Methylation of estrogen receptor β promoter correlates with loss of ER-β expression in mammary carcinoma and is an early indication marker in premalignant lesions

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Abstract

The function of estrogen receptor beta (ER-β) in mammary tissue is not completely understood. While early observations were often conflicting, more recent data suggest an important role as a tumor-suppressor gene. A decrease of ER-β expression has been observed in ductal carcinoma *in situ* and invasive carcinoma as compared with benign mammary epithelial cells. The loss of ER-β resulted in abnormal growth of mammary epithelial cells. We have previously shown that the mRNA expression of the ER-β gene is almost totally suppressed in breast carcinomas from patients with a poor prognosis. Here we analyzed whether methylation changes in the different promoters of ER-β are responsible for the loss of expression of the gene. A methylation assay with high specificity and sensitivity was developed, and a panel of breast tissue samples ($n = 175$) was characterized for methylation status. In contrast to benign breast, more than two-thirds of invasive breast cancers showed a high degree of methylation. Importantly, increased methylation was also detectable in numerous premalignant lesions. By analysis of breast tumors, previously characterized by gene-expression profiling, methylation was predominantly detected in a subgroup of patients with an unfavorable prognosis, suggesting a possible prognostic value of the ER-β methylation status. We also investigated the structural characteristics of the two ER-β promoters, which were both found to be closely associated with a second, downstream, localized and opposite-oriented promoter. However, we could not detect endogenous antisense RNA transcribed from these promoters, which may be involved in epigenetic gene silencing. We also failed to induce ER-β promoter methylation by expressing siRNAs in cell lines. Interestingly, by comparing the promoter sequences of ER-β with other genes known to be epigenetically inactivated in breast cancers, we identified a sequence motif possibly involved in promoter methylation.

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Introduction

DNA methylation is a ubiquitous process of gene inactivation in nature (reviewed in Bird 2002). It occurs preferentially at CpG dinucleotides. In normal cells, most of the 5-methyl-cytosine is found to be associated with repetitive elements such as Alus or LINEs, whereas CpG islands (CGI), GC-rich stretches, hundreds of base pairs long, remain hypomethylated. These CGI often become densely methylated as cells undergo malignant transformation. It has been shown that methylation of CpGs can interfere with gene transcription and induce genomic imprinting, such as X chromosome inactivation. Although it is clear that methylation of DNA is driven by at least three functionally and structurally related methyltransferases, little information is currently available on which proteins participate in the guiding of methyltransferases to target sequences. One model for regulation of epigenetic gene silencing is that of elements

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encoding antisense RNA, a mechanism involved in X chromosome inactivation and autosomal imprinting (reviewed in Ogawa & Lee 2002). In X chromosome inactivation, TSIX transcripts serve as an antisense regulator of the silencer element XIST, which itself makes a noncoding transcript. Imprinting by naturally occurring antisense transcripts (NATs) has also been detected in various autosomally loci (O'Neill 2005), as in the Beckwith–Wiedemann syndrome locus (Fitzpatrick *et al*. 2002), the Prader–Willi/Angelman syndrome locus (Mann & Bartolomei 1999) and the IGF2R/AIR locus (Zwart *et al*. 2001). However, exactly how these transcripts contribute to epigenetic silencing remains to be elucidated. Recently, Brenner *et al*. (2005) have shown that Myc binds the corepressor Dnmt3a and associates with DNA methyltransferase activity *in vivo*, suggesting a new mechanism in which targeting of DNA methyltransferases via transcription factors may establish specific cellular CpG methylation patterns.

It is commonly accepted that estrogens and their receptors play a pivotal role in development and growth of invasive breast cancer. The role of estrogen receptor beta (ER-β) in breast cancer is not completely understood, but there is strong evidence that ER-β may act as a tumor-suppressor gene (reviewed in Matthews & Gustafsson 2003, Bardin *et al*. 2004). By gene-expression profiling, we have previously demonstrated that in breast cancer with an unfavorable prognosis (Ahr *et al*. 2002) the expression of the ER-β gene is almost completely suppressed (Ahr *et al*. 2001). Skliris *et al*. (2003) found a gradual decrease of ER-β protein in normal mammary epithelial cells, ductal carcinoma *in situ* and invasive breast cancer. A complete loss of ER-β expression was seen in 21% of invasive carcinomas, but more often in invasive-ductal than in invasive-lobular cancers. Furthermore, by introducing the ER-β gene coding sequence, Paruthiyil *et al*. (2004) demonstrated an inhibition of proliferation of MCF-7 cells through repression of c-myc, cyclin D1 and cyclin A gene expression and an increased expression of $p21^{cip1}$ and $p27^{kip1}$, resulting in G2 cell-cycle arrest. Roger *et al*. (2001) found a decreased expression of ER-β protein by immunohistochemistry in premalignant breast lesions. All these data underline the function of ER-β as a tumor-suppressor gene and its crucial role in breast cancer development. Regarding possible mechanisms for regulation of ER-β mRNA expression, two different promoters, 0K and 0N, have been detected for the human ER-β gene (Zhao *et al*. 2003, Zhu *et al*. 2004). Zhao *et al*. reported that while promoter 0N of the ER-β gene is not methylated in normal mammary epithelial cells, it is highly methylated in breast cancer cell lines. Promoter 0K, in contrast, seems to be methylated neither in normal nor in cancer cells. Moreover, re-expression of the ER-β mRNA in cell lines was obtained by experimental demethylation. These results are also in good agreement with data of Zhu *et al*. (2004) concerning methylation of promoter 0N in prostate carcinoma cells. In the analyses presented here, we examined the methylation status of the ER-β promoters in benign human breast tissue, corresponding premalignant epithelial lesions and ductal carcinoma *in situ*, as well as in invasive breast cancer. Our results confirm that methylation is confined to promoter 0N. A methylation assay with high specificity and sensitivity for the CGI associated with promoter 0N was developed, and it revealed a close correlation between ER-β mRNA expression and methylation. In contrast to benign breast, more than two-thirds of invasive breast cancers show a high degree of methylation. Importantly, methylation changes are already detectable in premalignant lesions. By analysis of a panel of breast tumors, previously characterized by gene-expression profiling, methylation was predominantly detected in a subgroup of patients with an unfavorable prognosis, suggesting a possible prognostic value of the ER-β methylation status. Furthermore, we investigated the structural characteristics of the two ER-β promoters, which were both found to be closely associated with a second, downstream localized and opposite-oriented promoter. However, we could not detect endogenous antisense RNA transcribed from these promoters, which may be involved in epigenetic gene silencing. We also failed to induce ER-β promoter methylation by expressing siRNA in cell lines. However, by comparing the promoter sequences of ER-β with other genes known to be epigenetically inactivated in breast cancers, we identified a sequence motif possibly involved in promoter methylation.

Materials and methods

Tissue samples

Tissue samples were obtained from patients undergoing surgical resection between June 1997 and June 2004 at the Department of Obstetrics and Gynecology of the J W Goethe University (Frankfurt). The samples were fresh frozen in liquid nitrogen and stored at −196 °C. Specimens included ductal and lobular carcinomas of different tumor size, lymph-node status, grade, ER-α status and distant metastasis. In addition, several types of premalignant lesions of the breast were analyzed, including papilloma, fibroadenoma,

mastopathy, ductal hyperplasia, and ductal carcinoma *in situ* (DCIS). Normal tissue samples were obtained from patients undergoing surgical breast reduction. Cell culture of cell lines was performed as described (Hock *et al*. 1998).

Analysis of mRNA expression by real-time PCR

Total RNA from human primary mammary carcinomas was isolated by the guanidinium isothiocyanate method, as previously described (Holtrich *et al*. 1994), in combination with affinity purification (Rneasy; Qiagen). Real-time PCR analyses were performed with the ABI 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA, USA). cDNAs were generated by random-primed reverse transcription (RT) (ProSTAR cDNA synthesis kit; Stratagene, La Jolla, CA, USA). PCRs were performed according to the manufacturer's protocols (PE-Applied Biosystems). VIC-fluorophore-labeled GAPDH TaqMan probes served as internal quantification markers in the multiplex PCRs. Each quantitation was reproduced three times and normalized by GAPDH.

DNA isolation and bisulfite conversion

DNA isolation from tissue samples and cultured cells was performed according to standard protocols after digestion with proteinase K, as described previously (Holtrich *et al*. 1991).

Prior to bisulfite modification, 500 ng DNA were boiled for 1 min and then treated with 0.3 M NaOH for 15 min at 37 °C. It is critical that the bisulfite modification of unmethylated cytosine is complete; otherwise, the unconverted cytosine residues will appear as pseudomethylated sites. Therefore, this step was optimized with control DNA templates. In the optimized protocol, freshly denatured DNA was embedded in 10 µl 2% agarose beads to inhibit reassociation of the DNA strands during the conversion reaction. The DNA was then reacted with 3 M bisulfite and 10 mM hydroquinone, pH 5.0. The conversion reaction was performed under oil in a thermocycler at 50 °C with repeated heating steps to 80 °C for 3 min each for 180 min (total of five cycles). The beads were then washed six times with 0.9 ml H_2O, incubated two times in 1 M NaOH for 15 min for alkaline desulfonation, and finally washed in Tris–HCl (pH 7.5) for 15 min.

Methylation-specific PCR (MSP) assay

After bisulfite treatment, 10 µl distilled water were added to the agarose beads containing the converted DNA. Beads were melted and 7 µl added to first-round PCR using primers U1 and L1 for 45 cycles: 1 min at 95 °C, 2 min at 55 °C and 2 min at 72 °C. The U1 and L1 primers are located at sites devoid of CpG dinucleotides to allow an unbiased amplification of both methylated and unmethylated DNA. PCR was allowed to reach a plateau, and then the amplicons were diluted 10^{-2} to give a normalized input amount for a second nested PCR. Second-round PCR was performed with SYBR green for real-time detection. The upper primer U2 was used in combination with lower primer L2 for detection of methylation. To assess the extent of conversion, the primer U3 was used with the L2 primer. Second-round PCR cycle conditions were 1 min at 95 °C, 1 min at 60 °C and 2 min at 72 °C for 40 cycles. Primer positions according to GenBank accession no. AL161756 were as follows: 82,194–82,215 (U1), 82,232–82,254 (U2), 82,285–82,306 (U3), 82,459–82,480 (L1) and 82,403–82,426 (L2).

Detection of antisense transcripts

An RT-based real-time PCR strategy was used to detect endogenous antisense transcripts originating from the reverse-oriented promoter. RNA was isolated from methylated (MDA-MB-435) and unmethylated (MCF-7) cell lines and from mammary carcinoma tissues positive and negative for methylation. Genomic DNA was removed from the RNA with two rounds of DNase digestion on Qiagen columns. The extent of DNA depletion was assessed by real-time PCR. Next, cDNA synthesis was specifically primed with several sense primers located 5′ from the reverse-oriented promoter in the region encompassing promoter 0N (nt positions 81,283, 81,822, 82,305, 82,797, 83,208 and 83,655 according to accession no. AL161756). Since RNA tends to self-prime during cDNA synthesis, controls without the addition of primer were included. Specificity of the priming was checked by real-time PCR of sense transcripts, using GPDH and ESR2 as negative controls.

shRNA transfection for induction of RNAi-mediated promoter methylation

Induction of promoter methylation through RNAi processes, as described by Kawasaki and Taira (2004) and Morris *et al*. (2004), was examined. shRNAs were expressed by the human U6 promoter in cell lines to generate type Ia siRNA (Schwarz *et al*. 2003, Ui-Tei *et al*. 2004) targeted on different CpG sites in the promoter 0N of ER-β. U6-shRNAs were generated by PCR according to Castanotto *et al*. (2002). Synthesis

of the U6 containing hairpin constructs was monitored by SYBR green fluorescence and stopped before the reaction reached saturation. After an initial incubation at 95 °C for 9 min, cycles consisted of 1 min at 95 °C, 1 min at 60 °C and 3 min at 79 °C. An elongation temperature of 79 °C was necessary to disrupt the hairpin structure in order to allow efficient amplification and monitoring of SYBR fluorescence during cycling. PCR products were purified on QiaQuick columns (Qiagen) and used for transfection of MCF-7, T47D, MDA-MB-468 and HEK293 cells with lipofectamine (Gibco). Efficiencies were monitored by cotransfecting pEGFP-C1 (Clontech) and varied from 20 to 100%. After harvest of the cells on days 2, 5 and 10, the methylation status of ER-β was determined by MSP.

Bioinformatic analysis and database searches

Online resources, including FASTA and BLAST services from NCBI (www.ncbi.nlm.nih.gov/BLAST/) and EBI (www.ebi.ac.uk/services/), were used for sequence analysis. CGI were identified by the method of Takai and Jones (2002) with the CGI finder program, version 10/29/04, available at http://cpgislands.usc.edu/. Promoter predictions were performed with PROSCAN (Prestridge 1995), Version 1.7 (http://thr.cit.nih.gov/molbio/proscan/). For further sequence analysis, the HUSAR sequence analysis package (http://genius.embnet.dkfz-heidelberg.de/menu/biounit/) was applied, including the FINDPATTERN program. The ONCOMINE 2.0 database (Rhodes *et al.* 2004, www.oncomine.org) was used as an interface to access published microarray data sets. To analyze downregulation of genes, data sets of Sorlie *et al.* (2001), comparing seven benign breast tissues (four normal breast samples and three fibroadenomas) with 78 breast cancer samples, and Perou *et al.* (2000), comparing three normal breast samples and one fibroadenoma with 55 ductal carcinomas, were used. To check for confounding effects of lymphocytes, a data set of van't Veer *et al.* (2002) from breast cancers either positive ($n = 28$) or negative ($n = 89$) for lymphocytic infiltration was used. Statistical analyses (Fisher's exact test and Student's *t*-test) were done with SPSS 11.5 (SPSS Inc., Chicago, IL, USA) and R 1.7 (www.R-project.org).

Results

5′-Structure of the human ER-β gene and development of a methylation assay

The sequence of the genomic context of the human ER-β gene (ESR2) located on chromosome 14 was obtained from BAC clone R-712C19 (accession no. AL161756; Heilig *et al.* 2001). A thorough analysis of the 90 kb DNA sequence upstream of the first exon of ESR2 revealed a 5′-structure of the gene as represented in Fig. 1. Two CGI-associated promoters, separated by approximately 40 kb (referred to as promoter 0K and 0N according to Li *et al.* 2000 and Zhao *et al.* 2003), were predicted in sense orientation by the PROSCAN program (Prestridge 1995). Interestingly, both promoters were associated with a 5′-Alu repeat and immediately followed 3′ by reverse promoters with opposite orientation. The CGI associated with promoter 0K contained 86 CpG sites; the one associated with promoter 0N contained 55 sites. We used MSP in a screening approach to compare the methylation status of both promoters in benign breast samples with those in breast cancer specimens with known loss of ER-β mRNA expression. Although we were not able to detect differences in the methylation status of promoter 0K, the CGI of promoter 0N seemed to be heavily methylated in tumor samples (data not shown). Since MSP analysis as typically performed is often imperfect in both specificity and sensitivity, we next optimized the methylation assay by focusing on a CpG site located in promoter 0N (nt position 82,254 of accession no. AL161756). This site displayed a consistently high methylation content among all analyzed breast cancer samples with lost ER-β expression in the first scan. As depicted in Fig. 1, this assay employs a two-step PCR strategy. One major concern of methylation analyses is the occurrence of pseudomethylated sites caused by incomplete C–U transition during bisulfite treatment (reviewed in Rein *et al.* 1998). Therefore, control amplifications were included in the second step, which tests for completeness of conversion of a non-CpG cytosine nucleotide (Fig. 1) (nt position 82,306 of accession no. AL161756), allowing for optimization of the bisulfite reaction conditions (see Methods). Under standard bisulfite reaction conditions (50 °C overnight incubation) (Frommer *et al.* 1992, Herman *et al.* 1996), we observed conversion efficiencies far below the optimum, making it difficult reliably to detect actually methylated CpG sites. Thus, we employed the modified method described by Olek *et al.* (1996), using agarose bead-embedded DNA, which was then further optimized by repeated heating steps during incubation, as proposed by Rein *et al.* (1997). Five intervening treatments at 80 °C for 3 min during the 50 °C incubation yielded high C–U conversion, moderate DNA degradation (<80%) and stability of ^{5m}cytosine. Conversion efficiencies were determined to be more than $1:10^4$, as judged by PCR.

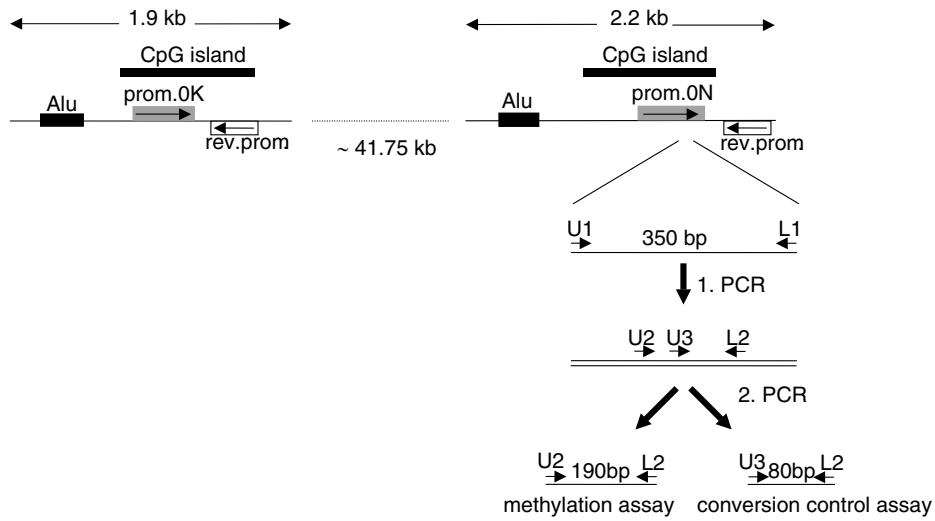

Figure 1 Structure of the 5′-region of the ER-β gene and location of the methylation assay. The 5′ region of the human ER-β gene (ESR2) contained in BAC clone R-712C19 (accession no. AL161756) is represented by a horizontal line. The relative positions of promoter 0K and 0N, separated by 41.75 kb intervening sequence, are depicted as gray boxes, and the associated Alu repeats and reverse promoters as black and white boxes respectively. Two CpG islands associated with the promoters are represented as filled black boxes above. The location of the MSP assay for promoter 0N involving two rounds of PCR is schematically shown on the right. Nucleotide positions of the primer are given in the Methods section. For detection of methylation, primer U2 is used in combination with primer L2. Primers U3 and L2 are used to monitor the bisulfite conversion reaction.

Characteristics of the methylation assay

Next to completeness of the bisulfite conversion, a second critical aspect of methylation analyses is the compromise between sensitivity and specificity. A high specificity can easily be obtained when several methylated sites are incorporated in the assay, a strategy mostly using upstream and downstream primers encompassing multiple methylated cytosine residues. However, since differences often exist between cells and tumors in methylation patterns, and as not all possible methylated sites are actually methylated in an individual cell, this strategy results in a loss of sensitivity. Therefore, to reach maximal sensitivity, we incorporated only one methylated CpG site in the assay and exhaustively optimized reaction conditions, using model templates to reach a Δct of more than 17 cycles between methylated and non-methylated templates (Fig. 2). This specificity is more than sufficient for detection of one methylated template among 10^5 unmethylated template molecules. To determine the sensitivity of the assay in the genomic context, decreasing amounts of methylated cells (MDA-MB-435) were diluted in a background of 4×10^4 unmethylated cells (MCF-7). The number of tests positive for methylation of total tests performed is reported in Table 1.

Approximately 100 methylated tumor cells can be reliably detected by the assay. To correlate the mRNA expression of ER-β and the methylation status as determined by our assay, real-time PCR data from cDNA were compared with the results of the methylation assay on DNA from the same tumor samples (Table 2). In comparing ER-β and GPDH signals, a Δct of 5.42–7.25 was observed for three of ten tumor samples. This range was also observed for benign breast tissue (data not shown), and it corresponds to an amount of ER-β mRNA about 30–150-fold lower than those of the higher expressed housekeeping gene GPDH. All these three tumors with persisting ER-β expression displayed a negative result of the methylation assay. In contrast, for six tumor samples, no amplification of ER-β was achievable. The resulting Δct of over 15 corresponds to an amount of the ER-β mRNA at least 3×10^4-fold lower than those of GPDH mRNA. All those tumors showed a positive result for methylation. In one sample, a residual expression of ER-β was detectable with Δct of 14.15 (more than 1.8×10^4-fold lower ER-β than GPDH) despite a positive result of the methylation assay. Presumably, this low ER-β expression resulted from residual benign epithelial cells in this tissue sample. Thus, seven of the ten breast tumors displayed a total or nearly total loss of ER-β mRNA expression, correlating with a positive

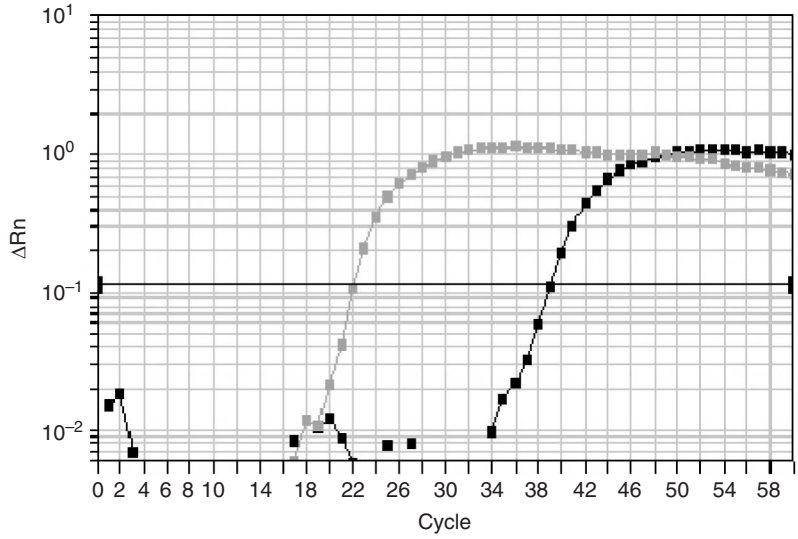

Figure 2 Specificity of the methylation assay using optimized reaction conditions on model templates. Model templates were generated by PCR incorporating the nucleotide changes resulting from either a methylated (C) or nonmethylated (T) cytosine at nucleotide position 82,306 according to BAC clone R-712C19 (accession no. AL161756). Subsequently, in order to determine cross-reactivity, equal amounts of both model templates were tested in PCR, using primer U2 and L2 for the detection of the 'methylated' model template (gray curve) in comparison with the 'nonmethylated' model template (black curve). As shown, optimization of PCR conditions resulted in a Δct of more than 17 cycles. The same results were obtained for reverse experiments using an upper primer specific for 'nonmethylated' molecules.

Table 1 Sensitivity of the ER-β methylation assay. Decreasing amounts of methylated cells (MDA-MB-435) were diluted in a background of 4×10^4 unmethylated cells (MCF-7), and five independent MSP assays were performed. The number and percentage of tests positive for methylation are given

No. of methylated cells (among 4×10^4 unmethylated cells)	no. positive tests/ no. of total tests
200–400	5/5 (100%)
100–200	5/5 (100%)
50–100	4/5 (80%)
20–50	2/5 (40%)

Table 2 Comparison of ER-β mRNA expression and promoter methylation status. Ten breast tumors were analyzed for ER-β mRNA expression by real-time PCR and the relative expression values (Δct using GPDH as endogenous control), and S.D. of three measurements are given. When no amplification of ER-β was achievable, a threshold value (>15) was used. The results of the methylation analysis obtained by MSP assay are given as '−' and '+' respectively

Tumor	ER-β mRNA expression $\Delta Ct_{ER\text{-}\beta}$-$Ct_{GPDH}$ (S.D.)	ER-β promoter methylation assay
1	5.42 (0.73)	−
2	7.25 (0.47)	−
3	6.38 (0.54)	−
4	14.15 (0.69)	+
5	>15	+
6	>15	+
7	>15	+
8	>15	+
9	>15	+
10	>15	+

result of the methylation assay ($P = 0.008$). These results are in good agreement with several reports showing loss of ER-β expression in the majority of breast cancers (Speirs *et al*. 1999, Iwao *et al*. 2000, Shaw *et al*. 2002, Park *et al*. 2003, Skliris *et al*. 2003). For example, only 7 out of 33 breast cancer samples analyzed by Zhao *et al*. (2003) by real-time PCR displayed levels of ER-β mRNA comparable to benign breast tissue. In summary, these results demonstrate that this methylation-specific PCR is a valid assay for evaluation of the epigenetic regulation of ER-β and can be used as a surrogate marker for ER-β expression status.

Analysis of ER-β methylation in breast cancer and premalignant lesions

The methylation status of ER-β from breast tissue samples ($n = 175$) was evaluated by the assay described above. Samples were classified by histology as 25 benign breast tissues, 21 benign breast tissues from patients with a mammary carcinoma, three papillomas, 28 fibroadenomas, 17 ductal hyperplasias, seven DCIS and 74 breast cancers. As shown in Table 3, an absence of methylation was seen in normal breast tissue, regardless of the existence of breast cancer in the environment. Furthermore, all papillomas showed no methylation. In contrast, in fibroadenoma and ductal hyperplasia, weak methylation could be detected ($P < 0.001$). In addition, 6/7 DCIS showed

Table 3 Frequency of ER-β methylation. Methylation status of 175 breast tissue samples was analyzed by MSP assay. For invasive carcinomas positive for methylation, 'strong' signals were detected by real-time PCRs with fluctuations in the Δct of <2 among samples. Real-time PCR signals with Δct values of 6–8, as compared with invasive carcinomas, were characterized as 'weak'. 'None' indicates signals below primer cross-reactivity (Δct >17)

		Signal strength of MSP assay		
Tissue	***n***	**Strong**	**Weak**	**None**
Normal breast	25			25
Normal breast from tumor patients	21			21
Papilloma	3			3
Fibroadenoma	28		26	2
Ductal hyperplasia	17		16	1
DCIS	7		6	1
Invasive carcinoma	74	52		22

weak methylation ($P<0.001$). Strong methylation was seen in 70.3% (52/74) of the invasive carcinomas. The remaining 29.7% showed no methylation. The observed differences in the signal strength of the MSP assay depend on the number of methylated cells in the sample. In contrast to mRNA and protein expression, which are metric values, the methylation state of a defined position on the DNA, as analyzed in the assay used here, is *a priori* a nominal value, which is either present or absent. However, if only a very small portion of the cells in the sample show methylation, a weak signal results, since no saturation of the primary amplification product from these cells will occur in the first-round PCR. It can be broadly estimated that in the benign/premalignant conditions exhibiting a weak methylation signal, about 0.1–1% of the total cells are methylated, in contrast to a range of 20–100% in the case of a strong signal in the methylation assay, as observed for invasive cancer cases.

ER-β methylation in breast cancer patients with unfavorable prognosis

To evaluate a potential prognostic value of ER-β promoter methylation, we tested a panel of breast tumors which had been previously characterized by gene-expression profiling (Ahr *et al*. 2001), and for which follow-up data were available (Ahr *et al*. 2002). As shown in Fig. 3, methylation was observed predominantly in the tumors which grouped in the branch marked by a gray bar ($P<0.001$). This subgroup contains most (9/12) of the patients with relapse during follow-up ($P=0.016$). In using ER-β promoter methylation alone as a prognostic factor, there is an inverse trend of the methylation status and prognosis, which is, however, not yet significant ($P=0.26$). While only 10.5% (2/19) of the tumors negative for methylation showed a relapse, this portion increased to 27.8% (10/36) among the tumors positive for methylation. These results agree with data from several recent studies showing a positive correlation of ER-β expression and prognosis (Omoto *et al*. 2001, Esslimani-Sahla *et al*. 2004, Myers *et al*. 2004, Palmieri *et al*. 2004).

Possible mechanisms for ER-β promoter methylation

Several mechanisms are conceivable that could result in promoter methylation of ER-β in the cell. The observation of associated promoters just 1 kb downstream of both promoter 0N and 0K, but with a

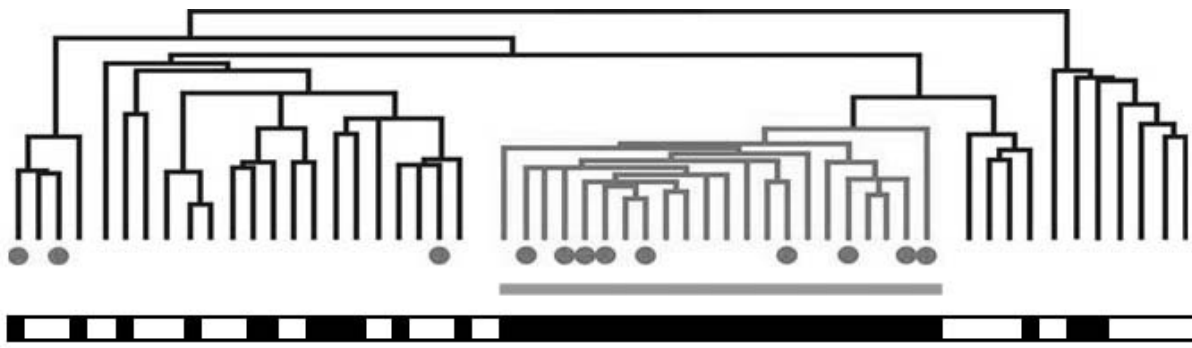

Figure 3 Correlation of gene-expression profiling and ER-β methylation. Fifty-five tumors from patients with known follow-up were analyzed by gene-expression profiling and grouped by unsupervised clustering, as described previously (Ahr *et al*. 2002). Patients with relapse are marked by dots, and the high-risk group by a gray bar. The ER-β methylation status of the tumor is indicated below. Black boxes mark samples positive for methylation.

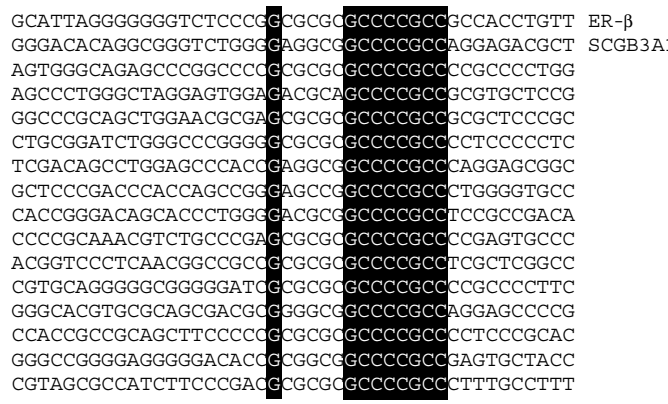

Figure 4 Sequence comparison of BLAST results from the homologous regions in the promoters of ER-β and SCGB3A1. Sequences from the promoter of ER-β and SCGB3A1 surrounding their region of homology were used as a query in a BLAST search of the human genome. Fourteen database hits from the output of the search are shown together with ER-β and SCGB3A1 in a multiple alignment. Nucleotide residues that are perfectly conserved are shown inverted.

reverse orientation (Fig. 1), might lead to an attractive model, where antisense transcripts are transcribed from these promoters. The generation of the antisense transcript could result in double-stranded RNA, leading to methylation of the upper-strand promoter, a mechanism which has been described for epigenetic silencing and imprinting (Ogawa & Lee 2002, Tufarelli *et al*. 2003, Tagoh *et al*. 2004). To detect such possible antisense transcripts originating from the reverse promoter downstream of promoter 0 N, we performed RT–PCR analyses, using cDNA specifically primed with different sense primers in the region encompassing and surrounding promoter 0N. Total RNA from several tumors, as well as cell lines with either methylated (MDA-MB-435) or unmethylated (MCF7) status of promoter 0N, was used for cDNA generation. RNA was depleted of residual genomic DNA by two rounds of DNase treatment, and the extent of DNA digestion was subsequently assessed by genomic PCR (see Methods). However, despite numerous efforts, we were unable to detect any antisense transcripts in this region. This was in line with BLAST and FASTA searches of EST databases that resulted in no hits. We next tested whether induction of *de novo* methylation in cell lines could be achieved by transfection with shRNA. Transcriptional silencing by targeting siRNAs against CpG sites was recently described by Kawasaki and Taira (2004) and Morris *et al*. (2004). We introduced PCR products containing shRNA targeted against various CpG sites in promoter 0N under the control of the human U6 promoter in various cell lines, and analyzed the DNA at days 2, 5 and 10 after transfection for methylation of the ER-β promoter. Neither a change in expression of the ER-β gene nor methylation of promoter 0N was detectable (data not shown).

Identification of a sequence motif putatively associated with promoter methylation

Although numerous genes have been shown to be methylated in breast cancer, only a limited number were reported to be epigenetically modified in premalignant lesions. Since SCGB3A1, also termed 'HIN-1' (Krop *et al*. 2001), has been shown to be methylated in a manner resembling ER-β, we compared the promoters of both genes for regions of homology. No extended similarities were identified except a short motif encompassing the 'APRT-mouse_US'-site (TFD# S00216). The 'APRT-mouse_US'-site, which contains an SP1 consensus sequence, was originally identified in the promoter of the APRT gene (Park & Taylor 1988 and has already been reported to be involved in methylation processes (Siegfried *et al*. 1999). BLAST searches with both promoter sequences of ER-β and SCGB3A1 respectively identified a number of other human genes containing similar sequences. In a multiple sequence alignment of the BLAST output (Fig. 4), this motif could be extended to a possible consensus sequence G-(N)5-GCCCCGCC. Since the BLAST algorithm is not suitable for pattern searches with short sequence motifs, we then performed a global pattern search of the assembled human genome sequence (GenBank Release 35.1: 3,149,005,344 nucleotides), using the above consensus sequence with the FindPatterns program. The search returned 17 605 hits representing 977 different motifs, most of which were at frequencies

expected by chance (about 24 times). The exact sequence of the ER-β motif was detected 45 times. To determine whether this sequence motif might be of functional relevance, we analyzed the surrounding 2 kb sequence of all 45 loci of the exact ER-β motif in the human genome. The results of these analyses are compiled in Table 4. Interestingly, all sequences were predicted to have promoter activity; moreover, 44 of these 45 promoters were associated with a CGI. Furthermore, all sequences were located immediately upstream of the start of described mRNAs. No strand specificity of the motif relative to the associated gene was observed. For 42 of the 45 sequences, the corresponding genes were identified (Table 4). We analyzed the expression of the 42 genes in breast cancer samples, using publicly available datasets from microarray analyses. We chose studies by Sorlie *et al*. (2001) and Perou *et al*. (2000) that allow comparison of gene expression in breast cancer and benign breast tissue. Data for 18 genes were available from those studies, and most of them consistently showed decreased expression in breast cancer samples (Table 4). For four genes (E2F1, TOB, SOS1 and BST1) that did not show reduced expression in breast cancer, a possible confounding effect of tumor-infiltrating lymphocytes was analyzed by using microarray data from van't Veer *et al*. (2002). Significant values for three of the genes (E2F1, TOB and SOS1) were observed. This suggests that the possible loss of their expression in breast cancer compared with benign breast tissue is overwhelmed by the expression in tumor-associated lymphocytes. STK11, whose expression was not analyzed in the Sorlie *et al*. and Perou *et al*. data sets, is also known as LKB1 (Marignani 2005), a well-known tumor suppressor kinase that is lost in Peutz–Jeghers syndrome (PJS). Patients with PJS develop cancer of epithelial tissue origin. Methylation of STK11 has already been described for cases of papillary breast carcinomas (Esteller *et al*. 2000) and colorectal carcinomas (Trojan *et al*. 2000). Of the genes for which microarray data were available, the most significant difference in expression was found for NFIB ($P<0.0001$), a transcription factor critical for lung and brain development (Steele-Perkins *et al*. 2005). Krop *et al*. (2003) reported that SCGB3A1 was found to be methylated only in sporadic cases of breast cancer in contrast to the hereditary forms resulting from BRCA1 mutation. Interestingly, in comparing the expression of NFIB in BRCA1 mutated ($n=18$) and sporadic ($n=97$) cases with microarray data from van't Veer *et al*. (2002), a significant difference ($P=0.0007$) was observed with loss of NFIB expression in sporadic cases.

Discussion

The highly sensitive MSP assay presented here demonstrated a strong inverse correlation of ER-β mRNA expression with the methylation status of the promoter. The analysis of methylation, moreover, offers important advantages over assaying for ER-β expression itself. While the analysis of ER-β methylation is a 'positive assay', the determination of the loss of expression itself would be a 'negative assay'. Detecting the loss of expression in only a small number of cells by such a negative assay is highly problematic if not impossible, since a high background signal is generated by the large number of cells still expressing the gene. This loss in sensitivity can be circumvented if the highly sensitive methylation assay is used as a surrogate marker to monitor the loss of ER-β expression. Methylation of CpG sites in promoter 0N of the ER-β gene seems to be a common event in breast cancer. More than two-thirds of all carcinomas showed methylation and an associated decrease in the expression of ER-β mRNA. Furthermore, methylation was already detectable in ductal hyperplasia and premalignant lesions such as DCIS. This result suggests that ER-β and regulation of its expression might play a pivotal role in the development of malignant breast tumors, and underlines its putative function as a tumor-suppressor gene. We hypothesize that methylation of the ER-β promoter region and the resultant suppression of mRNA expression is an early event in the development of endocrine-dependent cancer. These results agree with Roger *et al*. (2001), who showed the early loss of expression of ER-β receptor protein in premalignant breast lesions. Similar data were also obtained for prostate tissue. Zhu *et al*. (2004) established that ER-β gene silencing by promoter methylation results in a decrease of expression, not only in cancer tissue but also in premalignant stages. Of special interest is that we detected no methylation in benign breast tissue from breast cancer patients, suggesting that methylation of the ER-β promoter is a focal event, and not a generally occurring phenomenon in the breast (as resulting from aging processes). The function of ER-β in mammary tissue is not completely understood. However, while early observations were often conflicting, more recent data suggest an important role as a tumor-suppressor gene. On the basis of these results, agonistic drugs which selectively bind ER-β could have potential as protective compounds. This view might be supported by data from hormone replacement therapy (HRT), since ER-β receptor is known to be constitutively expressed in colorectal tissue, and its stimulation could be

Table 4 Characteristics of all human genes with an exact match to the ER-β promoter motif. All perfect matches to the ER-β promoter motif in the human genome sequence are compiled. Contig accession number, relative position of the motif in the contig, and chromosomal location are given. To check for associated genes, a region of 2 kb sequence surrounding each motif was analyzed for the presence of a CpG island (CGI) and predicted promoters. Results are indicated by '+' or '−'. If associated genes are known, the gene name, its putative functional classification and mRNA accession number is given. Then, the observed repression in breast cancer according to two microarray data sets is given with the associated *P* value for positive results. Lastly, for selected genes, a possible confounding effect of tumor-infiltrating lymphocytes was analyzed with microarray data from appropriate breast cancer samples

								repressed in breast cancer vs benign				perturbation by lymphocytes	
contig accession no.	nt-pos.	chromosome	CGI	promoter	gene name	functional classification	mRNA accession no.	Sorlie *et al.* (78 vs 7)	*P*-value	Perou *et al.* (55 vs 4)	*P*-value	van t'Veer (28 vs 89)	*P*-value
NT_026437	45760581	14p	+	+	ESR2	estrogen receptor beta	AY785359	n.d.		n.d.			
NT_011255	1146098	19p	+	+	STK11	tumour suppressor protein kinase	NM_000455	n.d.		n.d.			
NT_008413	14304149	9p	+	+	NFIB	transcription factor	BX648845	yes	3.9E-07	yes	1.5E-04		
NT_011520	18658816	22q	+	+	CBX6	chromatin modification	NM_014292	yes	1.6E-04	yes	0.007		
NT_011786	19440894	Xq	+	+	FHL1	transcription factor	NM_001449	yes	0.001	yes	0.032		
NT_010783	6786688	17q	+	+	ITGA3	integrin alpha 3,	AB209658	yes	0.008	yes	0.055		
NT_009714	19926237	12p	+	+	TM7SF3	membrane protein	AK023085	n.d.		yes	0.027		
NT_077531	3065125	8p	+	+	SOX7	transcription factor	NM_031439	yes	0.029	yes	0.094		
NT_007819	26466222	7p	+	+	HOXA4	transcription factor	U41755	yes	0.04	yes	0.09		
NT_077812	572988	19p	+	+	MAP2K7	signal transduction	AF013588	yes	0.051	yes	0.012		
NT_005403	67707756	2q	+	+	IGFBP2	cell growth regulation	AY398667	yes	0.313	yes	0.447		
NT_011651	32857714	Xq	+	+	AMMECR1	not known	NM_015365	yes	0.344	yes	0.178		
NT_008470	38636050	9q	+	+	SPTAN1	integrin pathway	AB191262	yes	0.645	yes	0.89		
NT_025741	47486341	6q	+	+	AIG1	androgen induced	NM_016108	yes	0.768	yes	0.679		
NT_033927	6716979	11q	+	+	E2IG4	estrogen induced	NM_015516	yes	0.8	yes	0.864		
NT_028392	2470468	20q	+	+	E2F1	transcription factor	AF516106	no		yes	0.114	yes	5.90E-07
NT_010783	7597000	17q	+	+	TOB1	antiproliferative erbB2 signal.	BC070493	no		yes	0.592	yes	0.012
NT_033903	10963881	11q	+	+	DIPA	not known	NM_006848	no		yes	0.651	n.d.	
NT_022184	18163326	2p	+	+	SOS1	signal transduction	AF106953	no		no		yes	0.039

Table 4 *continued*

contig accession no.	nt-pos.	chromosome	CGI	promoter	gene name	functional classification	mRNA accession no.	repressed in breast cancer vs benign: Sorlie *et al.* (78 vs 7)	*P*-value	Perou *et al.* (55 vs 4)	*P*-value	perturbation by lymphocytes: van t'Veer (28 vs 89)	*P*-value
NT_006316	6380207	4p	+	+	BST1	signal transduction	NM_004334	no		no		yes	0.14
NT_004836	9756862	1q	+	+	FAM36A	not known	NM_198076	n.d.		n.d.			
NT_007592	25075306	6p	+	+	MGC57858	not known	NM_178508	n.d.		n.d.			
NT_007914	9704869	7q	+	+	KIAA1285	transcription factor	BC023985	n.d.		n.d.			
NT_008705	4584851	10p	+	+	COMMD3	signal transduction	NM_012071	n.d.		n.d.			
NT_010194	40013072	15q	+	+	NOX5	nadph oxidase	AY358983	n.d.		n.d.			
NT_010393	20204834	16p	+	+	ATP2A1	atpase	U96774	n.d.		n.d.			
NT_010799	2632702	17p	+	+	LOC90313	not known	AB110214	n.d.		n.d.			
NT_011295	1804430	19p	+	+	MECT1	transcription factor	NM_025021	n.d.		n.d.			
NT_011295	10057542	19p	+	+	PDE4A	signal transduction	AF178570	n.d.		n.d.			
NT_011333	1199291	20q	+	+	BTBD4	transcription factor	BC073800	n.d.		n.d.			
NT_022184	4081098	2p	+	+	KIAA0953	not known	XM_039733	n.d.		n.d.			
NT_022184	43497959	2p	+	+	HSPC159	not known	NM_014181	n.d.		n.d.			
NT_024000	160549	9q	+	+	FLJ36779	not known	BC036221	n.d.		n.d.			
NT_024040	393730	10q	+	+	STK32C	protein kinase	BC045760	n.d.		n.d.			
NT_026437	74651039	14p	+	+	MOAP1	proapoptotic	AK024029	n.d.		n.d.			
NT_026437	81070550	14p	+	+	C14orf65	not known	XM_496005	n.d.		n.d.			
NT_037887	4106604	16p	+	+	ADCY9	signal transduction	AY028948	n.d.		n.d.			
NT_077812	349658	19p	+	+	TRAPPC5	cellular trafficking	XM_058961	n.d.		n.d.			
NT_086939	675986	Xp	−	+	AKAP4	signal transduction	NM_003886	n.d.		n.d.			
NT_009952	13348603	13q	+	+	CLYBL	not known	NM_206808	n.d.		n.d.			
NT_011109	19372730	19q	+	+	LOC400708	not known	AK098294	n.d.		n.d.			
NT_008818	3222562	10q	+	+	AY271017								
NT_005612	100509870	3q	+	+	not known								
NT_011519	1002352	22q	+	+	not known								
NT_029419	28279104	12q	+	+	not known								
total: 45			44	45	43		42	12 of 17 (71%)		16 of 18 (89%)			

responsible for the protective effect of HRT against the development of colorectal cancer (Chlebowski *et al.* 2004). Our data suggest that methylation of ER-β promoter is an early event in malignant transformation of breast tissue. As a risk marker for the malignant potential of breast epithelial cells, it could be useful in classifying benign and premalignant breast lesions. Moreover, even if we could not yet detect a significant correlation, the methylation status of ER-β might have prognostic potential. By analyzing breast cancer patients classified previously by gene-expression profiling, we detected ER-β methylation predominantly in a subgroup of patients characterized by unfavorable prognosis. It still remains unclear how DNA methyltransferases establish specific methylation patterns within a cell. Epigenetic regulation of the genome might involve several different pathways that contribute to methylation. One mechanism described for a number of genes is the generation of antisense transcripts from a reverse promoter (Ogawa & Lee 2002, Tufarelli *et al.* 2003, Tagoh *et al.* 2004). This possibility is especially intriguing since we detected reverse promoters in close proximity with both promoter 0K and 0N of ER-β. Although we were unable to detect antisense transcripts from the reverse promoter, it is possible that those transcripts exist *in vivo* only for a short window of time immediately preceding the methylation process. A second hypothesis of the induction of DNA methylation is the involvement of RNAi-dependent mechanisms resulting from endogenous noncoding RNA (ncRNA). The experimental induction of methylation by such an approach has been described recently (Kawasaki & Taira 2004, Morris *et al.* 2004). Several causes could account for our failure to induce methylation of the ER-β promoter by this strategy. First, both Morris *et al.* (2004) and Kawasaki & Taira (2004) transfected synthetic siRNA, in contrast to the shRNA used in our experiments, which has to be transcribed and processed to siRNAs in the cell. Secondly, in contrast to Kawasaki and Taira, Morris *et al.* observed a stringent dependency of the observed effect on nuclear transport of the siRNA by a shuttle protein (MPG). Finally, the obtained results may also differ depending on the cell type used. A third model for promoter methylation involves the targeting of DNMTs to the promoter by association with transcription factors, as described for Myc in recent work by Brenner *et al.* (2005) and Di Croce *et al.* (2002). Hints for such a mechanism could come from shared binding sites for distinct transcription factors in the promoter of genes that share a common methylation profile. Accordingly, we used homology comparisons of the promoters of ER-β and SCGB3A1 and succeeded in identifying such a shared motif. Interestingly, a global search of the human genome found that all 45 occurrences of the motif with the exact sequence from ER-β are in the promoter region of human genes. Further data on these genes from microarray analyses suggest that most of them even show decreased expression in breast cancer tissue and might be prone to methylation in this disease. Intriguingly, the majority (6/9) of those genes with most significant downregulation ($P<0.05$) in mammary carcinomas are themselves directly implicated in transcriptional regulation (ESR2, NFIB, CBX6, FHL1, SOX7 and HOXA4), while the remaining three are involved in signal transduction (STK11, ITGA3 and TM7SF3), and we might speculate that loss of these factors may trigger further changes in expression patterns in the tumor cell.

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