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Journals Club

Review of:
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 of the original article
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 carcinomas 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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Review

DNA methylation is a ubiquitous process of gene inactivation in nature, occurring preferentially at CpG dinucleotides [1]. Approximately 70–80% of CpG sites in the human genome are methylated and nearly half of all human genes (~45 000 genes) contain CpG islands [2]. Methylation of CpG islands is associated with loss of transcription of the target gene and can also induce genomic imprinting such as X chromosome inactivation. While DNA methylation alterations are now widely recognized as a contributing factor in human carcinogenesis, and even though the role of estrogen receptor beta (ERβ) in human breast cancer is not completely understood [reviewed in 3], some studies in the literature suggest that ERβ may act as a tumor suppressor gene. We and others have suggested this by showing a gradual decrease of ERβ protein from normal to pre-invasive lesions [4] to invasive cancers [5], while it has also been shown by gene expression profiling that in breast tumors with unfavorable prognosis, ERβ expression is almost completely suppressed [6,7]. In addition, a significant number of genes are transcriptionally silenced by promoter hypermethylation in breast cancer such as ERα, PR, and BRCA1 [8]. Two different promoters have been reported for the ERβ gene in recent studies. While promoter 0N of the ERβ gene is not methylated in normal breast epithelial cells, it is highly methylated in breast cancer cell lines and tumors [9]. Identical observations for promoter 0N were confirmed in prostate cancer cells, where methylation reached 80 to 90% in grade 4/5 carcinomas, but declined to less than 20% in bone metastases [10]. Promoter 0K is generally unmethylated in both normal and malignant breast epithelial cells.

In their recent article, Rody *et al*., investigated whether methylation changes in the two previously reported promoters of ERβ were responsible for loss of gene expression in benign, pre-malignant, ductal carcinoma in situ (DCIS) and corresponding invasive breast carcinomas. Total RNA/DNA was isolated from tissue samples and cultured cells according to standard protocols, while Real-time PCR analysis, Methylation-specific PCRs, detection of antisense transcripts and shRNA transfection for induction of RNAi-mediated promoter methylation were performed as described. Initially after optimizing and validating the specificity of methylation-specific PCR (MSP), Rody *et al*., demonstrated that seven out of ten breast cancers had a total or nearly total loss of ERβ mRNA expression, which correlated with a positive result of the methylation assay. These preliminary results are in agreement with other studies showing loss of ERβ expression in breast tumors [5,11] and suggest that MSP can be a valuable tool for epigenetic evaluation of ERβ regulation in breast cancer.

The ERβ 0N promoter methylation status was then evaluated by MSP in 175 breast tissue samples. No methylation signals were detected in normal breast samples originating from either normal mammoplasties ($n = 25$) or tumor patients ($n = 21$) or pre-malignant papillomas ($n = 3$), however 26/28 fibroadenomas, 16/17 ductal hyperplasias and 6/7 DCIS showed weak methylation (real-time PCR signals with Δct values of 6–8). In addition, 70% of the invasive carcinomas tested showed strong methylation signals ($n = 52/74$; real-time PCR signals with Δct values <2). In order to evaluate the potential prognostic value of ERβ promoter methylation, the authors next investigated a panel of breast cancers previously characterized by gene expression profiling [6,7]. Although only 10.5% of tumors (2/19) negative for methylation showed a relapse, the percentage increased to 28% (10/36) among breast cancers positive for ERβ promoter methylation. These data are consistent with previously published studies, demonstrating a positive correlation between ERβ expression and better clinical outcome [12–16].

Rody *et al*., further investigated the structural characteristics of promoters 0N and 0K, both of which were associated with a second, downstream, opposite-oriented promoter. Their hypothesis was that generation of antisense transcripts from these promoters could result in double-stranded RNA, leading to methylation of the upper-strand promoter, a mechanism which has been previously described for epigenetic imprinting and silencing for a number of genes [17–19]. RT-PCR analysis using cDNA specifically primed with different sense primers in the region surrounding promoter 0N was performed. These analyses failed to detect any endogenous antisense transcripts, which might be involved in epigenetic gene silencing. However, the authors suggested that it was possible that such antisense transcripts exist *in vivo* only for a short period of time immediately preceding the methylation process. Furthermore, by transfecting cell lines with shRNA, Rody *et al*., tested the possibility of inducing de novo promoter methylation. They introduced PCR products containing shRNA targeted against various GpC sites, in the 0N promoter, in MCF-7, T-47D, MDA-MB-468 and HEK293 cell lines, but these analyses failed again to show either promoter methylation or changes in ERβ gene expression. The authors discuss reasons that could account for their negative results one of them being that other studies have utilized siRNA compared to the shRNA used in their study [20]. However, at this point in time the mechanism(s) of ERβ promoter methylation in breast cancer cells remains unknown.

The sensitive MSP assay conducted by Rody *et al*., demonstrated a strong, inverse correlation of ERβ

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mRNA expression with the methylation status of the promoter. Promoter 0N methylation seems to be common in breast cancer, since methylation signals were detected in more than two thirds of all cancers. The authors suggest that the detection of methylation in some ductal hyperplasia and DCIS, indicates that ERβ might play an important role in breast cancer development. Since no methylation was detected in benign tissue from breast cancer patients, these data suggest that methylation of the ERβ promoter is not a generally occurring phenomenon in the breast.

Limitations of this study include the small numbers of samples analyzed and the lack of survival analyses (overall and/or relapse free survival using Kaplan–Meier curves) for patient samples with and without methylation signals based on the MSP results. We believe that such analyses would add information to our understanding of the biological significance of the ERβ promoter methylation status. To conclude, the present study proposes that methylation status of the ERβ promoter may have clinical value as a prognostic factor, since ERβ methylation was detected predominantly in a sub-cohort of breast cancer patients characterized by unfavorable prognosis. This is an important preliminary finding, but requires further investigation and validation in detailed future studies, encompassing increased numbers of samples associated with clinical outcome information and analysis.

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