CBP truncating mutations in ovarian cancer

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The *CBP* gene (OMIM #600140) encodes the 2441 amino acid cyclic AMP response element binding protein (CREB) binding protein (Mr 265 000) that functions as an essential co-activator for a number of transcription factors.¹ In particular, CBP, like its homologue EP300, enhances gene transcription by linking sequence specific transcription factors to transcription factor IIB and the RNA polymerase II holoenzyme.² CBP can also promote gene transcription by acetylation of histones through its histone acetyltransferase activity (HAT)³ and by acetylation of specific transcription factors such as p53.^{4–6} Several lines of evidence suggest the involvement of CBP in tumour formation. CBP interacts with the human T cell leukaemia virus (HTLV) Tax protein and v-myb to induce viral and cellular genes that promote cell transformation, while adenovirus E1A, simian virus SV40 Tag, and human papillomavirus (HPV) E6 bind to CBP and inhibit its co-activator function. CBP is also known to modulate the actions of the *hMDM2* and *AML1* cellular proto-oncogenes and the *BRCA1* and p53 tumour suppressors.⁷ More direct evidence comes from genetic studies of translocation events involving *CBP* in leukaemia. In particular, the t(8;16)(p11;p13) translocation in AML involves a fusion of the *CBP* and *MOZ* genes,⁸ a novel t(10;16)(q22;p13) translocation in a childhood acute myelogenous leukaemia (AML-M5a) generates a MORF-CBP chimera,⁹ and the t(11;16)(q23;p13.3) translocation in topoisoemerase II treated acute leukaemia or myelodysplasia results in a MLL-CBP fusion.¹⁰ In addition, haploinsufficiency of CBP in humans due to chromosomal rearrangements, microdeletions, and point mutations results in Rubenstein-Taybi Syndrome (RTS), and is associated with an increased risk for a variety of tumours.¹¹

As a result of these observations, a number of studies have evaluated whether CBP is a direct mutational target in cancer and has tumour suppressor activity. One recent mutation screening study of CBP in breast, ovarian, and colorectal tumours and cell lines identified two CBP truncating mutations in ovarian cancer cell lines, but failed to detect mutations in the tumours.¹² Haematological malignancies displaying loss of heterozygosity (LOH) at the *CBP* locus have been detected in aged CBP heterozygous mice,¹³ and in chimeric mice derived from CBP^{−/−} embryonic stem cells.¹⁴ In addition, recent studies using an MMTV driven conditional knockout mouse model demonstrated that truncation of the CBP protein in the thymus results in development of T cell lymphomas,¹⁵ suggesting that targeted mutations in CBP can predispose to cancer. To determine whether CBP is a mutational target in human epithelial cancers, and specifically to evaluate the presence of CBP mutations in breast and ovarian tumours, we conducted a mutational analysis of the *CBP* gene.

MATERIALS AND METHODS

Sample selection

A total of 75 unselected surgically resected fresh frozen ovarian tumour specimens and 61 unselected fresh frozen breast tumour specimens were obtained from the ovarian and

Key points

- Inactivation of the CBP transcriptional co-activator in mice results in haematological malignancy, indicating that *CBP* is a tumour suppressor gene.
- We performed *CBP* mutation analysis of 75 ovarian and 61 breast tumours, and detected in ovarian tumours two somatic frameshift mutations that truncate the CBP protein, but none in breast tumours.
- Five unique missense mutations of unknown effect on the CBP protein were also identified in breast and ovarian tumours.
- Both tumours with truncating mutations displayed loss of the wild type *CBP* allele, suggesting that the *CBP* tumour suppressor contributes to ovarian tumour development.
- Deregulation of p53 was observed in the CBP mutant tumours, suggesting that the contribution of inactivated CBP to ovarian tumorigenesis is independent of its role in regulation of p53 signalling.

breast tumour banks at the Mayo Clinic. Use of these samples was approved by the Mayo Clinic institutional review board. Each tumour specimen was defined by haematoxylin and eosin analysis as containing at least 30% tumour cells. Genomic DNA was prepared from sections of each tumour using the PureGene DNA extraction kit (Gentra). Genomic DNA was also prepared from 1×10⁶ cells from each of 8 ovarian cancer cell lines (SKOV3, OVCAR5, OVCAR8, OV17.17, Ov167, Ov177, Ov202, Ov207) and eight breast cancer cell lines (MCF7, BT474, ZR75-1, UACC893, UACC812, MB-MDA468, MB-MDA157, MB-MDA361).

Mutational analysis

Selection of primer pairs for PCR amplification of the *CBP* coding sequence and splice sites from genomic DNA was based on previous studies¹⁶ and on the genomic structure of *CBP*.¹⁷ The sequences of the 36 primer pairs used for PCR and the associated PCR conditions are available upon request. Briefly, 20 ng of tumour genomic DNA was used as template for PCR. PCR products were heteroduplexed, loaded onto a Transgenomic WAVE dHPLC system, and analysed using product specific melting temperatures. When altered peaks were identified on the dHPLC, the original tumour DNA was re-amplified by PCR and sequenced in both directions.

Germline DNA analysis

Genomic DNA was extracted from 5 µm sections of paraffin blocks of normal ovaries and/or endometrial tissue. Briefly, the sections and cores were deparaffinised with xylene and treated with proteinase K at 55°C overnight. Genomic DNA was isopropanol precipitated. These DNAs were used as

Table 1 Mutations in the *CBP* gene in breast and ovarian tumours

Mutation type	Amino acid change	Tumour sample	Protein domain	Germline/somatic	<i>CBP</i> allele status
Frameshift/truncation					
765delC	L297X	ov623	NTD	Somatic	LOH
4675delA	L1548X	ov519A	HATD	Somatic	LOH
Missense					
1597G→A	A467T	ov519A	CBD	Somatic	LOH
2876C→T	S893L	BT0167		?	?
3409G→A	A1081T	OVCAR5		?	?
4259C→T	A1354V	ov51	HATD	Somatic	LOH
6130A→G	N1978D	ov735	GR and CTD	Germline	LOH
6130A→G	N1978D	B110	GR and CTD	?	?
Silent					
2151T→C	Y651Y (polymorphism)	B73, B143, B103, ov519A, and ov141	CBD and KIX		
2755C→T	L852L	BT0167			
3171C→T	D1049D	B73 and ov323	HATD		
5250C→T	S1741S	B78			
5634C→G	T1812T	B93	HATD		
5651G→A	V1818V (polymorphism)	ov542, ov985, ov177, and OV177			
7047C→T	S2283S	MDA463			
7410A→G	E2404E	ov182			
3'-UTR					
7552insC	-	B61	3'-UTR		
7549T→C	-	UACC812	3'-UTR		
7610C→A	-	B113	3'-UTR		
7631G→A	-	B92	3'-UTR		
Intronic					
IVS2-31A→G	-	B80 and B101			
IVS5-33G→T	-	B81			
IVS8-39delA	-	B92 and ov69			
IVS9-24A→G	-	MDA468			
IVS17-16delTTT	-	ov647			
IVS21-8C→T	-	B81, B96, and UACC812			
IVS27+37A→C	-	ov742			
IVS27+46G→A	-	ov742			
IVS28-15C→T	-	B80			
IVS28-14G→C	-	ov990			
IVS28-14G→C	-	MDA361			
IVS28-12C→G	-	ov526			

HATD, histone acetyltransferase domain; NTD, N terminal transactivation domain; CTD, C terminal transactivation domain; GR, Gln rich region; CBD, CREB binding domain; KIX, KID interacting domain.

templates for PCR of the exons containing the *CBP* mutations.

Biallelic inactivation of *CBP*

The complete inactivation of the *CBP* locus was established by immunohistochemistry of sections from paraffin blocks of tumour specimens using anti-*CBP* antibodies A22 (sc-369) and C20 (sc-583). The A22 antibody detects an epitope in the N terminus of *CBP* and the C20 antibody detects an epitope in the C terminus. Briefly, 5 µm sections were deparaffinised and stained with A22 (1:1000) or C20 (1:800) and the EnVision+ HRP labelled polymer (Dako, Glostrup, Denmark) on a Dako Autostainer. LOH analysis at the *CBP* locus was also performed using PCR techniques. Briefly, genomic DNA was extracted from 0.6 mm cores of ovarian tumour paraffin blocks as described above. Cores were used to enrich for tumour cells over infiltrating normal cells. The tumour and normal DNA were used as templates for PCR of the exons containing the *CBP* mutations, and the products were evaluated by dHPLC. Tumour DNAs displaying little or no mutant heteroduplex formation were considered to have LOH of the *CBP* locus.

Immunohistochemistry of p53 and p27^{KIP1}

Sections from the paraffin block tumour specimens were also stained for the presence of stabilised p53 and p27^{KIP1} using

anti-p53 clone DO-7 (Dako) and anti-p27 clone SX53G8 (Dako) antibodies as described above.

RESULTS

Determination of the intron-exon structure of *CBP*

The detailed genomic structure of the *CBP* gene was analysed by comparing the genomic sequence of the two bacterial artificial chromosomes and two cosmid clones RP11-420F6, LA16c-RT191, LA16c-RT102, and RP11-316H7 (GenBank AC005564, AC004509, AC004651, AC004760) with the known *CBP* cDNA sequence. The sequence alignment revealed that *CBP* is comprised of 31 exons ranging in size from 45 to 3306 bp. This predicted gene structure matched exactly with the observations from another recent study.¹⁷ The N terminal transactivating domain of *CBP* is encoded by exons 2-4, the three cysteine histidine rich domains by exons 4-5, 19-28, and 30-31, the KIX domain by exons 6-10, the bromodomain by exons 17-18, the HAT domain by exons 18-30, and the C terminal transactivating domain by exon 31.

CBP mutation screening in primary tumours

To determine whether mutations in *CBP* were associated with human breast or ovarian cancer, we conducted a mutation screen of all *CBP* coding regions and splice acceptor and donor sites in genomic DNA from 75 unselected fresh frozen ovarian tumour specimens, 61 unselected fresh frozen breast tumour specimens, and 16 breast and ovarian cancer cell

Figure 1 Expression of CBP, p53, and p27^{KIP1} in ovarian tumours containing CBP mutations. Deparaffinised 5 µm sections of ovarian tumours containing CBP mutations were subjected to immunohistochemistry using primary antibodies against CBP N terminus (A22), CBP C terminus (C20), p53, and p27^{KIP1}, along with an EnVision+ HRP labelled polymer (Dako). Positive staining is shown in brown, negative in blue. Staining of ov519 (L1548X CBP mutation), ov623 (L297X CBP mutation), and ov735 (N1978D CBP mutation) tumour sections with the different antibodies are shown in columns 1–3, respectively.

lines. Each genomic DNA sample was PCR amplified using 36 independent PCR primer sets. PCR products were heteroduplexed, or in the case of cell line products were mixed with PCR products from normal lymphoblastoid cell DNA and subsequently heteroduplexed, and subjected to dHPLC analysis (Transgenomics Inc.). Samples with altered peak structure on the dHPLC were re-amplified from the original genomic DNA and sequenced to identify the specific sequence alterations.

Two unique frameshift mutations, six missense mutations, twelve intronic sequence alterations, four alterations in the 3' untranslated region, and eight silent mutations were identified in the tumours and cell lines (table 1). The frameshift mutations (765delC and 4675delA) were found in two ovarian tumours. These mutations result in truncation of CBP at residue 297 in the N terminal transactivation domain and residue 1548 in the histone acetyltransferase domain (HATD), respectively. Both mutations are expected to inhibit CBP function.

Three of the missense mutations were detected in ovarian tumours, two were found in breast tumours, and one was identified in the OVCAR5 ovarian cancer cell line. The A467T mutation was detected in the ovarian tumour (ov519) that

also carries the 4675delA frameshift. It was unclear whether this missense mutation was in *cis* or *trans* with the frameshift variant or whether this variant in the CREB binding domain could influence CBP function. An A1354V variant in the HAT domain of CBP and an N1978D variant in the Gln rich region and C terminal transactivation domains were also identified in ovarian tumours, while the OVCAR5 cell line contained the A1081T variant. Interestingly, the N1978D alteration was also observed in an unrelated breast tumour (B110). A separate S893L variant was detected in another breast tumour (BT0167). It is not known whether these missense mutations have any influence on CBP activity.

CBP mutation analysis in germline DNA

Genomic DNA was extracted from paraffin blocks of normal ovaries and/or endometrial tissue from the individuals with missense or frameshift CBP mutations in their ovarian tumours. The exons containing these mutations were PCR amplified using the normal genomic DNA as template as described above and the PCR products were directly sequenced. The 765delC, 4675delA, 1597G→A (A467T), and 4259C→T (A1354V) mutations were not detected in the germline DNA, suggesting that these mutations occurred

Table 2 Pathological characteristics of *CBP* mutant ovarian tumours

Tumour	<i>CBP</i> mutation	Stage	Grade	Histology
Ov51	A1354V	1C	2	Endometrioid
Ov519	4675delA	1C	1	Transitional cell
Ov623	765delC	3C	3	Clear cell
Ov735	N1978D	3C	3	Serous

somatically. However, the 6130A→G (N1978D) variant from ov735 was detected in germline DNA, indicating that this mutation was inherited. These studies were not possible for the mutations identified in breast tumours because the tumours had been de-identified prior to use and we could not identify the relevant patients in order to obtain normal DNA from blood or paraffin blocks of normal tissue.

Biallelic disruption of *CBP*

To determine if all alleles of *CBP* were disrupted in the tumour tissue containing *CBP* mutations we obtained paraffin block specimens of the ov623, ov519, ov51, and ov735 ovarian tumours and evaluated the presence of *CBP* by immunostaining. Using the A22 antibody against an N terminal *CBP* epitope we detected *CBP* protein in all tumour cells (fig 1). However, when the C-20 antibody, which recognises a C terminal *CBP* epitope, was used, no *CBP* protein was detected in tumour cells from ov623 and ov519, which contain truncated forms of *CBP*, although staining was evident in normal stromal cells in these samples (fig 1). This result suggests that these tumour cells express only the truncated form of the *CBP* protein and therefore display either LOH at the *CBP* locus or inactivation of the second allele by other genetic or epigenetic mechanisms. Immunohistochemistry analysis of ov51 and ov735 that contain *CBP* missense mutations detected *CBP* protein in tumour and normal cells. LOH analysis at the *CBP* locus in these four tumours was also performed using dHPLC analysis of genomic DNA from tumour cells isolated from paraffin block tumour specimens. All tumours displayed LOH in this region.

p53 and p27^{KIP1} status of *CBP* mutant tumours

CBP has been shown to interact with and regulate the transcriptional activity of p53,¹⁸ suggesting that *CBP* loss might contribute to tumorigenesis through failure to correctly induce p53 expression and activity. To test this possibility, we evaluated p53 expression in ovarian tumours with truncating and missense mutations of *CBP* by immunostaining. Stabilisation or overexpression of p53 was observed in ov735, ov623, and ov519, which harbour the N1978D, L297X, and L1548X mutations, respectively (fig 1), while ov51, containing the A1354V missense mutation, showed no p53 staining (data not shown). We noted that p53 was overexpressed and probably mutated in tumours that also contained *CBP* inactivating mutations. As inactivation of both proteins is needed for formation of some ovarian tumours, it appears that these proteins are not part of the same signalling pathway, and that inactivated *CBP* contributes to ovarian cancer in a p53 independent manner.

We also evaluated p27^{KIP1} levels by immunostaining in these tumours because previous work showed that p27^{KIP1} downregulation strongly synergises with *CBP* loss in T cell lymphomagenesis.¹⁵ Staining for p27^{KIP1} was evident in the ov623 tumour with the L297X mutation but not in the ov519 tumour with the L1548X mutation. Thus, while p27^{KIP1} downregulation is not a requirement for development of all

CBP deficient human ovarian cancers, in contrast to its uniform contribution to *CBP* deficient lymphomagenesis in the mouse, it may contribute to development of a subset of *CBP* mutant ovarian tumours.

DISCUSSION

In this study, we report that two frameshift mutations of *CBP* were detected in a mutation screen of 75 ovarian epithelial tumours. Both mutations truncate the *CBP* protein N terminal of the HAT domain, and are expected to result in expression of inactive partial *CBP* proteins. We demonstrated by immunostaining with *CBP* antibodies that the second *CBP* allele was inactivated in both tumours with frameshift/truncating mutations. Thus, we have established for the first time that *CBP* is a biallelically inactivated tumour suppressor in at least 2/75 (3%) of ovarian tumours. This finding is in strong agreement with mouse model studies that have shown tumour development in animals deficient in *CBP* function,^{13, 15} and with the detection of truncating mutations in two ovarian cancer cell lines.¹² The discovery of inactivating *CBP* mutations identifies *CBP* as one of a small number of genes including p53, *BRCA1*, and *BRCA2* that are mutated in ovarian tumours. Indeed, the frequency of truncating mutations in *CBP* in ovarian tumours is at least as high as that of *BRCA2*.

Although two truncating mutations were found, it is difficult to accurately establish the frequency of mutations in this gene based on this study. The recent description of internal *CBP* tandem duplications in human oesophageal cancer¹⁹ suggests that other mutations, either in the form of large genomic rearrangements or mutations in regulatory regions of the gene, undetected by the dHPLC approach described here, may be present in *CBP* in ovarian and/or breast tumours, and that the actual frequency of *CBP* mutations in ovarian cancer is higher.

We also report on the identification of three *CBP* missense mutations in ovarian tumours, one missense mutation in an ovarian cancer cell line, and two missense mutations in breast tumours.

Of these, the 1597G→A (A467T) variant was detected in the same ovarian tumour as the 4675delA variant. It is not known whether this mutation can alter *CBP* function or *CBP* mRNA and/or protein stability, but we do know that both alleles of *CBP* in this tumour fail to encode a full length *CBP* protein. The absence of *CBP* protein encoded by the second allele appears to be due to LOH within the tumour. The 6130A→G (N1978D) variant was found in an ovarian and a breast tumour from unrelated individuals and was also detected in germline DNA from one of the individuals with the mutation in their tumour DNA. LOH at the *CBP* locus was found in the ovarian tumour containing this mutation, and the residue is conserved throughout evolution at least as far as chicken, suggesting that it might be functionally important. However, the individual carrying the mutation in the germline does not have Rubenstein-Taybi syndrome, as would be expected of someone with a *CBP* inactivating mutation, suggesting that the mutation does not disrupt *CBP* function sufficiently to cause this syndrome and to predispose to breast and/or ovarian cancer. Interestingly, a missense mutation of the same residue (N1978S) has been detected in an individual with a mild form of Rubenstein-Taybi syndrome. Thus, different alterations in the same residue of *CBP* may have significantly different effects on *CBP* function. We were unable to derive any useful information about the disease relatedness of the 4259C→T (A1354V) missense mutation from studies of germline DNA and immunostaining of the mutant tumour for *CBP*, p53, and p27^{KIP1}. Similarly, we were unable to determine the relevance of the S893L and A1081T variants to development of the

BT0167 breast tumour and OVCAR5 cell line, respectively. Additional functional studies of these variants may help to clarify the effects of the various missense mutations on CBP activity and on ovarian tumorigenesis.

Eleven unique intronic variants in the *CBP* gene in breast and ovarian tumours and in cell lines were detected. RT-PCR analysis of RNA from the tumours with these mutations failed to detect any splicing alterations in the *CBP* transcript (data not shown), suggesting that these mutations have no influence on the *CBP* gene or protein and make no contribution to tumour development.

Of considerable interest is the finding that the mutations detected in ovarian tumours were found in four histologically different forms of the disease (table 2). This suggests that *CBP* mutations are not restricted to specific histological subtypes of ovarian cancer. In addition, *CBP* mutations appear to be present in both early and late stage ovarian tumours. Interestingly, truncating *CBP* mutations have now been found in ovarian but not breast tumours, while *p300* mutations have been detected in breast tumours but not in ovarian tumours,²⁰ suggesting a level of specificity in the relative contributions of the CBP and *p300* paralogues to hormonally dependent epithelial tumours. However, *CBP* and *p300* mutations have been found in breast and ovarian cancer cell lines, respectively.¹² Thus, further studies are needed to establish the relevance of *CBP* and *p300* mutations to ovarian and breast cancer development.

Using immunostaining approaches, we were able to demonstrate that both of the tumours containing truncated forms of CBP also expressed stabilised forms of p53. The identification of *CBP* mutations in association with *p53* mutation/overexpression suggests that disruption of CBP does not inactivate the p53 signalling pathway and that certain tumours need additional genetic events to further disrupt p53. Thus, it appears that mutant CBP contributes to ovarian tumorigenesis in a currently undefined p53 independent manner. Furthermore, the finding of *CBP* and *p53* mutations in the same ovarian tumours suggests that *p53* mutation/deregulation can synergise with mutant CBP during ovarian tumorigenesis. These findings are in full agreement with recent studies in conditional knockout models of the *CBP* gene in which it was found that disruption of p53 accelerates tumorigenesis in CBP deficient animals.¹⁵

In summary, the identification of CBP inactivating mutations in conjunction with disruption of the second *CBP* allele strongly suggests that *CBP* is a tumour suppressor gene that contributes to ovarian tumorigenesis in humans.

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