Biallelic Inactivation of the Thyroid Hormone Receptor β1 Gene in Early Stage Breast Cancer

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Abstract

Loss of heterozygosity within the short arm of chromosome 3 is a common molecular event in several types of solid tumors. In breast cancer, 3p loss of heterozygosity occurs in invasive tumor cells as well as in morphologically normal terminal ductal lobular units adjacent to carcinoma in some cases [G. Deng et al., Science (Wash. DC), 274: 2057–2059, 1996]. The most frequent region of allelic loss at 3p24.3 in morphologically normal terminal ductal lobular units encompasses the thyroid hormone receptor β1 (TRβ1) gene. Here we have observed a variable degree of TRβ1 promoter hypermethylation in all 11 cases of primary breast cancer examined. Moreover, hypermethylation occurred at the same CpG sites in nonmalignant tissue peripheral to carcinoma in 4 of 11 cases. The lack of TRβ1 nuclear staining, a likely result of biallelic gene inactivation, was observed in 25% (22 of 85) of primary tumors. This is a first demonstration of promoter hypermethylation and a concurrent reduction of TRβ1 transcripts in breast cancer cell lines, although specific CpG sites targeted for gene silencing remain to be determined. Gene expression was restored by treatment with 5-aza-deoxycytidine in such cases. The observation of early, frequent, and multiple mechanisms of TRβ1 inactivation suggests a potential role for this gene in the suppression of breast tumorigenesis.

Introduction

Thyroid hormone receptors are ligand-mediated transcription factors, which form complexes with other nuclear receptors and multiple effector proteins to regulate growth, differentiation, and development (1). Loss of gene function associated with v-erb A, a mutated variant of thyroid hormone receptor α (2) arrests normal differentiation of avian erythroblast progenitors resulting in virally induced leukemic transformation (3). Deletions encompassing TRβ3 are suspected to play a role in the genesis of small cell lung cancer (4). In breast cancer, LOH is a common occurrence at chromosome 3p in the general vicinity of the TRβ1 gene (5, 6). Most notably, LOH encompassing the TRβ1 gene occurs before the manifestation of morphological changes in TDLUs of cancerous breast tissue (7, 8). We demonstrate here that epigenetic changes in the promoter region of TRβ1 are also involved in the inactivation of this gene in breast cancer cell lines and possibly in early stage breast tumors.

Materials and Methods

RT-PCR Analysis. Established breast cancer cell lines and second passage normal mammary epithelial cells isolated from reduction mammaplasty tissue were propagated under routine culture conditions. Total cellular RNA was isolated from cells in the logarithmic growth phase using the RNAasy Mini kit (Qiagen). Up to 2 μg of total RNA was reverse transcribed using Superscript II RT RNase H–Reverse Transcriptase (Life Technologies, Inc.) and random hexamers (Operon, Inc.) in a reaction volume of 50 μL. In subsequent PCR reactions, 2 μL of cDNA were amplified. To normalize the relative amount of the cDNA synthesized for each test transcript, GAPDH was used as an internal competitive control within the same PCR reaction as the genes of interest. Gels were scanned, and the ratio of test gene:GAPDH was determined using ImageQuant (Molecular Dynamics). The PCR conditions were as follows: 94°C for 4 min 30 s to 30–35 PCR cycles at 94°C, 15 s; 58–61°C, 15 s; 72°C, 30 s; and a final 5 min extension at 72°C. PCR products were resolved in 2% agarose gel stained with ethidium bromide. Primer sequences were as follows:

GAPDH-F: TGATGACATCAAGAAGGTGGTA;
GAPDH-R: TCCTTGAGGCCCATGTGGCCAT; (250 bp product)
TRβ1-F: GAAACATGTCGCAATCATCTC;
TRβ1-R: TTAACCTCCATTTCCTGCT; (721 bp product)
RARβ2-F: AAGATTTGATGGAGTTGGGTGGAC;
RARβ2-R: GGCTGCAGGGATGAGGGAAGATTT (721 bp product).

For restoration of TRβ1 expression, cell lines were incubated with medium supplemented with 1 μM 5-aza-2-deoxycytidine for 5 days.

FISH, LOH, and Immunoperoxidase Analysis. For FISH, nuclear signals generated from two color hybridization of FITC-conjugated chromosome 3 centromere probe and Cy3-conjugated TRβ1 probe (prepared and validated in the Division of Cancer Genetics, University of California, San Francisco Cancer Center) were visualized and recorded from 50 to 100 cells of each test culture. The proportion of nuclei displaying fewer copies of orange-colored TRβ1 signal in comparison with the green colored centromeric signal was determined. Before LOH analysis of tumors, control DNA from nonmalignant skin of the patient was used to determine informative status (presence of heterozygosity) at the EABMD and EABH loci within the TRβ1 region. Tumor DNA was isolated from manually microdissected, H&E-stained paraffin sections. PCR conditions were as described in Deng et al. (7). Samples were scored as positive for LOH if ≥30% reduction was observed in the allelic ratio of tumor compared with control DNA. A mouse monoclonal antibody (J51) specific for human TRβ1 (Santa Cruz Biotechnology Biotech. Inc.) was used at 1:50 dilution for immunoperoxidase staining of multituform tissue array sections from 0.8 mm cores of paraffin-embedded samples of 106 stage I invasive ductal breast carcinoma (assembled at the University of California, San Francisco Cancer Center). Antibody binding to tissue sections was visualized with the ABC immunoperoxidase kit (Vector Labs). Normal breast tissue served as a positive control for nuclear signal specificity. Tumor samples, which displayed ≥10% nuclear positive cells in a ×10 microscope field, were recorded as TRβ1 positive.

DNA Extraction and Bisulfite Treatment. Fresh tissue from 7 cases of reduction mammaplasty and frozen blocks from 12 cases of pathologically confirmed stage I and II ductal breast carcinoma, and nonmalignant peripheral tissue were collected at the California Pacific Medical Center, San Francisco, CA, under Institutional Review Board approved guidelines. Genomic DNA was isolated from tissues and cell lines by the standard method of proteinase K digestion and phenol-chloroform extraction. A

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minimum of 10 ng (1000 cells) genomic DNA was treated with sodium bisulfite as described (9). On the basis of the TRβ1 promoter sequence (Human Genome Project Working Draft), a set of universal primers (CVT3F/CVT4R) was designed to amplify both the methylated and the unmethylated strands of bisulfite converted DNA. Additional analysis included MSP, and COBRA, as reported (9, 10). In the second round of the nested PCR assay, for MSP, primers M1F/M1R and U1F/U1R, and for COBRA (in tissue samples only), primers CVT3F/CVT3R were used. In COBRA, the PCR product was digested with TaqI to distinguish between methylated and unmethylated DNA. In each assay, absence of DNA template served as negative control, whereas the MDA435 cell line, where methylation was confirmed by sequence analysis of converted DNA, was used as a positive control.

MSP primers:
M1F: GGTAATTGGTAAAGGACGCGC;
M1R: CACCCCTCCGATTCTTACGACG;
U1F: TATTGGTAATTGGTAAATGTGT; and
U1R: CACACCCCTCCAATT CTTACAACA

COBRA primers:
CVT3F: GTTTTAGGGTATTGGTAATTGGT; and
CVT3R: CAAACTAATAACACCCCCACCA

The relative positions of the CpG sites covered by the above-mentioned primers are shown in Fig. 1a.
Results

**TRβ1 mRNA Expression and Gene Copy Number in Breast Cancer Cell Lines.** Toward a full understanding of the mechanisms of TRβ1 inactivation during tumorigenesis, initially we analyzed relative gene expression in breast cancer cell lines by RT-PCR, using primers encompassing exons 4–7. Moderate TRβ1 expression was observed in noncancerous breast epithelium before and after cell culture. Transcript levels below those of normal breast epithelial cells were observed in 7 of 10 cell lines (Fig. 1b; Table 1). Concurrently, we determined whether reduction in gene copy number was an underlying factor in the level of gene expression. In the majority of cell lines, FISH analysis with site-specific probes for interphase signal enumeration demonstrated equal numbers of chromosome 3 centromere and TRβ1-specific signals. In BT474 cells, reduction in copy number did not appear to influence expression level. In those cultures where gene expression was undetectable, such as MDA435 and SKBR3, fewer TRβ1 gene copies were observed in the nuclei of a large proportion of cells (Table 1).

Homozygous deletions encompassing TRβ1 were not found in any of 10 breast cancer cell lines examined. Similarly, in primary tumors, analyzed here (n = 12) and previously (64 stage I and II cases examined by LOH analysis, Ref. 8), chromosomal deletions were limited to loss of a single allele. To determine whether the remaining allele harbored inactivating gene mutations, cDNA of cell lines and primary tumors (12 cases) was analyzed by single-strand conformational polymorphism analysis of exons 9 and 10. This region consists of the ligand-binding domain, a common site of TRβ1 germ-line mutations in syndromes unrelated to cancer (11). Because TRβ1 mutations were not detected, here we have focused on the possible role of epigenetic mechanisms in gene inactivation.

**Methylation of the TRβ1 Promoter in Breast Cancer Cell Lines.** On the basis of the presence of a CpG island in the 5’ region of the gene (Fig. 1a) we examined the role of promoter hypermethylation in TRβ1 inactivation. Toward a rapid screen for TRβ1 hypermethylation in noncancerous breast epithelial cells isolated from cosmetic reduction mammaplasty tissue and breast cancer cell lines, we first analyzed bisulfite converted DNA by the MSP assay (Fig. 1c). High gene expression levels were associated with the absence of DNA hypermethylation in MCF7, T47D, and BT474 cell lines. Notably, a lack of detectable transcripts and promoter hypermethylation was concordant in MDA435 and SKBR3 cells. Subsequent sequence analysis of the MDA435 cell line demonstrated 27 of 27 potential sites in a 325-bp region within the CpG island of the gene promoter to be methylated. Four CpG sites downstream of the MSP region, where complete methylation was observed, are shown in Fig. 1d. Incomplete methylation evident at several sites (not shown) most likely accounts for the presence of unmethylated DNA in this cell line.

The detection of abnormally methylated sites in the promoter region is generally considered to be a strong indication of aberrant gene expression, although the target sites of methylation, which effectively inactivate the gene, may be far removed from the observed site of methylation. We have taken additional approaches to identify other methylation sites and additional links between methylation and lack of TRβ1 expression. In methylation analysis by the COBRA method, the cell lines found to be methylated by MSP once again displayed hypermethylation (Fig. 1e).

Moreover, we have showed the restoration of TRβ1 expression on treatment with a demethylating agent, 5-aza-deoxycytidine, demonstrating that silencing of the gene by this mechanism is partially reversible (Fig. 1e). We have observed a 43% and 20% reduction in DNA methylation status of 5-aza-deoxycytidine treated cultures of MDA435 and SKBR-3 cells, respectively. These results demonstrate a direct functional link between methylation and loss of expression, and demethylation and restoration of expression (Fig. 1e). Simultaneous analysis of another methylated gene in the 3p24 region, RARβ2, displayed discordant hypermethylation of the two genes in these samples suggesting that these events were not necessarily linked during tumor progression. Transcript levels of RARβ2 were unaltered by treatment with 5-aza-deoxycytidine alone as reported previously (12, 13).

**TRβ1 Promoter Methylation, LOH, and Immunolocalization in Primary Breast Cancer.** In addition to breast cancer cell lines, we evaluated 11 matched cases of primary tumor and breast tissue peripheral to carcinoma for epigenetic alterations in the TRβ1 promoter. We observed TRβ1 hypermethylation in all 11 of the breast tumors concurrently by MSP and COBRA (Fig. 2, a and b). Considerable intertumor variability in the degree of methylation was evident by the intensity of the PCR products. In 7 of 7 independent cases of normal tissue from women without breast cancer, no methylation was detected by any of the methods used (representative examples shown in Fig. 1, c and d). Whereas it was surprising that the frequency with which TRβ1 methylation occurred in established breast cancer cell lines was remarkably lower than tumor tissue, this finding may be related to years of *in vitro* selection. Methylation was not restricted only to the tumor cells but also occurred in nonmalignant tissue peripheral to carcinoma. In 4 of 11 cases, both CpG test sites in the promoter DNA were methylated. In the remaining 7 cases, no methylation was observed in the nonmalignant tissue or it was detected only within one of the two PCR-amplified regions of the CpG island. In one case (394T), in the quantitative COBRA assay, tumor DNA displayed less methylation than the peripheral tissue. Although the clonal relationship between the tumor and peripheral epithelium is not known in these samples, the TRβ1 methylation data suggest the presence of heterogeneity in the nonmalignant component of the afflicted breast in this regard.

To evaluate the concordance between TRβ1 promoter methylation, LOH, and expression of nuclear protein, we immunostained tumor sections of cases where TRβ1 methylation and allelic loss was confirmed previously (n = 7). These cases exhibited complete loss of expression (4 cases) or a mixture of nuclear positive and negative tumor cells present within a single microscopic field (Fig. 2, c, left panel) indicating biallelic TRβ1 silencing to be an emerging aberration in an evolving population of tumor cells.

To extend the analysis of aberrations in TRβ1 gene expression to a

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**Table 1** Status of TRβ1 copy number and relative gene expression in breast epithelial cultures

<table>
<thead>
<tr>
<th>Cell sample</th>
<th>FISH % nuclei with reduction in TRβ1 signals in TRβ1 relative TRβ1 intensity</th>
<th>RT-PCR Relative TRβ1 intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonmalignant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast epithelium</td>
<td>ND</td>
<td>0.6</td>
</tr>
<tr>
<td>(passage 2)</td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>Breast cancer cell lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DU 4475</td>
<td>ND</td>
<td>0.0</td>
</tr>
<tr>
<td>MDA435</td>
<td>43.0</td>
<td>0.0</td>
</tr>
<tr>
<td>SKBR-3</td>
<td>37.0</td>
<td>0.0</td>
</tr>
<tr>
<td>CAMA 1</td>
<td>3.0</td>
<td>0.2</td>
</tr>
<tr>
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<td>0.4</td>
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<td>BT 474</td>
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</tr>
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<td>MCF-7</td>
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</tr>
<tr>
<td>T47D</td>
<td>6.0</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* Percent nuclei = ([Nuclei where no. of signals of 3c > TRβ1]/(All nuclei evaluated) × 100.
* Relative TRβ1 intensity = (Density of 500-bp TRβ1 RT-PCR product)/(Density of 250 bp GAPDH RT-PCR product).
* ND, not done.
larger group of tumor samples, we evaluated tumor tissue arrays comprised of >100 cases of stage I primary breast carcinoma by immunoperoxidase localization of TRβ1 gene product. Samples, which were uniformly negative for all of the cell types, were excluded based on the assumption of protein degradation. A lack of characteristic TRβ1 nuclear immunostaining was observed in 18 of 78 cases (Fig. 2, c, middle panel). In these cases, nonmalignant constituents of the tumor sample, such as TDLU, lymphocytes, fibroblasts, and blood vessels continued to display TRβ1-positive nuclei (Fig. 2, c, right panel). The overall incidence of tumors lacking TRβ1 nuclear expression in the patient subset studied here was 25% (22 of 85). At this time it remains unknown whether hypermethylation is involved in biallelic inactivation and gene silencing of all or a proportion of these cases.

Discussion

TRβ1 regulates gene expression when bound to thyroid response elements in the proximity of target genes (14). On the basis of the presence or absence of the ligand, thyroid hormone (triiodothyronine, T3), TRβ1 can act as a transcriptional activator or silencer (15). In vitro studies have shown that T3 treatment of TRβ1-overexpressing cultures arrests proliferation in the G1/S phase of the cell cycle, and induces morphological and functional differentiation (16). Interestingly, T3-induced differentiation is preceded by a rapid decrease in the expression of the oncogene c-myc (17). Mechanisms, which inactivate TRβ1, may thus play an important role in the up-regulation of thyroid response elements harboring oncogenes during malignant progression. To our knowledge, this is the first direct demonstration of an epigenetic mechanism associated with TRβ1 gene silencing in cancer.

Our analysis of the TRβ1 gene in breast cancer cell lines provides evidence of promoter hypermethylation, decreased DNA copy number, and reversible reduction in transcript expression. TRβ1 status of clinical tumor samples demonstrates that hypermethylation and LOH occur more frequently than the complete absence of nuclear protein. This suggests the possibility that the full extent of methylation required for gene inactivation was most likely under-represented because of the limited MSP and COBRA sites examined here. However, the detection of any abnormally methylated site is a strong indication that this mechanism could alter expression levels of the target gene. Nonexpressing tumors could serve as an important tool in mapping target CpG sites in the TRβ1 promoter. A partially inactivating event, LOH in the region of the TRβ1 gene, occurs before detectable morphological changes in normal TDLU adjacent to carcinoma (7, 8).

Here, we have shown that the epigenetic alteration of TRβ1 is also an early event, which occurs in nonmalignant tissue peripheral to carcinoma. Similar findings of estrogen receptor gene hypermethylation in normal colon tissue of cancer patients are postulated to be “field defects” (18).

Ligand-mediated prevention strategies for breast cancer have targeted RARβ (19), another epigenetically inactivated nuclear receptor superfamily member (12, 13). Our findings regarding multiple mechanisms of TRβ1 inactivation support the importance of exploring this target as an additional approach for breast cancer control. However, a thorough understanding of the cellular consequences of modulating a ligand-induced master switch, such as TRβ1, is an essential prerequisite. Notably, the incidence of thyroid diseases, although controversial, is reportedly higher in breast cancer patients (20). An evaluation of thyroid function in the context of underlying breast biology in such cases could provide important clues regarding TRβ1 as a potential factor that links disease manifestation in the two organ systems.

Acknowledgments

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References


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