Frequent Loss of Imprinting of PEG1/MEST in Invasive Breast Cancer

Inge S. Pedersen, Peter A. Dervan, Dennise Broderick, Michèlè Harrison, Nicola Miller, Emma Delany, Donal O’Shea, Paul Costello, Alo McGoldrick, George Keating, Brendan Tobin, Tom Gorey, and Amanda McCann

Department of Pathology, Biotechnology Centre, University College Dublin, Belfield, Dublin 4 [I.S.P., P. A. D., D. B., E. D., P. C., A. McG., A. Mc.C.]; Departments of Histopathology [P. A. D., M. H., G. K., B. T.] and Surgery [T. G.], Mater Hospital, Dublin 7; National Centre for Medical Genetics, Crumlin, Dublin 12 [N. M.]; and Bio Research Ireland, Biotechnology Centre, University College Dublin, Belfield, Dublin 4 [D. O.], Ireland

Abstract

The human PEG1 gene is a newly identified imprinted gene on 7q32. Genetic aberrations of this chromosomal region are often detected in invasive breast carcinomas. In this study, we show monoallelic PEG1 expression in normal breast tissue, indicating the presence of a functional imprint, and more importantly, we demonstrate loss of imprinting (LOI) in all of seven informative invasive breast carcinomas. In contrast to this, in one case of atypical ductal hyperplasia (ADH) found in residual breast, imprinting was maintained. This raises the possibility that aberrant imprinting of PEG1 may be involved in the progression from hyperplasia to invasive breast cancer.

Introduction

Genomic imprinting refers to the mechanism by which a number of genes throughout the genome are monoallelically expressed according to their parental origin. Epigenetic alteration in the form of reactivation of a normally silent/imprinted allele (LOI) is a well-established mechanism for overexpression of the growth-promoting gene, IGF2, in cancer (1-5). A detailed knowledge of the involvement of imprinted genes in cancer is of utmost importance, because aberrant imprinting appears far more accessible to therapeutic intervention than genetic changes. The in vitro treatment of tumor cells with 5-aza-2’-deoxycytidine, a specific inhibitor of cytosine DNA methyltransferase, has been reported to reverse LOI of IGF2 and H19 (6).

In this study, we have investigated the allelic usage of the imprinted gene PEG1 in breast cancer. PEG1 is located on chromosome 7. Several lines of evidence suggest the involvement of genes on chromosome 7 in tumor progression and invasion. Cytogenetic studies show that trisomy 7 is one of the most frequent numerical chromosomal aberrations in breast tumor cell lines (7-9). Structural changes of chromosome 7 also appear in breast cancer, one of the more frequent being the LOH of several markers in the 7q31–32 region. One group reported 40.5% LOH on 7q31 and suggested that this might be the site of a breast tumor or metastasis suppressor gene (10). More recently, an international collaborative study group (The Breast Cancer Somatic Genetics Consortium) reported on their combined results on 683 breast tumors. LOH scorings for three polymorphic markers on 7q31–32 gave an average rate of 19% (11). The only imprinted gene identified on chromosome 7 thus far is PEG1, located at 7q32 (12). The biological function of PEG1 is unknown. However, the putative protein shares amino acid homology with the α/β hydrolase fold family, which also includes the lysosomal enzyme cathepsin A. This could suggest a possible role in degradation of the extracellular matrix in the invasive state of tumor development. Peg1/Mest knockout mice show abnormal maternal behavior and growth retardation and have smaller placenta (13). This indicates that Peg1 is either directly involved in the regulation of growth of the fetus and/or placenta.

Materials and Methods

Tissue Samples. Frozen breast tumor samples and adjacent normal breast tissue were prospectively collected from the Mater Misericordiae Hospital (Dublin, Ireland). Tissue samples from a total of 53 patients were analyzed. The sample cohort consisted of 20 infiltrating carcinomas (16 ductal, 1 lobular, and 3 miscellaneous), 3 pure DCIS, 19 mixtures of both invasive and in situ components, and 5 benign lesions (2 fibrocystic changes, 2 phyllodes tumors, and 1 myoepithelioma). In addition to these 47 primary lesions, 6 residual breast samples were analyzed. DCIS was found in one sample, and the remaining five were benign lesions (three fibrocystic changes, one combined fibroadenoma and fibrocystic change, and one ADH).

Histology. All invasive carcinomas were graded using a modified Bloom and Richardson grading system (14), and the DCIS were graded by nuclear morphology (15). The proportion of the tumor occupied by a DCIS component was assessed visually. The lymphocytic response to the tumor was assessed using a grading system as follows: mild, a small number of lymphocytes scattered around tumor cells; severe, an intense lymphocytic infiltrate as might be seen in medullary carcinoma; and moderate, midway between the two. The infiltrate was said to be focal if it occupied <10% of the tumor area and was confined to one or two areas.

PCR Amplification of Genomic DNA and RT-PCR. Primers were designed to encompass the A/III RFLP in the 3’ untranslated region of the PEG1 cDNA (Genbank accession no. D78611). High molecular weight DNA was extracted from snap frozen tissue using standard methods. PCR reactions were set up on 0.5 μg of genomic DNA using primers PegF (5’TAC TAA ACC AGA ATA CCC TTA C-3) and PegR (5’GCA GTC ATA ATC AAAG TCA G-3) in 50-μl reactions containing 1× Taq buffer, 250 μM deoxynucleotide triphosphate mixture, 80 pmol of each primer, and 1.25 unit of Taq polymerase (Promega). The reagents were subjected to 5 min of initial denaturing at 95°C, followed by 35 cycles (95°C for 30 s, 50°C for 1 min, and 72°C for 2 min) with a final extension for 7 min at 72°C. PCR was carried out on a DNA Engine (PTC-2000).

RNA from informative patients was extracted using the TRIzol RNA extraction kit (Life Technologies, Inc.), according to manufacturer’s instructions. To eliminate any contaminating genomic DNA, 1 μg of RNA was DNase treated using DNasel (Life Technologies, Inc.), and 0.5 μg was used as a template in the Access RT-PCR kit (Promega) using primers PegF and PegR. The remaining 0.5-μg aliquot of DNase-treated RNA was set up in the absence of the AMV enzyme and hence served as a control for DNA contamination. Cycling conditions were as follows: 48°C for 45 min, followed by 95°C for 5 min, and 40 cycles of 95°C for 30 s, 50°C for 1 min, and 68°C for 2 min, and finally, 7 min at 68°C.

Determination of Heterozygosity and Allelic Usage. A/III restriction digestion of 10 μl of PCR or RT-PCR product was carried out in 15-μl reactions containing 1 unit of A/III enzyme and 1× RExact3 buffer (Life Technologies, Inc.) for 4 h at 37°C.

Image Analysis. Digital images of gels were captured in 8-bit grayscale (256 gray values) and were processed in the following manner. The image

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1 To whom requests for reprints should be addressed, at Department of Pathology, Biotechnology Center, University College Dublin, Belfield, Dublin 4, Ireland. E-mail: amcann@ollamb.ucd.ie.

2 The abbreviations used are: LOI, loss of imprinting; IGF, insulin-like growth factor; LOH, loss of heterozygosity; DCIS, ductal carcinoma in situ; ADH, atypical ductal hyperplasia; RT-PCR, reverse transcription-PCR.

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grayscale values were inverted, resulting in an image with dark bands on a light background. The resultant image was normalized by stretching the histogram, setting the median grayscale value of the image (background) to 255 (white), and adjusting all other values upward on a factorial basis. This resulted in an image with dark bands on white background. Bands of interest were selected, and the following parameter was calculated:

\[ P = \sum_{i=1}^{N} \log_{10}\left( \frac{255}{I_i} \right) \]

where \( P \) is the integrated log inverse grayscale value (absorbance) for the band, \( N \) is the number of pixels in the area, and \( I_i \) is the intensity of pixel \( i \). Ratios of bands were calculated by comparing the calculated \( P \) of the less intense band to the \( P \) of the more intense band.

**Results and Discussion**

The \( PEG1 \)-specific PCR product was amplifiable from genomic DNA of 53 paired normal and tumor samples. Of these 53 patients, 12 (23%) were informative for the \( AflIII \) polymorphism (9 invasive carcinomas, 1 DCIS, 1 fibrocystic change, and 1 ADH in residual breast). All but one sample had retained heterozygosity in the abnormal tissue (Fig. 1). The one sample displaying LOH was a high-grade DCIS, comedo type. RNA analysis of this sample established that the lost allele was the inactive copy (Fig. 2). \( PEG1 \) RT-PCR products from 8 of the 11 samples with retained heterozygosity were analyzable by restriction digestion. Seven tumors, all invasive, showed biallelic expression (LOI), whereas a functional imprint, evident by monoallelic expression, was in place in the paired normal samples (Fig. 2). One sample had maintained imprinting in the abnormal tissue. This was a case of ADH.

LOI was measured semiquantitatively by comparing the absorbance of the gel bands representing the two alleles. All but one of the cases showing reactivation of the silent allele had ratios of the less abundant to the more abundant allele of more than 1:2 (Table 1). These cases would be classified as LOI using the arbitrarily chosen threshold of 1:3 applied by Cui et al. (16) for \( IGF2 \). One sample, however, had a ratio of 1:4, which could be a result of contaminating normal tissue or maintained imprinting in the coexisting DCIS component (DCIS accounts for 30% of the tumor mass in this case). Of note is the observation that three samples with allelic ratios approaching or reaching 1:1 were the only tumors without a DCIS component. This indicates the possibility of a normal imprinted phenotype in the noninvasive DCIS tumors, which would further support the theory that LOI of \( PEG1 \) is involved in tumor invasion. In agreement with previous reports, we found that the repression of \( PEG1 \) is incomplete (12), with transcription levels of the silent allele reaching 1:20 to 1:10 of the expressed allele.

\( PEG1 \) is biallelically expressed in adult lymphocytes (17). To ensure that the LOI observed in the tumors is not an artifact resulting from a lymphocytic response to the tumor, all tissue samples were stained with H&E, and an assessment was made of the number of lymphocytes present (Table 1). The degree of lymphocytic infiltrate present in the tumors appears unlikely to account for the biallelic profiles.

The consistent finding of \( PEG1 \) LOI in invasive breast carcinomas suggests a possible role for this imprinted gene in breast carcinoma progression and/or invasion. We were surprised by this finding, because of earlier reports of LOH in the 7q31–32 region, suggesting the existence of a tumor suppressor gene at this site. Because LOH in many cases is accompanied by duplication of the retained allele (as a result of gene conversion or mitotic recombination), it is possible that the outcome of LOH at an imprinted locus equals the outcome of LOI, i.e., two active gene copies. Alternatively, the existence of a reciprocal imprinted gene in close proximity to \( PEG1 \), the imprinting status of which is coregulated in a manner similar to \( H19 \) and \( IGF2 \), would also account for the apparent involvement of both LOH and LOI of \( 7q31–32 \). Whether \( PEG1 \) is part of a larger imprinting domain remains to be determined.

Investigation of the mechanism of \( PEG1 \) LOI in breast cancer would be facilitated by methylation analysis of the \( PEG1 \) promoter. In fetal samples, the promoter of the inactive maternal allele is methylated, whereas the paternal promoter is unmethylated (17). Interestingly, although this differential methylation is conserved in adult lymphocytes, these cells display biallelic expression (17). It would be of interest to establish whether the methylation of the promoter region is altered in cancer tissue with biallelic expression. This may suggest that different mechanisms underlie the normal tissue-specific biallelic expression of \( PEG1 \) and the biallelic expression observed in breast cancer.

\( PEG1 \) is also known as mesodermal-specific transcript (\( MEST \)) because of its preferential transcription in tissue of mesodermal origin. We did, however, detect \( PEG1 \) expression in the epithelial breast cancer cell lines MCF7 and MCF10 using Northern blot analysis (data not shown). The expression levels were ~4-fold higher in the invasive MCF7 cell line compared with the immortalized MCF10 cells. This further supports the possibility of \( PEG1 \) being one of the genes on chromosome 7 involved in the stages of local tumor invasion.
Table 1  Histological details of tumors

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Allelic usage</th>
<th>Allele ratios</th>
<th>Invasive carcinoma grade</th>
<th>DCIS grade (proportion)</th>
<th>Lymphocytic response</th>
<th>Lymphovascular space invasion</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>Biallelic</td>
<td>0.76</td>
<td>2</td>
<td>Intermediate (15%)</td>
<td>Focal mild</td>
<td>None</td>
</tr>
<tr>
<td>11</td>
<td>Biallelic</td>
<td>1.0</td>
<td>3</td>
<td>None</td>
<td>Moderate</td>
<td>None</td>
</tr>
<tr>
<td>18</td>
<td>Biallelic</td>
<td>1.0</td>
<td>2</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>39</td>
<td>Biallelic</td>
<td>0.83</td>
<td>NA</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>53</td>
<td>Biallelic</td>
<td>0.66</td>
<td>3</td>
<td>Intermediate (10%)</td>
<td>Focal moderate</td>
<td>Present</td>
</tr>
<tr>
<td>56</td>
<td>Monallelic</td>
<td>0.05</td>
<td>None</td>
<td>None</td>
<td>Moderate</td>
<td>None</td>
</tr>
<tr>
<td>74</td>
<td>Biallelic</td>
<td>0.50</td>
<td>NA</td>
<td>Intermediate (&lt;5%)</td>
<td>Focal mild</td>
<td>None</td>
</tr>
<tr>
<td>89</td>
<td>Biallelic</td>
<td>NA</td>
<td>None</td>
<td>High (100%)</td>
<td>Multifocal mild</td>
<td>None</td>
</tr>
<tr>
<td>98</td>
<td>Biallelic</td>
<td>0.25</td>
<td>3</td>
<td>High (30%)</td>
<td>Moderate</td>
<td>None</td>
</tr>
</tbody>
</table>

* Cases not otherwise designated were all infiltrating ductal carcinomas.
1 Mucinous carcinoma.
2 Squamous cell carcinoma (cannot be graded using the Bloom and Richardson scheme).
3 NA, not applicable.
4 ADH found in residual breast.
5 Moderately differentiated papillary carcinoma (cannot be graded using the Bloom and Richardson scheme).
6 DCIS displaying LOH.

Acknowledgments

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References

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