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Localization of the VHR Phosphatase Gene and Its Analysis as a Candidate for BRCA1

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The VH1-related human protein (VHR) gene was localized to human chromosome 17q21 in a region thought to contain the BRCA1 locus, a locus that confers susceptibility to breast and ovarian cancer. VHR encodes a phosphatase with dual specificity for tyrosine and serine residues. Thus it is a plausible candidate for a tumor suppressor gene such as BRCA1. To test this possibility, the VHR coding sequence was screened in individuals with familial breast cancer and in sporadic breast tumor and breast cancer cell lines. No mutations were detected, suggesting that the VHR gene is not BRCA1.

INTRODUCTION

BRCA1, a gene that controls genetic susceptibility to breast and ovarian cancer, segregates with markers on chromosome 17q21 (Hall *et al.*, 1990, 1992; Easton *et al.*, 1993). Recent studies of recombinant chromosomes from affected individuals in linked families have placed BRCA1 between D17S776 and D17S78 (Goldgar *et al.*, 1993; Simard *et al.*, 1993). This region may encompass several million base pairs of genomic DNA.

We have localized a gene to this region that encodes a previously identified phosphatase called VH1-related human protein or VHR (Ishibashi *et al.*, 1992). VHR has been shown *in vitro* to dephosphorylate a variety of substrates at both serine and tyrosine residues. Because of the involvement of tyrosine phosphorylation in cell growth, differentiation, and tumorigenesis and because of the VHR gene's genomic location, the VHR gene is an appealing candidate for BRCA1.

To test the possibility that the VHR gene is BRCA1, we determined the nucleotide sequence of nearly the entire VHR mRNA, including the untranslated regions. We also mapped the positions of the two introns that interrupt the coding region. The coding sequence of VHR has been screened for polymorphisms in eight unrelated individuals who carry BRCA1 susceptibility alleles. In addition, PCR-amplified VHR sequences from 24 breast primary tumor samples of sporadic origin and 12 breast cell lines have been examined by single-strand conformation analysis (SSCA). The results suggest that VHR is not BRCA1.

MATERIALS AND METHODS

BRCA1 pedigrees and DNA samples. Lymphocytes were separated from whole blood extracted from members of Utah BRCA1 kindreds 2081, 2092, 2039, 1910, 1911, 2090, 2300, 2303, and 1925 using Ficoll-Paque (Pharmacia) according to the manufacturer's instructions. mRNA samples were prepared according to standard procedures (Chomczynski and Sacchi, 1987).

Single-stranded, random-primed cDNA was prepared as follows: 10 μg of total RNA was mixed with reverse transcriptase buffer (final concentration: 50 m*M* Tris, pH 8.5, 70 m*M* KCl, 10 m*M* $MgCl_2$), dNTPs (Pharmacia; final concentration: 200 μM of each), dithiothreitol (final concentration: 33 m*M*) and random hexamers (Boehringer-Mannheim; final concentration: 250 μg/ml) and brought up to a volume of 28 μl with water. The sample was heated at 65°C for 3 min. After cooling, 40 units RNase inhibitor (Boehringer-Mannheim) and 24 units of avian myoblastosis virus reverse transcriptase (Boehringer-Mannheim) were added. The sample was incubated at 42°C for 2 h.

Recovery of VHR cDNA clones. The P1-489 clone, part of a P1 contig that covers a portion of the region known to contain BRCA1, was used for the hybrid selection experiments. The method of hybrid selection is described in detail elsewhere (Futreal *et al.*, 1994b) and is similar to a technique developed by Lovett and co-workers (1991).

From 45 hybrid selected, cloned inserts whose sequences were determined using dye terminator chemistry on an ABI 373 sequencer (Applied Biosystems), 7 contigs consisting of groups of overlapping cDNA sequences were constructed. One of these, composed of 11 independent cDNA inserts, proved to include sequences identical to the VHR coding region. Two other contigs were shown subsequently

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to comprise sequences from the 3′ untranslated region of the VHR gene. A cDNA library prepared in λZAP from mRNA extracted from human breast tissue (Clontech) was screened with the VHR coding sequence according to standard procedures (Sambrook *et al.*, 1989).

Mutation screening. Two fragments were amplified from pedigree lymphocyte cDNA: (1) primers 227F and 861R were used in an initial amplification, followed by dilution and reamplification with primers 227F and 838R; (2) primers 47F and 490R were used initially, followed by dilution and reamplification with primers 47F and 411R. One fragment was amplified from pedigree genomic DNA with primers −29F and 153R without reamplification. The PCR cycling conditions were 95°C, 5 min (1 cycle); 95°C, 10 s, 55°C, 10 s, 72°C, 30 s (35 cycles). The reaction mixture consisted of 1 ng genomic or cDNA, 200 m*M* dNTPs, 0.5 units *Taq* polymerase, 1 μ*M* primers, 10 m*M* Tris, pH 8.3, 50 m*M* KCl, 2 m*M* $MgCl_2$, and 0.01% gelatin in a total volume of 50 μl.

cDNA samples prepared from 14 primary breast tumors obtained from Duke University and 8 breast cancer cell lines (BT20, BT474, BT483, MDA175, MDA231, MDA468, MCF7, and ZR751) were used as templates for PCR as described previously (Futreal *et al.*, 1994a). Amplification primers are shown in Fig. 1. The initial amplification product was 614 bp and included all but 19 bp of the coding sequence. This product was used in a subsequent set of PCR reactions to generate 3 smaller fragments for SSCA: a 201-bp fragment, a 249-bp fragment, and a 312-bp fragment (see Fig. 1). Likewise, genomic DNA from an additional 10 breast tumors and from 12 cell lines, including the 8 above as well as 4 others (MDA435, SKBR3, T47D, and Hs578T), was amplified with intron-based primers (I1F and I2R). Gels for SSCA included 5% glycerol/5% acrylamide or 0.5× MDE (J. T. Baker) and were run at 8 W overnight at room temperature. The gels were dried and exposed overnight with or without intensifying screens.

Southern and Northern blot analysis. Multiple-species DNA blots, tumor DNA blots, and multiple-tissue RNA blots (Clontech) were hybridized with a probe consisting of all but 19 bp of VHR coding sequence. For the multiple species blots, the filters were washed in 0.1× SSC/0.1% SDS at 60°C for 15 min. The Northern blots were washed in 0.1× SSC/0.1% SDS at 65°C for 15 min.

RESULTS

Localization and characterization of VHR. To search for candidate genes within the BRCA1 region, P1 genomic clones from the region were used as hybridization probes in hybrid selection experiments (Futreal *et al.*, 1994b). One P1 clone (P1-489), whose distal end was located approximately 100 kb proximal to D17S78, selected at least 3 separate expressed sequences, among these a sequence (489:1) identical to a cDNA sequence in GenBank called VHR.

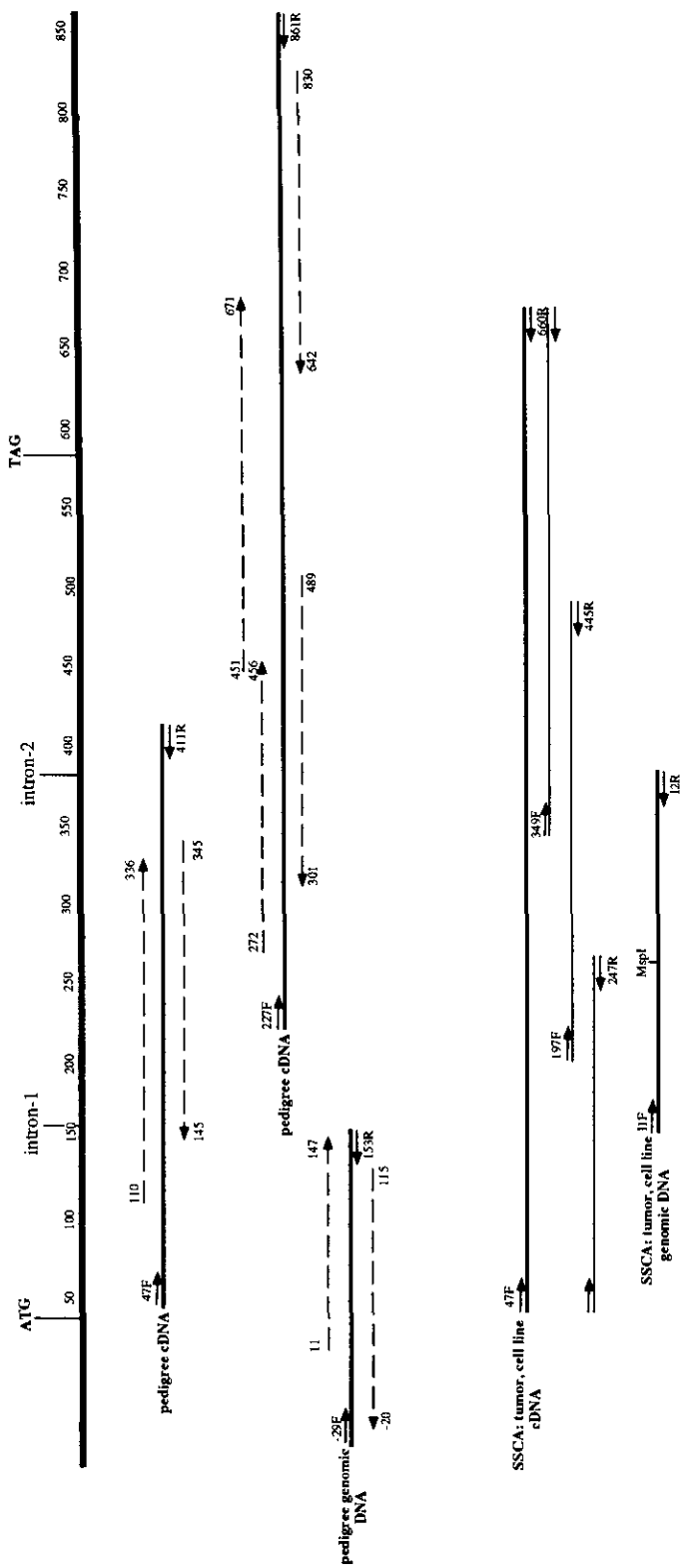
To obtain the complete nucleotide sequence of the VHR mRNA, a mixed random-primed and oligo(dT)-primed cDNA library was screened using a portion of the VHR coding sequence as a probe. Four cDNA clones were obtained that together with hybrid selected fragments spanned 3.2 kb of the transcribed sequence of VHR. The nucleotide sequence of most of the 5′ and 3′ untranslated regions of VHR were determined by assembly of DNA sequences obtained from both cDNA clones and hybrid selected fragment clones. We were unable to isolate cDNA clones that definitely extended to the extreme 5′ end of the mRNA or the extreme 3′ end. Thus, genomic clones were used to obtain sequence upstream and downstream of the furthest reaching 5′ and 3′ cDNA sequences. The VHR mRNA is roughly 4.2 kb. Assuming that the extreme 5′ and 3′ ends of the VHR gene contain no introns, the entire mRNA sequence is probably contained within the overlapping set of sequences that we examined. DNA sequences from genomic clones also identified 2 introns located within the coding sequence (Fig. 1).

A candidate for BRCA1 should be expressed in breast and ovary because BRCA1 influences breast and ovarian cancer susceptibility. Since the original VHR hybrid selected clones were obtained from human breast cDNA, the VHR gene was expected to be expressed in breast tissue. Northern blots of human breast and ovarian mRNA showed that the VHR gene is indeed expressed in breast and ovary (Fig. 2A). A Northern blot of other tissues revealed a varied pattern of expression with expression levels highest in heart, brain, and skeletal muscle (Fig. 2B).

To test whether the VHR gene is conserved through evolution, a Southern blot prepared from DNA extracted from a wide variety of species was probed with VHR coding sequences. All mammalian DNAs showed hybridization to the probe (Fig. 2C). This suggested that VHR has persisted through the course of mammalian evolution because it fulfills an important functional role.

Mutation screen in pedigrees. To test whether the VHR gene is BRCA1, 8 lymphocyte cDNA samples from unrelated individuals, members of Utah kindreds who carry BRCA1 predisposing alleles, were screened for mutations (Goldgar *et al.*, 1993). PCR primers were chosen to amplify fragments of 634 and 364 bp from cDNA (Fig. 1). Together the 2 fragments covered all but 19 bp of the VHR coding region. In every case these primers amplified fragments of the expected size. A third fragment of 182 bp that included the remaining few nucleotides of VHR coding sequence as well as 29 bases of 5′ UTR was amplified from genomic DNA from the same pedigree individuals. The 3 amplified fragments were used as DNA sequencing templates and examined for the presence of heterozygous polymorphisms. The area of clearly discernible DNA sequence information included the entire VHR coding region and 245 bp of 3′ untranslated region. Nearly 85% of the coding sequence was examined on both strands. None of the fragments from any sample contained detectable polymorphisms. Control sequencing experiments using other DNA fragments showed the predicted heterozygous polymorphisms, indicating that heterozygous nucleotide substitutions could be detected when present (data not shown).

Mutation screen in tumors. Tumor samples and breast cancer cell lines were screened for VHR mutations in different ways (Figs. 1 and 3). First, 14 primary human breast tumors were used as sources for cDNA. Three overlapping fragments comprising over 90% of the VHR coding sequence were amplified from cDNA and checked for mutations and polymorphisms using SSCA. No mobility shifts indicative of sequence vari-



ants were detected. A panel of 8 human breast cancer cell lines was examined for VHR mutations and polymorphisms in the same way. Again, no mobility shifts were detected. Second, genomic DNA instead of cDNA was used as a template for PCR amplification. A 328-bp fragment containing the VHR exon 2, including the entire active site and representing over 40% of the coding sequence, was amplified from 10 primary breast tumor DNA samples and 12 breast cancer line DNAs. The amplified fragments were examined by SSCA both whole and after digestion with *Msp*I to generate a pair of fragments (157 and 171 bp). No mutations or polymorphisms were observed. Finally, 22 breast tumor DNA samples were examined for rearrangements in the vicinity of the VHR gene. Southern blots prepared from tumor DNA digested with *Bam*HI were probed with the VHR coding sequence. Here again, no abnormal fragments were seen (data not shown).

DISCUSSION

BRCA1 and tumor suppressor loci. Tumor suppressor genes, or recessive oncogenes, are genes that antagonize the transformed state of cells. Their inactivation through chromosome loss and/or mutation is thought to be required for progression through the various stages of malignancy. Indeed, reintroduction of wild-type copies of tumor suppressor genes diminish the tumorigenic potential of cancer cells (Sumegi *et al.*, 1990; Goyette *et al.*, 1992). Based on the inhibitory effect of

FIG. 1. Schematic diagram of the VHR coding sequence and adjacent flanking DNA. The start (+28) and stop codons (+583) and intron positions (+152, +380) are marked above with an approximate nucleotide scale. For the mutation screen in pedigrees, the initial PCR products are depicted by heavy horizontal lines, the primers used for amplification listed at the ends. The primer numbers reflect the nucleotide positions of their 5′ ends. DNA sequencing runs are shown by dashed arrows, with the extent of the read given by the first and last nucleotide numbers by the dashed arrow. For SSCA, the initial amplification product is shown with a heavy horizontal line, and the secondary amplification fragments are shown below with lighter lines. The primers used in the analysis are:

47F	TCTCGGTGCAGGATCTCAAC
660R	CTAAACATGGCAGCTCGGAC
861R	ACAGGCCTTCCCCTCTGAG
411R	ATAACCTTCCCGGCAGTGG
153R	CGCGTTGCCCACGTAGATC
227F	CCTTCATGCACGTCAACACC
−29F	ATCCGCCGCTCTCCGCCT
I1F	CATTTGGTTGGTTAATCATCCC
I2R	CCAGAGGGACAGTCCAGTCAA
197F	ACCCATGTGCTGAACGCGGC
247R	GGTGTTGACGTGCATGAAGG
349F	CATTGACCAGGCTTTGGCTC
445R	GAGGTAGGCGATAACTAGCG
838R	ATCTCAGGGAAAAGACATCATA
490R	CACGATGCTCAGGGCAGA

Primer names ending with "F" lie on the VHR sequence in the 5′ → 3′ direction; names ending with "R" lie in the opposite orientation.

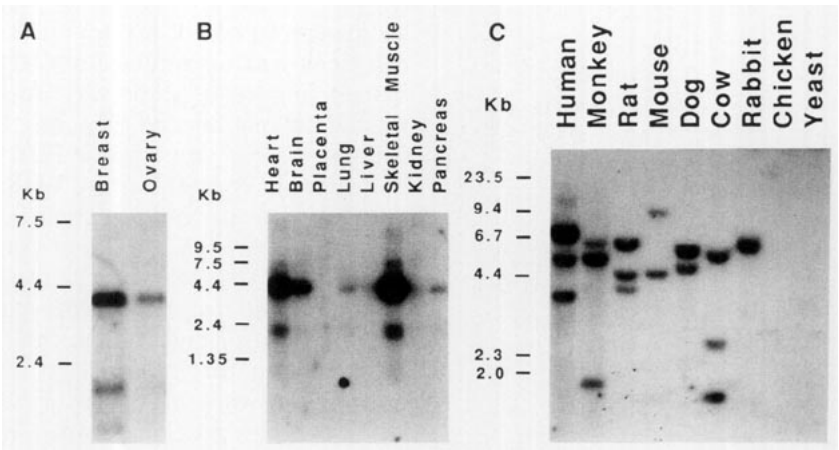


FIG. 2. Nucleic acid blots using VHR coding sequences as a hybridization probe. (**A**) Northern analysis of VHR demonstrating expression in breast and ovary tissue. (**B**) Multiple tissue Northern blot showing tissue-specific expression of VHR. (**C**) Multiple species Southern blot indicating conservation of VHR sequences in all mammalian species examined.

tumor suppressors on cell division and other characteristics of the transformed state, it is reasonable to suppose that certain types of gene products might act as tumor suppressors; for example, proteins that regulate transcription, proteins that inhibit ras activity such as GTPase activator proteins (GAPs), and proteins that antagonize tyrosine kinases such as phosphatases. Several tumor suppressor loci have been characterized, among them a GAP-like gene (NF1), and several transcription regulators (WT1, RB, p53). As yet no phosphatases have been demonstrated to function as tumor suppressors, although at least one phosphatase gene, the PTPγ gene, has been proposed as a tumor suppressor gene (Algrain *et al.*, 1993). Nevertheless, because of the prevalence of tyrosine kinases in the large set of oncogenes that have been identified, phosphatases are attractive candidates for tumor suppressors.

The evidence that BRCA1 is a tumor suppressor gene comes primarily from loss of heterozygosity studies. In breast tumors, heterozygous loss of the chromosomal region including BRCA1 is frequently observed (Futreal *et al.*, 1992). In tumors from individuals that carry BRCA1 susceptibility alleles, the region that is deleted invariably corresponds to the wildtype homolog (Smith *et al.*, 1992). This fits with the classical model of tumor suppressor genes in which the susceptibility is inherited as a dominant Mendelian trait, but acts somatically as a recessive mutated gene (Knudson, 1971). The inherited, nonfunctional allele for susceptibility is neutralized by the wildtype homolog until the functional copy of BRCA1 is lost. Because BRCA1 is likely to be a tumor suppressor gene, sequences such as the VHR phosphatase gene are prime candidates.

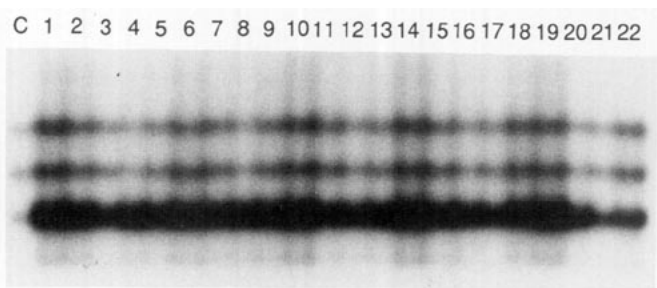


FIG. 3. Single-strand conformation analysis of VHR in breast cancer cell lines and sporadic breast cancers. The catalytic domain of VHR was reamplified from the initial cDNA PCR product with the 197F and 445R primers, and the resulting products were resolved on a 5% acrylamide/5% glycerol gel at room temperature. Samples are normal breast (**C**), sporadic breast carcinomas (**1–14**), and breast cancer cell lines (**15–22**).

VHR is probably not BRCA1. Compelling evidence that BRCA1 and the VHR gene are identical would follow from a demonstration that mutations in the VHR gene segregate with BRCA1 susceptibility in families. Predisposing alleles of many other tumor suppressor loci that control genetic susceptibility to rare cancers have primarily nonsense mutations, insertions or deletions of several nucleotides, or mutations that affect mRNA splicing. For example, the spectrum of germline mutations in the familial adenomatous polyposis (APC) gene is heavily skewed toward mutations that cause production of truncated forms of the APC protein: Over 90% of the familial APC mutations are frameshift mutations or nonsense mutations (Miyoshi *et al.*, 1992). This general pattern holds in tumors of sporadic origin as well. Thus, we expect that a significant proportion of BRCA1 germline and tumor mutations would not only occur in the coding sequence of the gene, but also be obviously disruptive to gene function. The search for germline mutations in individuals carrying BRCA1 predisposing alleles revealed no polymorphisms whatsoever in VHR coding sequences. In addition, we found no evidence for abnormal VHR sequences in breast tumors and breast cancer lines.

Could the relevant mutations reside outside the coding region and therefore have escaped detection? This possibility cannot be excluded. However, it seems unlikely that all eight independent, pedigree germline mutations in BRCA1 would fall, for example, in the promoter region of VHR, which probably presents a much smaller target size for deleterious mutations

than the coding sequence. The 3′ untranslated region of VHR is very large, nearly 3.5 kb. It is possible that mutations might exist in this region that may, for instance, affect mRNA stability. Examination of the sequence revealed no unusual features such as triplet repeats that might confer unusual instability. Thus, because no mutations were detected in the VHR coding region in any of 8 pedigrees and none in 24 tumors or 12 cell lines, and because the untranslated regions of VHR do not contain any sequences that appear peculiar, it seems unlikely that VHR is BRCA1.

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