X-Linked Ectodermal Dysplasia Receptor Is Downregulated in Breast Cancer via Promoter Methylation

Vasu Punj, Hittu Matta, and Preet M. Chaudhary

Abstract

**Purpose:** The X-linked ectodermal dysplasia receptor (XEDAR) is a novel receptor of the tumor necrosis factor receptor family that binds to ectodysplasin-A2 (EDA-A2) and induces cell death. The purpose of this study was to determine the tumor-suppressive potential of XEDAR in the development of breast cancer.

**Experimental Design:** We analyzed the expression of XEDAR in breast cancer cell lines and tumor samples using quantitative real-time PCR analysis and immunoblotting. We analyzed the human XEDAR gene promoter for the presence of any CpG island and examined its methylation status using methylation-specific real-time PCR. We examined the effect of 5-aza-2'-deoxycytidine on the expression of XEDAR and sensitivity to EDA-A2–induced apoptosis in breast cancer cell lines.

**Results:** Expression of XEDAR, but not EDA-A2, was downregulated in most tumorigenic breast cancer cell lines and tumor samples. Loss of XEDAR expression correlated with the hypermethylation of its promoter. Ectopic expression of XEDAR in MDA-MB-231 cells resulted in significant induction of apoptosis and reduction in colony formation. Treatment with 5-aza-2'-deoxycytidine restored XEDAR expression in breast cancer cell lines with methylated XEDAR promoter and sensitized them to EDA-A2–induced cell death.

**Conclusions:** Our results suggest that XEDAR expression is downregulated in most breast cancers via promoter methylation, which may contribute to accelerated tumor development by blocking EDA-A2–induced cell death. XEDAR may represent a novel breast tumor suppressor gene, and restoration of its expression by treatment with DNA demethylating agents may represent an attractive approach for the treatment of breast cancer. *Clin Cancer Res; 16(4); 1140–8. ©2010 AACR.*
Translational Relevance

The expression of the death receptors of the tumor necrosis factor receptor family is frequently downregulated during cancer development. X-linked ectodermal dysplasia receptor (XEDAR) is a recently characterized member of the tumor necrosis factor receptor family that binds to ectodysplasin-A2 (EDA-A2) and induces apoptosis in a caspase-8-dependent manner. In this article, we report that expression of XEDAR, but not EDA-A2, is downregulated in most tumorigenic breast cancer cell lines and tumor samples due to methylation of its promoter. XEDAR expression can be restored in cell lines with methylated XEDAR promoter by treatment with 5-aza-2′-deoxycytidine, a demethylating agent, which sensitizes them to EDA-A2–induced cell death. Our results suggest that XEDAR may be a novel tumor suppressor gene for breast cancer, and restoration of XEDAR expression may represent an attractive approach for the treatment of breast cancer.

Materials and Methods

Tissue samples, cell lines, and reagents. Breast cancer cell lines were obtained either from the American Type Culture Collection or from Dr. Adi F. Gazdar (University of Texas southwestern Medical Centre, Dallas, TX) and maintained as described previously (14, 15). Human mammary epithelial cells (HMEC) were obtained from Cambrex and maintained according to the instructions of the supplier. Human breast tissue specimens were obtained from Health Sciences Tissue Bank, Magee-Womens Hospital (Pittsburgh, PA). A rabbit polyclonal antibody against XEDAR was obtained from ProSci. EDA-A2 and 5-aza-2′-deoxycytidine (5-Aza-dC) were purchased from R&D Systems and Sigma, respectively.

Gene expression in cell lines and tissue samples. Expression of human XEDAR and EDA-A2 mRNAs was studied by quantitative real-time PCR (qRT-PCR) as described previously (16). Briefly, RNA was isolated using the RNeasy Mini kit (Qiagen), and qRT-PCRs were done in triplicate using an ABI Prism 7000 System and SYBR Green Taq polymerase mix to determine the relative change in the expression of XEDAR/EDA-A2 genes. β-Actin was used as a housekeeping control. qRT-PCR data (Ct values) were analyzed using the 2^−ΔΔCt method (17), and the data were presented as fold change in target gene expression ± SEM. A cDNA from K562 cell line was used as negative control in RT-PCR. Gene-specific primers used in RT-PCR are listed in Supplementary Table S2. 5-Aza-dC treatment of cell lines was done using a protocol described previously (18). Briefly, cells were grown to 50% to 60% confluency in culture medium (without 5-Aza-dC) for 24 h before harvesting for RNA isolation to check the expression of XEDAR.

Promoter analysis and identification of CpG island. A database search of transcriptional start sites (TSS) suggested an alternate promoter on chromosome X, with TSS at 65775833. We analyzed 1-kb region around TSS using MethPrimer (19) and Methyl Express (Applied Biosystems) softwares and identified a 317-bp CpG island located −64 to +253 bp upstream of the TSS.

DNA extraction and methylation-specific RT-PCR. Genomic DNA was extracted from primary tumors, cell lines, and nonmalignant cells using DNaseasy Tissue kit (Qiagen), and bisulfite modification of genomic DNA was done using Imprint DNA Modification kit (Sigma) following the manufacturer's instructions. RT-PCR primers (Fig. 2) were designed using the MethPrimer software (20). Real-time methylation-specific PCR was done using sets of primers for methylated and unmethylated regions of XEDAR as described by Bastian et al. (21). Genomic DNA and water (blank) were used as controls, whereas unmethylated MyoD1 was used as an internal reference standard (21). Quantitative methylation ratio (QR) was calculated as percent of the ratio of fluorescence emission of PCR product using methylated primer at the promoter of interest to that of MyoD1, as reported previously (22).

Cell viability (MTS) assay. Cell viability was measured using the MTS reagent as described previously (23).

Statistical analysis. Receiver operator characteristic (ROC) curve, a plot of sensitivity and specificity across all cutoff values, was used to study the accuracy of XEDAR methylation in discriminating cancer from nonmalignant tissues. The CpG island methylation data were used to generate ROC curve using MedCalc software (MedCalc Software; ref. 24). The quantitative methylation data for XEDAR were correlated with tumor diagnosis using nonparametric Mann-Whitney U test using Analyse-it statistical software (Analyse-it Software Ltd.). All tests were two-tailed and P values of <0.05 were considered significant.

Results

Expression pattern of XEDAR and EDA-A2 in human breast cancer cell lines. We began by examining the expression of XEDAR using RT-PCR analysis in a panel of 10 breast cancer cell lines developed by Dr. Adi F. Gazdar (14, 15). The clinical and pathologic features of these cell lines are summarized in Supplementary Table S1. All the cell lines, with the exception of HCC1008 and HCC1428, were derived from primary tumor samples and had the pathologic diagnosis of ductal carcinoma. The HCC1008 cell line was derived from the lymph node of a patient with metastatic ductal carcinoma, whereas the HCC1428 cell line was derived from malignant pleural effusion of a patient with metastatic adenocarcinoma, not otherwise specified. The cell lines varied with respect to the expression of estrogen receptors (ER) and progesterone receptors (PR) and the oncogene HER2/neu (Supplementary Table S1). As shown in Fig. 1A, significant expression of XEDAR

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mRNA was detected only in the HCC712 cell line, which was derived from primary breast cancer (stage IIB, grade 2, ductal carcinoma), and has low tumorigenic potential. The expression of XEDAR protein in the HCC712 cell line was confirmed by Western blotting (Fig. 1B).

The HCC712 cell line is partially adherent, ER+, PR+, and HER2/neu− and possesses near-diploid number of chromosomes (ploidy index = 1.2; ref. 15). It forms duct-like structures that are similar to the in vivo ductal morphology during normal breast development and forms domes representing hemispherical elevations of monolayer resulting from vectorial fluid transport (15). The above features have led to its classification as a differentiated breast cancer cell line (15). Our results showing high-level expression of XEDAR in HCC712 led us to hypothesize that expression of XEDAR may correlate with the degree of differentiation in breast cancer. To provide further support for this hypothesis, we examined XEDAR mRNA expression in an additional panel of six breast cancer cell lines representing different stages of differentiation and consisting of MCF7, MCF-10F, MCF-10-2A, MDA-MB-231, MDA-MB-435, and SKBR3. As shown in Fig. 1C, high-level XEDAR mRNA expression was observed in the MCF-10F cell line, a nontumorigenic cell line that was derived from floating cells in the initiating population. The MCF-10F cells display characteristics of luminal ductal cells by electron microscopy, exhibit three-dimensional growth in collagen culture, and form domes in confluent culture (25). In contrast, low-level XEDAR mRNA expression was seen in the MCF-10-2A cell line (Fig. 1C) that is derived from adherent cells in the starting population and infrequently forms domes in confluent cultures (25). Low-level expression of XEDAR mRNA was also observed in the MCF7 cells (Fig. 1C), which are also known to retain some characteristics of well-differentiated mammary epithelium, including the ability to process estradiol via cytoplasmic ERs and the capability of forming domes (26). However, no XEDAR expression was observed in the tumorigenic cell lines representing different stages of differentiation.
MDA-MB-231, MDA-MB-435, and SKBR3 that lack ER and PR expression (Fig. 1C; ref. 26). Collectively, the above results suggest that although \( \text{XEDAR} \) is expressed in the well-differentiated or nontumorigenic breast cancer cell lines, it is downregulated in most poorly differentiated or tumorigenic breast cancer cell lines.

We next examined the expression of \( \text{EDA-A2} \), the ligand for \( \text{XEDAR} \), in breast cancer cell lines by qRT-PCR analysis. Similar to the situation with \( \text{XEDAR} \), highest expression of \( \text{EDA-A2} \) was observed in the HCC712 cell line (Fig. 1D). In contrast to the situation with \( \text{XEDAR} \), the expression of \( \text{EDA-A2} \) was more prevalent and was seen in several cell lines, such as HCC1187, HCC1419, and HCC1428, which lacked \( \text{XEDAR} \) expression (Fig. 1D).

Because cell lines may acquire \textit{de novo} genetic and epigenetic lesions during cultivation (14, 27), it is mandatory to confirm the clinical relevance of such aberrations in primary tissue samples. Therefore, we next examined the expression of \( \text{XEDAR} \) in malignant (\( n = 10 \)) and nonmalignant (\( n = 6 \)) breast tissue samples by qRT-PCR analysis. Consistent with the results with breast cell lines, the expression of \( \text{XEDAR} \) was significantly downregulated in the malignant breast tissue samples as compared with the normal or benign breast tissue samples (\( P < 0.05; \) Fig. 1E).

\textbf{Mapping of 5’ flanking region of \( \text{XEDAR} \) and its aberrant methylation in malignant breast cancer cell lines}. Analysis of human \( \text{XEDAR} \) gene promoter on chromosome Xq12 using genomic DNA information contained in Ensembl Contig ENSG00000131080 revealed a CpG island between base position −64 to +253 (relative to the expected transcription site +1) according to the CpG island definition of Takai and Jones (28). In addition, examination of the \( \text{XEDAR} \) promoter region using DBTSS database and MethPrimer software (19) and using stringent criteria for CpG island (as explained in Materials and Methods) also identified a single CpG-enriched region of 317 bp between −64 and +253 bases across the TSS (Fig. 2). We next examined the methylation status of the \( \text{XEDAR} \) promoter in different human breast cancer cell lines using methylation-specific RT-PCR primers (MSP). Ten of the 12 breast cell lines that lacked \( \text{XEDAR} \) expression (HCC38, HCC70, HCC202, HCC1008, HCC1419, HCC1569, HCC1954, MDA-MB-231, and SKBR3) showed expression or showed low-level expression (MCF7) showed methylated \( \text{XEDAR} \) promoter, whereas the 2 cell lines that showed high-level \( \text{XEDAR} \) expression (HCC712 and MCF-10F) lacked methylation of \( \text{XEDAR} \) promoter (Table 1). MCF-10-2A cell line, which showed low-level \( \text{XEDAR} \) expression, also lacked methylation of \( \text{XEDAR} \) promoter (Table 1). HCC1187 was the only cell line in the panel that completely lacked \( \text{XEDAR} \) expression but possessed unmethylated \( \text{XEDAR} \) promoter (Table 1). Collectively, the above results show that methylation of \( \text{XEDAR} \) promoter correlates with its gene silencing in a majority of breast cancer cell lines.

\begin{table}
\centering
\caption{A representative pattern of hypermethylation status of various breast cancer cell lines for \( \text{XEDAR} \)}
\begin{tabular}{|c|c|c|}
\hline
\textbf{Cell line} & \textbf{Methylated} & \textbf{Unmethylated} \\
\hline
MCF7 & \textbullet & \textbullet \\
MCF-10-2A & \textbullet & \textbullet \\
MCF-10F & \textbullet & \textbullet \\
MDA-MB-231 & ND & ND \\
MDA-MB-435 & ND & ND \\
SKBR3 & \textbullet & \textbullet \\
HCC38 & \textbullet & \textbullet \\
HCC70 & \textbullet & \textbullet \\
HCC202 & \textbullet & \textbullet \\
HCC712 & \textbullet & \textbullet \\
HCC1008 & \textbullet & \textbullet \\
HCC1187 & ND & ND \\
HCC1419 & \textbullet & \textbullet \\
HCC1428 & ND & ND \\
HCC1569 & \textbullet & \textbullet \\
HCC1954 & \textbullet & \textbullet \\
\hline
\end{tabular}
\end{table}

\textbf{NOTE}: The DNA was extracted from cell lines and tissue specimens after bisulfite modification as explained in Materials and Methods. The modified DNA was used to run quantitative MSP using methylation-specific and unmethylation-specific primers. The closed boxes represent positive PCR, whereas the open boxes represent negative reaction. Abbreviation: ND, not determined.
Methylation of XEDAR in breast tumors. We next examined the methylation status of XEDAR promoter in human breast cancer tumor and normal breast tissue samples using real-time methylation-specific PCR. Figure 3 shows the QRs for the cancerous and malignant breast tissues. We found increased methylation of XEDAR promoter in breast tumors (QR, 1.19-95.68) and much lower or undetectable levels (QR, 0-1.2) in the nonmalignant breast tissues (Fig. 3A and B). Based on the highest quantitative ratio in benign or nonmalignant tissues as the cutoff (QR, 1.2), 8 of 10 (80%) of breast tumor samples were found to have hypermethylated XEDAR promoter (Fig. 3). Because a collection of cancerous and normal breast tissues was studied for methylation, we determined sensitivity, specificity, and positive and negative predictive values to assess the usefulness of hypermethylation of CpG island of XEDAR in distinguishing cancerous from benign tissue samples. This analysis was done using ROC curve and showed that XEDAR methylation has an excellent discriminatory capacity at separating breast cancer from normal tissues (area under the curve (AUC) = 0.870), with specificity of 100% and sensitivity of 80% (Fig. 3C).

Induction of apoptosis by EDA-A2/XEDAR signaling. As EDA-A2 and XEDAR are known to induce apoptosis, it was conceivable that loss of XEDAR expression in breast cancer samples and cell lines reflected an escape from their tumor-suppressive effect. To test this hypothesis, we examined the expression of XEDAR in normal HMECs and their response to EDA-A2. As shown in Fig. 4A, we observed significant expression of XEDAR in HMECs, as determined by qRT-PCR analysis. More importantly, treatment with recombinant EDA-A2 resulted in a dose-dependent decline in the viability of HMECs, supporting the argument that EDA-A2/XEDAR signaling negatively regulates the survival of normal breast epithelial cells (Fig. 4B).

We next asked the question whether ectopic expression of XEDAR in the MDA-MB-231 breast cancer cells with silenced expression of endogenous XEDAR will result in induction of apoptosis. To answer this question, the MDA-MB-231 cells were transfected with an expression vector encoding XEDAR along with a green fluorescent protein–encoding plasmid, and the effect of ectopic XEDAR expression on
cell survival was examined by fluorescence microscopy. We included transfection with an empty vector as a negative control and transfection with a plasmid encoding death receptor 4 (DR4), a known inducer of apoptosis (29), as a positive control. As shown in Fig. 5A and B, transfection with plasmids encoding XEDAR or DR4, but not the empty vector, resulted in nuclear condensation, cellular rounding, detachment, and fragmentation into apoptotic bodies, all features suggestive of cell death.

Lastly, to further confirm the tumor-suppressive effect of XEDAR, we evaluated the effect of its stable expression on the growth of MDA-MB-231 cells using liquid colony formation assay. MDA-MB-231 cells were transfected with an empty vector or vectors encoding XEDAR or DR4 and subsequently selected in Zeocin for 14 days. We found significantly reduced number of colonies in MDA-MB-231 cells that had been transfected with XEDAR- or DR4-encoding plasmids as compared with the cells that had been transfected with an empty vector (Fig. 5C and D).

Restoration of functional XEDAR expression after treatment with 5-Aza-dC. Studies in the preceding sections showed that ectopic expression of XEDAR can induce apoptosis in the MDA-MB-231 cell line, in which the expression of endogenous XEDAR had been silenced due to promoter methylation. Therefore, we asked whether XEDAR expression can be reinduced in cells, in which its promoter is methylated by treatment with demethylating agents and the consequences of its reexpression on cell survival. To address this question, we examined XEDAR mRNA expression in seven representative human breast cell lines before and after treatment with two doses (2 and 5 μg/mL) of methyltransferase inhibitor 5-Aza-dC. Total RNA was isolated from these cell lines with and without 5-Aza-dC treatment, and mRNA level of XEDAR was checked by qRT-PCR. As shown in Fig. 6A and B, the XEDAR expression was significantly enhanced in the three human breast cancer cell lines with methylated XEDAR promoter in the basal state (i.e., MDA-MB-231, SKBR3, and HCC1419) but did not change in the cell lines containing the nonmethylated XEDAR promoter (i.e., MCF-10F and MCF-10-A).

We next asked if restoration of XEDAR expression by 5-Aza-dC will sensitize breast cancer cell lines to EDA-A2-induced cell death. To address this question, we treated the HCC1419, SKBR3, and MDA-MB-231 cell lines, which contain methylated XEDAR promoter, with 5-Aza-dC for 4 days and then treated them with recombinant EDA-A2 for 72 hours. Cell viability was measured using an MTS assay. As shown in Fig. 6C to E, EDA-A2 treatment had no effect on cell viability in the untreated or PBS-treated HCC1419, SKBR3, and MDA-MB-231 cell lines, reflecting the lack of XEDAR expression in these cells in their basal state. However, EDA-A2 treatment led to significant decline in the viability of cell in which XEDAR expression had been restored by pretreatment with 5-Aza-dC (Fig. 6C–E). Thus, EDA-A2 reduced the viability of HCC1419, SKBR3, and MDA-MB-231 cells that had been treated with 2 μg/mL 5-Aza-dC to approximately 60%, 40%, and 80%, respectively, of the PBS-treated cells. The reduction in cell viability...
on EDA-A2 treatment was even more pronounced in cells that had been pretreated with 5 μg/mL 5-Aza-dC (i.e., to 30%, 5%, and 25%, respectively), reflecting higher-level induction of XEDAR expression.

Discussion

Multiple genetic and epigenetic alterations are involved in the development of cancer. Loss of expression of certain genes in tumor cells is often linked to the potential of their gene products to act as tumor suppressors. Furthermore, aberrant methylation of CpG islands in gene promoters has been described as a primary mechanism for the inactivation of tumor suppressor genes in human malignancies (30). The identification of genes that are prone to abnormal methylation and consequently become downregulated during cancer development is of clinical significance, as these genes can serve as novel tumor bio-

markers (31) and potential targets for therapeutic interventions (32, 33). In the present investigation, we present lines of evidence that XEDAR is a novel tumor suppressor gene that is inactivated by DNA methylation in breast cancer cell lines and tumors.

Our RT-PCR analysis suggests that XEDAR is expressed in normal breast epithelial cells (e.g., HMEC) and premalignant and/or differentiated breast cancer cell lines (e.g., MCF-10F and HCC712) and its expression is often lost in tumorigenic and/or poorly differentiated breast cell lines. The role of XEDAR as a tumor suppressor gene in breast cancer is supported further by our studies showing loss of its expression in breast cancer tissue samples. In contrast, we found no clear correlation between the expression of EDA-A2, the XEDAR ligand, and the degree of differentiation and/or malignant potential of different breast cell lines, suggesting that it is the XEDAR receptor itself that is differentially silenced in cancerous cell lines and reinforcing its
possible role as a tumor suppressor during breast cancer development.

Inactivation of tumor suppressor genes is an important event for carcinogenesis of malignant tumors. In addition to classic genetic alterations (deletion or inactivating point mutations), these tumor suppressor genes can be functionally inactivated by aberrant methylation (34, 35). We identified ∼300-bp CpG-rich segment in the promoter of XEDAR. We specifically targeted this short CpG-rich region and developed quantitative MSP assay suitable for quantitative methylation analysis. Using this assay, we observed that XEDAR methylation is specific for breast cancer cells and there is a strong correlation between the methylation status of the breast cancer cell lines and their level of expression of XEDAR, suggesting that XEDAR methylation may be causally linked to the loss of XEDAR mRNA expression. This hypothesis was further supported by our results showing restoration of XEDAR expression in the MDA-MB-231, SKBR3, and HCC1419 cell lines following treatment with 5-Aza-dC. MCF-10-2A and HCC1187 were the only cell lines that lacked significant XEDAR expression in the absence of its promoter methylation. It is conceivable that methylation-independent mechanisms of gene inactivation, such as gene rearrangement or splice site mutation, may contribute to the loss of XEDAR expression in these two cell lines. Consistent with this notion, 5-Aza-dC treatment failed to restore XEDAR expression in the MCF-10-2A cell line.

The correlation between silencing of XEDAR expression and promoter methylation, however, was not limited to breast cancer cell lines and was also observed in breast tumor samples. Furthermore, the ROC curve analysis suggested that methylation status of XEDAR promoter has an excellent discriminatory capacity in separating breast cancer from benign lesions. While this manuscript was in preparation, a recent study reported loss of XEDAR expression due to promoter methylation in colorectal carcinoma (36). Thus, the utility of loss of XEDAR expression as a cancer biomarker may not be limited to breast cancer and it may serve as a biomarker for other epithelial cancers as well.

What is the mechanism by which XEDAR acts as a tumor suppressor? Several death domain-containing receptors of the TNFR family have been recently shown to negatively affect the development of cancer, and this activity has been linked to their ability to promote cell death (9, 10). Although XEDAR lacks a discernible death domain, we had previously shown that it is capable of inducing apoptosis via caspase-8–dependent and FADD-dependent mechanism (8). Recently, we also reported that activation of signaling via XEDAR leads to induction of apoptosis in cell lines derived from osteosarcoma (37). Consistent with the above results, we observed significant induction of apoptosis and reduction of colony formation by ectopic expression of XEDAR in the MDA-MB-231 cells, in which the expression of endogenous XEDAR had been silenced by promoter methylation. Moreover, EDA-A2 treatment induced cell death in normal mammary epithelial cells with endogenous XEDAR expression and in MDA-MB-231, HCC1419, and SKBR3 cell lines in which XEDAR expression had been reinduced by treatment with 5-Aza-dC. Collectively, the above results suggest that loss of XEDAR expression promotes breast cancer development via inhibition of apoptosis.

In summary, we have shown that XEDAR expression is frequently lost in breast cancer cell lines and tumor samples. In a majority of cases, this loss is due to the aberrant methylation of XEDAR promoter, which can be reversed by treatment with DNA demethylating agents. These results may have considerable implications for the diagnosis and treatment of breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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