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Advances in Brief

The Human Prohibitin Gene Located on Chromosome 17q21 Is Mutated in Sporadic Breast Cancer[1]

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Abstract

A gene called "prohibitin" was isolated as a candidate antiproliferating gene in rat liver cells. We have isolated the human homologue of the rat prohibitin gene and mapped it to chromosome 17q12-21 where a gene responsible for hereditary breast cancer was localized. DNA sequence analysis of 2 exons in this gene in 23 sporadic breast cancers, which showed loss of heterozygosity on the long arm of chromosome 17 or developed in patients 35 years old or younger, identified 4 cases of somatic mutation; 2 of these were missense mutations; 1 showed a 2-base deletion resulting in truncation of the gene product due to a frame shift; the other had a C to T transition in an intron adjacent to an intron-exon boundary. These results suggest that this gene may be a tumor suppressor gene and is associated with tumor development and/or progression of at least some breast cancers.

Introduction

Breast cancer is the most common cancer in women. One in 10 Caucasian women and one in 60 Japanese women will develop breast cancer in their lifetime. The highest risk factor for breast cancer is family history (1) which reflects dominantly inherited susceptibility (2–5) caused by at least three susceptibility loci, the p53 locus on chromosome 17p13 (6), a 17q-linked susceptibility locus (7), and one or more unmapped loci. Our studies based on LOH[3] in breast tumors identified 17q21 as one of the commonly deleted regions (8, 9), indicating the possible presence of a tumor suppressor gene, in the same region as a gene responsible for familial breast cancer (7).

The rat prohibitin gene was isolated for its ability to negatively regulate cell proliferation (10). McClung *et al.* (11) isolated a rat prohibitin gene as one of a set of cDNAs derived from mRNAs which were more frequently expressed in normal liver than in regenerating liver. The gene caused an arrest of DNA synthesis by an *in vitro* assay in normal human fibroblast and HeLa cells, further demonstrating its antiproliferative activity (10). Moreover, it showed significant homology to the *Cc* gene, which was considered to be important for development and differentiation of *Drosophila melanogaster* (12). Hence, the function of the prohibitin gene is considered as an antiproliferating factor. The rat cDNA with a complete open reading frame was subsequently cloned (10) and used to isolate a fragment of human genomic DNA which was mapped by *in situ* hybridization to 17q12–17q21 (13). Because the prohibitin gene was assigned to this region, we undertook an analysis of it as a candidate for one of the tumor suppressor genes associated with breast cancer.

Materials and Methods

Materials. Tumor and normal tissues removed by mastectomy from 23 patients with primary breast cancer at the Cancer Institute Hospital, Tokyo, were analyzed for somatic mutations of the prohibitin gene. Extraction of DNAs from tumor and normal tissues was carried out according to the method described previously (8). Allelotype study of these 23 tumors was reported previously (8, 9).

RT-PCR and cDNA Cloning. To isolate human prohibitin cDNA, two oligonucleotides were synthesized for a RT-PCR reaction. Single-stranded cDNA was synthesized using 0.1 μg of human normal liver mRNA, 200 units of Moloney murine leukemia virus-reverse transcriptase (BRL) and a (5′-TCACCCTCAGCAGAGATGAT-3′) primer. Double-stranded cDNAs were then synthesized and amplified with 2.5 units Taq polymerase (Cetus), the above primer, and the (5′-CGCTCTCGACCACGTAATGT-3′) primer for 35 cycles (94°C, 1 min; 55°C, 2 min; and 72°C, 2 min). A 443-base pair product was cloned in pBluescript II SK(−) (Stratagene, La Jolla, CA) and sequenced. A human fetal brain cDNA library (approximately 3.5×10^5 clones) (Clontech, Palo Alto, CA) was screened with the PCR product. Two positive clones were isolated and then sequenced. Intron-exon boundaries were determined by comparing the genomic DNA sequence derived from cosmid and/or phage clones with that of the cDNA clone.

Mutation Analysis. Sequence analysis was undertaken for examining somatic mutations in sporadic primary breast cancer. A pair of tumor and its corresponding normal DNAs was sequenced by the following method. Briefly, PCR products of exon 4 (nucleotides 301 to 443) which is highly conserved in the *Drosophila*, *Cc* gene (12), were amplified using two primers in the introns flanking exon 4 (5′-GTACTCCAGCCTAGGCAAC-3′ and 5′-CAGGAAACTAGCAGCCACAT-3′; 94°C, 1 min; 55°C, 2 min; and 72°C, 2 min, 35 cycles). Products were cloned in pBluescript II SK(−) and at least 100 independent clones were pooled. Both strands were sequenced (14) using two internal primers in introns (5′-ACACTTGTTTTCCTACAG-3′ and 5′-GTGCTCTGGGCTCGAGC-3′) and Sequenase (T7 DNA polymerase; Pharmacia, Piscataway, NJ). The samples found alterations were tested the whole procedure more than twice to confirm the mutations.

Results

Isolation of Human Prohibitin Gene. To examine a possible role in human cancer, human prohibitin cDNA was isolated, based on its homology to the rat cDNA. First, a pair of oligonucleotides derived from the region of rat prohibitin gene, which was conserved in the *Cc* gene of *D. melanogaster* were used to create a 443-base pair RT-PCR product using human normal liver mRNA as a template. This PCR product was then

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[3] The abbreviations used are: LOH, loss of heterozygosity; RT-PCR, reverse transcriptase-polymerase chain reaction; cDNA, complementary DNA.

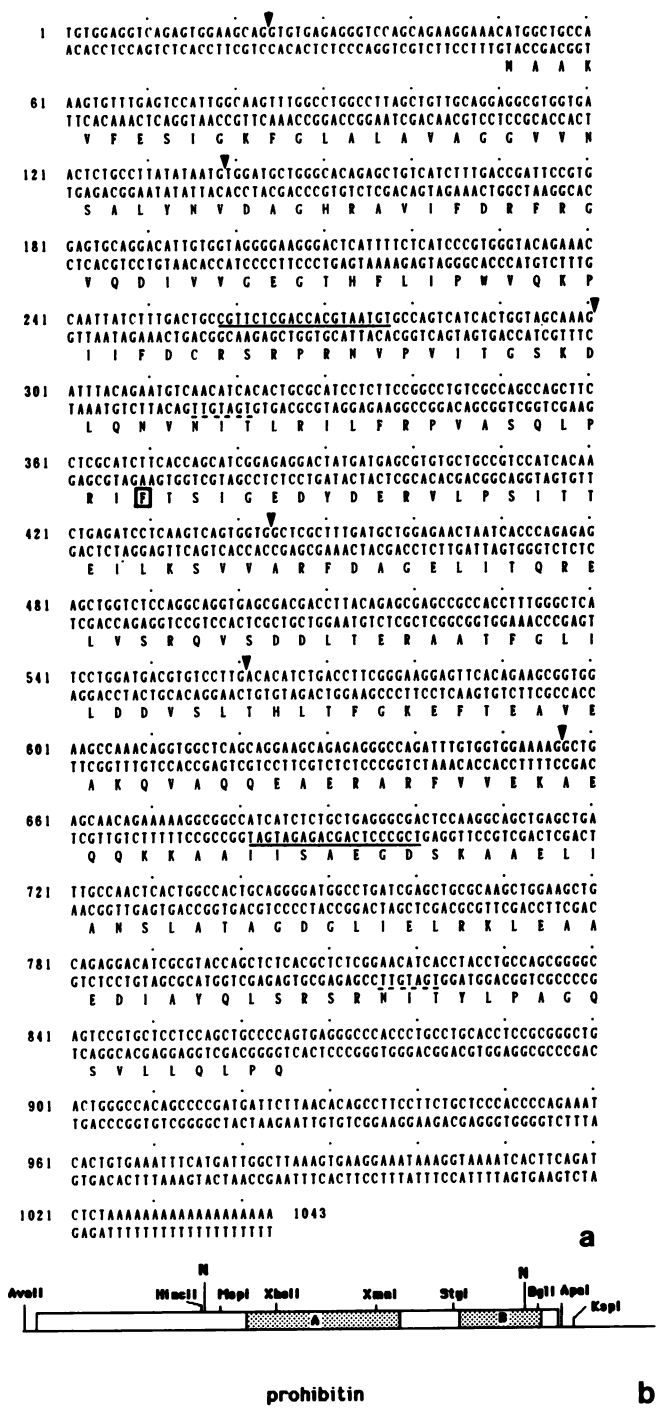

Fig. 1. *a*, human prohibitin nucleotide sequence and predicted amino acids sequence. Nucleotides are numbered at the *left*. Two oligonucleotides used to isolate human cDNA by the RT-PCR method are shown *underlined* and two potential sites of *N*-linked glycosylation are indicated by *dashed lines* above the amino acid sequence. The single amino acid difference at codon 107 between human (phenylalanine) and rat (tyrosine) prohibitin protein is enclosed in a box. *Arrows*, intron-exon junctions. *b*, a schematic representation of the prohibitin gene. *A* and *B* in a *hatched box*, region homologous to *NF1* and *IRA2*, respectively. The 272 residues of human prohibitin share a 99.6% identity with rat prohibitin (4) and residues 117–265 share a 50.7% identity with the *Cc* protein of *D. melanogaster* (12). Two *N*s indicate the potential *N*-glycosylation sites. *c*, alignment of the amino acid sequence of the human prohibitin and *GAP* catalytic domains of yeast *IRA2* and neurofibromatosis type 1 (*NF1*) protein (15, 16). Residues 221–269 share a 28.6% identity (the *B* region in Fig. 1*b*) with the yeast *IRA2* protein (17) which is a homologue of neurofibromatosis type 1 (*NF1*) (15, 16) and residues 112–190 shared a 21.5% identity with *NF1* (the *A* region in Fig. 1*b*). The regions of similarity were identified by the GCG sequence analysis software package (version 7.0; Madison, WI). *Vertical bars* and *dots*, identical amino acid residues and conservative replacement, respectively. Residue numbers of each protein are shown to the *left*.

Table 1 *Prohibitin gene mutations in sporadic breast cancers*

Tumor	Age (yr)	LOH on ch17q[a]	Codon	Mutation[b] nucleotide	Amino acid
136	38	+	88	GTC-GCC	Val-Ala
342	60	+	105	CGC-CAC	Arg-His
120	35	+	91–92	TCACACT-TCACT	Frame-shift
218	39	+		GTG/gtgagtgaaca-GTG/gtgagtgaata	

[a] LOH was determined by RFLP analysis as described previously (8, 9).
[b] The underlined nucleotides were mutant: lowercase letters represent introns; uppercase letters represent exons.

used to screen human cDNA, and genomic cosmid and phage libraries. The cDNA sequence containing the entire coding region is shown in Fig. 1*a*. This gene was mapped to chromosome 17q21–22 by *in situ* hybridization (13) and has been confirmed to the region 17q12–21 by fluorescent *in situ* hybridization[4] giving most likely assignment of 17q21.

The size of the predicted protein is 272 residues. It was expressed in all rat tissues tested by RT-PCR: liver, heart, colon, brain, kidney, adrenal gland, uterus, thymus, stomach, spleen, pancreas, skeletal muscle, lung, and ovary (data not shown).

Prohibitin is highly conserved (99.6% identical at amino acid level and 90.3% identical at nucleotide level between rat and human). Furthermore, it showed significant homology to the *Cc* gene which is considered to be important in the development and differentiation of *D. melanogaster* (12) and is also homologous to the human *NF1* gene (15, 16) and the yeast *IRA2* gene (17) as shown in Fig. 1, *b* and *c*.

Analysis of Somatic Mutation of the Prohibitin Gene in Sporadic Breast Cancer. We screened for somatic mutations in the prohibitin gene in DNAs from 23 breast tumors; 7 of these tumors developed in patients 35 years old or younger, 11 of the tumors showed significant reduction or loss of one of the polymorphic alleles on the long arm but not on the short arm of chromosome 17, and 5 tumors with LOH on chromosome 17q developed in patients 35 years old or younger. LOH in chromosome 17 was determined by restriction fragment length polymorphism analysis as described previously (8, 9). Furthermore, the *Eco*RI polymorphism of the human prohibitin gene (13) was also tested for the detection of LOH. We first examined the fourth exon since it is highly conserved in the homologous *Drosophila Cc* gene. The exon was amplified by PCR, subcloned, and then sequenced. The results are shown in Table 1 and Fig. 2, *a* and *b*. In one tumor, somatic mutation from CGC to CAC at codon 105 that resulted in amino acid change from arginine to histidine was detected as shown in Fig. 2*a* although a normal G is still observed in tumor because of the contamination of the normal cells. In another tumor, a 2-base deletion was found at codon 90–92 (Fig. 2b) which caused a

[4] K. Okui and Y. Nakamura, unpublished data.

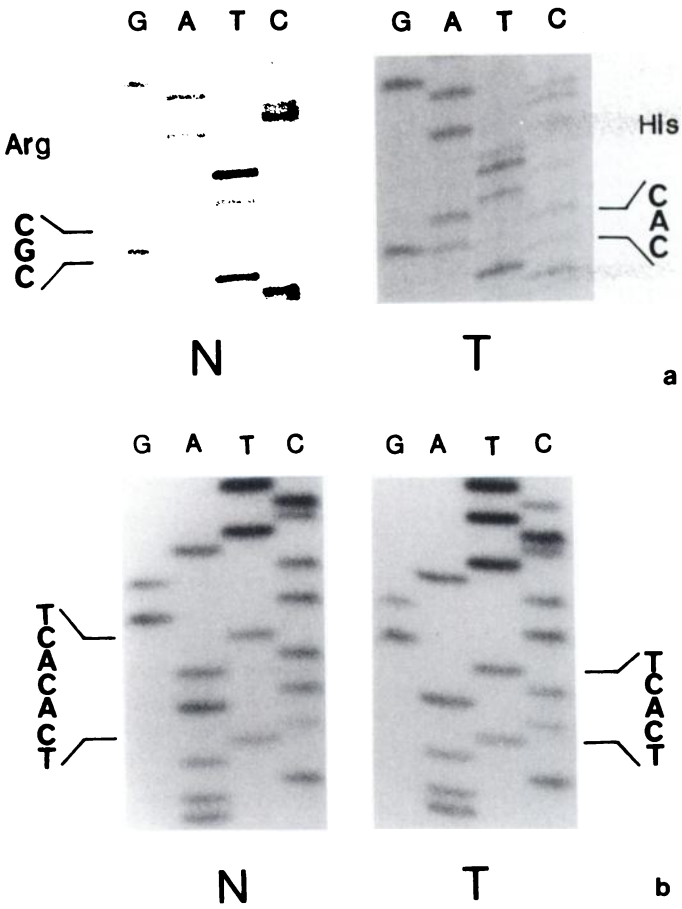

Fig. 2. Sequence analysis of PCR products from sporadic primary breast cancers. DNAs extracted from tumor and its corresponding normal tissues were tested for mutation analysis of exon 4 in the prohibitin gene. PCR primers in introns flanking exon 4 were used to amplify prohibitin nucleotide 301 to 443 as described in "Materials and Methods." *a*, sequence analysis of the T342 PCR product shows a G (in the normal allele) to A (in the mutated allele) transition in codon 105. The autoradiography represents the result from pooled clones. In *b*, the T120 product shows a 2-base deletion from TCACACT (in the normal allele) to TCACT (in the mutated allele) at codon 90–92, resulting in a frame-shift mutation.

frame shift and generated a new stop codon at nearly 70 base pairs downstream. As shown in Table 1, two other somatic mutations have been also detected in this region; one is a missense mutation from valine (GTC) to alanine (GCC) at codon 88 and the other is a C to T change in an intron near the intron-exon boundary. All tumors listed in Table 1 were detected the loss of one of polymorphic bands with one or more restriction fragment length polymorphism markers on the long arm of chromosome 17. However, somatic alteration in exon 5 was detected in none of the 23 tumors (data not shown).

Discussion

In this report, we present the cloning of the human prohibitin gene and its somatic mutations in primary breast cancer. In one case (T120), it is clear that tumor cells could produce no normal prohibitin gene product due to the loss of one allele and frame-shift mutation on the other allele. In another three tumors, it is still uncertain that missense mutations in tumors 136 and 342 caused a significant effect on the prohibitin function or that the point mutation in the intron in tumor 218 induced an abnormal splicing. However, it is notable that a change from arginine to histidine found in tumor 342 is the same as one of the most frequent mutations (at codon 273) observed in the *p53* gene (18).

These results suggested (*a*) that the defect of the prohibitin gene may have a significant role for development and/or progression of at least some breast carcinomas and (*b*) that the region in the prohibitin gene which was evolutionally conserved in the *Drosophila Cc* gene might be one of the important functional domains of this gene. Although the function of this gene or whether it acts in dominantly negative fashion (19, 20) is still unclear, it is suspected that this gene works as a negative growth factor (21) or *GAP* like protein (22–25) since this gene product has homology to the *Drosophila Cc* protein, the yeast *IRA2* protein, and the *NF1* protein. The human gene appears to be a member of a gene family because Southern blot analysis of genomic DNA detected several bands (data not shown). Taken together, the prohibitin gene could be a new tumor suppressor gene in breast cancer. Further analysis of somatic and germ line mutations in the prohibitin gene will be able to reveal the significant association with tumor development and/or progression in primary breast cancer.

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