The Characterization of CpG Methylation of *ERα* and *ERβ* Gene in the Breast Cancer

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Purpose: Aberrant methylation of promoter cytosine guanine dinucleotide (CpG) islands is known to be responsible for the alteration and silencing of cancer genes. The data presented here show that most methylations of Estrogen Receptorα (*ERα*) and *ERβ* are found at or near the transcriptional factor binding sites in the breast cancer tissues.

Methods: Fifty archival breast cancer tissues and twenty-five normal tissues were selected and the status of the methylation and the transcription were investigated by bisulfite genomic sequencing and reverse transcription (RT) PCR.

Results: Consequently, the hypermethylation of *ERα* and *ERβ* genes was found in 66.0% and 50.0% of 50 breast cancers, respectively. In particular, the methylation sites were frequently located near the CCAAT box (-363 and -375) for the *ERα* gene, and at or adjacent to binding sites of GATA (-217, -302) and Sp1 (+224, +227, +160) for the *ERβ* gene. The methylations at or near the binding sites were observed in most of the methylated cancers (*ERα* 87.9%, and *ERβ* 84.0%). The methylated cases were negatively correlated with the expression of *ERα* and *ERβ* RNA ($P < 0.01$). In particular, tumors with CpG methylation of *ERα* and *ERβ* at or near the binding sites did not express mRNA, whereas those CpG methylation outside the sites showed moderate expression. Four tumors with methylated *ERα* genes at sites unrelated to the binding sites showed higher levels of protein expression than those with methylation at or near the sites ($P=0.01$).

Conclusion: Although the number of samples was relatively small, our results suggest that DNA methylation in *ERα* and *ERβ* appears to take significant effect on transcriptional silencing and is most often present in the CpG sites at or near the putative transcriptional factor binding sites. We believe this finding offers a clue to the initiation or spread pattern of CpG methylation in human breast cancer. **(Journal of Korean Breast Cancer Society 2004;7:8-16)**

Key Words: Breast cancer, ERα, ERβ, Methylation, Transcription

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INTRODUCTION

Among the genes involved in sporadic human breast cancers, those related to estrogen action are good candidates for investigation because estrogen exerts as tumor promoter, through estrogen receptor.(*1*) Estrogen receptors belong to a superfamily of transcription activators and have two types of *ERα* (Estrogen receptor) and *ERβ* in the human tissue, showing differential expression in various tissues.(*2-4*) Their protein products are transcriptional factors that control the expression of estrogen-responsive genes by binding to a specific DNA sequence within their regulatory regions.(*5*,*6*)

There is increasing evidence that de novo methylation of promoter CpG (cytosine guanine dinucleotide) islands, contributes to the alteration of gene expression in cancer and is associated with gene silencing.(*7*)

Although numerous reports have been published on the characterization of *ERα* promoter region and the status of the CpG methylation in various types of cancers,(*8*,*9*) little information is available for the methylation of *ERβ* gene.(*10*,*11*) The transcription of human ERα gene occur from at least two different promoters and it was shown that the levels of expression of total ERα RNA and of transcript from distal promoter correlated well with the amount of ERα protein in

the human breast cancer.(*12*) Furthermore, the methylation of distal promoter of the ER alpha gene is important for loss of ER alpha gene expression in human breast cancer.(*13*) The promoter region of *ERα* gene possesses relatively few potential binding sites, whereas the human *ERβ* promoter region includes various consensus binding sites as illustrated in Fig. 1.(*11*)

We previously reported that p53 gene, a tumor suppressor gene, is methylated in the breast cancer tissues and four CpG sites with methylation are located at or near the potential binding sites of transcriptional factors such as AP1 or YY-1.(*14*) However, there have been few reports on the actual methylation status at or adjacent to the putative binding sites of transcriptional factors in the *bona fide* cancer tissues.(*15*,*16*) Furthermore, it is obscure to what extent the CpG methylation at or near the putative binding sites of transcriptional factors will influence the transcriptional silencing, compared with the methylation outside the binding sites.

We have now evaluated the methylation status of *ERα* (distal promoter) and *ERβ* promoter regions in the breast cancer tissues, including the region of potential the transcriptional factors binding sites by use of bisulfite genomic sequencing. Our data indicate that most CpG methylations in the ER genes are positioned at or adjacent to the putative binding sites of transcriptional factors and this type of methylation might exert more significant effect on the gene silencing.

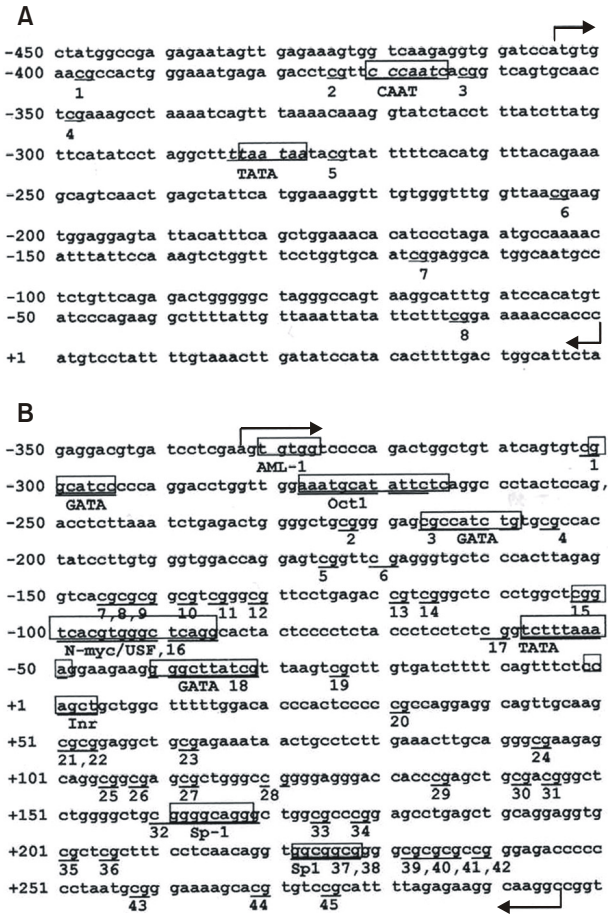

Fig. 1. Structure and sequence of the 5'-flanking region of the human *ERα* (GenBank accession number X62462, A) and *ERβ* gene (GenBank AF191544, B). The 5' transcription start site is designated as +1. Each promoter region under investigation is shown with the bent arrows. The individual CpG's are presented by numbering and underlined. The putative binding sites of transcriptional factors are indicated by boxes with the names of their matching factors.

METHODS

1) Tissue samples and nucleotide extraction

Fifty archival breast cancer tissues and twenty-five normal tissues were selected consecutively from the patients undergoing partial or total mastectomies from Nov. 2000 to Feb. 2002 at the National Cancer Center in Goyang City, Korea. The normal tissues were comprised of fifteen tissues adjacent to the tumor, and ten specimens far from the cancer. Sections were cut 5 mm thick from formalin-fixed, paraffin-embedded tissues and mounted on a microscope. To collect cancer tissues for DNA extraction, microdissection was performed as described previously.(*17*) DNA from microdissected tissues was extracted in 80 L of lysis buffer (50 mM Tris-HCl pH8.0, 1 mM EDTA, 0.5% Tween 20, 200 g/ml of proteinase K) at 55°C for 72 hours.

Thirty frozen breast tissues out of 50 cancer tissues were available for the RT-PCR and were disrupted using FastRNA Green kit (Qbiogene, Carisbad, CA) on a Ribolyser cell disruptor (Qbiogene). Purification was performed following the supplier's protocol and the RNA was finally suspended in 50 L of RNase-free water.

2) Bisulfite genomic sequencing

Bisulfite modification of genomic DNA was carried out as previously described(*14*) with minor modification. Briefly, cDNA extracted from the microdissected tissues was digested with *Eco*RI and then subjected to bisulfite treatment. Bisulfite-treated DNA was subjected to two rounds of nested PCR to amplify the *ER* promoter region. Primer sequences for *ER* are 5'-GGGTATTTTAGGAGTATTTTAG-3' and 5'-ACCAATC-AAAAATATATAAATA-3' for the primary PCR, and 5'-

AAAGTGGTTAAGAGG TGGATTT-3' and 5'-TCAAATTT-ACAAAATAAAACAT-3' for the nested PCR. For the *ER* promoter, its 654 bp promoter region (-332 to +322 in Fig. 1B) was amplified by two overlapping fragments, 349 bp and 337 bp. Primer sequences for the upstream 349 bp fragment are 5'-TATGTGAGTTAGGGGTTGAGGA-3' and 5'-AAA-AATAAATATCCAAAAAACCAAC-3' for the primary PCR, and 5'-AGTGTGGTTTTTAGATTGGTTG-3' and 5'-CCAA-AAAACCAACAACTAAAAAAAC-3' for the nested PCR. Primer sequences for the downstream 337 bp fragment are 5'-GTTTTTAAAAGGAAGAAGGGGTTTA-3' and 5'-ATAA-ACAAATATAATAACTTACA-3' for the primary PCR, and 5'-TTTTTTTAGTTTTTTTAGTTGTTGG-3' and 5'-ATAATA ACTTACAAATAAACACAC-3' for the nested PCR. All the PCR conditions were 94°C for 2 minutes, 30 cycles of 94°C for 20 seconds, 55°C for 20 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. The resulting products were subjected to agarose gel electrophoresis and purified using Qiaex II gel extraction kit (Qiagen, Valencia, CA). Sequencing was performed for each PCR product using the primers for the nested PCR on an ABI automated sequencer with Dye terminators (Perkin-Elmer, Foster City, CA). DNA sequences were confirmed by analyzing both direction of each PCR product and at least three PCR products were analyzed for each cancer tissue. By definition, the term methylation at or near the putative binding sites was methylation which occurs within two base pairs up- or downstream of binding sites,(*15*) corresponding to 375, -363 and 275 in the *ERα* (the transcriptional start site is designated as +1: GenBank accession number X62462, Fig. 1A) and 302, -207, -206, -103, -97, -61, -33, +159, +224, +227 in the *ERβ* (the transcriptional start site is designated as +1: GenBank accession number AF191544, Fig. 1B). The methylation levels of individual CpG site were calculated as percentages of 5-methyl cytosine among the whole cytosine residues (methylated plus unmethylated) at the same site.(*18*)

3) Reverse transcription (RT)-PCR

First-stranded cDNA was synthesized from 5 g of total RNA using a reverse transcription kit (Promega, Madison, WI) according to the manufacturer's protocol. To amplify double-stranded cDNA, PCR was performed in a 50 L reaction mixture containing 1 L of the reverse transcribed cDNA, 5 L of 10× PCR buffer, 1.25 mM dNTP, 1.5 mM $MgCl_2$, 1 M of each primer and 1 U of *Taq* polymerase (Roche Biochemicals, Germany). Sequence of the PCR primers used were as follows: *ERα*, 5'TCTGCCAAGGAGACTCGCTA-3' and 5'-TTGGCC-AAAGGTTGGCAGCT-3'; *ER*, 5'-TGTGCGGAGACAGAG-AAGTG-3' and 5'-GGTGGTCAATTGAGCGCCAC-3'.

To verify the integrity of mRNA, the *G3PDH* gene was amplified by use of the following primers: *G3PDH*F: 5'-ACCACAGTCCATGCCATCAC-3' and *G3PDH*R: 5'-TCCA-CCACCCTGTTGCTGTA-3'. PCR reactions were performed in a Primus thermal cycler (MWG-Biotech, Germany) at 94°C for 1 minute, 30 cycles at 94°C for 20 seconds, 57°C for 20 seconds, and 72°C for 30 seconds, followed by an extension step at 72°C for 5 minutes. After PCR, 10 L of the samples was electrophoresed in 1.5% agarose gels and visualized by ethidium bromide staining. All reactions included negative controls where RNA was used as template.

4) Immunohistochemical staining

The immunohistochemical staining was performed on paraffin-embedded tissue and sections were scored positive as previously reported.(*19*) The protein level of *ERα* was measured immunohistochemically by a combination of the staining intensity and the percentage of positively stained cells. In brief, a score of 0 to 3 for the carcinoma in the majority of the entire section was given (0, no staining; 1, weak; 2, intermediate; 3, strong). The percentage of positively stained cells was an average after counting the stained and the total number of cells from four high-magnification fields with the software IMAGE-PRO PLUS 4.1 (Media Cybernetics, Silver Spring, MD). The ERα protein level was expressed as the product of the staining intensity and the percentage of staining cells.

5) Statistical analysis

The chi-squared test and Fisher's exact test were used to analyze the differences in the rate of each variable and the student t-test was to detect differences in the mean values of variables. P-values <0.05 were considered to be statistically significant. All calculations were performed using SPSS for Windows release 7.0 (SPSS Inc., Chicago, IL).

RESULTS

1) CpG Methylation of the *ERα* and *ERβ* Gene in the normal tissue and the breast carcinoma

A total of 50 breast cancers and 25 normal tissues were analyzed for the methylation status of *ERα* and *ERβ* gene by bisulfite modification DNA sequencing (Fig. 2A-F). Hypermethylation of the *ERα* and *ERβ* was found in 33 (66.0%) and 25 (50.0%) of 50 breast cancer tissues, respectively. Eleven of

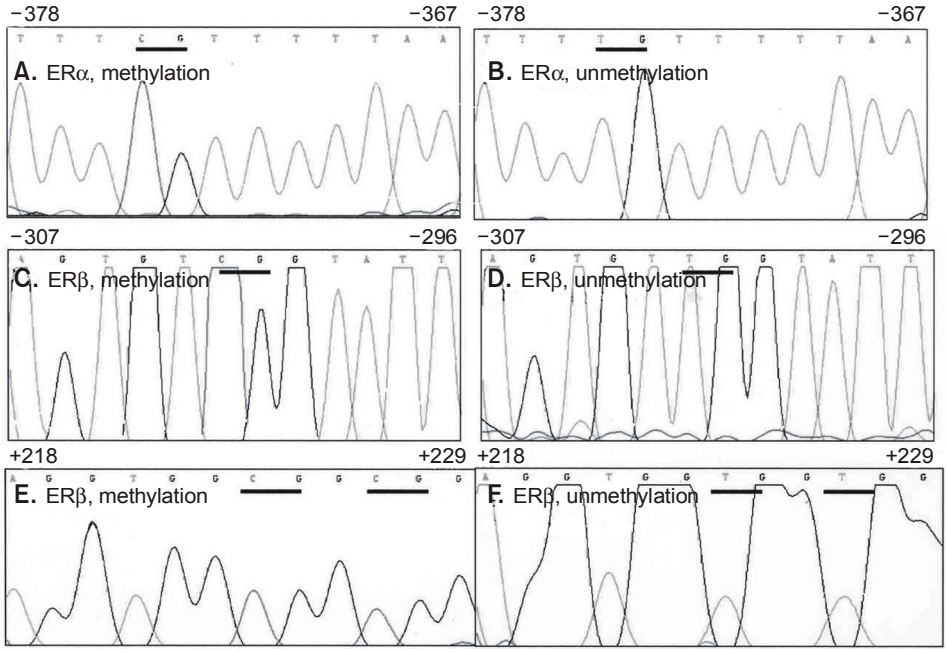

Fig. 2. The methylated (A, C, E) and unmethylated (B, D, F) samples of ERα and ERβ by use of bisulfite genomic sequencing. cDNA extracted from the microdissected tissues was digested with EcoRI and then subjected to bisulfite treatment. After treatment with bisulfite, DNA was PCR amplified and the resulting products were sequenced on an ABI automated sequencer (Perkin- Elmer Corp., Foster City, CA). The original sequences are a follows; A, B-cctcgttcccaat, C, D-agtgtcggcatc, E, F-aggtggcggcgg. All the unmethylated cytosines are changed to T by the bisulfite treatment but not the methylated cytosines. The CpG sites are indicated by underlines. Upper numbers correspond to the numbering of the genes as in Fig. 1.

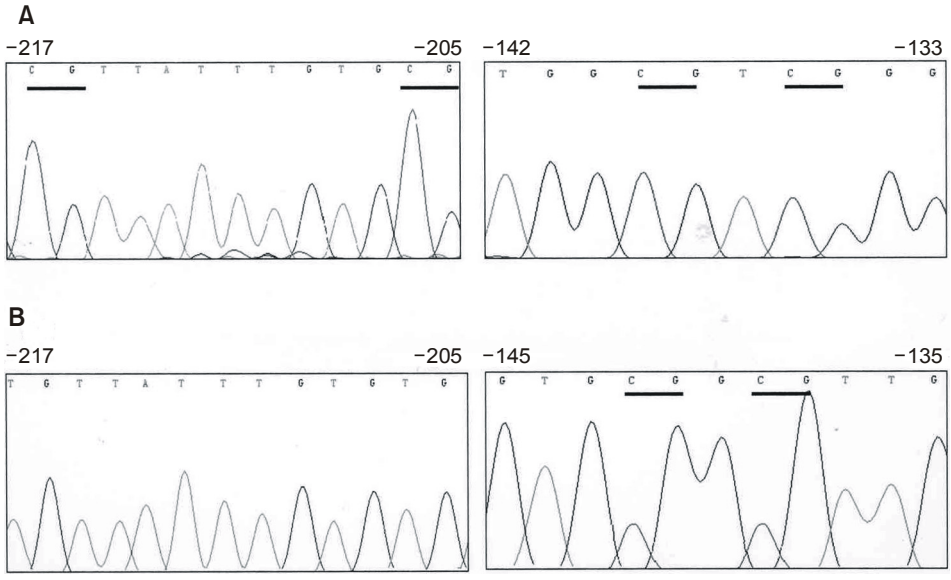

Fig. 3. The methylation profiles of ERβ gene in the cancer (No 44, Table 1, A) and the adjacent normal tissue (B). The methylation pattern in the normal tissue is different from that in the cancer tissue. Upper numbers correspond to the numbering of the genes as in Fig. 1.

50 samples (22.0%) did not show any methylation of either *ERα* or *ERβ*, whereas 19 samples (38.0%) showed methylations of both *ERα* and *ERβ*. In contrast to methylation pattern found in the cancer tissue, no methylation in the CpG site was detected in the normal tissues except one normal tissue adjacent to the cancer. Two CpG sites (-142, -139) in the *ERβ* gene

Table 1. Methylation profiles of CpG sites in the promoter regions of ERα and ERβ

No.	Histology	ERα methylation	ERα mRNA	ERα protein level[a]	ERβ methylation	ERβ mRNA
1	Invasive ductal	1,2,3,4,6[b]	-[c]	0	1,3[b]	-
2	Invasive ductal	1,2,3,5,7	-	0	1,2,3,23,24,25,27,32,33,34	-
3	Cribriform	3,8	-	0	3,38	-
4	Invasive ductal	1,2,3,4,5,7,8	-	0	3,32,37	-
5	Invasive ductal	None[d]	++	60	23	+
6	Invasive lobular	None	++	21	None	++
7	Invasive ductal	None	++	84	None	++
8	Invasive ductal	1,3,4,5,7	-	0	3,28	-
9	Papillary	1,2,6,7,8	-	0	None	++
10	Invasive lobular	4	+	18	None	++
11	Invasive ductal	1,2,3,4,8	-	0	25	-
12	Invasive ductal	1,2,3,4,5,6,7,8	-	0	None	++
13	Invasive ductal	None	++	45	30,37,38,39,40,41	-
14	Invasive ductal	1,2,3,4,7	-	0	38	-
15	Invasive ductal	1,2,3,5	-	15	None	++
16	Invasive ductal	1,2,3,4,6,8	-	0	1	-
17	Invasive ductal	None	++	30	1,3,37	-
18	Invasive ductal	None	+	0	None	+
19	Invasive ductal	1,2,3,5	-	0	23,28	-
20	Invasive ductal	1,2,3,4,7,8	-	0	None	++
21	Papillary	None	++	15	3,32	-
22	Invasive lobular	None	++	69	None	++
23	Invasive ductal	None	+	6	1	-
24	Invasive ductal	8	+	15	1,2,3,4	-
25	Invasive ductal	1,2,3,4,5,6,7,8	-	0	None	++
26	Invasive ductal	1,2,3,4,5,6,7,8	-	0	None	++
27	Invasive ductal	1,2,3,5	-	0	None	+
28	Invasive ductal	1,2,3,4,5,7	-	0	None	++
29	Invasive ductal	1,2,3,6	-	3	None	+
30	Invasive ductal	1,3,6,8	-	0	20,36	+
31	Invasive ductal	3,8	NA	0	None	NA
32	Invasive ductal	5	NA	3	1,5,23	NA
33	Medullary	6,7	NA	9	4,32	NA
34	Invasive ductal	1,2,3,4,5	NA	0	None	NA
35	Invasive ductal	None	NA	72	None	NA
36	Invasive ductal	1,2,3,4,6,7,8	NA	6	4,6	NA
37	Invasive ductal	None	NA	54	None	NA
38	Invasive ductal	1,3,5,6	NA	0	None	NA
39	Invasive ductal	None	NA	0	None	NA
40	Papillary	1,2,3,5,6,8	NA	6	1,3	NA
41	Invasive ductal	1,2,3,5	NA	0	37,38,39,40	NA
42	Invasive ductal	None	NA	30	None	NA
43	Invasive ductal	None	NA	60	None	NA
44	Invasive ductal	6, 7	NA	24	1,3,4,10,11	NA
45	Invasive ductal	1,2,3,4,5,6,8	NA	0	32, 37, 38	NA
46	Invasive ductal	None	NA	72	1,3,32	NA
47	Invasive ductal	None	NA	150	None	NA
48	Invasive ductal	1,2,3,4,5,6,7,8	NA	0	None	NA
49	Medullary	None	NA	180	None	NA
50	Invasive ductal	1,2,3,5	NA	0	1,2,3,4	NA

[a]The ERα protein level was expressed as the product of the staining intensity and the percentage of staining cells. [b] indicates the numerical order of CpG out of total CpG sites located in the promoter regions of ERα (8 CpG's) and ERβ (45 CpG's) as in Fig. 1A and Fig. 1B. [c]: -, negative; +, weak expression; ++ = positive expression. [d]denotes for absence of the methylated CpG.

were shown to be methylated in that normal tissue, which was different from the methylation pattern found in the cancer tissue (Fig. 3, Table 1, No 44).

Among all methylation-positive cancers analyzed, -363 site out of total 8 CpG sites in the *ERα* gene was the most frequent site of methylation followed by -398 and -375 sites (Fig. 4A). The methylation in the *ERβ* gene was identified as follows in the order of frequency: -217, -302, +160, +224, and +227 (Fig. 4B). Surprisingly, -375 and -363 CpG sites in the *ERα* gene are located in close proximity to the CCAAT box, while the frequent methylated CpG sites in the *ERβ* gene are corresponding to the site at or adjacent to the binding sites of GATA (-217, -302) and Sp1 (+160, +224, +227) (Fig. 4A and Fig. 4B). The methylations at or adjacent to the potential binding sites were observed in 29 cases (87.9%) of 33 *ERα* methylated cancers, and twenty-one (84.0%) out of tumors with *ERβ* methylation, respectively (Table 1).

2) Correlation between hypermethylation and differential expression of *ERα* and *ERβ*

To study whether the aberrant methylation actually correlates with gene silencing, the expression status of *ERα* and *ERβ* was determined by RT-PCR for 30 tumors with (*ERα*, 21 cases; *ERβ*, 16 cases) or without methylation of these loci (*ERα*, 9 cases; *ERβ*, 14 cases). Representative results for mRNA expression of *ERα* and *ERβ* are shown in Fig. 5. The expression level of ERα protein was also measured for all the 50 cases by immunohistochemical staining, as described in the section of Materials and Methods (Table 1). As a result, the tumors that showed aberrant methylation of *ERα* and *ERβ* did not express mRNA, compared with unmethylated cases (Fig. 5A and 5B, Table 2: $P < 0.01$). In particular, the tumors with methylation at position outside the binding sites of the transcriptional factors (*ERα*, #10, #24; *ERβ*, #5, #30) showed a low mRNA band, whereas those at or near the binding sites

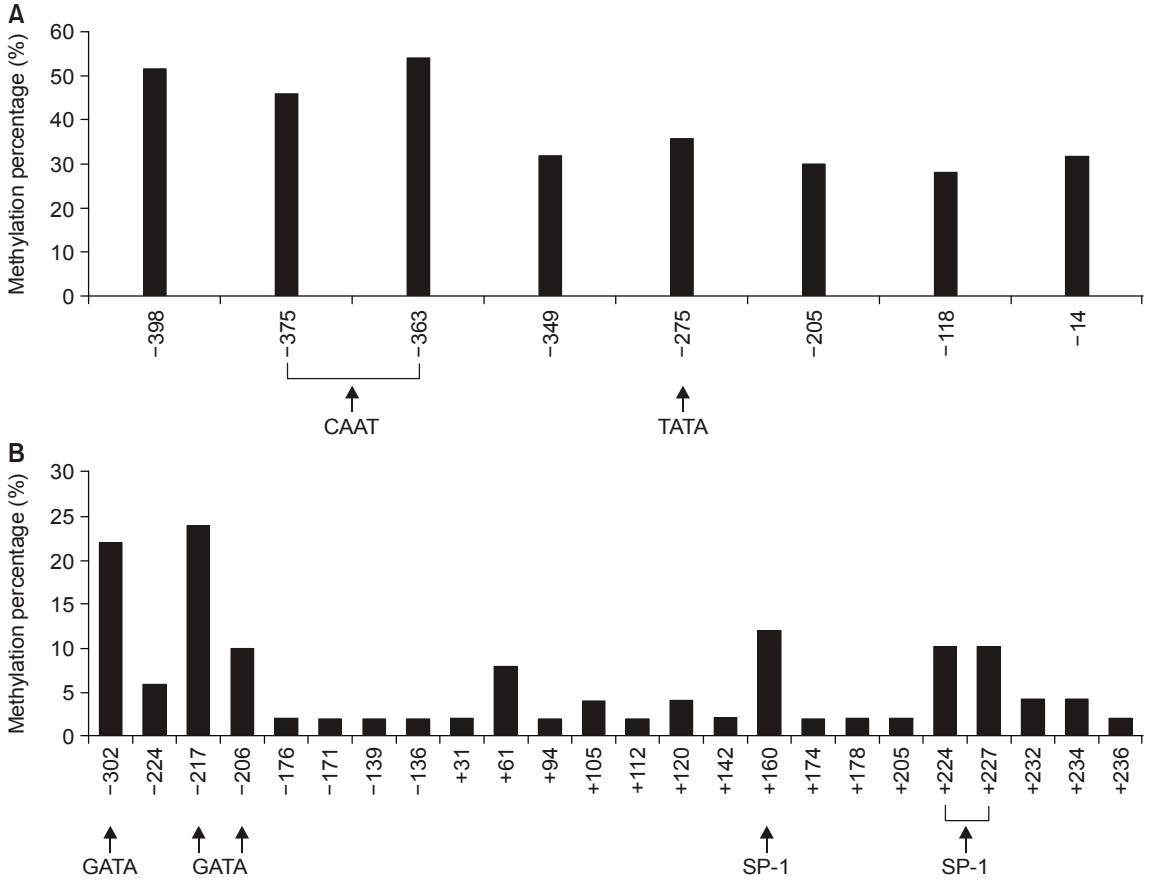

Fig. 4. The percentage of methylation at individual CpG site in *ERα* (A) and *ERβ* (B) genes. The CpG sites at or near the putative binding sites of transcriptional factors are noted by arrows and their matching transcriptional factors. Numbers on the X-axis correspond to the numbering in Fig. 1A and Fig. 1B.

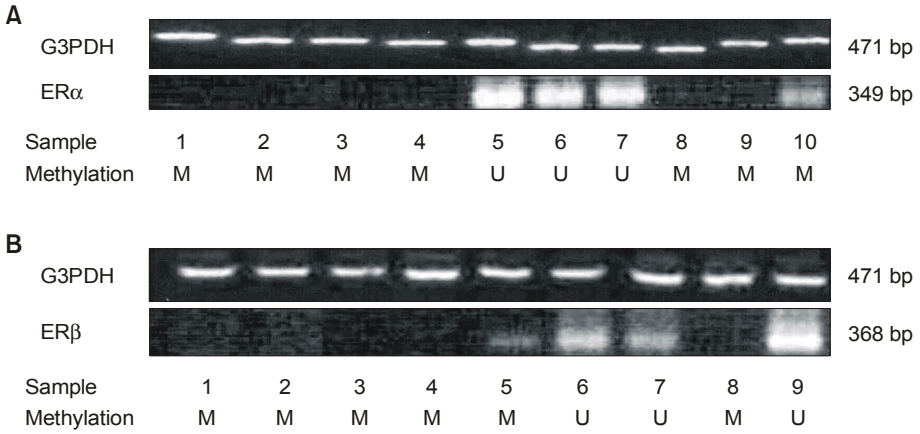

Fig. 5. Representative figures of RT-PCR products for *ERα* (A) and *ERβ* (B) mRNA in the breast cancer tissue. Total cellular RNA (5g) was reverse transcribed, and the resulting cDNA was amplified by PCR using specific primers for each gene. *G3PDH* expression demonstrates relatively equal amounts of initial mRNA. The results are summarized in Table 1. The letters M and U in the row of methylation indicate the presence and the absence methylation, respectively. In addition, M* means methylation outside the transcriptional factor binding sites.

Table 2. The correlation of methylation and transcriptional expression in *ERα* and *ERβ* gene

	RNA expression	P-value
ERα methylation(+)(N=21)	2 (9.5%)	$<0.01^a$
ERα methylation(-)(N=9)	9 (100%)	
ERβ methylation(+)(N=16)	2 (12.5%)	$<0.01^b$
ERβ methylation(-)(N=14)	14 (100%)	

Total number presented in this table was not 50 but 30 because RT-RCR was performed in 30 frozen tissues. Statistical methods: [a]Fisher's exact test, [b]chi-squared test

did not. The incidence of methylated case was significantly correlated with negative expression of ERα protein (24.2% vs 88.2% $P<0.01$). The expression level of ERα protein was lower among patients with *ERα* methylation than without methylation (2.82±1.08 vs 55.76±11.95, $P<0.01$), and also four tumors with *ERα* methylation outside the binding sites showed higher level of expression than those at or near the sites (16.50±3.12 vs 0.93±0.56, P=0.01).

DISCUSSION

Bisulfite genomic sequencing is the method of choice for the determination of methylation status with single-base resolution and eliminates the possibility of incomplete digestion by the methylation sensitive restriction enzymes, which would yield an inaccurate picture of DNA methylation.(*20*) And yet, there have been few reports published that the presence of CpG site methylation in *ERα* and *ERβ* gene was confirmed by bisulfite genomic sequencing.(*10*,*18*) The studies presented indicate that the promoter regions of *ERα* and *ERβ* are methylated in the breast cancer tissues and CpG methylation within the promoter region of the genes induces the transcriptional inactivation by use of bisulfite genomic sequencing and RT-PCR. In particular, we established an in vivo evidence that among the methylation positive cancers of *ER* gene, the methylation at or adjacent to the potential binding sites of transcriptional factors occurs in most breast cancers (*ERα* 87.9%; *ERβ* 84.0%). Exploring the previous results on the *ERα* and *ERβ* methylation in the prostatic cancer by the same technique,(*10*,*18*) the most frequent sites of methylation in *ER*α gene was found near the CCAAT box (-375 and -363), whereas methylation of *ERβ* detected at the putative binding site of Sp1 (+224) although a part of the transcriptional factor binding sites in promoter region of *ERβ* was not included in that study.(*18*) We previously reported that *p53* gene is methylated in the breast cancer tissue and the CpG sites with methylation are located at or in vicinity of the potential binding sites of transcriptional factors such as AP1 or YY-1. Mancini *et al.* demonstrated that the methylated CpG in the breast cancer tissues occurs at a putative CREB (cAMP-responsive element binding) transcription factor binding site in the BRCA1 promoter and this site is sensitive to the site-specific CpG methylation.(*16*) Similar methylation interference studies directed at other genes have shown that CpG methylation of CREB abolishes CREB binding.(*21*) Moreover, the most frequently methylated CpG of

connexin gene, a putative tumor suppressor gene, was reported to be in an Sp1 site known to be important for *connexin 26* gene expression in the breast cancer tissue.(*22*)

Two major hypotheses have been proposed to explain the finding that DNA methylation is responsible for gene silencing. First, the binding between the methylated DNA and a kind of methyl-CpG binding proteins induces the transcriptional suppression, leading to the alteration of the chromatin structure.(*7*) Second, DNA methylation at or in vicinity to the putative binding sites of transcriptional factors play a role by impeding specific interactions between transcription factors and their matching DNA control elements.(*23*) Since not many transcription factor binding sites contain CpG, the second hypothesis has been considered to be infrequently involved. Recently, the methylation at a CpG site two base pairs upstream of CCAAT box was reported to hinder the binding of the transcriptional factor CBF to CCAAT box, leading to suppression of hMLH1 expression.(*15*) This mechanism may be mediated by the induction of the changes in the DNA double helix configuration, or by interfering with the binding of cofactors. Furthermore, the methylation seems to be stabilized when the promoter region is confined by the spread of DNA methylation near or at its transcription factor binding sites and to occur in the organized and sequential, but not the chaotic, manner.(*24*) The potential role of the hypermethylation at the putative binding sites needs further investigation, as this might be a clue to the initiation or spread pattern of CpG methylation in the human cancer.

Our second concern is that CpG methylation at specific sites might exert an effect on the transcriptional silencing. In this study, the tumors with methylation outside the binding sites in *ERα* and *ERβ* gene showed a mRNA expression, whereas those at or near the binding sites did not. And also the tumors with methylation of *ERα* at other than putative binding sites had higher level of protein expression than those at or near the sites (16.50±3.12 vs. 0.93±0.56, P=0.01). In spite of the small number of samples, this finding implies that the DNA methylation present at or in close proximity to putative binding sites of the transcriptional factors may more strongly exert its effect on transcriptional silencing. The transcriptional silencing seems to be diverse according to the location of methylated CpG. Deng *et al.* demonstrated that the extent of gene silencing by methylation in *hMLH1* promoter region shows region-specific pattern in the colorectal cell line, and CpG methylation at the certain part of the promoter region is not critical in silencing the gene expression,(*15*) supporting our results.

In addition as illustrated in the experiment on the expression of ERβ in the rodent mammary gland, a considerable numbers of proliferating cells contain ERβ protein.(*25*) The mechanism of the supposed effect of ER on cell proliferation is unknown. Functional studies in cancer cell lines have shown differences in stimulating the transcriptional activating function (AF1) of the receptor and activator protein (AP1) cross talk(*26*); however, the master genes involved in the mitogenic activity of estrogens are still being debated.

In conclusion, aberrant DNA methylation at regulatory regions in *ERα* and *ERβ* genes plays a role in the transcriptional silencing of the genes within subsets of breast carcinomas. Although the number of samples was so limited that our results might take on a sort of hypothesis at this time, it is suggested that the DNA methylation in the *ERα* and *ERβ* appears to be most often present in the CpG sites at or near the putative binding sites of the transcriptional factors, and this type of methylation may influence more significantly on transcriptional silencing. This finding is believed to be a clue to the initiation or spread pattern of CpG methylation in the human breast cancer.

ACKNOWLEDGEMENTS

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