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

Ju Hee Kim, Tae Woo Kim & Sun Jung Kim[*]

Department of Life Science, Dongguk University-Seoul, Seoul 100-715, Korea

To identify novel genes that are regulated by promoter methylation, a combinational approach involving *in silico* mining followed by molecular assay was performed. From the expression microarray data registered in the European bioinformatics institute (EBI), genes showing downregulation in breast cancer cells were initially screened and then selected by e-Northern analysis using the Unigene database. A series of these *in silico* methods identified CAMK2B and ARFGEF1 as candidates, and the two genes were revealed to be hypermethylated in breast cancer cell lines and hypomethylated in normal breast cell lines. Additionally, cancer cell lines showed downregulated expression of these genes. Furthermore, treatment of the cancer cell lines with a demethylation agent, 5-Aza-2'-deoxycytidine, recovered expression of CAMK2B and ARFGEF1, implying that hypermethyaltion silenced gene activity in cancer cells. Taken together, promoter methylations of CAMK2B and ARFGEF1 are novel epigenetic markers identified in breast cancer cell lines and can be utilized for the application to clinical cancer tissues. [BMB reports 2011; 44(8): 523-528]

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.

*Corresponding author. Tel: 82-2-2260-3975; Fax: 82-2-2268-3975;
E-mail: sunjun0gk@dongguk.edu

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.
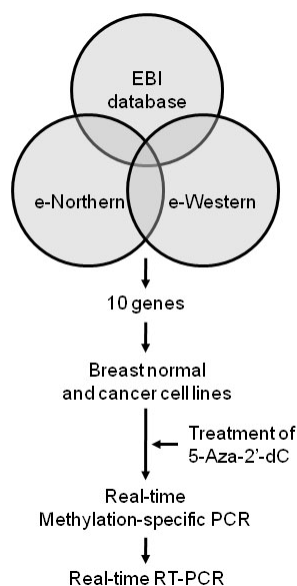
In this study, we screened expression databases, including ex-

pression 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 (MCF-7, MDA-MB-231, 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
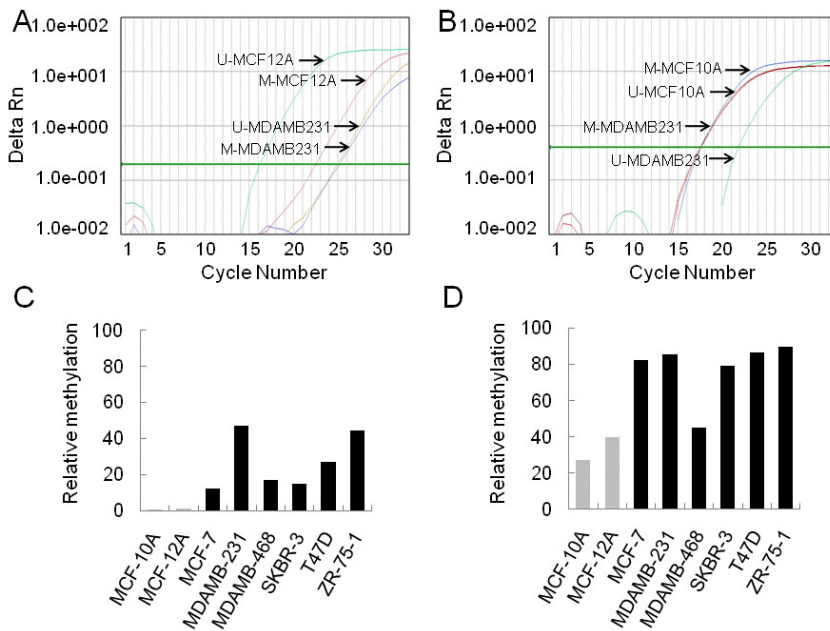
gene not expressed in the breast tissue or expressed at a higher level in cancer tissue was excluded. Forty-two genes were filtered through the Unigene database. To further confirm the lower expression of the genes in cancer tissue, e-Western analysis was applied by monitoring protein expression. A total 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 ARFFEF1 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

Fig. 1. Experimental scheme of identifying novel epigenetic markers in breast cancer cell lines. Genes showing downregulation were *in silico* screened from the Gene Expression Atlas interface. e-Northern and e-Western analysis was carried out using the Unigene Database platform and the human protein atlas database, respectively. *In silico* screened genes were undergone molecular approaches including real-time MSP, RT-PCR, and 5-Aza-2'-deoxycytidine treatment.

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.
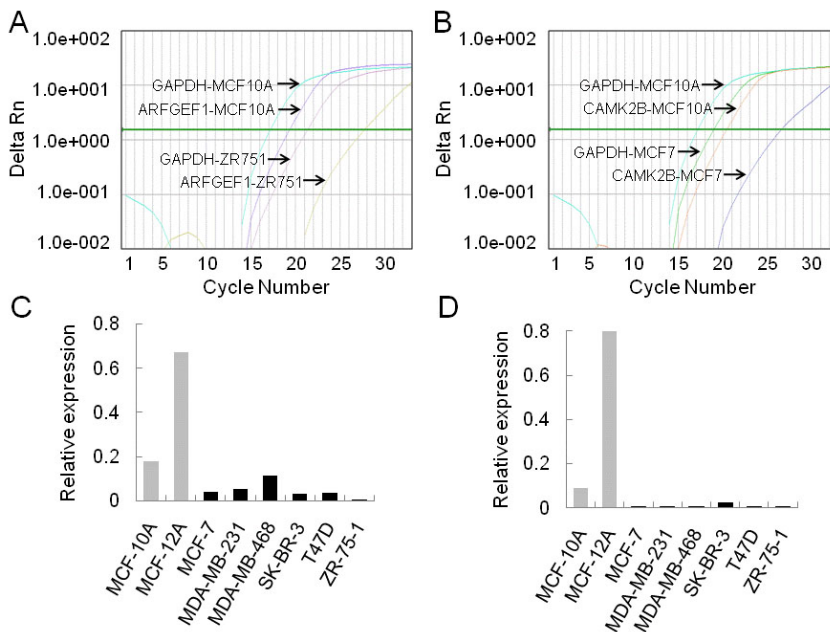

Fig. 3. Expression analysis of ARFGEF1 and CAMK2B in normal and cancer cell lines. (A, B) Representative real-time RT-PCR amplification curves for ARFGEF1 in MCF-10A and ZR-75-1 (A) and CAMK2B in MCF10A and MCF-7 cells (B). (C, D) Relative expression level of ARFGEF1 (C) and CAMK2B (D) in normal (gray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

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).

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
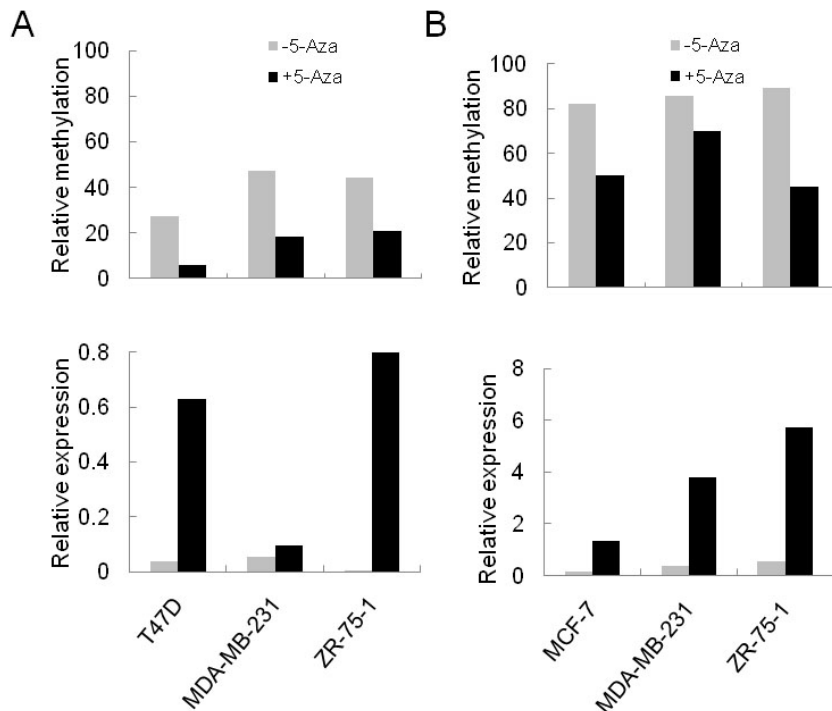
Fig. 4. Recovery of expression of ARFGEF1 and CAMK2B after treatment of 5-Aza-2'-deoxycytidine (5-Aza) in breast cell lines. Cell lines were treated with 5-Aza, and methylation (top) and expression level (bottom) were examined for ARFGEF1 (A) and CAMK2B (B) by real-time MSP and real-time RT-PCR, respectively. 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-Aza-2'-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 promoter 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 con-

sistency 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 (ARF1-3) 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 EpiTect 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 = $[1 / (1 + 2^{-(CTu - CTme)}] \times 100\%$, as previously described (20), where CTu is the average cycle threshold (CT) obtained from duplicate quantitative PCR analyses using the unmethylated primer pair and CTme 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 microliter 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 $2^{-\Delta Ct}$ method.

REFERENCES

1. Kulis, M. and Esteller, M. (2010) DNA methylation and cancer. *Adv. Genet* **70**, 27-56.

2. Dworkin, A. M., Huang, T. H. and Toland, A. E. (2009) Epigenetic alterations in the breast: Implications for breast cancer detection, prognosis and treatment. *Semin. Cancer Biol.* **19**, 165-171.

3. Ai, L., Kim, W. J., Kim, T. Y., Fields, C. R., Massoll, N. A., Robertson, K. D. and Brown, K. D. (2006) Epigenetic silencing of the tumor suppressor cystatin M occurs during breast cancer progression. *Cancer Res.* **66**, 7899-7909.

4. Nabilsi, N. H., Broaddus, R. R. and Loose, D. S. (2009) DNA methylation inhibits p53-mediated survivin repression. *Oncogene* **28**, 2046-2050.

5. Singh, P., Yang, M., Dai, H., Yu, D., Huang, Q., Tan, W., Kernstine, K., Lin, D. and Shen, B. (2008) Overexpression and hypomethylation of flap endonuclease 1 gene in breast and other cancers. *Mol. Cancer Res.* **6**, 1710-1717.

6. Ito, Y., Koessler, T., Ibrahim, A., Rai, S., Vowler, S., Abu-Amero, S., Silva, A., Maia, A., Huddleston, J., Uribe-Lewis, S., Woodfine, K., Jagodic, M., Nativio, R., Dunning, A., Moore, G., Klenova, E., Bingham, S., Pharoah, P., Brenton, J., Beck, S., Sandhu, M. and Murrell, A. (2008) Somatically acquired hypomethylation of IGF2 in breast and colorectal cancer. *Hum. Mol. Genet.* **17**, 2633-2643.

7. Tommasi, S., Karm, D. L., Wu, X., Yen, Y. and Pfeifer, G. P. (2009) Methylation of homeobox genes is a frequent and early epigenetic event in breast cancer. *Breast Cancer Res.* **11**, R14.

8. Van der Auwera, I., Yu, W., Suo, L., Van Neste, L., van Dam, P., Van Marck, E. A., Pauwels, P., Vermeulen, P. B., Dirix, L. Y. and Van Laere, S. J. (2010) Array-based DNA methylation profiling for breast cancer subtype discrimination. *PLoS One* **5**, e12616.

9. Zeller, C., Hinzmann, B., Seitz, S., Prokoph, H., Burkhard-Goettges, E., Fischer, J., Jandrig, B., Schwarz, L. E., Rosenthal, A. and Scherneck, S. (2003) SASH1: a candidate tumor suppressor gene on chromosome 6q24.3 is downregulated in breast cancer. *Oncogene* **22**, 2972-2983.

10. Hesson, L., Dallol, A., Minna, J. D., Maher, E. R. and Latif, F. (2003) NORE1A, a homologue of RASSF1A tumour suppressor gene is inactivated in human cancers. *Oncogene* **22**, 947-954.

11. Thirlwell, C., Eymard, M., Feber, A., Teschendorff, A., Pearce, K., Lechner, M., Widschwendter, M. and Beck, S. (2010) Genome-wide DNA methylation analysis of archival formalin-fixed paraffin-embedded tissue using the Illumina Infinium HumanMethylation27 BeadChip. *Methods* **52**, 248-254.

12. Novak, G., Seeman, P. and Tallerico, T. (2006) Increased expression of calcium/calmodulin-dependent protein kinase IIbeta in frontal cortex in schizophrenia and depression. *Synapse* **59**, 61-68.

13. Pacheco-Rodriguez, G., Moss, J. and Vaughan, M. (2002) BIG1 and BIG2: brefeldin A-inhibited guanine nucleotide-exchange proteins for ADP-ribosylation factors. *Methods Enzymol.* **345**, 397-404.

14. Teschendorff, A. E., Menon, U., Gentry-Maharaj, A., Ramus, S. J., Gayther, S. A., Apostolidou, S., Jones, A., Lechner, M., Beck, S., Jacobs, I. J. and Widschwendter, M. (2009) An epigenetic signature in peripheral blood predicts active ovarian cancer. *PLoS One* **4**, e8274.

15. Padilla, P. I., Uhart, M., Pacheco-Rodriguez, G., Peculis, B. A., Moss, J. and Vaughan, M. (2008) Association of guanine nucleotide-exchange protein BIG1 in HepG2 cell nuclei with nucleolin, U3 snoRNA, and fibrillarin. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 3357-3361.

16. Pfeffer, S. R. (1992) GTP-binding proteins in intracellular transport. *Trends Cell Biol.* **2**, 41-46.

17. Gaertner, T. R., Kolodziej, S. J., Wang, D., Kobayashi, R., Koomen, J. M., Stoops, J. K. and Waxham, M. N. (2004) Comparative analyses of the three-dimensional structures and enzymatic properties of alpha, beta, gamma and delta isoforms of Ca2+-calmodulin-dependent protein kinase II. *J. Biol. Chem.* **279**, 12484-12494.

18. Takeuchi, Y., Nomura, K. and Fukunaga, K. (2004) Differential subcellular distribution of Ca2+/calmodulin-dependent protein kinase II isoforms in the striatum and NG108-15 cells. *J. Neurosci. Res.* **75**, 480-490.

19. Luedi, P. P., Dietrich, F. S., Weidman, J. R., Bosko, J. M., Jirtle, R. L. and Hartemink, A. J. (2007) Computational and experimental identification of novel human imprinted genes. *Genome Res.* **17**, 1723-1730.

20. Lu, L., Katsaros, D., de la Longrais, I. A., Sochirca, O. and Yu, H. (2007) Hypermethylation of let-7a-3 in epithelial ovarian cancer is associated with low insulin-like growth factor-II expression and favorable prognosis. *Cancer Res.* **67**, 10117-10122.