biol*, 9:300-306.
33. Pissios P. (2017) Nicotinamide N-Methyltransferase: More Than a Vitamin B3 Clearance Enzyme. *Trends Endocrinol Metab*, 28, 340-353.
34. Mateuszuk Ł, Campagna R, Kutryb-Zając B, Kuś K, Słominska EM, Smolenski RT, Chlopicki S. (2020) Reversal of endothelial dysfunction by nicotinamide mononucleotide via extracellular conversion to nicotinamide riboside. *Biochem Pharmacol*, 178:114019.

35. Sartini, D., Seta, R., Pozzi, V., Morganti, S., Rubini, C., Zizzi, A., Tomasetti, M., Santarelli, L., Emanuelli, M. (2015). Role of nico-tinamide N-methyltransferase in non-small cell lung cancer: in vitro effect of shRNA-mediated gene silencing on tumour-igenicity. *Biol Chem*, 396, 225-234.
36. Wu, Y., Siadaty, M.S., Berens, M.E., Hampton, G.M., Theodorescu, D. (2008). Overlapping gene expression profiles of cell migration and tumor invasion in human bladder cancer identify metallothionein 1E and nicotinamide N-methyltransferase as novel regulators of cell migration. *Oncogene*, 27, 6679-6689.
37. Tang, S.W., Yang, T.C., Lin, W.C., Chang, W.H., Wang, C.C., Lai M.K., Lin, J.Y. (2011). Nicotinamide N-methyltransferase induces cellular invasion through activating matrix metalloproteinase-2 expression in clear cell renal cell carcinoma cells. *Carcinogenesis*, 32, 138-145.
38. Zhang, J., Wang, Y., Li, G., Yu, H., Xie, X. (2014). Down-regulation of nicotinamide N-methyltransferase induces apoptosis in hu-man breast cancer cells via the mitochondria-mediated pathway. *PLoS One*, 9, e89202.
39. Xie, X., Yu, H., Wang, Y., Zhou, Y., Li, G., Ruan, Z., Li, F., Wang, X., Liu, H., Zhang, J. (2014). Nicotinamide N-methyltransferase enhances the capacity of tumorigenesis associated with the promotion of cell cycle progression in human colorectal cancer cells. *Arch Biochem Biophys*, 564, 52-66.
40. Yu, T., Wang, Y.T., Chen, P., Li, Y.H., Chen, Y.X., Zeng, H., Yu, A.M., Huang, M., Bi, H.C. (2015). Effects of nicotinamide N-methyltransferase on PANC-1 cells proliferation, metastatic potential and survival under metabolic stress. *Cell Physiol Biochem*, 35, 710-721.
41. Bach, D.H., Kim, D., Bae, S.Y., Kim, W.K., Hong, J.Y., Lee, H.J., Rajasekaran, N., Kwon, S., Fan, Y., Luu, T.T., Shin, Y.K., Lee, J., Lee, S.K. (2018). Targeting Nicotinamide N-Methyltransferase and miR-449a in EGFR-TKI-Resistant Non-Small-Cell Lung Cancer Cells. *Mol Ther Nucleic Acids*, 11, 455-467.
42. You, Z., Liu, Y., Liu, X. (2018). Nicotinamide N-methyltransferase enhances the progression of prostate cancer by stabilizing sirtuin 1. *Oncol Lett*, 15, 9195-9201.
43. Liang, L., Zeng, M., Pan, H., Liu, H., He, Y. (2018). Nicotinamide N-methyltransferase promotes epithelial-mesenchymal transition in gastric cancer cells by activating transforming growth factor-β1 expression. *Oncol Lett*, 15, 4592-4598.
44. Cui, Y., Zhang, L., Wang, W., Ma, S., Liu, H., Zang, X., Zhang, Y., Guan, F. (2019). Downregulation of nicotinamide N-methyltransferase inhibits migration and epithelial-mesenchymal transition of esophageal squamous cell carcinoma via Wnt/β-catenin pathway. *Mol Cell Biochem*, 460, 93-103.
45. Xie, X., Liu, H., Wang, Y., Zhou, Y., Yu, H., Li, G., Ruan, Z., Li, F., Wang, X., Zhang, J. (2016). Nicotinamide N-methyltransferase enhances resistance to 5-fluorouracil in colorectal cancer cells through inhibition of the ASK1-p38 MAPK pathway. *Oncotarget*, 7, 45837-45848.
46. Cui, Y., Yang, D., Wang, W., Zhang, L., Liu, H., Ma, S., Guo, W., Yao, M., Zhang, K., Li, W., Zhang, Y., Guan, F. (2020). Nicotina-mide N-methyltransferase Decreases 5-fluorouracil Sensitivity in

Human Esophageal Squamous Cell Carcinoma Through Metabolic Reprogramming and Promoting the Warburg Effect. *Mol Carcinog*, 59, 940-954.

47. Wang, Y., Zeng, J., Wu, W., Xie, S., Yu, H., Li, G., Zhu, T., Li, F., Lu, J., Wang, G.Y., Xie, X., Zhang, J. (2019). Nicotinamide N-methyltransferase enhances chemoresistance in breast cancer through SIRT1 protein stabilization. *Breast Cancer Res*, 21, 64.
48. van Haren, M.J., Taig, R., Kuppens, J., Sastre Toraño, J., Moret, E.E., Parsons, R.B., Sartini, D., Emanuelli, M., Martin, N.I. (2017). Inhibitors of nicotinamide N-methyltransferase designed to mimic the methylation reaction transition state. *Org Biomol Chem*. 15, 6656-6667.

FIGURE LEGEND

Figure 1. Evaluation of NNMT silencing. A375 and WM-115 cells were treated with different shRNA plasmids against NMMT (pLKO.1-164, pLKO.1-330, pLKO.1-711 and pLKO.1-mix), with empty vector (pLKO.1-puro) or with transfection reagent only (Mock). NNMT expression was evaluated at mRNA (panel a for A375 cells; panel d for WM-115 cells) and protein level (for A375 cells, blot is reported in panel b and densitometry is reported in panel c) by Real Time PCR and Western blot, respectively. Values are expressed as mean ± standard deviation (*p<0.05).

Figure 2. In vitro effect of NNMT silencing on cell proliferation. A375 (panel a) and WM-115 (panel b) cell proliferation was analyzed by MTT assay in Mock and NNMT downregulating cells (pLKO.1-164, pLKO.1-330, pLKO.1-711 and pLKO.1-mix) at 0h, 24h, 48h and 72h. Values are expressed as mean ± standard deviation (*p<0.05; ns = not significant).

Figure 3. In vitro effect of NNMT silencing on cell migration. A375 cells were subjected to wound healing assay to evaluate their migration potential. A375 cells transfected with plasmids (pLKO.1-164, pLKO.1-330, pLKO.1-711, pLKO.1-mix or pLKO.1-puro) and Mock were photographed immediately after scratch (0h) and at different time points, ranging between 4h and 28h. For each sample, cell migration ability was evaluated at different time points by measuring percentage of wound recovery compared with 0h. Panel a displays images of wound recovery of Mock, pLKO.1-puro, pLKO.1-164, pLKO.1-330, pLKO.1-711 and pLKO.1-mix. Panel b reports bar diagram showing time course of wound recovery of all samples. Values are ex-pressed as mean ± standard deviation (*p<0.05).

Figure 4. Effect of chemotherapy treatment on melanoma cell proliferation. MTT assay was used to evaluate the effect of dacarbazine on proliferation of mock and NNMT downregulating cells (panels a-d for A375; panel e for WM-115). Measurements were performed at different time points (0h, 24h, 48h and 72h). All values are expressed as mean ± standard deviation (*p<0.05).

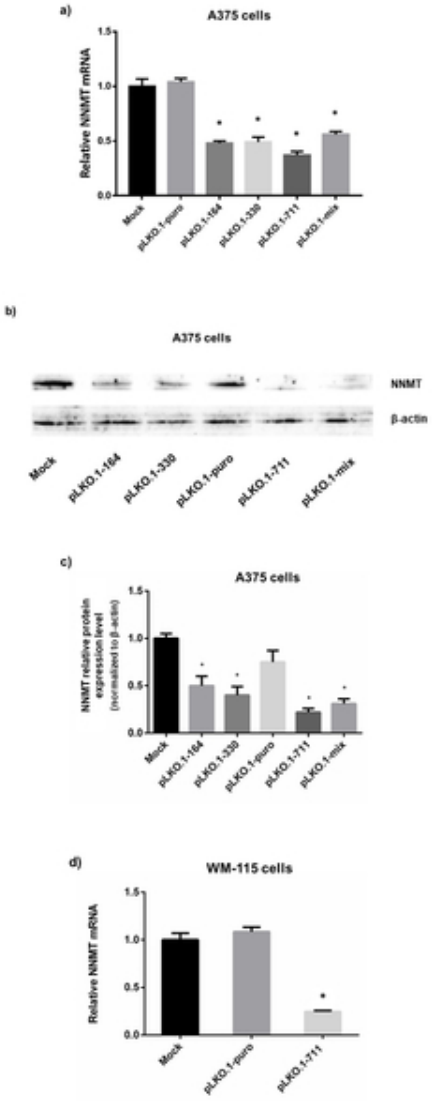

Figure 1. Evaluation of NNMT silencing. A375 and WM-115 cells were treated with different shRNA plasmids against NMMT (pLKO.1-164, pLKO.1-330, pLKO.1-711 and pLKO.1-mix), with empty vector (pLKO.1-puro) or with transfection reagent only (Mock). NNMT expression was evaluated at mRNA (panel a for A375 cells; panel d for WM-115 cells) and protein level (for A375 cells, blot is reported in panel b and densitometry is reported in panel c) by Real Time PCR and Western blot, respectively. Values are expressed as mean ± standard deviation (*$p<0.05$).

30x54mm (300 x 300 DPI)

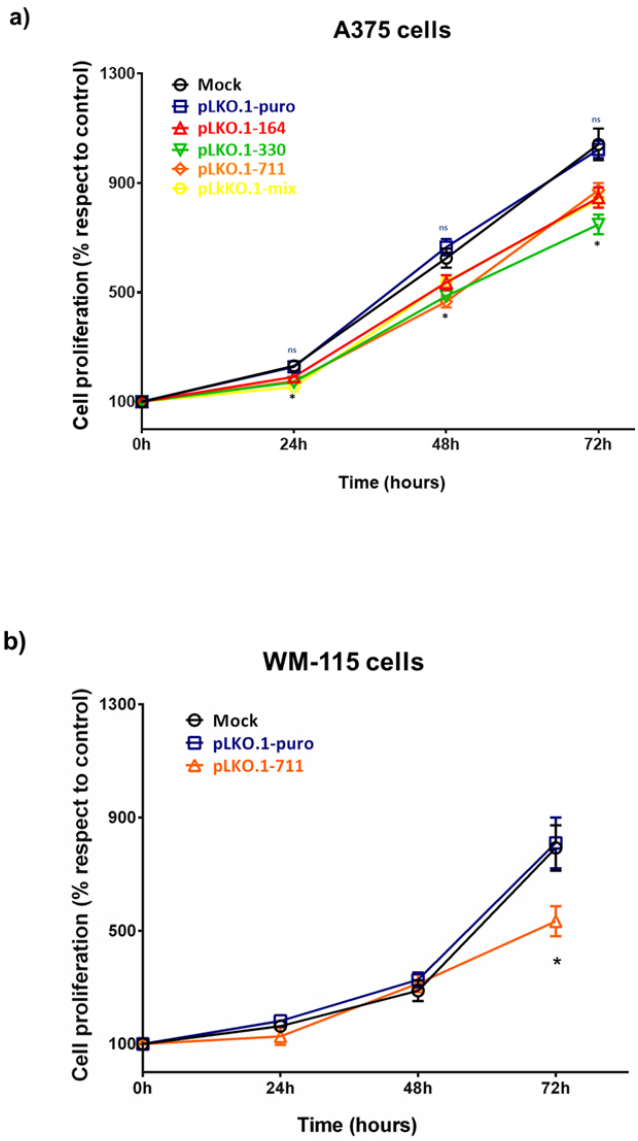

Figure 2. In vitro effect of NNMT silencing on cell proliferation. A375 (panel a) and WM-115 (panel b) cell proliferation was analyzed by MTT assay in Mock and NNMT downregulating cells (pLKO.1-164, pLKO.1-330, pLKO.1-711 and pLKO.1-mix) at 0h, 24h, 48h and 72h. Values are expressed as mean ± standard deviation (*$p<0.05$; ns = not significant).

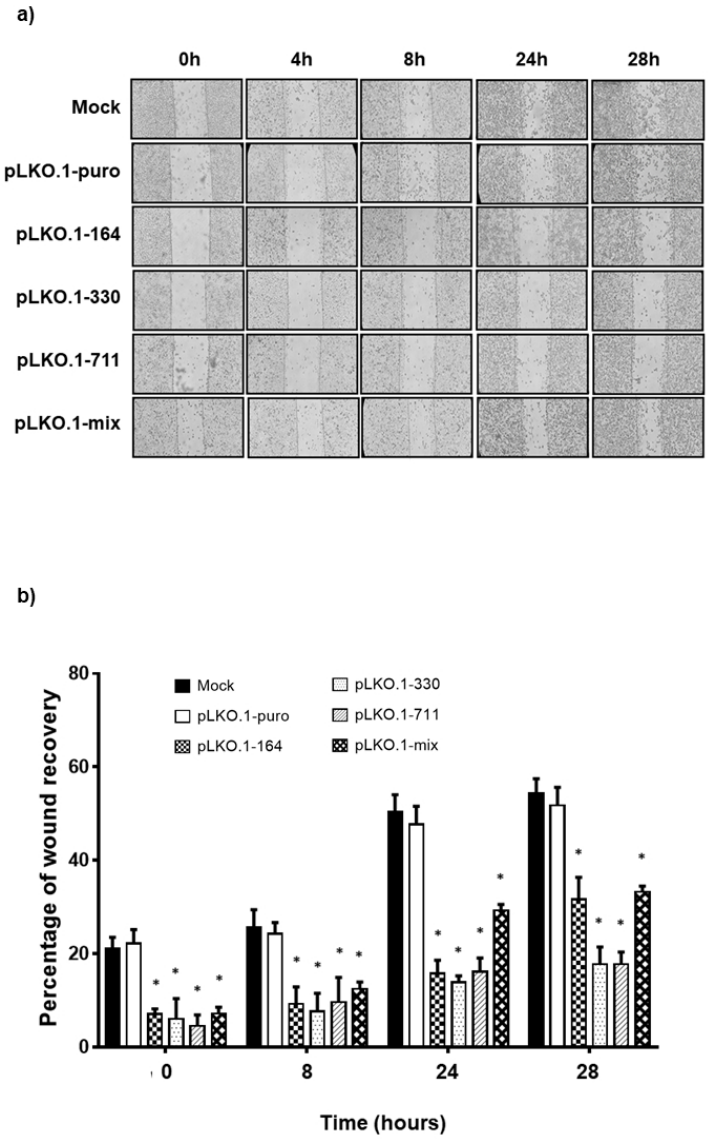

Figure 3. In vitro effect of NNMT silencing on cell migration. A375 cells were subjected to wound healing assay to evaluate their migration potential. A375 cells transfected with plasmids (pLKO.1-164, pLKO.1-330, pLKO.1-711, pLKO.1-mix or pLKO.1-puro) and Mock were photographed immediately after scratch (0h) and at different time points, ranging between 4h and 28h. For each sample, cell migration ability was evaluated at different time points by measuring percentage of wound recovery compared with 0h. Panel a displays images of wound recovery of Mock, pLKO.1-puro, pLKO.1-164, pLKO.1-330, pLKO.1-711 and pLKO.1-mix. Panel b reports bar diagram showing time course of wound recovery of all samples. Values are ex-pressed as mean ± standard deviation (*$p<0.05$).

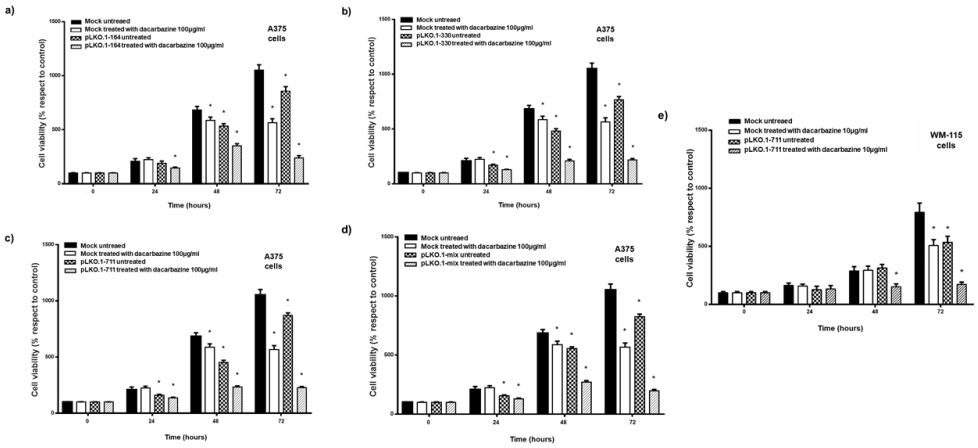

Figure 4. Effect of chemotherapy treatment on melanoma cell proliferation. MTT assay was used to evaluate the effect of dacarbazine on proliferation of mock and NNMT downregulating cells (panels a-d for A375; panel e for WM-115). Measurements were performed at different time points (0h, 24h, 48h and 72h). All values are expressed as mean ± standard deviation (*$p<0.05$).