Arch Biol Sci. 2019;71(3):393-401 https://doi.org/10.2298/ABS181008020A

TGF-β downregulates CAIII expression via MAPK and PI3K signaling pathways in colon carcinoma and osteosarcoma cells

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Received: October 8, 2018; **Revised**: February 26, 2019; **Accepted**: February 27, 2019; **Published online**: March 25, 2019

Abstract: Identification of cancer-associated genes is critical for developing effective treatments of colorectal cancer (CRC). A limited number of studies have examined the mechanisms and genes underlying CRC. Abnormal transforming growth factor beta (TGF-β) expression was observed at different stages of carcinoma. We examined the effect of cancer-related cytokine TGF-β on carbonic anhydrase (CA) III gene expression in colon cancer HT-29 cells. TGF-β (500 U/mL) downregulated CAIII gene expression at both the mRNA and protein levels. Transient transfection experiments indicated that different CAIII promoter constructs were active in HT-29 cells. TGF-β reduced transcriptional activity of all promoter constructs, indicating that the potential response element for TGF-β-directed transcription lies within the -108/+86 region of the CAIII promoter. According to the non-Smad pathway inhibitory assay, TGF-β downregulated the CAIII gene through mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and phosphoinositide-3-kinase (PI3K) pathways. The same decreasing effect was determined in the Saos-2, osteosarcoma cell line, indicating that the effect of TGF-β on CAIII was not tissue-specific. However, examination of PI3K and MAPK/ERK signaling pathways with suitable inhibitors revealed that the PI3K but not the MAPK/ERK pathway was responsible for TGF-β downregulation.

Keywords: CAIII; TGF-β; transcriptional regulation; colon carcinoma; osteosarcoma

INTRODUCTION

Colorectal cancer (CRC) is a heterogeneous disease with distinct genetic and epigenetic alterations. The mechanisms leading to CRC development, progression and recurrence are complex. CRC is caused by the accumulation of multiple genetic and epigenetic changes. Somatic mutations in genes encoding for adenomatous polyposis coli (APC), B-Raf proto-oncogene, serine/threonine kinase (BRAF), Kirsten rat sarcoma 2 viral oncogene homolog (KRAS), phosphoinositide-3-kinase, catalytic, alpha polypeptide (PIK3CA) and tumor protein p53 (TP53) have been frequently observed in CRCs [1-4]. Apart from mutations in these genes, chromosome instability (CIN) such as aneuploidy, amplification and deletion of subchromosomal genomic regions and loss of heterozygosity (LOH) have been observed in the majority of sporadic CRCs (65-70%). Recent studies [1,5] have also shown that DNA methylation and covalent histone modifications are common in CRC. Downregulation of the tumor suppressor genes caused by hypermethylation of CpG islands in promoter regions is one of the crucial mechanisms in CRC development and progression.

The TGF-β family constitutes a large group of extracellular growth factors that control tumor development [6]. Abnormal TGF-β expression was observed at different stages of colon carcinoma. TGF-β plays a tumor-suppressor role in normal colonic epithelial tissue by activating apoptosis. Interestingly, the tumor-enhancing function of TGF-β is observed in late stages of colorectal carcinogenesis and is often overexpressed. In primary tumors, abnormal expression of TGF-β has been observed to be associated with advanced-stage tumor recurrence. TGF-β can induce neoplastic transformation [6,7,8]. The TGF-β pathway has become an interesting target for novel therapies [9], and thus the

detection of the genes affected by TGF-β and detailed analysis of these genes are very important.

Carbonic anhydrase (CA) enzymes are very important for maintenance of pH balance. Some members, CAIX and CAXII, have been proposed as a cancer biomarker in a number of studies. CAIX was shown to be a potential hypoxic CRC biomarker. It has also been suggested that serum CAIX can serve as a potential tool in CRC clinical practice. CAIV is a novel tumor suppressor in CRC, acting through inhibition of the Wnt signaling pathway by targeting the WTAP-WT1-TBL1 axis [10]. CAIII that has lower hydratase activity than other members of the CA family is involved in acute myeloid leukemia and liver carcinoma. It has also been shown that CAIII in hepatocellular carcinoma provides cells with an invasive character via the focal adhesion kinase (FAK) signaling pathway and/or intracellular and extracellular acidification. Overexpression of CAIII increases the resistance to anticancer drugs such as paclitaxel [11] in rat fibroblast cells [11-13]. These activities suggest a potential therapeutic role of CAIII inhibitors in combination therapy with anticancer medication [13,14]. Moreover, there is nothing known about the link between TGF-β and CAIII in CRC. The underlying mechanisms are not well understood, and requires more in-depth characterizations of CAIII regulation in CRC.

When considering the different processes in which TGF-β is involved, the contribution to the regulation of key genes in different cancer types requires examination. Different studies have shown that several CA family members are important in different types of cancer. In particular, CAIX has been proposed as a biomarker in colon carcinoma and many other cancers [15-20]. CAIV has been shown to be hypermethylated in colon carcinoma [21]. Reduction of CAI, CAII, and CAIII in in HCC (hepatocellular carcinoma) has been demonstrated, and this reduction has been shown to increase tumor cell motility and to contribute to tumor growth and metastasis [22]. However, there is no information on the status of CAIII in colon cancer and its regulation by TGF-β. For this purpose, we investigated the effect of TGF-β on human CAIII expression in human colon and sarcoma cell lines. Our data show that TGF-β reduces the expression of CAIII at mRNA and protein levels and also causes transcriptional repression of the promoter.

MATERIALS AND METHODS

Strategies for cloning human CAIII promoter constructs

Genomic DNA was amplified using primers designed for the 939 bp upstream region of the translation start site of the CAIII gene (Access. No. MF374499.). CAIII promoter primer sequences were as follows: 5'-CTC GAG TTG CAA TCT CTC ATT GTA TCT T-'3, 5'-TTC GAA CAT GGT CGC CTT CCT CCG-'3. The 1025 bp CAIII promoter fragment was cloned into a pGEM-T Easy vector (Promega, USA) with a T:A cloning system. Truncated-deletion mutants of the human CAIII promoter region were prepared by a PCR-based technique. HindIII and XhoI restriction sites as possible cloning sites were inserted into the primers used for PCR amplification; 1025 bp, 785 bp, 322 bp, and 194 bp promoter fragments were designated as P1 (-939/+86), P2 (-699/+86), P3 (-236/+86), P4 (-108/+86), constructs, respectively. All PCR-amplified CAIII promoter fragments were confirmed by Sanger sequencing in Refgen, Ankara, Turkey.

Cell culture, transient transfection assays, and luciferase/SEAP assays

Human hepatoma cell line (Hep3B), human osteogenic sarcoma cell lines (MG-63 and Saos-2), human prostate cancer cell lines (PC3 and DU145), human breast carcinoma cell line (MCF-7), human colon carcinoma cell line (HT-29), human endometrial adenocarcinoma cell line (Ishikawa) and human umbilical vein endothelial cells (HUVEC) were used. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, USA) containing 2 mM L-glutamine and 10% heat-inactivated fetal calf serum (56°C for 1 h). Cells were grown in the incubator containing 5% CO_2 at 37°C. Cell viability was followed using trypan blue [23].

Transient transfection assay was performed by the calcium-phosphate precipitation method as described [23,24]. For the basal activity of constructs, cotransfections were carried out in 12-well plates (250000 cells per well) with 0.5 μg of all promoter constructs and 0.5 μg SEAP control plasmid. SEAP control vector was used as an internal control in each transfection. Secreted luciferase and alkaline phosphatase (SEAP)

activities were measured using the luciferase reporter systems (Clontech, USA) in Luminometer (Thermo, USA). Luciferase activity was normalized to the SEAP values. pMetLuc-control vector was also transfected in HT-29, and Saos-2 cells as a positive control. pMetLuc-Reporter and SEAP reporter vectors served in the transfections as a negative control.

To determine the effects of TGF-β on the CAIII transcriptional activity, cells were grown with bovine serum albumin (BSA; 0.1%) after transfection. TGF-β (500 U/mL) cytokine was administered to the cells at different time intervals. The medium was collected, and luciferase and SEAP activity were measured at each time point (48 and 72 h). Luciferase activity was normalized to the SEAP values. Each transfection experiment was performed in triplicate and independently at least three times.

MTT Assay

The MTT assay was used for the determination of cytotoxicity of TGF-β [25] as follows: 15000 cells/well were plated in 96-well plates after trypan blue application. Five hundred U/mL TGF-β were applied to wells at different time intervals (24 to 72 h) in DMEM with BSA. At each time point, 0.5 mg/mL MTT was added into each well and the cells were further incubated for 4 h at 37°C in a medium containing 5% CO_2. After 4 h, the medium was removed and the formazan crystals were dissolved in isopropanol containing 0.004 HCl. Absorbance was measured at 550 nm. The results were compared with the absorbance values obtained from the non-treated group. MTT experiments were independently performed at least two times.

RNA isolation and verification of cDNAs

Cell pellets were used for RNA isolation. Total RNA was obtained from pellets using an RNA isolation kit (Thermo, USA). RNAs were stored at -80° C. RT-PCR was used in two separate steps. In the first step, reverse transcriptase (RT) was used for cDNA synthesis. In the second step, amplification of the gene region and polymerase chain reaction (PCR) were carried out using the cDNA obtained from the first step with gene-specific primers. The primer sequences for CAIII were as follows: 5'-TGG GAA GAC CTG CCG AGT TGT ATT TGA TG-'3 and 5'-TTG ATA GGC TGT GAG GTC GCC AGT TGC-3' were used for RT-PCR; 5'-ACC ACT GGC ATG AAC TTT TCC CAA A-'3 and 5'-TCA GAG CCA TGA TCA TCC GAA GAG C-3' primers were used for RT-PCR for CAIII amplifications. Internal controls primers were: 5'-TCC CTG GAG AAG AGC TAC GA-'3 and 5'-AAG AAA GGG TGT AAC GCA AC-'3 for β-actin; 5'-TTT CTG GCC TGG AGG CTA TC-'3 and 5'-CAT GTC TCC ATC CCA CTT AAC T-'3 for the β-2 microglobulin (B2M) gene. The PCR products were run on a 1% (w/v) agarose gel and analyzed in the gel-doc system (UVP, DigiCam 130). Densitometric analyses were performed with the Image J image processing program, and the expression of CAIII was normalized relative to β2M expression.

Quantitative real time-PCR (qRT PCR)

qRT PCR was used for the expression of CAIII mRNA under cytokine and cytokine-free conditions. In this study, 96-well plates were loaded in a final volume of 10 μL by adding 1 μL cDNA, 5 μL SYBR Green PCR master mix, 0.5 μL forward and reverse primers (50 ng/μL) and 3 μL dH_2O. Three replicates of CAIII were performed for each cDNA sample and three replications were performed with β2M primers as an internal control. The results were analyzed according to the LIVAC method [26]. Statistical analyses were performed using the Minitab 14 program.

Immunoblotting

Proteins were extracted and prepared from cell pellets as described [23,27]. The 30-μg protein extract was applied on a 10% polyacrylamide gel and transferred to PVDF membranes (Millipore, USA). The membranes were blocked in solution of Tris-buffered Saline (TBS) containing 5% (w/v) fat-free milk-powder and 0.1% (v/v) Tween-20 to prevent non-specific binding. The membranes were first treated with primary antibody CAIII (sc-53425, Santa Cruz, USA) for 1 h at room temperature, and after three washes with a secondary antibody (HRP-conjugated anti-mouse antibody) for 1 h. The membranes were treated with chemiluminescence substrate (Pierce, USA) and transferred to films. Image J Software was used to analyze the bands. The amount of CAIII protein was normalized against the amount of β-actin protein.

Treatment of cells with inhibitors

Wortmannin, a PI3K inhibitor (99515), and PD98059, mitogen-activated protein kinase 1 (MEK-1) inhibitor (9900S), were obtained from Cell Signaling, USA and were used for inhibition of different cellular signaling pathways. Cells were grown in 6-well plates and were then treated with 1 μM wortmannin and 10 μM PD98059 for 30 min. RNA was isolated from the cells, and the changes in CAIII levels were evaluated by RT-PCR [28]. Statistical analyses were performed using the Minitab 14 program.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA). A probability (P) of 0.05 or less was deemed as statistically significant.

RESULTS

Analysis of the CAIII mRNA levels in different cell lines and the effect of TGF-β on HT-29 cell proliferation

There is limited knowledge of the expression of CAIII in cancer cells. Therefore, our initial aim was to determine the expression profiles of CAIII in different cancer cells, as follows: the human breast cancer cell line (MCF-7), human colon carcinoma cell line (HT-29), human hepatoma cell line (Hep3B), human prostate cancer cell lines (PC3 and DU145), human osteogenic sarcoma cell line (MG-63), human umbilical endothelial cell line (HUVEC), human endometrial adenocarcinoma cell line (Ishikawa) and human osteogenic sarcoma cell line (Saos-2). RT-PCR was performed using 1 μg of RNA after optimizing for non-saturated cycle conditions; β2M was used as an internal control. The resulting bands were analyzed densitometrically. Maximum CAIII mRNA levels were determined, revealing an increase in HUVEC (~1.71-fold), Saos-2 (~1.4-fold) and MG-63 (~1.49-fold) cells, regardless of their origin., HT-29 (~0.86) and ISC (~0.83-fold) indicated the high expression level compared to other cells (Fig. 1A).

Most tumor cells in the neoplastic stage are resistant to TGF-β proliferation inhibitory signals. However, TGF-β might exhibit a growth inhibitory effect

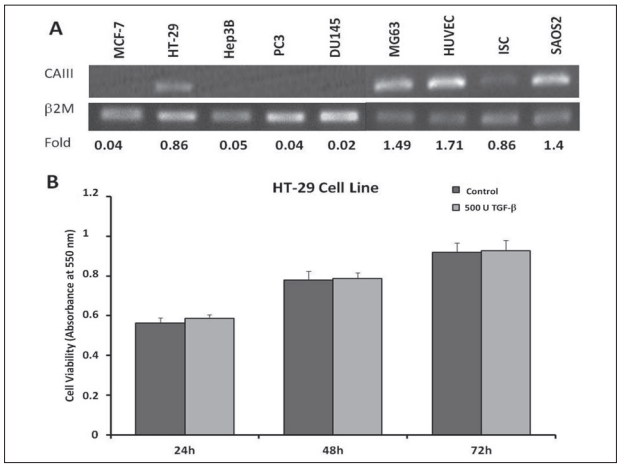

Fig. 1. CAIII is expression in different cancer cell lines and the cell proliferative effect of TGF-β in HT-29 cells. **A** – Cell lines used in our study: human breast cancer cell line (MCF-7), human colon carcinoma cell line (HT-29), human hepatoma cell line (Hep3B), human prostate cancer cell lines (PC3 and DU145), human osteogenic sarcoma cell line (MG-63), human umbilical endothelial cell line (HUVEC), human endometrial adenocarcinoma cell line (Ishikawa), human osteogenic sarcoma cell line (Saos-2). CAIII mRNA expression was examined by RT-PCR. B2M was used as an internal control. PCR results were visualized on an agarose gel; the bands were analyzed densitometrically. **B** – MTT assay used to determination the cytotoxicity of 500 U/mL TGF-β; 15000 cells/well were plated in 96-well plates after trypan blue application; 500 U/mL TGF-β were applied to the wells at different times (24 to 72 h) in DMEM with BSA. The absorbance values obtained from the untreated group were compared. MTT experiments were performed in six replicates and independently at least two times. The results were statistically tested with ANOVA.

through TGF-β receptor I and TGF-β receptor II at the beginning of tumorigenesis. It has been shown that the inhibitory effect of TGF-β on cells leads to loss of TGF-β receptors I and II) [29]. In our study showing TGF-β-related CAIII tissue expression, human colon carcinoma (HT-29) cells were used as model cells. Therefore, we first determined the effect of 500 U/mL of TGF-β in HT-29 cells by the MTT assay. We did not observe any statistically significant growth inhibitory or proliferative effect of TGF-β in HT-29 cells (Fig. 1B).

TGF-β downregulates the expression of CAIII gene in HT-29 cells

In our study, four different CAIII promotor constructs were used as follows: P1: 1025 bp (-939/+86), P2: 785 bp (-699/+86), P3: 322 bp (-236/+86), P4: 194 bp (-108/+86) (Fig. 2A). The basal activities of CAIII promotor con-

structs in HT-29 cells were higher than in Saos-2 cells (Fig. 2A). This result shows that CAIII is more transcriptionally active in colon carcinoma than in osteosarcoma cells. However, the smallest promoter construct, P4 (194 bp (-108/+86)), had a higher basal activity in Saos-2 cells than in HT-29 cells. This showed that cell type-specific regulatory elements regulated CAIII promoter activity in HT-29 and Saos-2 cells (Fig. 2A).

To the best of our knowledge, nothing is known about cytokine-mediated CAIII expression in CRC. To investigate the role of TGF-β in CAIII gene expression, CAIII promoter fragments were transiently transfected to observe transcriptional regulation of CAIII by TGF-β in HT-29 cells. Luciferase and SEAP activities were measured 48 h after transfection. We observed that TGF-β decreased the transcriptional activity of all CAIII promoter constructs (Fig 2B). Cells were treated with serum-free media containing 0.5% BSA for 24 h and then with 500 U/mL TGF-β for 48 h. Total RNA was isolated from these cells, and RT-PCR studies were performed with CAIII and β2M as an internal control. As shown in Fig. 2C, the concentration of 500 U/mL TGF-β lowered the level of CAIII mRNA 100-fold compared to the control at all time points.

Immunoblotting was used to observe the effect of TGF-β at the protein level. Cell lysates obtained from cytokine-treated and untreated cell pellets were subjected to Western blotting with rabbit anti-CAIII antibody or β-actin antibody. The level of CAIII protein was decreased, and densitometric analysis showed that application of 500 U/mL TGF-β at 24 h lowered the level of CAIII protein 1.3-fold (Fig. 2D).

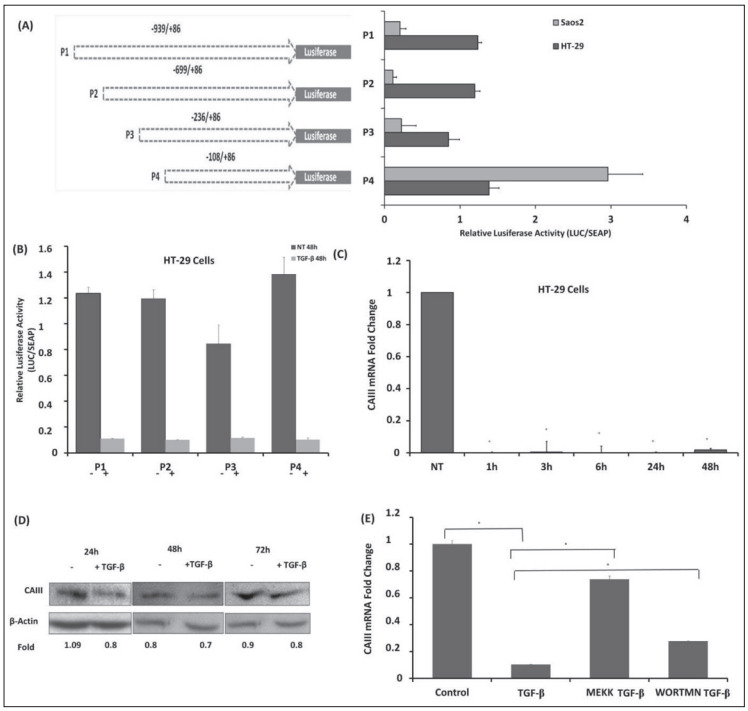

Fig. 2. Transcriptional regulation of CAIII by TGF-β. **A** – Basal transcriptional activities of human CAIII promoter in colon cancer and sarcoma cells. HT-29 and Saos-2 cells were transiently transfected with the human CAIII promoter fragments P1: 1025 bp (-939/+86), P2: 785 bp (-699/+86), P3: 322 bp (-236/+86), P4: 194 bp (-108/+86). pMetLuc reporter vector was used as a negative control, and pMetLuc control vector was used as a positive control. Luciferase activity and SEAP activities were analyzed at 48 h. Luciferase activity was normalized to SEAP activity (Luc/SEAP). The left side of the figure shows representative human CAIII promoter fragments, the right side shows basal activities. **B** – TGF-β stimulation decreased human CAIII gene promoter activity in HT-29 cells. In transient transfection experiments, P1, P2, P3 and P4 CAIII promoter fragments were used. Basal transcriptional activities were compared with the activities of promoter fragments treated with 500 U/mL TGF-β. Luciferase activity was analyzed at 48 h. **C** – CAIII mRNA was lowered in HT-29 cells treated with TGF-β (500 U/mL). Groups that were not administered the cytokine were used as controls. CAIII mRNA levels were measured by qRT-PCR and normalized to human B2M as described. Experiments were repeated 3 times. The results were analyzed with ANOVA (one-way). The asterisk indicates that CAIII mRNA levels were significantly lowered by TGF-β (*P≤0.05). **D** – CAIII protein expression was analyzed by Western blotting. Total cell lysates were prepared from TGF-β treated and untreated cells at 24, 48 and 72 h. The blot was probed with antibodies against CAIII (top) and β-actin (bottom). Bands were analyzed densitometrically by Image J analysis. **E** – Analysis of different inhibitor applications on HT-29 cells by Real-Time PCR.

MAPK and PI3K pathways are involved in TGF-β repression of CAIII

TGF-β represses CAIII expression at the mRNA and protein levels. The pathway responsible for this reduction was examined using specific inhibitors. TGF-β binds to membrane-bound TGF-β receptors I and II, resulting in the activation of the downstream effectors, STAT3, PI3K [30] and MEK pathways. Five hundred U/mL TGF-β was decreased by CAIII mRNA at 6 h. PD98059 and wortmannin partially increased the

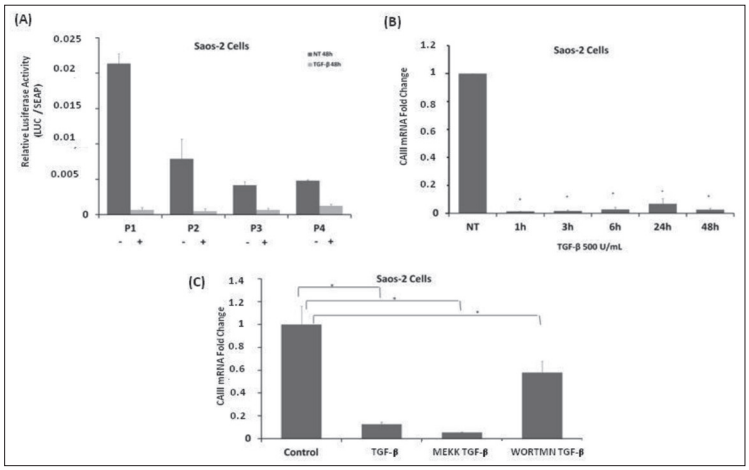

Fig. 3. CAIII expression in Saos-2 cells. **A** – TGF-β administration transcriptionally lowered human CAIII gene promoter activity in Saos-2 cells. Saos-2 cells were transiently transfected with the human CAIII promoter fragments P1: 1025 bp (-939/+86), P2: 785 bp (-699/+86), P3: 322 bp (-236/+86), P4: 194 bp (-108/+86). The transfected cells were incubated with 500 U/mL of TGF-β for 48 h; cells without cytokine administration were used as controls. Luciferase activity was normalized to SEAP activity. **B** – TGF-β (500 U/mL) lowered CAIII mRNA in the Saos-2 cell line. TGF-β was administered to cells maintained at serum starvation. After 48 h, cell pellets were obtained and the RNA was isolated. CAIII mRNA levels were measured by qRT-PCR and normalized to human B2M. Experiments were repeated 3 times. The results were analyzed with ANOVA (one-way). The asterisk indicates that CAIII mRNA levels were significantly reduced by TGF-β (*P≤0.05).

reduction of TGF-β-directed CAIII mRNA expression (Fig. 2E). We concluded that TGF-β reduced CAIII mRNA expression via the MEK-1 and PI3K pathways in colon cancer cells.

TGF-β also suppressed CAIII expression in different cancer cells

Expression analysis of CAIII mRNA showed that the osteosarcoma Saos-2 cell line expressed a high level of CAIII. Saos-2 cells were used in order to determine whether the contribution of TGF-β to CAIII expression in colon cancer cells was tissue-specific. Studies in this cell line examined transcriptional activity and the level of mRNA. CAIII promoter fragments were transfected into Saos-2 cells, which were then treated with 500 U/mL TGF-β. As shown in Fig. 3A, the activity of all promoter fragments was reduced in this cell line. To determine the effect of TGF-β on the level of mRNA, total RNA was isolated in transfected and control Saos-2 cells. CAIII mRNA levels displayed a 100-fold reduction after 48 h (Fig. 3B).

As for the pathway inhibition assay, we determined that the TGF-β-directed reduction of CAIII expression uses a different pathway in Saos-2 cells. Treatment with 500 U/mL TGF-β decreased the expression of CAIII mRNA after 6 h. Wortmannin partially recovered the level of CAIII expression, while the MEK inhibitor did not affect the CAIII expression (Fig. 3C). These results indicate that TGF-β represses CAIII mRNA expression via the PI3K pathway in Saos-2 cells.

DISCUSSION

CA enzymes are Zn ion-containing metalloenzymes that catalyze the reversible reaction of CO_2 and HCO_3^-, which is very important for physiological and pathophysiological processes. Recent studies have suggested that CAIX and CAXII contribute to cancer progression and can be used as biomarkers in many cancer types. Aberrant expression of CAI, II, CAIV, IX, XII, and XIII has been reported in CRC [15-21,31,32], and CAIX is used as a biomarker for colon cancer. CAIV has been identified as a tumor-suppressor gene in colon cancer. In another study, the expression of CA I and II was shown to be important for aggressive distant metastasis in the colon and rectum [33]. However, there is a need to clarify the regulation of each enzyme when different members of the family are thought to play different roles in the cancer process. CAIII is involved in certain cancers such as acute myeloid leukemia and liver carcinoma. Overexpression of CAIII increases the resistance of anticancer medication such as paclitaxel [12]. It was shown in this study that overexpression of CAIII in hepatocellular carcinomas provides the cells with an invasive character through the FAK signaling pathway. However, the role of CAIII in CRC was not elucidated.

In the present study, the role of TGF-β in the regulation of the CAIII gene was investigated. Studies have emphasized the heterogeneity of colon cancer cell lines in response to TGF-β due to the presence of different TGF-β receptors [33]. Herein we revealed the effect of TGF-β on colon cancer cells. This protein exerts its

functions by binding to TGF-β type I, type II and type III receptors. It was shown that the inhibitory effect of TGF-β on cells leads to the loss of these cytokine receptors (receptor I and receptor II) at the onset of CRC. The TGF-β signal in CRC has not been adequately characterized due to the high mutation rates in the TGF-β signaling pathway in human colon cancer cell lines [30]. In our study, TGF-β did not show any statistically significant cytotoxic effects in the related colon cancer model of HT-29 cells. However, TGF-β treatment led to a dramatic decrease in CAIII mRNA and protein expression in HT-29 cells. This decrease was regulated at the transcriptional level, and it could be related to the defense function of CAIII, which was recently reported [34]. Results of work on the liver and skeletal muscle suggest that CAIII can function as a scavenger of reactive oxygen species (ROS) [12,35,36]. In fact, CAIII protein has lower hydratase activity than other members of the CA family. In addition, this protein is more resistant to sulfonamides, which are specific CA inhibitors. Therefore, it is thought that CAIII is also involved in other functions in the cell [37-42]. CAIII carries two reactive sulfhydryl groups that are linked to glutathione by disulfide bonds [43,44]. This S-glutathionylation reaction is probably an important component of some sort of cellular defense mechanism that prevents irreversible oxidation of proteins [43,44]. It has also been shown that CAIII plays an antioxidant role in skeletal injury and protects cells from hydrogen peroxide-induced apoptosis [36]. In this respect, TGF-β might downregulate CAIII gene expression to prevent the cell from protecting itself. Thus, CAIII can be a candidate for a tumor-suppressor gene, like the CAIV gene. The downregulation of CAIII by TGF-β is regulated at the transcriptional level, indicating that it is effected via intracellular pathways. Pathway inhibition studies showed that TGF-β uses the MEK and PI3K signaling pathways. TGF-β regulates gene expression via Smad-dependent and Smad-independent routes [29]. TGF-β uses MEK and PI3K signaling pathways through the non-Smad-dependent mechanism in CRC model cells.

To test whether downregulation of CAIII by TGF-β is tissue-specific, osteosarcoma Saos-2 cells were used for TGF-β treatment. The TGF-β superfamily is involved in virtually every aspect of osteoblast cellular activity. Osteoblast differentiation is regulated by the TGF-β signaling pathway [45]. Studies have demonstrated that members of the CA family of enzymes have also been reported in osteoblasts. CAIX expression was shown to be associated with higher grade tumors, metastasis and poor prognosis of osteosarcoma patients [46]. Additionally, CAVIII, which does not possess enzymatic activity due to lack of zinc-binding histidine residues, was overexpressed in more aggressive types of human OS cells [47]. Similarly, the same decreasing effect was obtained in the osteosarcoma model, Saos-2 cells. Unlike HT-29 cells, TGF-β used differential pathways in Saos-2 cells. TGF-β repressed CAIII mRNA expression only via the PI3K pathway in Saos-2 cells.

Redox imbalance or oxidative stress results from increased production of reactive oxygen or nitrogen species and a reduced antioxidant capacity [48]. Results obtained in the liver, skeletal muscle and NP (nucleus pulposus) cells suggest that CAIII can function as an oxyradical scavenger [12,34-36]. CAIII protects NP cells from oxidative stress-mediated cell death, mediated by Cas3 [36]. On the other hand, TGF-β has been shown to increase ROS production and suppress the antioxidant system, thereby inducing oxidative stress or redox imbalance [48].

To conclude, in both colon cancer and osteosarcoma cells, TGF-β that increased ROS in the cells caused a decrease in CAIII expression that played a role in the mechanism of cell protection from ROS, contributing to the process of carcinogenesis.

Funding: This work was supported by the Turkish Research Council (TUBITAK) project (TBAG 112T669).

Acknowledgments: The authors would like to thanks Dr. Kenneth Brown and Dr. Dipak Ramji University of Cardiff, UK for the human osteogenic sarcoma cell lines (MG-63 and Saos-2) and Human hepatoma cell line (Hep3B) The authors would like to thank Dr. Kemal Sami Korkmaz, Ege University (EBILTEM) for the prostate cancer cell lines (PC3 and DU145) and human breast carcinoma cell line (MCF-7). The authors would like to thank the Culture Collection of Animal Cells, Foot and Mouth Disease Institute, Ankara (HUKUK) for the human colon carcinoma cell line, HT-29. The authors also wish to thank Dr. Ayhan Bilir, Istanbul University for the Human endometrial adenocarcinoma cell line (Ishikawa) and human umbilical endothelial cell line (HUVEC).

Author contributions: SAT and FK designed the research; DOA and SAT performed the experiments; SAT and FK checked the results; SAT wrote the paper. FK checked the language of the paper.

Conflict of interest disclosure: All authors claim no conflict of interest.

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