The human plakoglobin gene localizes on chromosome 17q21 and is subjected to loss of heterozygosity in breast and ovarian cancers

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ABSTRACT The gene encoding human plakoglobin was mapped to chromosome 17q12–q22. An intragenic restriction fragment length polymorphism was used to localize the plakoglobin gene distal to locus KRT10 and proximal to the marker D17S858. The plakoglobin gene colocalizes with the polymorphic 17q21 marker UM8 on the same cosmid insert. This subregion of chromosome 17 is known to be particularly subjected to genetic alterations in sporadic breast and ovarian tumors. We show loss of heterozygosity of the plakoglobin gene in breast and ovarian tumors. We have identified a low-frequency polymorphism in the plakoglobin coding sequence which results in an arginine to histidine substitution at amino acid position 142 of the protein, as well as a silent mutation at nucleotide position 332 of the coding sequence. This polymorphism allowed us to demonstrate an allelic association of plakoglobin with predisposition to familial breast and ovarian cancers. Our results, together with the present knowledge about the biological function of plakoglobin, suggest that plakoglobin might represent a putative tumor suppressor gene for breast and ovarian cancers.

Plakoglobin was first identified in purified desmosomal preparations, and the coding sequence for human plakoglobin has been determined (1, 2). Desmosomes serve as membrane-attachment sites for intermediate filaments, and plakoglobin associates with the cytoplasmic region of desmoglein I, one of the transmembrane desmosomal proteins (3). More recently, it has become evident that plakoglobin is also a component of the cadherin–catenin complex, which is predominantly localized where actin filaments anchor in adherens junctions of epithelial cells (4–6). Catenins have been identified in association with the Ca²⁺-dependent cell adhesion molecule, E-cadherin (uvomorulin), and have been termed α-, β-, and γ-catenin, with Mᵣ of 102, 88, and 80 kDa, respectively (7). Molecular cloning and comparison of primary structures revealed similarities of catenins with other peripheral cytoplasmic proteins known to be localized in prominent cell-contact sites. α-Catenin is homologous with vinculin, β-catenin is homologous with plakoglobin and the protein of the Drosophila armadillo gene, while γ-catenin is very likely identical to plakoglobin (for review, see ref. 8). Catenins are of central importance for cadherin function. They mediate the connection of cadherins to actin filaments and are part of a higher order submembranous network by which cadherins are linked to other transmembrane and peripheral cytoplasmic proteins. Of particular interest are recent results indicating that β-catenin mediates the interaction of the cadherin–catenin complex with the epidermal growth factor (EGF) receptor and that β-catenin and plakoglobin are substrates for tyrosine phosphorylation following EGF stimulation of cells (9). These results, together with the finding that catenins are associated with the tumor suppressor protein APC, open the possibility that catenins might be involved in signaling pathways and tumorigenesis (10).

We have recently used the cDNAs encoding mouse αE- and β-catenin and mouse plakoglobin to map the respective genes in the mouse genome (11). We found that the αE-catenin gene maps to chromosome (chr) 18, the β-catenin gene maps to chr 9, and the plakoglobin gene maps to chr 11 close to the HoxB cluster. For the chromosomal localization of αE- and β-catenin, these results are in agreement with the assignment of the respective human genes to chr 5q31 and chr 3p21 (12, 13). However, the human plakoglobin gene had been previously localized to chr 7 (14), which represented an apparent contradiction to the localization of mouse plakoglobin to chr 11. Due to the well-known homology of synteny between mouse chr 11 and human chr 17, our results suggested that the human plakoglobin gene might, in fact, localize to chr 17. Here, we show that indeed the human plakoglobin gene maps to the long arm of chr 17 in the vicinity of the BRCA1 gene. This has stimulated a more detailed analysis on the relationship between plakoglobin and the BRCA1 region for breast cancer predisposition.

We have localized the plakoglobin gene proximal to the BRCA1 gene and provide a physical link between plakoglobin and known DNA markers. We show loss of heterozygosity (LOH) for plakoglobin in tumors and have determined a low-frequency polymorphism in the gene which might be helpful in analyzing a possible involvement of plakoglobin in the development of breast and ovarian cancers.

MATERIALS AND METHODS

Hybrid Cell Lines. Mouse × human hybrid cell lines MH-22/6 (15), X17-41 (16), and MH-41/3 (15) and hamster × human hybrid cell line UMHG-17/2 (17) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM), containing 10% (vol/vol) fetal calf serum (FCS), 15 μg of hypoxanthine per ml, 0.2 μg of aminopterin per ml, and 5 μg of thymidine per ml (HAT), in an atmosphere of 10% CO₂/90% air at 37°C.

Screening for Genomic Cosmid Clones. A human cosmid library (Stratagene, catalog no. 951202) was screened with human plakoglobin full-length cDNA (kindly provided by W. Abbreviations: LOH, loss of heterozygosity; RFLP, restriction fragment length polymorphism; chr, chromosome; EGF, epidermal growth factor. *H.A. and C.B. contributed equally to this work. ††To whom reprint requests should be addressed.
W. Franke; German Cancer Research Center, Heidelberg). One million independent clones were plated and transferred to nylon membranes (Hybond-N+; Amersham, Braunschweig, Germany). All screening procedures were carried out according to instructions provided by Stratagene. Hybridization-positive clones were analyzed for the DNA marker Um8 by PCR amplification as described (18).

**Southern Blots.** DNA (20 µg) from hybrid cell lines or from Epstein–Barr virus-transformed lymphocytes was digested with appropriate restriction enzymes, size separated electrophoretically on a 1% agarose gel, and transferred onto nylon membranes (Hybond-N+; Amersham). Hybridization was performed in 2× SSC (1× SSC = 0.15 M NaCl/0.01 M sodium citrate), 1% SDS, 10% Dextran sulfate, and 0.5% low-fat milk at 65°C, overnight. Filters were washed twice in 2× SSC/0.1% SDS for 2 min at room temperature and twice in 0.2× SSC/0.1% SDS at 65°C for 15 min. The blots were exposed to Kodak XAR film with an intensifying screen at −70°C for 1–3 days.

**Reverse Transcription.** Total RNA (10 µg) isolated from tumor tissue was used for oligo(dt)-primed (Pharmacia) reverse transcription by using Moloney murine leukemia virus reverse transcriptase (BRL) and RNase-inhibitor RNasin (Promega, via Serva) in a 30-µl reaction mixture for 1.5 h at 37°C. For a control reaction, no reverse transcriptase was added. PCR was performed by using 4 µl of the first-strand cDNA product. After an initial denaturation step of 2 min at 98°C, two DNA polymerase (Boehringer Mannheim) was added. Amplification was carried out over 30 cycles as follows: 94°C for 45 s, 60°C for 30 s, and 72°C for 45 seconds.

**Cloning of Plakoglobin cDNA from Tumor Tissue.** To clone the full-length plakoglobin gene from tumor tissue, the transcript was divided into three parts. The N-terminal coding part (aa 1–246) was amplified from reverse-transcribed RNA with primer pair Plako120 (5'-ATATGGATCCATGGGATGTGATGAACTCTGATGG and Plako856r (5'-GGCCATGACCGAGCGCTCC) and inserted into the BamHI/Sph I restriction sites of pQE40 (Diagen, Hilden, Germany). The core coding region (aa 221–659) was amplified with primer pair Plako779 (5'-CGCCATCTTCAAGTCCGG) and Plako2096r (5'-GCTCTGGTTCTGTGTTCTC), double digested with Sph I and Bgl II, and inserted into the corresponding sites of pQE40. The C-terminal coding part (aa 554–745) was amplified with primer pair Plako1778 (5'-GTGAGGATGGAGGAGATTTGG and Plako2559r (5'-ACAAAGACATAAGGAAGCAG), double digested with Bgl II and Xba I, and inserted into the corresponding sites of pBluescript II SK(+) (Stratagene). Five independent plasmid clones were sequenced from each region.

**Sequencing Analysis.** Plasmid clones were sequenced by using a Sequenase 2.0 kit (United States Biochemical) or an automated sequencer (ALF, Pharmacia). Primers were complementary to flanking vector sequences or to plakoglobin cDNA. Primers for automated sequencing were fluorescein labeled. PCR products were purified with JetPure PCR purification kit (Genomed, New York) and were directly sequenced by using nested primers or, in some cases, the same primers as used for PCR. Heat-denatured PCR products were placed into liquid nitrogen for 2 min, and sequencing primers were pipetted onto the frozen sample. Samples were then thawed slowly to room temperature, and sequencing procedures were carried out by using the Sequenase 2.0 kit. Dried sequencing gels were exposed to Kodak XAR film at room temperature for 1–3 days.

**Amplification of Exons 2–5.** Plakoglobin N-terminal coding exons 2–5 from affected persons of 13 different BRCA1 families were amplified by PCR with primers binding to flanking intron sequences and sequenced directly. The exons-specific primers used were as follows: exon 2, E2F-77 (5'-CGGAATTTCTGGAGGCTTGAGCT) and E2R+87 (5'-AGCGATCTGGGCTTTTCTAG); exon 3, E3F-105 (5'-AGGATGCTCCCCAGAGGAGC) and 696r (5'-TGTGACGGACGCGGCGG); exon 4, E4F-70 (5'-GTAATTGACCTCTCTGGAAGGTATC) and E4R+72 (5'-CTCGGATCTCCTGGAGGAGG); and exon 5, ES-X (5'-AACCCATGCTGGCCTC) and ES5+72 (5'-CAAGGCCTGTCGAGATTAGAG).

**Restriction Fragment Length Polymorphism (RFLP) Analysis.** Genomic DNA (100 ng) was amplified with primers E3F-105 and 696r. Sequences were amplified over 30 cycles as follows: 45 s at 94°C, 30 s at 62°C, and 35 s at 72°C for 30 cycles. A single amplified band (680 bp) was visible after ethidium bromide staining of a 1% agarose gel. The PCR product was purified from the reaction mixture with JetPure PCR purification kit. Purified PCR product was subjected to restriction enzyme analysis with BstUI (New England Biolabs) or Hha I (BRL) to detect the Arg142 → His substitution at position 544. To detect the GAC/GAT polymorphism at position 332, the PCR products were overlayered with mineral oil and digested with BstEII (Biolabs, Northbrook, IL) at 60°C. Restriction enzyme analysis was carried out in buffers provided by the manufacturers. Digested bands were analyzed on an ethidium bromide-stained 3% MetaPhor agarose gel (FMC). Results of the restriction analysis were in some cases confirmed by nucleotide sequence analysis.

**RESULTS**

**Plakoglobin Maps to Human chr 17q12–q22.** DNA from human × hamster or human × mouse hybrid cell lines that contained only human chr 17 or parts thereof were probed with human plakoglobin cDNA in a Southern blot analysis (Fig. 1). Mouse and hamster DNA were included as controls, and hybridization and washing were carried out under high-stringency conditions. Plakoglobin is evolutionarily well conserved, and homologs have been isolated by cross-species hybridization (20). As seen in Fig. 1, human plakoglobin cDNA hybridizes to human DNA but also to mouse and hamster DNA. However, the results obtained with DNA from hybrid cells unambiguously demonstrate that the human plakoglobin gene maps to chr 17q12–q22. The previously reported localization of plakoglobin to human chr 7 (14) was based on PCR analysis using synthetic oligonucleotides and is not confirmed by our independent experimental approach.

**Plakoglobin Maps to the BRCA1 Region.** Extensive studies to elucidate the genetic basis of familial breast cancer have led to a high-density map of polymorphic markers and genes in chr 17q12–q21 (21, 22). A possible linkage between these polymorphic markers and the plakoglobin gene was investigated by studying the segregation of a Bgl II RFLP in breast cancer families. Digestion of random DNA with Bgl II identified an RFLP for the plakoglobin gene, namely a polymorphic fragment of 11 kb in addition to the 14-, 5-, and 1.3-kb fragments, with a frequency of about 20% (data not shown). The segregation of this RFLP with BRCA1-locus markers was followed in two BRCA1-linked families displaying meiotic recombinants in the chr 17q21 region (23). The results, summarized in Fig. 2, show that the plakoglobin gene lies below KRT10 and above the D17S858 marker, an interval which also contains the BRCA1 gene (19). A single recombination event in family P2850 separates the plakoglobin gene from the BRCA1 gene (data not shown). To gain more precise information about the localization of the plakoglobin gene within this region, a human cosmid library was screened with the plakoglobin cDNA. Six independent positive clones were isolated, and the inserts were probed with markers from the breast cancer region. Three plakoglobin-positive clones also contained the PCR-amplified marker Um8 (18), which places the plakoglobin-
bin gene at the proximal border of the breast cancer region in close physical proximity (<40 kb) to the 17q21 marker locus \textit{UM8} (Fig. 2).

\textbf{Arginine/Histidine Polymorphism.} Plakoglobin-positive clones isolated from a human genomic library in \textit{EMBL3A} allowed us to identify intron/exon boundaries of exons 2–5 of the plakoglobin gene (data not shown). Synthetic oligonucleotides flanking the intron/exon boundaries were used to amplify individual exons, which were then subjected to direct sequence analysis. In this way, sequence information was obtained from 20 (13 for exons 2 and 3 and 7 for exons 4 and 5) independent breast cancer families. No changes in the nucleotide sequences were observed for exons 2, 4, or 5. However, in 2 of 13 families (families F1816 and F2619), heterozygosity at nucleotide position 544 in exon 3 was observed, leading to the substitution of arginine (CGC) by histidine (CAC) at amino acid position 142 (designated R142H) (Fig. 3A). This base-pair substitution led to a loss of the recognition sites for restriction enzymes \textit{BsrU1} and \textit{Hha I} and thereby created a new RFLP which was convenient to use to follow the segregation of this allele in breast cancer families (Fig. 3B) and in testing random DNA samples. In some instances the RFLP was directly verified by sequence analysis of the respective DNA fragments, which confirmed heterozygosity for the Arg-142 and His-142 alleles. Breast cancer family F1816 turned out to be informative for the R142H substitution, and DNA from members of this family was analyzed for the presence or absence of the \textit{Hha I} site. The results summarized in Fig. 3B led to the following conclusions. In DNA from carrier individuals, the plakoglobin His-142 allele was always linked with the haplotype associated with cancer predisposition. However, some of the family members with genetic predisposition were homozygous for the His-142 allele in their

\begin{figure}[h]
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\caption{Assignment of the human plakoglobin locus to the chromosomal region 17q12–q22 by Southern analysis of somatic cell hybrids. Human plakoglobin DNA was used to probe a somatic cell hybrid panel containing \textit{Pst I}-digested genomic DNA from the cell lines MH-22.6 (17-only hybrid) (15), Y17-41 (17q12–qter) (16), UMHG-17/2 (17q22–qter) (17), and MH-41 (17q23–qter) (15), as well as human, mouse, and hamster genomic DNA. Hybrid UMHG-17/2 is a hamster hybrid; all other cell lines are mouse hybrids. Human-, mouse-, and hamster-specific signals are indicated by the respective arrows. Marker, \textit{HindIII}-digested a DNA.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{Fig2.png}
\caption{Genetic localization of the plakoglobin gene. \textit{Bgl II} RFLP intragenic to the plakoglobin gene has been typed in two \textit{BRCA1}-linked families displaying meiotic recombination in the chromosome 17q21 region (23). RFLP analysis shows that the plakoglobin gene (PLAKO) lies below the \textit{KRT10} and above the D17S858 markers. A recombination event in family F2850 separates the plakoglobin gene from the breast cancer susceptibility locus.}
\end{figure}
in three different segments. Since an amplification of plakoglobin mRNA from nontumor cells—i.e., interstitial and/or endothelial cells—could not be completely ruled out, five independent clones were sequenced from each portion. Remarkably, only sequences transcribed from the His-142 allele were obtained. In agreement with this result, when the primary reverse transcription-PCR product was digested with restriction enzyme Hha I, most of the transcripts detected were from the His-142 allele (Fig. 3C). The low-abundance transcripts from the Arg-142 allele seen in Fig. 3C were most likely derived from plakoglobin mRNA of nontumor cells. The results suggest that the plakoglobin gene is subjected to LOH in tumor cells from this individual.

A LOH for the plakoglobin gene was also identified in an individual (no. 3) from breast cancer family F2770 from whom germ-line DNA, as well as DNA and mRNA from a breast tumor, was available for comparison. Family F2770 is not polymorphic for the R142H substitution, but was heterozygous for a silent mutation in the germ line (Fig. 4A). The silent mutation induced a new RFLP for restriction enzyme BstEII, and this allele was again found to segregate in family F2770 with haplotypes associated with genetic breast cancer predisposition (Fig. 4B). Sequence comparison of the germ-line and tumor DNA from individual 3 revealed LOH for the plakoglobin gene in the tumor DNA (Fig. 4A). Concordant with this, when primary reverse transcription-PCR products were digested with restriction enzyme BstEII most of the transcripts were from the allele carrying the silent mutation (Fig. 4C). The low-abundance transcript from the other allele is again likely to be derived from nontumor cells since this tumor contained about 30% interstitial cells and blood vessels, as judged by histological examination. Cloning and sequencing of plakoglobin cDNA by using a strategy similar to that described above for the F2619 tumor demonstrated that only transcripts from the allele carrying the silent mutations were detectable. Thus, the results from two independent BRCA1-linked families demonstrate that the plakoglobin gene is subject to LOH in tumors of individuals carrying the BRCA1 predisposition.

**DISCUSSION**

Here we report the localization of the human plakoglobin gene to chr 17q12–q22 and show that the plakoglobin gene maps to the centromeric border of the breast cancer region. The colocalization of the plakoglobin gene with the 17q21 DNA marker UM8 on the same cosmid insert places the plakoglobin gene centromeric to BRCA1 in a region which is subjected to
genetic alterations in sporadic breast and ovarian tumors (24). We have identified a polymorphism of the gene which resulted in an Arg-142 → His substitution. This is a low-frequency polymorphism since only 10 chromosomes contained the His-142 allele from a total of 240 randomly tested chromosomes. The biological implication of this polymorphism is presently not well understood and is somewhat unexpected since plakoglobin is considered to be a structural gene which is highly conserved during evolution (20). The arginine residue is particularly well conserved in different species from man to hydra and is located within or very close to the α-catenin-binding site in plakoglobin (H. Schwartz, H.A., and R.K., unpublished observation). Interestingly, the low-frequency His-142 allele segregated in breast cancer families with marker loci for genetic breast cancer predisposition. This suggests that the BRCA1 gene might be associated with a particular plakoglobin allele. Finally, we demonstrate that the plakoglobin gene is subject to LOH in breast and ovarian tumors. Our results must be discussed in light of the accumulating data provided by an international effort to characterize the genetic predisposition for breast and ovarian tumor development. The BRCA1 gene has recently been identified and very likely encodes a nucleic acid-binding protein since the protein contains a zinc-finger domain (19). The search for mutations in the BRCA1 gene in sporadic breast and ovarian carcinomas led to the identification of mutations in the BRCA1 gene in 4 of 44 cases (25). However, the four mutations all turned out to be germ-line alterations, and no somatic mutations were detected, which raises the possibility that other tumor suppressor genes on chr 17q incidentally included in the breast cancer region may contribute to tumor development. Recently, Albertsen et al. (21) have proposed that two separate loci on 17q12–q21 are equally important in breast cancer development. In addition, analysis of LOH in sporadic breast tumors defined a minimal region centering around the GAS locus (24). We know that the plakoglobin gene is located in this region since both GAS and UMS8 have been found on the same P1 clone (21). These findings, together with our results, open the possibility that plakoglobin may represent a susceptibility gene for sporadic breast and ovarian cancers. This view is supported by current knowledge about the biological function of plakoglobin: (i) plakoglobin is a component of the cadherin–catenin complex (4–6); (ii) plakoglobin and β-catenin are phosphorylated at tyrosine residues after EGF-stimulation of cells, and β-catenin (and most likely plakoglobin) mediate(s) the interaction of the cadherin–catenin complex with the EGF-receptor (9); and (iii) plakoglobin and α- and β-catenin have been found in association with the tumor suppressor protein APC (10). Catenins might represent more general linker proteins involved in connecting membrane receptors with the cytoskeleton. They are also likely to be part of signal-transduction pathways. Obviously, mutations in these molecules could have dramatic effects on cell morphology and cell proliferation. It is intriguing to note that the genes for both β-catenin and plakoglobin map to chromosomal regions known to represent hot spots of genetic alterations, such as LOH. We have shown here that plakoglobin localizes to the breast cancer region and that the gene for β-catenin was mapped to human chr 3p21 (13), a genetic region known to be affected during lung carcinogenesis.

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