The Human Mammary-Derived Growth Inhibitor (MDGI) Gene: Genomic Structure and Mutation Analysis in Human Breast Tumors

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The mammary-derived growth inhibitor (MDGI) gene is a candidate tumor suppressor gene for human breast cancer. It has been shown to reduce the tumorigenicity of breast cancer cell lines in nude mice, and loss of expression of this gene has been shown in primary breast tumors. Furthermore, the human MDGI gene has been mapped to human chromosome 1p32–p35, a common region of deletion in sporadic breast tumors. We have determined the genomic structure of the human MDGI gene from a cosmid clone mapping to chromosome 1p32–p35 and have more finely mapped the MDGI gene relative to chromosome 1p microsatellite markers. The gene covers approximately 8 kb of genomic DNA and is divided into four exons. In an attempt to identify possible inactivating mutations in the MDGI gene in human breast cancer, we have sequenced all four exons and their surrounding splice junctions in 30 sporadic breast tumors. Ten of these tumors showed loss of heterozygosity (LOH) in the 1p32–p35 region, with 5 tumors showing LOH in the subregion containing the MDGI gene. No mutations were found in this analysis. A polymorphism was identified in exon 2 in the constitutional DNA of 1/30 cases in this study, which resulted in the conversion of a lysine to an arginine residue at codon 53. This variant was present in the constitutional DNA of a further 3/26 women with sporadic breast cancer and 2/90 control individuals ($P = 0.20$). Despite experimental evidence that MDGI has tumor suppressor activity, our data suggest that mutations in the coding region are uncommon in human breast tumorigenesis.

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INTRODUCTION

Breast cancer is a common neoplasm affecting women in the western world. The frequency of loss of heterozygosity (LOH) at different chromosomal loci in sporadic breast tumors suggests that a number of tumor suppressor genes are involved in the development of the disease (Callahan *et al.*, 1992). One such gene may be the mammary-derived growth inhibitor (MDGI) gene, which is located at 1p32–p35 (Hung *et al.*, 1995), a common region of loss in sporadic breast cancer (Borg *et al.*, 1992; Bieche *et al.*, 1993, 1994; Munn *et al.*, 1995).

Based on the complete cDNA and protein homologies between the mouse MDGI and fatty acid-binding protein 3-muscle and heart (FABP3) genes (Yang *et al.*, 1994; Treuner *et al.*, 1994), it is assumed that these are the same genes. Furthermore, two independent reports have mapped the human FABP3 and MDGI genes to the same region of chromosome 1 (1p32–p35) (Troxler *et al.*, 1993; Hung *et al.*, 1995), providing further evidence that the human FABP3 and MDGI genes are identical.

MDGI was first identified as a growth inhibitor in lactating bovine mammary gland and was shown to play a role in mammary gland differentiation (Böhmer *et al.*, 1987; Spener *et al.*, 1990; Grosse *et al.*, 1991). Recent studies on the transfection of an MDGI expression construct into breast cancer cell lines have shown reduced proliferation, promotion of cellular differentia-

tion (Yang *et al.*, 1994), and reduced tumor formation in nude mice (compared to mock-transfected or untransfected controls) (Hung *et al.*, 1995). Immunostaining of sections of human breast tumors showed loss of immunoreactive FABP3 protein in the neoplastic cells compared to its presence in adjacent normal epithelial cells (Hung *et al.*, 1995). Together these data suggest that MDGI/FABP3 is a candidate tumor suppressor gene involved in breast cancer.

We have previously isolated a cosmid clone containing the entire MDGI gene (Hung *et al.*, 1995). In this report we describe the genomic structure of this gene, including the number of exons, intron sizes, and sequence and location of intron/exon boundaries. We also map the gene relative to chromosome 1p microsatellite markers. To determine if human breast tumors contain inactivating mutations in the MDGI/FABP3 gene, we have sequenced the entire coding region of the gene including intron/exon boundaries in 30 sporadic breast tumors, 5 of which show loss of heterozygosity at microsatellite markers in the region containing the MDGI gene.

MATERIALS AND METHODS

Cloning and characterization of the human MDGI gene. A single cosmid clone (MDGI.3) containing the entire MDGI gene was isolated as described previously (Hung *et al.*, 1995). Sequencing of segments of this clone was performed using an ABI 373A automated sequencer using the methodology of Chen *et al.* (1993). The entire MDGI/FABP3 gene was sequenced in addition to a few kilobases of the flanking regions on both sides of the gene. The TED and autoTED programs (Gleeson and Hiller, 1991) edited the primary DNA sequence data. Assembly of the data was performed using the XBAP program of the Staden package (Dear and Staden, 1991). Alignments to known sequences and further analysis of the sequence were performed using the GCG sequence analysis software (University of Wisconsin Genetics Computer Group). The gene sequence revealed 100% identity with the previously published FABP3 cDNA. Intron/exon boundaries were determined by comparing the sequence of the FABP3 cDNA and the cosmid clone (MDGI.3). The MDGI/FABP3 genomic sequence is available in GenBank, and the structure is shown in Fig. 1. The intron/exon border sequences are given in Table 1, with exon sizes given in Table 2.

Patient material. The 30 tumors in this study were obtained from surgery prior to irradiation and chemotherapy and immediately frozen at −70°C. Peripheral blood was also collected for each case. Twenty-four cases were collected at the Karolinska Hospital, Stockholm, Sweden and the remaining six cases at Duke University Medical Centre, North Carolina, USA. DNA was isolated from the tumor tissue and blood by standard techniques (Larsson *et al.*, 1990; Futreal *et al.*, 1992). Histopathological examination of tumor tissue was performed on tumor pieces adjacent to those studied, and classification was made according to the World Health Organization guidelines (Hutter, 1987). Seventeen of the 30 tumors were ductal, 3 mucinous, 3 medullary, 2 lobular, 2 papillary, 1 comedo, and 2 of unknown histopathological subtype. The tumors were of malignancy stages I, II, or III, and the age of onset of breast cancer in the 30 women varied from 30 to 71 years.

Control population. The control population consisted of 90 Swedish individuals aged 50 and above who have shown no signs of breast cancer.

Loss of heterozygosity analysis of 1p. The following polymorphic microsatellite repeat markers from the 1p32–p35 region were typed on the constitutional and tumor DNA of the 30 sporadic breast cancer cases shown in Table 1: D1S233, L-MYC, D1S197, D1S209, D1S438, and D1S207 (Fig. 2). The oligonucleotide primer sequences, allele sizes, and order of markers along the chromosome are according to the Généthon genetic linkage map (Gyapay *et al.*, 1994). Genomic DNA (100 ng) was amplified by PCR in 50 m*M* KCl, 10 m*M* Tris–HCl, pH 9.0, 1.0–2.0 m*M* MgCl (BRL) (depending on primer set), 0.2 m*M* dTTP, dCTP, and dGTP, 0.05 m*M* dATP, [α-^{35}S]dATP (Amersham) at 1 μCi/reaction, and 2 units of *Taq* DNA polymerase (BRL) in a final volume of 25 μl. Thermocycling conditions consisted of 35 cycles of 40 s at 94°C, 40 s at 55/58°C (depending on primer set), and 40 s at 72°C, followed by one cycle of 3-min extension at 72°C. Reaction products were then diluted 1:2 with a stop buffer (90% formamide, 10 m*M* EDTA, bromophenol blue, and xylene cylanol) and heated to 94°C for 10 min prior to loading on 6% polyacrylamide sequencing gels. Gels were dried at 80°C and exposed to Kodak XAR-5 film.

Fine mapping of the MDGI/FABP3 gene relative to 1p microsatellite markers. A Research Genetics PCR-based radiation hybrid panel was used to map the gene relative to the microsatellite markers used for the LOH analysis. Exon 2 of MDGI/FABP3 (primers described below) and the microsatellite markers (above) were amplified in each of the hybrid DNAs (according to the manufacturer's instructions). By combining the data on chromosome 1p Généthon microsatellite markers previously run on the panel (accessed at the following web site address: ftp://shgc.stanford.edu/pub/hgmc/RH_data/rh_scores) and the data generated from this study, a common chromosomal region was identified. The region of common overlap suggested that MDGI was located centromeric to D1S233 and telomeric to MYCL1, as shown in Fig. 2.

Direct sequencing of the MDGI/FABP3 gene in sporadic tumors. To sequence the gene, oligonucleotide primers were designed for flanking intron sequences for each exon, so as to include intron/exon splice sites. Double-stranded DNA was PCR amplified for each exon, with the anti-sense primer 5′-biotinylated. Each reaction consisted of 35 cycles in a Perkin–Elmer 9600 thermocycler (Perkin–Elmer/Cetus), employing the following temperatures and times: denaturation, 94°C for 40 s; annealing, 58°C (exons 2, 3, and 4)/60°C (exon 1) for 40 s; and elongation, 72°C for 40 s. The primer sequences and sizes of amplified products for each exon are given in Table 3. The PCR products were then purified by agarose gel electrophoresis, and the band of appropriate size was excised from the gel. The DNA was isolated from the gel by freeze/thawing (Phelan *et al.*, 1995), and the biotinylated DNA was bound to strepavidin beads (Dynabeads, Dynal AS, Norway). Single-stranded DNA was then isolated by denaturing the DNA with 0.2 *M* NaOH and separating the nonbiotinylated DNA strand from the biotinylated (attached to the beads) using a magnetic rack (Magnetic Particle Concentrator, Dynal AS, Norway) (Phelan *et al.*, 1995). Both strands were sequenced using the Sanger dideoxy method with a USB Sequenase Version 2.0 kit. (USB, OH). The sequencing reaction products were separated on 6% denaturing polyacrylamide gels. Gels were transferred to filter paper (Whatman) and dried prior to exposure to Kodak XAR-5 film.

Single-strand confirmation (SSC) analysis of sequence variants. To determine the frequency of the sequence variant in exon 2, SSC analysis was developed for the exon 2 fragment. Genomic DNA (100 ng) was amplified using the exon 2 primers (Table 2) and under conditions as described above in the presence of 0.2 m*M* dTTP, dCTP, and dGTP, 0.05 m*M* dATP, [α-^{35}S]dATP (Amersham) at 1 μCi/reaction, and 2 units of *Taq* DNA polymerase (BRL) in a final volume of 12.5 μl. The reaction products were then diluted 1:3 with a stop buffer (described above) and heated to 94°C for 10 min prior to loading on 6% nondenaturing polyacrylamide gels with/without 5% glycerol. The gels were run at 4°C for 4–6 h and then were dried at 80°C and exposed to Kodak XAR-5 film.

RESULTS

The MDGI.3 cosmid clone that contains the gene (see Materials and Methods) was sequenced, and alignment

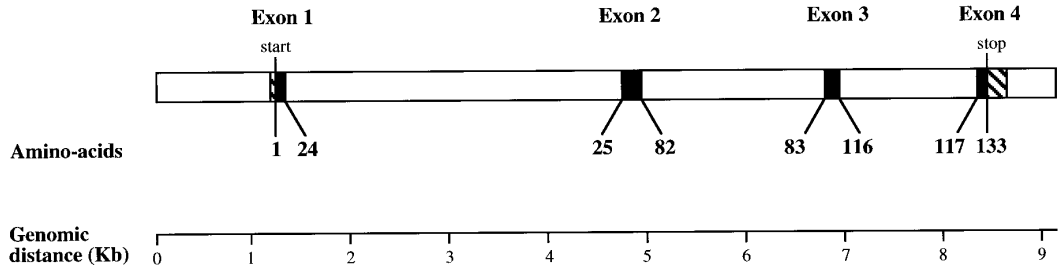

FIG. 1. Genomic structure of the human MDGI/FABP3 gene. The translated exons are depicted as solid boxes, the introns as open boxes, and untranslated exons as shaded boxes. The gene spans approximately 8 kb of genomic DNA. The translation start and stop sites are shown. Exon 1 spans 104 bp and contains the 5′-UTR region; exon 2, 173 bp; exon 3, 102 bp; and exon 4, 284 bp and the 3′-UTR region.

with the human FABP3 cDNA revealed 100% identity in the coding regions, implying that MDGI and FABP3 are the same gene. This gene consists of four exons (exon 1, 104 bp; exon 2, 173 bp; exon 3, 102 bp; exon 4, 284 bp) and three introns (intron 1, 3.38 kp; intron 2, 1.89 kb; intron 3, 1.44 kb) spanning approximately 8 kb of genomic DNA (Fig. 1). Exons 1 and 4 contain approximately 40 and 210 bp of 5′- and 3′-untranslated sequence, respectively. Each of the intron/exon boundaries was identified and sequenced (Table 1) to facilitate the search for mutations in the coding region and splice sites of the MDGI/FABP3 gene.

Six microsatellite repeat markers localized to chromosome 1p22–p35 (Fig. 2) (including the 1p32–p35 interval where MDGI/FABP3 has been mapped by FISH) were evaluated for LOH in the constitutional and tumor DNA of 30 breast cancer cases. Ten cases showed either complete or partial loss of this region (Fig. 2). Two cases appear to have shown LOH of the entire region (cases D3 and 100), while the remaining 8 cases showed partial losses involving both proximal portions (cases 8, 11, 36, 105, D177, and D176) and distal portions (cases D27, D49, D176) of this interval. This observation is consistent with previously published chromosome 1p microsatellite LOH data in breast cancer (Hoggard *et al.*, 1995). The localization of the MDGI/FABP3 gene relative to the microsatellite repeat markers was determined using a PCR-based hybrid panel (Research Genetics) (see Materials and Methods). The gene appears to map between the loci of markers D1S233 and MYCL1, where five of the tumors (D3, 100, D176, D27, and D49) show LOH.

In an attempt to identify mutations in the MDGI/FABP3 gene in 30 sporadic breast tumors, the four exons and flanking exon/intron junctions of this gene were sequenced (Tables 1 and 2). No mutations were found in the breast tumors. A single novel sequence polymorphism was identified (Fig. 3), which results in the conversion of lysine to arginine in codon 53 (the amino acid positions are according to Peeters *et al.* (1991). This rare polymorphic variant is found in 1/30 women included in this study and in the constitutional DNA of a further 3/26 women with sporadic breast cancer (data not shown). The variant was also detected in the constitutional DNA of 2/90 control individuals ($P = 0.20$), who do not have breast cancer (Fig. 3).

DISCUSSION

The MDGI/FABP3 gene has been demonstrated to have tumor suppressor activity in breast cancer in the following ways (Böhmer *et al.*, 1987; Hung *et al.*, 1995). (1) *In vitro* studies involving transfection of an MDGI-expression construct into MCF-7 breast cancer cell lines resulted in reduced proliferative rates, altered morphology, and differentiation, compared to mock-transfected controls. (2) Transfected breast cancer cell lines also showed a reduced ability to form tumors in nude mice compared to the untransfected controls (Hung *et al.*, 1995). (3) Immunostaining with an anti-FABP3 antibody revealed lack of expression of the protein in neoplastic breast cells compared to adjacent normal epithelia consistent with a role in breast tumor development (Hung *et al.*, 1995). In addition, the treatment of mouse mammary epithelial cells with antisense MDGI phosphorothioate oligonucleotides resulted in an inhibition of DNA synthesis, suppression of alveolar budding, and impaired β-casein synthesis, suggesting a block in cell differentiation (Yang *et al.*, 1994).

We have elucidated the complete sequence and genomic structure of the human MDGI/FABP3 gene (Fig.

TABLE 1

Intron/Exon Border Sequences

Intron	Intron size (bp)	5′ junction sequence	3′ junction sequence
1	3385	ATGAAGTCACTCGgtgagcaagccgcggggctcagga	tatactcataaccttccccctaccctcagGTGTGGGTTTTG
2	1890	AGGAAGGTCAAGgtaagtcaggaaacaggggtgggg	agctcaggcctctaccctctttccacagTCCATTGTGACAC
3	1443	AAACTCATCCTGgtaagatgggcaactttggagctatatct	cccccatctcactctgtctttccttccagACACTCACCCACG

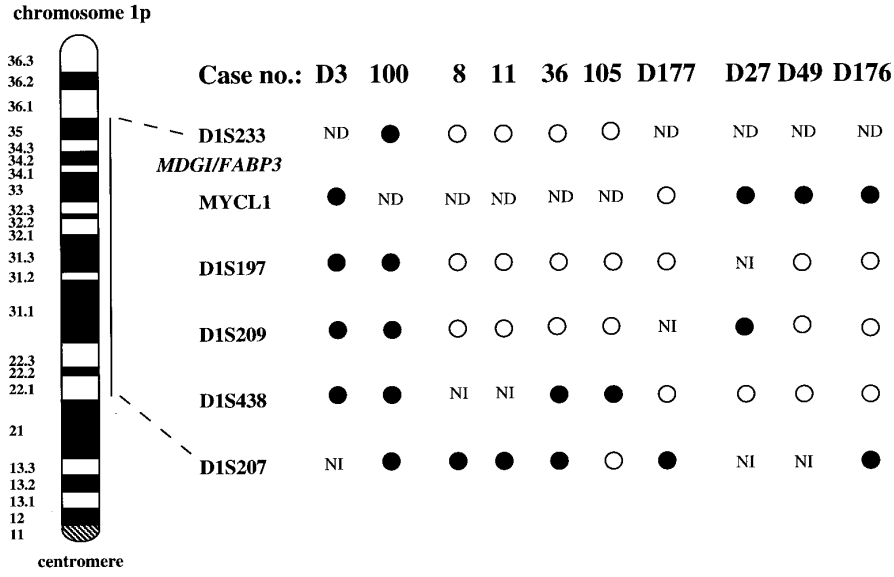

FIG. 2. A summary of loss of heterozygosity (LOH) results of six microsatellite repeat markers mapping to 1p22–p35 for 10 cases of sporadic breast cancer. The marker loci and their order are given to the right of the chromosome. Solid circles denote LOH; open circles denote retention of both alleles. The case numbers for each tumor are given at the top of the figure: cases 8, 11, 36, 100, and 105 are from the Karolinska Hospital; cases D3, D27, D49, D176, and D177 are from Duke University. NI, noninformative, ND, not determined.

1) so as to sequence all exons and their corresponding splice junctions to identify potential inactivating mutations in 30 sporadic breast cancers. The MDGI/FABP3 gene has been previously localized by FISH to 1p32–p35 (Troxler *et al.*, 1993; Hung *et al.*, 1995). We have more finely mapped the gene relative to 1p microsatellite markers in this region between D1S233 and MYCL1, consistent with the cytogenetic localization. This report and others have shown LOH in breast tumors in this region, suggesting that this chromosomal region may harbor tentative tumor suppressor genes. These studies have shown varying LOH frequencies ranging from 21 to 62% (Borg *et al.*, 1992; Bieche *et al.*, 1993; Munn *et al.*, 1995) and complicated patterns of allele loss (Hoggard *et al.*, 1995).

Tumor suppressor genes are thought to induce cancer by being inactivated, often by loss of one allele and mutation of the other (Knudson, 1993). It is expected that tumors showing loss of the first allele (as shown by LOH studies) are more likely to harbor point mutations in the second allele (Ruttledge *et al.*, 1994). Elucidation of the genomic structure of the human MDGI/FABP3 gene allowed us to look for mutations in the constitutional and tumor DNA of 5 breast tumors showing LOH in the MDGI region (Fig. 2). In addition, we sequenced 25 tumors that appeared to show retention of this region. No mutations were found. The lack of gene mutation in these 30 breast tumors suggests that this is not a common mechanism of inactivation of MDGI/FABP3 in these neoplasms.

The rare exon 2 polymorphism was observed more frequently (4/56) in breast cancer cases than in controls (2/90) ($P = 0.20$) (see Results). This missense amino acid change (lysine to arginine) affects a highly conserved residue at codon 53 in one of the protein's two fatty-acid binding sites (Offner *et al.*, 1988). The significance of this observation is the subject of further investigation. The detection of the rare polymorphism in exon 2 proved that our technique was sensitive and efficient (Fig. 3). Thus, we feel it is unlikely that small deletions and insertions or other single base substitutions would have escaped our detection. We also have analyzed 18 breast cancer cell lines for homozygous deletions of human MDGI/FABP3, using PCR amplification of exon 2 and an internal control from chromosome 22, but none were found (data not shown).

TABLE 2

Oligonucleotide Primers Used for SSC and Sequencing Analysis

Exon No.	Exon size (bp)	Sense primer	Antisense primer	Size of PCR product (bp)
1	104	5′-cctggggcttcctatttc-3′	5′-caagccaacatcctgagc-3′ 5′-cttcatgtagtcatagaa-3′	261
2	173	5′-tgtacactgcttctctaccc-3′	5′-gtctcagcactctccattcc-3′	271
3	102	5′-tcacagctcaggcctctacc-3′	5′-gatatagcgccaaagttgcc-3′	160
4	284	5′-gtagaatgctcgcaatggtg-3′	5′-ggataaaccaagactcccagag-3′	402

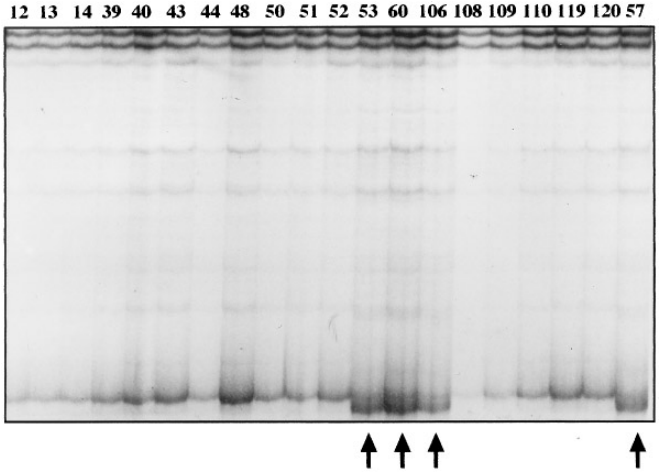

FIG. 3. Single-strand confirmational (SSC) analysis of constitutional DNA from 20 women with breast cancer showing 4 cases in which the codon 53 polymorphism was identified, as indicated by the arrows at the bottom of the figure.

In conclusion, although transfection experiments provide biological evidence for a suppressor activity of MDGI/FABP3 in breast cancer, we did not observe structural mutations in the coding region of the MDGI/FABP gene in 30 sporadic breast tumors. However, this finding is not unprecedented. Loss of expression of the breast–ovarian cancer gene BRCA1 has been reported in sporadic breast cancers (Thompson *et al.*, 1995) but no point mutations have been found in 32 cases of sporadic human breast tumors, all of which show LOH at the BRCA1 gene (Futreal *et al.*, 1994). It may be possible that the MDGI/FABP3 gene is not the target of losses of the 1p32–p35 region. On the other hand, alternative mechanisms may result in the loss of gene expression, including alterations in the methylation of the promoter. This phenomenon has been observed in the von Hippel Lindau gene (VHL) (Herman *et al.*, 1994) and the retinoblastoma gene (RB) (Ohtani-Fujita *et al.*, 1993). The evidence for decreased expression of MDGI in human breast cancer motivates further work regarding promoter methylation and regulation of expression of MDGI.

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