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Nicotinamide *N*-Methyltransferase and Its Precursor Substrate Methionine Directly and Indirectly Control Malignant Metabolism During Progression of Renal Cell Carcinoma

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Abstract. *Background/Aim: Renal cell carcinoma (RCC) is one of the most common tumor diseases in adults, and new specific biomarkers are urgently needed to define diagnosis and prognosis of patients with RCC as well as monitor the outcome of therapeutic interventions. The enzyme nicotinamide N-methyltransferase (NNMT) is believed to represent such a marker molecule in RCC therapy. Materials and Methods: NNMT expression was examined by western blotting in samples from patients with RCC and in RCC cell lines. Effects of NNMT on cell growth and metabolism were assessed using the Hoechst 33342 reagent assay and Vita-Orange cell viability assay. Incubation experiments were performed to study the influence of methionine and interleukin-6 (IL6) on expression of NNMT. Results: In patient samples, NNMT was up-regulated depending on the stage of progression. Investigations in an RCC cell culture model showed that after modulation of NNMT expression, cellular metabolism, but not cell growth was affected. This regulatory function was also dependent on the presence of the NNMT precursor substrate methionine and IL6. Conclusion: The metabolism-regulatory activity of NNMT depends on the precursor substrate methionine and the presence of IL6. The function of methionine appears to be dependent on the stage of progression, since in individual RCC cell lines, opposing effects on metabolism were demonstrated. This, in turn, reflects the thoroughly complex situation in the clinic.*

Renal cell carcinoma (RCC) is one of the most common tumor diseases in adults and to a large extent occurs as the clear-cell histological type. Due to the lack of early symptoms and despite the development of modern imaging methods, RCC is usually diagnosed late, often only in the metastatic stage (1). By characterizing molecular pathogenesis mechanisms, new targets for targeted therapy of RCC have been identified, *e.g.* inhibitors of tyrosine kinases (sunitinib, sorafenib), mechanistic target of rapamycin (temsirolimus, everolimus), and vascular endothelial growth factor (bevacizumab). In addition, new specific biomarkers are needed to define diagnosis and prognosis of patients with RCC and monitor the outcome of therapeutic interventions.

The protein nicotinamide *N*-methyltransferase (NNMT) might be such a marker molecule in RCC. Under physiological conditions, this enzyme is mainly located in the liver and catalyzes the biotransformation of low-molecular weight compounds. In RCC, NNMT appears to be up-regulated in cancer tissue and may represent a biomarker (2-4). However, the cellular function of NNMT during RCC initiation and progression is still largely unknown. First data correlated NNMT activity with cell mobility and metastasis (5, 6). Furthermore, whether NNMT participates in methylatory processes of epigenetic-regulatory mechanisms has been discussed. The enzyme is involved in the NAD metabolism *via* its substrate nicotinamide and in methyl metabolism through the consumption of S-adenosyl methionine (7, 8).

The aim of the study presented here was to characterize NNMT involvement in progression of RCC. Since enzyme functionality is linked to energy and methionine metabolism, NNMT properties in cell growth and metabolism are primarily addressed. Moreover, the expression levels of

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NNMT in patient samples were determined in order to evaluate its suitability as an RCC biomarker.

Materials and Methods

Patient samples and protein preparation. Tissue samples and related clinical data were collected based on approval from the Ethics Committee at the Medical Faculty of the University of Greifswald (III UV 12/03). For western blot analyses, tissue samples from patients with clear cell RCC were preserved at −80˚C immediately after resection. After histological evaluation, eight samples each were classified into three subgroups pT1, pT2 and pT3, as well as the non-malignant control group. Protein preparation was performed using peqGOLD TriFast™ (Peqlab, Erlangen, Germany) according to the manufacturer's instruction. Tissue (50-100 mg) was homogenized with 1 ml peqGOLD TriFast™. To ensure the dissociation of nucleotide complexes, samples were incubated at room temperature for 5 min. After addition of 0.2 ml chloroform, samples were shaken vigorously for 15 s. After a 20-min incubation at room temperature, the samples were centrifuged (5 min at 12,000 × *g*) to achieve phase separation. To remove genomic DNA, the interphase and the phenolic phase were incubated with 300 μl of ethanol for 3 min and centrifuged at 2,000 × *g* for 15 min. The supernatant was used to precipitate the proteins by adding 1.5 ml isopropanol. After centrifugation at 12,000 × *g* for 10 min, pellets were washed twice with 0.3 M guanidinium chloride in 95% ethanol and finally twice with 100% ethanol each for 20 min. Protein samples were dried, resuspended in sample buffer (8 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 40 mM Tris and 50 mM dithiothreitol and the total amount of protein was determined by modified Bradford assay.

Cell culture and treatment. Human RCC cell lines 769-P, 786-O, and Caki-2, as well as the human embryonic kidney cell line HEK-293, were obtained from the Leibniz Institute DSMZ–German Collection of Microorgansims and Cell Cultures (Braunschweig, Germany) and propagated in RPMI 1640 medium supplemented with 20 μM methionine (Carl Roth, Karlsruhe, Germany), 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA), 11 mM glucose (B. Braun Melsungen, Melsungen, Germany), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (PAN-Biotech, Aidenbach, Germany) at 37˚C in a humified atmosphere with 5% CO_2. In the case of Caki-2 cells, 16.5 mM glucose (B. Braun Melsungen) was used.

The amino acid methionine is essential for cell growth. Since the plasma concentration of methionine in humans is 20-40 μM, RCC cells were propagated with a minimum concentration of 20 μM methionine. In studies on the influence of methionine, the concentration in cell culture media was increased to 100 μM (7). The specified concentrations of methionine were obtained by supplementing an appropriate amount of methionine solution (Carl Roth).

Cloning of pcDNA3.1-NNMT. The vector pcDNA3.1+ (Invitrogen AG, Carlsbad, USA) was linearised with *Hind*III and *Xho*I. The human NMMT encoding DNA (position 159-953, accession number NM_006169) was amplified by polymerase chain reaction (PCR) using Caki-1 complementary DNA (cDNA) as template and primer with internal *Hind*III/*Xho*I sites as well as a Kozak sequence in front of the ATG start codon. The PCR product was ligated into the *Hind*III/*Xho*I sites of pcDNA3.1(+) vector. All purification steps were carried out by QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany) following the supplier's instructions. All required enzymes (restriction endonucleases, Phusion® High Fidelity DNA Polymerase, T4 DNA Ligase) were purchased from New England Biolabs (Frankfurt a.M., Germany). Finally, the cloning product was confirmed by sequencing.

Transfection experiments. Transient transfection was performed with jetPRIME® transfection reagent (Polyplus Transfection, Illkirch, France). The plasmid pcDNA3.1_NNMT was used to overexpress NNMT in HEK-293, Caki-2 and 769-P cells. The knockdown of *NNMT* was performed by application of siNNMT (5'-CGUCG UCACUGACUACUCdTdT-3') in Caki-1 and 786-O cells. As controls, the plasmid pcDNA3.1 or scrambled siRNA (5'-GCUCCC UCCUUCGAUUUAUdTdT-3') were used in mock transfections.

For transfection, cells were seeded 24 hours prior to treatment and used according to the manufacturer's instructions. In detail, 2 μg nucleic acid was diluted into 200 μl jetPRIME® buffer, and mixed by vortexing for 10 s. Then 4 μl jetPRIME® was added and the mixture vortexed for 10 s, and spun down briefly. After an incubation of 10 min at room temperature, 200 μl of transfection mix were added per well dropwise onto the cells in serum-containing medium, and distributed evenly before incubation at 37˚C. When necessary, the transfection medium was replaced after 4 h by cell growth medium and the plates were returned to the incubator.

Cell viability assay. To determine cellular viability, different cell numbers were used for analyses of treatment with variable methionine concentrations (20 μM and 100 μM methionine) or with 50 ng/ml IL6 (1.5×10^3 cells for HEK-293, 786-O and 769-P; 1×10^3 cells for Caki-2) or analyses after transfection of cells (5×10^3 cells for HEK-293, 6×10^3 cells for 786-O and 769-P; 4×10^3 cells for Caki-2). The cells were seeded in a 96-well cell culture plate with 100 μl of media, incubated for 24 h and 72 h, and analyzed according to the manufacturer's instructions using Vita-Orange Cell Viability Assay (Biotool, Houston, TX, USA). Briefly, to the medium, 11 μl of Vita-Orange solution were added and evenly distributed by panning. After 1 h of incubation at 37˚C, absorption was measured with an Infinite 200 Plate Reader (Tecan, Männedorf, Switzerland) at 450 nm.

Cell growth assay. Cell growth was determined utilizing Hoechst 33342 Reagent (Sigma-Aldrich, St. Louis, MO, USA). Cells were treated as described in the cell viability assay, frozen, and incubated after thawing for 10 min with 10 μl Hoechst solution. After dilution with 100 μl of PBS, fluorescence was detected using an Infinite 200 Plate Reader (Tecan) at 350/480 nm.

Preparation of protein from cell lines. After washing with 5 ml PBS, cells were detached from the cell culture plate with a cell scraper and transferred to a 1.5 ml Eppendorf tube and centrifuged at 500×g for 5 min. The supernatant was discarded. Cells were lysed with 200 μl lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, 5 mM EDTA, 0.5% NP-40, 0.5% Triton X-100, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM NaF, 1 mM Na_3VO_4, and complete protease inhibitor cocktail from Roche Applied Science (Mannheim, Germany), frozen at −80˚C and then centrifuged after thawing (for 10 min at 17,000 × *g* and 4˚C). Total protein concentration was determined by Bradford assay.

Western blot analysis. Equal amounts of protein were separated by Mini-Protean system (BioRad, Munich, Germany), transferred onto a Protran nitrocellulose membrane (70 min at 1 mA/cm^2; Whatman, Dassel, Germany), and subsequently blocked with Roti-Block (Carl Roth) for 1 h. Proteins of interest were detected by primary antibodies (4˚C for 16 h, diluted in 5% BSA in TBST with NaN_3) directed against NNMT (1:5000, 15123 1 AP; Proteintech, Rosemont, IL, USA), IL6 receptor (IL6R) (1:1000, 23457 1 AP; Proteintech), α-tubulin (1:50000, ab7291; Abcam, Cambridge, UK) as control, and peroxidase-coupled secondary antibodies (30 min, 1:30000, #7074, #7076; Cell Signaling Technology, Frankfurt, Germany). After incubation with 2 ml Enhanced Chemiluminescence Detection Substrate (Thermo Fisher Scientific) for 3 min, signals were visualized by X-ray films and subsequently quantified in digital form (ImageJ Software, https://imagej.nih.gov/ij/index.html). The quantification of the respective proteins was relative to the associated total protein amount of the lane.

Statistics. Statistical analyses were performed using the nonparametric Mann–Whitney test. Results with $p \leq 0.05$ were considered significant. Data are given as median values and were prepared using Prism 8 Software (GraphPad Software, La Jolla, CA, USA).

Results

NNMT expression in samples from patients with RCC and in RCC cell lines. Little is known about the cellular functionality of NNMT during the formation and progression of RCC. Therefore, the protein expression of NNMT in samples from 24 patients with RCC of different stages (eight samples each of stages pT1, pT2 and pT3) were evaluated and compared to eight samples from healthy tissues (Figure 1). Analysis demonstrated an increase in NNMT expression during progression from pT1 to pT3 compared to the non-malignant samples. This increase was statistically significant in stages pT2 (p=0.0006) and pT3 (p=0.0002) and may indicate a biological function of the protein in tumor. For further experimental investigations, an RCC model system was established consisting of the non-malignant renal cell line HEK-293 and the RCC cell lines Caki-2, 786-O and 769-P. In all cell lines, NNMT was detected at different amounts (Figure 2A), whereby non-malignant HEK-293 cells had the lowest NNMT protein content compared to RCC cells (compared to HEK-293 with median=1.0): Caki-2: 6.55-fold; 786-O: 52.1-fold; 769-P: 4.82-fold).

NNMT-dependent modulation of cellular metabolism and cell growth in RCC cells. Enhanced cell growth and cancer-specific alterations in cellular metabolism are among the most significant malignant events during cancer progression. A possible involvement of NNMT was investigated applying a metabolic activity assay and a proliferation assay. Owing to the low expression of NNMT in HEK-293, Caki-2, and 769-P cells, transient overexpression was established for further experiments. After transfection, overexpression of

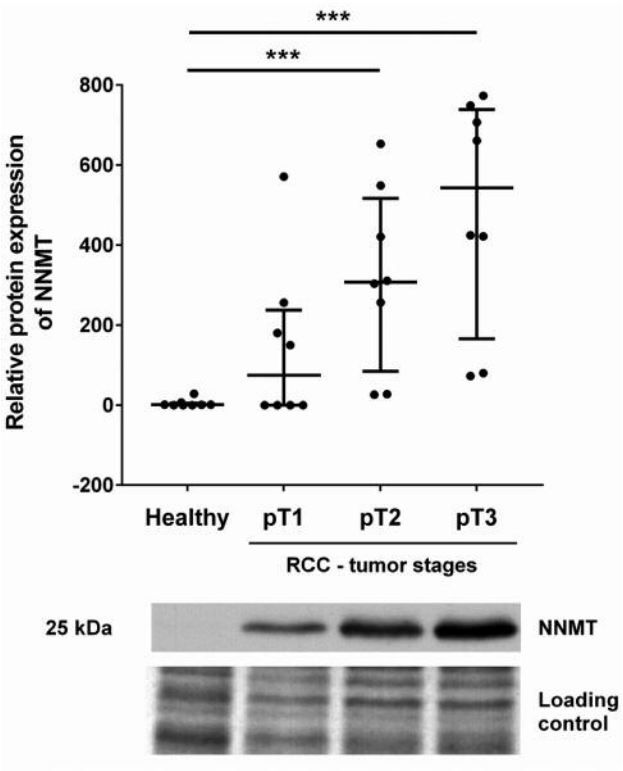

Figure 1. *Relative protein expression of nicotinamide N-methyltransferase (NNMT) in samples from patients with renal cell carcinoma (RCC). Quantitative analysis of western blots from samples from 24 patients' different stages of RCC (eight samples each) compared to eight samples from healthy donors. Total protein staining of a membrane is shown as loading control. Data are presented as scatter dot plot with median (lines) and interquartile range (bars). ***Significantly different at p≤0.001.*

NNMT protein was achieved by a factor of 7.0 in HEK-293 and 14.5 in Caki-2 and 769-P cells (Figure 2B).

Overexpression experiments revealed a significant decrease in metabolic activity in *NNMT*-transfected HEK-293 (p<0.0001), Caki-2 (p<0.0001), and 769-P (p<0.0001) cells (Figure 3A-C, left panel). However, this had an inhibitory effect on cell growth only in the case of non-malignant HEK-293 cells (p=0.0006) (Figure 3A, right panel); no changes were observed in Caki-2 and 769-P RCC cells (Figure 3B and C, right panel). The knockdown of *NNMT* in transfected 786-O cells confirmed these observations. Here, siRNA-mediated reduction of NNMT protein expression led to an increase in metabolic activity (p<0.0001), which had no effect on the cell growth rate (Figure 3D).

NNMT functionality is controlled by the NNMT precursor substrate methionine. For the methylation of substrates, NNMT requires the precursor substrate methionine which is converted by methionine adenosyltransferase to the methyl

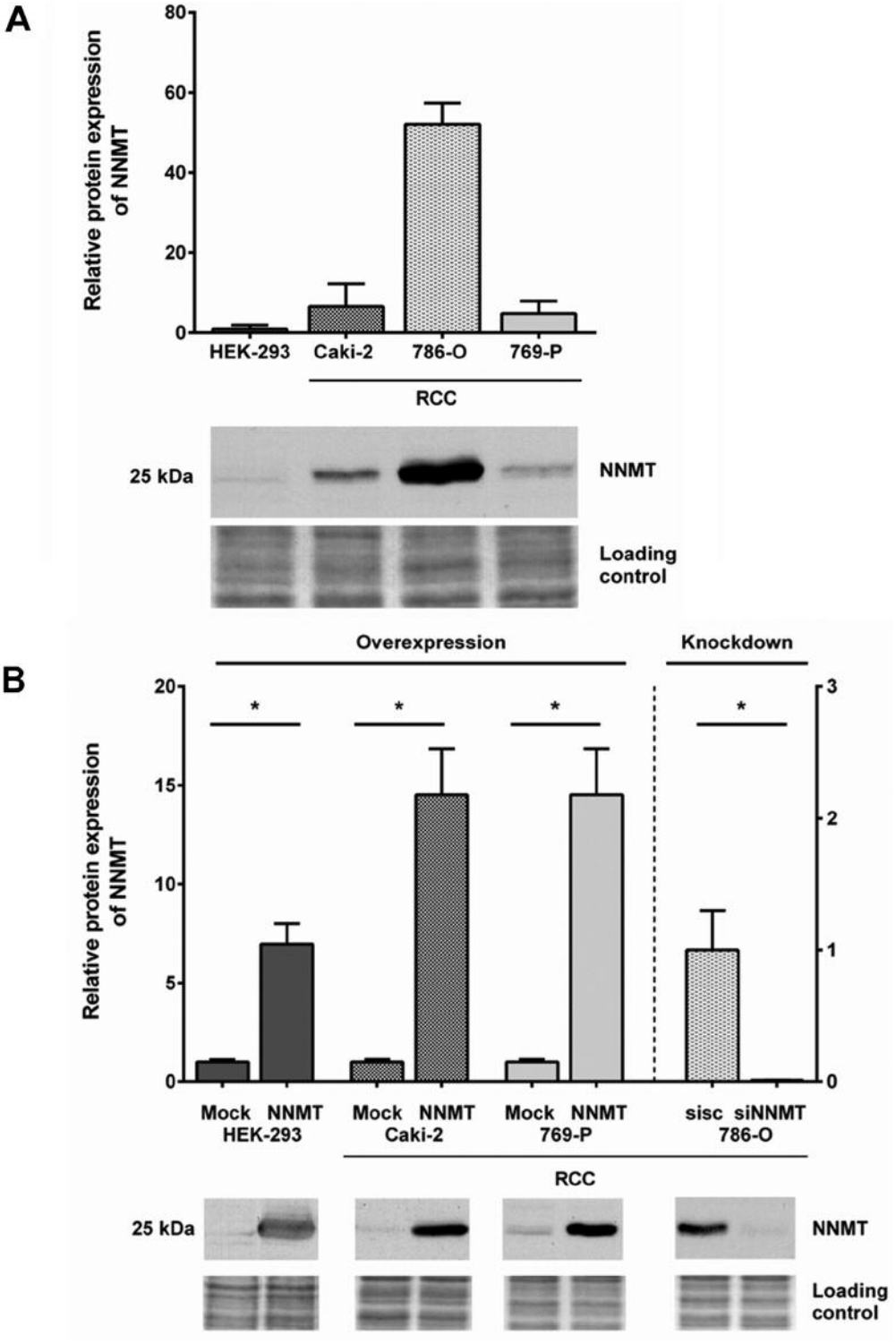

Figure 2. *Protein expression of nicotinamide N-methyltransferase (NNMT) in different renal cell carcinoma (RCC) cell lines. Quantitative analysis of western blots from Caki-2, 786-O, and 769-P RCC cells and human embryonic kidney cell line HEK-293. A: NNMT expression was evaluated relative to that of HEK-293 cells. B: Relative protein expression after overexpression of NNMT in HEK-293, Caki-2, and 769-P cells, compared to mock transfection. B: Relative protein expression after knockdown of NNMT in 786-O cells compared to scrambled siRNA (sisc) transfection. Representative western blots for NNMT detection are shown (n=4). Total protein staining of membranes are given as loading control. Data are presented as mean values with standard deviation. *Significantly different at p≤0.05.*

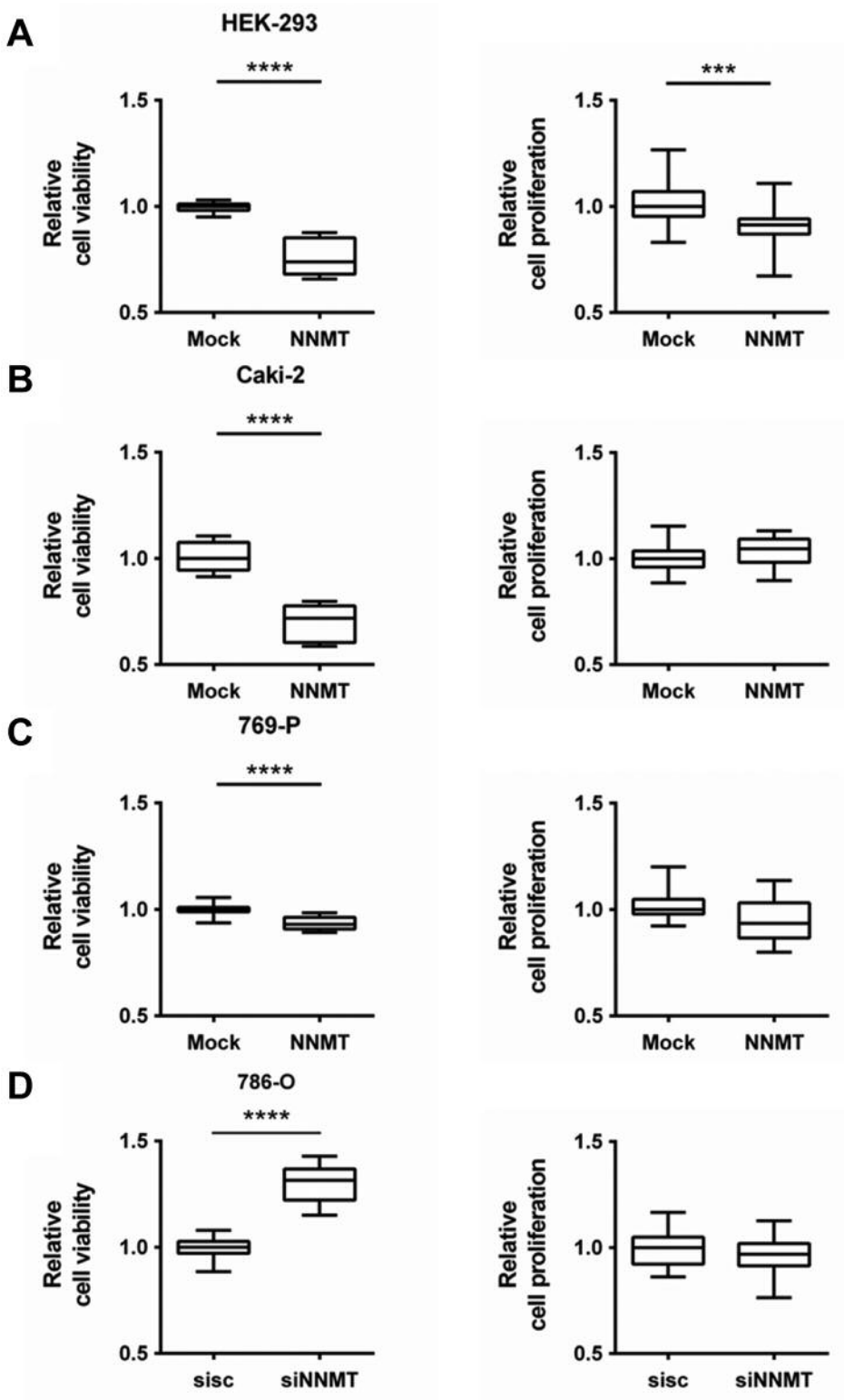

Figure 3. *Metabolic activity and proliferation of different renal cell carcinoma (RCC) cell lines after transfection with nicotinamide N-methyltransferase (NNMT) overexpression vector and NNMT-specific small interfering RNA (siNNMT). Left panel: Relative viability of cell lines with low expression of NNMT human embryonic kidney cell line HEK-293 (n=18) (A), and Caki-2 (n=12) (B) and 769-P (n=18) (C) RCC cells after forced overexpression of NNMT and high-level NNMT-expressing 786-O cells after knockdown of NNMT (n=18) (D). Right panel: Relative cell proliferation of corresponding cells (n=18). Data are presented as box blots with median (lines) and interquartile range (bars). Significantly different at ***p≤0.001 and ****p≤0.0001.*

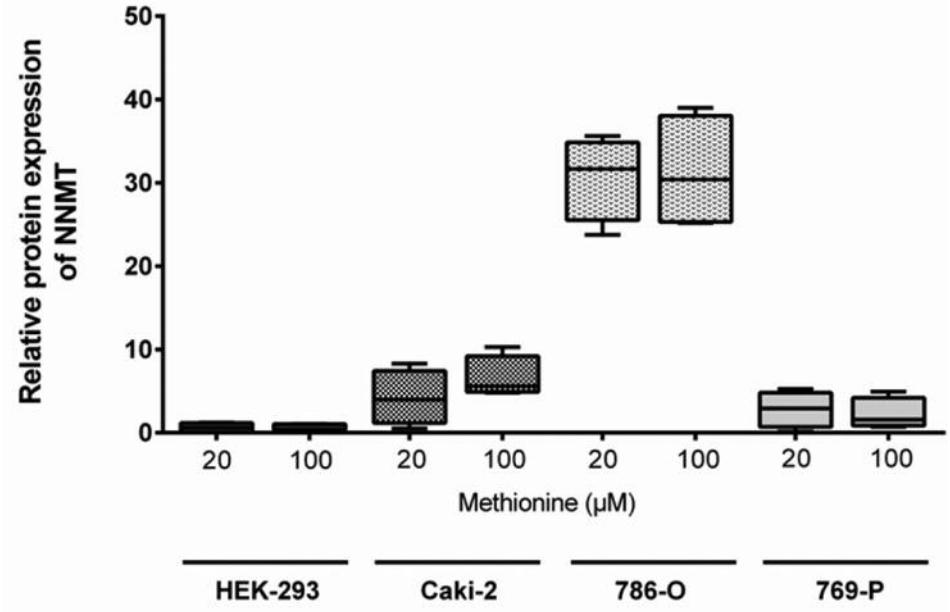

Figure 4. *Relative expression of nicotinamide N-methyltransferase (NNMT) protein in different renal cell carcinoma (RCC) cell lines – influence of methionine concentration. Quantitative analysis of western blots from HEK-293, Caki-2, 786-O, and 769-P cells. Expression was evaluated compared to that of HEK-293 cells at the specified methionine concentration (n=4). Data are presented as box blots with median (lines) and interquartile range (bars).*

donor S-adenosyl methionine. Alterations in concentrations of methionine to 100 μM did not lead to a significant change in NNMT expression (Figure 4). However, incubation experiments over 3 days showed a significant increase in metabolic activity in HEK-293 ($p<0.0001$) 786-O ($p<0.0001$) and 769-P ($p<0.0001$) cells on the third day in the presence of 100 μM methionine (Figure 5A, C and D, left panel), with the exception of Caki-2 (Figure 5B). In contrast to *NNMT* overexpression and knockdown approaches (Figure 3), effects on cell growth were also observed during incubation with the higher concentration of methionine. The increased metabolic activity in the presence of 100 μM methionine led to a reduction in growth of HEK-293 (Figure 5A, right panel; $p<0.0001$) and 786-O (Figure 5C, right panel; $p=0.0096$) cells. For Caki-2 and 769-P cells, on the other hand, no differences were detectable between growth at the two methionine concentrations used (Figure 5B and D, right panel).

NNMT-dependent modulation of the metabolic activity is mediated by IL6. The activity of NNMT is partially regulated by interleukins and is integrated into cellular signal transduction pathways. All four renal cell lines used expressed IL6R, however, IL6R expression was only dependent on methionine concentration in the case of 769-P cells (Figure 6A). Incubation experiments with 50 ng/ml IL6 confirmed the hypothesis that the IL6 signaling pathway is involved in the regulatory activity of NNMT. While metabolic activity was regulated by IL6 (Figure 6B), in the presence of the interleukin there was no influence on cell growth (Figure 6C). It was highly noteworthy that incubation with IL6 led to a significant decrease in metabolic activity of Caki-2 cells, but that under comparable conditions, it led to an increase in metabolic activity in 786-O cells.

Discussion

NNMT expression in tumor tissue is up-regulated in some entities such as prostate, bladder, pancreatic, and gastric cancer (9-13). In bladder cancer, the NNMT concentration in urine even correlates with tumor progression and may be used as a diagnostic biomarker in the future (14). In RCC, the biomarker function of NNMT has already been discussed (2, 15, 16), but the underlying studies are mainly based on proteomic or transcriptomic approaches. The cellular and molecular role of NNMT in RCC, however, is still largely unknown.

The analysis of patient material in this study demonstrated significant up-regulation of *NNMT* expression in RCC and confirms the findings of the only study to date on NNMT expression in patients with RCC (17). This fact was reflected in the *in vitro* model of the non-malignant cell line HEK-293 and the three RCC lines Caki-2, 769-P and 786-O. NNMT expression was many times higher in the three malignant cell lines than in HEK-293 cells. Furthermore, NNMT seemed to have a greater influence on cell growth in non-malignant HEK-293 cells than in the malignant cell lines. Both after

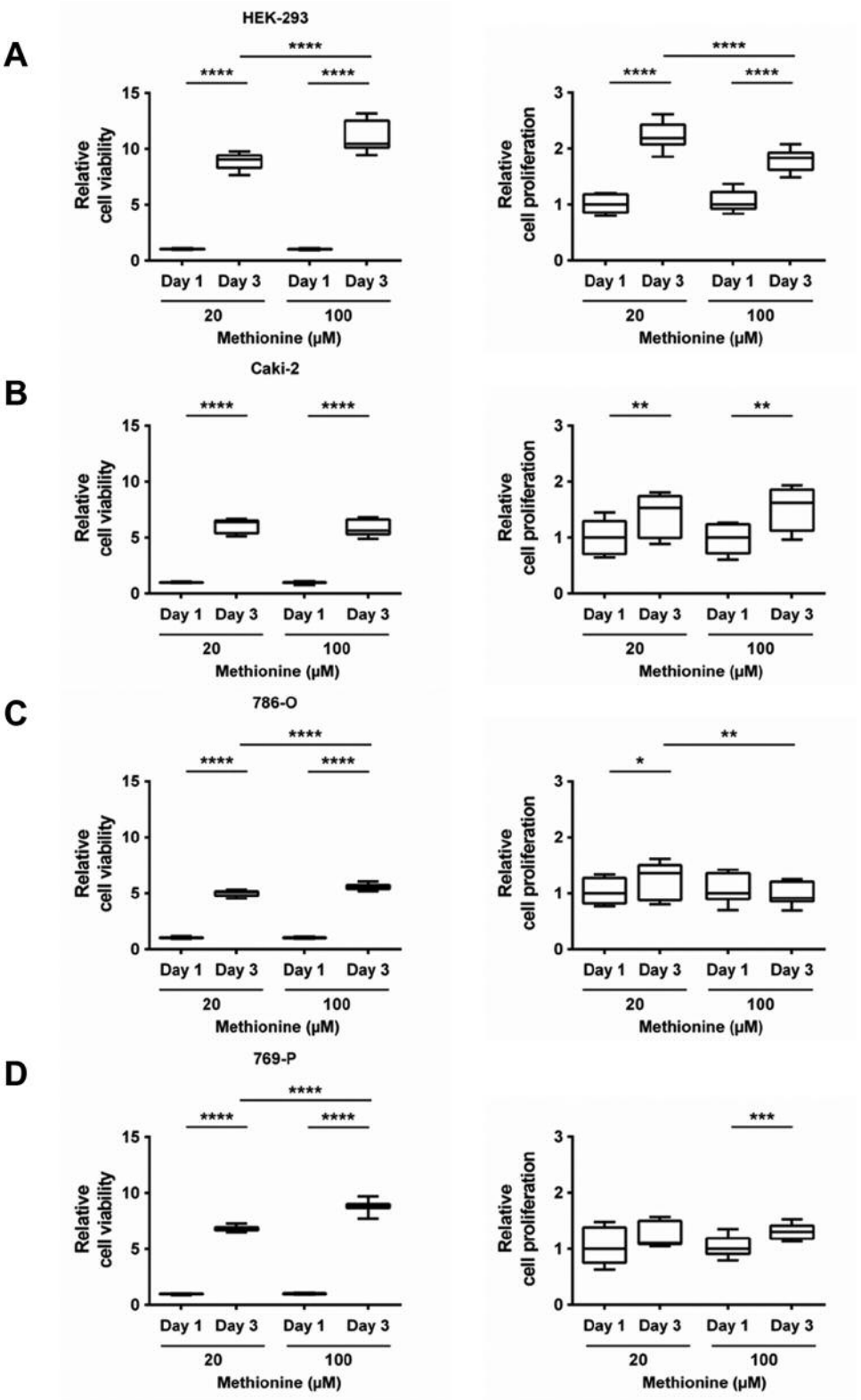

Figure 5. *Metabolic activity and proliferation in different renal cell carcinoma (RCC) cell lines due to the influence of methionine concentration. Left panel: Relative viability of human embryonic kidney cell line HEK-293 (A), and Caki-2 (B) and 769-P (C) RCC cells after 1 or 3 days applying methionine concentrations of 20 μM or 100 μM in the medium (n=18). Right panel: Relative cell proliferation of corresponding cells (n=12). Data are presented as box blots with median (lines) and interquartile range (bars). Significantly different at *p≤0.05, **p≤0.01, ***p≤0.001, and ****p≤0.0001.*

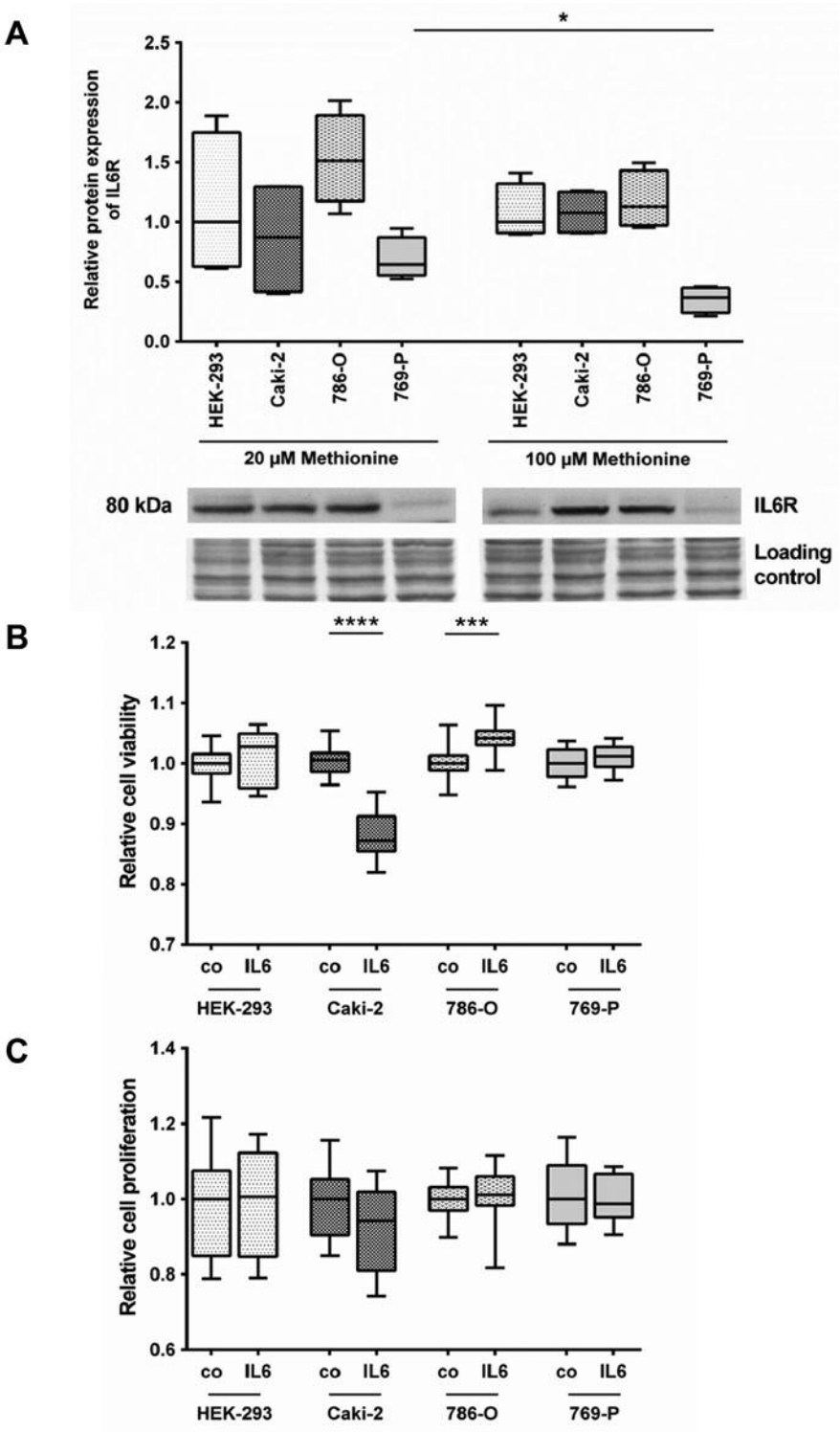

Figure 6. *Influence of interleukin-6 (IL6) treatment on the metabolic activity and cell proliferation of different RCC cell lines. A: Protein expression of the IL6 receptor (IL6R) in an human embryonic kidney cell line HEK-293, and Caki-2, 786-O, and 769-P RCC cells at methionine concentration of 20 µM or 100 µM in the medium. Relative expression was evaluated compared to that of HEK-293 cells at the specified methionine concentration. A representative western blot for IL6R detection is shown (n=4). Total protein staining of a membrane is given as a loading control. B: Relative viability of HEK-293, Caki-2, 786-O, and 769-P cells after treatment with 50 ng/ml IL6 compared to controls (co; n=18). C: Relative proliferation of HEK-293, Caki-2, 786-O and 769-P cells after treatment with 50 ng/ml IL6 compared to controls (co; n=18). Data are presented as box blots with median (lines) and interquartile range (bars). Significantly different at *p≤0.05, ***p≤0.001 and ****p≤0.0001.*

overexpression of NNMT and at higher concentrations of the NNMT precursor substrate methionine, the influence was more pronounced in HEK-293 cells than in RCC cells. Notably, in the *in vitro* RCC model, antiproliferative properties of NNMT were demonstrated. These observations are in contrast to current data attributing oncogenic and proliferative properties to NNMT in breast, pancreatic, lung, and oral squamous cell carcinoma (18-22). On the other hand, the antiproliferative properties of NNMT shown in this study correspond to its function in metabolism. The overexpression of NNMT led to a reduction in metabolic activity and this would be expected to result in reduced cell growth. Thus, NNMT might possibly play a different role in RCC cells than in cells of other entities. Interestingly, overexpression and knock-down experiments demonstrated that metabolic activity did not always correlate with cell growth. This fact sheds critical light on colorimetric assays such as WST or 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assays that determine enzyme substrate turnover and are interpreted as indirect detection of biomass and thus cell growth.

Besides the expression level of NNMT, the availability of the precursor substrate methionine also plays a role in protein function. With the exception of Caki-2 cells, an increase in available methionine concentration also led to an increase in metabolic activity. The methionine product S-adenosyl methionine is a universal methyl donor in numerous enzyme-catalyzed reactions and is being tested for the treatment of various diseases, including cancer (23, 24). In colorectal cancer, *S*-adenosyl methionine has been shown to control cell growth in a concentration- and time-dependent manner. Higher *S*-adenosyl methionine concentrations and longer incubation times led to the inhibition of cell growth, while lower concentrations and short incubation times stimulated proliferation (25). Thus, the experimental conditions used are crucial in the assessment of the proliferative effects of methionine. Furthermore, we cannot exclude from the present study that methionine- and S-adenosyl methionine-mediated but NNMT-independent mechanisms led to an effect on the demonstrated metabolic activity and cell growth. Both the expression level of NNMT and the availability of the S-adenosyl methionine precursor methionine seem to be important for the malignant activity of RCC cells.

NNMT is an important factor in energy metabolism (26). Alterations in NNMT activity due to modulation of protein expression or concentration of the precursor substrate methionine therefore also have an effect on cell growth. IL6 is a key factor in the progression and proliferation of RCC (27-29) and we therefore analyzed whether this factor is also involved in the cellular functionality of NNMT. The data showed both methionine-dependent expression of IL6R and IL6 induced modulation of metabolic activity. However, this was not detectable in all cell lines, so that the function of IL6 in NNMT-regulated RCC progression remains unclear. It is remarkable, however, that IL6 had pro-metabolic and anti-metabolic activity depending on the cell line. Thus, the hypothetical signal axis NNMT–methionine–IL6 can be considered to be unique in different patients, which makes the use of NNMT as a diagnostic or prognostic biomarker unlikely.

In summary, NNMT plays a multifunctional role in RCC cells. Its up-regulation during tumor progression suggests an important oncogenic function. This does not affect tumor growth, but cellular metabolism. In contrast to other cancer entities, increased intracellular concentration of NNMT leads to reduced metabolism in RCC. Additionally, the concentration of the precursor substrate methionine seems to be of importance for NNMT activity. With increasing methionine concentration, the metabolic activity of RCC cells also increased. The regulatory function of NNMT in RCC appears to be progression-dependent, depends on the precursor substrate methionine and appears very individual in the clinical context. Furthermore, cellular function is mediated by additional factors, possibly IL6.

References

1 Rini BI, Campbell SC and Escudier B: Renal cell carcinoma. Lancet *373*: 1119-1132, 2009. PMID: 19269025. DOI: 10.1016/S0140-6736(09)60229-4

2 Sartini D, Muzzonigro G, Milanese G, Pierella F, Rossi V and Emanuelli M: Identification of nicotinamide N-methyltransferase as a novel tumor marker for renal clear cell carcinoma. J Urol *176*: 2248-2254, 2006. PMID: 17070307. DOI: 10.1016/j.juro.2006.07.046

3 Takahashi M, Rhodes DR, Furge KA, Kanayama H, Kagawa S, Haab BB and Teh BT: Gene expression profiling of clear cell renal cell carcinoma: Gene identification and prognostic classification. Proc Natl Acad Sci USA *98*: 9754-9759, 2001. PMID: 11493696. DOI: 10.1073/pnas.171209998

4 Yao M, Tabuchi H, Nagashima Y, Baba M, Nakaigawa N, Ishiguro H, Hamada K, Inayama Y, Kishida T, Hattori K, Yamada-Okabe H and Kubota Y: Gene expression analysis of renal carcinoma: Adipose differentiation-related protein as a potential diagnostic and prognostic biomarker for clear-cell renal carcinoma. J Pathol *205*: 377-387, 2005. PMID: 15682440. DOI: 10.1002/path.1693

5 Tang S-W, Yang T-C, Lin W-C, Chang W-H, Wang C-C, Lai M-K and Lin J-Y: Nicotinamide N-methyltransferase induces cellular invasion through activating matrix metalloproteinase-2 expression in clear cell renal cell carcinoma cells. Carcinogenesis *32*: 138-145, 2011. PMID: 21045016. DOI: 10.1093/carcin/bgq225

6 Egeblad M and Werb Z: New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer *2*: 161-174, 2002. PMID: 11990853. DOI: 10.1038/nrc745

7 Ulanovskaya OA, Zuhl AM and Cravatt BF: NNMT promotes epigenetic remodeling in cancer by creating a metabolic methylation sink. Nat Chem Biol *9*: 300-306, 2013. PMID: 23455543. DOI: 10.1038/nchembio.1204

8 Sperber H, Mathieu J, Wang Y, Ferreccio A, Hesson J, Xu Z, Fischer KA, Devi A, Detraux D, Gu H, Battle SL, Showalter M, Valensisi C, Bielas JH, Ericson NG, Margaretha L, Robitaille AM, Margineantu D, Fiehn O, Hockenbery D, Blau CA, Raftery D, Margolin AA, Hawkins RD, Moon RT, Ware CB and Ruohola-Baker H: The metabolome regulates the epigenetic landscape during naive-to-primed human embryonic stem cell transition. Nat Cell Biol *17*: 1523-1535, 2015. PMID: 26571212. DOI: 10.1038/ncb3264

9 You Z, Liu Y and Liu X: Nicotinamide N-methyltransferase enhances the progression of prostate cancer by stabilizing sirtuin 1. Oncol Lett *15*: 9195-9201, 2018. PMID: 29805651. DOI: 10.3892/ol.2018.8474

10 Sartini D, Muzzonigro G, Milanese G, Pozzi V, Vici A, Morganti S, Rossi V, Mazzucchelli R, Montironi R and Emanuelli M: Up-regulation 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, 2013. PMID: 23097023. DOI: 10.1007/s12013-012-9451-1

11 Chen C, Wang X, Huang X, Yong H, Shen J, Tang Q, Zhu J, Ni J and Feng Z: Nicotinamide N-methyltransferase: A potential biomarker for worse prognosis in gastric carcinoma. Am J Cancer Res *6*: 649-663, 2016. PMID: 27152242.

12 Xu Y, Liu P, Zheng D-H, Wu N, Zhu L, Xing C and Zhu J: Expression profile and prognostic value of NNMT in patients with pancreatic cancer. Oncotarget *7*: 19975-19981, 2016. PMID: 26942567. DOI: 10.18632/oncotarget.7891

13 Zhou W, Gui M, Zhu M, Long Z, Huang L, Zhou J, He L and Zhong K: Nicotinamide N-methyltransferase is overexpressed in prostate cancer and correlates with prolonged progression-free and overall survival times. Oncol Lett *8*: 1175-1180, 2014. PMID: 25120681. DOI: 10.3892/ol.2014.2287

14 Pozzi V, Di Ruscio G, Sartini D, Campagna R, Seta R, Fulvi P, Vici A, Milanese G, Brandoni G, Galosi AB, Montironi R, Cecati M and Emanuelli M: Clinical performance and utility of a NNMT-based urine test for bladder cancer. The Int J Biol Markers *33*: 94-101, 2018. PMID: 29148015. DOI: 10.5301/ ijbm.5000311

15 Kim DS, Choi YP, Kang S, Gao MQ, Kim B, Park HR, Choi YD, Lim JB, Na HJ, Kim HK, Nam Y-P, Moon MH, Yun HR, Lee DH, Park W-M and Cho NH: Panel of candidate biomarkers for renal cell carcinoma. J Proteome Res *9*: 3710-3719, 2010. PMID: 20455597. DOI: 10.1021/pr100236r

16 Teng P-n, Hood BL, Sun M, Dhir R and Conrads TP: Differential proteomic analysis of renal cell carcinoma tissue interstitial fluid. J Proteome Res *10*: 1333-1342, 2011. PMID: 21142074. DOI: 10.1021/pr101074p

17 Zhang J, Xie X-y, Yang S-w, Wang J and He C: Nicotinamide N-methyltransferase protein expression in renal cell cancer. J Zhejiang Univ Sci B *11*: 136-143, 2010. PMID: 20104648. DOI: 10.1631/jzus.B0900249

18 Seta R, Mascitti M, Campagna R, Sartini D, Fumarola S, Santarelli A, Giuliani M, Cecati M, Lo Muzio L and Emanuelli M: Overexpression of nicotinamide N-methyltransferase in HSC-2 OSCC cell line: Effect on apoptosis and cell proliferation. Clin Oral Investig *23*: 829-838, 2018. PMID: 29882109. DOI: 10.1007/s00784-018-2497-8

19 Bach D-H, Kim D, Bae SY, Kim WK, Hong J-Y, Lee H-J, Rajasekaran N, Kwon S, Fan Y, Luu T-T-T, Shin YK, Lee J and Lee SK: Targeting nicotinamide N-methyltransferase and miR-449a in EGFR-TKI-resistant non-small-cell lung cancer cells. Mol Ther Nucleic Acids *11*: 455-467, 2018. PMID: 29858080. DOI: 10.1016/j.omtn.2018.03.011

20 Yu T, Wang Y-T, Chen P, Li Y-H, Chen Y-X, Zeng H, Yu A-M, Huang M and Bi H-C: Effects of nicotinamide N-methyltransferase on PANC-1 cells proliferation, metastatic potential and survival under metabolic stress. Cell Physiol Biochem *35*: 710-721, 2015. PMID: 25592232. DOI: 10.1159/000369731

21 Sartini D, Seta R, Pozzi V, Morganti S, Rubini C, Zizzi A, Tomasetti M, Santarelli L and Emanuelli M: 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, 2015. PMID: 25204218. DOI: 10.1515/hsz-2014-0231

22 Zhang J, Wang Y, Li G, Yu H and Xie X: Down-regulation of nicotinamide N-methyltransferase induces apoptosis in human breast cancer cells *via* the mitochondria-mediated pathway. PLoS ONE *9*: e89202, 2014. PMID: 24558488. DOI: 10.1371/journal.pone.0089202

23 Hoang BX, Tran HQ, Vu UV, Pham QT and Shaw DG: Palliative treatment for advanced biliary adenocarcinomas with combination dimethyl sulfoxide-sodium bicarbonate infusion and S-adenosyl-L-methionine. J Pain Palliat Care Pharmacother *28*: 206-211, 2014. PMID: 25102038. DOI: 10.3109/15360288.2014.938882

24 Roje S: *S*-Adenosyl-L-methionine: Beyond the universal methyl group donor. Phytochemistry *67*: 1686-1698, 2006. PMID: 16766004. DOI: 10.1016/j.phytochem.2006.04.019

25 Modis K, Coletta C, Asimakopoulou A, Szczesny B, Chao C, Papapetropoulos A, Hellmich MR and Szabo C: Effect of S-adenosyl-L-methionine (SAM), an allosteric activator of cystathionine-beta-synthase (CBS) on colorectal cancer cell proliferation and bioenergetics *in vitro*. Nitric Oxide *41*: 146-156, 2014. PMID: 24667534. DOI: 10.1016/j.niox.2014.03.001

26 Komatsu M, Kanda T, Urai H, Kurokochi A, Kitahama R, Shigaki S, Ono T, Yukioka H, Hasegawa K, Tokuyama H, Kawabe H, Wakino S and Itoh H: NNMT activation can contribute to the development of fatty liver disease by modulating the NAD (+) metabolism. Sci Rep *8*: 8637. 2018. PMID: 29872122. DOI: 10.1038/s41598-018-26882-8

27 Wang Y, Fu D, Chen Y, Su J, Wang Y, Li X, Zhai W, Niu Y, Yue D and Geng H: G3BP1 promotes tumor progression and metastasis through IL6/G3BP1/STAT3 signaling axis in renal cell carcinomas. Cell Death Dis *9*: 501, 2018. PMID: 29717134. DOI: 10.1038/s41419-018-0504-2

28 Ishibashi K, Haber T, Breuksch I, Gebhard S, Sugino T, Kubo H, Hata J, Koguchi T, Yabe M, Kataoka M, Ogawa S, Hiraki H, Yanagida T, Haga N, Thuroff JW, Prawitt D, Brenner W and Kojima Y: Overriding TKI resistance of renal cell carcinoma by combination therapy with IL6 receptor blockade. Oncotarget *8*: 55230-55245, 2017. PMID: 28903416. DOI: 10.18632/oncotarget.19420

29 Chen Y, Liu J, Lv P, Gao J, Wang M and Wang Y: IL-6 is involved in malignancy and doxorubicin sensitivity of renal carcinoma cells. Cell Adhesion Migration *12*: 28-36, 2018. PMID: 28328292. DOI: 10.1080/19336918.2017.1307482

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