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ORIGINAL ARTICLE

Transcriptional activation of Carbonic Anhydrase III (CAIII) mediated by SP1

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Abstract

Background Carbonic anhydrase III (CAIII) is among the least characterized carbonic anhydrase isoforms, particularly in cancer. Although CAIII has been suggested to contribute to cellular antioxidant defense due to its high cysteine content, limited data exist regarding its expression patterns and transcriptional regulation in malignancies. Previous studies have shown that CAIII can be regulated by hypoxia and signaling pathways such as PI3K and MAPK/ERK; however, its regulation under normoxic conditions remains largely unknown.

Methods and Results Here, we investigated the transcriptional regulation of the human CAIII gene. In silico analysis demonstrated high sequence conservation within the proximal promoter region of CAIII among human, mouse, and rat, and no CpG island was detected. Promoter deletion assays identified the −236 to +86 region as the core active promoter, which contains multiple SP1 binding sites. Overexpression of SP1 significantly increased CAIII mRNA and protein levels. Transient transfection assays further confirmed that SP1 enhances the activity of CAIII promoter constructs. Electrophoretic mobility shift assays demonstrated direct binding of SP1 to the CAIII promoter, while mutation of SP1 binding elements abolished DNA–protein complex formation, confirming the specificity of this interaction.

Conclusions This study provides the first detailed characterization of the human CAIII promoter and identifies SP1 as a critical transcriptional regulator of CAIII through direct promoter binding. These findings provide new insights into the molecular regulation of CAIII and establish a foundation for future studies exploring its biological and pathological roles.

Keywords Carbonic Anhydrase III · Promoter · SP1 · Oxidative stress · Transcriptional regulation

Introduction

Human Carbonic Anhydrases (hCAs) belong to the α-class of CA family with fifteen isoforms that differ in cellular localization, tissue distribution, catalytic activity [1–4]. CAs are associated with different physiological processes, and abnormal expression levels and activities have been implicated with various pathological conditions [5–11].

CAIII is one of cytosolic CA isoforms which is mainly present in skeletal muscle since it has a high oxygen consumption rate condition. Therefore, they are involved in cell defence processes against oxidative damage [1, 12–14]. The expression profile of CAIII has been determined at lower levels in other tissues [15–18]. Cytosolic CAs possess a single cysteine whereas CAIII has 5 cysteine residues. Thus, CAIII inhibited apoptosis in NIH/3T3 cells and H_2O_2-stressed mature osteocytes CAIII was also involved in protecting cells from hypoxic stress, along with in diminishing ROS production [19–21]. High CAIII expression was implicated with osteoblast differentiation. In addition, Evi1 overexpression, a transcription factor in cancer progression, led to the decrease of CAIII transcription in Rat cells [20, 22]. Even though several studies suggest the role of CAIII in cellular defence mechanisms against oxidative stress, limited knowledge is known on the processes involved in this behavior. One of the studies from our group demonstrated that the hypoxic condition upregulated CAIII gene expression by direct binding to HRE elements present in the promoter region in prostate models [23]. However, there

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is virtually no information available on the transcriptional regulations of CAIII under the normal oxygen level. In the present study, we determined the CAIII promoter region with putative SP1 binding sites along with GC box-binding elements by bioinformatics methods. In order to determine the functional role of SP1 on the CAIII promoter activity, SP1 overexpression was carried out. SP1 overexpression resulted in the increase CAIII mRNA and protein expression. In order to functionally dissect promoter, SP1 overexpression was analyzed with truncated deletion constructs of CAIII promoter. SP1 activates at least three constructs indicating that minimal SP1 responsive site is located − 236/+86 region. EMSA analysis of this region revealed SP1 binds to multiple sites in this region indicating that Sp1 may play a predominant role in the regulation of CAIII gene activation.

Materials and materials

Cell culture

Human prostate cancer cells (PC3), Human colon cancer cells (HT-29), and osteosarcoma cells (MG-63 and Saos-2) were cultivated in DMEM (Invitrogen) with 2 mM L-Glutamine and 10% Fetal Calf Serum (Invitrogen) in a humidified atmosphere with 5% CO_2 at 37˚C. Cell viability was determined by trypan blue exclusion.

Transient-transfection and luciferase and SEAP assays

250.000 cells/well were plated out in 12-well plate. For basal activities, transient transfection assay was performed with 0.5 µg promoter constructs and pSEAP plasmid DNA using a modified-Calcium Phosphate precipitation method [24]. pSEAP vector was used as a transfection efficiency control. Four truncated CAIII Reporter plasmids, P1 (-939/+86), P2 (-699/+86), P3 (-236/+86) and P4 (-108/+86), were constructed by PCR-based approach reported [25]. After transfection, reporter gene expression was assessed from medium at 48 h and 72 h using Ready-To-Glow™ Secreted Luciferase Reporter Systems (Clontech) as described in [26]. Luciferase activity was normalised against SEAP activity. As a positive control pMetLuc control vector were transfected into HT-29 and Saos-2 cells. To analyse the effect of overexpression of SP1 transcription factor on CAIII promoter activity, SP1 expression plasmids were co-transfected with promoter constructs. and pMetluc reporter and pCDNA 3.1 vectors were also transfected in every transfection as negative control.

qRT- PCR

Total RNA was extracted from cells using RNA isolation kit (Thermo) according to the manufacturer's instructions. Firstly, 1 µg of total RNA was revers-transcribed with reverse transcriptase using oligo dT primers (Thermo). qRTPCR was carried out SYBR® Green PCR Master Mix with gene-specific primers (*supp Table 1*). For normalisation of qRTPCR, hß2 microglobulin was used. qRT-PCR results were analysed according to the LIVAC method [27]. Statistical analyses were carried-out with the Minitab 14 program. p values ≤ 0.05 were considered statistically significant. All data are expressed as mean ± SD.

Immunofluorescence microscopy

Immunofluorescence labeling was performed on PC3 and Saos-2 cells. 125 × 10^3 cells/ well were seeded in 24-well plates. Immunofluorescence staining was carried out according to Alper et al., 2015. Cells were briefly cultured on cover slips for 24 h. Cells were fixed for 15 min at 25 °C with 4% paraformaldehyde in PBS. Anti-CAIII (Invitrogen, Carlsbad, CA, PA525977) polyclonal antibody was treated with the cells for an overnight period at + 4 °C in a 1/50 dilution. The cells were then treated for 1 h at 25 °C with Alexa Fluor 488 secondary antibody and stained with DAPI following the incubation of the second antibody. After then, the cells were examined with an Olympus microscope [28].

Western blotting

PC3 and Saos-2 were plated in 25 cm^2 flask prior to one day. Transient-transfection was carried out with 10 µg of *SP1* expression vector. Parallel controls were transfected with control empty vector. 24 h later, protein extraction and concentration was carried out as described in [29]. 50 µg of total protein extracts were resolved in 10% SDS-PAGE and transferred onto PVDF membrane (Millipore, USA). The membrane was treated with CAIII primary antibody (Invitrogen, Carlsbad, CA, PA525977) (1:200) in blocking buffer for ON for CAIII detection. After incubation, a secondary anti-mouse peroxidase-conjugated antibody (Abcam, ab6708) (1:5000) was incubated for 1 h at RT. Anti-β actin polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA sc47778) were used to probe the membrane as a loading control. Following treatment, the membrane was cleaned and developed using a Thermo ECL kit to produce enhanced chemiluminescence. Using ImageJ 1.38x software, the intensity of bands belonging to distinct proteins was assessed [14]. Protein concentration was calculated as the ratio of each band's intensity to the -β actin bands. Gel and Blot raw images are given in Supplementary Figure-1.

Electrophoretic Mobility Shif Assay (EMSA)

Nuclear extracts of Saos-2 and PC3 cells were obtained using the method prepared by Poyrazlı et al. [30]. To analyze the SP1 binding sites in the CAIII promoter region, 3 different regions were selected: (-181/-153), (-158/-134) and (+ 28/+62). Oligonucleotides probes were obtained from MacroGen and shown in Supp Table 1. The biotin 3' end DNA Labelling Kit (Pierce, Cat. No: 89818) was used for labelling of the oligonucleotides according to the protocol provided. The binding reactions include 3 µL poly (dI: dC), 5 µg nuclear extracts, 2 µL binding buffer, 50 fmol biotin-labelled probes, 1 µL $MgCl_2$ and 1 µL KCl. The mixture was combined with 1000 times unlabelled probes for the competition reactions. The reaction was incubated for 60 min at RT. The binding samples were loaded on a 6% native polyacrylamide gel, transferred on to nylon membrane and then fixed by UV cross-linkingembrane. Protein-DNA complexes were detected by chemiluminescent detection methods using streptavidin-horseradish peroxidase (Pierce, Cat. No: 21132).

Results

Human CAIII promoter is transcriptional active in Saos-2 and HT-29 cells

To identify regulatory elements in the human CAIII gene, a 1027 bp promoter sequence was cloned, sequenced, and deposited in the GenBank database under accession number MF374499.1. Transcriptional start Site (TSS) and Translation Start codon (TSC) were indicated in the 1027 bp of the CAIII promoter sequence (Fig. 1A). 5'UTR region is about 86 bp. Several potential TF recognition sites, such as Kruppel-like transcription factors, Myo-D, activator protein 2, and activator protein 4 (Data not shown) were identified using MathInspector program. The most interesting feature of the promoter is to possess many consensus sequences of GC-box factors SP1/GC (Fig. 1A and B). There are 9 putative SP1 binding elements. To determine the conserved regions of CAIII promoter, 429 bp of human, rat and mouse CAIII promoters were aligned using BioEdit program. Aligned region of CAIII promoter site showed the high similarity in all species (Fig. 1B). CAIII promoters form three species were compared and the results showed 73% identity between rat and human sequences, 85% identity between rat and mouse sequences, and 75% identity between mouse and human sequences. (Fig. 1A). The human CAIII gene promoter does not possess classical TATA box but includes many putative transcription factor binding elements as well as classical GC box (Although the TATA box and GC boxes are conserved between mouse and rat CAIII promoter, Fig. 1A and B).

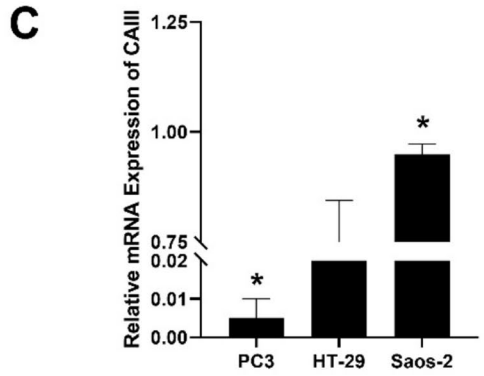
To determine the CAIII mRNA expression pattern, we used prostate cancer cells (PC3), colorectal cancer cells (HT-29), and osteosarcoma cells (Saos-2). CAIII mRNA expression was analyzed using cDNA obtained from the selected cell lines (Fig. 1C). Using qRT-PCR analysis, we determined that CAIII had the highest expression in Saos-2 and HT-29 cells and showed moderate expression in PC3 cells. Immunofluorescence analysis was used to determine the same effect at the protein level. With immunofluorescence analysis, we both determined the location of human CAIII protein and determined the protein expression in the two cell lines showing the lowest and highest mRNA expression. According to this analysis, CAIII was expressed in PC3 and Saos-2 cells and was found in both the nucleus and cytoplasm (Fig. 1D).

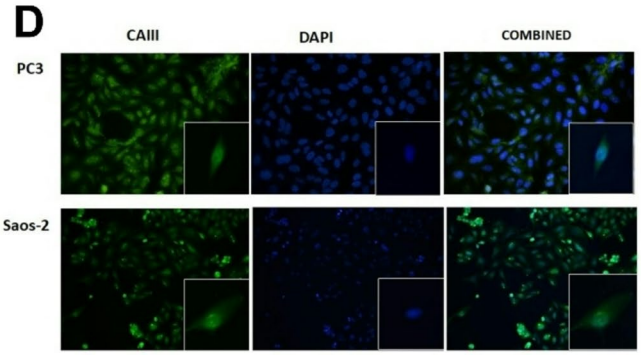
To determine the minimal promoter region for human CAIII gene promoter, four truncated deletion plasmids were constructed in pMetluc vector with progressively larger deletions from the 5' end of the promoter containing –939/+86, -699/+86, -236/+86, and −108/+86 (Fig. 1E). All promoter constructs are active in human colon carcinoma cells, HT-29 cells and human osteosarcoma, Saos-2. 194 bp fragment P4 (-108/+86) displayed the most active promoter activity in both cells (Fig. 1E). In addition, a central functional promoter of the human CAIII gene is located within the −108/+86 region. However, other promoter fragments are more active than the activities of fragments in HT-29 cells (Fig. 1E). The difference of the promoter activity in Saos-2 and HT-29 cells might be resulted from the presence of cell type-specific regulatory elements.

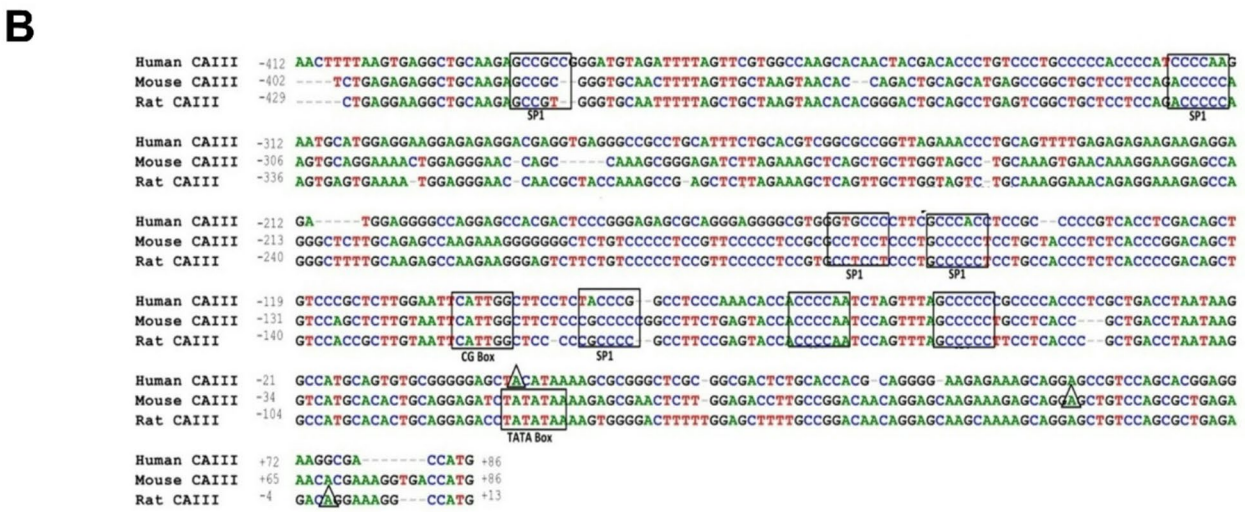
Overexpression of SP1 transcription factor upregulates CAIII gene expression

The −941/+86 sequence of the CAIII promoter was analyzed using potential transcription factor binding analysis. Multiple SP1 binding elements were identified on the CAIII promoter, and these binding sites were specified on the −941/+86 sequence of the CAIII promoter (Fig. 1A). Due to the presence of numerous SP1 binding sites on the CAIII promoter, we hypothesized that the human CAIII gene might be regulated via the SP1 transcription factor. Therefore, to demonstrate whether the SP1 transcription factor regulates CAIII transcription, we prepared four different CAIII promoter constructs: -939/+86, 699/+86, -236/+86, and −108/+86. The SP1 binding sites and their numbers on these four different CAIII promoter constructs are schematically shown (Fig. 2A). To analyze the activity of the schematically represented SP1 binding sites in four different contracts, Saos-2 cells were co-transfected with the


A
-941 TTGCAATCTCTCATTGTATCTTGGAGACCTAGACCACAGGTTTTAAGTAA -890
CAIII Pro Forward
-891 ATAGGACTCCATTTTATGTTTGGGGCCTTCCAATCCTTCAGAAAAATCTC -840
-841 GAAATTTCAAGAACAGAGGGACAGCTCAGGGAGGGGGGCTAAATCAATCC -790
-791 CAGTCTCCAGCTCCGCGTGAACCTGGGATCCAGACATCTCCTGGATATCT -740
-741 GGCGCTCTCTGAGATCCAGCCCTCGGTTTCCTGCAAGCCAAAAGGATCCG -690
-691 CTAGGTTGGGAAATGCCGCCTGGTGCGATTCCAGAAAGGGCCATCTCTTT -640
GC Box Factor SP1
-641 AAGGATAACGGACTTGGAGGGCCTCTTCACCCTCTCACCCTGCTCCGGAC -590
-591 CCTTTCTTCCCATCCTTTGCTCCTAGGATTTTACATGTTGCCTGCAAAGG -540
-541 GAGTCAAACTTAGGGGGCAGGCAAACAAACGAGTTCTTTCCAGCCTCTGT -490
-491 AACCGGATCGCTAGAGCGAAATAAACTCGCACAAGTGTCCAGAGATCGTA -440
-441 GCCAGACAGCCAGCCTGCGCTTGAAGCAACTTTTAAGTGAGGCTGCAAGA -390
GC Box Factor SP1
-391 GCGGCCGGGATGTAGATTTTAGTTCGTGGCCAAGCACAACTACGACACCC -340
GC Box Factor SP1
-341 TGTCCCTGCCCCCACCCCATCCCCAAGAATGCATGGAGGAAGGAGAGAGG -280
-291 ACGAGGTGAGGGCCGCCTGCATTTCTGCACGTCGGCGCCGGTTAGAAACC -230
-241 CTGCAGTTTTGAGAGAGGGGAAGAGGAGATGGAGGGGCCAGGAGCCACGA -190
-181/-153 probe
-191 CTCCCGGGAGAGCGCAGGGAGGGGCGTGGGTGCCCTTCGCCCACCTCCG -140
GC Box Factor SP1
GC Box Factor SP1
-158/-134 probe
-141 CCCCCGTCACCTCGACAGCTGTCCCGCTCTTGGAATTCATTGGCTTCCTC -90
-158/-134 probe
GC Box Factor SP1
-91 TACCCGGCCTCCCAAACACCACCCCAATCTAGTTTAGCCCCCGCCCCAC -40
GC Box Factor SP1
-41 CCTCGCTGACCTAATAAGGCCATGCAGTGTGCGGGGCAGCTACATAAAAG +10
GC Box Factor SP1
GC Box Factor SP1
+9 CGCGGGCTCGCGGCGACTCTGCACCACGCAGGGGAAGAGAAAGCAGGAGC +60
+28/+62 probe
+59 CGTCCAGCACGGAGGAAGGCGACCATG +86
CAIII Pro Reverse


C
Relative mRNA Expression of CAIII
1.25
1.00
0.75
0.02
0.01
0.00
*
*
PC3
HT-29
Saos-2


D
CAIII
DAPI
COMBINED
PC3
Saos-2


B
Human CAIII -412 AACTTTTAAGTGAGGCTGCAAGAGCCGCCGGGATGTAGATTTTAGTTCGTGGCCAAGCACAACTACGACACCCTGTCCCTGCCCCCACCCCATCCCCAAG
Mouse CAIII -402 TCTGAGAGAGGCTGCAAGAGCCGC GGGTGCAACTTTTAGTTGCTAAGTAACAC CAGACTGCAGCATGAGCCGGCTGCTCCTCCAGACCCCCA
Rat CAIII -429 CTGAGGAAGGCTGCAAGAGCCGT GGGTGCAATTTTTAGCTGCTAAGTAACACACGGGACTGCAGCCTGAGTCGGCTGCTCCTCCAGACCCCCA
SP1
SP1
Human CAIII -312 AATGCATGGAGGAAGGAGAGAGGACGAGGTGAGGGCCGCCTGCATTTCTGCACGTCGGCGCCGGTTAGAAACCCTGCAGTTTTGAGAGAGAAGAAGAGGA
Mouse CAIII -306 AGTGCAGGAAAACTGGAGGGAAC CAGC CAAAGCGGGAGATCTTAGAAAGCTCAGCTGCTTGGTAGCC TGCAAAGTGAACAAAGGAAGGAGCCA
Rat CAIII -336 AGTGAGTGAAAA TGGAGGGAAC CAACGCTACCAAAGCCG AGCTCTTAGAAAGCTCAGTTGCTTGGTAGTC TGCAAAGGAAACAGAGGAAAGAGCCA
Human CAIII -212 GA TGGAGGGGCCAGGAGCCACGACTCCCGGGAGAGCGCAGGGAGGGGCGTGGGTGCCCCTTCGCCCACCTCCGC CCCCGTCACCTCGACAGCT
Mouse CAIII -213 GGGCTCTTGCAGAGCCAAGAAAGGGGGGGCTCTGTCCCCCTCCGTTCCCCCTCCGCGCCTCCTCCCTGCCCCCTCCTGCTACCCTCTCACCCGGACAGCT
Rat CAIII -240 GGGCTTTTGCAAGAGCCAAGAAGGGAGTCTTCTGTCCCCCTCCGTTCCCCCTCCGTGCCTCCTCCCTGCCCCCTCCTGCCACCCTCTCACCCCGACAGCT
SP1
SP1
Human CAIII -119 GTCCCGCTCTTGGAATTCATTGGCTTCCTCTACCCG GCCTCCCAAACACCACCCCAATCTAGTTTAGCCCCCCGCCCCACCCTCGCTGACCTAATAAG
Mouse CAIII -131 GTCCAGCTCTTGTAATTCATTGGCTTCTCCCGCCCCCGGCCTTCTGAGTACCACCCCAATCCAGTTTAGCCCCCTGCCTCACC GCTGACCTAATAAG
Rat CAIII -140 GTCCACCGCTTGTAATTCATTGGCTCC CCCGCCCC GCCTTCCGAGTACCACCCCAATCCAGTTTAGCCCCCTTCCTCACCC GCTGACCTAATAAG
CG Box
SP1
Human CAIII -21 GCCATGCAGTGTGCGGGGGAGCTACATAAAAGCGCGGGCTCGC GGCGACTCTGCACCACG CAGGGG AAGAGAAAGCAGGAGCCGTCCAGCACGGAGG
Mouse CAIII -34 GTCATGCACACTGCAGGAGATCTATATAAAAGAGCGAACTCTT GGAGACCTTGCCGGACAACAGGAGCAAGAAAGAGCAGGAGCTGTCCAGCGCTGAGA
Rat CAIII -104 GCCATGCACACTGCAGGAGACCTATATAAAAGTGGGGACTTTTTGGAGCTTTTGCCGGACAACAGGAGCAAGCAAAAGCAGGAGCTGTCCAGCGCTGAGA
TATA Box
Human CAIII +72 AAGGCGA CCATG +86
Mouse CAIII +65 AACACGAAAGGTGACCATG +86
Rat CAIII -4 GACAGGAAAGG CCATG +13

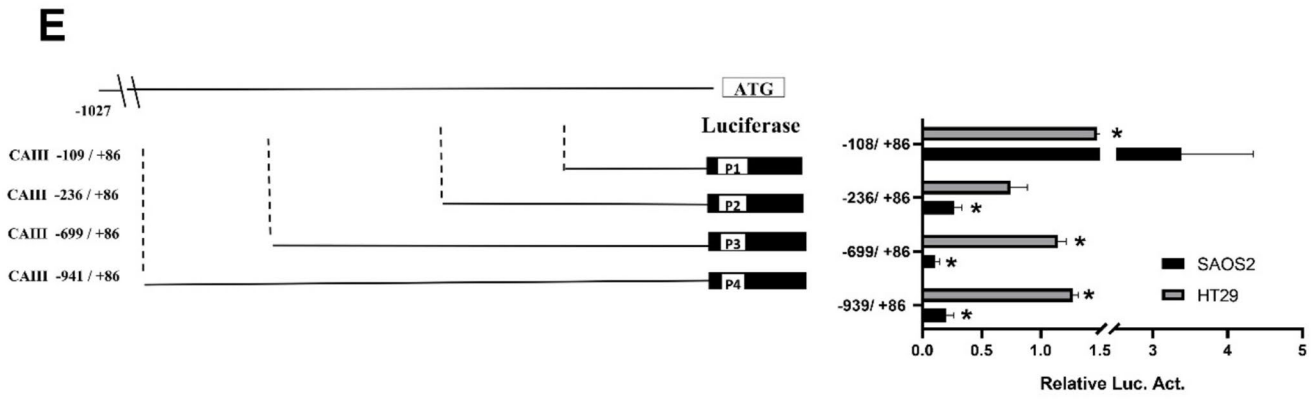

E
-1027
ATG
Luciferase
CAIII -109 / +86
CAIII -236 / +86
CAIII -699 / +86
CAIII -941 / +86
P1
P2
P3
P4
-108/ +86
-236/ +86
-699/ +86
-939/ +86
SAOS2
HT29
0.0
0.5
1.0
1.5
3
4
5
Relative Luc. Act.

Fig. 1 Transcriptional activity and characterization of the human CAIII promoter. (**A**) Schematic representation of the 1027 bp CAIII promoter sequence. The transcription start site (TSS) and transcription start codon (TSC) are indicated in bold. Primers used for promoter amplification are shown as CAIII-pro forward and CAIII-pro reverse. Ten putative SP1 binding sites (GC boxes) are indicated within the promoter sequence. EMSA probes containing selected SP1 binding elements are also highlighted. (**B**) Sequence alignment of mammalian orthologues of the CAIII promoter. The relative positions of the putative TATA box and conserved transcription factor binding sites are boxed. Putative transcription start sites of rat, mouse, and human CAIII genes are indicated by triangles. (**C**) CAIII mRNA expression analysis. Quantitative real-time PCR (qRT-PCR) was performed to assess CAIII mRNA expression in PC3, Saos-2, and HT-29 cells. Results are presented as the mean ± SD of three independent experiments. (**D**) Immunofluorescence analysis of endogenous CAIII protein expression. DAPI staining was used to visualize cell nuclei.(**E**) Transcriptional activities of 5′-truncated CAIII promoter constructs. Transcriptional activities of four promoter constructs were determined by transient transfection in Saos-2 and HT-29 cells. A schematic diagram of the CAIII promoter region showing putative transcription factor binding sites is presented. Data are expressed as the mean ± SD of three independent experiments

SP1 expression vector. Upregulation by SP1 was observed in these contracts, but the highest level of activity was determined in the −108/+86 promoter contract (Fig. 2A). Therefore, it was determined that SP1 binding sites located on the promoter may play a role in the activation of gene transcription.

To demonstrate whether this activation would continue at the transcriptional and translational levels, CAIII mRNA and protein levels were determined upon SP1 overexpression. First, SP1 overexpression was confirmed by qRT-PCR in Saos-2 cells, an osteosarcoma model, using SP1-specific primers (Fig. 2B). As shown in Fig. 2B, SP1 was successfully overexpressed in Saos-2 cells. Only vector-transfected cells were used as control groups for the overexpression studies. While SP1 effectively upregulated CAIII mRNA expression, it increased approximately 6-fold at the late timeframe of 72 h. SP1 overexpression led to a time-dependent increase in CAIII mRNA levels (Fig. 2C). We performed Western Blot analysis to determine translational regulation (Fig. 2D). Consistent with mRNA levels in experiments performed using CAIII-specific antibodies, SP1 was found to increase CAIII protein levels twofold in Saos-2 cells (Fig. 2E).

SP1 activates CAIII not only activate in osteosarcoma but also in prostate cancer cells

SP1-mediated regulation of CAIII was evaluated at the promoter, mRNA, and protein levels. EMSA experiments were performed to identify specific SP1 binding sites within the CAIII promoter. Among the three promoter fragments analyzed (−699/+86, −236/+86, and −108/+86), the −236/+86 region exhibited the highest SP1 density and showed a statistically significant increase in SP1-dependent transcriptional activity; therefore, this region was selected for further analysis. For EMSA, the P3 (−236/+86) fragment was subdivided into three overlapping regions (−181/−153, −158/−134, and +28/+62) (Fig. 3A).To investigate the tissue-specific regulation of CAIII, SP1 overexpression experiments were performed in PC3 cells. As shown in Fig. 3B, efficient SP1 overexpression was achieved and remained stable for up to 72 h. Consistent with the findings in Saos-2 cells, SP1 overexpression induced a time-dependent increase in CAIII expression in PC3 cells (Fig. 3C).

For EMSA analysis, three promoter probes were synthesized and biotin-labeled. To examine SP1-mediated regulation of the CAIII promoter, DNA–protein interaction assays were performed using the −158/−134 probe and nuclear extracts from osteosarcoma (MG-63 and Saos-2) and prostate cancer cells (PC3 under normoxic and hypoxic conditions). Distinct binding patterns were observed depending on the cellular origin of the nuclear extracts. Notably, the −158/−134 probe formed a strong and specific DNA–protein complex exclusively with nuclear extracts from normoxic PC3 cells (Fig. 3D). These findings suggest that SP1 binding to the CAIII promoter is regulated in a tissue- and context-dependent manner.

SP1 directly binds on multiple putative SP1 sites of CAIII promoter

Nuclear extracts obtained from PC3 were analyzed using three different biotin-labeled oligonucleotide probes (-181/-153), (-158/-134), and (+28/+62) (Fig. 4A). In the EMSA analysis performed for the −158/-134 region, a DNA-protein complex was formed as a result of the binding reaction with the biotin-labeled probes. To determine that the resulting complex belonged to CAIII, a competition assay was set up by adding unlabelled oligonucleotide sequence-specific probes 500-fold. The same reaction was subjected to competition using the SP1 consensus sequence. The results showed that the resulting complex was CAIII-specific, that the sequence contained SP1 binding sites, and that this region contained specific SP1 binding sites (Fig. 4B). The same reactions were performed separately for the −181/-153 and +28/+62 regions (Fig. 4C and D). The presence of SP1 binding sites in these sequences was also proven by competition assays. As further evidence for SP1 binding, directed mutagenesis studies were conducted. For this purpose, bases in the SP1 consensus region on the CAIII probe were deleted, and competition experiments were performed using this EMSA probe. When comparing the Wild CAIII probe with the Mutant CAIII probe, a reduction in the DNA-protein complex was determined (Fig. 4E). This result reveals that the SP1 transcription factor directly binds

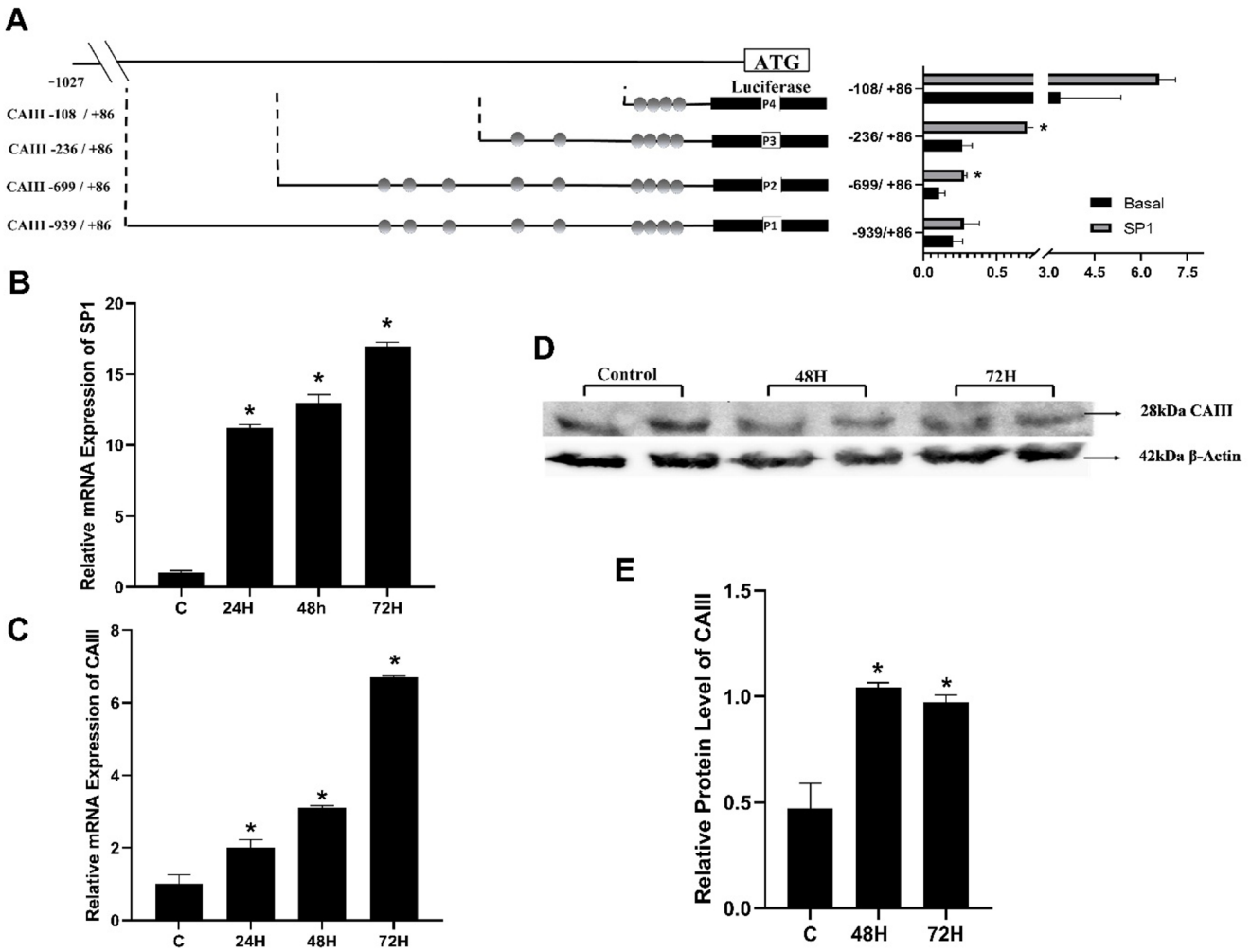


Fig. 2 SP1 overexpression upregulates CAIII gene expression. (**A**) CAIII promoter activity in Saos-2 cells. Cells were co-transfected with 1 µg of CAIII promoter constructs (−939/+86, −699/+86, −236/+86, and −108/+86) together with 1 µg of either an SP1 expression plasmid or an empty vector. CAIII promoter activity was measured 48 h after transfection, and the ratio of secreted luciferase to SEAP activity was calculated. Data are presented as the mean ± SD of three independent experiments. Statistical significance was assessed using one-way ANOVA (*p ≤ 0.05, **p ≤ 0.01). (**B**) SP1 mRNA expression levels in Saos-2 cells following transfection with the SP1 expression plasmid. (**C**) CAIII mRNA expression levels in Saos-2 cells after co-transfection with SP1 and CAIII promoter constructs. Results are shown as mean fold changes ± SD from three independent experiments (*p ≤ 0.05, **p ≤ 0.01). (**D**) SP1-induced CAIII protein expression in Saos-2 cells. Representative densitometric analyses of CAIII protein levels are shown. Data are expressed as mean fold changes ± SD from three independent experiments (*p ≤ 0.05, **p ≤ 0.01). (**E**) CAIII protein expression following SP1 overexpression, as determined by Western blot analysis. Protein levels are expressed as the ratio of CAIII to β-actin

to the hypothetical SP1 binding sites located between −153 and −158 bp in the CAIII promoter sequence.

Discussion

CAIII is one of the least understood CA isoforms especially in cancers. It has been recommended that CAIII could be part of cellular antioxidant defence system due to higher number of cysteine residues. Limited studies are available on CAIII expression in cancers. Kuo et al., discovered that CAI, CAII and CAIII protein expression levels are reduced in human HCC cells compared to the normal tissues [31]. Dai and his colloquies hypothesized that the expression of CAIII was upregulated in later phases in HCC's metastasis, and this may play a significant role in liver cancer metastasizes [32]. They discovered CAIII is overexpressed in more acidic pH in the extracellular and intracellular environments. This leads to activate the focal adhesion kinase (FAK) signalling pathway, which is crucial for cell motility, spreading, and survival. It has been also implicated that CAIII may influence the EMT process by inhibiting the epithelial marker E-cadherin gene transcription binding site affinity, and thus decreasing E-cadherin expression in squamosa cancers [33].

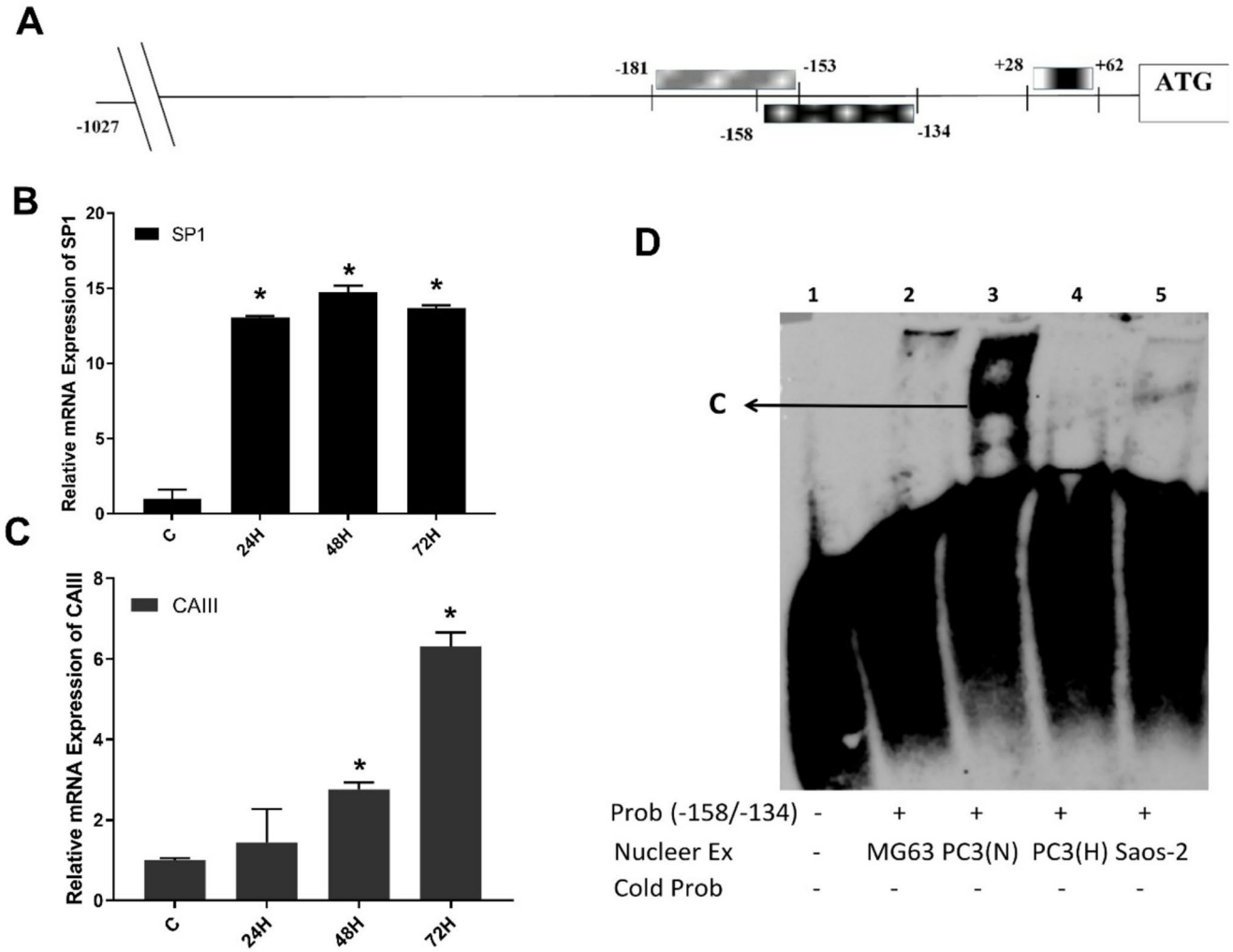


Fig. 3 SP1 activates CAIII expression in prostate cancer cells in addition to osteosarcoma cells. (**A**) Schematic representation of EMSA probes used for the analysis of SP1 binding sites within the CAIII promoter. Three probes corresponding to the −181/−153, −158/−134, and +28/+62 regions were designed. (**B**) SP1 mRNA expression levels in PC3 cells following SP1 overexpression. (**C**) CAIII mRNA expression levels in PC3 cells after SP1 overexpression. The time-dependent increase in CAIII mRNA expression indicates SP1-mediated transcriptional upregulation. Results are expressed as the mean percentage of CAIII activity ± SD from three independent experiments (*$p \leq 0.05$, **$p \leq 0.01$). (**D**) Analysis of SP1 binding sites within the −158/−134 region by EMSA. The −236/+86 promoter region was subdivided into three fragments (−181/−153, −158/−134, and +28/+62), and corresponding biotin-labeled probes were synthesized. Nuclear extracts were prepared from MG-63, Saos-2, and PC3 cells under normoxic and hypoxic conditions. A DNA–protein complex was detected exclusively in nuclear extracts from normoxic PC3 cells and is indicated as complex C

Although the expression of CAIII is mainly restricted in skeletal muscle, there is wide expression pattern in other tissues. CAIII expression was highly regulated by hypoxia. In our previous study we found that CAIII is upregulated by hypoxia in prostate cancer cells. HIF1-α directly binds on to Hypoxia response elements in CAIII promoter [23]. Moreover, in our different study, in colon cancer cells, TGF-beta inhibited the CAIII gene via the phosphoinositide-3-kinase (PI3K) and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathways [28]. Thus, there is virtually no information of transcriptional regulation of CAIII in normal oxygen condition. Therefore, the main purpose of the study is to elucidate the regulation of CAIII transcriptional activity in cells that possess CAIII expression. In silico analysis revealed high sequence homology amongst rat, mouse and human CAIII proximal promoter region. Sequence analysis of the CAIII promoter region indicated that there is no CpG island in the promoter. -939 to + 86 of CAIII 5'-regulatory region showed that the active promoter sequence was resides from − 236 to + 86 which contains multiple SP1 binding sites The widely recognized GC box-associating transcription factors include Specificity Protein 1 (Sp1), the Krüppel-like factor, and other Sp family members. Sp family could recognize the conserved

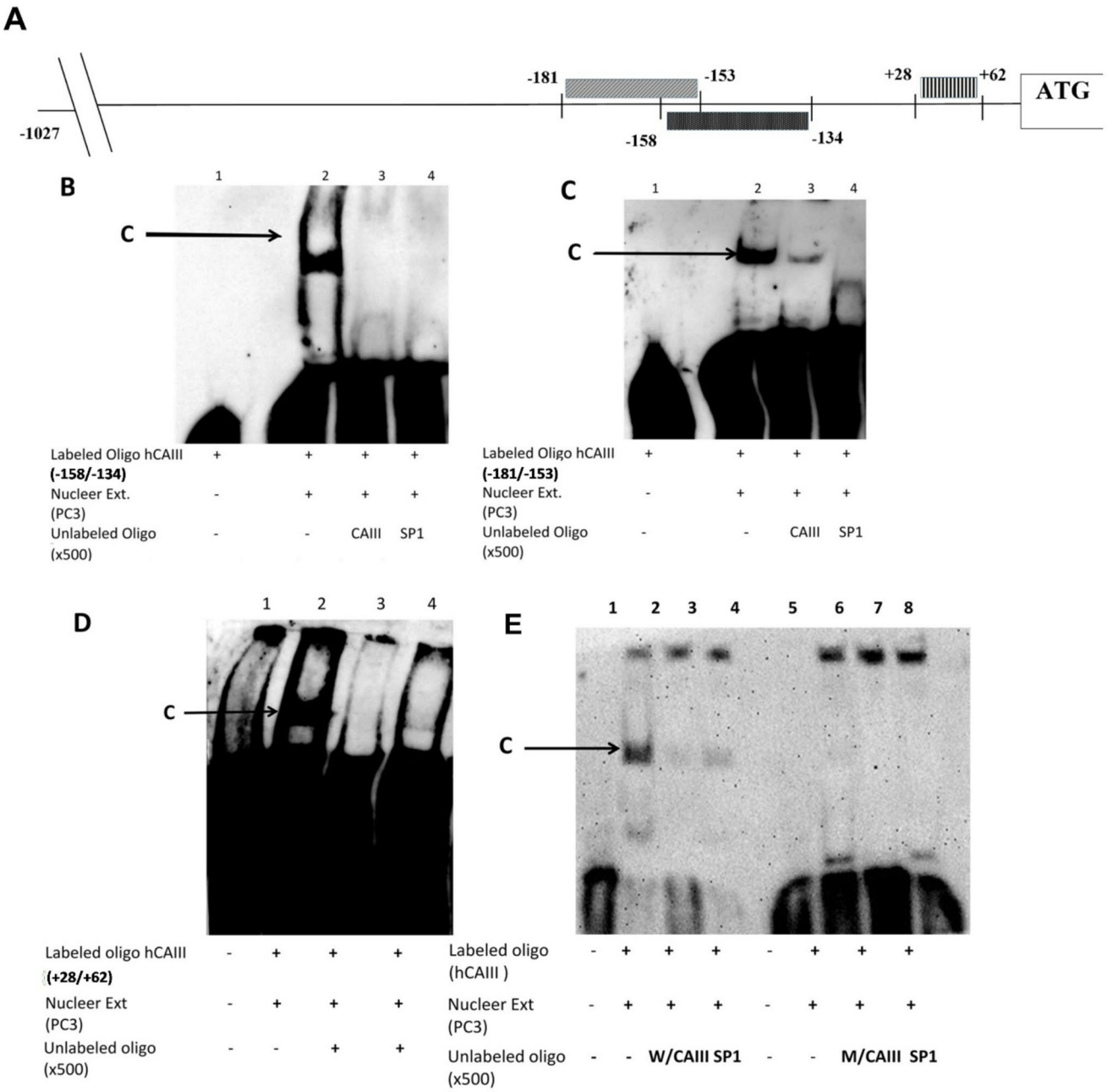


Fig. 4 SP1 directly binds to multiple putative SP1 sites within the CAIII promoter. (**A**) Schematic diagram of EMSA probes used to analyze SP1 binding sites in the CAIII promoter. (**B**–**D**) EMSA analysis of SP1 binding to the −158/−134 (**B**), −181/−153 (**C**), and +28/+62 (**D**) promoter regions using nuclear extracts from PC3 cells. Biotin-labeled probes were incubated with nuclear extracts and separated on a native polyacrylamide gel. The first lane represents the free probe. A specific DNA–protein complex was observed and is indicated as complex C. Competition assays were performed using a 500-fold molar excess of unlabeled corresponding oligonucleotides or SP1 consensus sequences. (**E**) Identification of the functional SP1 binding site by mutational analysis. The common SP1 binding motif shared by the −158/−134 and −181/−153 probes (−158/−153) was deleted. EMSA was performed using wild-type probes (lanes 1–4) and SP1-mutated probes (lanes 5–8). Mutation of the SP1 binding site markedly reduced DNA–protein complex formation, confirming direct SP1 binding

GGGCGG sequence [34]. There are numerous SP1 binding sites in CAIII promoters. SP1 overexpression studies also confirmed that Sp1 overexpression increase CAIII mRNA, protein level. Transient-Transfection studies of the promoter constructs also show SP1 upregulate three truncated deletion constructs of CAIII gene. EMSA analysis indicates that SP1 could directly bind to the CAIII promoter within − 236 to + 86 region. The specificity of complexes was tested by the competition of unlabelled oligonucleotide SP1 consensus site and unlabelled oligonucleotide. Mutation of SP1

Binding elements resulted in disappearing the DNA-protein complex. This strongly suggested the involvement of SP1 in the regulation of CAIII expression. Numerous genes required for cellular function have been demonstrated to be regulated by SP1 [35, 36]. Sp1 may also serve as a transactivator by itself or in conjunction with other recruited co-activators to increase the expression of some particular genes. Moreover, Sp1 has been shown to regulate carbonic anhydrase members, namely CA9 and other CA genes [37–40].

Despite the strengths of the present study, several limitations should be acknowledged. First, the transcriptional regulation of CAIII was investigated primarily using in vitro cell line models, which may not fully recapitulate the complexity of CAIII regulation in vivo. Second, although SP1 binding to the CAIII promoter was demonstrated by EMSA and mutational analyses, additional approaches such as chromatin immunoprecipitation (ChIP) assays would further strengthen the evidence for SP1 occupancy under physiological conditions. Finally, the functional consequences of SP1-mediated CAIII regulation on cellular phenotypes were not explored in the present study and should be addressed in future investigations.

In summary, we have for the first time characterized promoter region of the human CAIII gene. This discovery should make it easier to conduct further research on the mechanisms that control the expression of the CAIII gene and the molecular actions of CAIII in numerous biological processes. SP1 plays a vital role in the regulation of human CAIII transcription through direct mechanism. Our findings lay the groundwork for a deeper comprehension of the transcriptional control and biological significance of the human CAIII gene.

Conclusions

This study provides the first comprehensive functional characterization of the human CAIII promoter and reveals a critical role for SP1 in the transcriptional regulation of CAIII under normoxic conditions. Our findings demonstrate that the core promoter activity of the CAIII gene is confined to the −236 to +86 bp region, which contains multiple functional SP1 binding sites. Using promoter deletion analysis, SP1 overexpression, EMSA, and site-directed mutagenesis, we show that SP1 directly binds to this region and positively regulates CAIII expression at both the mRNA and protein levels. Given the limited understanding of CAIII regulation, particularly outside hypoxic contexts, our results fill an important gap by identifying SP1 as a key transcriptional regulator of CAIII independent of hypoxia-driven HIF-1α signaling. These data suggest that CAIII expression may be finely controlled by GC box–binding transcription factors in cells where CAIII is constitutively expressed. Overall, this work establishes a molecular framework for understanding CAIII gene regulation and provides a foundation for future studies exploring the biological functions of CAIII in cancer progression, redox regulation, and cell signaling pathways. Elucidating SP1-mediated control of CAIII may also contribute to a broader understanding of carbonic anhydrase regulation in both physiological and pathological conditions.

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Data availability The data presented in this study are available on request from the corresponding author.

Declarations

Competing interests The authors declare no competing interests.

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