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SHORT COMMUNICATION

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Cloning and mutation analysis of *ZFP276* as a candidate tumor suppressor in breast cancer

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Abstract Loss of heterozygosity (LOH) involving chromosome 16q23.4 occurs frequently in breast tumors, which suggests that this region may contain a tumor suppressor gene. Since *ZFP276* is located in this region, we have therefore cloned and performed mutation analysis of its coding region in 70 breast tumors. One silent polymorphism and two alterations predicted to result in amino acid changes were detected. Absence of the wild-type allele in tumors carrying the E530D variant suggests a possible role for this change in tumorigenesis.

Keywords Zinc finger protein · Breast cancer · Tumor suppressor · 16q24.3 · Polymorphism

Introduction

Chromosome 16q is frequently associated with loss of heterozygosity (LOH) in sporadic breast cancer, suggesting that a tumor suppressor gene is located in this region (Chen et al., 1996; Cleton-Jansen et al., 1994, Cleton-Jansen et al. 2001). A number of detailed mapping studies have identified three regions on chromosome 16q (16q22.1, 16q23.2-q24.1, and 16q24.3) with LOH (Brenner and Aldaz, 1997), of which a 650 kb region between genetic markers D16S303 and D16S3026 has been identified as the minimum LOH region at 16q24.3 (Savino et al., 1999; Whitmore et al., 1998).

During the cloning of the mouse Fanconi Anemia Group A cDNA, a novel overlapping penta zinc finger protein Zfp276 was identified (Wong et al., 2000). In this paper, we report the cloning of the human homolog of Zfp276 (ZFP276). *ZFP276* maps within the region of LOH to chromosome 16q24.3 in breast tumors, suggesting that it could be a breast cancer tumor suppressor gene. We therefore performed mutation analysis of *ZFP276* in 70 breast cancer patients by single strand conformation polymorphism (SSCP) analysis and subsequent sequencing.

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Materials and methods

cDNA cloning

EST AA504834 was used as a probe to screen a Caco-2 cDNA library (generously provided by J. Rommens, The Hospital for Sick Children, Toronto, Canada) and a human lymphoblast cDNA library (Strathdee et al., 1992) using conditions as described (Wong et al., 2000).

Northern analysis

The human breast tumor multisample mRNA Northern blot was purchased from Biochain Institute, Inc. Single-strand antisense RNA probe corresponding to nucleotides 122–1084 of the ZFP276 cDNA was synthesized using the MAXIscript In vitro Transcription Kit (Ambion) and labeled with ^{32}P-dUTP (Amersham). Hybridization was performed as described (Wong et al., 2000).

Specimens

Primary tumor samples from randomly selected axillary-node-negative breast cancer patients were immediately snap frozen and stored in liquid nitrogen. RNA was extracted by the guanidinium thiocyanate-cesium chloride gradient method (Chomczynski et al., 1987) Genomic DNA extracted from the peripheral blood lymphocytes of noncancer individuals were used as controls.

Mutation analysis

Ten primer pairs were used to analyze the coding region of *ZFP276* (Table 1). cDNA was reverse-transcribed from 100 ng of total cellular RNA with random hexadeoxynucleotide primers and Moloney murine leukemia reverse transcriptase (MMLV-RT). The following PCR conditions were used for each primer set: 2 min at 94°C for denaturation, followed by 30 cycles of 15 s at 94°C for denaturation, 15 s at 56°C for annealing, and 20 s at 72°C for extension. The ^{33}P-ATP-incorporated PCR products were analyzed as described (Gokgoz et al., 2001).

Results

Cloning and analysis of the human homolog for Zfp276

A Caco-2 cDNA library and a human lymphoblast cDNA library were screened with an EST clone showing significant homology to Zfp276. One and four positive clones were isolated from each library, respectively. The clone from the Caco-2 library was 2.1 kb in length, while the four clones from the human lymphoblast cDNA library were identical to each other and were 3.4 kb in length. Sequence alignment shows that the 3.4-kb clone is a longer version of the 2.1-kb clone due to a longer 3'UTR (Genbank Accession numbers AF354755 and AF354756), which probably results from the usage of two different polyadenylation sites at nucleotides 2010 and 3280 respectively.

Sequence analysis predicts an open reading frame of 1619 bp starting from nucleotide 86, which translates to a protein of 539 amino acids in length (Fig. 1a). Sequence alignment with the mouse Zfp276 protein shows that the human ZFP276 homolog is longer in the N-terminal region and has 81% amino acid identity in the region that aligns with Zfp276. All five Cys-Cys-His-His zinc finger domains are conserved, suggesting that this region is functionally significant.

As with the mouse homolog, nucleotides 1332–3300 of the ZFP276 cDNA overlaps with the Fanconi Anemia Group A cDNA in a tail-to-tail manner. BLAST analysis of ZFP276 cDNA sequence with the human genome mapped the gene to contig NT_010542.13, which lies within the minimum LOH region in chromosome 16q24.3. Comparison of cDNA and genomic sequences shows that *ZFP276* contains ten exons and spans approximately 17 kb of genomic DNA (Fig. 1b).

Expression and mutation analysis of ZFP276

Northern analysis of ZFP276 demonstrates that three transcripts of approximately 5.0 kb, 3.8 kb and 2.4 kb are expressed in breast tumors and in normal breast and lung tissues, and the clones we isolated probably correspond to the two shorter transcripts (Fig. 2a). Although the expression levels of ZFP276 are not significantly different between normal and tumorigenic breast tissues, but since the LOH status at 16q24.3 has not been evaluated in the tissues used for Northern analysis, it is possible that ZFP276 does not play a direct role in the tumorigenesis of these particular samples. However, the expression of ZFP276 in breast tumors suggests that it functions in breast cancer cells.

We therefore investigated tumor specimens from sporadic breast cancer patients by analyzing the coding region of *ZFP276* by SSCP and subsequent sequencing. Three different DNA variations were detected in this study: a silent polymorphism at amino acid position 241 (GCG → GCA); an arginine to tryptophan change (R200 W) at position 200 (CGG → TGG), and a glutamic acid to aspartic acid change (E530D) at position 530 (GAG → GAC). R200 W was found to be a common polymorphism occurring in 63 out of 82 patients (77%), of which 23 are heterozygotes (28%) and 40 (49%) are hemi/homozygotes. We were unable to

Table 1 Primer pairs used for SSCP analysis of ZFP276

Fragment	Primer pair	Region (nucleotide in cDNA)
1	ZFP-1F: ATGGGTCACTGTCGCCTC ZFP-1R: TTGAGAAGGCTGTGGCACT	86–313
2	ZFP-2F: TTGTCTGCAAGAGCTGCCA ZFP-2R: GCATGTCAATGCACCAACC	261–480
3	ZFP-3F: GATCTGATCACATCCAGC ZFP-3R: AAGGCCTTGCAGCTGGAG	419–612
4	ZFP-4F: CACGACTACACCATGGATA ZFP-4R: CGTCGCTGTCCGAAGGA	589–793
4A	ZFP-4AF: GGCTGAGACCAAGACCCT ZFP-4AR: GAAAGGTCACTAACTCGT	748–939
4B	ZFP-4BF: TCTTCAACCTCGGATGATTG ZFP-4BR: CTTCGAAGCAACACTCTCC	893–1141
5	ZFP-5F: GAATTCGGAAGAAGCAGGG ZFP-5R: TTCGTCACAGATATAGTTCC	1083–1360
6	ZFP-6F: GCATGAAGAAGCACATCAAG ZFP-6R: ACACTGCAAAGGCTTGGCTC	1209–1438
7	ZFP-7F: TTCTCGTCCACCAAATGCG ZFP-7R: TTGTCCTGGGTCTGTGTCA	1392–1614
8	ZFP8F: TCAATGTACACATGTCCATG ZFP8R: CAAGCTGCTAGAGTGCTCA	1566–1739

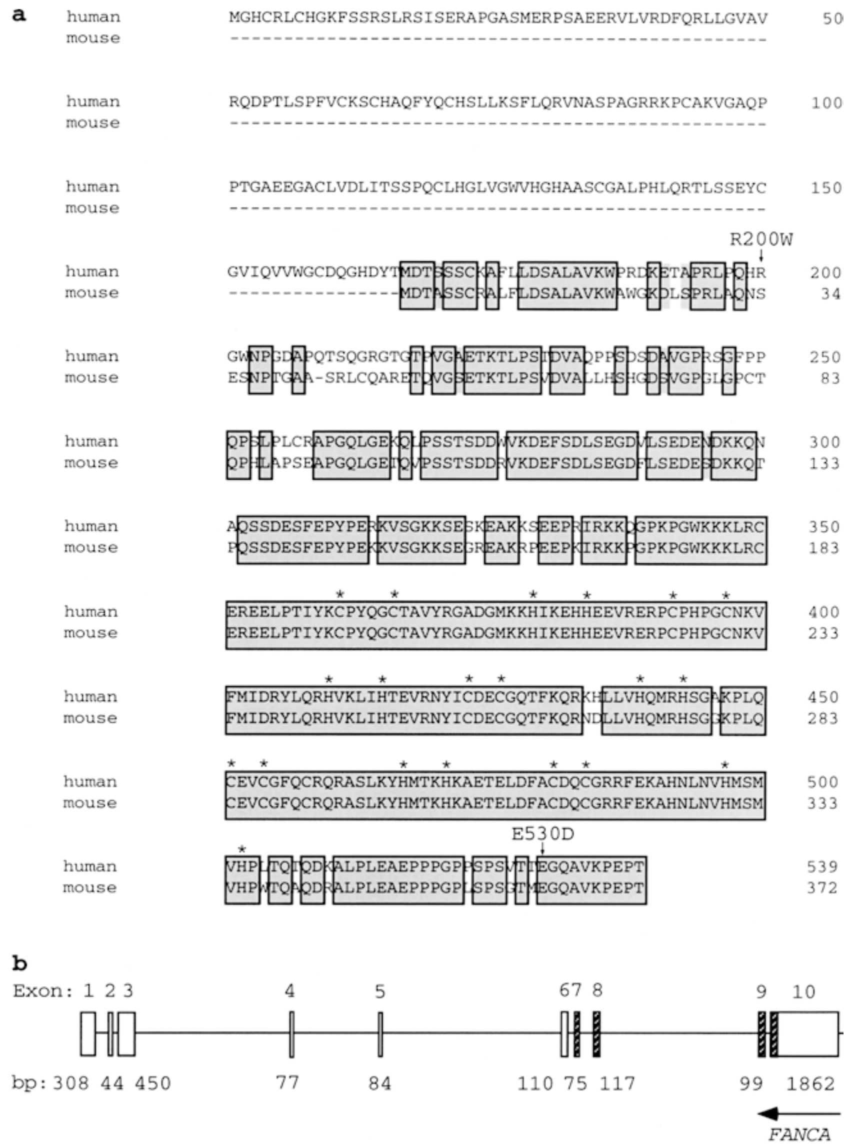

Fig 1 **a** Comparison of the ZFP276 proteins: Matches between sequences are *boxed* and *shaded*, similar amino acids are *shaded only*. Gaps (indicated by *dashes*) are introduced to maintain alignment. Conserved cysteines and histidines forming the C_2H_2 zinc finger domains are marked with *asterisks*. Amino acid changes detected by SSCP analysis are indicated with *arrows*. **b** Genomic structure of *ZFP276*: *Boxes* indicate exons, *hatched area* represents the region that encodes for the C_2H_2 zinc finger domain, *arrow* indicates the region that overlaps with *FANCA* gene

calculate the allelic frequency for this variant, since we could not conclusively determine the LOH status of the hemi/homozygous samples due to unavailability of normal DNA from these patients. R200 W also occurred in similar frequency in the control individuals, as 81 of 97 controls showed this sequence variation (84%), of which 32 were heterozygotes (33%) and 49 were homozygotes (51%). In contrast, the silent polymorphism at amino acid position 241 was found only in one of 70 patients (1.4%), and this patient was hemi/homozygous both for this sequence variation and for arginine at codon 200.

E530D was present in three out of 70 patients. To determine whether this variation is a polymorphism, we analyzed the genomic DNA of 100 individuals from a noncancer population and found the E530D change in two. In the three patients with the E530D change, the wild-type band was not present in the tumor, while both controls had equal intensity of the shifted and nonshifted band in their blood DNAs (Fig. 2b). Interestingly, all three patients with the E530D change were also hemi/homozygous for arginine at codon 200.

Discussion

In this study, we cloned and evaluated *ZFP276* as a potential candidate gene in sporadic breast cancer tumorigenesis. As is the case for the mouse homolog, the human *ZFP276* gene also overlaps with the Fanconi Anemia Group A gene. Human ZFP276 shares a strong amino acid similarity to its mouse counterpart, especially in the zinc finger domain, suggesting that this gene is functionally important.

We have also identified three sequence alterations in ZFP276, two of which are predicted to result in amino acid changes. While the R200 W variation was common in both the cancer and control groups, the E530D variation was rare. Although the frequency of occurrence of E530D in the cancer group did not deviate significantly

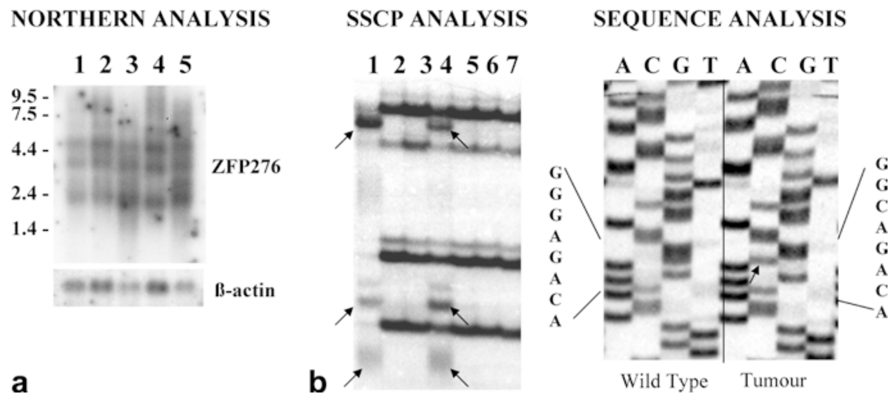

Fig 2 **a** Expression of ZFP276 in breast tumor, normal breast and lung tissues: Tissues are indicated by *1* breast tumor, moderately differentiated invasive ductal carcinoma, *2* breast tumor, poorly differentiated invasive ductal carcinoma, *3* breast tumor, moderately differentiated invasive ductal carcinoma, *4* normal breast, *5* normal lung. The same blot was hybridized with human β-actin to control for differences in lane loading. Size markers are indicated on the *left* of the autoradiograph. **b** SSCP analysis of E530D (*left*): Electrophoretic mobility shifts were detected in lane 1 (breast cancer patient) and lane 4 (control), as indicated by *arrows*. Sample 1 displayed only the altered band. Sequencing analysis of sample 1 on SSCP gel (*right*): The *arrow* indicates the base change (G to C)

from that in a normal control population, it is possible that this nucleotide change may have an effect on tumorigenesis. First, the glutamic acid at position 530 is conserved in the mouse, suggesting possible functional importance. Second, tumours with this variation have only the altered band, while both controls are heterozygous for this change, suggesting that there may be LOH. However, the sample size in this study was too small for us to conclude whether E530D is associated with the occurrence of breast cancer.

It has been documented that single nucleotide polymorphisms (SNPs) can potentially affect the way genes function (Lumb and Danpure, 2000). Nonsynonymous changes (amino acid changes) may affect protein folding and thus the function of the proteins. Single base substitutions can alter or create essential sequence elements for splicing, processing, or translation of human mRNA (Shen et. al., 1999). E530D may confer an increase in cancer risk by affecting gene function. Further studies on the function of the E530D variant in a larger number of breast tumors may clarify the relationship between E530D and sporadic breast cancer.

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