Demethylation of the Estrogen Receptor Gene in Estrogen Receptor-negative Breast Cancer Cells Treated with 5-aza-2′-deoxycytidine Can Reactivate Functional Estrogen Receptor Gene Expression

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Abstract: To study demethylation action of 5-aza-2′-deoxycytidine and its effect on the expression of functional estrogen receptor (ER) genes in the human ER-negative breast cancer cell. The methylation status of ER gene in the ER-negative breast cancer cell was evaluated by methylation specific PCR(MSP) and genomic sequencing. The expression of ER and progesterone receptor (PR) mRNA and production of ER protein were detected by RT-PCR and Western-blot method, respectively. MTT assay was used to examine the function of re-expressed ER protein. The ER gene promoter was highly methylated in the ER negative breast cell line MDA-MB-231 and ER mRNA and ER protein were not expressed in the ER-negative breast cancer cell. The ER-negative breast cells treated with demethylating agent 5-aza-2′-deoxycytidine(5-aza-2′-deoxyC) were restored the expression of ER mRNA and PR mRNA and ER protein. The methylation of ER gene was simultaneously decreased and cytosine demethylated in 17/18 CpG island . The growth of cells treated with tamoxifen was inhibited significantly after MDA-MB-231 was treated with 5-aza-2′-deoxyC ($P<0.05$). The abnormal methylation of ER gene promoter plays an important role in the inactivation of ER gene. 5-aza-2′-deoxyC may lead to demethylation and reactivate functional ER expression silenced by aberrant hypermethylation. [Life Science Journal. 2006;3(1):40-44] (ISSN: 1097-8135).

Keywords: estrogen; receptor; methylation; breast cancer

1 Introduction

Breast cancer has been threatening women's health these years. It has been demonstrated that estrogen plays an important role in the initiation and progression of breast cancer. Its mechanism is that estrogen can combine with estrogen receptor and stimulate the occurrence of breast cancer. Approximately two thirds of breast cancers express estrogen receptor(ER) and their growth is stimulated by estrogen (Ferguson, 1995). For these patients, hormonal therapies target ER pathway are taken via a variety of mechanism including depletion of endogenous estrogen, interference with ligand-receptor interactions, or destruction of ER (Keen, 2003). Compared with combination chemotherapy, endocrine therapy is cheaper and less toxic, which becomes advantageous and promising. However, the remaining fraction of primary breast cancers lack of detectable ER protein and are rarely responsive to hormonal therapy (Ferguson, 1995). ER negative phenotype is associated with increased tumor grade and proliferation.

Many previous study proved that methylation plays an important role in loss of ER expression. In this report we provide evidence that 5-aza-2′-deoxycytidine(5-aza-2′-deoxyC) can lead to demethylation and re-expression of ER mRNA and functional ER protein.

2 Materials and Methods

2.1 Cell culture and reagents

Human ER negative breast cancer cell MDA-MB-231 and ER positive breast cancer cell MCF-7 were maintained in DMEM with 10% FCS, and the cell dense was 5×10^5/ml ~ 1×10^6/ml. The first day, MDA-MB-231 cell was treated with 0.75μmol 5-aza-2′-deoxycytidine. The second day the medium was changed. The third and fifth day, cells were treated repeatedly as the first day. On the sixth

day, cells were harvested. 5-aza-2′-deoxyC (Sigma) was freshly prepared in DMEM.

2.2 Extraction of DNA and RNA

Total RNA was prepared from cells by the method of TRIZOL and quantified by measuring absorbance at 260 nm. The integrity of the RNA and the accuracy of the spectrophotometric determinations were assessed by visual inspection of the ethidium bromide stained 28S and 18S ribosomal RNA bands after agarose gel electrophoresis. DNA was extracted with standard method as described previously (Blin, 1976).

2.3 RT-PCR

RT-PCR was performed as described previously (Issa, 1994). 11.5 μl total cellular RNA were used for each reverse transcription reaction. Primers were designed as follows: ER (5′-ATG-GAGT CTGGTCCTGTG-3′ sense; 5′-TTCG-TATCCCACCTTTCA TC-3′ anti-sense) and PR (5′-CCAGTGCCTCAGTCTCGT-3′ sense; 5′-CCTTCCATTGCCCTCTTA-3′ anti-sense). The length for ER and PR PCR amplification product was 181 bp and 460 bp respectively. β-actin (5′-ACCATGGATGATGAT ATCGC-3′ sense 5′-A-CATGGCTGGGGTGTTGAAG-3′ anti-sense). The length for β-actin PCR amplification product was 400 bp. The PCR sample was subjected to electrophoresis in 1% agarose gel, stained with ethidium bromide and visualized by UV light. The PCR reaction (50 μl) contained 10 × PCR buffer 5 μl, dNTP 4 μl, CDNA 5 μl, ER sense and anti-sense primer 1 μl respectively, β-actin primer sense and anti-sense primer 1 μl respectively, Taq polymerase 0.5 μl, H_2O 33.5 μl. PCR reaction was performed in UNOⅡ Biometra PCR.

2.4 Western blot analysis

Total cellular proteins extracted from breast cancer cells were resolved by electropheresis in a 12% denaturing polyacrylamide gel and proteins were electrotransferred to nitrocellulose membranes. The ER protein was then identified by using rabbit polyclonal antibody, which was specific for the ER protein, and the standard ABC Kit. Diaminobenzidine in a buffer was used in the coloration step indicate the presence of the ER protein.

2.5 Methylation specific polymerase reaction (MSP) and genomic sequencing

DNA was bisulfite modified as described previously (Herman, 1996). According to CpGenome™ DNA Modification Kit (Catalog # S7820), both genomic sequencing and MSP rely on chemical modification of DNA samples. The first step of genomic sequencing was bisulfite modification of the DNA sample followed by PCR. The PCR products amplified with primers specific either for the methylated or for the unmethylated DNA were purified and cloned on an ABI PRISM 377 DNA Sequencer-D Sangon by using M13 primers. Specific primers were designed to distinguish methylated from unmethylated DNA (Blin, 1976). The specific methylated primer ER(m): (5′-CGAGTT GGAGTTTT TGAA TCGTTC-3′; 5′-CTACGCGTTAACGACG ACCG -3′) The length for PCR product was 151 bp; ER (u): (5′-ATGAGTTGG AGTTTTTGAA TTGTTT -3′; 5′-ATAAACCTACACAT TAACA A CAACCA -3′). The length for PCR product was 158 bp. The PCR reaction (50 μl) contained 2 × GC buffer 25μl, 8 μl dNTP Mixture (each 2.5 Mm), sense and anti-sense primer were 1μl respectively, modified DNA 5 μl, LA Taq polymerase 0.5 μl, ddH_2O 9.5 μl, mix briefly and centrifuge. The PCR sample was subjected to electrophoresis in 1% agarose gel.

2.6 MTT

MDA-MB-231 cells treated with 5-aza-2′-deoxyC were detached by 0.25% trypsinization and seeded into 96-well plates (Costar, Cambridge, Mass.) at $10^4 - 10^5$/well in 100 μl of medium contained with estrogen and incubated for 24 h at 37℃. After 24 h, the medium was changed. 10^{-6} mol/L tamoxifen (TAM) or 10^{-8} mol /L E_2 in 200 μl medium were added and cells were incubated for an additional 48 h before quantification of cell growth. The inhibitory effect on cell growth was examined using the MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium) assay according to the previously described method (Scudiero, 1988).

3 Results

3.1 The methylation status of ER gene CpG Island Region

By MSP, PCR primers were designed to amplify a 158 bp fragment containing 18 CpG sites. Extensively methylation exited within ER gene CpG island and PCR products were observed (M group: 151 bp) in breast cancer cell MDA-MB-231, after treated with demethylating agent 5-aza-2′-deoxyC ER gene promoter demethylated and products were observed (U group: 158 bp); DNA from ER-positive breast cancer cell line MCF-7 was unmethylated within ER gene promoter. The result was shown in Figure1.1.

The PCR products amplified with primers specific either for the methylated or for the unmethylated DNA were purified and cloned by using M13 primers. Cytosine residues outside of the CpG sites were converted to thymine after bisulfite treatment. In untreated breast cancer cell MDA-MB-

231 cytosine residues at CpG sites remained unchanged. In 18 CpG island, cytosine residues at 17 CpG sites cytosines were deaminated and converted to thymine after bisulfite treatment. The result was shown in Figure 1.2.

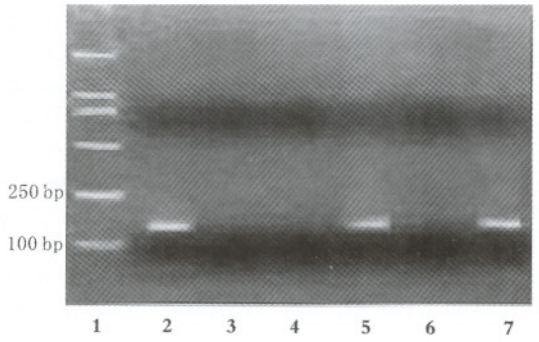

Figure 1.1 The methylation status of ER 5′CpG island by MSP method
From left to right 1: 100 bp marker; 2: MDA-MB-231 breast cancer cell: M group; 3: MDA-MB-231 breast cancer cell : U group; 4: MDA-MB-231 breast cancer cell treated with demethylating agent MDA-MB-231 breast cancer cell: M group; 5: MDA-MB-231 breast cancer cell treated with demethylating agent MDA-MB-231 breast cancer cell: U group; 6: Positive control MCF-7 cell: M group; 7: Positive control MCF-7 cell: U group.

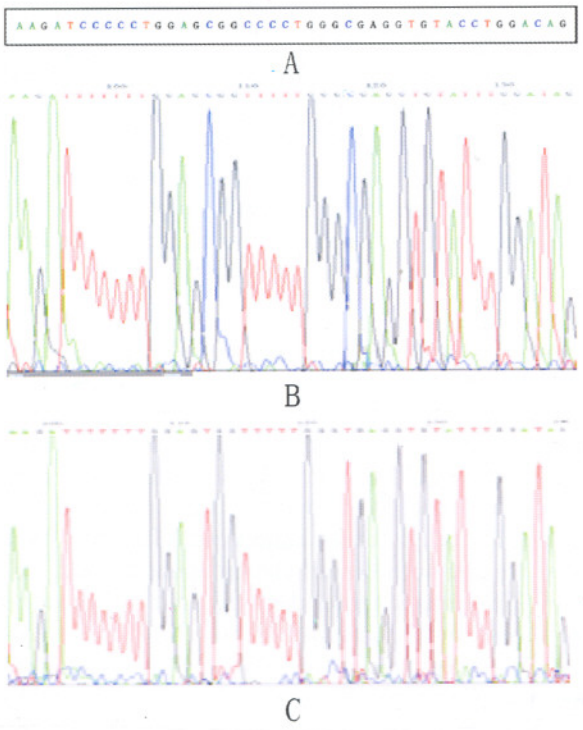

Figure 1.2 (A) Original sequence: before bisulfite treatment. (B) untreated MDA-MB-231 breast cancer cytosine residues at CpG sites remained unchanged although other cytosines were converted to thymine.
(C) MDA-MB-231 breast cancer cell treated with 5-aza-2′-deoxyC: cell cytosines were deaminated and converted to thymine.

3.2 Expression of the ER gene

As shown in Figures 2 and 3, ER gene expression was undetectable by RT-PCR using RNA from MDA-MB-231. After treatment with 5-aza-2′-deoxyC, the cells began to express the gene at levels detectable by RT-PCR. In addition, ER protein re-expressed and was detected by Western blot methods. These data were consistent with the previous findings and affirm that DNA methylation was one participant in the regulation of ER gene expression (Herman, 1996). The β-actin transcripts in each sample were also amplified as internal controls to normalize the amount of ER specific products.

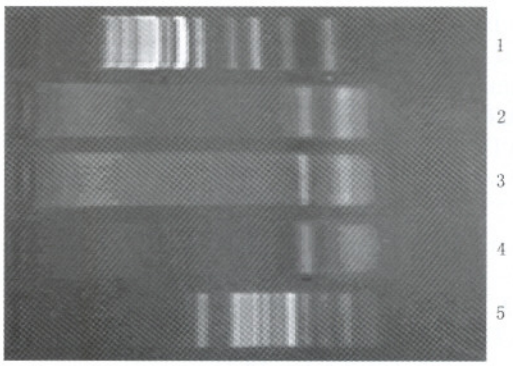

Figure 2. The expression of ER mRNA by RT-PCR method
From top to bottom 1: 200 bp marker; 2: positive control ER(+) MCF-7 cells; 3: MDA-MB-231 cells treated with 5-aza-2′-deoxyC; 4: untreated ER(-)MDA-MB-231 cells; 5: 100 bp marker

ER protein production was detected by Western blot analysis. The result is consistent with ER mRNA expression.

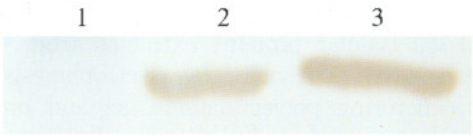

Figure 3. The ER protein by Western blot analysis
Lane 1: untreated ER(−)MDA-MB-231 cells;
Lane 2: MDA-MB-231 cells treated with 5-aza-2′-deoxyC
Lane 3: positive control ER(+) MCF-7 cells

3.3 Functional analysis of ER induced by 5-aza-2′-deoxyC

As shown in Figure 4, PR gene was unexpressed at mRNA level. After treatment, PR gene was re-expressed. Re-expression of an estrogen responsive gene-PR (460 bp) indicated induced ER was functional.

The growth and sensitivity to estrogen and tomoxifen were investigated by MTT, as shown in Table 1. There was statistical significance between

5-aza-2′-deoxyC (control) group and TAM + 5-aza-2′-deoxyC group ($P < 0.05$). After ER protein was re-expressed, TAM could inhibit the growth of MDA-MB-23 cells.

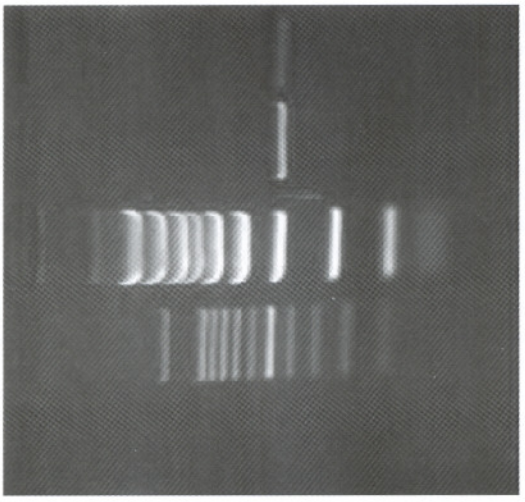

Figure 4. The expression of PR mRNA by PT-PCR method. Lane 1: deoxyC treated MDA-MB-231 cells; Lane 2: positive control: ER(+) MCF-7 cells; Lane 4: 100bp marker; Lane 5: untreate ER(−) MDA-MB-231 cells

Table 1. The inhibition rate of MDA-MB-231 cells in dillorent treated mert groups by MTT

	N	OD(x+/−s)	The rate of inhibition	*P*
5-aza-2′-deoxyC (control)	16	0.1672+/−0.0091	—	
TAM+5-aza-2′-deoxyC	16	0.1530+/−0.0168	8.49%	$P<0.05$
E2+5-aza-2′-deoxyC	16	0.1677+/−0.0121	−0.30%	$P>0.05$

As MTT was shown, there was statistical significance between 5-aza -2′-deoxyC (control) group and TAM + 5-aza-2′-deoxyC group($P<0.05$).

3.4 Statistical analysis

All results were expressed as mean ± SD. $P<0.05$ was considered statistically significant. All statistical analysis was performed by using SPSS 11.0 for Windows. Un-paired T test was adopted.

4 Discussion

The mechanisms involved in suppression of transcription of genes via hypermethylation at CpG islands is an area of active research (Jenuwein, 2001). Abnormal DNA methylation of CpG island is an early event in the progression of some human cancers (Baylin, 1991). Many previous work has demonstrated that ER expression is associated with rearrangement and re-modeling of the chromatin structure surrounding the ER gene (Yang, 2001; Iwase, 2003). Epigenetic modification including DNA methylation is tightly linked with expression of ER in human breast cancer cells, suggesting that chromatin conformation is an essential component of ER expression (Asch, 2001; Jones, 2002; Yang, 2001). CpG island hypermethylation may inhibit transcription by interfering with the recruitment and function of basal transcription factors or transcriptional coactivators. Also, hypermethylation of CpG dinucleotides near the transcriptional regulatory region may initiate the recruitment of the methyl-CpG binding domain (MBD) family proteins that mediate silencing of genes via facilitation of a repressive chromatin environment(Bird, 1999; Wade, 2001). With respect to breast cancer, ER (−) human breast cancer cells have up to a 40-fold higher level of DMT mRNA and up to a 9-fold higher level of DMT activity than ER(+) cells (Ottaviano, 1994). CpG methylation of the ER promoter results in transcriptional silencing (Lapidus, 1998) and inhibition of DNMT activity reactivates ER (Yang, 2001; Yang, 2000). Absence of estrogen receptor expression characterizes 25% of invasive breast cancer. 25% of cancers absent of estrogen receptor have hypermethylation in their promoter (Juttermann, 1994). 5-aza-2′-deoxyC is widely used as DNA methylation inhibitor to induce gene expression and cellular differentiation (Keen, 2003), but fewer reports are on the function of re-expressed ER protein.

To test whether ER induced in 5-aza-2′-deoxyC treated cells was functional, cells grown in the presence of E2 were treated with 0.75 μM 5-aza-2′-deoxyC for the indicated number of days . As is shown by MTT, after MDA-MB-231 cell was treated with demethylating agent 5-aza-2′-deoxyC, OD value in 5-aza-2′-deoxyC control group was 0.1672 +/− 0.0091, OD value in 5-aza-2′-deoxyC + TAM group was 0.1530 +/− 0.0168($P<0.05$) and the rate of inhibition was 8.49%; OD value in E2 + 5-aza-2′-deoxyC group was 0.1677 +/−0.0119($P>0.05$). In MTT experiment, (Table 1) MDA-MB-231 cells were maintained in medium contained with phenol red and phenol red possess estrogen-like function. TAM as anti-estrogen agent can block estrogen to exert the function of stimulating growth and be used as endocrine therapy for ER (+) breast cancers. MTT demonstrated that TAM could inhibit the growth of MDA-MB-231 cell with re-expressed ER protein and reached significant difference($P<0.05$). E2 could stimulate slightly the growth of MDA-MB-231 cell with re-expressed ER protein and reached no significant difference($P>0.05$). The main reason may be as follows. First, cells adapt to medium contained with estrogen-like

substances and are not sensitive to exogenous estrogen. Alternatively, estrogen receptor can be partially re-expressed and reach saturation in endogenous estrogen. The function of stimulating cell growth has exerted the maximum. All the results demonstrated that ER was functional. In addition, we further investigated the ability of the drug-induced ER to activate expression of the endogenous ER-responsive PR gene (Figure 4).

In our experiment, MSP method is a most sensitive method to detect methylation. The result demonstrated that there was extensively methylation in breast cancer cell MDA-MB-231 ER gene 5′ CpG island. Treatment of the cells with demethylating agents led to demethylation and re-expression of ER mRNA and subsequent production of the functional protein (Figures 2,3).

Chao et al (Hongxia, 2000) demonstrated that 5-aza-2′-deoxyC could inhibit tumor growth by reactivating regulatory-genes silenced by hypermethylation on endometrial carcinoma xenografted nude mice. Our further study is being under investigation on xenograft nude breast cancer mice. Therefore, it is conceivable that the demethylating agents can render ER(−) breast cancers responsive to hormonal therapies.

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Received September 29, 2005