의학석사 학위논문

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RNA editing modulates the transcriptional repression activity of CtBP1

By

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A Thesis Submitted to the Department of Biomedical Sciences in Partial Fulfillment of the Requirements for the Degree of Master of Philosophy in Biomedical Sciences at the Seoul National University

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Approved by thesis committee:

Professor	Yong-Sung Juhnn	**Chairman**
Professor	Hong-Duk Youn	**Vice Chairman**
Professor	Ju-Hong Jeon	

ABSTRACT

RNA editing modulates the transcriptional repression activity of CtBP1

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RNA modification includes alternative splicing and RNA editing, which increases the diversity of transcripts and proteins. The C-terminal binding protein (CtBP), a transcriptional corepressor, has alternative splicing forms but there is no information about involving RNA editing in CtBP function. Here we found a unique transcript variant of CtBP1 with an A-to-G conversion at codon 528 in breast cancer cell lines. *CtBP1* mRNA editing produces protein in which threonine-176 is changed into alanine. This CtBP1 (T176A) mutant lacks *in vitro* dehydrogenase activity and dimerization ability compared with wild-type CtBP1. Microarray analysis showed that many oncogenes were upregulated in *CtBP*-knockout mouse embryonic fibroblast. These candidate target genes were repressed by restoring wild-type CtBP1, but not by CtBP1 (T176A) mutant, indicating that CtBP1 (T176A) mutant is defective in transcriptional repression activity. We propose that RNA editing modulates the role of CtBP1 in transcription repression of oncogenes involved in breast cancer cells.

Keywords: CtBP1, mRNA editing, cancer, transcription
Student Number: 2010-21915

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

CtBP1	C-terminal binding protein 1
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced form
MEF	mouse embryonic fibroblast
shRNA	short hairpin RNA
KMTB	2-keto-4-methylthiobutyrate
HMTB	2-hydroxy-4-methylthiobutyrate
PTN	pleiotrophin
MDK	midkine
CDH1	cadherin 1 (E-cadherin; epithelial cadherin)

INTRODUCTION

CtBP proteins mainly function as transcriptional corepressors through interactions with various transcription factors and chromatin modifiers (1), which affect a variety of cellular processes such as development and tumorigenesis (2). CtBP is highly conserved in vertebrates and invertebrates and shares amino acid sequences and structural homologies to NAD^+-dependent D-isomer-specific 2-dehydrogenase (D2-HDH), especially having a conserved NAD(H) binding domain ($GXGXXGX_{17}D$) and a putative catalytic site (His315/Glu295/Arg266) (3-5). It was verified that CtBP binds with NAD^+ and NADH in practice (4, 5), and that it has a weak dehydrogenase acivity *in vitro* (5-7). However, it is not clear whether this enzymatic activity contributes to the CtBP function.

CtBP was originally identified as an adenoviral E1A-binding protein. E1A mutants defective in interaction with CtBP enhanced transformation of cells cooperating with Ras, leading to highly tumorigenic and metastatic pheynotypes. These studies strongly support the importance of CtBP in tumorigenesis (3, 8, 9). Several evidences showed that CtBP represses epithelial genes as an antagonist of epithelial phenotype and E1A-CtBP interaction de-represses these genes (10). Along with this, other studies suggested that CtBP plays an important role in promoting epithelial-to-mesenchymal transition (EMT) by repressing epithelial genes. Furthermore, CtBP mediates repression of proapoptotic genes and tumor suppressor genes. In addition, CtBP is down-regulated by tumor suppressors (11). Despite the studies supporting the role of CtBP in tumor development, some reports suggested the possibility that CtBP acts as a negative regulator in melanoma. CtBP1 expression was lost or significantly reduced in melanoma (12). Melanoma cells

expressed an alternative splice variant of CtBP1 that could not bind TCF4, promoting expression of LEF/TCF-regulated genes which are involved in progression and metastasis of malignant melanomas (13). Taken together, CtBP may influence both positively and negatively on cancer development. We report here global gene expression patterns of *Ctbp*-knockout MEF compared with *Ctbp*-heterozygous MEF and found several oncogenic genes regulated by CtBP.

There are two CtBP-coding genes, *CtBP1* and *CtBP2* in human. *CtBP1* locus codes for two isoforms (CtBP1-L and CtBP1-S) and *CtBP2* locus encodes three isofoms (CtBP2-L, CtBP2-S and RIBEYE) (14). These isoforms result from alternative splicing. Other variants have been reported such as the exon4-lacking splice variant CtBP1 in melanoma (13) and the CtBP2 variant resulted from post-transcriptional regulation in breast cancer (15).

Recently, RNA editing attracts a lot of interest as a mechanism that increases the diversity of proteins. This process has been known to be associated to various pathologies such as cancer (16, 17). Adenosine-to-inosine (A-to-I) conversion is the most frequent type of RNA editing. We found A-to-G substitution of a single nucleotide at codon 528 of *CtBP1* mRNA from breast cancer cell lines. The substitution of mRNA generates CtBP1 proteins with a single amino acid change (T176A). The consequent CtBP1 mutant showed different activities from wild-type CtBP1 protein. These results suggest that partial population of *CtBP1* mRNA is edited by A-to-I conversion and may be associated with cancer development.

MATERIALS AND METHODS

Cell culture and transfection

MCF-7, MDA-MB-231, HeLa and HEK293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and penicillin-streptomycin. *Ctbp*$^{+/-}$ and *Ctbp*$^{-/-}$ mouse embryonic fibroblast (MEF) cells were cultured in DMEM containing 10% FBS, penicillin-streptomycin and 2 mM L-glutamine. *Ctbp*$^{-/-}$ (WT) and *Ctbp*$^{-/-}$ (TA) MEF cells were established as previously described (18). Transfections were done using Lipofectamine reagent (Invitrogen).

Plasmids

pcDNA3.1-CtBP1-myc, pRSET-CtBP1 and pGEX-CtBP1 plasmids were previously described (19). pcDNA3-flag-CtBP1 was generated by subcloning of full-length CtBP1 PCR product. For the T176A mutant plasmids of CtBP1, the PCR products of mutated CtBP1 were obtained from pcDNA3.1-CtBP1-myc using pre-designed mutagenesis primer sets. The PCR products were treated with *Dpn I* and then transferred to XL-1 blue competent cells. The mutant plasmid was purified from a transformed single colony and verified by sequencing. Other T176A mutant-expressing vectors were generated by subcloning of mutated CtBP1 PCR products from pcDNA3.1-CtBP1 (T176A)-myc into applicable plasmids.

RNA editing analysis

Total RNA and genomic DNA samples were extracted with TRIzol Reagent

(Invitrogen). cDNA was synthesized from total RNA by reverse transcription using SuperScriptⅡ Reverse Transcriptase (Invitrogen) and Oligo-dT primers according to the manufacturer's protocol. Fragments of cDNA or genomic DNA encoding CtBP1 protein were amplified by PCR with Ex Taq polymerase (Takara) and primers. PCR products were purified with AxyPrep DNA Gel Extraction kit (Axygen) and then sequenced using an ABI 3730xl DNA analyzer with ABI BigDye Terminator cycle sequencing (Applied Biosystems). Editing was also validated by restriction fragment length polymorphism. Purified cDNA fragments were digested with *Stu I* (New England Biolabs) at 37℃ overnight and visualized by ethidium bromide staining on a 3% agarose gel.

Immunofluorescent staining

HeLa cells were grown on coverslips. After 24 hr of transfection, the cells were then stained with monoclonal anti-myc (Covance) and fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody (Jackson Immunoresearch). Staining and detection was performed as previously described (19).

Protein purifications

$(His)_6$-tagged and GST-fused CtBP1 proteins were purified as previously described (19). Briefly, $(His)_6$-tagged and GST-fusion proteins were expressed in the BL21(DE3)pLysS and DH5α, respectively. Both proteins were induced by adding 1 mM IPTG for 4 hr. $(His)_6$-tagged CtBP1 proteins were bound to TALON metal affinity resin (Clontech) and eluted with a gradual increase of imidazole concentration from 150 to 250 mM. GST-fused CtBP1 proteins were purified using Glutathione Sepharose (Amersham)

and eluted with 10 mM of reduced glutathione. Fractions containing proteins were concentrated using Centricon (Millipore).

In vitro GST-pull down assay

$(His)_6$-tagged proteins were incubated with GST-fusion proteins and Glutathione Sepharose 4B bead (GE Healthcare) for 4 hr at 4℃ in the binding buffer (20 mM Tris-Cl, pH 7.4, 0.5% (v/v) Nonidet P-40, 150 mM NaCl, 1 mM phenylmethyl fluoride and protease inhibitors). Precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted using anti-His (Cell Signaling). Proteins were visualized by chemiluminesccence (Pierce).

Dehydrogenase activity assay

Dehydrogenase activity assay was performed with two different known substrates, pyruvate and 2-keto-4-methylthiobutyrate (KMTB), respectively. Assay was carried out in 0.2 M Tris-Cl (pH 7.4) containing 0.15 mM NADH and 1 mM pyruvate or 0.075 mM KMTB with 20 μg of $(His)_6$-tagged CtBP1 proteins (WT or T176A). The absorbance was measured by monitoring the disappearance of NADH at 340 nm for 1 hr every one minute.

Microarray analysis

Microarray analysis was conducted by Macrogen Inc. Briefly, total RNA was isolated from *Ctbp*$^{+/-}$ and *Ctbp*$^{-/-}$ MEF cell lines, and then amplified using the Illumina Amplification Kit (Ambion) and purified by RNeasy kit (Qiagen) to synthesize

biotinylated cRNA according to the manufacturer's instructions. Labeled cRNA samples were hybridized to Sentrix MouseRef-6 Expression BeadChip v1.1 (Illumina). Arrays were scanned with an Illumina BeadArray Reader confocal laser scanner. The scanned image was analyzed with BeadStudio program (Illumina). The array data were filtered by a detection *p*-value < 0.05 in at least 50% of the samples. Selected gene signal values were transformed using a logarithm and normalized by the quantile method. The comparative analysis between the test and control groups was performed using fold-change values and the *p*-value obtained from LPE test. Hierarchical cluster analysis was carried out using complete linkage and Euclidean method. Data analysis was conducted with ArrayAssist® (Stratagene) and R statistical language v. 2.5. Ontology-based analysis was performed using the Panther classification system.

RNA purification and quantitative real-time PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen) as manufacturer's instructions. cDNA synthesis was conducted using AMV-XL reverse transcriptase (Takara) with random hexamer (Takara). Quantitative real-time PCR (qRT-PCR) was performed as described in manufacturer's instructions (DyNAmo HS SYBR® Green qPCR Kit, Finnzymes). The accumulation of specific product in the reaction was continuously monitored by iCycler Real-Time PCR (Bio-Rad).

Lentivirus-based shRNA

Human CtBP1 shRNA (TRCN0000013738, TRCN0000013742) was purchased from Open Biosystems. Lentivirus production was carried out as previously described (18). To

treat lentivirus containing hCtBP1 shRNA, when MCF-7 cells were 30% confluent, lentiviral stock was treated with polybrene at a concentration of 6 μg/ml. After 48 hr post-treat of lentivirus, cells were harvested and further subjected to other experiments.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as previously described (18). HEK293T cells were transfected with flag-tagged CtBP1 (WT or T176A). After 24 hr, cells were harvested and applied to assay. Anti-flag (Sigma) or mouse normal IgG (Santa Cruz Biotechnology) was used for ChIP. Purified DNA was quantified by qRT-PCR using pre-designed primer sets.

Primers

Primers used in this study are as follows:

Primers for site-directed mutagenesis

T176A CtBP1	Forward: ATCCGCGGGGAGGCCTTGGGCATCATC
	Reverse: GATGATGCCCAAGGCCTCCCCGCGGAT

Primers for RNA editing analysis

CtBP1 gDNA fragment	Forward: GGCATTGCCGTCTGCAAC
	Reverse: TGCGCACTCAGTCCTGTCCT
CtBP1 cDNA fragment	Forward: GGCATTGCCGTCTGCAAC
	Reverse: AAGGCCTTGGCCCGCAGC

Primers for qRT-PCR

Mouse β-Actin	Forward: ATCACTATTGGCAACGAGCG
	Reverse: TCAGCAATGCCTGGGTACAT
Mouse Fxyd3	Forward: TTGTGCAGGGATTCTCTGTG
	Reverse: AGTTGTGAGCTGAGCCTGGT
Mouse Fgf5	Forward: GCGACGTTTTCTTCGTCTTC
	Reverse: GATGCCCACTCTGCAGTACA
Mouse Fgf13	Forward: CATTTTCTGCCCAAACCACT
	Reverse: CTCATGGATTTGCCTCCATT
Mouse Igfbp3	Forward: CAGGCAGCCTAAGCACCTAC
	Reverse: GCATGGAGTGGATGGAACTT
Mouse Grb7	Reverse: GACTTTCTGCAGATGGCACA
	Forward: GGTTTCATGGACGCATCTCT
Mouse Ptn	Forward: GAAAATTTGCAGCTGCCTTC
	Reverse: ACACTCCACTGCCATTCTCC
Mouse Mdk	Forward: CCTGCAACTGGAAGAAGGAA
	Reverse: CTTAGTCACGCGGATGGTCT
Mouse Cysltr1	Forward: CCTCACCACCTATGCCTTGT
	Reverse: AATTCCAATGCAAACGAACC
Mouse Tacstd2	Forward: CGCTGCTACTGCTACTGCTG
	Reverse: AATACCTGTGAGCCCATTGC
Human β-Actin	Forward: ACGTTGCTATCCAGGCTGTGCTAT
	Reverse: TTAATGTCACGCACGATTTCCCGC
Human CtBP1	Forward: TTCACCGTCAAGCAGATGAG
	Reverse: GGCTAAAGCTGAAGGGTTCC

Human PTN	Forward: CAGGCTCAACAGTACCAGCA
	Reverse: ACTCCACTGCCATTCTCCA
Human MDK	Forward: GGAGCCGACTGCAAGTACA
	Reverse: CTTTCTTGGCTTTGGCCTTT

Primers for ChIP assay

Human PTN promoter	Forward: ACCATCTCATGGCCTCTTGGTTCA
	Reverse: ACGGAAGGGAAATGGAGAATGGGA
Human CDH1 promoter	Forward: TAGAGGGTCACCGCGTCTAT
	Reverse: TCACAGGTGCTTTGCAGTTC

RESULTS

1. *CtBP1* mRNA editing in breast cancer cell lines

CtBP proteins have been widely studied as the contributor to tumorigenesis and tumor progression (11). However, some reports demonstrated that CtBP functions as a negative regulator in melanoma (12, 13). Although CtBP1 is known to down-regulate tumor suppressors such as Brca1 and E-cadherin in breast cancer and its expression seems to be increased in breast cancer compared with normal breast (20, 21), we questioned the possibility that CtBP1 may act as a tumor suppressor in breast cancer as in melanoma.

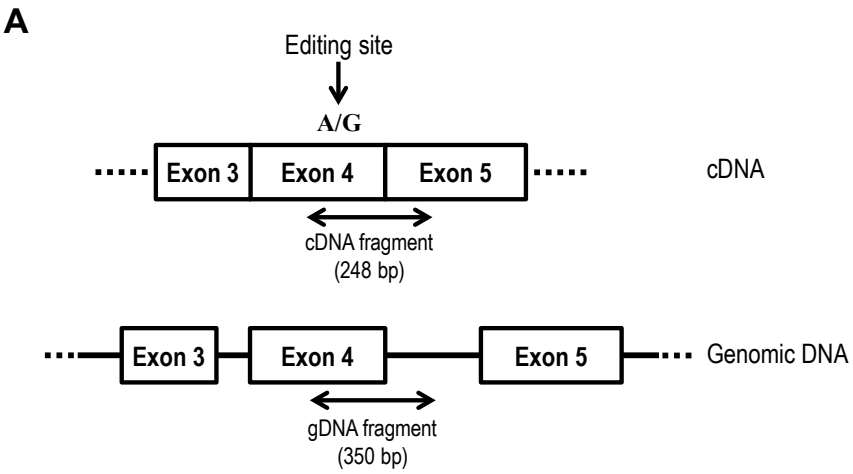
First, we expected that a mutation of *CtBP1* gene would lead to breast cancer in spite of the abundance in expression, but no mutation was detected in exons of *CtBP1* genomic DNA (gDNA) from breast cancer cell lines (data not shown). To explore the possibility that there are *CtBP1* transcript variants in cancer cell lines, the full-length cDNA encoding CtBP1 protein was cloned and individual clones were sequenced. Interestingly, a single nucleotide substitution of adenosine-to-guanosine at codon 528 of cDNA was found (data not shown). This was further confirmed by direct sequencing of 350-bp gDNA and 248-bp cDNA fragments including the edited site in MCF7 and MDA-MB-231 (Figure 1A and 1B). In consistent with the full-length sequencing, mixed signals of adenosine and guanosine were observed in the cDNA sequences, where the gDNA sequences exhibit only adenosine signals. Inosine (I) resulted from A-to-I RNA editing which is a site-specific RNA modification mediated by adenosine deaminase acting on RNA (ADAR) enzymes is recognized as guanosine by the splicing, translational

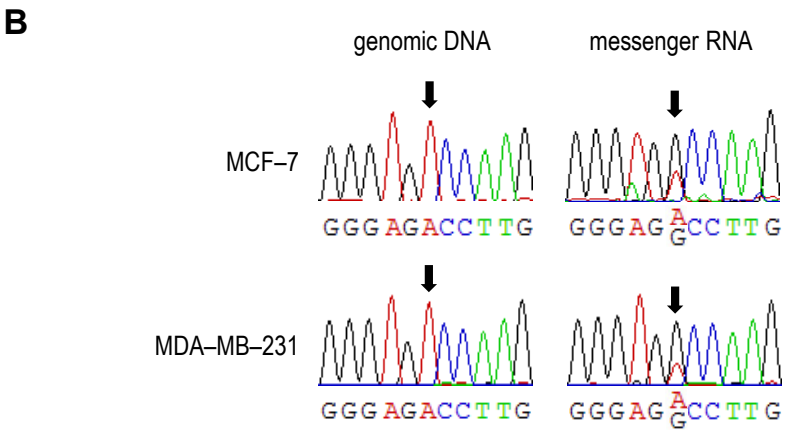
machineries and even the sequencing reaction (22). Accordingly, these A-to-G mismatches are regarded as the result of A-to-I mRNA editing.

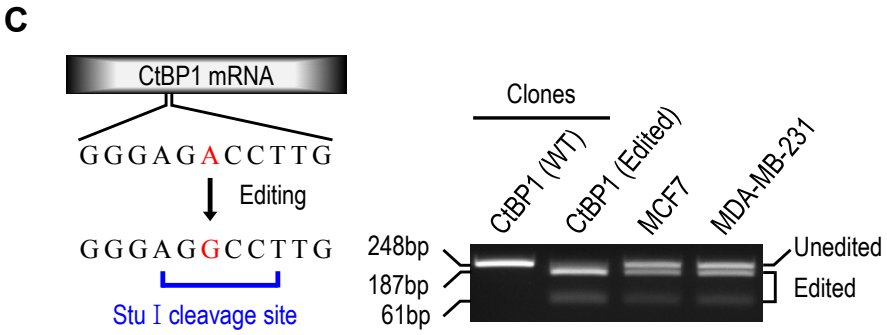
As this nucleotide conversion generates a single *Stu I* restriction enzyme site, editing of *CtBP1* mRNA was also demonstrated by *Stu I* enzyme digestion (Figure 1C). 248-bp cDNA fragments were partially digested with *Stu I*, producing 187-bp and 61-bp fragments. It further supports that *CtBP1* transcripts undergo mRNA editing at the specific codon in breast cancer cell lines and this is not occurred by a sequencing error.

Figure 1 *CtBP1* mRNA editing in breast cancer cell lines

(A) Diagram of PCR strategy for a comparison of sequences in gDNA and cDNA of *CtBP1*. gDNA and cDNA fragments were amplified from the region surrounding editing site. (B) Comparison of cDNA sequences to corresponding gDNA in various breast cancer cell lines. cDNA was synthesized using total RNA isolated from MCF7 and MDA-MB-231 cells. The region containing the editing site of cDNA and gDNA were amplified by PCR (Figure 1A). The PCR products were purified and directly sequenced. The arrow indicates the editing site (codon 528). (C) The analysis of *CtBP1* mRNA editing by *Stu* Ⅰ digestion. The editing region of *CtBP1* mRNA is depicted as a diagram (left). Editing produces a single *Stu* Ⅰ cleavage site. cDNA fragments obtained by PCR were digested with *Stu* Ⅰ and then visualized by ethidium bromide staining on agarose gel (right). For control, PCR products amplified from CtBP1 clone (wild type and T176A mutant) were used. When the cDNA fragment (248-bp) is edited, two fragments (187-bp and 61-bp) are generated by *Stu* Ⅰ.


A
Editing site
A/G
Exon 3
Exon 4
Exon 5
cDNA
cDNA fragment
(248 bp)
Exon 3
Exon 4
Exon 5
Genomic DNA
gDNA fragment
(350 bp)


B
genomic DNA
messenger RNA
MCF–7
GGGAGACCTTG
GGGAGACCTTG
MDA–MB–231
GGGAGACCTTG
GGGAGACCTTG


C
CtBP1 mRNA
GGGAGACCTTG
Editing
GGGAGGCCTTG
Stu I cleavage site
Clones
CtBP1 (WT)
CtBP1 (Edited)
MCF7
MDA-MB-231
248bp
187bp
61bp
Unedited
Edited

2. The characteristics of an edited CtBP1 protein

A528G conversion in *CtBP1* mRNA changes threonine (T)-176 into alanine (A) in CtBP1 protein. To explore if the edited form of CtBP1 protein is functional in cells, T176A mutant CtBP1 were exogenously expressed, and its biological characteristics were compared with those of wild-type CtBP1.

CtBP1 is predominantly localized in the nucleus (23), but some CtBP1 mutants fail in proper nuclear localization (24). Thereby, we examined the subcellular localization of exogenously expressed T176A CtBP1 mutant by immunofluorescence analysis (Figure 2A). T176A mutant was localized in the nucleus as wild-type CtBP1. This result suggests that the editing of CtBP1 do not affect the nuclear localization.

As the edited site is adjacent to the putative NAD(H)-binding site and the active site cleft, the editing of CtBP1 is may influence the dehydrogenase activity. To examine this possibility, we compared the dehydrogenase activity of T176A mutant CtBP1 with that of wild-type CtBP1. For the dehydrogenase activity assay, $(His)_6$-tagged CtBP1 proteins were bacterially expressed and purified (Figure 2B). Enzymatic activity was measured by changes of the absorbance at 340 nm induced by the oxidation of NADH to NAD^+ using pyruvate or 2-keto-4-methylthiobutyrate (KMTB) as a substrate (5-7) (Figure 2C). Edited forms of CtBP1 proteins exhibited a reduced dehydrogenase activity compared to wild-type CtBP1.

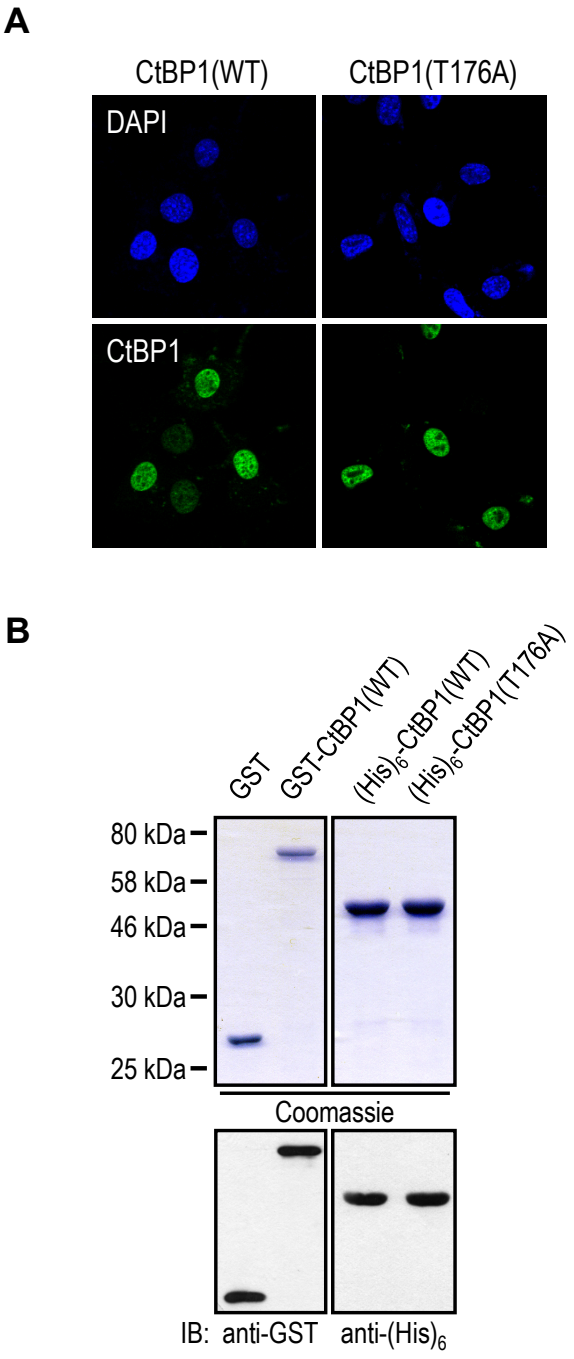
Several studies suggested that NAD(H)-binding ability is required for CtBP dimerization. Both CtBP1 mutants in the NAD(H)-binding motif (G183A/G186A) and in the dimerization interface (R141A/R163L) are deficient in dimerization with wild-type CtBP1 (24). In addition, CtBP1 dimerization is increased in an NADH-dependent manner

(19). Therefore, we examined the dimerization properties of the T176A mutant with wild-type CtBP1 by *in vitro* GST-pull down assay (Figure 2D). The dimerization ability of the mutant was significantly reduced.

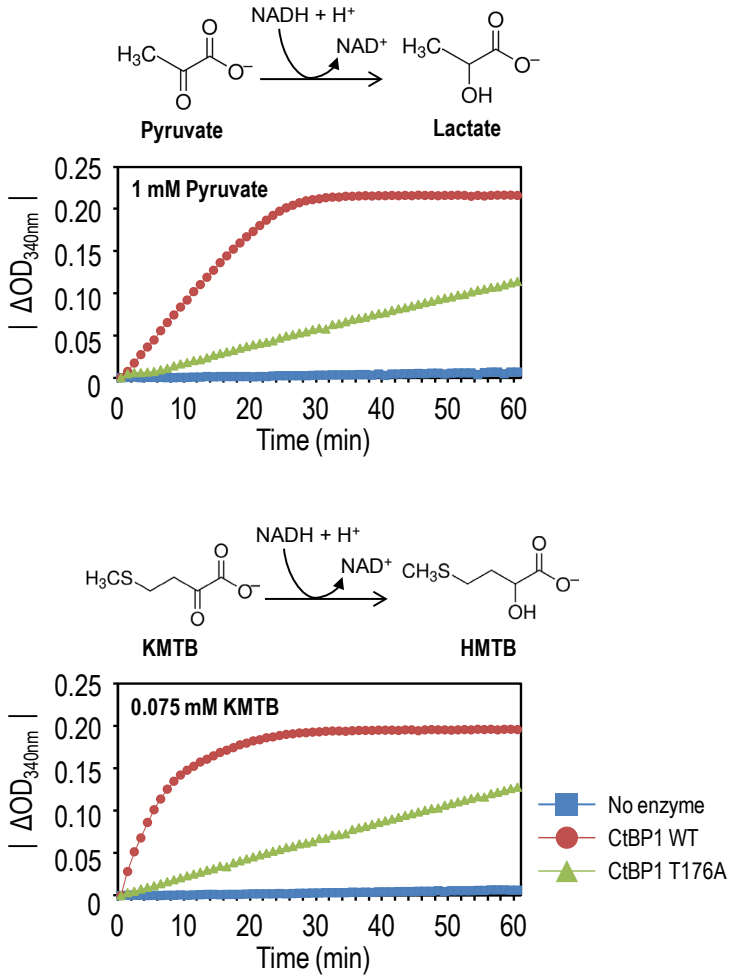
Although the relationship between the enzymatic activity of CtBP1 and its corepression function has not been clearly elucidated, the loss of dehydrogenase activity and dimerization ability suggests the distinguished function of the edited form of CtBP1 from the wild type.

Figure 2 The characteristics of an edited CtBP1 protein

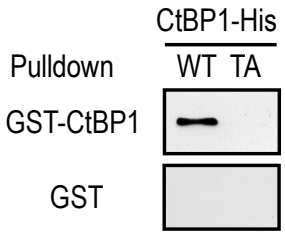
(A) The localization of wild-type and T176A mutant CtBP1. HeLa cells were grown on coverslips and transiently transfected with myc-tagged CtBP1 (WT or T176A). After 24 hr of transfection, cells were stained with anti-myc and FITC-conjugated anti-mouse antibody. Cells were observed under a laser scanning microscope. (B) Purification of GST-fused CtBP1 and $(His)_6$-tagged CtBP1 (WT and T176A) proteins. CtBP1 proteins were bacterially expressed and purified (19). Purified proteins were confirmed by coomassie staining (up) and immunoblot (down). (C) *In vitro* dehydrogenase activity of wild-type and T176A mutant CtBP1. Dehydrogenase activity assay was performed with 20 μg $(His)_6$-tagged CtBP1 proteins (WT or T176A). The reaction couples the reduction of pyruvate or KMTB to lactic acid or 2-hydroxy-methylthiobutyrate (HMTB), respectively, with the oxidation of NADH to NAD^+. The absorbance was measured by monitoring the disappearance of NADH at 340 nm for 1 hr every one minute. (D) Dimerization of wild-type and T176A mutant CtBP1. $(His)_6$-tagged and GST-fused CtBP1 proteins were incubated in the binding buffer with glutathione sepharose bead for 4 hr at 4℃. Precipitated proteins were detected by immunoblotted using anti-$(His)_6$.


A
CtBP1(WT)
CtBP1(T176A)
DAPI
CtBP1
B
GST
GST-CtBP1(WT)
(His)6-CtBP1(WT)
(His)6-CtBP1(T176A)
80 kDa
58 kDa
46 kDa
30 kDa
25 kDa
Coomassie
IB: anti-GST
anti-(His)6

C


NADH + H+
NAD+
H3C
O
O−
OH
Pyruvate
Lactate
1 mM Pyruvate
| ΔOD340nm |
0.25
0.20
0.15
0.10
0.05
0
0 10 20 30 40 50 60
Time (min)
H3CS
CH3S
KMTB
HMTB
0.075 mM KMTB
No enzyme
CtBP1 WT
CtBP1 T176A

D


CtBP1-His
Pulldown
WT TA
GST-CtBP1
GST

3. The change of gene expression patterns by CtBP in mouse embryonic fibroblast.

We hypothesized that the transcriptional corepressor activity of wild type CtBP1 is essential in repressing the oncogenic genes, while the edited form of CtBP1 is deficient in the transcriptional repression activity, leading to the cancer development.

In order to assess the possibility that CtBP regulates oncogenes, we identified genes regulated by CtBP through microarray analysis. We compared gene expression patterns of *CtBP*-knockout ($Ctbp^{-/-}$) MEF with those of $Ctbp^{+/-}$ MEF cell line. We found statistically significant candidate genes which are directly or indirectly regulated by CtBP. Among them, a subset of genes were selected based on the > 2 fold change and the < 0.05 *p*-value, and they were depicted as a heat map (Figure 3A). These genes were further analyzed for classification of biological processes (Figure 3B). Categorization based on biological processes revealed that the selected genes were related to 29 biological processes, and the 19% of genes was included in the category of signal transduction. It was also shown that the second largest number of genes was related to developmental processes in accordance with the previous study (25).

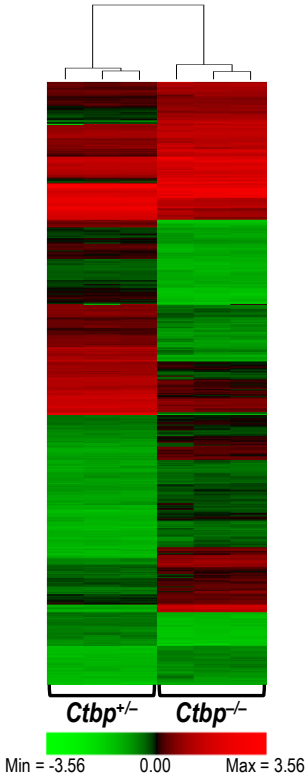
In order to validate microarray data, we performed qRT-PCR. We narrowed the range of candidate genes down by selecting up-regulated genes in $Ctbp^{-/-}$ MEF, because CtBP generally functions as a corepressor. Among them, we focused on nine up-regulated genes with relatively higher fold changes; *Fxyd3* (FXYD domain-containing ion transport regulator 3), *Fgf5* (Fibroblast growth factor 5), *Fgf13* (Fibroblast growth factor 13), *Igfbp3* (Insulin-like growth factor binding protein 3), *Grb7* (Growth factor receptor bound protein 7), *Ptn* (Pleiotrophin), *Mdk* (Midkine), *Cysltr1* (Cysteinyl leukotriene receptor 1)

and *Tacstd2* (Tumor-associated calcium signal transducer 2). In consistent with microarray data, $Ctbp^{-/-}$ MEF cells showed significantly higher expression levels of these genes than $Ctbp^{+/-}$ MEF (Figure 3C). Moreover, the gene expression level in rescued $Ctbp^{-/-}$ MEF with wild-type CtBP1 ($Ctbp^{-/-}$ (WT)) revealed markedly lower than $Ctbp^{-/-}$ MEF, and they were comparable to those in $Ctbp^{+/-}$ MEF. All verified genes are related to a signal transduction process (Table 1). Several genes are also associated with cell proliferation and differentiation. *Fgf5* is known to be related to oncogenesis (26), and Ptn and Mdk were known to be highly expressed in some cancers (27-30). The negatively regulation of these genes by CtBP suggest the tumor suppressive role of CtBP.

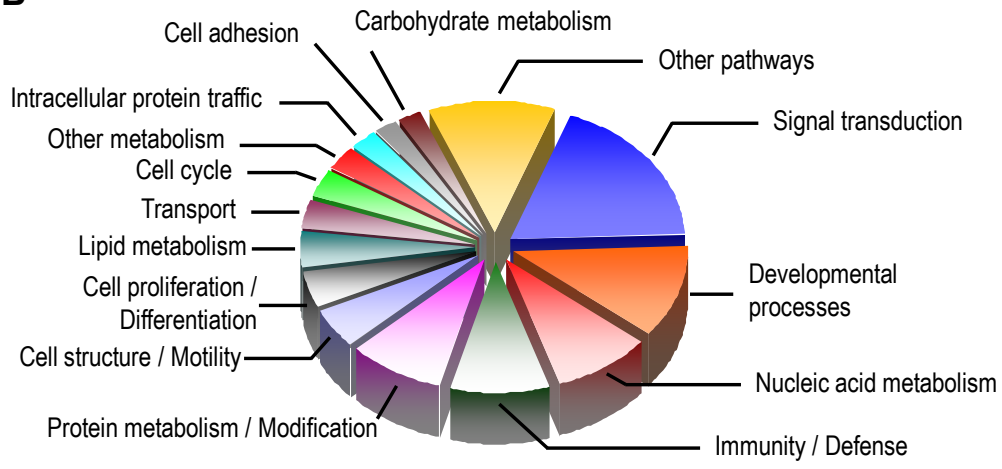
Figure 3 The change of gene expression patterns by CtBP in mouse embryonic fibroblast

(A) Heat map and clustering analysis of gene expression by microarray analysis. Data was filtered by appropriate criteria and normalized by the quantile method. Among them, data with fold-change > 2 and p-value < 0.05 were selected and used for hierarchical clustering. In heat map, a row means a gene and a column means a sample. (B) Biological processes of genes analyzed by microarray. Genes selected in (A) are classified according to biological processes and depicted as the pie chart. Ontology-based analysis was performed using the Panther classification system. (C) Relative mRNA expression levels in $Ctbp^{+/-}$, $Ctbp^{-/-}$ and $Ctbp^{-/-}$ (WT) MEF cells. Total RNA was purified from MEF cell lines and then mRNA levels were analyzed by qRT-PCR. Data are presented as mean ± standard deviation (n=3).

A


Ctbp+/–
Ctbp–/–
Min = -3.56
0.00
Max = 3.56

B


Cell adhesion
Carbohydrate metabolism
Other pathways
Intracellular protein traffic
Signal transduction
Other metabolism
Cell cycle
Transport
Lipid metabolism
Cell proliferation / Differentiation
Developmental processes
Cell structure / Motility
Nucleic acid metabolism
Protein metabolism / Modification
Immunity / Defense

C

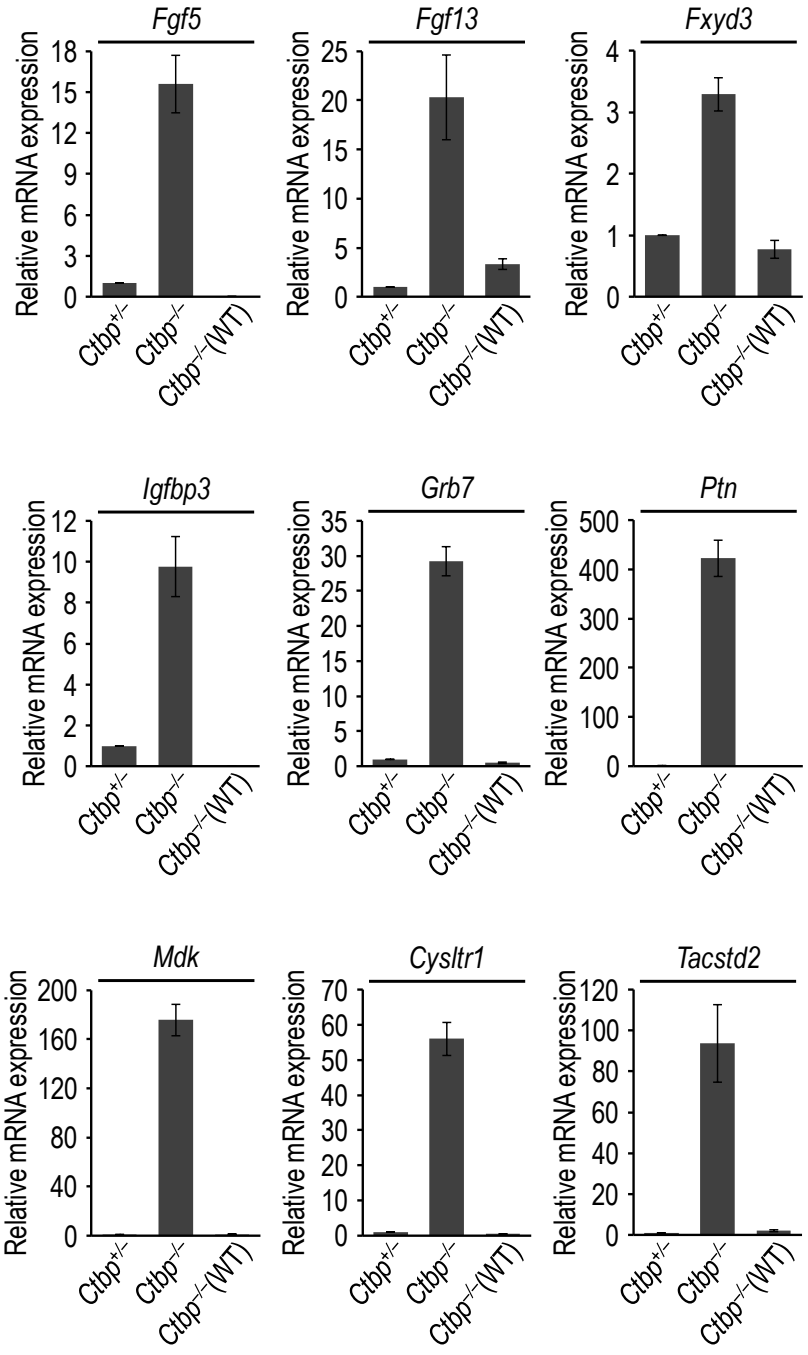

Fgf5
Fgf13
Fxyd3
Igfbp3
Grb7
Ptn
Mdk
Cysltr1
Tacstd2
Relative mRNA expression
Ctbp+/-
Ctbp-/-
Ctbp-/-(WT)

Table 1 The candidate genes up-regulated by CtBP

The table indicates candidate genes up-regulated by CtBP and their molecular and biological classifications. The list includes some genes with relatively higher fold change in the microarray analysis of *Ctbp*$^{-/-}$ MEF. Ontology-based analysis was performed using the Panther classification system.

Gene	Symbol	Accession	Panther classification	
			Biological process	Molecular function
FXYD domain containing ion transport regulator 3	Fxyd3	NM_008557	Signal transduction Transport	Ion channel Select regulatory molecule
Fibroblast growth factor 5	Fgf5	NM_010203	Signal transduction Developmental processes Cell proliferation & differentiation Cell cycle Oncogenesis	Signaling molecule
Fibroblast growth factor 13	Fgf13	NM_010200	Signal transduction	Signaling molecule
Insulin-like growth-factor binding protein 3	Igfbp3	NM_008343	Signal transduction Homeostasis	Miscellaneous function
Growth factor receptor bound protein 7	Grb7	NM_010346	Signal transduction	Miscellaneous function
Pleiotrophin	Ptn	NM_008973	Signal transduction Cell proliferation & differentiation	Signaling molecule
Midkine	Mdk	NM_010784	Signal transduction Cell proliferation & differentiation	Signaling molecule
Cysteinyl leukotriene receptor 1	Cysltr1	NM_021476	Signal transduction Immunity & defense	Receptor
Tumor-associated calcium signal transducer 2	Tacstd2	NM_020047	Signal transduction Cell proliferation & differentiation	Receptor

4. Transcriptional repression activity of edited CtBP1

We established stable cell lines expressing T176A mutant CtBP1 (*Ctbp*$^{-/-}$ (TA)) to examine whether edited CtBP1 is able to repress the target genes. mRNA expression levels of 6 of 9 candidate genes in *Ctbp*$^{-/-}$ (TA) MEF were compared with other cells by qRT-PCR (Figure 4A). *Ptn, Fgf5* and *Fgf13* showed comparable expression levels in *Ctbp*$^{-/-}$ (TA) MEF to those in *Ctbp*$^{-/-}$ MEF. The expression of *Mdk*, *Cysltr1* and *Grb7* were reduced in *Ctbp*$^{-/-}$ (TA) MEF than *Ctbp*$^{-/-}$ MEF, but still higher than *Ctbp*$^{+/-}$ and *Ctbp*$^{-/-}$ (WT) cells. This reflects that the transcriptional repression activity of edited CtBP1 protein was abolished compared to wild-type CtBP1.

PTN and MDK are expressed in human cancer cells, contributing to cancer development, including breast cancer (31-33). To examine whether CtBP1 regulates *PTN* and *MDK* gene expression in breast cancer cells, mRNA levels of the two genes were investigated in CtBP1-knockdown MCF7 breast cancer cells by qRT-PCR (Figure 4B and 4C). The results revealed that mRNA expressions of *PTN* and *MDK* were increased by the reduction of CtBP1 protein level although fold changes were relatively lower than MEF. This implies that the loss of transcriptional repression activity of CtBP1 induces PTN and MDK.

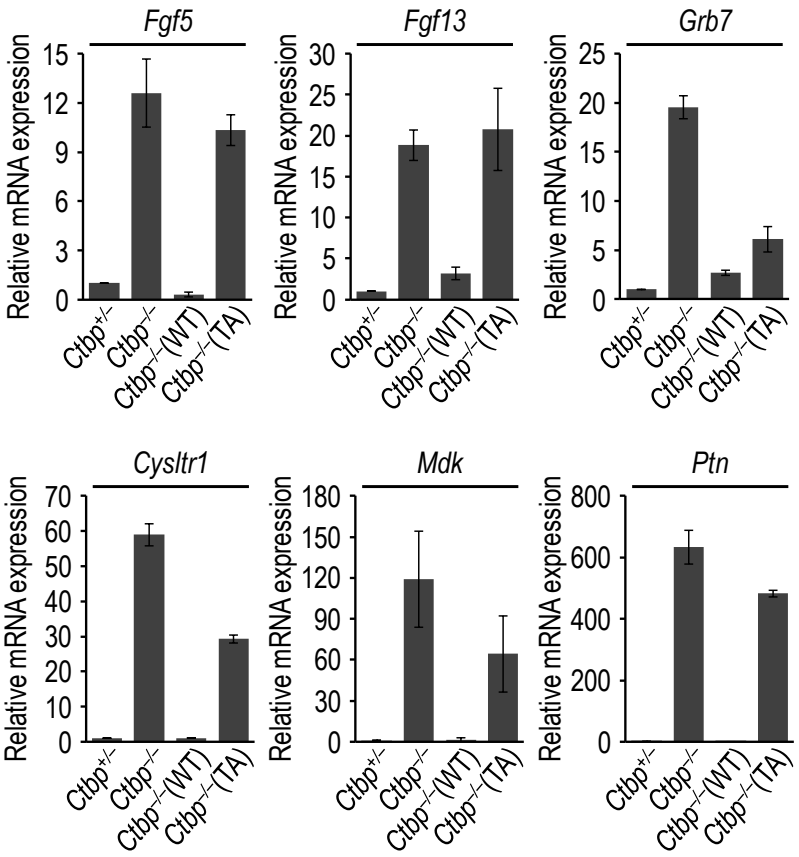
We performed ChIP assay to further investigate the regulation of *PTN* expression by CtBP1. As CtBP1 typically binds near the transcriptional start site (34), we assessed whether CtBP1 is recruited to the proximal *PTN* promoter (-182 to +5). CtBP1 occupied the proximal *PTN* promoter, while occupancy of T176A CtBP1 mutant was decreased (Figure 4D). CtBP1 is recruited to the proximal promoter of the E-cadherin gene (*CDH1*)

to repress transcription (1, 35). Thus, we also examined binding of T176A mutant to the *CDH1* proximal promoter. As the PTN promoter, occupancy of T176A mutant was reduced compared with wild-type CtBP1. Therefore, the edited forms of CtBP1 found in breast cancer cells may affect the expression of target genes, resulting in the cancer development.

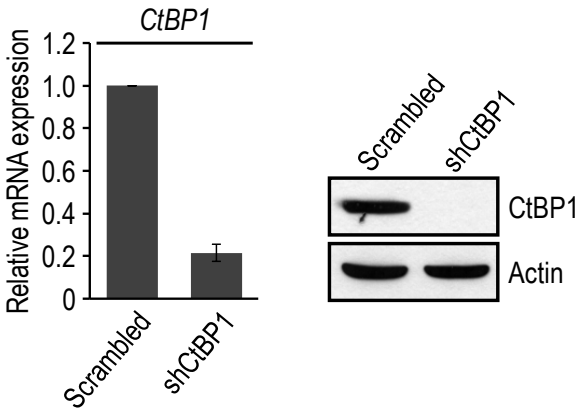
Figure 4 Transcriptional repression activity of edited CtBP1

(A) Relative mRNA expression levels in *Ctbp*$^{+/-}$, *Ctbp*$^{-/-}$, *Ctbp*$^{-/-}$ (WT) and *Ctbp*$^{-/-}$ (TA) MEF cells. mRNA levels were confirmed by qRT-PCR. (B) Knockdown of CtBP1 in MCF7 breast cancer cells. MCF7 cells were infected with CtBP1 shRNA-expressing lentivirus. Control cells were infected with scrambled shRNA-expressing lentivirus. After 48 hr, cells were harvested and then mRNA levels of CtBP1 were analyzed by qRT-PCR. Protein levels of CtBP1 were also assessed by immunoblot using anti-CtBP. (C) mRNA expression levels of *PTN* and *MDK* in CtBP1-knockdown MCF7 cells. (D) The binding of wild-type and T176A mutant CtBP1 proteins to *PTN* and *CDH1* promoters. HEK293T cells were transfected with empty (control) or flag-CtBP1 (WT or T176A) expression vector. After 24 hr, cells were harvested and then ChIP assay was performed with anti-flag or mouse normal IgG. Expression of flag-tagged WT and T176A CtBP1 was confirmed by immunoblot (up). DNA was amplified using pre-designed primer sets for *PTN* (-182 to +5) and *CDH1* (-171 to +30) proximal promoters and analyzed by qRT-PCR (down). Binding is expressed relative to the nonspecific binding of empty vector control. Data are presented as mean ± standard deviation (n=3) in (A)-(D).

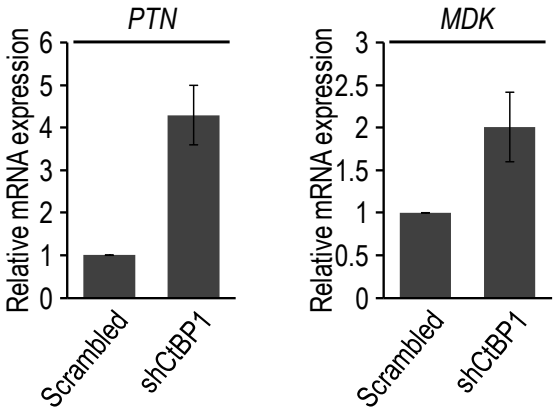
A


Fgf5
Fgf13
Grb7
Cysltr1
Mdk
Ptn
Relative mRNA expression
Ctbp+/-
Ctbp-/-
Ctbp-/-(WT)
Ctbp-/-(TA)

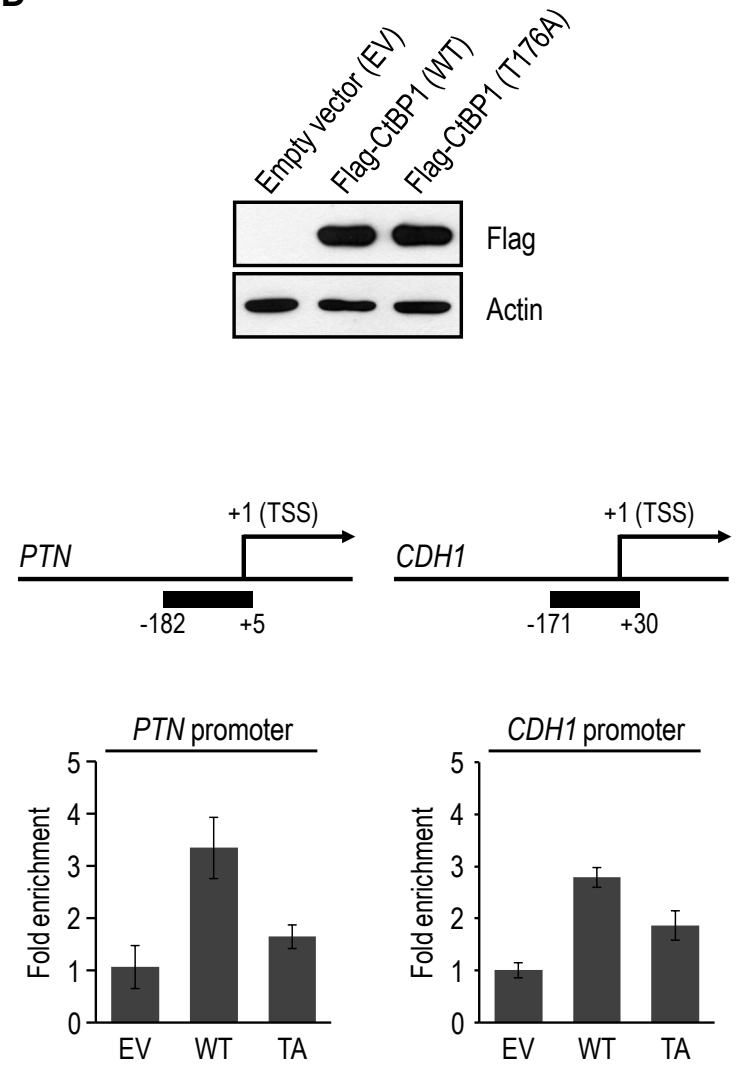
B


CtBP1
Relative mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0
Scrambled
shCtBP1
Scrambled
shCtBP1
CtBP1
Actin

C


PTN
Relative mRNA expression
6
5
4
3
2
1
0
Scrambled
shCtBP1
MDK
Relative mRNA expression
3
2.5
2
1.5
1
0.5
0
Scrambled
shCtBP1

D


Empty vector (EV)
Flag-CtBP1 (WT)
Flag-CtBP1 (T176A)
Flag
Actin
+1 (TSS)
PTN
-182
+5
+1 (TSS)
CDH1
-171
+30
PTN promoter
Fold enrichment
0
1
2
3
4
5
EV
WT
TA
CDH1 promoter
Fold enrichment
0
1
2
3
4
5
EV
WT
TA

DISCUSSION

RNA molecules are modified after synthesis of the primary transcript. These modifications include different post-transcriptional events such as alternative splicing and RNA editing. These processes provide the diversity of transcripts and proteins from the limited number of genes. *CtBP* transcripts also undergo RNA modification generating several isoforms. CtBP1 proteins have two isoforms, CtBP1-L and CtBP1-S, resulted from alternative splicing (14). Recently, other splice variants of CtBP are identified in breast cancer and melanoma (13, 15).

Although CtBP is widely accepted to promote tumorigenesis, it was recently demonstrated that the inactive CtBP1 splicing variant contributes to melanoma development (24). This suggested that CtBP1 functions as a tumor suppressive protein. Therefore, we questioned if a functionally defective mutant CtBP1 results in the breast cancer as in melanoma.

We report a unique CtBP1 variant, in which a single nucleotide of mRNA was changed from corresponding genomic DNA. This variant showed A-to-G conversion in the cDNA sequence. A-to-I RNA editing mediated by adenosine deaminase acting on RNA (ADAR) enzymes is a site-specific modification in RNA (22). The splicing, translational machineries and even the sequencing reaction recognize inosine as guanosine. Accordingly, we elucidated A-to-G conversion is resulted from A-to-I mRNA editing. The possibility of a sequencing error was excluded by further performing restriction enzyme digestion of cDNA against an enzyme site generated by editing.

A528G editing of CtBP1 results in the alteration of an amino acid sequence

(threonine-to-alanine substitution at the residue-176). Although T176A mutant CtBP1 proteins were localized in nucleus as wild-type CtBP1 they were defective in the *in vitro* dehydrogenase activity, dimerization and transcriptional repression activity. Recently, it was reported that the threonine-176 of CtBP1 is phosphorylated by Akt (36). This study showed somewhat conflicting results in experiments using T176A mutants. In this paper, T176A demonstrated no significant difference from wild-type CtBP1 in the dimerization ability and the transcriptional repression activity. The inconsistent results may be due to the different experimental conditions. These characteristics should be further determined by more elaborately designed work. However, the loss of normal function of T176A mutant, at least in part, is inevitable, because the residue lies near the NAD(H)-binding site (5, 37, 38).

To identify cancer-related genes regulated by CtBP, gene expression patterns of CtBP-knockout MEF were analyzed by microarray compared with CtBP-heterozygous MEF. A previous study revealed CtBP corepresses epithelial and proapoptotic genes through the microarray analysis of CtBP-knockout MEF (39). Based on the finding, CtBP1 is mainly recognized as an oncogenic protein. Although we found genes consistent with previous data, we focused on the genes up-regulated in *CtBP*-knockout MEF and their relationship with cancer. Of candidate genes, *PTN* and *MDK* are closely related to cancer development, especially breast cancer. The results from qRT-PCR revealed that these two genes may be regulated by CtBP1 and de-repressed by edited CtBP1 in cancer. From the results, CtBP1 may play an ambivalent role in cancer development. In breast cancer, CtBP1 represses tumor suppressor genes and the edited forms of CtBP1 may de-repress oncogenic genes.

To identify RNA editing sites, genome-wide studies have been conducted with bioinformatic analyses and high-throughput sequencing (40-42). These analyses identified putative A-to-I editing sites of CtBP2. Most sites are located in non-coding regions and each studies predicted different sites. Although RNA editing of *CtBP1* was not founded in the studies, we serendipitously observed a single nucleotide substitution of *CtBP1* mRNA, leading to the change of an amino acid. Because the edited forms of CtBP1 is functionally defective, if RNA editing mechanism is further studied, the founding may be useful for diagnosis and/or prognosis of diseases.

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국문 초록

RNA 변형 기작에는 선택적 스플라이싱 (alternative splicing)과 RNA 편집 (RNA editing) 등이 있다. 이러한 기작을 통해 전사체와 단백질의 다양성이 증가된다. 전사 보조 억제 인자인 CtBP는 선택적 스플라이싱에 의해서 다양한 isoform이 만들어진다. 그러나 아직까지 RNA 편집과 CtBP의 기능간의 연관성에 대해서는 밝혀진 바가 없다. 본 연구에서는 유방암 세포에서 528번 코돈 (codon)이 adenosine에서 guanosine으로 치환된 CtBP1의 독특한 전사 변이체를 발견하였다. 이렇게 RNA 편집이 일어난 CtBP1 전사체는 176번 아미노산 서열이 threonine에서 alanine으로 변화된 T176A 돌연변이 단백질을 생성하게 된다. 이 돌연변이 단백질의 특성을 조사해 본 결과 탈수소효소 활성과 이합체를 형성하는 능력이 야생형의 CtBP1에 비해 결여되어 있었다. 한편, microarray를 통해 CtBP가 없는 마우스 배아섬유아세포의 유전자 발현 양상을 분석한 결과 CtBP가 존재하지 않을 때 발현이 증가하는 유전자들이 발견되었고 이들 중 일부는 암과 관련이 있었다. CtBP가 발현되지 않는 세포에 야생형의 CtBP1을 회복시켜주면 이 표적유전자들은 다시 발현이 억제되었으나 T176A 돌연변이 CtBP1을 발현시켜주면 표적유전자들의 발현이 억제되지 못하고 여전히 높은 수준을 유지하였다. 이 결과를 통해 T176A 돌연변이 단백질은 전사 억제 능력에 결함이 있다는 것을 알 수 있었다. 따라서 CtBP1 단백질은 RNA 편집을 통해 암과 관련된 유전자들의 발현을 억제하는 능력을 조절하는 것 같다.

주요어: CtBP1, mRNA 편집, 암, 전사

학번: 2010-21915