Down-regulation of MTUS1 in human colon tumors

CHRISTINA ZUERN1,2, JUTTA HEIMRICH1, ROLAND KAUFMANN2, KONRAD K. RICHTER2, UTZ SETTMACHER2, CHRISTOPH WANNER1, JAN GALLE1 and STEFAN SEIBOLD1

1Department of Medicine, Division of Nephrology, University Clinic of Wuerzburg, Wuerzburg; 2University Hospital, Department of General and Visceral Surgery, Friedrich Schiller University, Jena; 3Department of Nephrology and Hypertension, University of Erlangen-Nürnberg, Erlangen, Germany

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Abstract. Loss of proliferative control and failure to undergo cellular differentiation are key events during carcinogenesis. We recently identified a new potential tumor suppressor gene named MTUS1 (mitochondrial tumor suppressor 1), down-regulated in undifferentiated tumor cell lines, inhibiting tumor cell proliferation after recombinant over-expression. The aim of this study was to investigate whether MTUS1 is also down-regulated in human tumor tissues, and whether reduced expression of MTUS1 enhances cellular proliferation. Expression of MTUS1 in human colon cancer tissues was compared with corresponding normal colon tissues using Western blot analysis and RT-PCR. Investigation of the DNA sequence and methylation pattern was performed using bisulfite reaction and DNA sequencing. Promotor activity was measured by promoter assays. Silencing of MTUS1 was carried out by siRNA transfection. Proliferation was measured by cell count. MTUS1 expression is significantly down-regulated in colon cancer tissues compared to the corresponding normal tissues, on protein and mRNA level. No mutations of MTUS1 were detected in the coding sequence or the predicted promotor region in cancer tissues. No difference of CpG methylation, but an altered CpNpG methylation was found in the predicted promotor region. Functional significance of the predicted promotor region was demonstrated by promoter assays. Down-regulation of the MTUS1 expression by siRNA transfection significantly increased cellular proliferation. This study demonstrates a significant down-regulation of the MTUS1 expression in human colon cancer tissues. Since reduced expression of MTUS1 results in increased cellular proliferation, these data suggest that MTUS1 could be involved in the loss of proliferative control in human colon cancer.

Introduction

The multistage process of carcinogenesis includes the progressive acquisition of genetic alterations, resulting in activation of proto-oncogenes and inactivation of tumor suppressor genes. Consequential critical events in the evolution of tumors include the loss of proliferative control and the failure to undergo cellular differentiation. Genes located at chromosome 8p21.3-22 near marker D8S254 participate in tumor progression in colorectal cancer, pancreatic cancer, breast cancer, esophageal cancer, hepatocellular carcinoma, lung cancer, prostate cancer, urinary bladder carcinoma and head and neck squamous cell carcinoma (1-15). We previously identified a new gene named MTSG1, only <0.9 Mb apart from the D8S254 marker locus, regulating tumor cell proliferation (16). In accordance with the Guidelines for Human Gene Nomenclature it was recommended to change the gene name to MTUS1 (mitochondrial tumor suppressor 1), as it meanwhile appears in most databases. MTUS1 was discovered by investigating the molecular regulation during cellular transition from proliferation to senescence and differentiation in a three-dimensional collagen type I cell culture model. We thereby observed a transient up-regulation of MTUS1 during the initiation of a differentiated and senescent cellular phenotype in human endothelial cells. Additionally a reverse correlation of the MTUS1 mRNA expression with cellular proliferation and differentiation was noted in various pancreatic tumor cell lines, showing low expression in undifferentiated proliferating cell lines and high expression in differentiated and slowly proliferating cell lines (16). Further support for the hypothesis that MTUS1 might influence tumor progression by regulating tumor cell proliferation arose from studies in a pancreatic tumor cell line expressing no native MTUS1 mRNA, since recombinant over-expression of MTUS1 in this cell line resulted in significant inhibition of tumor cell proliferation (16). Previously, the inhibitory effect of MTUS1 on cell proliferation was linked to the growth inhibitory signaling cascade by trans-inactivation of receptor tyrosine kinases in insulin, bFGF, PDGF and EGF-induced ERK2 activation (17). A previously published investigation demonstrated a significant down-regulation of MTUS1 in ovarian carcinoma tissues, implicating a possible role of MTUS1 on the development of ovarian carcinoma (18). In addition, new studies revealed lower expression of MTUS1 in...
head and neck squamous cell carcinoma, down-regulation in colon polyps and copy number variant in breast cancer (19-21).

The gene responsible for tumor progression in colon cancer, located at chromosome 8p21.3-22 near marker D8S254, would be expected to be down-regulated in colon cancer (1,10). Therefore, the aim of the present study was firstly to confirm down-regulation of MTUS1 expression in colon cancer tissues, secondly to investigate the underlying genetic background responsible for the down-regulation of MTUS1, and thirdly to investigate whether down-regulation of MTUS1 results in increased cellular proliferation. This study supports the hypothesis that MTUS1 acts as a candidate tumor suppressor gene, responsible for tumor progression in human colon cancer.

Materials and methods

Tumor tissue acquisition. Colon tissue was obtained from ten unrelated patients admitted to the university hospital in Jena, Germany for the surgical removal of colon cancer. Small tissue parts from the tumor tissue itself, as well as from the surrounding normal non-tumor tissue, were removed for protein and total RNA isolation. The tissue sections were routinely investigated by the local pathologist. The local Ethics Committee approved the study, and all the patients gave their informed consent prior to surgery.

Generation of an MTUS1 specific polyclonal antibody. A polyclonal rabbit anti-MTUS1 antibody was established in collaboration with Eurogentec (Seraing, Belgium). Therefore, the peptide H2N-CPRNSGSPFPSisp-COOH (amino acids 422-436 of the MTUS1 protein sequence) was coupled to hemocyanin. Rabbits were immunized with the modified peptide on days 0, 14, 28 and 56, and the final bleed was carried out at day 80 of the immunization procedure. Subsequently the rabbit serum was affinity purified with the antigen peptide coupled to an AF-Amino Toyopearl 650 M matrix (Tosoh Bioscience, Stuttgart, Germany).

Recombinant expression of MTUS1. Primers 5'-cgccgggtaccc gtcttctgtaaatctgcg-3' and 5'-cgccggtacccgtttctgtaaatctggaactgggtgtagctg-3' were used for RT-PCR. The PCR product was cloned into the pcDNA3 vector (Invitrogen, Karlsruhe, Germany) using the introduced KpnI and BamHI restriction sites, and the correct sequence was confirmed by sequence analysis. MiaPaCa-2 cells (ATCC, USA) (10^5 cells/well) were transfected with 2 μg plasmid DNA per 6-well using Lipofectamine Plus transfection reagent (Invitrogen) as recommended by the manufacturer. pcDNA3 vector alone was used as a control. Cells were harvested for protein isolation 24 h after transfection.

Western blot analysis. Proteins were isolated from tumor tissue, as well as from the corresponding normal colon tissue from the same patients. Therefore, tissues were homogenized and directly lysed in 50 mM HEPES pH 7.5, 50 mM NaCl, 20 mM EDTA, 1 mM MgCl2, 2% Triton X-100, and protease inhibitor (complete mini, Roche Diagnostics, Mannheim, Germany). Proteins were isolated under the same conditions from cells, transfected with either pcDNA3 vector or siRNA. Subsequently, protein concentration was determined with the Lowry reaction. Ten micrograms of protein were loaded per lane, transferred to a PVDF membrane (Amersham, Freiburg, Germany) and blocked with 5% non-fat dried milk. Primary antibodies were polyclonal rabbit anti-MTUS1 (described above) diluted 1:2,000 in TBS-T containing 5% non-fat dried milk, monoclonal mouse anti-PCNA (Transduction Laboratories, Lexington, KY, USA) diluted 1:2,000 in TBS-T containing 5% non-fat dried milk, polyclonal goat anti-Ki-67 (Santa Cruz, Santa Cruz, USA) diluted 1:500 in TBS-T containing 5% non-fat dried milk, and monoclonal mouse anti-β-actin (Sigma, Taufkirchen, Germany) diluted 1:20,000 in TBS-T containing 5% non-fat dried milk. Secondary antibodies were HRP-conjugated goat anti-rabbit (Dako, Hamburg, Germany), goat anti-mouse (Dako), and mouse anti-goat (Sigma), all diluted 1:2,000. For detection ECL Plus (Amersham) was used.

Tumor tissue mRNA expression. Total RNA isolation from tumor and corresponding normal surrounding tissue was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer. First strand cDNA synthesis was performed at 37°C for 1 h applying 1 μg of total RNA using oligo-dT primers. Two microliters of the cDNA reaction were subsequently applied in a multiplex PCR with 5'-gctcttctgtaaatctgcg-3' and 5'-cgccggtacccgtttctgtaaatctggaactgggtgtagctg-3' as MTUS1 specific primers and 5'-tataaacttgagcatggggtgg-3' and 5'-gagcatggggtgg-3' as β-actin specific primers (35 cycles, 57°C annealing temperature). Multiplex RT-PCR was established testing various cycles to ensure absence of a plateau phase (data not shown). PCR products were separated on 2% agarose gels, and the fluorescence intensity of the appropriate bands was detected and evaluated with the Quantity One quantization software (Bio-Rad, Munich, Germany).

Sequence analysis of the colon tumor tissue. Cytoplasmatic RNA of two colon tumor tissues was isolated and cDNA synthesis was performed as described above. For sequence analysis 1 μl cDNA was amplified using 5'-gagcgcttcaagtggtcagcggagagga-3' and 5'-ggagaaatacctgaaccgctattc-3' as MTUS1-specific primers (66.5°C annealing temperature, 38 cycles), generating a DNA fragment containing the sequence of the 10 exons of MTUS1 (16). These PCR products were separated on a 1% agarose gel, bands were subsequently isolated using the Gel extraction kit (Qiagen) and the DNA was repeatedly sequenced (MWG, Ebersberg, Germany) with the reverse primer 5'-gagcgcttcaagtggtcagcggagagga-3' and the following forward primers: 5'-ggagctttgcagaagttc-3', 5'-gatccctttgcagaagttc-3', 5'-gatccctttgcagaagttc-3', 5'-gatccctttgcagaagttc-3', 5'-gatccctttgcagaagttc-3', 5'-gatccctttgcagaagttc-3', 5'-gatccctttgcagaagttc-3', 5'-gatccctttgcagaagttc-3'.

For the analysis of the predicted promoter region the MTUS1-specific primers 5'-ccttggagaggaagttc-3' and 5'-gatccctttgcagaagttc-3' were amplified with genomic DNA (60.5°C annealing temperature, 38 cycles). The PCR products were separated on a 2% agarose gel, bands were isolated, and the DNA was sequenced (MWG Biotech).

Sequence analysis of the MiaPaCa-2 cells. DNA of MiaPaCa-2 cells was isolated using the DNA Tissue Kit (Qiagen). Respec-
tively, exon sequences were amplified with 200 ng of DNA using MTUS1-specific primers (60°C annealing temperature, 36 cycles). For exon 1 forward primer 5'-ctcgtagacgctacctctctct-3' and reverse primer 5'-catctcggggtgctacgagtcctc-3' were used, for exon 2 the primers 5'-tacaacatagctacctctctct-3' and 5'-cagcttgggagcgttggtggtcag-3', for exon 3 the primers 5'-ctcgtagacgctacctctctct-3' and 5'-atgctcctggggtgctacgagtcctc-3', for exon 4 the primers 5'-gcttcaggtgtcatcactagcc-3' and 5'-aatgggtgctgctgagtact-3', for exon 5 the primers 5'-ctcgtagacgctacctctctct-3' and 5'-agctggctggtcagcaggtcagacctctctct-3' were used. For the predicted promoter region the specific primers 5'-gtttatgttaagt-3' were used (60.5°C annealing temperature, 38 cycles). The PCR products were separated on 2% agarose gels, bands atgcgc-3' were used (60.5°C annealing temperature, 38 cycles). For the predicted promoter region the specific primers 5'-cctgtgaccttgtgcaggat-3' and 5'-tactgtgctgtcacagggct-3' were used, for exon 2 the primers 5'-tccaaccatctagatctccg-3' and 5'-tcggcttcatcatcaacccct-3', for exon 3 forward primer 5'-cctgagaccacccactt-3' (including an XhoI restriction site) and reverse primer 5'-catcccccgtgcaacactaa 3' were used, for exon 4 the primers 5'-gcttcaggtgtcatcactagcc-3' and 5'-aatgggtgctgctgagtact-3', for exon 5 the primers 5'-ctcgtagacgctacctctctct-3' and 5'-taaagtaggtggattataaggttaggatattg-3' were used. For both constructs 5'-gga-3' were used as a reverse primer. Both fragments were cloned into the XhoI and HindIII restriction sites into the pG3s basic vector (Promega, Mannheim, Germany) and the correct construct insertion was confirmed by sequence analysis.

MiaPaCa-2 cells were grown at 37°C, 5% CO2, in DMEM supplemented with 10% fetal bovine serum (FBS) and 2 mM glutamine. For the reporter assay 1x10^4 cells were seeded per well on a 96-well plate and the cells were transfected with 0.5 μg of the different plasmid DNAs using GenePORTER 2™ (Gene Therapy Systems) according to the manufacturer's recommendations. Four hours after transfection 1 ml of growth medium was added. Twenty-four hours after transfection cells were washed with PBS, and harvested for protein isolation. Luciferase siRNA (MWG) transfected cells were used as a control.

Proliferation assay. HUVEC (1x10^5 per 6-well) were transfected with 25 nM MTUS1 siRNA, using GenePORTER 2 (2.5 μl/ml medium) according to the manufacturer's recommendations. Four hours after transfection 1 ml of growth medium was added. Forty-eight hours after transfection, cells were harvested and counted using a Coulter Z2 (Beckman Coulter, Krefeld, Germany). Luciferase siRNA (MWG) transfected cells were used as a control.

Statistical analyses. Data are presented as mean ± SEM and analyzed by ANOVA or Student's t-test. A P<0.05 was considered significant.

Results

Characterization of the MTUS1 antibody. After affinity purification of the MTUS1 antibody, the final quality control showed >85% purity in SDS-PAGE and a high specificity in ELISA (data not shown). Western blot analysis confirmed the sensitivity and specificity of the MTUS1 antibody, demonstrating detection of the MTUS1 protein (50 kDa) (Fig. 1), and inhibition of the MTUS1 signal by addition of the blocking peptide (data not shown).
MTUS protein expression is down-regulated in human colon cancer. We compared the MTUS1 protein expression in human colon cancer tissue with the MTUS1 protein expression in the corresponding normal surrounding colon tissue in each patient using Western blot analysis. We observed a >25% down-regulation of the MTUS1 protein expression in the tumor tissue as compared to the corresponding normal surrounding tissue in 5 out of 10 patients (Fig. 2). The mean MTUS1 protein expression in the tumor tissues of these 5 patients was 44.8% (range 25.2-63.6%) compared to normal colon tissue. The tumor samples of the other 5 patients investigated showed no significant down-regulation of the MTUS1 protein expression level (mean expression 92.8%; range 82.1-107.4%), compared to the normal surrounding colon tissue (data not shown). The absolute expression levels of the MTUS1 protein varied clearly between different patients.

The observed expression of the proliferation marker PCNA confirms a higher proliferative index in the tumor tissue compared to the corresponding normal tissue. Fig. 2A reveals a negative correlation between the MTUS1 and PCNA protein expression. The higher Ki-67 protein expression in each tumor tissue compared to the corresponding normal tissue of the same patient confirms the correct assignment of the tissues to the ‘tumor’ and ‘normal’ category and presents a prognostic marker for overall and disease-free survival in colon cancer (23). The β-actin protein expression was determined in order to adjust the MTUS1 protein expression to the amount of total protein loaded per lane (Fig. 2).

MTUS mRNA expression is down-regulated in human colon cancer. We next investigated whether significant down-regulation of the MTUS1 protein expression results from reduced MTUS1 mRNA expression, by comparing the MTUS1 mRNA expression in the colon cancer tissue with the respective normal surrounding tissue. We established a multiplex RT-PCR analysis to adequately adjust the MTUS1 mRNA expression to the simultaneously obtained β-actin mRNA expression (Fig. 3A). In all patients with significant down-regulation of the MTUS1 protein expression, we observed a significant down-regulation of the MTUS1 mRNA expression in the colon cancer tissues as compared to their corresponding normal colon tissue (Fig. 3B). The mean MTUS1 mRNA expression in the tumor tissues was 46.4% (range 36.7-53.3%) compared to the normal colon tissue.

MTUS1 down-regulation is not a consequence of mutations. To analyze the reasons for the down-regulation of MTUS1 in colon tumor tissue and different pancreatic tumor cell lines (16), sequence analysis was carried out on tumor tissues and the MiaPaCa-2 cell line. The exon sequencing of the MiaPac-2 cells revealed no relevant mutation in the coding sequence of MTUS1 and in the predicted promoter sequence. Also colon tumor tissues displayed no mutations in the coding sequence of MTUS1 or in the sequence of the predicted promoter region. Therefore, mutations in the exon sequence or the predicted promoter region are unlikely the cause for the low MTUS1 expression in MiaPaCa-2 cells and colon tumor tissue.
The predicted promoter sequence regulates transcription of MTUS1. To substantiate that the analyzed promoter region executes transcriptional regulatory properties, we performed a promoter assay to test the promoter activity of two different constructs, including or excluding the predicted promoter area. The MiaPaCa-2 cells transfected with the promoter construct thereby revealed a significantly (P=0.018) higher luminescence (RLU 0.0166±0.0011) as compared to the control transfected cells (0.0109±0.0015) (Fig. 4). This investigation therefore approved the hypothesis that the examined promoter sequence displays regulatory properties for MTUS1 transcription.

Variable DNA methylation of the predicted promoter sequence. We next investigated whether methylation of the MTUS1 promoter sequence could cause the down-regulation of the MTUS1 expression in tumor cells. Therefore bisulfite reactions were performed with DNA of MiaPaCa-2 cells, of HUVEC cells and of four colon tumors and corresponding normal tissues. The analysis regarding the methylation pattern of the CpG and CpNpG islands did not reveal differences in the methylation of the CpG islands, but a clear hypomethylation of the CpNpG islands of the MiaPaCa-2 cells (100%) in contrast to the HUVEC cells (82.1%). In the colon tissue, the methylation pattern of the CpG islands also showed no differences between tumor and normal tissue. But as already seen in the MiaPaCa-2 cells, hypomethylation of the CpNpG islands was observed in the tumor tissues (mean of unmethylated cytosines 94.7%, range 92.9-100%) compared to the corresponding normal tissue (mean 67%, range 28.6-89.3%) (Fig. 5).

MTUS1 down-regulation results in increased cellular proliferation. In order to provide further evidence that MTUS1 plays an active role and is not simply a bystander of tumor development, we investigated the impact of MTUS1 down-regulation on cellular proliferation using MTUS1 silencing by transfection of specific siRNA. Transfection efficacy of the cells was 85%, as measured by FACS analysis using fluorescence-labeled siRNA (data not shown). Compared to luciferase transfected controls, cells transfected with 25 nM of MTUS1 siRNA showed a 58±4% reduction in MTUS1 protein expression (Fig. 6). Similar degrees of MTUS1 protein down-regulation were obtained using 10 and 50 nM of MTUS1 siRNA respectively (Fig. 6). Transfection of cells with 25 nM of MTUS1 siRNA resulted in a significantly increased total cell number (438,000±22,000) after 48-h incubation with growth medium compared to luciferase transfected controls (388,000±16,000) (Fig. 7). Thus, the relative difference of cells between both groups was 17% (P=0.02).

Discussion

In this study we discovered a significant down-regulation of MTUS1 in patients suffering from colon cancer, and an increased cellular proliferation resulting from reduced MTUS1 expression. Thus, taking these results into consideration...
Together with previously published data, there are now several lines of evidence supporting the hypothesis that MTUS1 is involved in the regulation of tumor progression in various malignant diseases including human colon carcinoma: 1), MTUS1 is located at chromosome 8p21.3-22 near marker D8S254, exactly where the presence of genes involved in tumor progression was recorded. In colon cancer, genes at this chromosomal localization are thought to be responsible for progression of the malignant disease in 44% of patients (1), concordant with the observed down-regulation of MTUS1 in 50% of the investigated patients. Furthermore, this chromosomal region is also associated with disease progression in breast cancer, esophageal cancer, hepatocellular carcinoma, pancreatic cancer, lung cancer, prostate cancer, urinary bladder carcinoma, and head and neck squamous cell carcinoma (2-9). 2), monochromosome 8 transfer into colorectal cancer cell lines reduced their tumorigenicity and the metastatic potential in prostate tumor cells, confirming tumor suppressor activity of chromosome 8 in malignant diseases (24,25). 3), we observed an up-regulation of MTUS1 during the initiation of a differentiated and quiescent cellular phenotype, and we observed a reverse correlation of the MTUS1 expression with cellular proliferation and differentiation in different tumor cell lines (16), 4), and of particular importance, the tumor suppressor potential of MTUS1 was confirmed by over-expression of MTUS1 in a tumor cell line expressing no native MTUS1 mRNA, resulting in significantly reduced tumor cell proliferation (16). 5), the study presented here further supports the hypothesis that MTUS1 is involved in tumor progression in human colon cancer, since we observed a significant down-regulation of the MTUS1 expression in 50% of patients suffering from colon carcinomas, as expected for the responsible gene at 8p21.3-22 (16). 6), in this study we showed that down-regulation of MTUS1 resulted in increased cellular proliferation.

Taken together, gain of function experiments, as well as loss of function experiments clearly support the hypothesis that MTUS1 regulates cellular proliferation. The exact mechanism by which MTUS1 controls cellular proliferation is currently unknown, but MTUS1 is believed to be an early component of a growth inhibitory signaling pathway. It was shown that MTUS1 inhibits ERK2 activation and proliferation in eukaryotic cells stimulated with growth factors such as insulin, bFGF, PDGF and EGF, and it was suspected that MTUS1 inhibits cell proliferation via trans-inactivation of receptor tyrosine kinases (RTK) (17). Since members of the RTK signaling pathway such as EGFR, FGFR, PDGFB, RAS, RAF are well known to be involved in carcinogenesis, it is also well conceivable that MTUS1 is involved in tumor progression (26).

We herein confirmed the expected down-regulation of the MTUS1 expression in human colon cancer tissue. The mechanisms responsible for down-regulation of MTUS1 in human colon cancer and as previously reported in pancreatic tumor cell lines (16) seem not to be due to mutations in the coding region or the predicted promoter region. Our results of the sequencing analysis are also confirmed by a published investigation of 109 HCC tumors and cell lines, where MTUS1 was not frequently mutated (27). However, the predicted promoter range was proved to have regulatory effects on the transcription and exhibited variable methylation patterns of the tumor tissue and cells compared to the normal tissue and cells. The CpNpG islands showed a hypomethylation in the MiaPaCa-2 cells and the tissues with down-regulated MTUS1 expression, while the CpG islands did not reveal any differences in methylation. Since it already has been reported that CpNpG methylation can occur in vivo and that methylation of these islands is suspected to stabilize the DNA (28), the hypomethylation of CpNpG islands could be a mechanism for the MTUS1 inactivation. Nevertheless, some published investigations have found deletion and some small mutations in the exon region of MTUS1, so that further analyses are needed to understand the mechanism of down-regulation in this gene (19,21).

The histological type, grading and pTNM classification of the colon carcinomas from patients with >25% down-regulation of MTUS1 are shown in Table 1.

Table 1. Histological type, grading and pTNM classification of the colon carcinomas from patients with >25% down-regulation of MTUS1.

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Tumor classification</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Adenocarcinoma G1 pT2N0M0</td>
</tr>
<tr>
<td>2</td>
<td>Adenocarcinoma G3 pT3N2M1</td>
</tr>
<tr>
<td>3</td>
<td>Adenocarcinoma G3 pT3N0M1</td>
</tr>
<tr>
<td>4</td>
<td>Adenocarcinoma G1 pT1N0M0</td>
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<tr>
<td>5</td>
<td>Adenocarcinoma G1 pT1N0M0</td>
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