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Overexpression of Evi-1 Oncoprotein Represses TGF- β Signaling in Colorectal Cancer

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Abstract

Human colorectal cancer (CRC) cells are resistant to the anti-proliferative effect of transforming growth factor- β (TGF- β), suggesting that disruption of TGF- β signaling plays an important role in colorectal carcinogenesis. Ecotropic virus integration site-1 (Evi-1) oncoprotein represses TGF- β signaling by interacting with Smads, but its role in CRC has not been established. The purpose of this study is to determine whether Evi-1 plays role(s) in CRCs and to characterize Evi-1 transcript(s) in CRCs. Evi-1 was overexpressed in 53% of human CRC samples, 100% of colon adenoma samples, and 100% of human colon cancer cell lines tested. Using 5' RACE, we cloned a novel Evi-1 transcript (Evi-1e) from a human CRC tissue and found that this novel transcript was expressed at a higher level in CRC tissues than in normal tissues and was the major Evi-1 transcript in CRCs. Transient Evi-1 transfection inhibited TGF- β -induced transcriptional activity and reversed the growth inhibitory effect of TGF- β in MC-26 mouse colon cancer cells. In conclusion, we have identified overexpression of Evi-1 oncoprotein as a novel mechanism by which a subset of human CRCs may escape TGF- β regulation. We have also identified a novel Evi-1 transcript, Evi-1e, as the major Evi-1 transcript expressed in human CRCs.

Keywords

colorectal cancer; ecotropic virus integration site-1; transforming growth factor- β ; Smad proteins; rapid amplification of cDNA ends; growth inhibition

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies worldwide and the third leading cause of cancer death in the United States [1]. CRC develops from apparently normal mucosa into a benign precursor stage, the premalignant polyp, which eventually

progresses to invasive disease. The molecular mechanisms driving this transformation and the important pathological landmarks that characterize CRC are being continually refined [2]. The development of CRC is associated with both genetic alterations such as activation of oncogenes or inactivation of tumor suppressor genes and altered production of, or responsiveness to, stimulatory or inhibitory growth and differentiation factors. Among these, transforming growth factor (TGF)- β signaling pathway acts as key determinants of CRC cell behavior [3].

TGF- β is a critical tumor suppressor in many solid tumors and is able to inhibit proliferation of a wide range of cell types including cells of the epithelial, endothelial, and hematopoietic origin [4]. Many tumor cells become resistant to TGF- β -induced growth inhibition and apoptosis through mutations or inactivation of TGF- β receptors and intracellular signaling proteins, Smads [5,6]. TGF- β plays a critical role in CRC, particularly in tumor progression, invasion and metastasis, and loss of TGF- β response occurs during the progression of CRCs [7–9]. However, the majority of CRCs that are resistant to TGF- β -induced growth inhibition have no identifiable alterations in the TGF- β signaling pathway [10]. Thus, the mechanisms that account for somatic loss of TGF- β responsiveness in CRCs are not completely understood.

The ecotropic virus integration site-1 (Evi-1) gene was originally identified in a common integration site of murine leukemia retrovirus [11]. Evi-1 plays important roles both in normal development and in oncogenesis [12]. Previous studies have provided direct evidence linking Evi-1 overexpression and activation to the development of genomic instability and clonal progression toward myelodysplastic syndrome (MDS) and eventually acute myeloid leukemia (AML) [13–18]. Patients with Evi-1-positive AML independently have a very unfavorable prognosis [19]. Drugs such as arsenic trioxide that selectively degrades the Evi-1 protein are being tested as a target therapy for patients with Evi-1-related MDS [20]. In addition to malignancies of the hematopoietic origin, Evi-1 is also overexpressed in and has been implicated in the occurrence and/or progression of solid tumors such as melanoma, prostate cancer, breast cancer, ovarian cancer [21–23], nasopharyngeal carcinoma [24], and infratentorial ependymoma [25]. The Evi-1 protein is expressed at very low levels in the adult human normal small intestine and colon [26] and the role of Evi-1 in human CRC development has not been investigated. Elucidation of the interaction of Evi-1 with the TGF- β signaling pathway will be of help to the development of novel therapies to treat CRCs.

Human Evi-1 gene is located on chromosome band 3q26 and encodes a 1051 amino acid protein with two DNA-binding zinc finger domains [11]. Through its first zinc finger domain, Evi-1 physically interacts with Smad2 and Smad3, thereby suppressing the transcriptional activity of Smad2 and Smad3 and TGF- β signaling [27–29]. Further, Evi-1 directly interacts with the transcriptional corepressor CtBP to repress TGF- β signaling [30]. Thus, the oncogenic effects of Evi-1 may be attributable in part to its ability to disrupt TGF- β signaling and TGF- β -mediated growth inhibition.

Attempts to understand the physiological and pathological roles of Evi-1 are complicated by the discovery that there are multiple forms of Evi-1 mRNA and protein, resulting from the use of alternative transcriptional initiation sites and alternative splicing. Transcription of the Evi-1 locus can be initiated from at least four different promoters [12], giving rise to Evi-1 transcripts that differ in 5' ends. For example, two well-characterized transcription start sites give rise to functional Evi-1 exon 1a and exon 1b, respectively [31]. MDS1/Evi-1, another Evi-1 5' end variant, has lost the ability to repress TGF- β signaling [32]. The cloning of Evi-1d was not published, but the sequence was deposited in GenBank (acc. no. AF164154). Alternative splicing also added to the complexity of Evi-1 gene regulation, resulting in

several splice variants of Evi-1 with addition or deletion of specific protein domains. For example, two MDS1 exons are spliced to Evi-1 exon 2 to form the MDS1/Evi-1 transcript [33]. However, the functional significance of these alternative forms of Evi-1, especially in the development of solid tumors, is not entirely understood. This study was undertaken to determine whether Evi-1 is upregulated in CRCs and to characterize Evi-1 transcript(s) expressed in CRCs.

MATERIALS AND METHODS

CRC Tissue Specimens

Human CRC tissues and colorectal adenoma samples were collected from the University of Texas Medical Branch (Galveston, TX) immediately following surgical resection. Adjacent morphologically normal tissues as control were taken from regions >10 cm away from bulk tumor tissues that had clearly defined margins. Tissues were snap frozen in liquid nitrogen for protein preparation, immersed in RNeasy lysis buffer (Qiagen, Inc., Austin, TX) for RNA preparation, or fixed overnight in 4% formaldehyde for immunohistochemistry. The use of human subjects was approved by the Institutional Committee for the Protection of Human Subjects and informed consent was obtained from all patients involved in the study.

Protein Sample Preparation and Immunoblotting

Human colorectal tissues were minced in a lysis buffer (25 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM NaVO₃, 1 mM PMSF, 1 mM benzamide, 1 mM DTT, 25 µg/mL aprotinin, 25 µg/mL trypsin inhibitor, 25 µg/mL pepstatin A, 25 µg/mL leupeptin) using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Cultured cells were lysed in the same lysis buffer. Whole cell lysates were prepared and quantified according to our standard procedure [34]. Protein samples were resolved on 3–8% NuPAGE Tris-Acetate gel (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride membrane (PVDF+, Millipore, Bedford, MA). The membrane was blocked with 5% nonfat dry milk followed by incubation with an anti-Evi-1 antibody (raised in rabbit against amino acids 719–735 and 805–1042 of Evi-1 of mouse origin) for tissue lysates or an anti-HA antibody (Babco, Berkeley, CA) for transfected cell lysates. The membrane was then detected with an HRP-conjugated secondary antibody, and visualized with enhanced chemiluminescent reagents (ECL-plus, Amersham-Pharmacia Biotech, Piscataway, NJ). For quantification, the images were scanned and analyzed with the KODAK 1D software (version 3.6) using β-actin as the normalization control.

Immunohistochemistry

CRC tissue samples were processed and subjected to immunohistochemistry as described previously [35]. Briefly, paraffin-embedded tissue sections of 4 µm thickness were deparaffinized, rehydrated and washed in TBS. Antigen retrieval was performed using DAKO target retrieval solution. H₂O₂ solution was used to block the endogenous peroxidase activity. The sections were incubated with an anti-Evi-1 antibody (same antibody as described in immunoblotting) at room temperature for 1 h. The sections were then visualized using DAKO LSAB 2 System with DAB as the chromagen, counterstained with Mayer's hematoxylin, and mounted. Sections incubated without primary antibody were used as a negative control.

Rapid Amplification of 5' cDNA Ends

Total RNAs were prepared using Trizol reagent (Invitrogen) from colorectal tissue samples. Rapid amplification of 5' cDNA ends (5' RACE) was performed with 1 µg of total RNA using the switching mechanism at 5' end of RNA transcript (SMART) RACE cDNA

Amplification Kit and moloney murine leukemia virus (MMLV) reverse transcriptase (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's protocol. This kit uses an Upstream Primer A Mix (UPM), which recognizes the SMART oligo sequence and, together with a gene-specific primer, amplifies the 5' cDNA ends of the target gene. For subsequent PCR amplification, 1 µl of a 1/100 dilution of the RACE products was used as template along with the specific primers as specified in the Results Section.

Regular RT-PCR and Real-Time Quantitative RT-PCR

Total RNAs prepared as above were transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Regular RT-PCR amplification of Evi-1e was performed with Platinum® *Taq* DNA Polymerase (Invitrogen) using the following primers: forward 5'-TTTCTATGTGGAAGCATGATGG-3' and reverse 5'-CCATATATGCTGTGTAGGAGCA-3'.

Real-time quantitative RT-PCR was performed with the Applied Biosystems 7900HT Real-Time PCR System using the following primers: forward 5'-TTT CTA TGT GGA AGC ATG ATG G-3' (located at nt 19–30 of exon 1e), and reverse 5'-GCG CAA TGT CTG CAA CTA CTC T-3' (located at 199–220 of Evi-1 exon 2; NM_005241). SYBR Green I (1/40 000) (Invitrogen) was used as the fluorescent probe. The cycling conditions included 95°C, 1 min followed by 40 cycles of 95°C, 12 s; 60°C, 5 s; and 72°C, 30 s. The results were expressed as fold changes compared to the calibrator sample (normal tissue of patient #3) using the Comparative C_t Method. The resulting data were analyzed using SDS 2.3 (7900) software (Applied Biosystems). All PCR reactions were run in triplicate and 18S rRNA was used as a normalization control.

Promoter Cloning and Promoter Activity Assay

The novel Evi-1 promoter (pEvi-1e) of about 600 bp in length was cloned from human genomic DNA libraries using a Genome Walker kit (BD Biosciences). The resultant PCR product was cloned into the luciferase reporter vector pGL3 (Promega, Madison, WI) to yield the pEvi-1e/Luc reporter. For promoter activity assay, pEvi-1e/Luc (firefly) was cotransfected together with 50 ng of pRL-SV40 (renilla) into MC-26 mouse colon cancer cells using FuGENE 6 transfection reagent (Roche, Indianapolis, IN) according to the manufacturer's instructions. After transfection, cells were allowed to recover in RPMI 1640 medium supplemented with 10% FBS for 24 h followed by assay for firefly and renilla luciferase using the dual-luciferase reporter assay system (Promega).

Measurement of TGF-β-Mediated Transcriptional Responses

MC-26 cells were seeded in triplicate at a density of 3×10^5 per well in six-well plates and cultured in RPMI 1640 medium supplemented with 10% FBS. Eighteen hours later, pSG5-Evi-1 or pSV40-β-gal was cotransfected together with p3TP-Lux [27] into MC-26 cells using FuGENE 6. Total amount of DNA was adjusted to be equal by adding the vector plasmid. Twenty-four hours after transfection, the cells were treated with TGF-β1 (120 pM) or vehicle for 12 h and whole cell lysates were prepared and assayed for luciferase and β-galactosidase activity using an Enhanced Luciferase Assay kit (Pharmingen, San Diego, CA) and a Galacto-Light Plus kit (Tropix, Bedford, MA), respectively. Light emission was detected using a Monolight Luminometer (Pharmingen).

Growth Study

MC-26 cells were transfected with pSG5-Evi-1-HA, pSG5-MDS1/Evi-1, or empty pSG5 plasmid using FuGENE 6 for 24 h and replated in triplicate at a density of 2×10^4 per well in 12-well plates for 6 h in RPMI 1640 medium supplemented with 10% FBS. Cells were

then treated with or without TGF- β 1 at 120 pM in serum-free medium, counted daily using a hemocytometer with 0.4% trypan blue staining to exclude dead cells. The results were expressed as percentage of cell growth in TGF- β -treated groups versus vehicle control.

Statistical Analysis

Data were expressed as means $\pm$ SEM. For growth study, differences were analyzed by Student's *t*-test. For Evi-1 luciferase assay, differences between groups were analyzed by ANOVA with Tukey-Kramer multiple comparison test. *P* < 0.05 was considered statistically significant.

RESULTS

Overexpression of Evi-1 Protein in Human Colorectal Tumors

To determine whether Evi-1 is upregulated in human colorectal tumors, Evi-1 protein expression was determined in 15 human CRC samples and 3 adenoma samples, which were pair-matched with adjacent normal mucosal controls. Immunoblot analysis using an Evi-1-specific antibody revealed that 53% (8/15) of human CRC samples showed a >2-fold increase in expression of Evi-1 protein compared to adjacent normal colonic mucosa (Figure 1A). The two immunoreactive bands may be due to difference in posttranslational modifications, such as phosphorylation [36]. In addition, 100% (3/3) adenoma samples showed a >2-fold increase in expression of Evi-1 protein compared to normal tissues (Figure 1B), suggesting that Evi-1 expression might also be important at early stages of colorectal carcinogenesis. Next, we determined whether Evi-1 is expressed in human colon cancer cell lines. All five human colon cancer cell lines tested, that is, Caco-2, DLD1, HCT-116, HT-29, and Lovo, expressed Evi-1 protein (Figure 1C), with the highest level in DLD1, and the lowest level in HCT-116. A comparison between cancer cell line DLD1 and CRC tissues 2 indicated that cancer cell line expressed Evi-1 at a similar level comparable to CRC cancer tissue but at a much higher level compared with the normal control tissue (Figure 1D). These results demonstrate that Evi-1 is really upregulated in colon cancer cell lines. We further performed immunohistochemistry to confirm Evi-1 protein expression in CRC tissues. Evi-1 staining was stronger in a representative cancer tissue of patient #2 than in its adjacent normal colon mucosa (Figure 1E). Furthermore, Evi-1 was mainly localized in the nucleus of epithelial cells of the colon cancer tissue, consistent with its role as a transcriptional factor [36]. These results suggest that Evi-1 protein expression is upregulated in at least a subset of human colorectal tumors.

Identification of a Novel Transcript at the 5' End of Evi-1

We performed 5' RACE to determine which Evi-1 isoforms are expressed in CRCs. The strategy to clone the 5' end of Evi-1 transcripts is illustrated in Figure 2A. The initial amplification was carried out using the primer P2 (5'-TTC AAA GAG CTG GTC ACA GTC TTC GCA G-3'), which primes from within the coding exon 4 of human Evi-1 cDNA (NM_005241, nt 17–44), and the UPM primer of the RACE kit, to anchor the 5' end of the Evi-1 transcripts (as described below). The PCR products were resolved by gel electrophoresis. We found a major band of about 700 bp in four out of the six CRC samples tested and in only one of the corresponding normal tissue samples (Figure 2B). Based on the molecular size, this major 700 bp band may represent a novel Evi-1 transcript which has not been reported before in the literature. Other less intense bands amplified by 5' RACE may represent other known isoforms of Evi-1 present in CRC or normal tissues. Since the 5' RACE we employed in our study theoretically amplifies all the Evi-1 isoforms, our results suggest that the predominant transcript in colon cancer originates from this novel Evi-1 promoter, although the possibility could not be excluded that Evi-1 expression in CRC can also originate from other previously known Evi-1 isoforms at relatively lower levels.

To characterize the major 700 bp Evi-1 transcript, we examined Evi-1 RNA transcripts from human CRC tissue sample #2, which had a significant increase in Evi-1 protein expression compared to its adjacent normal mucosa control as shown by immunoblotting and immunohistochemistry (Figure 1A and E). The ~700-bp amplification product from CRC tissue #2 was excised and partial sequence was obtained, which included the junction between exon 2 and a novel sequence that was not found in the National Center for Biotechnology Information (NCBI) human transcript database. The novel sequence was confirmed by further PCR amplification of the 5' RACE products using the UPM primer and a nested primer, P20 (5'-CTT TGT TGC AGT CGC TGA AGT TCT C-3'), which spans the junction between exon 2 and the novel sequence. The resultant PCR product was sequenced to identify a novel 164-bp-long first exon of Evi-1, which was designated Evi-1e. The sequence of this novel transcript is shown in Figure 2C. The presence of the Evi-1e transcript in CRC cancer tissues as shown in Figure 2B was confirmed using primers specific for the Evi-1e exon using RT-PCR, which gave rise to a 101-bp amplification product from all four samples tested (Figure 2D). The complete genomic organization of the Evi-1 locus is shown in Figure 2E. BLAST search against the NCBI human chromosome 3 genomic database located a sequence that is 100% identical to exon 1e. Exon 1e maps 139 646 bp upstream of MDS1 and defines a novel Evi-1 exon that has not been described previously.

Next, we screened 15 human CRCs and their paired normal controls for the presence of the Evi-1e transcript by quantitative real-time RT-PCR. Because the normal tissues of patient #1 and #2 had C_t values too high to be valid, therefore, normal tissue of patient #3 was used as the calibrator sample, to which all the rest of samples were compared. Evi-1e mRNA was overexpressed in 45% (6/15) of CRC tissue samples compared to adjacent normal mucosa (Supplementary Table 1). Evi-1e mRNA was undetectable or very low in most of normal adjacent colonic tissue samples. These results confirmed that the novel Evi-1e mRNA transcript is overexpressed in a subset of human CRCs. We noticed that Evi-1e mRNA expression in CRC tissues #3 and #4 detected by quantitative RT PCR was not detected by 5' RACE (Figure 2B). We do not know the exact reason for this discrepancy but this may be related to the efficiency of the RACE method itself, which is influenced by a number of variables such as the quality and the secondary structure of the target mRNA [37]. Nevertheless, there is no doubt that the novel Evi-1 transcript is expressed in a considerable portion of human CRCs.

Cloning and Activity of the Exon 1e Promoter

In order to confirm that the novel Evi-1e transcript is the result of a novel Evi-1 promoter, we cloned by PCR a 626 bp genomic fragment which includes 164 bp of the Evi-1 exon 1e and 462 bp of the 5'-flanking genomic sequence. Sequence analysis confirmed that this PCR product was identical to the 5'-flanking sequence immediately upstream of the position of exon 1e on human chromosome 3. This novel sequence was deposited in GenBank (accession #JN836552). Using a web-based promoter prediction software (http://www.fruitfly.org/seq_tools/promoter.html), we found a TATA-like (TATAGA) promoter sequence in the 462-bp promoter region, located 168-bp upstream of the Evi-1e exon, which is similar to the conserved prokaryotic TATA box (TATAAA) [38]. This presumptive promoter was cloned into the pGL3 reporter plasmid and this Evi-1e/Luc plasmid was transiently transfected into a mouse colon cancer cell line, MC-26. As shown in Figure 3, transfection of Evi-1e/Luc at 750 or 1500 ng resulted in a 5.3- or 4.8-fold increase in luciferase activity as compared to the control pGL3 control plasmid. These data confirmed the activity of the Evi-1e promoter, which drives Evi-1 expression in human CRC.

Evi-1 Represses TGF- β Signaling in Colon Cancer Cells

Evi-1 binds to Smad2 and Smad3 and blocks TGF- β signaling through repression of Smad-induced transcription [27,28]. Therefore, we determined whether Evi-1 blocks TGF- β signaling in colon cancer cells. Since most human colon cancer cell lines have defective or attenuated TGF- β signaling, we used a mouse colon cancer cell line, MC-26, which has intact TGF- β signaling [39] but with low level of endogenous Evi-1 (data not shown). MC-26 cells were transiently transfected with an Evi-1 expression plasmid, pSG5-Evi-1, which contains the full-length mouse Evi-1 driven by the SV40 promoter, and TGF- β signaling was analyzed by the standard luciferase reporter assay using the p3TP-Luc TGF- β reporter plasmid plus SV40 β -galactosidase as an internal control [40,41]. TGF- β induced a 3.1-fold increase in p3TP-Luc luciferase activity in MC-26 cells transfected with the control plasmid. Co-transfection with pSG5-Evi-1 resulted in a dose-dependent suppression of TGF- β -induced luciferase activity (Figure 4). Significant inhibition was observed beginning at 100 ng of pSG5-Evi-1 plasmid, and 1000 ng of pSG5-Evi-1 plasmid almost completely suppressed TGF- β -mediated activation of p3TP-Luc. We also observed some suppression of the basal-level luciferase activity by Evi-1 in the absence of TGF- β , which was consistent with a previous report by Sood et al. [42]. This suggests that Evi-1 also affects the activity of the promoter independently of TGF- β . Alternatively, MC-26 cells may be under autocrine or paracrine TGF- β stimulation, which is attenuated by Evi-1.

Evi-1 Antagonizes the Growth Inhibitory Effect of TGF- β

Because Evi-1 inhibits TGF- β signaling in MC-26 cells, we determined whether Evi-1 antagonizes the anti-proliferative effect of TGF- β on MC-26 cells. Plasmid expressing Evi-1 was transiently transfected into MC-26 cells, and the cells were then treated with TGF- β or vehicle. Addition of TGF- β significantly inhibited the growth of control cells, with 16–17% growth inhibition on day 1 and day 2. Transient Evi-1 transfection reversed the growth inhibitory effect of TGF- β on day 1 and day 2 (Figure 5A). Evi-1 expression following transfection was monitored by immunoblotting. Evi-1 protein expression was detected 1 or 2 d after transient transfection in either TGF- β -treated or vehicle-treated MC-26 cells (data not shown). We further compared the suppression of the growth inhibitory effect of TGF- β between Evi-1 and MDS1/Evi-1. As shown in Figure 5B, both Evi-1 and MDS1/Evi-1 similarly reversed the growth inhibitory effect of TGF- β in MC-26.

DISCUSSION

In the present study, we found that Evi-1 protein was overexpressed in human colorectal tumors and cell lines. Furthermore, we identified a novel Evi-1 transcript, Evi-1e, using 5' RACE and found that Evi-1e expression was upregulated in a subset of human CRC samples compared to the corresponding adjacent normal mucosa. We also confirmed that Evi-1e was the major Evi-1 transcript in human CRCs. The Evi-1e we cloned in the current study is an alternative transcription product initiated from a new promoter. This new exon uses the same coding sequence as other Evi-1 variants to code for the same gene product which begins with exon 3. The cloning of this new Evi-1 exon/promoter reveals a new method of regulation of the Evi-1 gene at the transcriptional level. Furthermore, transient Evi-1 expression repressed TGF- β signaling and blocked TGF- β -induced growth inhibition in colon cancer cells. Our studies suggest that overexpression of Evi-1 is a novel mechanism by which a subset of human colorectal tumors can escape the tumor suppressor effects of TGF- β which is important for the initiation and progression of CRCs.

Although Evi-1 expression is increased in all three adenoma samples, we are unable to establish a correlation between Evi-1 expression and the different TNM classifications and pathologic stages because of the sample size limitation. It is possible that Evi-1 is

overexpressed beginning from the initial stage and plays a role in different stages of colorectal carcinogenesis. However, this hypothesis needs further validation in a larger sample set from different stages of colorectal carcinogenesis. Also, it will be important in future studies to determine whether the status of Evi-1 overexpression can be used to predict clinical outcomes or to tailor chemotherapeutic treatments. In this respect, we have previously shown that Evi-1 can affect drug response through an AKT-mediated anti-apoptotic signaling pathway in colon cancer cell lines [43]. It will be of interest to determine if overexpression of Evi-1 predicts drug resistance in human CRCs.

It has been shown that the majority of CRCs are resistant to TGF- β -mediated growth inhibition [10,44]. The underlying mechanisms may involve alterations of the TGF- β signaling pathway including mutations of the receptors and the intracellular effector molecules, Smads [45–49]. However, many CRC tumors that are insensitive to TGF- β -induced growth inhibition have no genetic defects in TGF- β receptors or Smad proteins [10]. It is thus possible that during initial phases of tumorigenesis, cells become selectively insensitive to TGF- β /Smad-induced growth inhibition, for example, through downregulation of TGF- β receptors or functional inactivation of Smads by other tumor-promoting molecules such as oncoproteins like Evi-1. The interaction between Evi-1 and the TGF- β signaling pathway may predispose the cells to further genetic or epigenetic alterations and promote their malignant phenotype [5].

As one of the candidate oncoproteins, Evi-1 attenuates transcriptional activation and counteracts cellular responses induced by members of the TGF- β family through regulation of TGF- β target genes [12]. In this study, we have shown that Evi-1 suppresses TGF- β -induced growth inhibition in CRC cells. Several mechanisms may account for the inhibitory effects of Evi-1 on TGF- β -induced actions. One possible mechanism is that Evi-1 may interfere with the transcriptional activity of the TGF- β responsive Smad2/Smad3/Smad4 transcription factor complex. For example, we [28] and others [30] have found that Evi-1 acts as a general Smad corepressor to inhibit TGF- β -inducible transcription. Other mechanisms may include promoting the activation of the PI3K/AKT signaling pathway, which is required for Evi-1's effect on inhibiting TGF- β -induced apoptosis [43].

In conclusion, we found that the Evi-1 oncoprotein is overexpressed in human colorectal tumors and cell lines. Using 5' RACE, we have identified a novel transcript of Evi-1 in a subset of human CRC tissues and studied the role of Evi-1 in the regulation of TGF- β signaling and its growth inhibition. Our studies define a role for Evi-1 in human CRC carcinogenesis and reveal a novel mechanism by which human CRC abrogates the growth inhibitory effect of TGF- β and contributes to human colorectal carcinogenesis. It is tempting to speculate that Evi-1 overexpression negates the cells' response to TGF- β signaling and promotes continuous proliferation of altered CRC cells. In this case, targeting Evi-1 gene may restore TGF- β responsiveness and interrupt the malignant transforming process in a subset of human CRCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CRC	colorectal cancer
TGF-β	transforming growth factor- β
Evi-1	ecotropic virus integration site-1
MDS	myelodysplastic syndrome
AML	acute myeloid leukemia
RACE	rapid amplification of cDNA ends
UPM	Upstream Primer A Mix

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Figure 1.

Evi-1 protein expression in human colorectal tissue samples and in human colon cancer cell lines. (A) Five representative pairs of cancer and normal samples, (B) three pairs of adenoma and normal samples, (C) five human colon cancer cell lines were shown for Evi-1 protein expression using immunoblotting, and (D), comparison of Evi-1 protein expression between DLD-1 and CRC cancer and normal tissues. Cell lysates from Cos-1 cells transfected with human Evi-1 were used as a positive control (PC) for Evi-1 protein. β -Actin was used as a loading control. Expected bands of Evi-1 at 145 kDa were detected in human CRC tissues, adenomas, and colon cancer cell lines. The bottom panels are quantification of the relative Evi-1 protein levels compared to the corresponding normal controls (A and B) or arbitrary relative Evi-1 protein levels (C and D). All values are normalized with β -actin. (E) Immunohistochemical staining of human colorectal tissue sections for Evi-1 expression. Paired human colon cancer and normal tissue sections of patient #2 were subjected to immunostaining for Evi-1 protein expression. Evi-1 was primarily stained in the nucleus of epithelial cells of the colon cancer tissue. Incubation without primary antibody was used as a negative control ($\times 200$). N = matched normal mucosal tissue; C = colorectal cancer; A = adenoma.

Figure 2.

Cloning and sequencing of the novel transcript at the 5' end of Evi-1. (A) A schematic representation of the strategy for cloning and sequencing of the novel transcript at the 5' end of the Evi-1 gene. (B) Expression of a major Evi-1 transcript of about 700 bp in human CRCs. 5' RACE was performed on RNA samples prepared from selected pairs of human colorectal tissues using the UPM/P2 primer pair. A major band of about 700 bp was detected in four out of the six cancer samples tested, which was detected in only one of the six normal colorectal tissues. (C) The sequence of the novel exon 1e. (D) Confirmation of expression of the Evi-1e-specific transcript in human CRCs. Regular RT-PCR was performed with RNA samples prepared from human CRC cancer tissues using the Evi-1e-specific primer pair. A 101-bp Evi-1e band was detected in all cancer samples tested. (E) Mapping of Evi-1 exons on chromosome 3. The nucleotide sequence of full-length Evi-1, including all known 5' end sequences was BLASTed against human chromosome 3 to locate the position of the various exons. Note that the Evi-1e exon is located 139.6 kb upstream of exon 1a in an intron of MDS1. The length of each Evi-1 exon is marked on the bottom of the corresponding exon.

Figure 3.

Activity of the Evi-1e promoter. The Evi-1 exon 1e promoter was cloned and inserted into a pGL3 reporter plasmid, and different amounts of Evi-1e/Luc or pGL3 were transfected into MC-26 cells using FuGENE 6 together with pRL-SV40 as described in the Materials and Methods Section. Promoter activity was measured as firefly luciferase activity relative to renilla luciferase activity 24 h after transfection.

Figure 4.

Evi-1 represses TGF- β signaling in colon cancer cells. The indicated amounts of Evi-1 expression plasmid, pSG5-Evi-1, were transiently transfected into MC-26, along with p3TP-Lux and pSV40- β -gal. Twenty-four hours later, cells were treated with TGF- β 1 at 120 pM or vehicle for 12 h in serum-free RPMI 1640 medium. Relative luciferase activity was measured as luciferase activity relative to β -galactosidase activity. * $P < 0.05$ as compared to TGF- β -treated control cells.

Figure 5.

Evi-1 blocks the growth inhibitory effect of TGF- β . (A) Suppression of TGF- β -induced growth inhibition by Evi-1. MC-26 cells were transiently transfected with pSG5-Evi-1 (HA-tagged) or control pSG5 plasmid for 24 h and replated for 6 h. Cells were then treated with TGF- β 1 at 120 pM in serum-free RPMI 1640 medium. Cells were counted on day 1 and day 2 after TGF- β 1 treatment using a hemocytometer, expressed as percentage of cell growth in TGF- β -treated groups versus vehicle control. (B) Similar effects on suppression of TGF- β -induced growth inhibition between Evi-1 and MDS1/Evi-1. MC-26 cells were similarly transfected with pSG5-Evi-1, pSG5-MDS1/Evi-1, or control pSG5 and treated with TGF- β 1 as in (A). Cells were counted 1 d after TGF- β 1 treatment. * $P < 0.05$ as compared to vector control.