Downregulation of ARFGEF1 and CAMK2B by promoter hypermethylation in breast cancer cells

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INTRODUCTION

Epigenetic regulation of gene expression through changes in CpG methylation in the promoter plays pivotal roles during cellular differentiation, aging, and tumorigenesis (1). To date, many genes have been found to be regulated in an epigenetic manner in breast cancer cells (2). Both hypermethylation and hypomethylation have been observed in tumor-related genes. Hypermethylation induces gene inactivation and can cause tumor development if present in tumor suppressor genes (3). However, the opposite has been found in a few genes (4). Well-known hypermethylated tumor suppressor genes in breast cancer cells are RASSF1A, BRCA1, PCDH8, and 15-Hydroxyprostaglandin. Hypomethylation can also provoke tumor development through activation of proto-oncogenes.

Examples of hypomethylated genes in breast cancer cells are FEN1 and IGF2 (5, 6).

Identification of genes that are regulated by promoter methylation or demethylation in breast cancer cells as well as other cancer cell types has been carried out using a few different approaches at both the genome-wide and individual gene levels. Characterization of individual genes is carried out by examining the methylation status of CpGs in the promoter using techniques such as plasmid sequencing after cloning of the PCR product, direct sequencing of the PCR product, combined bisulfite restriction analysis (COBRA), and pyrosequencing.

Genome-wide searches for hypermethylated or hypomethylated genes have been performed in both breast cancer cell lines and tissues. HOXB13 and HNF1B are tumor suppressors and were identified from breast cancer tissue by the differential methylation hybridization (DMH) method (7). MOGAT2 and NTSR2 were also identified from breast cancer tissue by Illumina Infinium methylation array (8). The hypermethylated and hypomethylated genes obtained by microarray assay are available at the public databases EBI (European bioinformatics institute) (http://www.ebi.ac.uk/gxa/) and GEO (http://www.ncbi.nlm.nih.gov/sites/geo).

Using the information from such databases, it is now feasible to identify candidate genes that are regulated either genetically or epigenetically before actually investigating experimentally. By following a combinatorial method featuring in silico and experimental analysis, several genes, including SASH1 and NORE1A, have been identified as methylation markers for breast cancer (9, 10). However, the mining of genes by the DMH method frequently results in false-positive results due to the non-specific binding of anti-CpG antibody or methyl-binding protein, which are used to recognize methylated CpGs. Recently, a genome-wide methylation array was developed, the so-called Infinium methylation assay, which is based on genotyping technology (11). This array, which is considered the most comprehensive so far, only covers one or a few CpG sites within the proximal promoter. Therefore, this method has a limitation in providing information on the general methylation pattern of CpGs. Considering these limitations, it is essential to verify any changes in the methylation of individual genes obtained by the genome-wide experiment for the development of methylation markers for cancer.

In this study, we screened expression databases, including ex-
expression array and the Unigene database, in order to mine candidate genes that are downregulated in breast cancer tissue. Using in silico approaches, ARFGEF1 and CAMK2B were finally identified, and real-time methylation-specific PCR (MSP) and RT-PCR revealed that they were hypermethylated and downregulated in breast cancer cells. These results suggest that methylations of the genes are possible methylation markers for breast cancer.

RESULTS

In silico analysis of ARFGEF1 and CAMK2B
To identify genes for which the relationship between expression level and promoter methylation in breast cancer cells has not yet been investigated, we carried out a series of in silico analyses followed by MSP and RT-PCR (Fig. 1). First, the expression microarray data registered at the EBI database were analyzed in order to identify genes exhibiting lower expression in breast cancer cells than in normal breast cells. For this, one normal cell (MCF-10A) and three cancer cells (MDAMB-231, MDAMB-468, and SK-BR-3) were submitted as a query. The result provided 173 genes showing lower expression in cancer cells. Second, the 173 genes were queried in the Unigene database in order to perform e-Northern analysis, which is based on the EST count of a specific tissue. This step enabled us to screen genes containing a higher number of ESTs in normal breast tissue and a lower number of ESTs in breast cancer tissue. Any gene not expressed in the breast tissue or expressed at a higher level in cancer tissue was excluded. Forty-two genes were filtered through the Unigene database. To further confirm the lower expression of the genes in cancer tissue, e-Western analysis was applied by monitoring protein expression. A total of 32 genes were excluded, leaving 10 genes. Third, promoter regions of the genes spanning from +1,000−200 were scanned for the presence of CpG islands. Ten genes satisfying all the criteria above were finally identified, as indicated in Supplementary Table 2. ACTG1 was included in the table since e-Western showed that it was downregulated in breast cancer tissue. However, this was in conflict with the e-Northern result, which indicated upregulation. Therefore, the in silico mined genes were possibly downregulated in breast cancer tissue due to the presence of CpG islands in their promoters.

Hypermethylation and downregulation of CAMK2B and ARFGEF1 in breast cancer cells
For the 10 genes selected from the EBI database, a preliminary experiment was carried out in order to examine the difference in methylation of CpG islands between normal and cancer breast cells. A normal cell line, MCF-10A, and a cancer cell line, MCF-7, were examined by real-time MSP analysis, and the result indicated that ADP-ribosylation factor guanine nucleotide-exchange factor 1 (ARFGEF1) and calcium calmodulin-dependent kinase IIB (CAMK2B) showed the largest differences in methylation. ARFGEF1 and CAMK2B were both hypermethylated in cancer cells by 22.5 and 3.5 fold, respectively.

These two genes were therefore selected for in depth characterization of expression and promoter methylation in cancer cells. ARFGEF1 and CAMK2B are known as key elements in cell signaling. CAMK2B, together with CAMK2A, comprises splicing variants of CAMK2, which is a central signaling molecule in neurons (12). ARFGEF1 plays a role in intracellular vesicular trafficking by accelerating the replacement of bound GDP with GTP on ARF (13). These two genes were thus examined in a wider range of breast cell lines. For the normal cells, MCF-10A and MCF-12A were used, whereas for the cancer cells, MCF-7, MDAMB-231, MDAMB-468, SKBR-3, T47D, and ZR-75-1 were used. Among the cells, MCF-12A, MCF-7, T47D, and ZR-75-1 were estrogen receptor (ER)-positive, whereas MDAMB-231, MDAMB-468, and SKBR-3 were ER-negative. Both ARFGEF1 and CAMK2B showed higher methylation in the cancer cell lines than in the normal cell lines (Fig. 2). The differences in methylation ranged from 9.2-86.2 fold and 1.1-3.3 fold for ARFGEF1 and CAMK2B, respectively. No significant correlation was found between the presence and absence of ER.

Next, expression of ARFGEF1 and CAMK2B in the breast cell lines was monitored by real-time RT-PCR analysis. The genes were expressed lower in cancer cells than in normal cells (Fig. 3). Normal MCF-12A cell line showed the highest expression of both genes. Even though there appeared to be no proportional correlation between methylation and expression level, the genes were generally hypermethylated and downregulated in cancer.
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Fig. 2. Real-time MSP analysis of the normal breast and cancer cell lines. (A, B) Representative MSP amplification curves for ARFGEF1 (A) and CAMK2B (B) in MCF12A or MCF10A, and MDAMB-231 cells. The prefix "U" and "M" in front of the gene name are unmethylated and methylated primer, respectively. (C, D) Relative methylation level of ARFGEF1 (C) and CAMK2B (D) in normal (gray bar) and cancer cell lines (black bar). Each sample was examined in duplicate and the average relative methylation level is presented.

Fig. 3. Expression analysis of ARFGEF1 and CAMK2B in normal and cancer cell lines. (A, B) Representative real-time RT-PCR amplification curves for ARFGEF1 in MCF-10A and ZR-75-1 (A) and CAMK2B in MCF10A and MCF-7 cells (B). (C, D) Relative expression level of ARFGEF1 (C) and CAMK2B (D) in normal (gray bar) and cancer cell lines (black bar) is indicated. Each sample was examined in duplicate and the average relative expression level is presented.

Induction of expression by demethylation of the CAMK2B and ARFGEF1 promoters

To examine the relationship between hypermethylation and downregulation of gene expression in cancer cells, MCF-7, MDAMB-231, and ZR-75-1 cells showing higher methylation were treated with the methyltransferase inhibitor 5-Aza-2'-deoxycytidine. MSP was then performed to measure demethylation-inducing efficacy of the inhibitor. Demethylation in the cells. ER status did not affect the expression of the two genes, implying that expression of the genes was regulated via an ER-independent pathway. Immunohistochemical images for ARFGEF1 and CAMK2B are available from the EBI database and they indicate that the two proteins showed moderate or weak expression in breast cancer cell and tissues with remarkable downregulation in case of CAMK2B (Supplementary Fig. 1).
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Fig. 4. Recovery of expression of ARFGEF1 and CAMK2B after treatment of 5-Aza2'-deoxycytidine (5-Aza) in breast cell lines. Cell lines were treated with 5-Aza, and methylation (top) and expression level (bottom) were examined for ARFGEF1 (A) and CAMK2B (B) by real-time MSP and real-time RT-PCR, respectively. Gray and black bars represent before and after treatment of 5-Aza, respectively. Each sample was examined in duplicate and the average relative level is presented.

treated cells was induced up to 15-44% of the untreated cells (Fig. 4). These induced methylation levels were close to those of the corresponding normal cells.

Alteration of expression by demethylation was monitored by real-time RT PCR-analysis. Increases in the gene expression of both ARFGEF1 and CAMK2B were observed in the 5-Aza2'-deoxycytidine-treated cells (Fig. 4). In the treated cells, ARFGEF1 and CAMK2B were upregulated by 1.8-169.5 fold. Recovery of gene expression upon demethylation of the CpG sites implies that hypermethylation at the promotor was responsible for the downregulation in the cancer cell lines.

DISCUSSION

Many genome-wide expression arrays as well as methylation arrays have been carried out using breast cancer tissue, and their results are available in databases such as EBI and GEO. At present, two different genome-wide methylation analyses are most widely used. One uses CpG-recognizing antibody or CpG-binding protein followed by immunoprecipitation and microarray. The other utilizes a genotyping-based principle that allows measurement of the absolute methylation level at specific sites. Each method has its own advantages and has been used in varieties of cancer types, including breast cancer. Even though genome-wide expression or methylation array is useful to collect information at the whole genome level, many genes do not show consistency with further individual gene expression analysis. Moreover, data from different databases do not display consistency for the same genes. Therefore, it is essential to confirm results from genome-wide approaches using further stringent methods such as real-time RT-PCR or MSP analysis.

To minimize false-positive results resulting from in silico data mining, we compared several databases, including expression arrays, Unigenes, and protein databases. The combinational approach for identifying candidate genes of which expression is confined to cancer tissue has proven useful by successfully mining and characterizing ARFGEF1 and CAMK2B. A previous genome-wide study in breast cancer that used an Illumina methylation array indicates that 7.8% of the total genes underwent methylation change in the tumor tissue (8). Another study on ovary cancers also showed ~7.8% of methylation change (14). Therefore, it is estimated that less than 10% of total genes are affected by methylation during carcinogenesis.

ADP-ribosylation factor, ARFGEF1 (also called BIG1), a brefeldin A-inhibited guanine nucleotide-exchange protein, activates class I ADP-ribosylation factors (ARF-1-3) by catalyzing the replacement of bound GDP with GTP, an action critical for the regulation of protein transport in eukaryotic cells (15). In addition to its ARF-activating capacity, ARFGEF1 has scaffolding functions and interacts with several proteins in other cellular compartments. In mammalian cells, dysregulation of proteins involved in vesicular membrane trafficking, including GTP-binding proteins such as ARFs that ensure coordinated fission-fusion or correct targeting of intracellular transport vesicles, may affect Golgi structural and functional integrity (16).

The product of CAMK2B belongs to the serine/threonine pro-
tein kinase family as well as to the Ca\(^{2+}\)/calmodulin-dependent protein kinase subfamily. In mammalian cells, the enzyme is composed of four different chains: alpha, beta, gamma, and delta (17). The product of the CAMK2B gene is a beta chain. It is possible that distinct isoforms of this chain have different cellular localizations and interact differently with calmodulin (18). Recently, it was found that all four isoforms are expressed in prostate cancer cells derived from bone metastases, whereas the alpha and beta forms are lacking in prostate cancer cells not derived from bone metastasis. This suggests that the isoforms are differentially expressed in carcinogenesis. Upregulation of CAMK2B has been observed in the cerebral cortices of patients suffering from schizophrenia or depression (12). Although it is known that the two genes are dysregulated in a few diseases, the relevant mechanism is unknown. To our knowledge, this study is the first report showing the involvement of methylation in breast cancer cell lines.

CAMK2B is supposedly imprinted by a bioinformatic approach (19), even though its precise imprinting mechanism has not yet been elucidated. Since cancer develops through genetic and epigenetic alterations, the CAMK2B gene possibly affects tumorigenesis depending on whether or not the parental allele undergoes alteration. Further research on the allele-specific promoter methylation status of the CAMK2B gene in breast cancer tissues should be performed to obtain detailed information relevant to the methylation and expression of CAMK2B in imprinting and breast cancer.

In conclusion, we identified that ARFGEF1 and CAMK2B were downregulated in breast cancer cell lines and further proved that the downregulation was due to promoter hypermethylation. Further, these genes could be used to develop epigenetic markers for the diagnosis of breast cancer.

MATERIALS AND METHODS

In silico mining of breast cancer-specific genes

To identify genes that are downregulated by promoter hypermethylation in breast cancer tissue, we first screened candidate genes in silico from the Gene Expression Atlas interface (http://www.ebi.ac.uk/gxa/). Expression profiles from the breast cancer cell lines MCF-7, MDA-MB-231, and SK-BR-3 and from the normal breast cell line MCF-10A were queried, and the genes showing downregulation were selected. e-Northern analysis was carried out using the Unigene Database platform (http://www.ncbi.nlm.nih.gov/UniGene/info_ddd.shtml) in order to select genes having high EST counts in normal breast tissue and low EST counts in cancer tissue. Presence of CpG islands was monitored using the Sequence Manipulation Suite: CpG Islands (http://www.ualberta.ca/~stothard/javascript/cpg_islands.html). e-Western analysis was carried out at the human protein atlas database (http://www.proteinatlas.org/) in order to compare in silico RNA and protein expression levels.

Cell culture and 5-Aza-2’-deoxycytidine treatment

Normal human breast cell lines, MCF-10A and MCF-12A, and cancer cell lines, MCF7, MDA-MB-231, MDA-MB-468, SK-BR-3, T47D, and ZR-75-1, were purchased from the American Type Culture Collection (ATCC; Manassas, VA) and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). To induce demethylation of the cytosine residues, a methyltransferase inhibitor, 5-Aza-2’-deoxycytidine, was added to the culture medium at 5 μM for 72 hr. Culture medium with or without treatment was changed every 24 hr.

Methylation-specific PCR (MSP)

Chromosomal DNA was isolated from the cell cultures in a 75 cm\(^2\) culture flask using a genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer’s protocol. The extracted DNA was eluted with 250 μl of distilled water. Sodium bisulfite modification of genomic DNA was carried out using an Epitexit Bisulfite kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol using 0.1 mg of purified DNA. PCR primers for CAMK2B and ARFGEF1 (Supplementary Table 1) were designed with one pair for amplification of methylated DNA and one pair for amplification of unmethylated DNA using the Methprimer program (http://www.urogene.org/methprimer/index1.html). Quantitative PCR was performed using a Power SYBR Green Kit (Applied Biosystems, Foster City, CA) according to the protocols of the manufacturer. To assign a quantitative measure to the level of methylation, a methylation index was calculated for each sample using the following formula: methylation index = \[1 / (1 + \frac{\Delta\text{Ct}_u}{\Delta\text{Ct}_\text{me}}) \times 100\%\], as previously described (20), where Ctu is the average cycle threshold (CT) obtained from duplicate quantitative PCR analyses using the unmethylated primer pair and Ctm is the average CT obtained using the methylated primer pair.

Real time RT-PCR

Total RNA from cell culture was prepared using Trizol reagent according to the manufacturer’s protocols (GibcoBRL, Carlsbad, CA). Reverse transcription was conducted using 10 μg of total RNA with a reverse transcription kit (Promega). Expression levels of CAMK2B and ARFGEF1 were measured by real-time quantitative RT-PCR analysis in order to confirm consistency with those from the microarray data. One microtiter of cDNA was used for the PCR, and duplicate reactions were performed for each sample using a Kapa SYBR Fast qPCR Kit (Kapa Biosystems, Woburn, MA) with gene-specific primers on an ABI 7500 instrument (Applied Biosystems). The primers used for these selected genes are listed in Table 1. RNA quantity was normalized to GAPDH content, and gene expression was quantified according to the 2\(^{-\Delta\Delta\text{Ct}}\) method.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2010-0011481).
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