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Mutation analysis of CBP and PCAF reveals rare inactivating mutations in cancer cell lines but not in primary tumours

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In this study we screened the histone acetyltransferases CBP and PCAF for mutations in human epithelial cancer cell lines and primary tumours. We identified two CBP truncations (both in cell lines), seven PCAF missense variants and four CBP intronic microdeletions. These data suggest that neither gene is commonly inactivated in human epithelial cancers.

Keywords: P300; CBP; PCAF; mutations; epithelial cancers

The addition of an acetyl group to specific lysine residues within the N-terminal region of the four core histone proteins by acetyltransferases, causes the destabilisation of the chromatin structure and enhances access of transcription factors and other DNA-binding components to DNA (Grunstein, 1997). The histone acetyltransferases CBP, P300 and PCAF also acetylate sequence-specific transcription factors such as P53. CBP was originally isolated on the basis of its interaction with CREB in response to cAMP signalling (Chrivita et al., 1993). P300 was purified as a cellular protein, which binds the adenoviral protein E1A (Eckner et al., 1994). PCAF, P300/CBP-associated factor, was the first mammalian histone acetyltransferase discovered on the basis of homology to yeast Gcn5p (Yang et al., 1996). The fact that histone acetyltransferases are involved in cell proliferation and differentiation suggests that they may be involved in cancer. Indeed P300 (also known as EP300) and CBP are fused to MLL in acute myeloid leukaemia. It is also known that P300, CBP and PCAF are targeted by viral oncoprotein E1A (Eckner et al., 1994; Yang et al., 1996; Chakravarti et al., 1999). In colorectal and gastric carcinomas two somatic P300 missense mutations coupled to deletion of the second allele of the gene were identified (Murukaa et al., 1996). The role of P300 as a tumour suppressor gene was later confirmed with inactivation of the second allele (Gayther et al., 2000). In this study we screened the whole coding sequence and intron-exon boundaries of both CBP and PCAF for somatic mutations in a series of human primary tumours and cancer cell lines. We also screened a panel of cell lines for truncating P300 mutations using Western blotting.

MATERIALS AND METHODS

Samples
The CBP gene was screened in 179 DNA samples isolated from 59 primary breast tumours, 37 primary ovarian tumours, 20 colorectal tumours, and 63 cancer cell lines. The PCAF gene was screened in 80 cancer cell lines (31 breast, 25 ovarian, 10 pancreatic, 6 SCLC, 5 colorectal, 1 NSCLC, 1 MIS, 1 BCLL) and 20 primary colorectal tumours. In all cases the collection of tumour material was done with Local Research Ethics Committee approval. All tumours were ‘flash’ frozen immediately following surgery. Cell lines were obtained from ATCC and ECACC cell repository or as a gift from collaborating laboratories.

Preparation of DNA and RNA
Frozen primary tumours were serially sectioned onto slides. Tumour tissue was microdissected and DNA extracted by SDS-proteinase K digestion followed by phenol-chloroform extraction. Germ-line DNA was prepared from either a matching blood sample or from normal tissue. Cell line DNA was extracted by either proteinase K or DNAzol™ (Gibco BRL). RNA was extracted with TriZol™ (Gibco BRL). cDNA was synthesized by reverse transcription of RNA using random hexamers and Superscript II (Gibco BRL).

Determination of the exon–intron structure of CBP and PCAF
The exon-intron structure of CBP and PCAF were determined from the available cDNA and genomic DNA sequences in Genbank (NCBI). CBP is a 8694 bp cDNA consisting of 32 exons distributed over 154 Kb of genomic sequence at chromosome band 16p13.3. PCAF is a 2957 bp cDNA consisting of 20 exons spread over 114 Kb of genomic sequence at chromosome band 3p24.
Polymerase chain reaction

CBP was amplified from gDNA in 43 fragments and PCAF was amplified from cDNA in 13 fragments of approximately 200–400 bp (oligonucleotide primer sequences are available on request, ho212@cam.ac.uk). PCAF sequence alterations were confirmed subsequently in genomic DNA. Amplification reactions (30 μl) contained 20 mM (NH₄)₂SO₄, 75 mM TrisHCl, pH 9.0 at 25°C, 0.1% (w/v) Tween, 2.5–3 mM MgCl₂, 200 μM dNTP, 10 pmol of each primer and 2.5 U of Red Hot DNA polymerase (Advanced Biotechnologies). The amplifications were done using a DNA Engine Tetrad, MJ Research PTC-225 Peltier Thermal Cycler.

Protein truncation test

PCAF coding sequence was analysed initially by PTT. Cell lines HCT15 and OVCAR8, which showed an altered sized P300 protein on Western blot were also analysed by PTT. RT–PCR amplification was done in overlapping fragments of approximately 1000–1200 bp in length each, using a 5′ oligo containing the appropriate sequences (oligonucleotide sequences are available on request). PTT reactions were performed following the manufacturer’s protocol (Promega). Alterations found in PTT were confirmed by sequencing.

SSCP/HA (Single Strand Conformation Polymorphism/ Heteroduplex Analysis)

Formamide loading buffer was added to PCR products. The mix was denatured at 95°C for 10 min and kept on ice until loading onto 0.8 × MDE (Mutation Detection Enhancement) gel (Flowgen), both with and/or without 10% Glycerol. Gels were run overnight at 120 V and 4°C.

Western blot analysis

Western blot analysis was used for P300 truncating mutations in a panel of 24 cell lines. We also performed Western blot in cell lines identified to have truncating CBP mutations. Cell extracts were prepared by direct lysis on cell culture plates (TBS, 0.5% NP-40, 5 mM EDTA, Complete Protease Inhibitor Coctail, Boehringer), then electrophoresed in pre-cast polyacrylamide Tris-Glycine gels (Novex). The separated proteins were transferred to nitrocellulose membrane (Millipore) and hybridised with the respective primary (CBP A-22 Santa Cruz, P300 N-15 Santa Cruz) and secondary antibodies (Dako). Detection employed the ECL kit (Amersham).

DNA Sequencing

Purified PCR products were sequenced using ABI Prism® BigDye terminators and an ABI377 sequencer or ABI3100 genetic analyzer (Applied Biosystems, Foster, CA, USA). All samples with a mutation were re-amplified and re-sequenced.

| Gene | Sample | Sequence alteration | Result |
|------|--------|---------------------|--------|
| CBP  | Shin3  | IV521-4delI22       | In frame deletion of exon 22 |
| CBP  | LK1    | IV531-7insA         | Stop1795 |
|      | LK2    |                     |        |
|      | PA1    |                     |        |
|      | CH1    |                     |        |
| P300 | HCT15  | 4239G>T             | E1014X |
| P300 | OVCAR8 | 6387delT            | S1733X |

RESULTS AND DISCUSSION

CBP mutations

Two different CBP truncating mutations were identified in the 63 cell lines analysed (Table 1). Shin3, an ovarian cancer cell line, was found to have a heterozygous 22 bp deletion in intron 21 at position –4 (Figure 1A). This intronic deletion was shown to cause an in-frame deletion of the whole exon 22 at the cDNA level (Figure 1B). In four cancer cell lines (LK1, LK2, PA1, CH1) an identical heterozygous insertion of an A was found in intron 31 at position –7 (Figure 1C). This insertion was shown to create an alternative splice donor site. This in turn caused a frame-shift and a premature stop codon at nucleotide 5457 (codon 1795). This heterozygous mutation was confirmed using Western blotting (Figure 1D). The finding of the identical mutation raised the suspicion of cross contamination between cell lines. HLA typing was performed and the results showed that these cell lines were indeed the same, despite originating from two different labs (data not shown). We considered these as a single cell line for purposes of mutation frequency analysis and therefore the truncating mutations were identified in two distinct cell lines out of 60 analysed (3%). No truncating mutations were identified in 116 primary tumours. Small intronic microdeletions in CBP were identified in four samples (Table 2). A primary colorectal cancer had a heterozygous deletion of T at position –2 in intron 18. This tumour had no molecular phenotype suggestive of microsatellite instability (MSI). Two cancer cell lines with MSI, OVIP and HCT15, had an identical microdeletion. These intronic microdeletions, which were very close to the splice donor site, had no apparent effect on mRNA splicing as tested by amplification of cDNA with primers flanking exon19. A breast cancer cell line, MT3, was found to have a deletion of T in intron 14 at position –82, with no apparent effect on splicing as tested by RT–PCR. This cell line is also MSI+. Uncommon CBP single nucleotide polymorphisms were also detected (Table 3).

Although we have characterised two truncating CBP mutations in cancer cell lines, the absence of inactivating mutations in the primary tumours analysed prevents us from unequivocally establishing the role of CBP in human primary cancers. Nevertheless the uncommon mutations identified, together with the increased tumour incidence in Rubinstein-Taybi syndrome (Petritj et al, 1995) and the tumorogenic phenotype in CBP mice (Kung et al, 2000) provide circumstantial evidence for a possible role of CBP as a tumour suppressor gene. We can speculate that the functional significance of the intronic microdeletions seen, but we note that we have previously identified similar intronic microdeletions in other genes in MSI cell lines and primary tumours (data not shown).

Missense PCAF sequence alterations

No truncating mutations were identified in the PCAF gene. We used cDNA to screen for truncating mutations and therefore nonsense mediated RNA decay (Maquat, 1995) could have contributed to a lower sensitivity of the mutation screen. Missense sequence alterations in PCAF were identified in 1 out of 20 primary tumours (5%) and 5 out of 80 cell lines (6%). In a colorectal cancer case the missense variant was a C to A transversion at nucleotide 2595 in exon 17, resulting in a proline to threonine substitution at codon 713. The same sequence alteration was also found in the germ-line DNA of the same individual (Table 2). The functional significance of this single nucleotide polymorphism was not tested but this residue is conserved in mouse and human GCN5, suggesting it might be important for protein function. Sequence analysis in DNA extracted from laser capture microdissected normal and tumour tissue samples confirmed the heterozygous mutation in both tumour and germ-line DNA, and
therefore the mutation is not associated with somatic allelic deletion. A missense alteration at nucleotide 1615 (N386S) was found in four cell lines (Ovmana, Hela, L23, MC4000/ Matu). The mouse homologue of this residue is serine, implying that this alteration is a polymorphism. An ovarian cancer cell line, OVI-P, had a C to T transition at nucleotide 2415 resulting in Arg653Trp substitution. This arginine residue is conserved in human GCN5, and therefore this substitution could impair protein function. In addition to these missense sequence alterations a single nucleotide deletion in the 3' untranslated region of PCAF was found in a colon cancer cell line, SW48. Three silent PCAF polymorphisms were also identified (Table 3).

Table 2  Intronic microdeletions in CBP and missense sequence alterations in PCAF

| Gene | Frequency (%) | Sequence alteration | Result |
|------|---------------|---------------------|--------|
| CBP  | 0.5           | IVS14-82delT        | –      |
| CBP  | 2             | IVS18-2DelT         | –      |
| PCAF | 5             | 1615A>G            | N386S  |
| PCAF | 1             | 2415C>T            | R653W  |
| PCAF | 1             | 2595C>A            | P713T  |

Truncating P300 mutations

We have previously shown that truncating mutations resulted in the production of stable protein, detectable by Western blot. Using
this approach we studied a panel of 24 cell lines and in two (8.3%) truncated protein was detected (Table 1). OVCAR8 had two bands on the Western blot, one of normal size and one from a truncated protein, suggesting a heterozygous mutation. Sequencing confirmed a heterozygous frameshift deletion (6387delT) resulting in truncation of the protein at codon 1733 (Figure 1E). HCT15 expressed a heterozygous frameshift deletion (IVS12–18G) resulting in a truncated protein, suggesting a heterozygous mutation. Sequencing confirmed this approach we studied a panel of 24 cell lines and in two (8.3%) truncated protein was detected (Table 1). OVCAR8 had two bands on the Western blot, one of normal size and one from a truncated protein, suggesting a heterozygous mutation. Sequencing confirmed a heterozygous frameshift deletion (6387delT) resulting in truncation of the protein at codon 1733 (Figure 1E). HCT15 expressed a heterozygous frameshift deletion (IVS12–18G) resulting in a truncated protein, suggesting a heterozygous mutation. Sequencing confirmed a heterozygous frameshift deletion (IVS12–18G) resulting in a truncated protein, suggesting a heterozygous mutation.

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Table 3 Silent polymorphisms in CBP and PCAF

| Gene | Frequency (%) | Sequence alteration |
|------|--------------|--------------------|
| CBP  | 0.5          | 122G>A             |
| CBP  | 0.5          | 131I>G             |
| CBP  | 0.5          | 181G>A             |
| CBP  | 0.5          | 215I>T             |
| CBP  | 0.5          | IVS12–37C>T        |
| CBP  | 0.5          | IVS12–18G>A        |
| CBP  | 1.6          | 4099C>A            |
| CBP  | 1.6          | IVS28–13G>A        |
| CBP  | 1.1          | IVS28–13G>A        |
| CBP  | 1.1          | IVS28–13G>C        |
| PCAF | 2            | 767G>A             |
| PCAF | 3%           | 1037C>T            |
| PCAF | 3%           | 172I>T             |