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ORIGINAL ARTICLE

Overexpression of nicotinamide *N*-methyltransferase in HSC-2 OSCC cell line: effect on apoptosis and cell proliferation

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Abstract

Objectives Oral squamous cell carcinoma (OSCC) is the most common malignancy of oral cavity. Despite advances in therapeutic approaches, the 5-year survival rate for oral cancer has not improved in the last three decades. Therefore, new molecular targets for early diagnosis and treatment of OSCC are needed. In the present study, we focused on the enzyme nicotinamide *N*-methyltransferase (NNMT). We have previously shown that enzyme expression is upregulated in OSCC and NNMT knockdown in PE/CA PJ-15 cells significantly decreased cell growth in vitro and tumorigenicity in vivo.

Material and methods To further explore the role of the enzyme in oral cancer cell metabolism, HSC-2 cells were transfected with the NNMT expression vector (pcDNA3-NNMT) and the effect of enzyme upregulation on cell proliferation was evaluated by MTT assay. Subsequently, we investigated at molecular level the role of NNMT on apoptosis and cell proliferation, by exploring the expression of β-catenin, survivin, and Ki-67 by real-time PCR. Moreover, we performed immunohistochemistry on 20 OSCC tissue samples to explore the expression level of NNMT and survivin ΔEx3 isoform.

Results Enzyme upregulation significantly increased cell growth in vitro. Moreover, a positive correlation between NNMT and survivin ΔEx3 isoform expression levels was found both in HSC-2 cells and in OSCC tissue samples.

Conclusion Taken together, our results indicate a possible involvement of NNMT in the proliferation and tumorigenic capacity of OSCC cells and seem to suggest that the enzyme could represent a potential target for the treatment of oral cancer.

Clinical relevance The involvement of NNMT in cell growth and anti-apoptotic mechanisms seems to suggest that this enzyme could be a new therapeutic target to improve the survival of OSCC patients.

Keywords NNMT · Survivin · Cell growth · Tumorigenicity · Oral cancer

Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignancy of head and neck, representing up to 90% of oral cavity cancers. The annual incidence and mortality of this neoplasm are approximately 275,000 and 145,000, respectively, making this the 11th most common tumor worldwide [1]. Although the cause of OSCC is unknown, several risk factors are strongly related to the development of this neoplasm, such as alcohol consumption, tobacco use, and HPV infection [2].

In the last decades, despite progress in therapeutic strategies of OSCC, the 5-year survival rates showed no significant improvement, remaining slightly below 50%. The main reason of this poor prognosis is the diagnostic delay, which leads the patients to be treated when the tumor has reached an advanced stage [3].

The study of the molecular mechanisms that are involved in the carcinogenesis is necessary to identify new markers suitable for early diagnosis and prognosis, as well as to identify new targets for molecular-based treatments.

Nicotinamide *N*-methyltransferase (NNMT; EC 2.1.1.1.) is a phase II drug-metabolizing enzyme which catalyzes the *N*-

Riccardo Seta and Marco Mascitti contributed equally to this work.

This paper is dedicated to the memory of my husband, Andrea L. Tranquilli (M. Emanuelli)

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methylation of nicotinamide and other structurally related compounds as pyridines. The conjugation reaction of this enzyme plays a critical role in the biotransformation and detoxification of many drugs and xenobiotic compounds [4, 5].

NNMT is highly expressed in the liver, although other tissues showed low expression levels, such as kidney, skeletal muscle, lung, bladder, placenta, heart, and brain [6].

In several tumors, NNMT overexpression is well known, and our previous reports showed that this enzyme was upregulated in clear cell renal cell carcinoma (ccRCC), urothelial carcinoma, non-small cell lung cancer, and OSCC [7–10]. In particular, NNMT mRNA overexpression in oral cancer inversely correlates with lymph node metastasis, pT, and pathological staging, and cytosolic protein levels inversely correlate with histological grading [8, 11]. In addition to the potential role of NNMT as a prognostic factor, the measurement of salivary NNMT could serve as biomarker for early diagnosis of OSCC [12]. Furthermore, the inhibition of KB cell proliferation due to the RNA interference-mediated downregulation of NNMT corroborates the hypothesis that this enzyme plays a role in tumorigenesis [6].

In this study, in order to further explore the biological function of NNMT in oral cancer cell metabolism, we investigate the effects of plasmid-mediated overexpression of NNMT in oral cancer cell line HSC-2. Real-time PCR, Western blot, and catalytic activity assay were used, and the assessment of cell proliferation was performed with MTT colorimetric assay. Furthermore, apoptosis and cell proliferation were investigated at the molecular level, evaluating the effects of NNMT upregulation on β-catenin, survivin, and Ki-67 expression. Subsequent immunohistochemical analyses, performed on a cohort of 20 tissue samples obtained from OSCC patients, were carried out to evaluate the expression of both NNMT and survivin ΔEx3 isoform.

Materials and methods

Cell lines and reagents

The human oral cancer cell line HSC-2, purchased from the America Type Culture Collection (ATCC; Rockville, MD, USA), was cultured in DMEM/F12 medium, supplemented with 10% fetal bovine serum and 50 μg/ml of gentamicin, at 37 °C in a humidified 5% CO_2 incubator.

Cloning

Total RNA was isolated from HSC-2 cells (1×10^6) using the RNeasy Micro Kit (Qiagen, Hilden, Germany). RNA (2 μg) was reverse transcribed with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) using random primers. One microliter of the reaction mixture was then subjected to PCR with Pfu DNA Polymerase (Biotools, Madrid, Spain) in a total volume 50 μl, using the primers 5′-TTCGGATCCATGGAATCAGGCTT-3′ (forward) and 5′-TTACTCGAGTCACAGGGGTCTGC-3′ (reverse) to amplify the human NNMT ORF and to insert *Bam*HI and *Xho*I restriction sites. The amplified and digested PCR product was cloned into the pcDNA3 plasmid vector (Life Technologies, Carlsbad, CA, USA) to obtain the plasmid construct pcDNA3-NNMT.

Plasmid transfection

HSC-2 cells were seeded in six-well plates (2.4×10^5 cells/well) the day before transfection and were transfected with the pcDNA3-NNMT plasmid vector (3 μg per well). Control cells were transfected with the empty vector (pcDNA3) or treated with transfection reagent only (mock). Transfection was performed using FuGENE HD Transfection Reagent (Promega, Madison, WI, USA), according to the manufacturer's instructions. Forty-eight hours after transfection, cells were harvested and subjected to further analyses.

Real-time PCR

To evaluate NNMT mRNA levels quantitatively, a real-time PCR assay was performed using CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). cDNA, generated as described above, was used as template. To avoid false-positive results attributable to the amplification of contaminating genomic DNA in the cDNA preparation, all primers were selected to flank an intron, and PCR efficiency was tested for all primer pairs and found to be close to 1. The oligonucleotide sequences of primers for NNMT, survivin isoforms, β-catenin, Ki-67, and β-actin are reported in Table 1.

All 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, Hercules, CA, USA). 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 PCR products was monitored by measuring the fluorescence produced by EvaGreen dye binding to double-strand DNA after every cycle. These measurements were then plotted against cycle numbers. The parameter threshold cycle (Ct) was defined as the cycle number at which the first detectable increase above the threshold in fluorescence was observed. Fold changes in relative gene expression were calculated by $2^{-\Delta\Delta Ct}$, where ΔCt = Ct (gene of interest) − Ct (reference gene), and ΔΔCt = ΔCt (pcDNA3 or pcDNA3-NNMT) − ΔCt (mock).

Table 1 Nucleotide sequence of primers used for quantitative real-time PCR

Target gene	Sequence
NNMT	Forward 5′-GAATCAGGCTTCACCTCCAA-3′
	Reverse 5′-TCACACCGTCTAGGCAGAAT-3′
Survivin	Forward 5-ATGACGACCCCATAGAGGAAC-3′
	Reverse 5′-CCTTTGCAATTTTGTTCTTGGC-3′
Survivin 2B	Forward 5′-CACTGAGAACGAGCCAGACT-3′
	Reverse 5′-ATGTTCCTCTCTCGTGATCC-3′
Survivin ΔEx3	Forward 5′-GACCACCGCATCTCTACATTC-3′
	Reverse 5′-ATTGTTGGTTTCCTTTGCATG-3′
β-Catenin	Forward 5′-AACAGGAAGGGATGGAAGGT-3′
	Reverse 5′-ATACCACCCACTTGGCAGAC-3′
Ki-67	Forward 5′-GACATCCGTATCCAGCTTCC-3′
	Reverse 5′-CCGTACAGGCTCATCAATAAC-3′
β-Actin	Forward 5′-TCCTTCCTGGGCATGGAGT-3′
	Reverse 5′-AGCACTGTGTTGGCGTACAG-3′

Western blot analysis

Western blot experiments were performed to evaluate NNMT protein expression level. Cell pellets (2×10^6 cells) were suspended in 200 μl lysis buffer (phosphate-buffered saline containing 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml aprotinin) and homogenized by passing 3–5 times through a 30-gauge needle attached to a 1-ml syringe. After centrifugation at 16,000×*g* for 10 min at 4 °C, the supernatant containing the protein extract was collected. Samples containing 50 μg protein were subjected to 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After regular blocking and washing, the membranes were incubated (1:1000 dilution) with rabbit polyclonal antibody against NNMT (Sigma-Aldrich, St. Louis, MO, USA) for 1 h, followed by incubation (1:2000 dilution) with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h. NNMT protein was visualized using enhanced SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA). The chemiluminescent signal of NNMT protein detected in blots was acquired using ChemiDoc XRS + System (Bio-Rad Laboratories, Hercules, CA, USA).

Enzyme assay

An HPLC-based catalytic assay was performed to analyze NNMT activity. Cell pellets (5×10^6 cells) were suspended in 200 μl of cold lysis buffer (50 mM Tris-HCl, pH 8.6, 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1% Nonidet P-40) and one-half volume glass beads. The suspension was vigorously vortexed for 2 min and then chilled on ice for 2 min. The homogenate was centrifuged at 16,000×*g* for 10 min at 4 °C and the supernatant was collected and kept on ice until assayed. The standard assay mixture contained 50 mM Tris-HCl, pH 8.6, 1 mM dithiothreitol, 5 mM nicotinamide, 0.5 mM S-adenosyl-L-methionine, and the appropriate amount of enzyme sample to reach a final volume of 350 μl. The reaction was started by adding S-adenosyl-L-methionine. Incubations were performed at 37 °C for 30 and 60 min. The reaction was stopped by adding 100 μl assay mixture to 50 μl ice-cold 1.2 M $HClO_4$. After 10 min at 0 °C, proteins were removed by 1 min of centrifugation in a microfuge and 130 μl perchloric acid supernatant was then neutralized by adding 35 μl 0.8 M K_2CO_3. The $KClO_4$ so formed was removed by centrifugation. One hundred microliters of the neutralized supernatant was injected into a high-performance liquid chromatography system 10 Dvp-UV-Vis photodiode array detector (Shimadzu, Duisburg, Germany) using a 250 × 4.6 mm inner diameter Supelcosil LC-18-S 5 μm reversed phase column. Elution conditions were as previously described [13]. Enzyme activities were tested by measuring the amount of N1-methylnicotinamide produced, as determined by the peak areas of the separated compound with 1 U activity representing the formation of 1 nmol N1-methylnicotinamide per hour of incubation at 37 °C. The lower detection limit for NNMT catalytic activity assay was 0.01 U/mg.

Protein assay

Protein concentration was measured by the Bradford method, using bovine serum albumin as the standard [14].

MTT assay

Cell proliferation was determined using a colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The MTT assay measures the conversion of MTT to insoluble formazan by dehydrogenase enzymes of the intact mitochondria of living cells. After transfection, HSC-2 cells were seeded in 96-well plates (5×10^3 cells/well). Cells were allowed to attach overnight and cell proliferation was evaluated for up to 4 days by measuring the conversion of the tetrazolium salt MTT to formazan crystals. Briefly, 10 μl of MTT reagent (5 mg/ml in phosphate-buffered saline) was added to the cells and incubated for 4 h at 37 °C. The medium was removed and 200 μl of isopropanol was added. The amount of formazan crystals formed correlates directly with the number of viable cells. The reaction product was quantified by measuring the absorbance at 570 nm using an ELISA plate reader. Experiments were repeated three times. Results were analyzed and expressed as percentage of the control

(control equals 100% and corresponds to the absorbance value of each sample at time zero) and presented as mean values ± standard deviation of three independent experiments performed in triplicate.

Patients

A total of 20 formalin-fixed and paraffin-embedded (FFPE) tissue specimens, obtained from OSCC patients who underwent surgical resection, were retrieved from the Department of Biomedical Sciences and Public Health (Section of Pathology) of Polytechnic University of Marche. Criteria for pathological staging assignment were based on the 2017 Tumor Node Metastasis (TNM) classification system of malignant tumors. Grade was assigned according to the 2005 World Health Organization (WHO) classification system for histologic differentiation. Table 2 lists the demographic and clinic-pathological characteristics of patients enrolled in the study.

Immunohistochemistry

For each case, 5-μm sections were cut from FFPE tissue blocks. Immunohistochemistry was performed on 5-μm histological sections mounted on poly-L-lysine-coated glass slides. After deparaffinization in xylene and rehydration in a graded series of alcohol, slides were incubated with EnVision FLEX Target Retrieval Solution, Low pH (Dako, Carpinteria, CA, USA), for antigen retrieval. Endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxide for 7 min. Sections were then washed for 5 min with EnVision FLEX Wash Buffer (Dako) and incubated with rabbit polyclonal antibody raised against amino acids 118–130 of human NNMT (1:1500 dilution) (Sigma-Aldrich, St. Louis, MO, USA) or rabbit polyclonal antibody raised against amino acids 50–150 of human survivin ΔEx3 (1:250 dilution), in a humidified atmosphere at room temperature for 1 h. After washing, sections were incubated with EnVision FLEX/HRP (Dako) for 20 min. After washing, sections were incubated with EnVision FLEX DAB+Chromogen (Dako) for 10 min. Sections were then counterstained using Mayer's hematoxylin (Bio-Optica, Milano, Italy), permanently mounted on slides, and examined by light microscopy. ccRCC and thyroid cancer were used as a positive control for NNMT and survivin ΔEx3, respectively, whereas negative control slides were obtained by replacing the primary antibody with rabbit IgG isotype.

To evaluate NNMT cytoplasmic expression, the percentage of positive cells was determined from the analysis of 100 cells in 10 random areas at × 400 magnification. NNMT expression was then scored using a five-tier grading system: 0 (0–10%), 1 (10–30%), 2 (30–50%), 3 (50–80%), and 4 (80–100%). The expression of survivin ΔEx3 was evaluated by assessing the percentage of cells displaying a positive-stained nucleus and scored following the above reported grading system.

The positivity for NNMT and surviving ΔEx3 was evaluated by an expert in the field, who was blinded to the clinico-pathological data. Each specimen was analyzed three times.

Table 2 OSCC patients and clinico-pathological findings

Categories	No.
Cases	20
Gender	
Males	18
Females	2
Age	
Mean	60
Range	45–85
Tumor localization	
Gingiva	8
Palate	4
Floor	6
Mucosa	2
Grading	
G1	4
G2	4
G3	12
Staging	
I	0
II	2
III	10
IV	8

Statistical analysis

Data were analyzed using GraphPad Prism software version 6.00 for Windows (GraphPad Prism Software, San Diego, CA, USA). Differences between groups were determined using the Kruskal-Wallis test. Correlation between variables was explored by the Spearman test. A p value < 0.05 was accepted as statistically significant.

Results

Efficiency of NNMT overexpression

In order to modulate NNMT expression for functional assays, HSC-2 cell line was transiently transfected with the NNMT expression vector (pcDNA3-NNMT), and control cells were transfected with the empty vector (pcDNA3) or treated with transfection reagent only (mock), as described in "Materials and methods." Forty-eight hours after transfection, cells were harvested. To evaluate enzyme overexpression, NNMT

mRNA, protein, and catalytic activity levels were analyzed by real-time PCR, Western blot, and enzyme assay, respectively. Compared with mock and pcDNA3-treated cells, HSC-2 transfected with pcDNA3-NNMT displayed significantly increased NNMT expression levels. Real-time PCR showed a significant ($p < 0.05$) upregulation of NNMT in cells transfected with pcDNA3-NNMT plasmid (10,087 ± 1495) compared with pcDNA3-treated (0.78 ± 0.08) and mock (1.00 ± 0.13) cells (Fig. 1a). NNMT overexpression was confirmed at protein level by Western blot analysis. Lanes loaded with equal protein amounts displayed markedly increased NNMT expression in cells treated with pcDNA3-NNMT (Fig. 1b) compared with both pcDNA3 and mock samples. In keeping with results obtained by real-time PCR and Western blot analysis, NNMT-specific activity levels, expressed in units per milligram protein, were significantly ($p < 0.05$) higher in cells transfected with the pcDNA3-NNMT (8.59 ± 0.46) compared with controls (Fig. 1c).

In vitro effect of NNMT overexpression on cell proliferation

To examine the role of NNMT in tumor cell metabolism, and analyze the biological effect associated with enzyme upregulation, pcDNA3-NNMT vector was introduced into HSC-2 cells, and cell viability was then assayed. The effect of NNMT overexpression on cell proliferation was evaluated by MTT assay. As shown in Fig. 2, pcDNA3-NNMT plasmid was able to increase cell growth of HSC-2 cells compared with controls. The results of MTT colorimetric assay were expressed as relative cell viability referred to control.

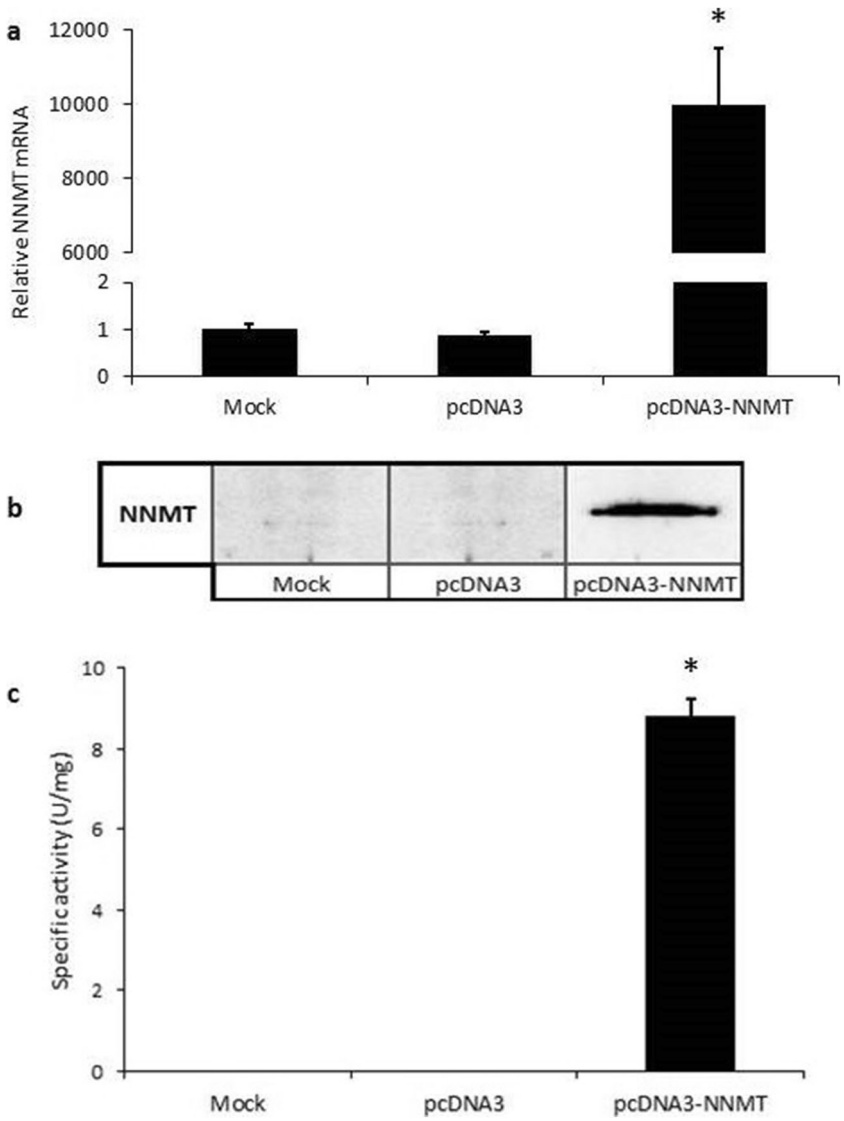

Fig. 1 NNMT expression level measurements. HSC-2 cells were transfected with pcDNA3-NNMT, with pcDNA3, or treated with transfection reagent only (mock). NNMT mRNA levels were evaluated by real-time PCR in transfected (pcDNA3 and pcDNA3-NNMT) compared with mock cells (**a**). Protein lysates, obtained from transfected and mock cells, were analyzed by Western blot to measure NNMT expression levels (**b**). NNMT-specific activity was determined using an HPLC-based method that measures the amount of N1-methylnicotinamide produced (**c**). All values are expressed as mean ± standard deviation (*$p < 0.05$)

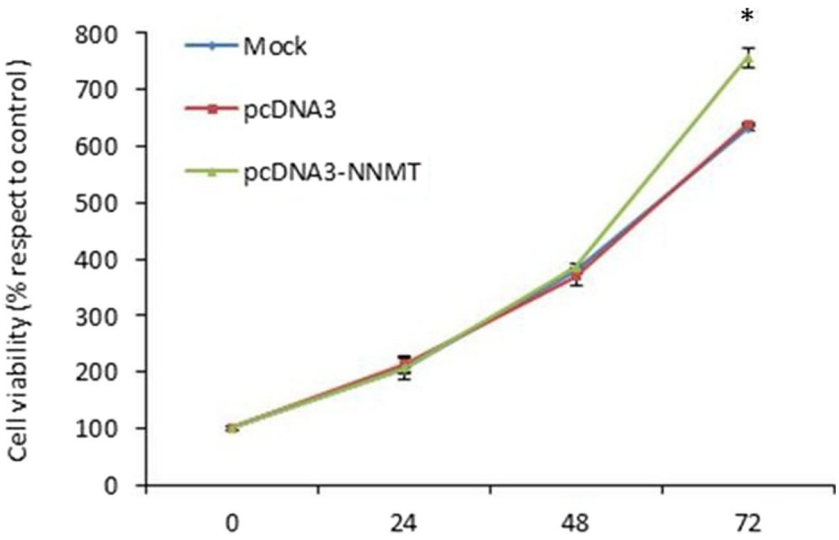

Fig. 2 Evaluation of cell proliferation. In vitro effect of NNMT overexpression on cell proliferation was assessed by MTT assay. Cell growth was evaluated in mock and transfected cells after 24, 48, and 72 h of incubation. All values are expressed as mean ± standard deviation (*$p < 0.05$)

Enzyme upregulation led to a significant ($p < 0.05$) increase of percentage value at 72-h time point.

Effect of NNMT upregulation on β-catenin, survivin, and Ki-67 expression

Subsequent analyses were performed to explore the potential involvement of the enzyme in cellular pathways, such as apoptosis, cell proliferation, and cell signaling. Therefore, we examined whether NNMT overexpression in HSC-2 was able to enhance or repress the expression of β-catenin, survivin, and Ki-67. Results reported in Fig. 3 showed that enzyme upregulation did not alter the expression level of both β-catenin and survivin main isoform. On the contrary, survivin ΔEx3 isoform levels were significantly ($p < 0.05$) higher in NNMT-overexpressing cells (pcDNA3-NNMT) compared with those detected in HSC-2 transfected with empty vector (pcDNA3) or treated with transfection reagent only (mock). Survivin 2B isoform was significantly ($p < 0.05$) overexpressed in HSC-2 transfected with pcDNA3-NNMT compared with those treated with pcDNA3. However, its expression levels did not differ significantly between pcDNA3-NNMT-treated and mock cells. The expression of survivin 3B and 2α isoforms was not detectable in the examined samples (data not shown). Both pcDNA3 and pcDNA3-NNMT treatments seemed to increase Ki-67 expression. Anyhow, there was no significant difference between empty vector-treated HSC-2 and those transfected with NNMT expression plasmid.

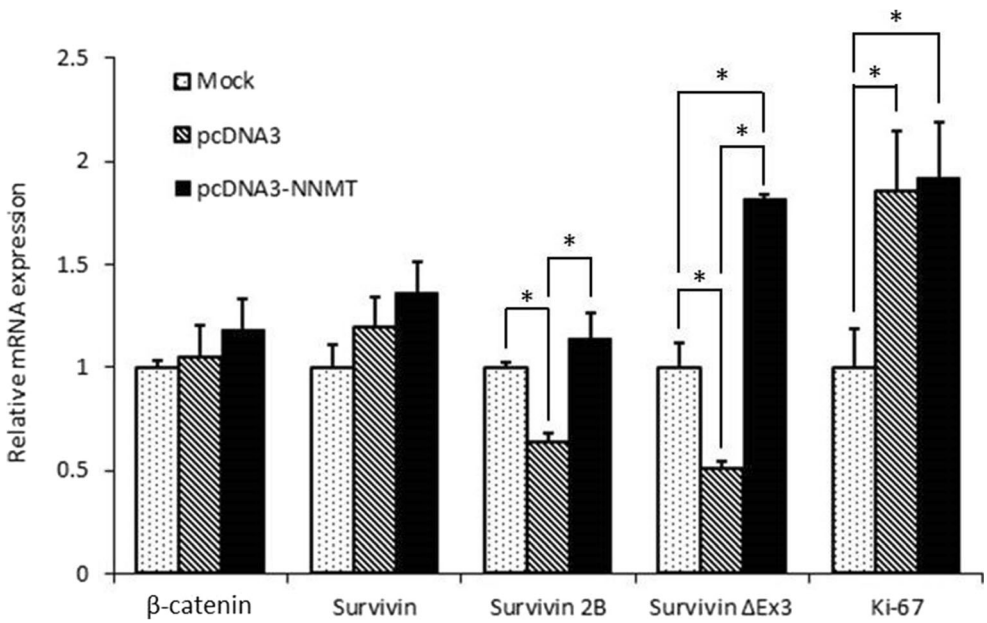

Fig. 3 Expression of β-catenin, survivin isoforms, and Ki-67. Real-time PCR was used to evaluate mRNA levels of β-catenin, survivin isoforms, and Ki-67 in cells, after transfection with pcDNA3-NNMT or pcDNA3. Control cells were treated with transfection reagent only (mock). All values are expressed as mean ± standard deviation (*$p < 0.05$)

Expression of NNMT and survivin ΔEx3 in OSCC tissue samples

The expression of NNMT and survivin ΔEx3 was analyzed by immunohistochemistry in 20 FFPE tumor tissue samples obtained from patients with primary OSCC. Specimens included 18 men (90%) and 2 (10%) women, whose age ranged between 45 and 85 years old, with a mean value of 60 years old at the time of diagnosis. Regarding the assessment of histological grading, 4 cases were well-differentiated (20%), 4 were moderately differentiated (20%), and 12 were poorly differentiated (60%) tumors. According to 2017 TNM classification system, there were no patient with stage I (0%), 2 patients with stage II (10%), 10 with stage III (50%), and 8 with stage IV (40%) (Table 1).

Results obtained from immunohistochemistry showed that cytoplasmic and nuclear positivity for NNMT and survivin ΔEx3, respectively, varied between 0 and 4 scores. Statistical analyses showed that the expression of both proteins did not significantly correlate with age, gender, staging, and tumor localization. Interestingly, there was a statistically significant ($p < 0.05$) inverse correlation between histological grading and the scored level of NNMT ($r = -0.9682$) and survivin ΔEx3 ($r = -0.4564$). This meant that the expression of both genes was markedly high in well-differentiated cases, while poorly differentiated tumors displayed low NNMT and survivin ΔEx3 levels. Results obtained from analyses aimed to compare the expression of both proteins revealed that there was a statistically significant ($p < 0.05$) positive ($r = 0.5692$) correlation between NNMT and survivin ΔEx3 expression. Indeed, NNMT upregulation correlated with high survivin levels and vice versa (Fig. 4).

Discussion

Oral squamous cell carcinoma (OSCC) is the most common malignancy of oral cavity, accounting for about 90% of oral cancers. Despite advances in cancer therapy, the 5-year survival rate for oral cancer has not improved over the past three decades and remains at approximately 50%. The poor prognosis of patients with oral cancer is primarily due to delayed diagnosis, whereas the survival rate increases up to 80% when the tumor is detected in the early stage (stage I). Therefore, the identification of biomarkers for early detection of OSCC as well as new molecular targets for the treatment of oral cancer are needed.

In the present study, we focused on nicotinamide *N*-methyltransferase, a phase II metabolizing enzyme which plays an important role in biotransformation and detoxification of many xenobiotic compounds.

To investigate the involvement of NNMT in oral cancer cell metabolism, we evaluated the effect of NNMT overexpression on cell proliferation in vitro in the oral cancer cell line HSC-2, and we found that upregulation of the enzyme led to a significant increase on cell viability.

Moreover, we explored the effect of NNMT on apoptosis and cell growth at molecular level, analyzing the expression of β-catenin, survivin, and Ki-67. Results obtained showed that NNMT upregulation in HSC-2 cells significantly enhanced the expression level of survivin ΔEx3 isoform.

NNMT is a cytosolic enzyme that catalyzes the *N*-methylation of nicotinamide, pyridine, and other structural analogs, using S-adenosyl-L-methionine as methyl donor. Upregulation of NNMT has been reported in several tumors, including glioblastoma, papillary thyroid cancer, colorectal cancer, ccRCC, bladder cancer, OSCC, and non-small cell

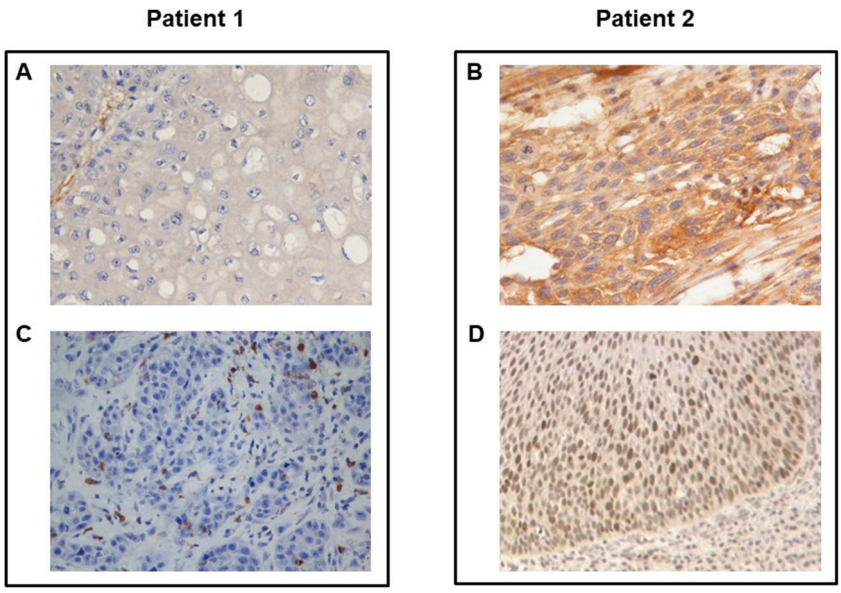

Fig. 4 Expression of NNMT and survivin-ΔEx3 in tissue samples. **a** and **b** (× 40 magnification) refer to low and high cytoplasmic NNMT immunoreactivity, respectively. **c** and **d** (× 25 magnification) refer to low and high survivin-ΔEx3 nuclear staining, respectively. **a** and **c** are related to patient 1; **b** and **d** were obtained from patient 2

lung cancer [7–11]. In oral cancer, we demonstrated that lymph node metastasis, pT, and pathological stage inversely correlate with NNMT mRNA levels, suggesting the possibility of NNMT as a prognostic factor for cancer, and a possible role of the enzyme in tumor growth [8]. Furthermore, we found that salivary NNMT expression levels were significantly higher in patients with OSCC compared to control, indicating that NNMT could be used as a biomarker for early and non-invasive diagnosis of oral cancer [12].

As already mentioned, a number of studies have shown that NNMT is overexpressed in a variety of diseases, but few of these have explored the biological effect associated with NNMT upregulation, and the role of the enzyme in cancer cell metabolism remains still unclear.

In several human tumors, NNMT expression has been associated with cellular invasion. Wu et al. found that NNMT is necessary for cell migration and proliferation in human bladder cancer cells [15], as Tang et al. reported a positive correlation between NNMT expression and the invasive capacity in ccRCC cells [16]. Moreover, a recent work revealed that NNMT downregulation significantly reduced cell proliferation, migration, and invasive capacity in the human pancreatic carcinoma cell line PANC-1 [17]. In order to explore the cellular effect of NNMT expression, Parsons et al. overexpressed NNMT in the human neuroblastoma cell line SH-SY5Y and demonstrated that NNMT expression significantly increased cell viability, correlated with increased complex I activity and intracellular ATP content. Furthermore, NNMT and N1-methylnicotinamide protected SH-SY5Y cells from the toxicity of CxI inhibitors MPP+ and rotenone [18]. Recent studies reported that NNMT downregulation inhibited cell growth in vitro and in vivo, and increased apoptosis and reactive oxygen species (ROS) level in Bcap-37 and MDA-MB-231 breast cancer cell lines [19], while overexpression of NNMT in SW480 colorectal cancer cell line promoted tumorigenicity, inhibited apoptosis, increased intracellular ATP level, and decreased ROS production [20]. Moreover, we have recently investigated the biological function of NNMT in lung cancer and demonstrated that the enzyme has an important role in tumorigenicity, affecting cell proliferation and anchorage-independent cell growth [21].

We have been studying the involvement of NNMT in oral cancer metabolism for many years. As demonstrated by our data already published, we investigated the NNMT expression levels in several human oral cancer cell lines. In the cell line PE/CA-PJ15, we have shown that NNMT downregulation significantly reduced in vitro and in vivo cell growth [6]. In this latest study, we choose wisely the cell line PE/CA to silence NNMT, since its very high NNMT expression level was very useful to evaluate the phenotypic changes associated with enzyme downregulation. On the contrary, in the present paper, we focused on HSC-2 cells, since they show a very low NNMT expression level that allows to better elucidate the effect of induced NNMT upregulation. In a previous paper, we have demonstrated that in Hep-2 cell line, NNMT overexpression is mainly associated with cancer stem cell population [22], therefore suggesting a pivotal role of this enzyme in tumor progression and metastases. The present results indicate that overexpression of NNMT increases cell proliferation in HSC-2 cells, confirming that the enzyme plays an important role in the tumorigenic capacity of oral cancer cells. Even though there are relevant differences between oral cancers related to their localization, NNMT upregulation and its involvement in oral tumorigenesis seems to be a common feature.

Furthermore, we investigated the effect of NNMT on apoptosis, exploring whether NNMT overexpression influences the expression level of survivin isoforms.

This hypothesis arises from a bulk of studies, as mentioned above, where we determined the expression level of NNMT and survivin proteins in different cohorts of oral squamous cell carcinoma specimens [8, 11, 12, 23] and found that both genes were upregulated. Both to confirm these data and to demonstrate the association between NNMT and survivin ΔEx3 isoform expression, immunohistochemistry was performed on 20 OSCC tissue samples, in order to evaluate the expression level of both genes in the same samples. Results obtained showed that there was a positive correlation between NNMT and survivin ΔEx3 expression.

Survivin is a member of the inhibitor of apoptosis protein (IAP) family, containing a single baculovirus IAP repeat (BIR) domain, and is involved in cell cycle progression and control of apoptosis [24].

A variety of studies have reported that survivin is overexpressed in several human tumors, while it is barely detectable in normal differentiated tissues [25, 26]. When overexpressed, survivin accumulates in cytosol, where it inhibits apoptosis by suppressing caspase-9 activation after association with HBXIP cofactor [27]. Furthermore, it has been shown that the interaction between survivin and Smac/DIABLO suppressed apoptosis induced by taxol [28].

In human cancers, survivin has been associated with tumor proliferative activity, poor overall survival, and resistance to chemotherapy and radiotherapy [29–31], suggesting a potential involvement in tumor progression.

The survivin gene consists of four exons encoding for a 16.5 kDa protein. In addition, other five alternative splice variants of survivin have been identified, including survivin-ΔEx3 (lacking exon 3 and containing part of 3′-UTR), survivin-2B (including part of intron 2, called exon 2b), survivin-3B (including part of intron 3, called exon 3B, containing a stop codon), survivin-2α, and survivin-3α. Main survivin isoform can heterodimerize with its splice variants, and it has been suggested that heterodimer formation can lead to the different biological functions observed in tumor cells [32].

Of particular interest is survivin-ΔEx3, an anti-apoptotic protein that has been reported to be upregulated in oral cancer [23] and in other malignancies [33]. In different human cancers, survivin-ΔEx3 has been associated with tumor aggressiveness, tumor staging, and poor prognosis. In colorectal cancer, elevated expression of survivin-ΔEx3 was associated with lymphoid metastasis and invasiveness [26], while Waligórska-Stachura et al. observed a higher survivin-ΔEx3 expression level in thyroid malignant nodules [34].

In the present work, we found that overexpression of NNMT in HSC-2 cells is associated with survivin-ΔEx3 expression. Moreover, although without reaching statistical importance, enhanced expression level of survivin-2B was observed in HSC-2 transfected with pcDNA3-NNMT compared with controls.

Our results demonstrate the involvement of NNMT in tumor cell proliferation, suggesting that the enzyme could represent a potential molecular target for the treatment of oral cancer and data obtained also indicate a possible correlation between NNMT and survivin-ΔEx3 expression levels. Although the effect of NNMT on survivin deserves further investigation, our findings seem to suggest a potential role of the enzyme in the regulation of apoptosis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent For this type of study, formal consent is not required.

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