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Original Research

Involvement of transforming growth factor beta 1 in the transcriptional regulation of nicotinamide N-methyltransferase in clear cell renal cell carcinoma

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This paper is dedicated to the memory of Elisabetta Renzi

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Abstract: Renal cell carcinoma (RCC) is the most common tumor of the kidney and its major histologic subtype is clear cell RCC (ccRCC). About 30% of diagnosed ccRCCs already have metastasis. Traditionally, localized ccRCC is treated with nephrectomy but the relapse rate is 30%. Thus, the discovery of effective biomarkers for early detection, as well as the identification of new targets for molecular-based therapy of ccRCC are urgently required. In this study, we focused on molecules that could modulate the trascription of the enzyme nicotinamide N-methyltransferase (NNMT) that is known to be up-regulated in ccRCC. Signal transducer and activator of transcription 3 (STAT3), interleukin 6 (IL-6), hepatocyte nuclear factor 1 beta (HNF-1β) and transforming growth factor beta 1 (TGF-β1) expression levels were determined in tumor and non tumor samples obtained from 30 patients with ccRCC, using Real-Time PCR. Results obtained showed that TGF-β1 is significantly ($p<0.05$) overexpressed in tumor compared with normal tissue samples of ccRCC patients. Conversely, we did not find any statistically significant difference concerning STAT3, IL-6, HNF-1β gene expression levels. TGF-β1 up-regulation could be responsible for the high levels of NNMT observed in ccRCC. Targeting TGF-β1 could improve the outcome of ccRCC patients due to its role in epithelial-mesenchymal transition (EMT), that is known to be associated with a worse overall survival (OS) in this neoplasm.

Key words: Renal cell carcinoma; NNMT; Biomarker; TGF-β1; Transcription factor.

Introduction

Renal cell carcinoma (RCC) is the most common malignancy of kidney, representing up to 3% of all global cancer cases and about 270,000 cases are expected to be diagnosed every year worldwide (1, 2).

Although the cause of RCC is unknown, multiple studies indicate that people have a twofold increased risk of RCC if there is a family history of RCC. However, the majority of RCC occurs sporadically and several risk factors seem to be related to the development of this neoplasm, such as cigarette smoking, obesity, hypertension and acquired cystic kidney disease (3).

Among the histologic subtypes, clear cell renal cell carcinoma (ccRCC) is the most common type with an incidence of about 75% of all RCC cases (4). Due to the fact that at initial stages RCC is often asymptomatic, there is a serious diagnostic delay, and approximately 30% patients with RCC have metastasis by the time it is discovered. Moreover, RCC is known to be resistant to traditional chemotherapy and radiation and this is one of the main reasons for its poor prognosis (5). The resistance to chemotherapy and radiation makes the metastatic RCC (mRCC) very difficult to be treated, although immunotherapy improved the outlook of patients affected with mRCC (6).

Thus, the investigation of the molecular mechanisms that are involved in carcinogenesis is essential to identify new specific markers suitable for early diagnosis and to make an accurate prognosis, as well as to identify new targets for molecular-based therapy.

Many efforts have been made to identify specific biomarkers for diagnosis or prognosis, but none of them is used in clinical practice (7-9).

In a previous work, our group explored the involvement of enzymes of drug metabolism in RCC. We found that the enzyme nicotinamide N-methyltransferase (NNMT, EC 2.1.1.1.) is upregulated in ccRCC and that its expression is significantly inversely correlated with tumor size (10).

NNMT is a Phase II drug-metabolizing enzyme that catalyzes the N-methylation of nicotinamide and other structurally related compounds as pyridines. The conjugation reaction catalyzed by this enzyme plays a critical role in the biotrasformation and detoxification of many drugs and xenobiotic compounds (11, 12).

Considering our previous results, we decided to investigate the transcriptional regulation of the NNMT in ccRCC. Therefore, we evaluated the gene expression level of signal transducer and activator of transcription 3 (STAT3), interleukin 6 (IL-6), hepatocyte nuclear factor 1 beta (HNF-1β) and transforming growth factor beta 1

(TGF-β1) by Real-Time PCR, in tumor and non tumor tissue samples obtained from 30 patients with ccRCC.

Materials and Methods

Patients

A total of 30 patients who underwent surgical treatment for ccRCC at our institution between November 2003 and June 2005 were included in this study. Patients included 17 men and 13 women, 33 to 88 years old (mean age 64 years). A total of 29 samples were obtained at radical nephrectomy and 1 was obtained at partial nephrectomy. Pathological stage and histological grading was assigned to the tumors according to the 2016 WHO/ISUP classification system (13). None of the patients had metastasis to regional lymph nodes or to a distant site at surgery. The study was carried out in accordance with the principles of the Declaration of Helsinki and met the ethical standards of the responsible regional committee on human experimentation. All patients provided informed consent. Table 1 lists characteristics of the 30 patients.

Surgical specimens

Fresh renal tissue was obtained during surgery. Normal and tumor renal tissue samples were collected, snap frozen in liquid nitrogen and stored at –80°C until use. All samples were collected according to the institutional committee guidelines.

Table 1. ccRCC patients and clinicopathologic findings

Cases	30
Mean age	64 (33-88)
Men-Women	17-13
Tumor size (cm):	
Mean	6.2
Range	2-11
pT classification:	
pT1	20
pT2	6
pT3	4
Histological grading	
G1	2
G2	16
G3	12

RNA extraction

An aliquot of frozen tissue (20 to 40 mg) was homogenized in lysis buffer. Total RNA was extracted using the SV Total RNA Isolation System (Promega). RNA samples were tested by ultraviolet absorption at 260nm to determine the concentration. The quality and concentration of RNA samples were further confirmed by electrophoresis on denaturated 1% agarose gel.

Real-Time Quantitative PCR

The total RNA was reverse transcribed using the First Strand cDNA Synthesis Kit II (Bio. Basic. Inc) and oligo$(dT)_{18}$ as primers.

To examine STAT3, IL-6, HNF-1β and TGF-β1 gene expression quantitatively, we performed Real-Time PCR assay using Rotor-Gene 6000 Real-time rotary analyzer (Qiagen). cDNA previously obtained was used for the Real-Time quantitative PCR. The oligonucleotide sequence of primers used for the Real-Time PCR are reported in Table 2.

For each sample the total volume of the reaction mix was 25µl including: 1µl cDNA, 2.5µl Buffer 10X, 3µl $MgCl_2$ 25mM, 0.5µl JumpStart Taq DNA Polymerase (Sigma-Aldrich), 2.5µl dNTP 2mM, 0.125µl SYBR Green 100X (Thermo Fisher Scientific) and the primers to a final concentration of 300nM.

For IL-6, HNF-1β and TGF-β1 genes, the thermocycler was programmed for 45 cycles of 94°C for 30s and 56°C for 30s. For STAT3 gene, the thermocycler was programmed for 45 cycles of 94°C for 15s and 60°C for 20s.

All samples were tested in triplicate using β-actin as the reference gene for data normalization and to correct for variations in RNA quality and quantity. Direct detection of PCR products was monitored by measuring the fluorescence produced by SYBR Green I 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 using the equation, $2^{-\Delta(\Delta Ct)}$ where ΔCt = Ct (Gene of interest) - Ct (β-actin) and Δ(ΔCt) = ΔCt (tumor tissue)-ΔCt (normal tissue).

Western blot

Protein extracts from renal tissues were prepared with lysis buffer (phosphate buffered saline containing

Table 2. Primers used for quantitative Real-Time PCR.

Target gene	Sequence
β-actin	forward 5'-CTCTTCCAGCCTTCCTTCCT-3'
	reverse 5'-TGTTGGCGTACAGGTCTTTG-3'
STAT3	forward 5'-GAGCTGGCTGACTGGAAGAG-3'
	reverse 5'-TGTTGACGGGTCTGAAGTTG-3'
IL-6	forward 5'-GGATTCAATGAGGAGACTTGC-3'
	reverse 5'-GCACAGCTCTGGCTTGTTC-3'
HFN-1β	forward 5'-AAATGATCTCAGTCTCAGGAGGA-3'
	reverse 5'-GGAGGTGTTGAGGCTTTGTG-3'
TGF-β1	forward 5'-GTACCTGAACCCGTGTTGCT-3'
	reverse 5'-GTATCGCCAGGAATTGTTGC-3'

1% Nonidet P40, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride and 2 µg/ml aprotinin). Aliquots of frozen tissue (20–40 mg) were suspended in 33 volumes of lysis buffer and homogenized on ice using Ultra-Turrax homogenizer (IKA) at medium speed. Tissue homogenates were then centrifuged at 16000xg for 10 min at 4°C and the supernatant represented the total protein extract. Samples containing 50 µg protein were subjected to 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After regular blockin band washing, the membranes were incubated with mouse monoclonal antibody against human TGF-β1 (Santa Cruz Biotechnology) (1:200 dilution) or with mouse monoclonal antibody against human β-actin (Sigma-Aldrich) (1:2000 dilution) for 2 hours, followed by incubation (1:1000 dilution) with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology) for 1 h. TGF-β1 protein was visualized using enhanced SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). The chemiluminescent signal of TGF-β1 protein detected in blots was acquired using ChemiDoc XRS+ System (Bio-Rad Laboratories).

Statistical analysis

Data were analyzed using GraphPad Prism software version 6.00 for Windows (GraphPad Prism Software). Differences in gene expression were determined using the Wilcoxon test. Correlation between variables was explored by the Spearman test. A p-value < 0.05 was considered as statistically significant.

Results

Real-Time quantitative PCR was used to evaluate STAT3, IL-6, HNF-1β and TGF-β1 expression in ccRCC in cohort of 30 patients. STAT3, IL-6, and HNF-1β did not show any significant alteration in gene expression level (tumor versus normal tissue). Interestingly, TGF-β1 was significantly up-regulated (4.2-fold increase, $p < 0.05$) in pathological tissue compared to control (Figure 1).

To confirm the above reported results, TGF-β1 expression was detected at protein level by Western blot analysis in a representative cohort of samples. Consistent with the results of Real-Time PCR, lanes loaded with equal protein amounts showed increased TGF-β1 expression in tumor samples compared to that in matched normal tissues (Figure 2).

To explore whether TGF-β1 expression was significantly correlated with that of NNMT, statistical analyses were performed in order to compare TGF-β1 mRNA levels, determined in this study, and NNMT mRNA expression, previously reported by Sartini et al. (10). Results obtained showed that there was a significant ($p<0.05$) positive correlation ($r=0.634$) between the expression levels of both genes.

Discussion

ccRCC is one frequent form of urologic malignancy and is the most lethal of the common urological cancers. Its diagnosis is often problematic due to the lack of symptoms in the early stages and is frequently diagnosed at the advanced stage only. The absence of biomarkers for early detection of ccRCC is a critical problem and complicates both early diagnosis and treatment. Thus, discovering efficient biomarkers for ccRCC would significantly improve early diagnosis and therapeutic effectiveness, and would lead to a decrease of number of mRCCs at time of diagnosis.

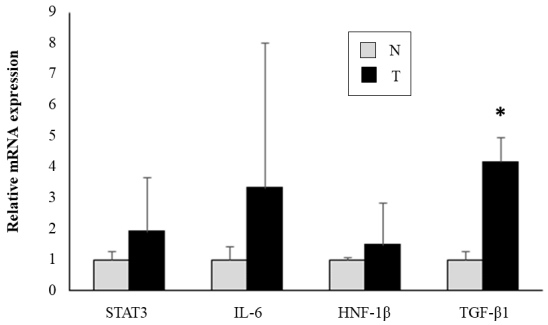

Figure 1. Real-Time PCR analysis of STAT3, IL-6, HNF-1β and TGF-β1. Expression levels were determined in tumor (T) and non tumor (N) samples obtained from 30 patients with ccRCC using Real-Time PCR, as described under Materials and Methods.

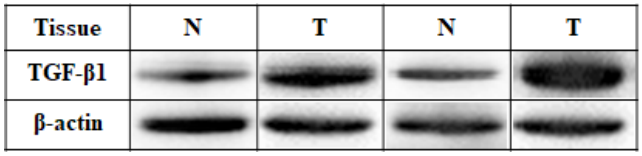

Figure 2. Western blot analysis of TGF-β1. Tumor (T) and matched adjacent healthy (N) tissue expression of TGF-β1 was evaluated at protein level by Western blot, as described under Materials and Methods.

In a previous work, our group demonstrated that NNMT is upregulated in ccRCC and that its expression significantly inversely correlated with tumor size (10).

To our knowledge, the mechanism of regulation of NNMT activity is still partially unknown. Thus, we decided to study the transcriptional regulation of the NNMT in the ccRCC.

In 2007, Tomida et al. investigated STAT3-regulated genes and found a correlation between activated STAT3 and the expression of NNMT in colon cancer tissues. Moreover, NNMT expression in Hep-G2 and SW480 cell lines increased after stimulating cells with IL-6 (14). STAT3 is a member of the STAT protein family. STAT family members are phosphorylated by the receptor associated kinases in response to cytokines and growth factors. In particular, STAT3 is activated by tyrosine phosphorylation in response to several cytokines and growth factors like IL-6, IFNs, EGF, IL-5, HGF, LIF and BMP2. Once activated, it dimerizes and translocates into the nucleus to regulate gene expression. It mediates the expression of a variety of genes in response to cell stimuli, and thus it plays a key role in many cellular processes such as cell growth and apoptosis (15-17).

Xu et al. cloned and studied NNMT promoter in papillary thyroid cancer cell lines, finding that it can be activated by hepatocyte nuclear factor-1β, although its influence on the NNMT promoter displays a variable efficacy (18). HNF-1β has been reported to function as a trascriptional factor with variable activity depending on the promoter and cell type but is highly expressed in the developing kidney (19).

Nabokikh et al. demonstrated that in human insulinomas there is a down-regulation of the TGF-β1 and its target genes, including NNMT (20).

Thus, in order to explore the transcriptional regulation of NNMT in ccRCC, we decided to investigate the role of these molecules evaluating their gene expression level by Real-Time PCR, in tumor and non tumor tissue samples obtained from 30 patients with ccRCC.

According to the results previously obtained by our group, having demonstrated that the enzyme is upregulated in ccRCC, in this study we expected to find an up-regulation of its transcriptional regulators. Surprisingly, STAT3, IL-6 and HNF-1β did not exhibit any significant difference in gene expression between tumor and non tumor samples. Interestingly, we found that TGF-β1 was significantly up-regulated (4.2-fold increase) in tumor than in normal tissue.

TGF-β1 is a widely expressed cytokine that requires, for its maturation, intracellular processing exerted by the proprotein convertase furin (21). The bioactive form of secreted TGF-β molecules is a dimer that binds type I and type II serine/threonine kinase receptors. Upon binding, the signal is able to propagate and amplify through a phosphorylation cascade, involving in the final step different transcription factors. As consequence of this combinatorial interaction, a TGF-β induced stimulus can lead to activation or repression of hundreds of target genes (22). The TGF-β signalling pathway plays a fundamental role in different cellular processes, since TGF-βs are able to switch from tumor suppressors in normal or dysplastic cell to tumor promoters in advanced cancers (23). Recent studies demonstrated that, in addition to a paracrine mechanism, TGF-β1 is also able to act in an autocrine manner (21), by binding to receptors and initiating a signal in the same cell secreting the cytokine. According to this, results reported in this work could demonstrate the existence of an autocrine regulation of renal cancer cell exerted by TGF-β1, whose signal could lead to increase the expression of several target genes including NNMT. In this light, TGF-β1 up-regulation, detected at both mRNA and protein level in ccRCC (tumor versus normal tissue), could be partially responsible for the high NNMT levels observed in the same cohort of tissue samples.

Moreover, TGF-β1 is known to be a powerful inducer of epithelial-mesenchymal transition (EMT) (24). In RCC, EMT is a central mechanism of invasiveness, metastasis and response to therapies. It was also demonstrated that EMT leads to an increased recurrence risk and worsening of overall survival (OS) in patients with RCC (25). Moreover, dissemination in mRCC is often triggered by EMT, modifying tumor cell polarity and cell-cell adhesion and enhancing tumor migration and invasion. In this light, it seems there is a correlation between EMT and the outcome of RCC patients (26, 27).

A recent study demonstrated that upregulation of NNMT in gastric cancer cells is not only associated with increased expression of TGF-β1 but also lead the cell to undergo epithelial-mesenchymal transition. In particular, upon NNMT overexpression BCG-823 gastric cancer cells acquired an elongated and spindle-shaped morphology. Moreover, enzyme upregulation significantly increased the expression of mesenchymal markers as well as the migratory and invasive abilities of BCG-823 cell line (28).

On the basis of obtained results, we are willing to think that TGF-β1 up-regulation could be involved in enhancing the EMT process in ccRCC, by enhancing the expression of NNMT. Thus, targeting TGF-β1, as an element that promotes EMT in ccRCC, could represent an effective strategy to improve the outcome of ccRCC patients.

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Conflict of interest
The authors declare that there is no conflict of interest.

Authors' contribution
ME conceived the study and was responsible for the experimental design. RC and MC performed most of the experiments and co-wrote the manuscript. DS co-wrote the manuscript and oversaw the results. ABG and GM participated in manuscript revision. VPoz performed statistical analyses. SF and VPom contributed to carry out Real-Time PCR analyses.

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