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Epigenetic silencing of the sulfotransferase 1A1 gene by hypermethylation in breast tissue

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Abstract. Sulfotransferase 1A1 (SULT1A1) is reported to be involved in the conjugation with sulfate, resulting in the inactivation of estrogens. Aberrant methylation of promoter CpG islands is known to be responsible for the alteration and silencing of the gene in cancers. This study was intended to evaluate the methylation status and transcriptional activity of SULT1A1 in breast cancer tissue (n=56), benign breast tissue (n=20) and morphologically normal breast tissue (n=20), examined by bisulfite genomic sequencing and reverse transcription (RT)-PCR. As a result, the methylation of the proximal promoter (P1) was identified in 64.3% of breast carcinomas, 15% of normal and 20% of benign breast tissues. In terms of the distal promoter (P0), 32 of 56 cancer tissues (57.1%) were methylated, while 4 normal (20%) and 6 benign tissues (30%) were methylated. Breast cancer tissue showed a higher methylation rate of SULT1A1 than normal and benign tissue at both P1 (p=0.001) and P0 (p=0.006) promoters with statistical significance. Furthermore, cancer tissue showed a higher methylation density rate than normal and benign breast tissue at both P1 and P0 promoters (P1, p=0.001; P0, p=0.001). The tissues that showed aberrant methylation of SULT1A1 did not express mRNA significantly, compared with the unmethylated cases (P1, p=0.003; P0, p=0.023). Although the number of samples was relatively small, our results suggest that DNA methylation in the SULT1A1 gene appears to be present in breast tissue including cancer and methylation significantly impacts transcriptional silencing of the gene. In addition, it can be suggested that progressive SULT1A1 methylation within the promoter area of the gene occurs during breast carcinogenesis.

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Abbreviations: CpG, cytosine guanine dinucleotide; SULT, sulfotransferase; RT, reverse transcriptase; PCR, polymerase chain reaction

Key words: sulfotransferase, methylation, breast cancer

Introduction

Carcinogen-metabolizing enzymes are involved in the activation and deactivation of a diverse group of chemical carcinogens (1,2). Sulfation is generally considered to be a high-affinity metabolic reaction (i.e., likely to be important in low exposure situations), which can result in either bio-inactivation or detoxification of xenobiotics as well as important endogenous chemicals such as iodothyronines, catecholamines and estrogens (3). Conjugation with sulfate gives rise to the inactivation of estrogens because the addition of the charged sulfonate group averts the binding of the steroid to its receptor, thereby terminating its mitogenic effects.

Sulfation reactions are catalyzed by the gene products of the cytosolic sulfotransferase (SULT) gene superfamily, which in humans comprises of at least 10 genes falling into three subfamilies (4,5). To date, at least seven characterized isoforms are known to make up the human phenol SULT family (6). In adult human tissue, the product of the SULT1A1 gene is the major form of phenol SULT. SULT1A1 protein is found almost ubiquitously in human tissues and is known to metabolize hydroxylated aryl amines (7) and heterocyclic amines (8).

The SULT1A1 gene has two specific promoters, distal (P0) and proximal (P1), which induce cDNAs heterogeneous in the 5'-untranslated region (UTR). Tissue specific promoter usage has been suggested for the separated cis-acting promoter sequences (9,10).

There is promising evidence that the *de novo* methylation of promoter cytosine guanine dinucleotide (CpG) islands, contributes to the alteration of transcriptional expression in cancer and is associated with gene silencing (11). To the best of our knowledge, no reports have been published with reference to the methylation of the SULT1A1 gene in bona fide human tissue.

Therefore, we assessed the methylation status of both proximal (P1) and distal promoters (P0) of the SULT1A1 gene in normal, benign and cancer tissues of the breast. We also evaluated the mRNA expression of the SULT1A1 gene in breast tissue with or without methylation of the gene.

Materials and methods

Tissue samples and nucleic acid extraction. Formalin-fixed and paraffin-embedded specimens were obtained from the

Table I. The clinicopathological features of the cancer patients.

	Cancer (n=56)
Mean tumor size (cm)	2.71±0.32
Presence of node metastasis (%)	21 (37.5)
Stage (%)	
Stage I	19 (33.9)
Stage IIA	26 (46.4)
Stage IIB	11 (19.7)
Nuclear grade (%)	
Grade 1	14 (25)
Grade 2	23 (41.1)
Grade 3	19 (33.9)

patients who received surgery at the National Cancer Center, Korea, between 2000 and 2001. The patients' informed consents for participation in the study were obtained. The normal tissue comprised 12 samples adjacent to the cancer, and 8 samples far from the cancer. The benign breast tissue was obtained from 20 histologically proven benign patients and classified as follows: fibrocystic disease (n=11), fibroadenoma (n=6), intraductal papilloma (n=3). All 56 cancer samples were infiltrating ductal carcinomas in their histological type and their nuclear grades were as follows: grade 1 (n=14), grade 2 (n=23), and grade 3 (n=19) (Table I).

Thick sections (5 μm) were cut from formalin-fixed, paraffin-embedded tissues and mounted on slide glasses. Microdissection was performed as described previously (9), and genomic DNAs were extracted from the microdissected tissues after lysis in 80 μl of buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5% Tween-20, 200 μg/ml of proteinase K) at 55˚C for 72 h.

Total RNAs were isolated from 30 frozen breast tissues (4 normal, 5 benign, 21 cancer) by using a FastRNA Green kit (Qbiogene, Carisbad, CA) according to the supplier's protocol and were suspended in 50 μl of RNase-free water.

Bisulfite genomic sequencing. Bisulfite genomic DNA sequencing was carried out as previously described (12) with a minor modification. Briefly, genomic DNA extracted from the microdissected tissues was digested with *Eco*RI and then subjected to bisulfite treatment. The bisulfite-treated DNA was subjected to PCR to amplify the SULT1A1 promoter region. The promoter region spanning exons 1B and 1A (base no. 2328 through 3629 of the Genbank accession no. U52852) and containing 16 CpG sites was sub-divided into five fragments of 394, 209, 205, 211, and 167 bp and each fragment was amplified by two rounds of primary and nested PCR (Fig. 1). The primer sets for the primary and nested PCR of the DNA fragments are as follows: fragment 1, 5'-TTTGGTAGGGTGGAGTTTAGGG-3', 5'-CCTTAATATACCAACTAAAAAC-3' and 5'-GGGGTAGGTTAGGAGTTTAGTG-3', 5'-CCATTACCCTCCTTAATATACC-3'; fragment 2, 5'-TTTAGTTGGTATATTAAGGAGG-3', 5'-CCACTATATCACTCACCTAAAC-3' and 5'-ATTAAGGAGGGTAATGGAGAAG-3', 5'-CTAAACTCTTAAAAACCTAACC-3'; fragment 3, 5'-GGTTAGGTTTTTAAGAGTTTAG-3', 5'-AAAATCCCACAACACTCCAACC-3' and 5'-GTTTAGGTGAGTGATATAGTGG-3', 5'-TAACAACAAAACCTAACCTCCC-3'; fragment 4, 5'-TAGTTTTGGTTTTTAGTAGTAG-3', 5'-AAACTCTAAAACCTTCCTATAC-3' and 5'-AGTAGTTTAGTTTTTTAATGGG-3', 5'-CTATACTATCTCCCTACCAACC-3'; fragment 5, 5'-GGTTGGTAGGGAGATAGTATAG-3', 5'-ATAATTCCCCAACCTAACCTCA-3' and 5'-TAGTATAGGAAGGTTTTAGAGT-3', 5'-CTAACCTCACCTTTCATTCACC-3'. The PCR conditions were 94˚C for 2 min, 30 cycles of 94˚C for 20 sec, 55˚C for 20 sec, and 72˚C for 30 sec, with a final extension at 72˚C for 5 min. The resulting products were subjected to agarose gel electrophoresis and were purified using a 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). The DNA sequences were confirmed by analyzing each PCR product in both directions and at least three PCR products were analyzed for each cancer tissue. A single 'C' at the corresponding CpG site was considered as 100% methylation, a single 'T' as no methylation, and overlapping 'C' and 'T' as partial methylation. In the last case, the percentage of methylation was expressed as the ratio of 'C' peak value to the peak values of 'C' plus 'T'. The methylation density rate of individual tumor specimen was calculated as percentages of 5-methyl cytosine among the whole cytosine residues of the promoter region under investigation.

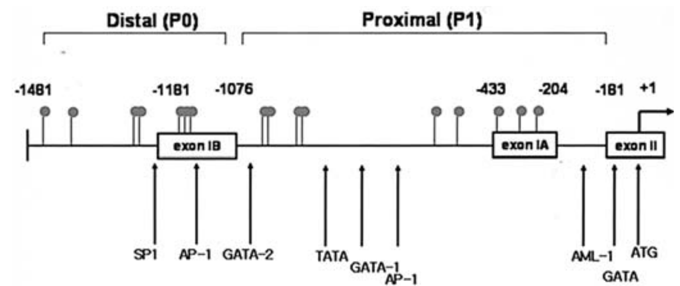

Figure 1. Structure of the 5'-flanking region of the human SULT1A1 gene (Genbank accession no. U52852). The putative transcriptional factor binding sites are indicated by the arrows. The numbers on the upper column of the figure are according to the numbering scheme regarding the transcriptional start site as +1. The individual CpG sites are indicated as gray circles.

RT-PCR. First-stranded cDNA was synthesized from 5 μg of total RNA using a reverse transcription kit (Promega, Madison, WI) according to the manufacturer's protocol. To amplify the double-stranded cDNA, PCR was performed in a 50 μl reaction mixture containing 1 μl of the reverse-transcribed cDNA, 5 μl of 10X PCR buffer, 1.25 mM dNTP, 1.5 mM $MgCl_2$, 1 μM of each primer and 1 U of *Taq* polymerase (Roche Biochemicals, Germany). The two transcripts derived from the two promoters of the SULT1A1 gene, the distal and proximal promoter, were amplified by PCR using transcript-specific upstream primers and a common downstream primer. The upstream primer sequences of the transcripts from the distal and proximal promoters are 5'-AGGCCAGGTTCCCAAGAGCT-3' and 5'-GTAAGGGAACGGGCCTGGCT-3', respectively. The

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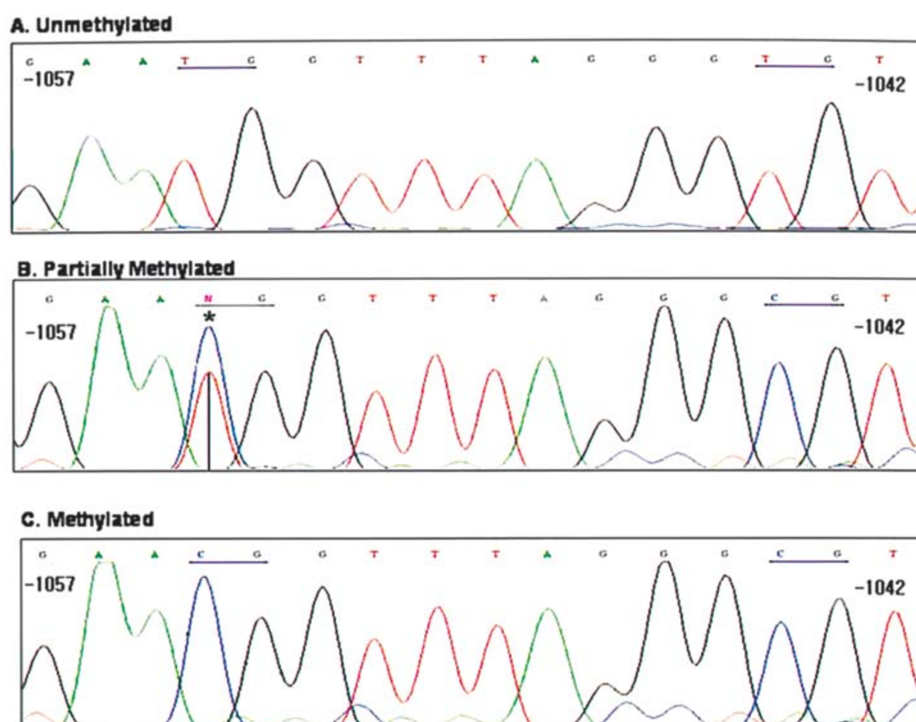

Figure 2. Examples of direct sequencing chromatogram. A CpG site is underlined, and the cytosine (C) and thymine (T) peaks are indicated by asterisks. A, no methylation; B, partial methylation; C, complete methylation of the SULT1A1 gene.

downstream primer sequence is 5'-AGTCGTGGGGCCGGT GTGTC-3'.

PCR was performed in a 50-μl reaction mixture containing 1 μl of the reverse-transcribed cDNA, 5 μl of 10X PCR buffer, 1.25 mM dNTP, 1.5 mM $MgCl_2$, 1 μM of each primer and 1 U of *Taq* polymerase (Roche Biochemicals). To verify the integrity of the mRNA, the *G3PDH* gene was amplified by means of the following primers: *G3PDH*F, 5'-ACCACAGTC CATGCCATCAC-3'; and *G3PDH*R, 5'-TCCACCACCCTG TTGCTGTA-3'. The PCR reactions were performed in a Primus thermal cycler (MWG-Biotech, Germany) at 94˚C for 1 min, 30 cycles at 94˚C for 20 sec, 57˚C for 20 sec, and 72˚C for 30 sec, followed by an extension step at 72˚C for 5 min. After PCR, 10 μl of each sample was electrophoresed in 1.5% agarose gel and visualized by ethidium bromide staining. All reactions included negative controls where RNA was used as a template.

Statistical analysis. The χ^2 test was used to analyze the differences in the rate of each variable and the Student's test and ANOVA test were used to detect differences in the mean values of the variables, appropriately. 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

The methylation rate and methylation density rate of the SULT1A1 gene in breast tissue. In order to determine the rate of aberrant DNA methylation in this gene, we studied the methylation pattern by bisulfite sequencing of the promoter

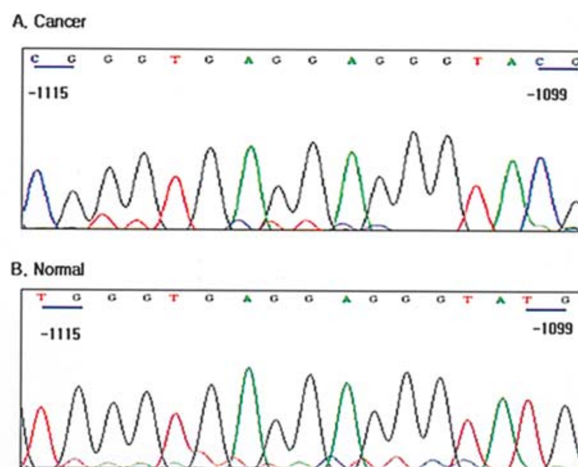

Figure 3. The methylated sample of the SULT1A1 gene in the cancer (A) and its corresponding normal tissue (B) by use of bisulfite genomic sequencing. All the unmethylated cytosines are changed to the thymine by the bisulfite treatment but not the methylated cytosines. The CpG sites are underlined.

sequence. Fig. 2 demonstrates a representative bisulfite DNA sequencing of the SULT1A1 gene that was studied. No significant difference in the mean ages was identified among the three groups in this study, indicating that the age-related effect on the methylation did not influence the methylation rate (p=0.666) (Table II). We have identified methylation of the P1 promoter in 64.3% of the breast carcinomas, 15% of normal and 20% of benign breast tissues. Regarding the P0 promoter, 32 of 56 cancer tissues (57.1%) were methylated, while 4 normal (20%) and 6 benign tissues (30%) were methylated (Fig. 3). The methylation rates of both P1 (p=0.001)

Table II. The methylation patterns of the SULT1A1 gene in normal tissue, benign breast disease and cancer.

	Normal (n=20)	Benign (n=20)	Cancer (n=56)	p-value
Age (years)	45.77±10.61	45.23±9.78	43.77±8.81	0.666[a]
SULT1A1 methylation rate (%)				
P1	3 (15)	4 (20)	36 (64.3)	0.001[b]
P0	4 (20)	6 (30)	32 (57.1)	0.006[b]
Mean methylation density rate (%)				
P1	6.11±2.61	10.56±5.06	40.28±3.13	0.01[c, d]
				0.01[c, e]
				0.440[c, f]
P0	7.85±3.51	7.14±4.94	47.19±3.12	0.01[c, d]
				0.01[c, e]
				0.893[c, f]

P1, proximal; P0, distal. Statistical method: [a]ANOVA, [b]χ^2 test, [c]Student's t-test. [d]Between cancer and normal, [e]between cancer and benign, [f]between normal and benign.

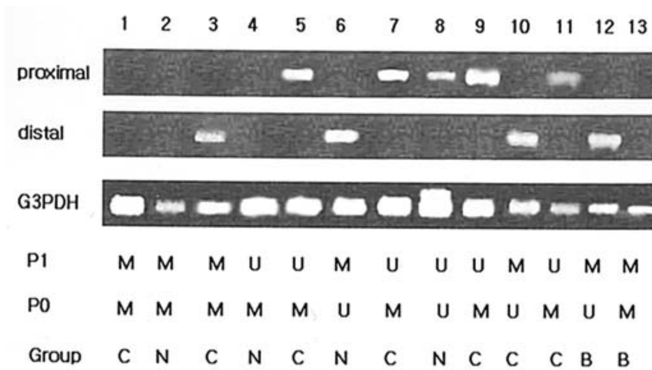

Figure 4. Representative figures of RT-PCR products for SULT1A1 mRNA in breast tissue. Total cellular RNA (5 μg) was reverse-transcribed, and the resulting cDNA was amplified by PCR using specific primers for each gene. G3PDH expression demonstrates relatively equal amounts of initial mRNA. The results are summarized in Table III. M and U in the row of P0 and P1 indicate the presence and absence of methylation, respectively. N, B and C in the group row indicate normal, benign and cancer tissues, respectively.

and P0 (p=0.006) promoters of the SULT1A1 gene were significantly higher in malignant tumors than in benign and normal tissues (Table II).

The mean methylation density rates of the P1 and P0 promoters in normal tissue were 6.11±2.61% and 7.85±3.51% respectively, and those in benign tissue were 10.56±5.06% and 7.14±4.94%, respectively. In contrast, the mean methylation density rates of P1 and P0 promoters in breast cancer tissue were 40.28±3.13% and 47.19±3.12% respectively. Cancer tissue showed a higher methylation density rate than normal and benign breast tissues at both P1 and P0 promoters (P1, p=0.001; P0, p=0.001) (Table II).

Transcriptional silencing by the SULT1A1 methylation in breast tissue. In order to examine the effect of SULT1A1 promoter methylation on transcriptional silencing, we tested for SULT1A1 mRNA expression in normal, benign and cancer tissues by RT-PCR. Representative results for the mRNA expression of the SULT1A1 gene are shown in Fig. 4. In terms of the P1 promoter, the mRNA expression of the SULT1A1 gene was evident in 9 of 14 tissues without methylation, whereas only 2 cases were found to have mRNA expression in 16 cases with methylation (Fig. 4). Tissues from aberrant methylation of the SULT1A1 gene did show a significantly lower rate of mRNA expression, as compared to unmethylated cases (p=0.003) (Table III). A similar negative correlation was observed at the P0 promoter region (p=0.023).

Table III. CpG methylation and mRNA expression of the SULT1A1 gene in breast tissue.

	SULT1A1 met[a] (+)	SULT1A1 met (-)	p-value
P1	(n=16)	(n=14)	
No expression (%)	14 (87.5)	5 (35.7)	0.003
P0	(n=17)	(n=13)	
No expression (%)	14 (82.4)	5 (38.5)	0.023

P1, proximal; P0, distal. [a]met, methylation.

Discussion

Gene silencing through aberrant DNA methylation of the CpG island of a gene has been reported in many tumor types, including breast cancer (11). Current methylation studies are focusing on tumor suppressor genes or genes related to tumor growth and invasion. However, the epigenetic events of the

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ated to drug metabolizing enzymes remain to be d in breast tumorigenesis. Here, we demonstrated that DNA methylation in the SULT1A1 gene, a member of the drug metabolizing enzyme gene family, was increased in breast cancer tissue when compared with benign and normal tissue. This methylation significantly impacts transcriptional silencing of that gene. To the best of our knowledge, this is the first study on methylation of the SULT1A1 gene in human tissue.

Sulfation gives rise to the inactivation of estrogens, which most human breast cancers require for the maintenance of growth during their development, thereby leading to the termination of its mitogenic effects. Sulfation reactions are catalyzed by the products of the SULT gene superfamily which, in humans, comprises of at least 10 genes falling into three subfamilies (4,5) and the major form of phenol SULT in the adult human is the product of the SULT1A1 gene. In this study, breast cancer tissue showed higher epigenetic gene silencing of the SULT1A1 gene in both proximal ($p=0.001$) and distal promoters ($p=0.006$) than normal and benign breast tissues. However, at present, the mechanism through which SULT1A1 activity influences the development of breast cancer is not known, although a polymorphism in the SULT1A1 gene (Arg213His) has been recognized (13). Individuals homozygous for the histidine allele have lower platelet SULT activity *in vitro* than wild-type and heterozygous individuals (13,14). Furthermore, the SULT1A1 variant allele is related with an elevated breast cancer risk among postmenopausal women (15).

It can be hypothesized that certain genetic and/or epigenetic events, including genetic polymorphism and DNA methylation, which lead to deactivation or a decrease in activity of the SULT1A1 gene, thereby interfering with the metabolism of endogenous steroid hormones, increase the probability of acquiring a mutation in oncogenes or tumor suppressor genes.

Interestingly, we also found CpG methylation in the promoters from normal and benign breast tissues, although the methylation frequency was lower than that of cancer tissue. This is in line with the previous result reporting that gene methylation, including that of tumor suppressor genes, is present in benign and normal breast tumors (16). In this study, the age effect in the methylation does not appear to be present because there was no significant difference in age among the three groups (17). Our data may signify that epigenetic SULT1A1 silencing occurs early during breast tumorigenesis and is required for the development of breast cancer.

In addition, our present study showed that CpG methylation of the SULT1A1 gene in cancer is denser than in normal and benign tissues. The higher methylation density of the SULT1A1 gene in cancer tissue and comparatively lower densities in normal and benign breast tissues implicates that the methylation process is dynamic and ever-increasing within the CpG sites of the promoter region during the development of breast cancer. This result is similar to RASSF1A gene methylation, which progressively accumulates in sequential passages of human mesothelial cells transfected with SV40 (18). In addition, another study reported the methylation spreading of the E-cad and VHL CpG islands in cultured fibroblasts overexpressing DNA methyltransferase DNMT1 (19). It is worthwhile to note that our results came from methylation analysis comparing tumor samples and corresponding normal tissue from the same patient.

These *in vitro* methylation spreads take place in a time-dependent manner, probably starting from the outer flanks of the CpG islands and gradually moving toward the transcription start sites of these genes (19). Increased density of DNA methylation in the promoter may attract the binding of methyl-CpG binding proteins, such as MeCP2 and other repressors, which may directly work together with histone methyltransferases or deacetylases, resulting in an alteration of the histone code (11). Yan *et al* suggested that aberrant DNA methylation, in company with a combination of histone modifications, establishes a repressive heterochromatin to result in stable silencing of the gene in breast cancer cells (20).

In conclusion, the studies reported here represent a first step in investigating whether an epigenetic event in the human SULT1A1 gene truly exists during breast carcinogenesis. The CpG methylation rate of the SULT1A1 gene in cancer was shown to be denser than in normal and benign tissues. On the basis of these results, we propose that a methylation wave occurs and progressively extends within the promoter area of the SULT1A1 gene during breast carcinogenesis.

Acknowledgements

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