SPANDIDOS PUBLICATIONS

Reduced expression of TANGO in colon and hepatocellular carcinomas

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Received April 2, 2007; Accepted May 25, 2007

Abstract. The TANGO gene was originally identified as a new family member of the MIA gene family. The gene codes for a 14-kDa protein of so far unknown function. Recently, we identified TANGO as a tumor suppressor in malignant melanoma. In this study we evaluated TANGO transcription in different colon and hepatocellular carcinoma cell lines and tissue samples, to analyze whether loss of TANGO expression is a more general process in tumor development. TANGO was down-regulated or lost in all hepatocellular and colon cell lines compared to primary human hepatocytes or normal colon epithelial cells, respectively, and in most of the tumor samples compared to non-tumorous tissue. These results were confirmed *in situ* by immunohistochemistry on paraffin-embedded sections of colon and hepatocellular tumors. Functional assays with exogenous TANGO treatment of colon and hepatoma cell lines revealed reduced motility and invasion capacity. Our studies present for the first time the down-regulation of TANGO in colon and hepatocellular carcinoma and provide the first indications for a tumor suppressor role of the TANGO gene in human colon and hepatocellular carcinoma. Thus, functional relevant loss of TANGO expression may contribute to general tumor development and progression, and may provide a new target for therapeutic strategies.

Introduction

Hepatocellular carcinoma (HCC) is one of the most frequent primary malignant tumors in the world (1). In recent years the incidence of HCC has risen in cirrhotic patients worldwide (2). Colon cancer is still one of the leading causes of cancer death worldwide (3). Therefore, it is very important to understand the molecular mechanisms leading to tumor development and progression. This understanding will lead to the development of new, effective therapeutic options and tumor markers.

A novel human gene, TANGO, encoding an MIA (melanoma inhibitory activity) homologous protein was identified by a gene bank search (4). TANGO, together with the homologous genes MIA, OTOR (FPD, MIAL) and MIA 2, defines a novel gene family (5). The protein MIA, which is strongly expressed in melanoma cells but not in melanocytes, is likely to represent a key molecule regulating melanoma progression (5,6). In 2000 OTOR [other designations: fibrocyte-derived protein (FDP), MIA-like (MIAL)], was cloned and shown to be a close homologue of MIA (7-9). Cohen-Salmon *et al* (7) identified OTOR via subtractive cDNA screening in a search for genes which are referentially expressed in the inner ear. Recently, MIA2 (Melanoma inhibitory activity-2) was identified and found to be specifically expressed in hepatocytes (10,11). All proteins of the MIA family have hydrophobic N-terminal regions with features characteristic for eukaryotic signal peptides. All four cysteine residues that are known to be essential for stabilizing the three-dimensional structure are conserved. In addition, all structural motifs important for the SH3 domain-like folding are highly conserved (5,12,13). TANGO encodes a mature protein of 103 amino acids. Interestingly, *in situ* hybridization, RT-PCR and Northern blotting revealed an almost ubiquitous TANGO expression in contrast to the highly restricted expression patterns determined for the other members of the MIA gene family. High levels of TANGO expression were observed both during embryogenesis and in adult tissues (4).

The present study was performed to evaluate the expression pattern of TANGO in colon and hepatoma carcinoma starting from our recently published study on TANGO in malignant melanoma, in which we present that TANGO is a tumor suppressor and could be used as a prognostic marker of progression in melanoma patients (14). Therefore, we screened the expression profiles of TANGO in three hepatoma cell lines (HepG2, HepB3 and PLC) and in six colon carcinoma cell lines (HT29, LoVo, CaCo-2, HCT116, SW480 and SW48), in ten different liver tumor samples *in situ* and in different colon and hepatoma tumor immunohistological sections. In addition, functional assays with TANGO-treated cells were performed to characterize the biological effects.

Materials and methods

Cell culture. The colon carcinoma cell lines CaCo-2 (ATCC HTB-37), HT29 (ATCC HTB-38), SW48 (ATCC CCL-231),

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Abbreviations: CEC, colon epithelial cells; PHH, primary human hepatocytes

Key words: TANGO, tumor-suppressor gene, migration, invasion, hepatocellular carcinoma, colon carcinoma, cancer

SW480 (ATCC CCL-228), HCT116 (ATCC CCL-247) and LoVo (ATCC CCL-229), the hepatoma cell line HepG2 (ATCC HB-8065), and the HCC cell lines PLC (ATCC CRL-8024) and Hep3B (ATCC HB-8064) were used. Cells were maintained in DMEM supplemented with penicillin (400 U/ml), streptomycin (50 μg/ml), L-glutamine (300 μg/ml) and 10% fetal calf serum (FCS; Sigma, Deisenhofen, Germany) and passaged at a 1:5 ratio every three days as described elsewhere (15,16). Primary normal human colonic epithelial cells (CEC) and primary human hepatocytes (PHH) were isolated and cultured as previously described (17,18).

RNA isolation and reverse transcription. Total cellular RNA was isolated from cultured cells or from microdissected tissues using the RNeasy kit (Qiagen, Hilden, Germany) and cDNAs were generated by reverse transcriptase reaction performed in 20 μl reaction volume containing 2 μg of total cellular RNA, 4 μl of 5X first-strand buffer (Invitrogen, Groningen, The Netherlands), 2 μl of 0.1 M DTT, 1 μl of dN_6-primer (10 mM), 1 μl of dNTPs (10 mM) and DEPC water (19). The reaction mixture was incubated for 10 min at 70˚C, 200 units of Superscript II reverse transcriptase (Invitrogen) were added and RNAs were transcribed for 1 h at 37˚C. Reverse transcriptase was inactivated at 70˚C for 10 min and the RNA was degraded by digestion with 1 μl RNase A (10 mg/ml) at 37˚C for 30 min.

Tumorous human tissues. HCC tissue and non-neoplastic liver tissue of the same patient were obtained from 10 HCC patients. Tissue samples were immediately snap frozen and stored at -80˚C. Informed consent was obtained from all patients and the study was approved by the local Ethics Committee. RNA isolation and cDNA generation were performed as described previously.

Analysis of expression by quantitative PCR. Quantitative real-time PCR for TANGO was performed on a Lightcycler (Roche, Mannheim, Germany). cDNA template (2 μl), 2.4 μl 25 mM $MgCl_2$, 0.5 μl (20 mM) of forward and reverse primers (hTANGO for: 5'-ggctcttgaagatttcac-3'; hTANGO rev: 5'-atccgtctcatctgttgg-3') and 2 μl of SYBR-Green LightCycler Fast-Start DNA Master SYBR-Green Mix in a total of 20 μl were applied to the following PCR program: 10 min at 95˚C (initial denaturation); 20˚C/sec temperature transition rate up to 95˚C for 15 sec, 3 sec at 60˚C, 5 sec at 72˚C, 81˚C acquisition mode single, repeated 40 times (amplification). The PCR reaction was evaluated by melting curve analysis and checking the PCR products on 2% agarose gels. ß-actin was amplified to ensure cDNA integrity and to normalize expression. Each real-time PCR was repeated at least three times.

Western blot analysis. Approximately seven million trypsinized cells were incubated in 200 μl RIPA-buffer (Roche) for 15 min at 4˚C. Insoluble fragments were removed by centrifugation at 13000 rpm for 10 min and the supernatant lysate was stored at -20˚C. RIPA-cell lysate (40 μg) was loaded and separated on 12% SDS-PAGE and subsequently blotted onto a PVDF membrane. After blocking for 1 h with 3% BSA/PBS the membrane was incubated for 16 h with a primary antibody against TANGO (generated by BioGenes GmbH, Berlin, 1:1000) or ß-actin (Sigma, MO, USA, 1:2500). Then the membrane was washed three times in PBS, incubated for 1 h with an alkaline phosphatase-conjugated secondary antibody (Chemicon, CA, USA, 1:5000) and then washed again. Finally immunoreactions were visualized by NBT/BCIP (Zytomed, San Francisco, CA, USA) staining. All Western blots were repeated at least three times.

Immunohistochemistry. Paraffin-embedded preparations of tissues from patients with colon carcinomas (n=10) and hepatocellular carcinomas (n=10) were screened for TANGO protein expression by immunohistochemistry. Normal colon and liver tissue (each n=5) served as a control. The tissues were deparaffinated, rehydrated and subsequently incubated with anti-TANGO antibody (BioGenes GmbH, Berlin, 1:1000) overnight at 4˚C. The secondary antibody (biotin-labelled anti-rabbit, anti-mouse; Dako, Germany) was incubated for 30 min at room temperature, followed by incubation with streptavidin-POD (Dako) for 30 min. Antibody binding was visualized using AEC-solution (Dako). Finally, the tissues were counterstained by haemalaun solution (Dako). The evaluation of the staining was performed semi-quantitatively by light-microscopy. The method was described elsewhere (14).

Expression of recombinant biotinylated TANGO protein. A TANGO prokaryotic expression vector with a 15-amino acid Avi-tag peptide sequence including an FXa cleavage site was constructed by overlap extension PCR. The TANGO full-length cDNA construct was cloned into the vector pIVEX2.3-MCS (Roche, Mannheim, Germany). The expression vector was used in the Rapid Translation System, a cell-free *E. coli*-based protein transcription/translation system (Roche). By adding biotin, ATP and the *E. coli* biotin protein ligase BirA during the procedure, the protein was biotinylated at the introduced Avi-tag at the N-terminus and called recombinant biotinylated TANGO.

Migration and invasion assay. Migration and invasion assays were performed using Boyden Chambers containing polycarbonate filters with 8 μm pore size (Costar, Bodenheim, Germany), essentially as described previously (14). Filters were coated with gelatine or matrigel (diluted 1:3 in H_2O; Becton Dickinson, Heidelberg, Germany), respectively. The lower compartment was filled with fibroblast-conditioned medium, used as a chemo-attract and recombinant biotinylated TANGO protein. SW480, SW48, CaCo-2 colon tumor cells and HepG2, Hep3B hepatoma tumor cells were harvested by trypsinization for 2 min, resuspended in DMEM without FCS at a density of $2x10^4$ cells/ml (migration) or $3x10^5$ cells/ml (invasion) and placed in the upper compartment of the chamber. After incubation at 37˚C for 4 h, the filters were collected and the cells adhering to the lower surface were fixed, stained and counted. All assays were repeated at least three times and results were calculated in percent.

Statistical analysis. Results are expressed as mean ± SD (range) or percent. Comparison between groups was made using the Student's paired t-test. A p-value <0.05 was considered statistically significant. All calculations were performed using the GraphPad Prism 4 software (GraphPad software Inc., San Diego, CA, USA).

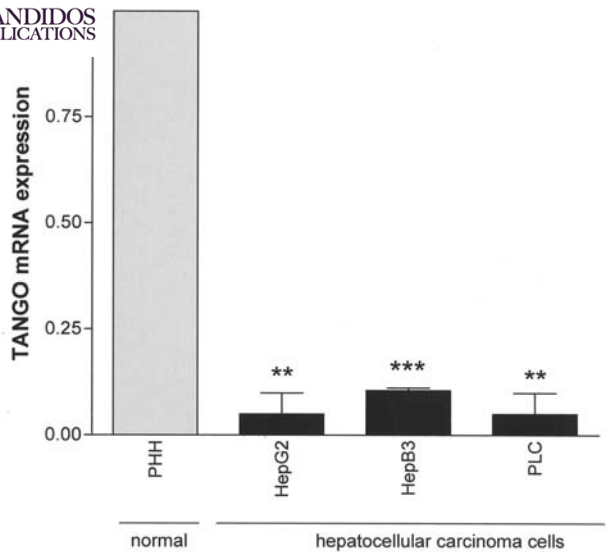


Figure 1. TANGO mRNA expression in hepatoma cell lines. By RT-PCR the amount of TANGO mRNA expression was quantified. All hepatoma cell lines (HepG2, HepB3 and PLC) showed a strong reduction in TANGO expression compared to primary human hepatocytes (PHH). $^{**}p<0.01$, $^{***}p<0.001$.

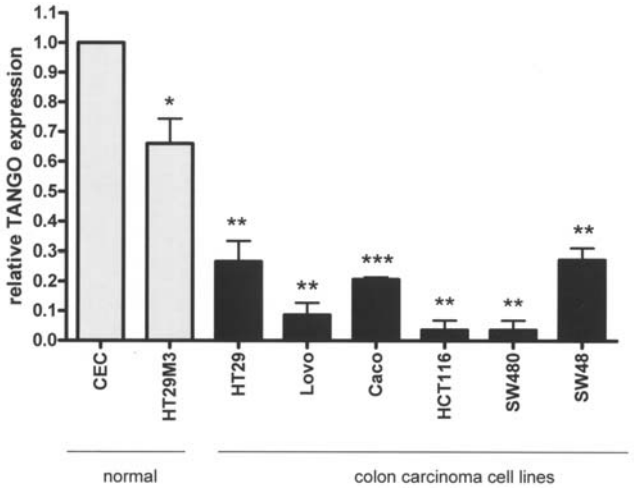


Figure 2. TANGO mRNA expression in colon carcinoma cell lines. By RT-PCR the amount of TANGO mRNA expression was quantified. All colon carcinoma cell lines (HT29, LoVo, CaCo-2, HCT116, SW480 and SW48) showed a strong reduction in TANGO expression compared to normal colon epithelial cells (CEC) and the modified HT29M3 cell line. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

Results

Reduction of TANGO transcription during tumor development. Three hepatocellular cell lines (HepG2, Hep3B and PLC) (Fig. 1) and six colon tumor cell lines (HT29, LoVo, CaCo-2, HCT116, SW480 and SW48) (Fig. 2) were evaluated for expression of TANGO using quantitative RT-PCR and compared to primary human hepatocytes (PHH) and to normal colon epithelial cells (CEC), respectively. A significant reduction or even loss of TANGO mRNA expression was found in all hepatocellular tumor cell lines compared to PHH and in all colon tumor cell lines compared to normal CECs.

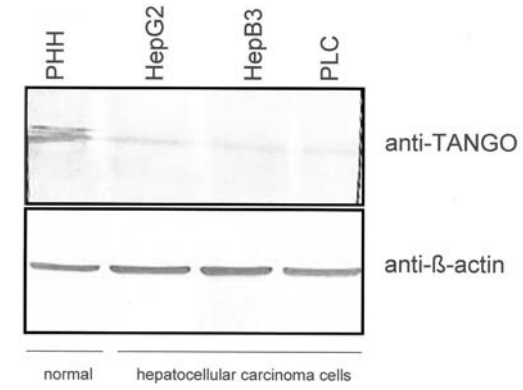


Figure 3. TANGO protein expression in hepatoma cell lines. By Western blot analysis the expression of TANGO was quantified. The reduction of TANGO in all hepatoma cell lines was confirmed on protein level compared to PHH. ß-actin was used for loading control.

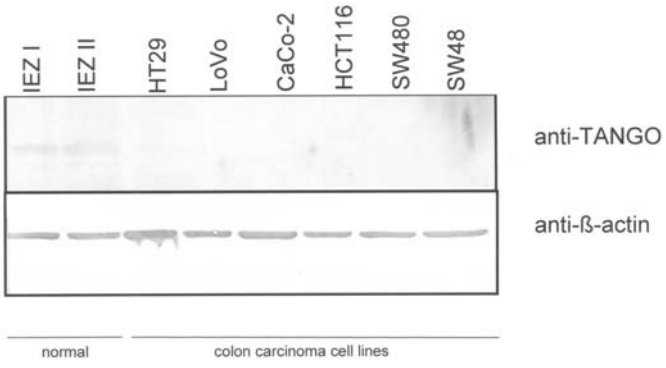


Figure 4. TANGO protein expression in colon carcinoma cell lines. By Western blot analysis the expression of TANGO was quantified. The reduction of TANGO in all colon carcinoma cell lines was confirmed on protein level compared to normal intestinal epithelial cells (IEZ) and HT29M3, a modified HT29 cell line. ß-actin was used for loading control.

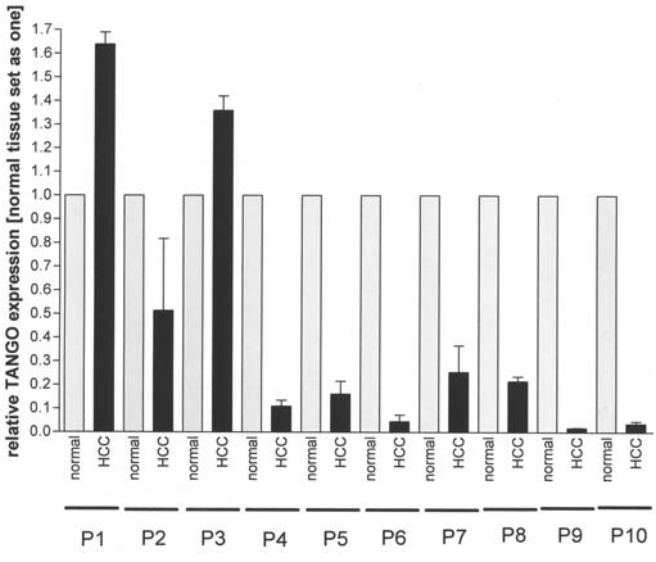
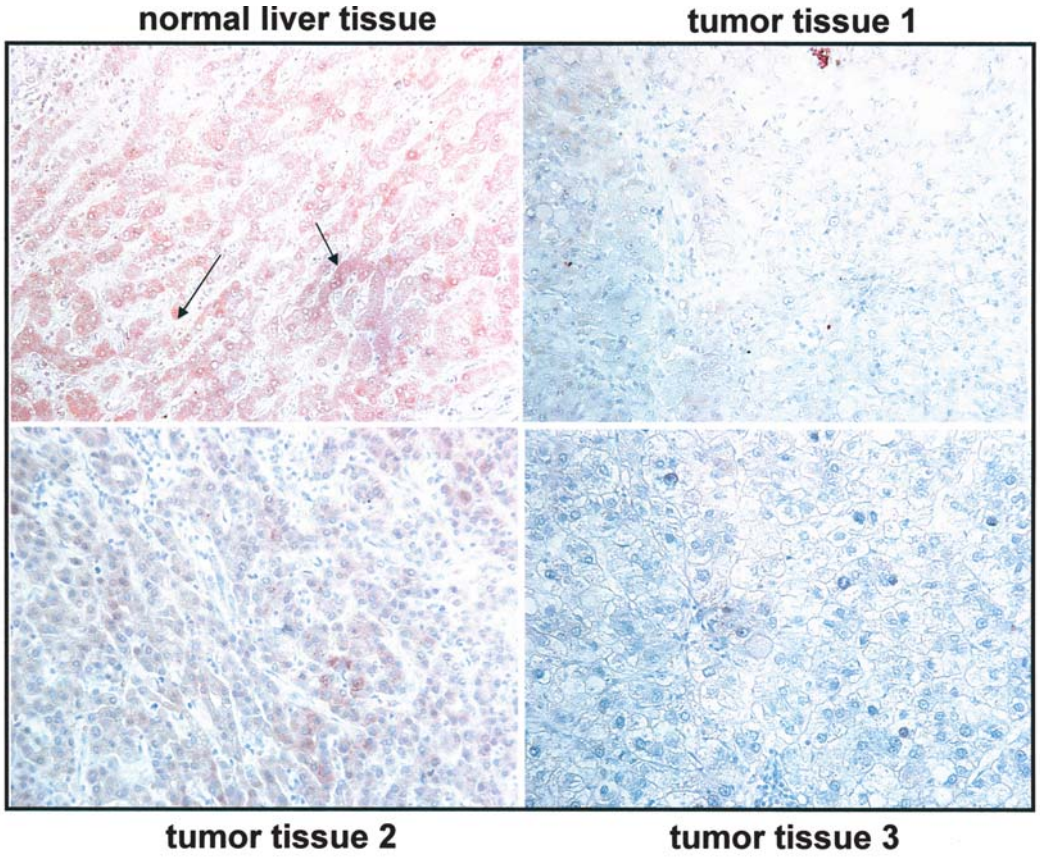


Figure 5. TANGO *in situ* expression of HCC patients. Microdissection from ten different patients with hepatocellular carcinoma (HCC) was screened by quantitative RT-PCR in comparison to normal liver tissue of the same patient. Reduction of TANGO transcription was observed in eight from ten analyzed tumor samples compared to cells of normal liver tissue

A



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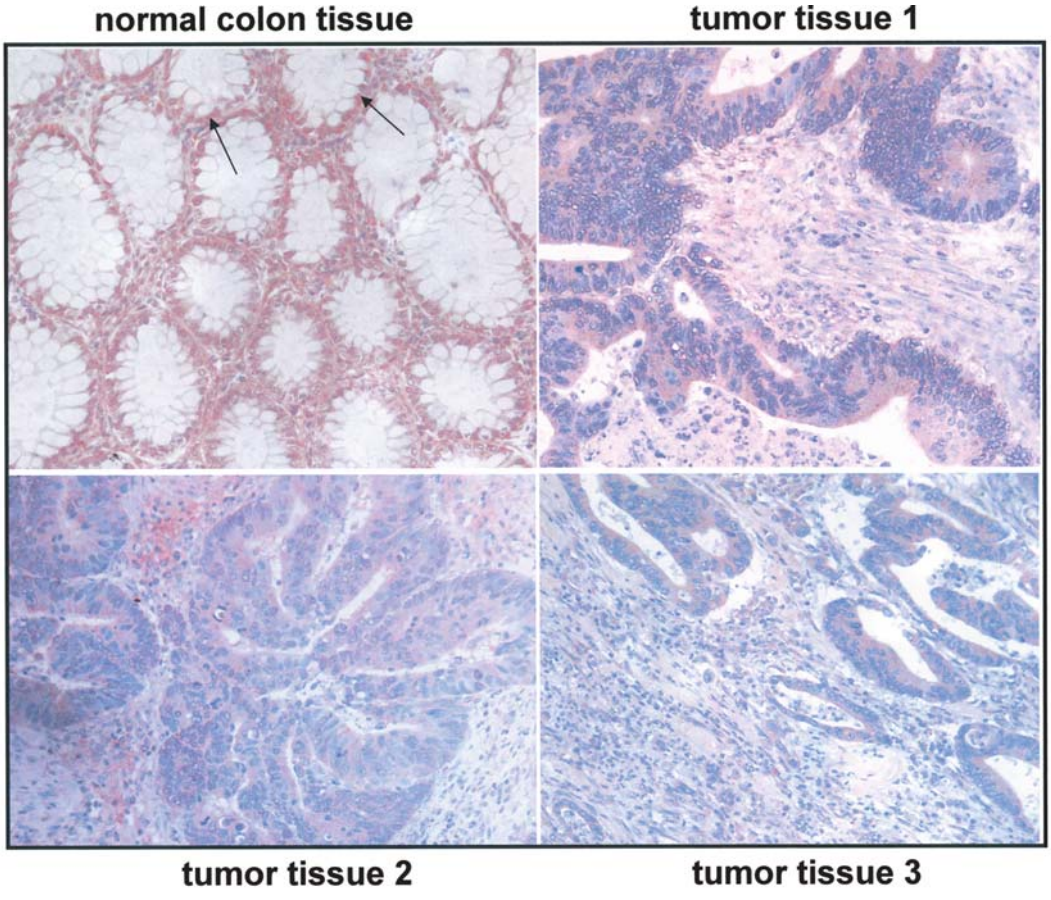

Figure 6. Immunohistological analysis of hepatoma and colon tumor tissues. (A) Immunohistological analysis of paraffin-embedded sections of different patients with hepatoma tumors confirmed a reduction of TANGO in the tumor compared to normal tissue. (B) In analogy to TANGO expression in hepatoma sections, colon tumor sections showed reduction of TANGO in all analyzed samples compared to normal healthy colon tissue.

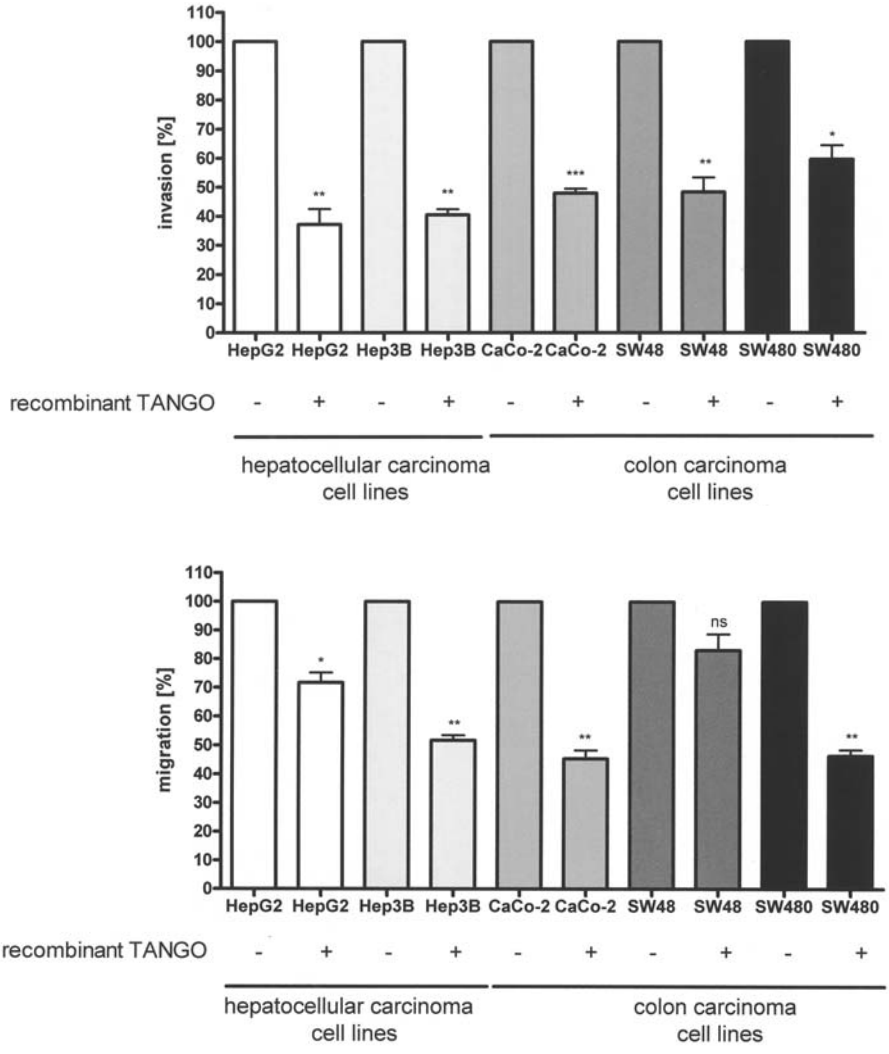

Figure 7. Invasion and migration reduction of hepatoma and colon carcinoma cells after treatment with recombinant human TANGO protein. (A) Boyden chamber invasion assay. TANGO-treated colon and hepatoma tumor cells exhibited a strongly reduced capacity to penetrate Matrigel, as compared to untreated cells. (B) Boyden chamber migration assay. Migration as estimated in a gelatine-coated Boyden chamber assay showed a decreased migration capacity of TANGO-treated hepatoma and colon carcinoma cell lines compared to untreated cells.

The cell line HT29M3 is a modified HT29 cell line resembling rather normal colon epithelial cells (20) and was added to the expression analysis. This cell line re-expresses high amounts of TANGO compared to the colon tumor cell line HT29.

To study TANGO protein expression in liver and colon tumors we analyzed tumor cell lines in comparison to isolated primary cells. Blot analysis loading 40 μg protein lysate of hepatoma cells (PLC, HepG2 and Hep3B) detected a reduction of TANGO expression in all hepatocellular carcinoma cells compared to normal PHH (Fig. 3). Compared to normal intestinal epithelial cells, all analyzed colon tumor cells revealed loss of TANGO expression (Fig. 4). To further study TANGO expression in HCC *in vivo*, we analyzed a panel of 10 paired specimens obtained from patients with HCC. From each HCC patient RNA was isolated from cancerous tissue and surrounding non-tumorous tissue, and TANGO mRNA expression was measured by quantitative RT-PCR (Fig. 5). TANGO mRNA expression was not significantly different compared to non-tumorous tissue in only one HCC specimen (P2) and two patients (P1 and P3) revealed increased TANGO mRNA expression levels compared to matched non-tumorous tissue specimens. In 7 HCC specimens (P4-P10) TANGO mRNA expression was significantly reduced or even lost compared to matched non-tumorous tissue (normal healthy liver set as 1.0) versus HCC. In order to assess TANGO expression in HCC and in colon tumors *in situ*, we performed immunohistochemical staining for TANGO in tumorous tissue of three HCC and three colon carcinoma patients. Furthermore, tissue samples obtained from healthy normal liver and normal colon were immunostained for comparison. Representative stainings are presented in Fig. 6. Paraffin-embedded sections of all analyzed patients with hepatoma (Fig. 6A) and colon tumors (Fig. 6B) confirmed a reduction of TANGO in tumor tissue sections compared to normal healthy tissue.

Functional relevance of TANGO. To analyze the functional role of TANGO in tumor cells we treated them with recombinant TANGO protein and determined the influence of exogenous TANGO on hepatoma and colon tumor cells. As shown in Fig. 7A, TANGO-treated SW480, SW48 and CaCo-2 colon

tumor cells, and HepG2 and Hep3B hepatoma tumor cells exhibited a strongly reduced capacity to penetrate Matrigel in a Boyden Chamber assay, compared to the untreated cells (set to 100%). Migration performed in a gelatine-coated Boyden Chamber assay also showed a reduction in migration capacity of the TANGO-treated cells compared to the untreated cells (set to 100%) (Fig. 7B), except of SW48.

Discussion

In this study, we investigated the expression pattern of TANGO in colon and hepatocellular carcinoma (HCC) because these are two of the most common human carcinomas worldwide. Colon carcinoma derives from intestinal epithelial cells and hepatocellular carcinoma derives from hepatocytes.

We initiated this study because we recently found that TANGO is reduced in malignant melanoma and has a tumor suppressor role in this kind of tumor (14). Specifically, we were interested whether TANGO plays a similar role also in other tumors and, therefore, analyzed the expression of TANGO in colon carcinoma and HCC and whether treatment with TANGO may change the migratory and invasive activity of the colon and hepatoma tumor cells.

Our data indicated that TANGO expression was reduced in colon and hepatocellular carcinoma in analogy to malignant melanoma. We showed that TANGO expression was reduced on mRNA and protein level in all analyzed colon and hepatoma carcinoma cell lines. Similar reduction or loss of TANGO expression on mRNA level was also found *in situ* in most of the patients with hepatocellular carcinoma compared to normal tissue samples. The treatment with exogenous TANGO on colon and hepatoma tumor cells reduced the migration and invasion ability, as we also observed recently for melanoma cells.

TANGO is the only MIA family member found to be expressed in colon cells, whereas in addition to TANGO Melanoma inhibitory activity 2 (MIA2) is expressed in hepatocytes (10). Hepatocytes were confirmed as the exclusive cellular source of MIA2 expression, with a granular, cytoplasmatic staining pattern without enhancement at the cell membrane. MIA2 expression levels in most HCC and hepatoma cell lines were also reduced, similarly to TANGO expression levels. Only in HCC that contained fibrous stroma or thick hyalinized bundles did adjacent atypical hepatocytes reveal strong MIA2 staining (10,11). These findings are in contrast to the regulation of the protein MIA which was found to be strongly up-regulated in malignant melanoma. Obviously, each of the MIA family members displays a defined function. Recently, we found that MIA interacts with integrin α4ß1 and α5ß1, leading to down-regulation of integrin activity and reduction of mitogen-activated protein kinase signaling (21). These findings also suggest that MIA may play a role in tumor progression and the spread of malignant melanomas via mediating detachment of cells from extracellular matrix molecules by modulating integrin activity. It could be possible that TANGO generally interacts also with integrins. We therefore suggest that TANGO could play an important role in cellular attachment, and that loss of TANGO promotes cell migration and invasion. Interaction studies must be performed to prove a possible TANGO-integrin relationship.

In conclusion, we present for the first time that TANGO is down-regulated or even lost in colon and hepatocellular carcinoma. In analogy to our recently published data on TANGO in malignant melanoma, we suggest a general tumor suppressor role for TANGO in tumors. Reduction or loss of TANGO expression is frequent in different tumors and may provide a new target for therapeutic strategies. Further studies must be performed to identify the molecular mechanism underlying the TANGO down-regulation in cancer.

Acknowledgements

We are indebted to Susanne Wallner and Sibylla Lodermeyer for excellent technical assistance and to Claus Hellerbrand for providing the HCC samples. This study was supported by a grant from the DFG.

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