Promoter hypomethylation of the N-acetyltransferase 1 gene in breast cancer

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Abstract. Arylamine N-acetyltransferase type 1 (NAT1) is reported to be involved in the transfer of an acetyl group from acetyl-CoA to the terminal nitrogen of hydrazine and arylamine drugs or carcinogens. Gene-specific hypomethylation frequently occurs in a range of cancers and hypomethylation of the genes often correlates well with increased transcription levels. This study was conducted in order to evaluate the methylation status and the transcriptional activity of NAT1 in breast cancer tissues (n=72), benign breast tissues (n=31) and morphologically normal breast tissues (n=30). Our findings showed that the methylation of the NAT1 gene was identified in 39 of the breast carcinomas (54.2%), 23 normal (76.7%) and 25 benign breast tissue samples (80.6%). The breast cancer tissues showed significantly lower methylation rates of the NAT1 promoters than the normal and benign tissues (P=0.012). Furthermore, cancer tissues showed lower methylation density rates than normal and benign breast tissues (P=0.012). The tissues that showed aberrant methylation of NAT1 showed significantly less mRNA expression compared with the unmethylated cases by a thousand fold (P<0.001). Twenty cancers from the methylated group showed positive staining for the estrogen receptor (ER) (51.3%), while 72.7% from the unmethylated group stained positive (P=0.063). Our results suggest that DNA hypomethylation in the NAT1 gene appears to be present in cancerous breast tissues thus indicating that this type of methylation may significantly influence the transcriptional activation of the gene. Therefore, hypomethylation of the NAT1 gene plays a significant role in breast carcinogenesis.

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Abbreviations: CpG, cytosine guanine dinucleotide; ER, estrogen receptor; NAT, N-acetyltransferase1; RT-PCR, reverse transcription-polymerase chain reaction

Key words: arylamine N-acetyltransferase type 1, methylation, breast cancer

Introduction

Human NAT1 (arylamine N-acetyltransferase type 1), a phase II drug-metabolizing enzyme, is found in most tissues, where it is responsible for the transfer of an acetyl group from acetyl-CoA to arylamine and hydrazine substrates (1,2), including the folate catabolite p-aminobenzoylglutamate (3). The gene encoding NAT1 is genetically variant and is located at 8p21.3-22, a site that is often deleted in human tumors (4,5). Together, the protein encoded by the gene is responsible for the acetylation of most exogenous arylamine, heterocyclic amine and hydrazine compounds. These compounds initiate breast cancer in rats and are implicated as factors in the etiology of various human cancers, including breast cancer (1).

The association between the NAT genotype and breast cancer risk has been examined in several studies, though these interactions were only observed among pre- or postmenopausal women (6-10). However, the results are not consistent across the studies and taken together, they do not support a strong role for NAT1 genotypes in breast cancer risk. NAT1 was recently reported to be one of the most consistently up-regulated proteins in breast cancer tissues compared to normal breast tissues (11).

Cytosine methylation of the regulatory sequences of DNA is an epigenetic mechanism that is associated with transcriptional inactivation of genes, while hypomethylation contributes to the activation of transcription (12). The methylation profiling of cancer cells and studies of individual genes reveal that gene-specific hypomethylation at the promoters of the genes occurs frequently in a range of cancerous tissues (13,14). As expected, hypomethylation of specific genes often correlates well with increased transcription levels (12). To the best of our knowledge, there has been no study published concerning the methylation pattern of the NAT1 gene in human tissues.

Therefore, we evaluated the methylation status of the NAT1 gene promoter in tissues from normal, benign and cancer tissues of the breast. We also evaluated the mRNA expression of the NAT1 gene in breast tissues with or

without methylation of the gene. Additional attention was paid to comparing the clinicopathological characteristics between the groups with and without methylation.

Materials and methods

Tissue samples and nucleic acid extraction. Formalin-fixed and paraffin-embedded specimens were obtained from patients who had surgery at the National Cancer Center in Korea, between 2000 and 2004. Informed consent for participation in the study was obtained from each patient. The normal tissues comprised of 21 tissues adjacent to cancer tissues and 9 specimens situated far from cancer tissues. The benign breast tissues were obtained from 31 histologically proven benign patients who had been operated on and were classified as: fibrocystic disease (n=16), fibroadenoma (n=12) and intraductal papilloma (n=3). The histological type of the 72 cancers was infiltrating ductal carcinomas (Table I).

Five-μm thick sections were cut from formalin-fixed, paraffin-embedded tissues and mounted on slide glasses. Microdissection was performed as previously described (15), 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 40 frozen breast tissues (4 normal, 9 benign, 27 cancer) using Trizol (Gibco BRL, Carlsbad, CA) according to the protocol of the supplier and were suspended in 50 μl of RNase-free water.

Bisulfite genomic sequencing. Bisulfite genomic DNA sequencing was carried out as previously described (15) with minor modification. In brief, genomic DNA extracted from the microdissected tissues was digested with EcoRI and then subjected to bisulfite treatment. The bisulfite-treated DNA was subjected to PCR in order to amplify the NAT1 promoter. The promoter region contained six CpG sites and was sub-divided into two fragments of 160 and 155 bp, respectively (Fig. 1). The primer sets were: fragment 1, 5'-TTATGTGTTTTAATTATTTTTG, 5'-ATAAATACAAAAAAAAAACTCC and 5'-TTTGTTTTTTGAATGTTTTTTG, 5'-AAACACACCAAAAAAAAAACAC; fragment 2, 5'-TGAGTTAATTAATTAATGGATG, 5'-AAAAAAATTCCAACTAATATCC and 5'-TGAATTAATATAGTTTTTTGAG, 5'-ACTAATATCCTTTAAAATAAAC. The PCR conditions were 94˚C for 2 min, 30 cycles at 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 purified using a PCR purification 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 to indicate 100% methylation, while a single 'T' indicated no methylation and the overlapping of 'C' and 'T' was regarded as partial methylation. In the latter case, the percentage of methylation was expressed as the ratio of the 'C' peak value to the peak values of 'C' plus 'T'. The methylation density rate of the individual tumor specimens was calculated as the percentage of 5-methyl cytosine among the whole cytosine residues of the promoter region being investigated.

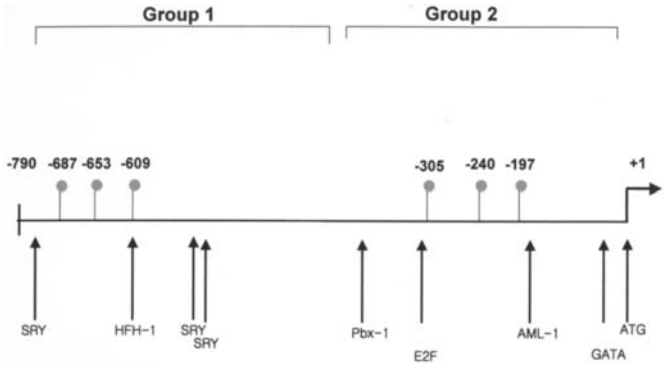

Figure 1. Structure of the 5'-flanking region of the human NAT1 gene (GenBank accession number AY338489). The putative transcriptional factor binding sites are indicated by arrows. The numbers in the upper column are the numbering scheme, with the transcriptional start site regarded as +1. The individual CpG sites are indicated by the grey circles.

Real-time quantitative RT-PCR. Real-time quantitative RT-PCR analysis of gene expression was carried out on first-strand cDNA derived from RNA isolated from samples of breast tumor tissues and normal tissue RNAs (16). All clinical samples were obtained with informed patient consent and ethical approval. One hundred ng of the template (cDNA), 300 nM of each forward and reverse primer, a dye-labeled Taq Man probe and 1 x PCR Master mix (Applied Biosystems, Foster City, CA) were combined in a volume of 20 μl. Each PCR plate contained triplicate test cDNA templates and samples, which were used to construct a standard curve. Samples were amplified for 40 cycles in an ABI Prism 7300 sequence detection system (Applied Biosystems) with an initial melt at 95˚C for 10 min, followed by 40 cycles, each carried out at 95˚C for 15 sec, then 60˚C for 1 min. The partial cycle giving a statistically significant increase in the NAT1 product was determined and normalized to glyceraldehyde 3-phosphate dehydrogenase (G3PDH).

The primer sequences used for NAT1 were as follows: forward primer, 5'-GATGGTGTCTCCAGGTCAATCAT, reverse primer 5'-CCAACATCGTGGTCTCAAAACC and probe, 5'-TCAGAGCCCAGTACAGAAG. G3PDH was amplified using a probe and primers synthesized by Applied Biosystems.

End-point RT-PCR. The primers, 5'-CTATTGGTTTTGAGACCACG and 5'-AATTAACTCCAGAGGCTGCC, amplified a 172 bp cDNA fragment. G3PDH primers were used to normalize NAT1 expression; their sequences were 5'-ACCACAGTCCATGCCATCAC and 5'-TCCACCACCCTGTTGCTGTA, and they amplified a 500 bp cDNA fragment. Twenty-five cycles of the PCR reaction were performed, each cycle being carried out at 94˚C for 45 sec, 62˚C for 1 min and then 72˚C for 40 sec.

Statistical analysis. The Chi-squared test was used to analyze the differences in the rate of each variable and the Student's

Table I. The methylation patterns of the NAT1 gene in normal tissue, benign breast disease and cancer tissue.

	Normal (n=30)	Benign (n=31)	Cancer (n=72)	P-value
Age (years)	43.91±7.59	40.90±6.11	42.77±6.87	0.360[a]
NAT1 methylation rate (%)	23 (76.7)	25 (80.6)	39 (54.2)	0.012[b]
Mean methylation density rate (%)	55.55±7.03	64.51±6.73	39.81±4.96	0.012[a]

Statistical methods: [a]ANOVA, [b]Chi-square test.

Table II. CpG methylation and mRNA expression of the NAT1 gene in breast tissues.

	NAT1 Met[a] (+) (n=22)	NAT1 Met (-) (n=18)	P-value
Expression	3 (13.6%)	14 (77.8%)	0.001

Met[a], methylation. Statistical method: Chi-square test.

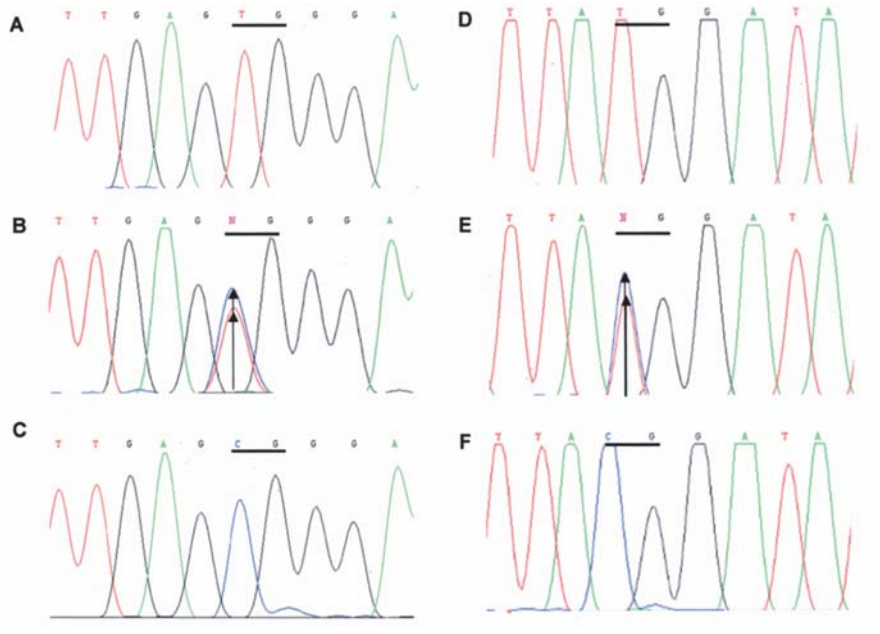

Figure 2. Examples of direct sequencing chromatogram. A CpG site is underlined. A and D, no methylation; B and E, partial methylation; C and F, complete methylation of the NAT1 gene.

t-test and the ANOVA test were used to detect differences in the mean values of the 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

Methylation pattern of the NAT1 gene in breast tissues. In order to determine the rate of aberrant DNA methylation in the NAT1 gene, we examined the methylation pattern through bisulfite sequencing of the promoter sequence. Figs. 2 and 3 demonstrate a representative bisulfite DNA sequencing of the gene. No significant difference in the mean ages was identified among the three groups, which indicates that the age-related effect on methylation did not influence our experiment (P=0.360) (Table II). We have identified the methylation of the promoter in 39 cases of breast carcinoma (54.2%), 23 normal tissues (76.7%) and 25 benign breast tissues (80.6%). The methylation rate of the NAT1 gene was significantly lower in malignant tumors compared to benign and normal tissues (P=0.012) (Table I).

The mean methylation density rates in normal tissues were 55.55±7.03%, while those in benign tissues were 64.51±6.73%. In contrast, the mean methylation density rates of the promoters in breast cancer tissues were 39.81±4.96%. Cancer

Table III. Clinicopathological features in breast cancer patients with and without methylation.

	NAT1 Met[a] (n=39)	NAT1 Met (-) (n=33)	P-value
Mean primary tumor size (cm)	2.78±0.34	2.80±0.28	0.959[b]
Presence of node metastasis	19 (48.7%)	10 (30.3%)	0.112[c]
Nuclear grade			0.161[c]
Grade 1	14 (35.9%)	16 (48.5%)	
Grade 2	13 (33.3%)	13 (39.4%)	
Grade 3	12 (30.8%)	4 (12.1%)	
ER-positive rate	20 (51.3%)	24 (72.7%)	0.063[c]
PgR-positive rate	19 (48.7%)	17 (51.5%)	0.813[c]
c-erbB2-positive rate	21 (53.8%)	13 (39.4%)	0.221[c]
Ki-67 expression	9.33±1.22	11.42±1.66	0.305[b]
p53-positive rate	10 (25.6%)	9 (27.3%)	0.876[c]

[a]Met, methylation. Statistical Methods: [b]Student's t-test, [c]Chi-square test.

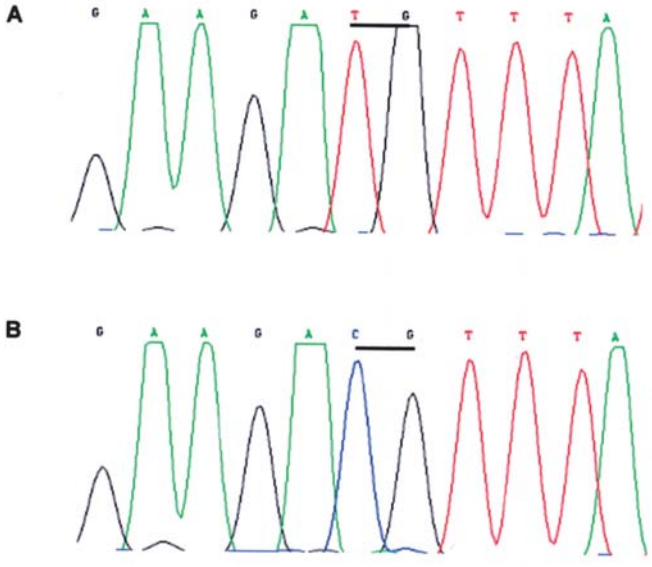

Figure 3. The unmethylated sample of the NAT1 gene in cancer tissue (A) and a methylated sample in its corresponding normal tissue. All of the unmethylated cytosines were changed to thymine by the bisulfite treatment, however, methylated cytosines were not changed. The CpG sites are indicated by underlining.

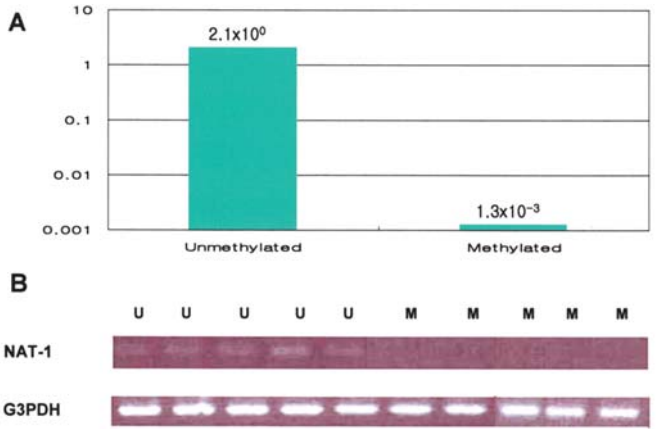

Figure 4. (A) NAT1 mRNA expression using quantitative real-time RT-PCR. The bar indicates the expression ratio of each sample. The quantified value was defined as the ratio of the NAT1 PCR products when compared with those of the G3PDH. (B) Representative figures of RT-PCR products for NAT1 mRNA in the breast tissues. 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. The letters M and U in the NAT1 row indicate the presence and absence of methylation, respectively.

tissues showed a lower methylation density rate than normal and benign breast tissues in terms of the NAT1 promoters (P=0.012) (Table I, Fig. 3).

Transcriptional effect of NAT1 methylation in breast tissues. In order to examine the effect of NAT1 promoter methylation on transcription, we measured NAT1 mRNA levels by end-point and quantitative real-time RT-PCR in a range of normal tissues and breast cancer/normal matched tissues. Representative results for the mRNA expression of the NAT1 gene are shown in Fig. 4. The results of real-time RT-PCR suggest that NAT1 mRNA expression of unmethylated tissues was about a thousand-fold upregulated compared with the methylated tissues (Fig. 4A). In the end-point RT-PCR, the mRNA expression of the NAT1 gene was evident in 14 out of 18 tissues without methylation, whereas only three out of 22 cases with methylation were found to express mRNA (Fig. 4B). Tissues with aberrant methylation of the NAT1 gene showed significantly lower rates of mRNA expression compared with the unmethylated cases (P=0.001) (Table II).

Clinicopathological features according to the status of NAT1 methylation. We compared the clinicopathological features between breast cancer tissues with or without methylation of the NAT1 gene, as summarized in Table III.

We found that 51% of cancers from the methylated group showed positive staining for ER, while 72.7% from the unmethylated group stained positive. This difference showed only a marginal significance (P=0.063). However, none of the other characteristics were significantly different between the two groups; mean primary tumor (P=0.959), presence of node metastasis (P=0.112), nuclear grade (P=0.161), c-erbB2 (P=0.221), Ki-67 expression (P=0.305) and p53 (P=0.876).

Discussion

Human N-acetyltransferase 1 (NAT1) plays an important role in the activation and/or deactivation of environmental carcinogens such as heterocyclic and aromatic amines found in well-cooked meats and tobacco smoke (1,2). Genetic polymorphisms in NAT1 may modify breast cancer risk following exposure to heterocyclic and aromatic amine carcinogens. Recent human epidemiological studies have reported an association between the NAT1 genotype and breast cancer in pre- and postmenopausal women (6-10).

DNA methylation of the promoter CpG islands has been recognized as an important mechanism for the regulation of gene expression and transcriptional modification in mammals (12). Cancer-associated DNA hypomethylation is as prevalent as cancer-linked hypermethylation, although its biological significance in carcinogenesis is not as well understood. Studies on DNA hypomethylation in cancer tissues have been focused strictly on growth regulatory genes and oncogenes. For example, there is a strong relationship between CDH3 promoter hypomethylation and P-cadherin expression that is evident in relation to histological grade and invasiveness in breast cancer (17); activation of cyclin D2 is associated with stage III and IV gastric cancers (18); activation of synuclein γ is associated with progression and metastatic potential in a range of solid tumors (19); and maspin expression in colorectal cancer is associated with microsatellite unstable tumors (20).

Our data support the theory that the NAT1 gene is hypomethylated in breast cancer tissues compared to normal and benign breast tissues. To the best knowledge of the authors, this is the first study on the methylation of the NAT1 gene, a drug metabolizing gene, in human tissues. We previously showed that DNA methylation in the SULT1A1 gene appears in cancerous breast tissue (21). Actually, many genes, including NAT1 and SULT1A1, were studied in terms of genetic polymorphisms that may modify breast cancer risk following exposure to a variety of carcinogens. However, our results raise the necessity that studies of epigenetics should be extended to the less vital genes, including genes related to drug metabolism.

The present study also demonstrated that the hypomethylation of the NAT1 promoter region resulted in aberrant mRNA expression levels in breast carcinomas. The methylation status of the DNA chromatin in the NAT1 promoter region plays a crucial role in the ability of transcription factors in binding to the transcription start site, thus regulating mRNA transcription. Conversely, abnormal gene-specific hypomethylation may potentially lead to the overexpression of genes, which can contribute to the development of disease. In addition, comparative 2-D proteomic analysis of normal and breast cancer tissues revealed elevated NAT1 protein levels in breast cancer (11). This is in line with our data, which suggest that DNA hypomethylation was more frequently found in breast cancer tissues and NAT1 mRNA transcription and, consequently, its protein expression was elevated in the cancer tissues.

Although NAT1 shows a relationship with carcinogenesis via polymorphisms and somatic changes in mRNA and protein expression, it remains unclear whether NAT1 methylation may be prognostic. The markers with maximal prognostic or predictive value were recently revealed to be GATA3, NAT1 and an estrogen receptor, using AQUA-based objective quantitative analysis of tissue microarrays (22). Perou *et al* have shown evidence of increased NAT1 mRNA expression in ER-positive breast cancers using DNA microarray analyses (23). In this study, we failed to find any relationship between NAT1 methylation and clinicopathological features in the breast. However, the unmethylated cancers showed higher ER-positive rates than the methylated cancers, though this difference showed only marginal significance (P=0.063). The ER-positive cell lines, T47D and MCF7, were reported to express less NAT1 than the ER-negative cell lines, BT20 and MB-MDA-468 (11). This means that although there may be a clinical association between the expression of NAT1 and ER, the expression of NAT1 is not dependent on ER expression.

Our results suggest that DNA hypomethylation in the NAT1 gene appears to be present in breast cancer and also indicates that hypomethylation may have a significant impact on the transcriptional activation of the gene. Therefore, hypomethylation of the NAT1 gene plays a significant role in breast carcinogenesis.

Acknowledgements

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