**Featured Article**

**Loss of Heterozygosity and Internal Tandem Duplication Mutations of the CBP Gene Are Frequent Events in Human Esophageal Squamous Cell Carcinoma**

Chi-Kwong So,¹ Yan Nie,¹ Yunlong Song,¹ Guang-Yu Yang,¹ Suzie Chen,¹ Caroline Wei,¹ Li-Dong Wang,² Norman A. Doggett,³ and Chung S. Yang¹

¹Susan Lehman Cullman Laboratory for Cancer Research, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, New Jersey; ²Laboratory for Cancer Research, Zhengzhou University, Henan, China; and ³Bioscience Division and Center for Human Genome Studies, Los Alamos National Laboratory, Los Alamos, New Mexico

**Abstract**

**Purpose:** Cyclic AMP response element binding protein (CBP), a nuclear transcriptional corepressor protein, is an important component of the cAMP signal transduction pathway. In this study, we systematically analyzed the pattern and frequency of CBP gene alterations in esophageal squamous cell carcinoma (ESCC) samples from Linzhou (Linxian), China.

**Experimental Design:** Using microsatellite markers D16S475, D16S2622, and D16S523 within the chromosome 16p13.3 locus flanking the CBP gene, we observed loss of heterozygosity (LOH), microsatellite instability (MSI), or homozygous deletion in 16 of 26 ESCC samples. Additional ESCC samples were analyzed using different sets of microsatellite markers (CS1–CS5) within the introns or in close proximity to the 3' end of the CBP gene.

**Results:** The data showed that CBP gene LOH or MSI occurred in 9 of 19 ESCC samples. A detailed genetic alteration map of the CBP gene showed that an LOH or MSI hot spot occurred within intron 2 of the CBP gene. Furthermore, ESCC samples were investigated for CBP gene mutation by conformation sensitive gel electrophoresis and DNA sequencing. These results revealed that most of the shifted fragments contained internal tandem duplication (ITD), frequently in the regions encoding the histone acetyltransferase domain and COOH-terminal transactivating domain one of the CBP gene. The presence of ITD within the CBP gene was additionally confirmed by Southern blot analysis and sequencing.

**Conclusions:** These studies show that LOH and ITD of the CBP gene are frequent genetic events in human ESCC. These alterations may have functional importance in the development of human ESCC.

**Introduction**

Cyclic AMP response element binding protein binding protein (CBP), has been shown to play important roles in multiple cellular signaling pathways, in particular the cyclic AMP pathway (1). Cyclic AMP response element binding protein (CREB), which is phosphorylated by protein kinase A (2), was the first transcription factor shown to interact with CBP (3, 4). Subsequently, other basal transcription machinery proteins such as transcription factor IIIB and RNA polymerase II holoenzyme complex were found to bind to the CBP protein (4). In addition to cyclic AMP response element binding protein, numerous other transcription factors, including c-jun (5), c-fos (6), and c-Myb (7, 8), are also known to interact with CBP. Furthermore, CBP has been shown to regulate gene expression at the chromatin level (9). CBP possesses histone acetyltransferase (HAT) activity and releases the repressive interaction between the acidic DNA backbone and the basic histone proteins by acetylating lysine residues (10, 11). The relaxed acetylated-chromatin structure is then able to interact with the basal transcription machinery proteins. Besides HAT activity, CBP also possesses nonhistone or transcription factor acetyltransferase activity (12, 13). The effects of posttranslational modification by CBP vary among the transcription factors. For example, under stress conditions, CBP-mediated acetylation converts p53 from the inert form to the active form (13) but represses Tcf transcriptional activity during normal cellular progression (14).

Mutations in the CBP gene have been shown to be involved in several diseases. In humans, germ-line point mutations and microdeletions of the CBP gene have been identified in Rubinstein-Taybi Syndrome patients. This disease is characterized by mental retardation, craniofacial malformations, broad thumbs, broad big toes, and an increased occurrence of malignancy (15, 16). CBP has been mapped to human chromosome 16p13.3 (17). Loss of heterozygosity (LOH) at the 16p13.3 locus has been observed in hepatocellular carcinomas (18). It has also been documented that somatic chromosomal translocation of the CBP gene occurs in various types of hematological malignancies. Fusion transcripts such as MOZ-CBP [t(8;16)(p11;p13)], MLL-CBP [t(11;16)(q23;p13)], and MORG-CBP [t(10;16)(q22;p13)] are observed in various types of leukemia such as acute myeloid leukemia, chronic myeloid leukemia, and myelodysplastic syndrome (19–22). Mice harboring a null mutation in one CBP allele are prone to developing hematological malignancies such as histiocytic sarcoma and mononuclear leukemia (23).
The CBP gene encodes a 7.3-kb transcript that is translated into a M, 265,000 nuclear protein of 2441 amino acids (1). The CBP protein comprises several functional domains, including three cysteine-histidine rich domains, a KIX domain, one bromodomain, and one glutamine rich domain at the COOH terminus (24). The exact genomic structure of CBP has not been well defined. However, it has been suggested that the CBP genomic sequence spans ~160 kb and is composed of at least 13 exons (17).

In the present study, we used the human chromosome 16 genomic sequence finished at Los Alamos National Laboratory and compared this to the completed CBP mRNA sequence available at National Center for Biotechnology Information 4 to generate a detailed genomic structure for the CBP gene, including the identification of 31 exons within the CBP structural gene and the intron-exon boundaries. Because Rubinstein-Taybi Syndrome patients have a high propensity for developing cancers (25), the status of the CBP gene in human esophageal cancer was examined; in particular, the specimens obtained from a population at high risk for esophageal squamous cell carcinoma (25), the status of the gene in human esophageal cancer was examined; in particular, the specimens obtained from a population at high risk for esophageal squamous cell carcinoma (ESCC) in Lixinian, China. Using five CBP intragenic microsatellite markers, LOH or microsatellite instability (MSI) genetic alterations were detected in 9 of the 19 ESCC cases examined. Samples that showed these genetic alterations were then further analyzed for mutations in the coding regions. Conformation-sensitive gel electrophoresis (CSGE; Refs. 26, 27) and DNA sequencing results showed that internal tandem duplication (ITD; Ref. 28) within the CBP gene was the predominant alteration event in these ESCC samples.

Materials and Methods

ESCC Specimens and KYSE Cell Lines. Forty-five surgically resected ESCC samples and neighboring nontumorous esophageal epithelia were used for the analysis. Use of human samples in this study was approved by Institutional Review Board from Rutgers University under protocol no. 94-138. All samples were collected from a high incidence area for esophageal cancer in Linzhou (formerly Lixinian), Henan, China. Twenty-six of the 45 specimens were fixed with formalin and embedded in paraffin. Serial sections (5 μm) were prepared from paraffin-embedded tissue blocks and stained with Haematoxylin and eosin (H&E staining) for histological examination. After the H&E staining, the tumors were dissected under a microscope for DNA extraction. Nontumor epithelium from the same esophagus was used as control. DNA samples were extracted from tissues using Dneasy Tissue kit (Qiagen, Valencia, CA). All procedures were performed according to the manufacturer’s instructions. Nineteen of the 45 specimens were fresh frozen in OCT (Finetech, Terrance, CA). The frozen samples were cryosectioned at −20°C into 14- or 20-μm sections for H&E staining followed by DNA/RNA extraction or H&E staining. Tissues were dissected from the slides, and DNA was extracted using the same procedure as above. Total RNA was extracted from frozen tissue using RNeasy Tissue kit (Qiagen). All procedures were performed according to manufacturer’s instructions. KYSE cell lines nos. 150, 190, 30, 450, 510, 790, and 810 (29) were cultured with 50% RPMI and 50% HAM containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. DNA and RNA extractions were performed as described above.

Single and Multiplex PCR-Based Microsatellite Analysis. Three microsatellite markers (D16S475, D16S2622, and D16S522) were used to study genetic alterations on locus 16p13.3. Primer sequences for all three markers were obtained from the Genomic Database. The microsatellite marker was considered homozygously deleted if the normalized signal intensity (microsatellite marker 1/microsatellite marker 2) in the tumor lane was <25% of the signal in the normal lane (30, 31) by considering ~25% normal tissue contamination in the tumor tissue. A sample was considered to contain LOH of a microsatellite marker when the band intensity of one allele was decreased at least 50% or absent in the tumor as compared with the normal DNA. LOH was determined by calculation of the band intensity of normal and tumor alleles according to the following formulation:

\[
\text{LOH} = \frac{(\text{band intensity of normal allele 2}) - (\text{band intensity of normal allele 1})}{(\text{band intensity of tumor allele 2}) - (\text{band intensity of tumor allele 1})}
\]

The sample was considered to contain LOH if the ratio was <0.5 or >2.0. The sequences of the five pairs of intragenic microsatellite markers used for LOH studies are available upon request. For genetic alteration studies, one of the primers in the pair was end-labeled with [γ-32P] ATP (ICN, Costa Mesa, CA) at 37°C using T4 Polynucleotide Kinase kit (USB, Cleveland, OH). The PCR reaction mixture contained 1× PCR buffer [100 mM Tris-HCl (pH 8.3), 50 mM KCl, and 2.5 mM MgCl2], 10 ng of human genomic DNA, 25 pmol of each primer, 0.4 mM dideoxynucleotide triphosphates, and 0.1 IU of Taq. The reaction mixture was placed in a Gene Amp PCR system 9700 (Perkin-Elmer, Boston, MA) at 37°C using T4 Polynucleotide Kinase kit (USB, Cleveland, OH). The PCR reaction mixture contained 1× PCR buffer [100 mM Tris-HCl (pH 8.3), 50 mM KCl, and 2.5 mM MgCl2], 10 ng of human genomic DNA, 25 pmol of each primer, 0.4 mM dideoxynucleotide triphosphates, and 0.1 IU of Taq. The reaction mixture was placed in a Gene Amp PCR system 9700 (Perkin-Elmer, Boston, MA) for 35 amplification cycles. The PCR profile temperature for denaturing was 94°C for 25–45 s, and the extension temperature was 72°C for 1–3.5 min. The annealing temperature was between 55°C to 65°C for 30–60 s. PCR-LOH analyses were repeated two additional times on all samples to verify the genetic alteration results. Multiplex PCR was performed using the procedure of Li et al. (32).

CSGE Analyses of CBP Transcripts. CSGE analyses were used to screen for mutations in CBP transcripts. The sequences of the 18 pairs of primers used for the CSGE mutation analysis are available upon request. Using 5 μl of total RNA, cDNA was synthesized using RT-Advantage kit (Clontech, Palo Alto, CA). Reverse transcription-PCR (RT-PCR) reactions included 1 μl of cDNA, 1× PCR buffer [100 mM Tris-HCl (pH 8.3), 50 mM KCl, and 2.5 mM MgCl2], 25 pmol of each primer, 0.4 mM dideoxynucleotide triphosphates, 1 μCi [α-32P] dCTP (ICN), and 0.1 IU of Taq. The reaction mixture was placed in a Gene Amp PCR system 9700 (Perkin-Elmer) for 35 amplification cycles. The PCR profile temperature for denaturing was 94°C for 25–45 s, and the extension temperature was 72°C for 1–3.5 min. The annealing temperature was between 55°C and 65°C for 30–60 s. Multiplex PCR reactions were performed according to the procedure of Li et al. (32). To facilitate heteroduplex formation, labeled PCR products were

---

denatured at 95°C for 5 min, renatured at 68°C for 30 min, and cooled at 4°C for 10 min. One volume of PCR product was mixed with 3 volumes of 10× loading buffer (30% glycerol, 0.25% bromphenol blue, and 0.25% xylene cyanol FF) before being resolved by CSGE for heteroduplex analysis. Samples were analyzed on a 15% polyacrylamide CSGE gel as described previously (26). The gel was run at 15–20 W for 8–12 h, transferred to blotting paper, dried under a vacuum at 80°C for 1–5 h, and exposed to autoradiography film for 6–24 h without an intensifying screen at room temperature.

**Mutational Analyses of p53 Gene.** Mutational hot spots within exons 5–8 of the p53 gene were screened for mutations (33). In brief, DNA samples were amplified by PCR. The PCR products were then resolved by single-strand conformational polymorphism. The shifted bands, which may indicate possible conformational polymorphism difference, were extracted from the single-strand conformational polymorphism gel and reamplified for DNA sequence analyses. DNA sequence results would verify whether the corresponding exon contains any mutations.

**Sequencing.** The CSGE shifted bands were excised and extracted with distilled water at 55°C and reamplified using the same sets of primers and PCR conditions. PCR fragments were then sent to the DNA Sequencing Core Facility. PCR products were sequenced from both directions to confirm sequencing accuracy. RT-PCR-CSGE and sequencing were repeated two additional times on all samples to verify the ITD results.

**Southern Blot Analysis of the CBP Gene in KYSE Cell Lines.** Genomic DNA was extracted from one confluent 100 mm dish for each of the seven KYSE cell lines (nos. 150, 190, 30, 450, 510, 790, and 810). KYSE cell lines DNA (10 μg) and human placental genomic DNA (10 μg; Novagen, Madison, WI) were digested with restriction enzymes BshuAI and BsiH-KAI. The double-digested DNA was electrophoresed through a 1% agarose gel. The gel was then depurinated, denatured, and neutralized. The denatured DNA was transferred to a Gene Screen Plus membrane (NEN, Boston, MA). Prehybridization and hybridization of 32P-labeled CBP exon 18 probe were performed at 59°C using Perfect Hyb Plus Hybridization Buffer (Sigma, St. Louis, MO) for 30 min and overnight, respectively. The PCR product of CBP exon 18 was cloned into pGEMT easy vector (Promega, Madison, WI) and confirmed by DNA sequencing. The probe for hybridization was prepared by PCR from the above clone and labeling with α32P-dCTP using All in One Random Prime Labeling Mix (Sigma). After washing, the membrane was exposed to autoradiograph film (Kodak, Rochester, NY) with an intensifying screen at −70°C.

**PCR Analyses of the CBP Gene.** Alterations in the CBP gene were confirmed by PCR. The sequences of the primers are available upon request. The PCR reaction mixture contained 1× PCR buffer [100 mM Tris-HCl (pH 8.3), 50 mM KCl, and 2.5 mM MgCl2], 10 ng of human genomic DNA, 25 pmol of each primer, 0.4 mM dNTPs, and 0.1 U of Taq. The reaction mixture was placed in a GeneAmp PCR system 9700 (Perkin-Elmer) for 35 amplification cycles. The PCR product size for denaturing was 94°C for 25–45 s, and the extension temperature was 72°C for 1–3.5 min. The annealing temperature was from 55°C to 65°C for 30–60 s. The PCR products were then sequenced to verify the presence of ITD in the CBP gene. PCR and DNA sequencing were repeated two additional times on all samples showing alterations to verify the ITD results.

**Results**

**Genomic Structure of CBP.** The detailed genomic structure of the CBP gene was analyzed by comparing the genomic sequence of a finished four clone contig of this region consisting of two BACs and two cosmids ordered from 16pter toward 16cen as RP11-461A8, LA16c-RT102, LA16c-RT191, and RP11-95J11 (GenBank accession nos. AC006111, AC004651, AC004509, and AC007151). The CBP cDNA was aligned with this genomic sequence to identify consensus splice sites and the number of exons. The analysis showed that the CBP gene is composed of 31 coding exons spanning ~154 kb. The exons range in size from 45–2157 bp (Table 1). Exons 2–4 code for the NH2-terminal transactivating domain. Exons 4–5, 19–28, and 30–31 code for three cysteine-histidine rich domains. Exons 6–10 code for the KIX domain. Exons 17–18 code for the bromodomain. Exons 18–30 code for the HAT domain. The COOH-terminal transactivating domain and glutamine rich regions are encoded by exon 31 (Fig. 1A). The CBP gene has 30 introns ranging in size from 0.3 to 40 kb. All three of the large introns are located at the 5′ end of the gene. Furthermore, the

<table>
<thead>
<tr>
<th>5′ intron</th>
<th>Exons</th>
<th>Introns 3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTT</td>
<td>Exon 1</td>
<td>893 bp</td>
</tr>
<tr>
<td>TC</td>
<td>Exon 2</td>
<td>713 bp</td>
</tr>
<tr>
<td>TTT</td>
<td>Exon 3</td>
<td>177 bp</td>
</tr>
<tr>
<td>TCA</td>
<td>Exon 4</td>
<td>241 bp</td>
</tr>
<tr>
<td>TTA</td>
<td>Exon 5</td>
<td>114 bp</td>
</tr>
<tr>
<td>TTA</td>
<td>Exon 6</td>
<td>243 bp</td>
</tr>
<tr>
<td>TTA</td>
<td>Exon 7</td>
<td>103 bp</td>
</tr>
<tr>
<td>CCT</td>
<td>Exon 8</td>
<td>147 bp</td>
</tr>
<tr>
<td>TTT</td>
<td>Exon 9</td>
<td>118 bp</td>
</tr>
<tr>
<td>TTT</td>
<td>Exon 10</td>
<td>172 bp</td>
</tr>
<tr>
<td>TTA</td>
<td>Exon 11</td>
<td>45 bp</td>
</tr>
<tr>
<td>TTA</td>
<td>Exon 12</td>
<td>125 bp</td>
</tr>
<tr>
<td>TCG</td>
<td>Exon 13</td>
<td>181 bp</td>
</tr>
<tr>
<td>TTA</td>
<td>Exon 14</td>
<td>417 bp</td>
</tr>
<tr>
<td>TCA</td>
<td>Exon 15</td>
<td>180 bp</td>
</tr>
<tr>
<td>TCT</td>
<td>Exon 16</td>
<td>190 bp</td>
</tr>
<tr>
<td>CTT</td>
<td>Exon 17</td>
<td>119 bp</td>
</tr>
<tr>
<td>TTA</td>
<td>Exon 18</td>
<td>240 bp</td>
</tr>
<tr>
<td>TTA</td>
<td>Exon 19</td>
<td>89 bp</td>
</tr>
<tr>
<td>TGA</td>
<td>Exon 20</td>
<td>81 bp</td>
</tr>
<tr>
<td>AAC</td>
<td>Exon 21</td>
<td>57 bp</td>
</tr>
<tr>
<td>TCC</td>
<td>Exon 22</td>
<td>78 bp</td>
</tr>
<tr>
<td>TTA</td>
<td>Exon 23</td>
<td>68 bp</td>
</tr>
<tr>
<td>TTA</td>
<td>Exon 24</td>
<td>151 bp</td>
</tr>
<tr>
<td>TTA</td>
<td>Exon 25</td>
<td>147 bp</td>
</tr>
<tr>
<td>CCA</td>
<td>Exon 26</td>
<td>114 bp</td>
</tr>
<tr>
<td>TCT</td>
<td>Exon 27</td>
<td>166 bp</td>
</tr>
<tr>
<td>ACT</td>
<td>Exon 28</td>
<td>168 bp</td>
</tr>
<tr>
<td>TTA</td>
<td>Exon 29</td>
<td>162 bp</td>
</tr>
<tr>
<td>CCG</td>
<td>Exon 30</td>
<td>282 bp</td>
</tr>
<tr>
<td>CAG</td>
<td>Exon 31</td>
<td>2157 bp</td>
</tr>
</tbody>
</table>

CAG: GTR AGT

Table 1 Exon sizes and exon-intron boundary sequences of the CBP gene

Both the 5′ and 3′ intron sequences are compared with the consensus donor and acceptor sequences (last row). R in the consensus sequence presents a purine nucleotide.
genomic sequence of CBP contained a relatively high density of Alu repeat sequences in the intron regions (averaging 1 Alu repeat/1.5 kb of sequence).

LOH Analyses of Locus 16p13.3 and the CBP Gene.

Three microsatellite markers, D16S475, D16S2622, and D16S523 (Fig. 2; from the telomere to centromere) were selected from the Genomic Database to assess genetic alterations within chromosome locus 16p13.3. These three markers are located within a 2-Mb region flanking the CBP gene (Fig. 2A).

A panel of 26 paired esophageal samples (ESCC and normal samples) were analyzed for genetic alterations on locus 16p13.3 (Table 2). LOH in at least one of the loci was detected in 7 of 26 cases (27%), MSI was detected in 5 of 26 cases (19%; Fig. 2B), and homologous deletion was detected in 9 of 26 cases (35%; Fig. 2C). Homologous deletion on locus 16p13.3 was additionally confirmed by multiplex PCR with two microsatellite markers (D16S475 and D16S523). Both microsatellite markers were amplified in all of the normal samples. Only one microsatellite marker (D16S475 or D16S523) was amplified in some tumor samples (Fig. 2C). Taken together, these results showed genetic instability within the locus of 16p13.3. However, results from this study did not show a correlation between p53 mutations and genetic instability within the locus of 16p13.3.

Additional experiments were performed to analyze the nature of these genetic alterations within the CBP gene. Five CBP specific microsatellite markers were identified and used for additional studies. The first, second and third, and fourth markers were located within the first, second, and thirteenth introns of CBP, respectively. The fifth marker was ~9 kb from exon 31. Because of the limited amount of DNA materials from the 26 paired ESCC samples, a separate panel of 19 paired esophageal samples was used for the studies using these five intragenic markers. LOH (Fig. 3) in at least one of the microsatellite marker was detected in 7 of 19 cases (37%) and MSI was detected in 4 of 19 (21%) cases of ESCC. These results were then used to construct a genetic alteration map of the CBP gene (Fig. 1B). Seven of 9 MSI or LOH cases were observed at microsatellite markers CS2 and/or CS3, located within intron 2. No genetic alterations were observed in the 19 ESCC samples using microsatellite marker CS4, which was located in intron 13.

CBP Fusion Transcripts Detected by RT-PCR-CSGE.

The entire coding region (31 exons) of the CBP gene was screened in 16 of the 19 ESCC samples and in paired normal
samples by RT-PCR-CSGE analysis. Shifted bands were observed in 12 of the 16 ESCC samples (75%), and no shifted bands were observed in the corresponding normal epithelial samples (Fig. 4A). The excised fragments were amplified and analyzed by DNA sequencing. Comparison of the transcript sequences with the sequences in the National Center for Biotechnology Information database was performed using BLAST. The results showed that 7 of the 12 excised fragments contained mosaic transcript sequences of the CBP coding region. Tandem duplication of partial sequences within the same exon was also detected. All fusion sequences were joined in the correct orientation but did not occur at the consensus splice sites of the intron-exon boundaries. In addition, some fusion transcripts contained 0–9 overlapping nucleotides (Fig. 4B). A total of six different fusion transcripts was obtained: mosaic exon 15–exon 14; mosaic exon 14–exon 10; mosaic exon 22–exon 14; mosaic exon 19–exon 17; partially duplicated exon 18–exon 18; and partially duplicated exon 31–exon 31. All breakpoints of the fusion transcripts were precisely mapped and are shown in Fig. 5.

Sequence analyses of three of the scrambled sequences revealed that the fusion product preserved an open reading frame, suggesting that protein products could be expected. Two of four out-of-reading frame transcripts contained a premature stop codon, possibly leading to truncated products (Fig. 6). Duplication hot spots of the CBP transcript occurred in exons 14, 18 (HAT/2nd cys-his domain), and 31 (C-TAD1 domain). No functional domain has been assigned for exon 14.

<table>
<thead>
<tr>
<th>Sample</th>
<th>p53 status</th>
<th>Microsatellite marker</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>D16S475</td>
</tr>
<tr>
<td>920922</td>
<td>With p53 mutations</td>
<td>NI*</td>
</tr>
<tr>
<td>91787</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>920925</td>
<td>LOH</td>
<td>LOH</td>
</tr>
<tr>
<td>91779</td>
<td>–</td>
<td>NI</td>
</tr>
<tr>
<td>910609</td>
<td>NI</td>
<td>–</td>
</tr>
<tr>
<td>910840</td>
<td>HD</td>
<td>LOH</td>
</tr>
<tr>
<td>910673</td>
<td>MSI</td>
<td>–</td>
</tr>
<tr>
<td>705</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>920928</td>
<td>–</td>
<td>LOH</td>
</tr>
<tr>
<td>920935</td>
<td>MSI</td>
<td>–</td>
</tr>
<tr>
<td>910715</td>
<td>LOH</td>
<td>–</td>
</tr>
<tr>
<td>910623</td>
<td>HD</td>
<td>–</td>
</tr>
<tr>
<td>920973</td>
<td>–</td>
<td>NI</td>
</tr>
<tr>
<td>910793</td>
<td>Without p53 mutations</td>
<td>LOH</td>
</tr>
<tr>
<td>91799</td>
<td>LOH</td>
<td>MSI</td>
</tr>
<tr>
<td>920947</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>704</td>
<td>HD</td>
<td>–</td>
</tr>
<tr>
<td>910618</td>
<td>MSI</td>
<td>–</td>
</tr>
<tr>
<td>933334</td>
<td>MSI</td>
<td>–</td>
</tr>
<tr>
<td>910666</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>920953</td>
<td>HD</td>
<td>–</td>
</tr>
<tr>
<td>910649</td>
<td>–</td>
<td>NI</td>
</tr>
<tr>
<td>910473</td>
<td>MSI</td>
<td>NI</td>
</tr>
<tr>
<td>910634</td>
<td>LOH</td>
<td>NI</td>
</tr>
<tr>
<td>91702</td>
<td>HD</td>
<td>NI</td>
</tr>
<tr>
<td>910783</td>
<td>–</td>
<td>NI</td>
</tr>
</tbody>
</table>

*NI, noninformative; –, normal; MSI, microsatellite instability; HD, homozygous deletion; LOH, loss of heterozygosity.
To additionally assess mutations of the CBP gene in human ESCC, RNA samples from seven KYSE human esophageal carcinoma cell lines (29) were used for RT-PCR-CSGE and sequencing analysis. Shifted bands were observed for all seven cell lines by CSGE analysis. Sequencing results showed that all shifted bands contained mosaic CBP transcripts. All of the fusion breakpoints in the KYSE cell lines occurred in exon 18 or 31, which correspond to the HAT/2nd cys-his and C-TAD domains, respectively (data not shown).

Southern Blot Analysis of CBP Genomic DNA in KYSE Cell Lines. To determine whether the observed CBP duplications occurred at the genomic level, specific primers were designed for PCR to amplify across the observed fusion breakpoints. DNA samples digested with BfuAI and BsiHKAI to excise a fragment spanning from the 3' end of intron 17 to the 5' end of intron 19. The membrane was probed with CBP exon 18, and a band with the expected size of 2 kb was observed in the human placental genomic DNA (positive control) and all seven of the cell lines. This 2-kb band contained part of intron 17, exon 18, intron 18, exon 19, and part of intron 19 of the CBP gene. An additional BfuAI and BsiHKAI 5-kb fragment with amplification/rearrangement was observed in KYSE nos. 150 and 810 (Fig. 7). Previous RT-PCR-CSGE and sequencing results showed that these two cell lines exhibit ITD involving exon 18, whereas the other five KYSE cell lines did not. Southern blot analysis did not show novel fragments in these five KYSE cell lines, confirming that the aberrant DNA 5-kb fragment was only detected in KYSE nos. 150 and #810 but not in the other KYSE cell lines.

PCR and Sequencing of CBP Genomic DNA. To additionally confirm that the observed CBP duplications occurred at the genomic level, specific primers were designed for PCR to amplify across the observed fusion breakpoints. DNA samples were double-digested with BfuAI and BsiHKAI, electrophoresed in 1% agarose gels, and blotted to nylon membrane. The membrane was hybridized with CBP exon 18 probe. The novel rearranged fragments that were indicated by solid arrows were observed in cell lines KYSE nos. 150 and 810. Samples without detectable rearrangement using exon 18 probe were KYSE nos. 190, 30, 450, 510, 790, and human placental control DNA.

Fig. 5 Schematic representation of selected CBP internal tandem duplicated fusion transcripts detected by reverse transcription-PCR. Tandem duplication involving two different exons or the same exon resulted in out-of-frame or in-frame fusion transcripts. The arrows indicate exon fusion breakpoints. The corresponding case number is on the right. Diagrams are not to scale. Duplicated exons are indicated in bold italic.

Fig. 6 Partial nucleotide sequence of conformation sensitive gel electrophoresis-excised shifted band from case no. 98010 was analyzed by sequencing. The arrow shows the duplication breakpoint of CBP exon 19 and exon 17. Nucleotide 4449 of CBP exon 19 was fused out-of-frame with nucleotide 4249 of CBP exon 17. A premature stop codon was created, indicated by dashed arrow and bold •.

To additionally assess mutations of the CBP gene in human ESCC, RNA samples from seven KYSE human esophageal carcinoma cell lines (29) were used for RT-PCR-CSGE and sequencing analysis. Shifted bands were observed for all seven cell lines by CSGE analysis. Sequencing results showed that all shifted bands contained mosaic CBP transcripts. All of the fusion breakpoints in the KYSE cell lines occurred in exon 18 or 31, which correspond to the HAT/2nd cys-his and C-TAD domains, respectively (data not shown).

Southern Blot Analysis of CBP Genomic DNA in KYSE Cell Lines. To determine whether the observed CBP duplications occurred at the genomic level, Southern blot hybridization was performed to detect possible genetic rearrangements using CBP exon 18, which was involved in ITD, as a probe. On the basis of the results from RT-PCR-CSGE analysis of KYSE cell lines, ITD of exon 18 was detected in both KYSE nos. 150 and 810. Genomic DNA from seven KYSE cells lines were double-digested with BfuAI and BsiHKAI to excise a fragment spanning from the 3' end of intron 17 to the 5' end of intron 19. The membrane was probed with CBP exon 18, and a band with the expected size of 2 kb was observed in the human placental genomic DNA (positive control) and all seven of the cell lines. This 2-kb band contained part of intron 17, exon 18, intron 18, exon 19, and part of intron 19 of the CBP gene. An additional BfuAI and BsiHKAI 5-kb fragment with amplification/rearrangement was observed in KYSE nos. 150 and 810 (Fig. 7). Previous RT-PCR-CSGE and sequencing results showed that these two cell lines exhibit ITD involving exon 18, whereas the other five KYSE cell lines did not. Southern blot analysis did not show novel fragments in these five KYSE cell lines, confirming that the aberrant DNA 5-kb fragment was only detected in KYSE nos. 150 and #810 but not in the other KYSE cell lines.

PCR and Sequencing of CBP Genomic DNA. To additionally confirm that the observed CBP duplications occurred at the genomic level, specific primers were designed for PCR to amplify across the observed fusion breakpoints. DNA samples were double-digested with BfuAI and BsiHKAI, electrophoresed in 1% agarose gels, and blotted to nylon membrane. The membrane was hybridized with CBP exon 18 probe. The novel rearranged fragments that were indicated by solid arrows were observed in cell lines KYSE nos. 150 and 810. Samples without detectable rearrangement using exon 18 probe were KYSE nos. 190, 30, 450, 510, 790, and human placental control DNA.
from ESCC containing fusion transcripts were analyzed for the presence of fusion exons. Because of the presence of large introns at the genomic-level, not all introns containing fusion transcripts were able to be amplified. For those primer pairs, which flanked <700 bp, PCR products were obtained from the tumor DNA samples, and no PCR products were amplified from the corresponding normal samples. Sequence analysis from these amplified PCR products revealed mosaic sequences in the genomic DNA (Fig. 8). Furthermore, the genomic DNA sequences corresponded with the cDNA sequence results.

Discussion

Sakai et al. (18) reported that LOH of 16p13.3 is observed frequently in hepatocellular carcinoma. These authors proposed that genetic alterations at 16p13.3 have a dominant negative effect, which may contribute to tumor development. Furthermore, in Rubinstein-Taybi Syndrome patients who have a high propensity for developing cancer, CBP mutations were found and mapped to 16p13.3 (25). A tumor-suppressing role for CBP has been additionally exemplified by the indicated predisposition to hematological malignancies observed in mice heterozygous null for CBP locus (23). These findings prompted us to investigate the role of CBP in ESCC development.

This is the first systematic study demonstrating that there are frequent genetic alterations within the CBP gene in human ESCC. Genetic alterations within 16p13.3 were assessed in 26 pairs of esophageal DNA samples. LOH, MSI, or HD at 16p13.3 were detected at a high frequency in these ESCC samples. High-resolution genetic alteration mapping of another 19 pairs of ESCC samples showed that LOH or MSI also occurred in 9 of the 19 cases within the CBP gene. Taken together, these results indicate that the CBP gene is a frequent target for genetic alterations, which may play a role in the pathogenesis of human ESCC.

From LOH or MSI studies, hot spots were identified within the second intron of the CBP gene. These results indicate that the genomic region around intron 2 of CBP is very unstable. The impact of genetic instability within this region on the CBP structural gene is not known. The genetic alteration hot spot identified from this study was in close proximity (within 40 kb) of a 13-kb chromosomal translocation breakpoint of CBP that has been reported earlier in leukemia (19–21, 34). The presence of a high copy number of Alu repeat elements may facilitate homologous recombination, including inter- and intrachromosomal recombination events (35–37). A review of the CBP genomic sequence in the noncoding region shows a high number of Alu repeat sequences, which may make the CBP gene prone to genetic alterations such as LOH. Both types of rearrangements have been observed within the CBP gene in various diseases. Interchromosomal recombination is a likely mechanism for complex chromosomal abnormalities, including the CBP-MLL, CBP-MORF, and CBP-MORF translocations that have been well documented in leukemia patients (19–21, 38). On the other hand, intrachromosomal recombination may also lead to deletion or duplication events. Microdeletions of the CBP gene have been observed in Rubinstein-Taybi Syndrome patients (16).

The present study showed that ITD occurred within CBP transcripts from these ESCC samples. To determine whether this rearrangement occurred at the genomic level, genomic DNA from seven KYSE cell lines were used for Southern blot analysis. The probe from the duplicated exon 18 revealed a normal band in all seven cell lines and one higher molecular weight, amplified/rearranged fragment in the two cell lines that also showed ITD of exon 18 by RT-PCR-CSGE and sequencing analysis. The sequence of this rearranged fragment is not known. However, the probe that was used for the Southern blots was CBP exon 18. Therefore, this amplified/rearranged fragment contained sequences homologous to CBP exon 18. Results from the Southern blot analysis agree with those of the earlier RT-PCR-CSGE and sequencing analysis. By RT-PCR-CSGE, the other five KYSE cell lines did not show rearrangements involving exon 18. By Southern blot analysis, no aberrant DNA fragments were detected. Therefore, we showed by both methods of Southern blot also RT-PCR-CSGE and sequencing analysis are in good agreement. ITD in the ESCC samples was additionally confirmed in genomic DNA by PCR and sequencing analysis. Primers flanking the mosaic sequences were used to amplify from the genomic DNA and the PCR products were verified by sequencing. The result confirmed that ITD of the CBP gene occurred at the genomic level. A similar spectrum of tandem duplications of the CBP gene was detected in tumor-derived ESCC cell lines (KYSE). KYSE cell lines showed a
high frequency of duplication in the HAT/2nd cys-his or C-TAD1 domains. Therefore, the scrambled CBP transcripts were most likely because of exon rearrangement and duplication events during DNA replication at the genomic level, rather than abnormal alternative splicing at the transcriptional level (39).

The frequent ITD mutations of the CBP gene may play an important role in the progression of human esophageal cancer. ITD has also been observed in the FLT3 and COL1A1 genes in which the alterations are associated with myeloid leukemia and osteogenesis imperfecta, respectively. ITD of the juxtamembrane domain of the FLT3 gene leads to a constitutively active FLT3 receptor in 20% of AML. In AML, both the ras and STAT5 pathways have been shown to be activated in the absence of the corresponding growth factors (40). ITD of exons 14–17 of the COL1A1 gene lead to duplication of 60 amino acid residues within the triple helical domain. This mutated COL1A1 protein disrupts the normal triple helical structure of the collagen fiber and may contribute to osteogenesis imperfecta (41). The high number of Alu repeats in the intron regions of the CBP gene may facilitate tandem duplication mutations (28). Alu-mediated tandem and non-tandem duplications have been observed in MLL (42, 43). These duplications occurred in both the exon and intron regions.

The fusions observed in this study resulting from ITD sequence of the CBP gene were found to be in-frame, out-of-frame, and out-of-frame with a known premature stop codon. In-frame fusion predicted a partial duplicated protein product. Out-of-frame fusion with a known premature stop codon suggested a truncated protein product. The consequence of out-of-frame fusion without any known premature stop codon is not known. All of the tandem duplicated rearrangements observed in the current study occurred between exons 14, 18, and 31, which code for an undefined domain, HAT/2nd cys-his, and C-TAD1 domains, respectively.

The functional consequence of the above rearrangement is not known. It is conceivable that tandem duplication of the HAT domain in CBP may alter acetyltransferase activity resulting in aberrant chromatin organization and deregulation of transcription (44). Previous findings have indicated the presence of MOZ-CBP or MLL-CBP fusion transcripts in leukemia patients. CBP and MOZ proteins both possess HAT activity, and an increase in HAT activity has been suggested for MOZ-CBP or MLL-CBP fusion proteins (19–21). Earlier studies by Kitabayashi et al. (45) showed that a MOZ-CBP fusion protein inhibited AML-1-mediated transcription and differentiation in M1 myeloid cells. They proposed that MOZ-CBP may antagonize the function of AML1 by either acetyllating protein(s) that bind to AML-1 and/or by interfering with the binding of acetylated protein/s to AML-1. In addition to the HAT domain, other regions such as the second and third cys-his domains, KIX domain, bromodomain, C-TAD1, C-TAD2, and the Q-rich domain possess important functions for CBP (12). Recently, results from several studies have suggested that different CBP functional domains interact with transcription factors in a cooperative rather than isolated manner. For example, IFN-β expression is mediated through CBP. Upon gene activation, CBP acetylulates HMG1, disrupting the stability of the IFN-β enhancerosome, and turns off IFN-β gene expression (46–48). Thus, for certain cellular events, both the transcriptional and acetyltransferase activities of CBP are critical for the activation and inactivation of the processes. Therefore, the cooperative interaction between different domains within the CBP protein is essential. Genetic alteration within an isolated region will probably affect the overall biological functions of CBP.

In summary, genetic alterations of the CBP gene is a frequent event in human ESCC samples from a high risk area of China. Additional genetic analyses showed that the CBP gene also frequently contained ITD mutations, which are a novel mutation of the CBP gene in human cancer.

Acknowledgments

We thank Drs. Xiaoxin Chen and Darren N. Seril for helpful discussions.

References


Loss of Heterozygosity and Internal Tandem Duplication Mutations of the CBP Gene Are Frequent Events in Human Esophageal Squamous Cell Carcinoma

Chi-Kwong So, Yan Nie, Yunlong Song, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/10/1/19

Cited articles
This article cites 48 articles, 17 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/10/1/19.full.html#ref-list-1

Citing articles
This article has been cited by 6 HighWire-hosted articles. Access the articles at:
/content/10/1/19.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.