Candidate target genes for loss of heterozygosity on human chromosome 17q21

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Loss of heterozygosity (LOH) on chromosome 17q21 has been detected in 30% of primary human breast tumours. The smallest common region deleted occurred in an interval between the D17S746 and D17S846 polymorphic sequences tagged sites that are located on two recombinant P1-bacteriophage clones of chromosome 17q21: 122F4 and 50H1, respectively. To identify the target gene for LOH, we defined a map of this chromosomal region. We found the following genes: JUP, FK506BP10, SC65, Gastrin (GAS) and HAP1. Of the genes that have been identified in this study, only JUP is located between D17S746 and D17S846. This was of interest since earlier studies have shown that JUP expression is altered in breast, lung and thyroid tumours as well as cell lines having LOH in chromosome 17q21. However, no mutations were detected in JUP using single-strand conformation polymorphism analysis of primary breast tumour DNAs having LOH at 17q21. We could find no evidence that the transcription promoter for JUP is methylated in tumour DNAs having LOH at 17q21. We suspect that the target gene for LOH in primary human breast tumours on chromosome 17q21 is either JUP and results in a haploinsufficiency for expression or may be an unidentified gene located in the interval between D17S846 and JUP.

Keywords: chromosome 17q21; loss of heterozygosity; plakoglobin

MATERIALS AND METHODS

Characterisation of 17q12–21

Two P1 phages clones were used to analyse the 17q12–21q21 region: 122F4 and 50H1 (generously provided by Dr Ray White, Department of Oncological Sciences, Huntsman Cancer Institute, University of Utah, Salt Lake City, UT, USA). Libraries of 122F4 and 50H1 were constructed using standard techniques with BamHI or SstI genomic restriction fragments, respectively, cloned into the pBluescript II KS (+) vector (Stratagene, La Jolla, CA, USA) (Miyazaki et al, 1999). Subclones were subsequently ordered by size, restriction enzyme and nucleotide end-sequence analysis using universal primers from the vector. Nucleotide sequence analysis was performed using the ABI Big Dye Terminator sequencing kit (PE Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions on an ABI 377 automated sequencer from PE Applied Biosystems (Foster City, CA, USA). The alignment of nucleotide sequences was determined with the Sequencher Analysis Software Package by Genetics
mutations in JUP genomic from breast tumours having LOH on chromosome 17q21. The primers used correspond to intron sequences located 5’ and 3’ of the particular exon (Table 1). After an initial denaturation cycle of 94°C for 5 min, amplification from 100 ng genomic DNA was carried out with two cycles each of (94°C 10 min, 66°C 10 min, 72°C 20 min), (94°C 10 min, 64°C 10 min, 72°C 20 min), (94°C 10 min, 62°C 10 min, 72°C 20 min), (94°C 10 min, 60°C 10 min, 72°C 20 min) and then 25 cycles of (94°C 10 min, 56°C 10 min, 72°C 20 min) with a final extension cycle at 72°C for 7 min. All SCCP products were analysed on 4–20% precast polyacrylamide gels (Novex, San Diego, CA, USA) in TBE at 325 V for 60 min at a primer set-dependent temperature (see Table 1) using the manufacturer’s conditions. The gels were stained with SYBR Green II (Molecular Probes, Inc., Eugene, OR, USA) using the manufacturer’s conditions, visualised using a 340 nm UV viewing box and photographed.

Methylation-specific PCR for the JUP transcription promoter

As a positive control for CpG methylation of genomic DNA, we have methylated a restriction fragment of recombinant genomic DNA containing the JUP transcription promoter. This was performed using the Sssl methylase (CpG methylase) (New England Biolabs, Inc., Beverly, MA, USA) and the manufacturer’s conditions. A PCR-based experimental protocol was used to detect hypermethylation of genomic DNA (Herman et al, 1996; Dracopoli et al, 1998). Briefly, 1 μg of primary breast tumour genomic or control recombinant DNA containing the JUP promoter was treated with sodium bisulphite. Modified and nonmodified control and tumour DNA were used as templates in PCR reactions with FastStart Taq DNA Polymerase (Roche Molecular Biochemicals, Indianapolis, IN, USA). The reaction mixture contained 5 μl of 10× PCR buffer (containing 2 mM MgCl2), 1 μl of 10 mM PCR Nucleotide Mix, 250 ng of each primer, 0.4 μl FastStart Taq DNA Polymerase (2 U), 1 μl of bisulphite modified DNA and distilled water to 50 μl total volume. The primer pairs for unmethylated DNA: PgU F, forward 5’-TTGAGTACGTTGTTGTGTTGT-3’; PgU reverse 5’-AAAACCAATATCCCAATACCAAAC-3’; and for methylated DNA: PgMeth F forward 5’-ACACCAACCTGCAATGATATCT-3’; PgMethR reverse 5’-AACCGGAATGGATACCC-3’, have been previously described by Potter et al (2001) and lead to the amplification of 84 and 76 bp fragments, respectively. The PCR reactions were placed in an Applied Biosystems GenAmp PCR System 9700 thermal cycler and activated at 95°C for 4 min, denatured at 95°C for 30 s, annealed at 56°C for 30 s and elongated at 72°C for 1 min for 35 cycles. A final extension of 72°C for 7 min and a 4°C indefinite hold

Reverse transcriptase–polymerase chain reaction (RT–PCR) analysis

For JUP expression, an RT–PCR assay was performed, using a Superscript One-Step RT-PCR kit with Platinum Taq Polymerase from Invitrogen (Carlsbad, CA, USA). Normal total RNA (10 ng), purchased from Clontech, was used as template. The JUP primers for exon 1A were: forward, 5'-CCGAGCT- CAGTTGGCTG7T-3'; reverse, 5'-TCGGTTGAGC AGTTTGGTGAG-3'; for Exon 1B were: forward, 5'-ACCCGGCT TTCCGTGAAAGAAT-3'; reverse, 5'-AGCAGAAAGGAAGACTG TC7T-3'. The GAPDH primers were used as a control for the amount of RNA tested: forward, 5'-CCCTTCTAGGACCTCAACTAC-3'; reverse, 5'- CCACCTTCCTGAGTGCTCAT-3' to amplify a 600 base pair (bp) fragment. A measure of 25 μl of 2× mixture containing 0.4 mM of each dNTP, 2.4 mM MgSO4, 10 μM of each primer, 1 μl of Ru/Platinum Taq mix and autoclaved (RNase-free) distilled water up to 50 μl of total reaction volume. The cDNA synthesis and predenaturation was performed at 52°C for 30 min for one cycle and the PCR amplification was performed for 35 cycles at 94°C for 15 s, 52°C for 30 s and 72°C for 1 min. A final extension was performed at 72°C for 10 min.

Mutation screening

‘Cold’ single-strand conformation polymorphism (SSCP) analysis was performed as described by Hongyo et al (1993) to identify

| Table 1 | Primers for the amplification of JUP exons |
|---|---|---|---|---|
| Exon | Intron location | Forward (5’ – 3’) | Reverse (5’ – 3’) | Gel running temp. (C) | Product (bp) |
| 1 | –16 | ATACCTAGACGGGACGAT | +13 | CCCCACCATCAACATGCCC | 15 | 254 |
| 2 | –15 | CTCGACCTAGGCGCCTGTC | +6 | ACCCTGCAGGAGGCTCG | 15 | 315 |
| 3 | –1 | CTCGTCCTTGTCCTCAG | +6 | AACCTCAGCTGCTACTGAGT | 15 | 279 |
| 4 | –6 | GATCTAACCCCTGGCACC | +13 | TCAGGCGTCGGGAGAGTT | 12 | 255 |
| 5 | –4 | CTCACTTCTGCTTACC | +3 | CCGTCTACAGGCAACTAACAT | 15 | 188 |
| 6 | –8 | TGAAGACCTGACATCT | +11 | CACCACAGCAGCGCAG | 15 | 157 |
| 7 | –24 | TTTGCGCTGCTTCTAGC | +22 | CTGTGCTAGCGGACTCTT | 15 | 420 |
| 9 | –2 | ACTGGCCCTTTGTTG | +175 | GCATGGACAGAGTGCCTT | 17–18 | 350 |
| 9 | –22 | AAGGCCACCTGCCCAG | +53 | TCTGGAGCTCTAAACCTT | 17–18 | 230 |
| 10 | –12 | TTCGCCGTGTCCTCCACGT | +55 | TTAAGTGGCGGCACCG | 12–13 | 249 |
| 11 | –4 | CCTGCTGGTTCCTTCCCTCT | +10 | ACTACTGTTGTCACCATC | 15 | 178 |
| 12 | –51 | ACCAGCTGAAATCCACG | +16 | TCTCCAGGCTGCTGAGA | 12–13 | 143 |
| 13 | –20 | TCGAGACCTGAGGACGCG | +43 | GGAAAGGCTCTCAAAGA | 15 | 232 |

The forward primer for Exon 1 starts 1.6 bp 5’ to the translation start signal, for the other Exons the number given is the base pair separating the last base of the primer and the beginning of the Exon. The number given is the base pair separating the last base of the Exon and the 3’ end of the reverse primer. The temperature at which the 4–20% polyacrylamide gel was run. The size of the PCR product is indicated base pairs.
completed the reaction. For analysis, 12 ml of each PCR reaction was mixed with 5/C2 Hi-Density TBE sample Buffer (Invitrogen, Carlsbad, CA, USA) and loaded onto a 6% TBE Gel (Invitrogen). The gel was run at 200 V for 30 min, stained with ethidium bromide and visualised on a UV light box.

RESULTS AND DISCUSSION

To identify new genes with a potential tumour suppressor function and determine their position within the region of chromosome 17q12–21 affected by LOH, we have determined the nucleotide sequence of portions of the 122F4 and 50H1 P1-phage clones of human genomic DNA that span the region between the D17S846 (centromeric) and D17S746 (telomeric) polymorphic markers (Figure 1). Nucleotide sequence analysis of BamHI and SStI fragments of 122F4 demonstrated the presence of the JUP gene. Previous studies (Aberle et al, 1995; Whittock et al, 2000) localised JUP within this area of chromosome 17. In the present study, we can now physically link JUP to SC65 (Exons 1–8), FK506BP10 (Exons 1–10) and MGC20781 (Exon 8). FK506BP10 is a member of a family of genes that encode proteins that are immunophils that bind FK506 and rifampysin and possess prolyl:prolyl isomerase activity (Siekierka et al, 1989; Sendaert et al, 1990). The normal function of FK506BP10, SC65- and MGC20781-encoded proteins is not known. Only the last exon (Exon 8) of MGC20781 is located on 122F4. The results of a PCR analysis of the exons for each of these genes on 122F4 are shown in Figure 1A.

We have also localised by PCR analysis D17S746 and D17S846 on these P1-phage clones (Figure 1B). D17S846 is 5.8 kb from one end of the recombinant genomic DNA in P1-phage 50H1 and is 2291 bp from the transcription promoter region of HAP1. The D17S746 locus is present on 122F4 but not on 50H1, suggesting that it is located near JUP. This observation was confirmed by nucleotide sequence analysis of a recombinant clone from a library of SStI-digested 122F4 DNA. D17S746 is located 3865 bp telomeric of JUP Exon 1A (Figure 1B). The HUM122F4-3'
STS locus is located 191 bp from one end of the genomic fragment in the 122F4 P1-phage clone.

The transcriptional orientation of the genes could be deduced by PCR and nucleotide sequence analysis. Thus of the genes on 122F4, JUP exon 13 is located ~11.5 kb from the HUM122F4-3' STS locus (Figure 1). This is consistent with the direction of transcription being from the telomeric to centromeric regions of the chromosome. This analysis was also anchored with the observation that only exon 8 of MGC20781 was found on 122F4 and is consistent with the transcriptional orientation of this gene being in the same direction as JUP (Figure 1). In addition, exon 8 of MGC20781 could be linked to a 3.4 kb PCR fragment containing exon 10 of FK506BP10, which is consistent with the transcriptional orientation of FK506BP10 being in the opposite direction as JUP. Similarly, exon 8 of SC65 and D17S746 were linked in an 11 kb PCR fragment (Figure 1) consistent with the transcriptional orientation of SC65 as being in the same direction as JUP. These results confirm the Human Genome Map and physically locate the STS loci HUM122F4-3' and D17S746, respectively.

As genes were identified in this study, we screened genomic DNA from 11 primary human tumours having LOH in this region of chromosome 17 by nucleotide sequence analysis. No missense, nonsense or frameshift mutations were detected in SC65, FK506BP10 or MGC20781 in these tumours DNAs. Gastrin (GAS) and HAP1 were not tested since they are not expressed in the mammary gland. When it was clear that among the genes that were analysed, only JUP was located between D17S746 and D17S846; JUP genomic DNA was tested by the more sensitive 'cold' SSCP analysis (Osborne et al., 1991; Hongyo et al., 1993) for mutations, none were found (data not shown).

JUP has two potential transcription promoters. JUP, exon 1A was expressed in 10 tissues tested (Figure 2), whereas exon 1B could not be detected in any of these tissues (Figure 2). However, five ESTs that contain exon 1B from a placenta cDNA library (NIH_MGC_21) have been reported in the GenBank. One of these is the positive control shown in Figure 2B (lane 12).

Winn et al (2002) demonstrated that plakoglobin expression is reduced or absent in a subset of human lung cancers. Further they showed that re-expression of plakoglobin inhibits transformed cell growth, suggesting JUP is a tumour suppressor gene. Blanco et al (2002) found that 76% (13 out of 17) invasive ductal carcinomas of the breast had reduced levels of plakoglobin. Potter et al (2001) have shown that in some thyroid tumours and cell lines, which express low or undetectable levels of plakoglobin, the JUP promoter is hypermethylated. To see if a similar phenomenon affects the remaining JUP allele in breast tumours having LOH at chromosome 17q21, we have examined the methylation status of the transcription promoter region for JUP. The specificity of the primers for methylated and unmethylated JUP exon 1A is shown in Figure 3A. No PCR product (Figure 3B, lanes 2 – 12) the size of the control fragment (lane 13) of methylated JUP genomic DNA was detected using the tumour DNAs as the template. However, as shown in Figure 3C, lanes 2 – 12, PCR products of the same size as the control (lane 13) for unmethylated DNA was detected with tumour DNA templates. JUP exon 1B was not tested for its methylation status.
methylation status since it is not active in the mammary gland (Figure 2).

In conclusion, we have focused on 122F4 and 50H1 P1-phage clones because they contain (Albertsen et al., 1994, and unpublished data) overlapping genomic DNA fragments that spanned a region of chromosome 17q21 affected by LOH. Our nucleotide sequence analysis of the ends of the genomic DNA in these P1-phage clones is consistent with an overlap of 2.3 kb. At the present time, this region of chromosome 17q12–q21 is ambiguous with respect to the presence and location of particular genes identified in the Human Genome Project. For instance, D17S746 has not been physically mapped relative to JUP in the Human Genome Project map. However, consistent with our data, Aberle et al. (1995), have identified three recombinant cosmids clones of this region of human chromosome 17q21 that contained both D17S846 and JUP. It is possible, therefore, that the target gene for LOH on chromosome 17q21, in primary human breast tumours, could be located in the intervening nucleotide sequences between D17S846 and JUP (17 489 bp, GenBank). At the present time, however, there is no evidence for a gene in this region of chromosome 17q21 in the Human Genome Project map.

Another possibility is that JUP is the target for LOH. JUP encodes γ-catenin or plakoglobin and is highly homologous to β-catenin (Gumbiner, 1996). Both are components of cell–cell adherens junctions linking cadherin receptors to the actin cytoskeleton (Ben-Ze’ev and Geiger, 1998). In addition, plakoglobin is a component of desmosomes (Franke et al., 1989; Ben-Ze’ev and Geiger, 1998). Immunohistochemical analysis of primary human breast tumours for the expression of the catenin family demonstrated that if one of these proteins is downregulated, the function of the others in suppressing metastasis is altered (Bukholm et al., 1998; Gonzalez et al., 1999).

In another study, it was shown that expression of plakoglobin was selectively downregulated in metastatic tumours (Bukholm et al., 2000). We have found no evidence that in tumours having LOH between D17S846 and D17S746 the remaining allele of JUP is mutated. Nor have we found evidence that the transcription promoter of JUP is methylated preventing transcription of the remaining allele in these tumours. Therefore, we speculate that haploinsufficiency of JUP caused by LOH is sufficient to contribute to breast tumour progression. A future study specifically aimed at correlating LOH of JUP and reduced plakoglobin levels in primary breast tumours seems warranted.

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