Securin \((hPTTG1)\) expression is regulated by \(\beta\)-catenin/TCF in human colorectal carcinoma

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Overexpression of the transcriptional activator \(\beta\)-catenin, mostly owing to loss-of-function mutations of the *adenomatous polyposis coli* (APC) tumour suppressor gene, is crucial for the initiation and progression of human colorectal carcinogenesis. Securin is a regulator of chromosome separation and its overexpression has been shown to be involved in different tumour-promoting processes, like transformation, hyperproliferation and angiogenesis, and correlates with tumour cell invasion. However, the molecular mechanism leading to securin overexpression in human colorectal cancer is unknown. Here we show a correlated high expression of \(\beta\)-catenin and securin \((hPTTG1)\) in colorectal adenomas and carcinomas and further demonstrate that securin is a target of \(\beta\)-catenin transcriptional activation. This implies that deregulation of the \(\beta\)-catenin/T-cell factor-signalling pathway leads to overexpression of securin in human colorectal cancer, which subsequently may contribute to tumour progression.

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**MATERIALS AND METHODS**

**Tissue specimen and immunohistochemistry**

Formalin-fixed, paraffin-embedded colorectal adenocarcinomas from patients who underwent surgery without additional treatments were retrieved from the archive of the Department of Pathology, University of Erlangen-Nürnberg. The study comprised 30 cases. Immunohistochemistry for \(\beta\)-catenin and Ki-67 was performed as described previously (Hlubek et al, 2004). For securin staining, a mouse anti-securin antibody (1:25; NovoCastra NCL-SECURIN clone DCS-280.2, Newcastle, UK) and microwave treatment for antigen retrieval was used.

**RNA isolation and DNA microarray analysis**

Detailed protocols and results will be published elsewhere (Hlubek et al, in preparation). This study comprised well characterised...
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Transfections and reporter assays

Human colon carcinoma cell lines SW480, DLD1 and HeLa (human cervix carcinoma) cells were obtained from the American Type Culture Collection (ATCC). Cells were cultivated under standard conditions in Dulbecco’s modified Eagle’s medium +10% fetal bovine serum. Transfections and reporter assays were carried out as described previously (Hlubek et al, 2004). Experiments were conducted in triplicate and repeated at least three times with similar results. Control plasmid (pcDNA3; Invitrogen, Karlsruhe, Germany) was used to equalise for the total amount of DNA and to keep the amount of background plasmid constant.

RNA interference and quantitative real-time reverse-transcription – PCR

Synthetic siRNA (Thermo Electron, Ulm, Germany) specific for β-catenin (CAGUUUGGUGUAAGACUCUdTdT) or GFP (AAG CUAACCUGUUCAUGGCCGdTdT) as control and Oligofectamine (Invitrogen, Karlsruhe, Germany) was used for transient transfection of the colorectal cancer cells according to the instructions of the manufacturer. After 72 h incubation time, cells were harvested, total RNA isolated and real-time reverse transcription—PCR (RT–PCR) was performed as described (Hlubek et al, 2004). The relative quantities of securin-specific mRNA in β-catenin-siRNA- and control-siRNA-transfected cells were determined for each sample based on the Ct value from a standard curve generated for each primer/probe set and normalised to the corresponding values of the house-keeping gene β-actin.

Electromobility shift assay

The following sense and corresponding antisense oligonucleotides were annealed and used as probes or for competition (500- and 100-fold molar excess): securin-TBE: 5’-ATAAAATCAGTATCAAAAG GATAGAATT-3’; securin-mutTBE: 5’-ATAAAATCA CTGGCGAAAG GAT AGAATT-3’. Probes were end labelled and incubated with 0.5 μg of bacterially expressed GST-TCF-4 DBD (DNA-binding domain codon 265–496) or GST alone as described previously (Hlubek et al, 2004).

DNA clones

The securin promoter–reporter plasmid (pGL-Sec. fl.) was constructed by amplification of the primers U1 and D1 described previously (Kakar, 1999) using the bacterial artificial chromosome clone #RP11-35508 (BioCat, Heidelberg, Germany) as template. The PCR product (nt −1336 to +34; GenBank accession number AF167560) was cloned into the pGL3basic (Promega, Mannheim, Germany) reporter vector. For construction of the securin promoter fragment (nt −1336 to 625; pGL-Sec frag.), the primers U1 and PTTG1-625 (5’-TTAAAAATAAATACGGAGGCTT-3’) were used. Plasmids provided by other researchers are as follows: pGST-TCF4(DBD), pDNA/hTCF4 and pDNA/DN-TCF4 from Bert Vogelstein (Johns Hopkins University, Baltimore, MD, USA); pcDNA/h-catenin from Hans Clevers (University Medical Center, Utrecht, The Netherlands).

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RESULTS

Overexpression of securin correlates with nuclear β-catenin and Ki-67 expression in human colorectal adenoma and carcinoma

DNA microarray analysis revealed significant overexpression of securin (threelfold, $P$-value <0.00001) in six human colorectal carcinomas compared to the corresponding normal mucosa (Figure 1A; detailed results will be published elsewhere (Hlubek et al, in preparation). Similarly, securin overexpression was found by real-time PCR analysis of seven different colorectal tumours compared to the corresponding mucosa (Figure 1B). Thus, securin shows a very similar differential expression pattern between tumour and mucosa tissue like 12 other well-characterised β-catenin/TCF target genes (fold changes between 3.3 and 1.8-fold, $P$-value <0.005; Figure 1A). This observation gave rise to the hypothesis, that securin is a target gene of β-catenin/TCF signalling, which is constitutively active in these tumours. Therefore, we analysed the expression pattern of β-catenin and securin protein in 30 colorectal adenocarcinomas by immunohistochemistry. We found a perfect correlation of pattern of active in these tumours. Therefore, we analysed the expression of securin, Ki-67 and β-catenin in 30 colorectal adenocarcinomas.

Correlated overexpression of nuclear β-catenin, securin and Ki-67 in colorectal adenoma and carcinoma. Immunohistochemical detection of β-catenin (first row), securin (second row) and Ki-67 (third row) using serial sections. Tissue sections show adenoma (a, a’, a’’) and carcinoma (c, c’, c’’) of the same case. Note that securin is already overexpressed in the adenoma tissue (arrowhead in a’’) and only weakly expressed in the underlying normal colon mucosa (arrow in a’’). A substantial overexpression of securin, Ki-67 and nuclear β-catenin can be seen in the carcinoma part (b, b’, b’’ and c, c’, c’’; the rectangle in b indicates the magnified area shown in c). Concordant with securin function in mitosis, the proliferation marker Ki-67 displayed the same expression pattern as securin. Nuclear accumulation of β-catenin in many of these tumour cells may suggest a function of this transcriptional activator protein for securin expression. Magnifications are indicated at the top of the figure.

β-Catenin binds to a TCF-binding site of the human securin promoter

We searched for potential TCF-binding elements (TBE) in the securin promoter/enhancer sequence (Kakar, 1999; Clem et al, 2003) as β-catenin/TCF might directly regulate securin expression. Two sites comprising the TBE motif (5’-WWCAAAG-3’) were identified (nt −1064 to −1058, nt −796 to −790) and tested for interaction with TCF-4 in electromobility shift assays (EMSA). One TBE (nt −1064 to −1058; Figure 3A) was found to bind recombinant TCF-4 (Figure 3B), which is consistent with the very recent report by Zhou et al (2005). The TCF–DNA interaction was abrogated by specific competitor DNA containing the wild-type TBE site (wt) whereas the mutant TBE motif competitor (mt) had no effect (Figure 3B). Competitor DNA containing the TBE of the c-myc promoter (myc) inhibited TCF binding to TBE to the same extent. Mutant TBE-DNA showed no TCF binding and GST protein alone did not bind to wild-type TBE (Figure 3B), indicating specific binding of TCF-4 to TBE of the securin promoter in vitro.

The human securin promoter is activated by β-catenin/TCF

The functional relevance of TCF-4 binding to the TBE motive was investigated in luciferase reporter assays using a full-length securin
promoter (−1336 to +34). In β-catenin-overexpressing colorectal carcinoma cell lines SW480 and DLD1, the securin promoter (pGL Sec fl.) exhibits strong transcriptional activity, 74 and 35-fold over background (pGLbasic). Addition of dominant-negative TCF-4 (dnTCF), which lacks the N-terminal β-catenin interaction domain, reduced the securin promoter activity in a dose-dependent manner by up to 51 and 44% (Figure 4A and B). As it is known that complete abrogation of β-catenin/TCF transcriptional activity by dnTCF can induce a G1-phase arrest of the cell cycle (van de Wetering et al, 2002) possibly influencing the cell cycle-dependent expression of securin, we determined cell cycle progression by fluorescence-activated cell sorting (FACS) analysis (Supplementary Figure 2). Unsynchronised DLD1 and SW480 cells showed no significant change in cell cycle phases after transient transfection with dnTCF compared to cells transfected with control vector.

Moreover, in HeLa cells, which do not have an activated β-catenin/TCF-signalling pathway, the activity of the securin promoter fragment (−1336 to −625) was enhanced by over-expression of TCF4 and β-catenin in a dose-dependent manner (Figure 4C). Neither β-catenin nor TCF alone was able to activate the securin promoter fragment. In addition, dnTCF abolished promoter activation by β-catenin (Figure 4C).

**Expression of endogenous human securin is regulated by β-catenin**

To test the regulatory function of β-catenin on the expression of the endogenous securin gene in colorectal carcinoma cells, we utilised
Figure 5: Endogenous securin expression is activated by β-catenin. HCT116, SW480 and DLD1 colorectal cancer cells were transfected with β-catenin-specific siRNA and control siRNA (GFP-siRNA). The effect of β-catenin knockdown on securin expression was measured by quantitative real-time RT–PCR and β-catenin expression was confirmed by Western blotting (Supplementary Figure 3). Inhibition of β-catenin expression by siRNA in colorectal cancer cells clearly reduces the endogenous securin expression. Black bars: β-catenin siRNA-transfected cells; grey bars: control cells (GFP-siRNA transfected); hatched bars: quantity of β-catenin mRNA; closed bars: quantity of securin mRNA. Quantification of securin and β-catenin-specific mRNA was done in triplicates, the amounts were normalised to the amount of β-actin mRNA, and expressed in percent relative to the control cells. The experiments were repeated twice with similar results.

siRNA knockdown technology. The expression of endogenous β-catenin was inhibited by transfection of HCT116, SW480 and DLD1 colorectal cancer cells with β-catenin-specific siRNA. β-catenin expression, measured by quantitative RT–PCR, was clearly reduced compared to the control cells (transfected with green fluorescent protein (GFP)-siRNA; Figure 5). Specific reduction of β-catenin expression by β-catenin-specific siRNA transfection was confirmed by Western blot in control experiments (Supplementary Figure 3; see also Hlubek et al., 2004). Importantly, transfection of β-catenin-specific siRNA decreased endogenous securin expression by 37, 43 and 56% compared to control cells, but not β-actin expression (Figure 5, and Supplementary Figure 3). To exclude the possibility that reduction of securin expression was caused by changes in cell cycle progression, we performed control experiments using FACS analysis. Transient transfection of HCT116 cells with β-catenin-specific siRNA did not result in significant changes in the cell cycle phase distribution (Supplementary Figure 4).

DISCUSSION

The results presented above demonstrate a correlated expression of β-catenin, securin and Ki-67 in human colorectal adenomas and carcinomas, indicating that securin is a potential β-catenin/TCF target gene. In support of this hypothesis, we show that securin, like 12 well-known β-catenin/TCF target genes (e.g. c-myc, c-Met, survivin and MMP7), is significantly overexpressed in colorectal cancer compared to normal mucosa using DNA microarray analysis, real-time PCR and immunohistochemistry. Moreover, we found a functional TCF-binding element in the securin promoter sequence identical to the TBE found to be functional in esophageal squamous cell carcinoma, published during the preparation of this manuscript (Zhou et al., 2005). In EMSA experiments, we show specific TCF–DNA interaction. The functional relevance of this interaction is shown by reporter-gene experiments in colorectal cancer cell lines demonstrating that dnTCF inhibits securin promoter activity in a dose-dependent manner. As securin expression is cell cycle dependent, peaking in mitosis (Ramos-Morales et al., 2000), and β-catenin can influence cell cycle progression by regulating c-myc expression (van de Wetering et al., 2002), we performed FACS analysis. However, we did not observe significant differences in cell cycle progression between dnTCF-transfected and control vector-transfected cells (Supplementary Figure 2). The transient transfection of dnTCF or β-catenin siRNA (see below) reduces β-catenin transcriptional activity, but does not completely abolish it, which would be necessary for inducing a G1-phase arrest of the cell cycle (van de Wetering et al., 2002, 2003).

Additionally, we show that securin promoter fragment containing the TBE is activated in HeLa cells by overexpression of TCF-4 and β-catenin, but not by either protein alone. However, we were unable to activate the full-length promoter construct significantly by overexpression of TCF-4 and β-catenin, because the promoter region (659 nucleotides) proximal to the transcriptional start site contains additional regulatory elements (Kakar, 1999; Clem et al., 2003) presumably obscuring this effect. We tested the function of β-catenin/TCF in the regulation of the endogenous securin gene by transfection of colorectal carcinoma cells with β-catenin-specific siRNA. Owing to the transient transfection procedure used, no complete abrogation of β-catenin expression was observed and cell cycle progression was not changed. However, a potent inhibition of β-catenin expression was induced, resulting in a significant reduction of the endogenous securin gene expression. Even though we do not exclude the possibility of an indirect effect of β-catenin on securin expression by affecting cell cycle progression, our results show the relevance of β-catenin for the direct regulation of securin gene expression in colorectal tumour cells.

The oncogene securin is deregulated in many human tumours and is able to contribute to several main aspects of tumorigenesis. In several tumours, overexpression of securin is associated with tumour metastasis and poor clinical outcome and has been identified as a significant prognostic marker in esophageal carcinoma (Zhang et al., 1999; Heaney et al., 2000; Shibata et al., 2002; Ramaswamy et al., 2003). The identification of securin as a Wnt target gene activated early in the adenoma–cancer sequence further supports a decisive role of aberrant overexpression of β-catenin in all phases of colorectal carcinogenesis.

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