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Transcriptional silencing of EphB6 receptor tyrosine kinase in invasive breast carcinoma cells and detection of methylated promoter by methylation specific PCR

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Abstract

The receptor tyrosine kinase EphB6 is expressed at reasonable levels in normal breast cells. It shows decreased abundance in non-invasive breast carcinoma cells and is transcriptionally silenced in invasive breast carcinoma cells. We have characterized EphB6 promoter and correlated the expression of EphB6 transcript to differential methylation of the promoter region. The demethylation of promoter sequence in vivo by growth in media containing 5-aza-2′-deoxycytidine restores the expression of EphB6 to normal levels in breast carcinoma cells, and the ability of the promoter to initiate transcription of a reporter gene is lost after methylation of the promoter sequence. The promoter region has binding sites for various factors such as SP1 and p300. The specific methylation of CpG dinucleotides has allowed us to design primers that can selectively amplify the methylated promoter and thus facilitate identification of normal, non-invasive, and invasive breast cells. The potential significance of EphB6 to serve as a diagnostic and prognostic indicator is discussed.

Keywords: EphB6; Transcriptional silencing; 5-Aza-2′ deoxycytidine; Breast carcinoma cell lines; Sodium bisulfite sequencing; Methylation specific PCR; Breast cancer

The transformation of a normal cell into a cancer cell can be attributed to the altered expression and activity of a variety of genes [1–3]. Similarly, a non-invasive tumor cell can become invasive by corresponding changes in a different subset of genes [4]. The genes involved in normal cellular functions as well as genes that may be involved in the progression of diseases including cancer are regulated at various levels of expression including their transcription. Therefore, the generation of an altered state of gene expression that may lead to cancer or lead to a more aggressive tumor may be the result of transcriptional misregulation [5–8]. The methylation of gene promoter is a well-characterized mechanism of transcriptional regulation. In general, hypomethylation is associated with gene expression, while hypermethylation can limit the transcription of a specific gene. If one allele of a tumor suppressor gene or a metastasis suppressor gene is silenced by a mutation (either germ line or somatic), then any change in the expression of the second allele would completely ablate the production of this gene. There are many cases of tumor suppressor or metastasis suppressor genes being silenced by the hypermethylation of the promoter sequences as well as cases of hypomethylation of a promoter leading to overexpression of genes involved in tumorigenesis [9,10]. The mechanisms of this transcriptional misregulation and the affected genes are important as diagnostic tools, prognostic models of disease progression, and potential therapeutic targets for treatment of these diseases [8–11].

One family of genes that has been shown to be involved in cancer progression is the Eph receptor tyrosine kinases [12–14]. These receptor tyrosine kinases and their ephrin ligands are involved in cell-to-cell signaling in many developmental processes including hindbrain development, axon

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guidance as well as vascular patterning [15–19]. Furthermore, these genes are also proven to play a role in the formation and progression of many cancers including colon cancer, lung carcinomas, prostate cancer, melanomas, and breast cancer [14,20–32]. A detailed analysis of the expression profiles of these genes in breast carcinoma cell lines by this laboratory has indicated a subset that may be of particular importance in the progression of breast cancer from a non-invasive to an invasive phenotype [33]. Specific Eph receptors and ephrin ligands have been reported to be useful as prognostic markers in neuroblastomas [29,30] and have some apparent value in prognosis/diagnosis in other cancers, such as melanomas, colon cancer, prostate cancer, non-small cell lung cancer, esophageal squamous cell carcinoma, and breast cancer [21–26,28–33]. Despite this information, there are currently no DNA based diagnostic tools that we are aware of that can predict the expression levels of any Eph receptor or ephrin ligand in cancer cells. The generation of a DNA based diagnostic tool predicting expression levels of an Eph receptor may be useful given: (1) the abundance of information indicating that many Eph receptors play a role in, or may be used to predict, the progression of various types of cancer and (2) the detection sensitivity of DNA based markers.

There are several reports which suggest that Eph receptors may be regulated by methylation [34,35] and we have also established methylation sensitivity of some Eph receptors in several breast carcinoma cell lines (unpublished results). The differences in methylation patterns at specific promoters have been reliably determined by methylation specific PCR (MSP) [36]. There is strong evidence in the literature correlating the loss of EphB6 with the progression of cancers including non-small cell lung carcinoma, neuroblastomas, melanomas, and breast cancer [29–33]. It is therefore important to characterize methylation mediated transcriptional silencing of EphB6 in breast carcinoma cell lines.

The results presented here indicate that EphB6 is strongly regulated by methylation, and the specific methylated residues in this promoter can be used in MSP to predict whether or not EphB6 is expressed in these cell lines. Based on the potential involvement of EphB6 in the invasiveness of breast cancer cell lines, we propose it to be a useful biomarker for the detection of breast cancer cells among normal cells using MSP.

Materials and methods

Cell culture. The breast cancer cell lines used in this study were: MCF-10A, a cell line established from normal breast, and seven breast carcinoma cell lines: MCF-7, BT-20, and SkBr3, which are non-invasive, and four invasive cell lines: MDA-MB-231, MDA-MB-435, BT-549, and Hs 578T. All cells were cultured at 37 °C/7% CO_2. MCF-10A cells were grown in 1:1 DMEM:F12 media (Gibco) with 5% Horse Serum (Gibco), 20 mM Hepes (Gibco), 10 ng EGF/ml (Invitrogen), PenStrep Glutamine (Gibco) (10 U penicillin/ml, 10 μg streptomycin/ml, and 29.2 μg L-glutamine/ml), 10 μg insulin/ml (Invitrogen), 0.1 μg cholera toxin/ml (Sigma), and 500 ng hydrocortisone/ml (Sigma). All other cell lines were grown in DMEM (Gibco) with 10% fetal bovine serum (Hyclone), 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Gibco), penstrep (Gibco) 25 U penicillin/ml, and 25 μg streptomycin/ml.

5-Aza-2′-deoxycytidine (Aza) treatments. The cells were treated with varying concentrations of 5-aza-2′-deoxycytidine (Calbiochem) from a stock solution of 10 mg/ml in 5% acetic acid. Exponentially growing cells were split in normal media and allowed to grow for 24 h. The following day, the normal media were removed and replaced with media containing either 5 or 100 μM Aza or a corresponding mock treatment (containing acetic acid). The cells were grown under these conditions for 72 h with fresh media added every 24 h.

Total RNA isolation. RNA was isolated from 10 cm tissue culture dishes when cells were 85–95% confluent using TRI reagent (Molecular Research Center) with slight modifications to the recommended protocol. Briefly, 1 ml reagent was used per 10 cm dish, and 200 μl chloroform was added to separate RNA in the aqueous phase. RNA was precipitated by adding 250 μl isopropanol. The pellet was then washed sequentially with 80% and 100% ethanol, dried and resuspended in DEPC-treated H_2O, and stored in aliquots at −80 °C. The quality of RNA was visualized by running on a formaldehyde gel. The appearance of ribosomal RNA bands indicated that RNA was not degraded during the procedure. The amount of RNA was determined by measuring its absorbance at 260 nm.

DNase treatment of total RNA. To remove DNA contamination, 20 μg RNA (quantified spectrophotometrically) was treated with 500 ng DNase I, 80 U RNasin (Promega), and 1 mM $MgCl_2$ in Tris buffer in a total volume of 50 μl. The reaction was carried out at 37 °C for one hour and the DNase inactivated by heating to 65 °C for 30 min. The elimination of genomic DNA was confirmed by the absence of any amplified intronic product in PCRs using primers specific for EphB6 and β-actin.

Semi-quantitative RT-PCR. The Qiagen one-step RT-PCR kit was used for all reactions. Equal amounts of RNA from each cell line were used in each reaction (normalized to actin). Except for the number of cycles, which was optimized for each primer pair, the conditions were as follows: 10 μl reactions containing 200 ng DNase-treated RNA, 6 pmol of each primer, 400 μM dNTPs, 0.4 μl enzyme mixture, and 1× supplied buffer were cycled in a Perkin-Elmer 9600 Gene Amp PCR System. The reverse transcriptase reaction was performed at 50 °C for 30 min, followed by a 15 min incubation at 95 °C to activate *HotStarTaq* DNA Polymerase. The PCR conditions were: 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min. The number of cycles used for each primer pair is listed in the Results. The primers used were as follows:

*EphB6 forward primer*5′-CCAGGAACCTGCCAGCGGTGGTG-3′ (bases 2026–2048 NCBI Accession No. NM_004445).
*EphB6 reverse primer*5′-GGTCAGGGTGAAGGAGTGGGATTCG TCTTCTG-3′ (bases 2404–2373 NCBI Accession No. NM_004445).
*Actin forward primer*5′-CTGACTGACTACCTCATGAAG-3′ (bases 626–646 NCBI Accession No. NM_001101).
*Actin reverse primer*5′-ATCCACATCTGCTGGAAGGTG-3′ (bases 1143–1123 NCBI Accession No. NM_001101).

5′RACE. A nested touchdown PCR approach was used to identify the 5′ end of the EphB6 mRNA using Ambion's FirstChoice RACE-Ready Brain cDNA. Both PCRs were performed using the TripleMaster PCR kit from Eppendorf in 50 μl reactions at the following conditions: 94 °C for 30 s/5 cycles of 94 °C for 5 s and 72 °C for 30 s/5 cycles of 94 °C for 5 s and 70 °C for 30 s/20 cycles of 94 °C for 5 s and 68 °C for 30 s. The first reaction contained the following components: 1× reaction buffer, 400 μM dNTPs, 400 nM each primer (5′RACE Outer F supplied by Ambion and EphB6 5′RACE Outer R consisting of bases 356–336 of EphB6 mRNA NCBI Accession No. NM_004445), 2.5 U enzyme, and 0.5 ng Ambion brain cDNA. The second PCR conditions were the same as the first with the following exceptions: the template was 1 μl of the primary PCR and the forward primer was 5′RACE Inner F supplied by Ambion and the reverse was EphB6 5′RACE Inner R (bases 250–220 of NCBI Accession No. NM_004445). The second PCR product was purified using a PCR

purification kit from Qiagen and cloned into pGemT Easy vector (Promega). Ten clones were selected and plasmid DNA sequenced using SP6 and T7 primers to determine the 5′ end of the EphB6 mRNA.

Promoter analysis. The region spanning the EphB6 mRNA start site (−285 to +115) was PCR amplified from genomic DNA (MCF-10A) and cloned into pGL3 basic reporter vector (Promega) in both orientations to generate two clones, pGL3 B6 F (−285 to +115) and pGL3 B6 R (+115 to −285). The pGL3 B6 R construct was used as a negative control for promoter activity in the promoter assay experiments. These constructs (pGL3 B6 F, or pGL3 B6 R) or empty pGL3 basic was transfected along with a control plasmid (pSVβGal) into MCF-7 or MDA-MB-231 cells with Lipofectamine reagent (Invitrogen) according to the manufacturer's specifications. The cells were lysed 36 h after transfection and the lysate was tested for light production (Luciferase Assay kit from Promega). The results were normalized to β-galactosidase activity (Promega). The same constructs were then tested for promoter activity following treatment with *M.Sss*I methylase (NEB) or a mock treatment lacking the methylase enzyme.

Sodium bisulfite sequencing. Genomic DNA was isolated using DNA isolation kit (Qiagen) and digested with *Dra*I enzyme overnight. The DNA was then purified with QiaexII DNA purification kit (Qiagen), eluted in 50 μl water, and denatured by adding 5.5 μl of 3 N NaOH and incubating at 42 °C for 30 min. The unmethylated cytosines were converted to uracils by adding 520 μl of freshly prepared 3 M sodium bisulfite, pH 5 (Sigma), and 30 μl of 10 mM hydroquinone (Sigma) and incubating at 55 °C for 8 h. The DNA was again purified with QiaexII kit and eluted in 50 μl water. The conversion was completed by adding 5.5 μl of 3 N NaOH and incubating at room temperature for 15 min. Next, the DNA was ethanol precipitated, dissolved in 50 μl water, and stored at −80 °C for use in PCRs. The PCR was performed in 50 μl volume with TripleMaster Taq kit (Eppendorf) using a nested PCR approach under the following reaction conditions: 1× reaction buffer, 400 μM dNTPs, 400 nM primers (see Results), 2.5 U enzyme, and 2 μl bisulfite-treated DNA. The nested PCR was performed using the same conditions as above except for the following changes: 1 μl primary PCR product was used as template, and both forward and reverse primers were internal to the forward and reverse primers used in the primary PCR. All cycling conditions were 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 68 °C for 1 min followed by a final extension at 68 °C for 10 min. The amplified products were purified with a PCR purification kit (Qiagen), cloned into pGemT Easy vector (Promega), and plasmid clones were sequenced to determine methylation status of EphB6 promoter in different cell lines.

Methylation specific PCR assay. A nested PCR strategy was used to generate a template for the MSP assay. Four primers were used to amplify the top strand of EphB6 AY280502 following bisulfite conversion. The first reaction contained B62017bs-F (bases 2017–2037 TAGAGAAGG GGTTAGAGGTGG) and B63364bs-R (bases 3364–3345 CCCCACTA CTCTTTCAAACC), and the nested reaction contained B62159bs-F (bases 2159–2179 GGGAGGAGGTATTTAGAGGTG) and B63338bs-R (bases 3338–3315 CCTCTATCCCCAAACATACTATCC). These regions were chosen because they contain no CG dinucleotides and can therefore be used to amplify any bisulfite converted DNA template regardless of methylation status. Both reactions were performed using the TripleMaster PCR kit (Eppendorf) in 50 μl reactions under the following conditions: 94 °C for 2 min/35 cycles of 94 °C for 30 s, 58 °C for 30 s and 68 °C for 1 min followed by a final extension of 10 min at 68 °C. The first reaction contained: 1× reaction buffer, 400 μM dNTPs, 400 nM of each primer (B62017bs-F and B63364bs-R), 2.5 U enzyme, and 2 μl bisulfite-treated DNA. The second reaction was performed under the same conditions with different primers (B62159bs-F and B63338bs-R) and 1 μl of primary PCR product was used as template. The control reactions containing no template DNA were performed in the same manner, with water used in the first reaction and 1 μl of this PCR product used as template in the second PCR. These nested PCR products were then column purified (Qiagen), diluted 1:1000, and used in MSP assay. The MSP assay was also performed using TripleMaster kit using the same conditions as above except that only 25 cycles were needed for amplification. Parallel PCRs were set up using primer sets that would recognize either methylated (MSPF/R-M) or unmethylated (MSPF/R-U) sequences. The sequence of these primers and the rationale for their design are listed in the Results section and illustrated in Fig. 6.

Results

EphB6 mRNA expression as compared to the invasive nature of breast cancer cell lines

We have recently analyzed the abundance of all Eph and ephrin transcript levels in breast carcinoma cell lines (BCCL) by semi-quantitative RT-PCR and correlated them with the invasive phenotypes of these cells. We have thus identified EphB6 as a transcript that may be related to the invasive phenotype of BCCL. The relative expression of EphB6 mRNA among varying BCCL is shown in Fig. 1 as determined by semi-quantitative RT-PCR. At 34 cycles, the EphB6 transcript is absent in 3 out of 4 cell lines characterized as invasive by either in vitro or in vivo assays. The three invasive cell lines showing an absence of EphB6 transcript are MDA-MB-231, MDA-MB-435, and BT-549. The EphB6 transcript is present in the invasive cell line, Hs578T as well as in all non-invasive cell lines, both normal (MCF-10A) and tumorigenic (MCF-7, BT-20, and SkBR3). With the exception of Hs 578T this analysis of EphB6 mRNA levels seems to support our previous assertion that EphB6 mRNA levels may be correlated to the invasive phenotype of BCCL due to the fact that all non-invasive BCCL express the EphB6 transcript while only one of the invasive cell lines shows any expression of this transcript under the conditions tested.

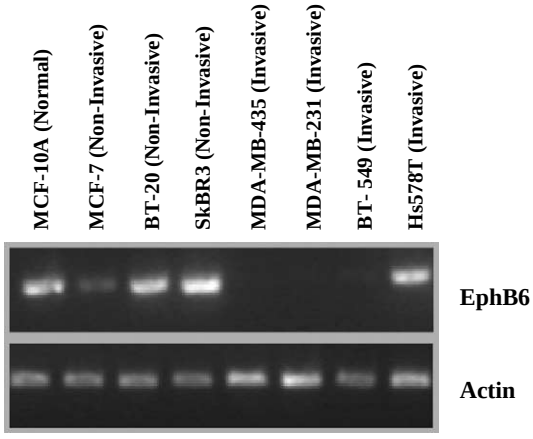

Fig. 1. Expression profile of EphB6 in a panel of breast carcinoma cell lines. One-step RT-PCR (Qiagen) was performed for 34 cycles using 200 ng DNase-treated RNA from each cell line with specific primers for EphB6 as listed in Materials and methods. These RNAs were normalized to actin to confirm equal amounts of RNA used per reaction. The actin RT-PCRs were performed for 18 cycles which we have confirmed to be in the exponential range of amplification. RT-PCR products were separated on 1.5% agarose gels and visualized and photographed using Gel Doc (Bio-Rad) imaging software. The experiments were performed three times and representative results are presented.

Promoter analysis of human EphB6

In order to study the transcriptional regulation of EphB6, we first needed to identify the transcriptional start site (TSS) of this gene. An EST database search proved inconclusive. Therefore, we decided to map the TSS using a 5′RACE approach. Fig. 2 shows the results of our analysis as compared to the previously documented 5′ end of the EphB6 mRNA. The genomic sequence from NCBI (Accession No. AY280502) lists base 2950 as the first base of the mRNA but not all ESTs listed in the NCBI database share the same first base. Our results show that 9/10 clones obtained from a 5′RACE Ready human brain cDNA library contain a TSS at base 2976 and 1/10 starting at base 2970. While not significantly different than the published data, to our knowledge we are the first to confirm the TSS of EphB6 and have based our promoter analysis upon these results.

The region upstream of the EphB6 TSS does not contain any TATA box. In order to determine if the DNA sequence just upstream of the start of the EphB6 transcript could act as a promoter, we used a luciferase based reporter gene system (pGL3 basic from Promega). We prepared two constructs and transfected them into MCF-7 and MDA-MB-231 cells to determine their ability to promote transcription of the luciferase gene. Both constructs were made by performing PCR on MCF-10A (normal) genomic DNA with primers specific to the region between −285 and +115 of the TSS. This region corresponds to the region from base 2691 to 3090 on NCBI Accession No. AY280502 as shown in Fig. 3. The construct pGL3 B6 F was made by cloning the putative promoter sequence in the forward direction (−285 to +115), while the control construct pGL3 B6 R contained the fragment in the opposite direction (+115 to −285), between *Kpn*I and *Hin*dIII sites of the vector. The

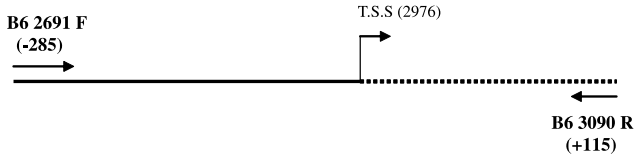

Fig. 3. Region spanning EphB6 TSS. A schematic of the region surrounding the TSS of EphB6 and the primers used to generate promoter constructs. The nucleotide numbers correspond to the EphB6 gene sequence in the database (NCBI Accession No. AY280502).

constructs, or an empty pGL3 basic vector, were transfected in triplicate into MCF-7 and MDA-MB-231 cells along with pSVβGal plasmid (to control for transfection efficiency) using Lipofectamine (Invitrogen) and grown for 36 h. The cell lysates were prepared from each well of a 24-well plate, and the lysates were tested for luciferase activity. Similarly, the lysates were also tested for β-galactosidase activity. The luciferase values were normalized between replicates by calculating the ratio of the RLU produced by the luciferase assay and the A_{420} of the lysate from the same transfection following the β-galactosidase assay. The fragment in the forward direction (−285 to +115) has strong promoter activity while the activity was barely detectable when the orientation of the fragment was reversed (Fig. 4). These results demonstrate that the region just upstream of the TSS can act as a promoter in a cell line-independent manner.

The human EphB6 promoter contains a CpG island and its activity is regulated by methylation

An analysis of the EphB6 promoter shows that a significant number of CG dinucleotides are present so we hypothesized that methylation may play a role in the transcriptional regulation of this gene in BCCL. We tested this

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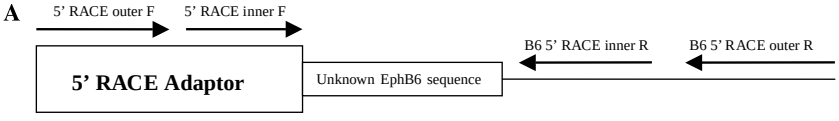


B

NCBI Accession Number	Start of mRNA as compared to reference clone NCBI Accession # **AY280502**
AL532843	**2849**
BX414750	**2920**
BX414749	**2920**
AY280502	**2950**
NM_0044555	**2969**
D83492	**2969**
BP348912	**2972**
BP201172	**2976**
BP251706	**2993**
BP250880	**2993**
BX419816	**3007**
5'end of EphB6 as determined by 5'RACE of Ambion RACE Ready Brain cDNA: 9/10 clones start at **2976** and 1/10 start at **2970** of **AY280502**	

Fig. 2. EphB6 transcriptional start site as determined by 5′RACE. (A) A schematic showing the nested PCR approach used to generate PCR products for 5′RACE cloning experiment. The primers used in the primary PCR were 5′RACE outer F and B6 5′RACE outer R and the primers used in the second PCR were 5′RACE inner F and B6 5′RACE inner R. One microliter of the primary PCR product was used in the second PCR. The secondary PCR product was purified and cloned into pGemTeasy (Promega) and sequenced to determine the 5′ end of the EphB6 mRNA (B) The first base of EphB6 mRNA generated by 5′RACE or those sequences present in NCBI database and its placement on the genomic sequence AY280502.

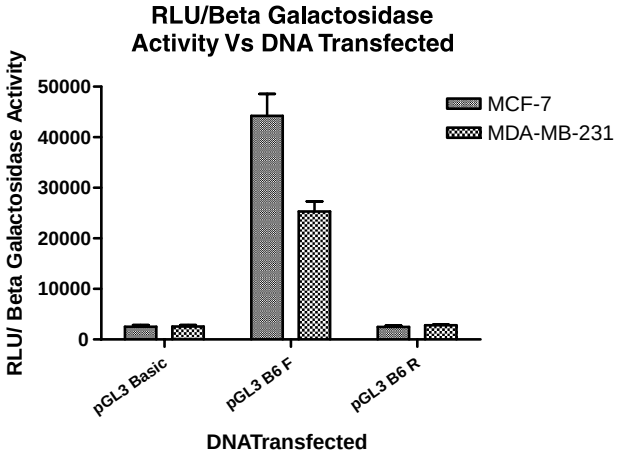


Fig. 4. Promoter activity of the region surrounding the EphB6 transcriptional start site in MCF-7 and MDA-MB-231 cells. Promoter constructs were made using primers shown in Fig. 2 with MCF-10A (normal) DNA as template. These PCR products were then cloned into pGL3 basic vector (Promega) to generate either the forward construct pGL3 B6F (primers B6 2691 F/B6 3090 R) or the reverse construct pGL3 B6R (primers B6 3090 F/B6 2691 R). The constructs, or an empty pGL3 basic vector, were transfected (Lipofectamine 2000) into MCF-7 or MDA-MB-231 cells along with pSVβgal vector (Promega) and lysates were prepared after 36 h. Luciferase activity and β-galactosidase activity were measured and results illustrated. The values plotted are the average of three experiments with standard deviation.

hypothesis initially by growing MDA-MB-231 cells in the presence of 5-aza-2′-deoxycytidine (Aza) for 72 h and then measuring the expression levels of EphB6 transcript by semi-quantitative RT-PCR. The expression of EphB6 transcript increased drastically in MDA-MB-231 cells after Aza treatment (Fig. 5). To demonstrate the inhibitory effect of methylation on promoter activity, we methylated the promoter construct with *M.Sss*I methylase (NEB) prior to transfection. As shown in Fig. 6, the methylated promoter construct has lost its ability to promote the transcription of the luciferase gene.

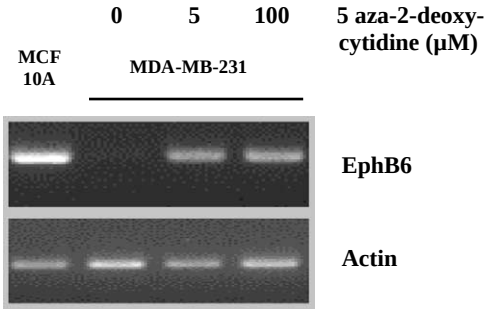


Fig. 5. EphB6 transcript levels in MDA-MB-231 cells following treatment with 5-aza-2′-deoxycytidine. MDA-MB-231 cells were cultured in the presence of varying concentrations of 5-aza-2′-deoxycytidine and the amounts of EphB6 and β-actin transcripts were determined by RT-PCR as previously described. RT-PCR products were separated on a 1.5% agarose gel and visualized and photographed using Gel Doc (Bio-Rad) imaging software. The experiments were performed three times and yielded similar results.

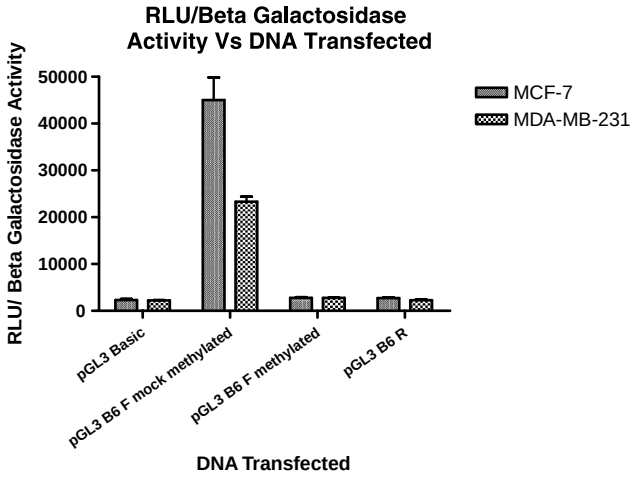


Fig. 6. Activity of methylated EphB6 promoter in MCF-7 and MDA-MB-231 cells. The EphB6 forward promoter construct (pGL3 B6F) was either methylated or mock methylated in vitro using *M.Sss*I (NEB) and transfected into MCF-7 or MDA-MB-231 cells along with pSVβgal (Promega) as described in Fig. 3 and Materials and methods. Luciferase activity and β-galactosidase activity were measured after 36 h. The values plotted are the average of three experiments with standard deviation.

Methylation status of EphB6 promoter in BCCLs

Once we had shown that the promoter for EphB6 could be controlled by methylation, we tested whether this mechanism was responsible for the absence of the EphB6 transcript in the invasive cell line. We employed a sodium bisulfite sequencing approach to compare the methylation status of the EphB6 promoter in BCCL. A nested PCR strategy was used to generate a template for this sequence analysis. Four primers were generated to amplify the top strand of EphB6 AY280502 following bisulfite conversion. The first reaction contained B62159bs-F (bases 2159–2179 GGGAGGAGGTATTTAGAGGTG), and B63364bs-R (bases 3364–3345 CCCCACTACTCTTTCAAACC) and the nested reaction contained B62568bs-F (bases 2568–2590 GGTGTGTGTAGGGTTTTGTTTTG) and B63338bs-R (bases 3338–3315 CCTCTATCCCCAAACA TACTATCC). These regions were chosen because they contain no CG dinucleotides and can therefore be used to amplify any bisulfite converted DNA template regardless of methylation status. Both reactions were performed using the TripleMaster PCR kit (Eppendorf) in 50 μl reactions at the following conditions: 94 °C for 2 min/35 cycles of 94 °C for 30 s, 58 °C for 30 s and 68 °C for 1 min followed by a final extension of 10 minutes at 68 °C. The first reaction contained: 1× reaction buffer, 400 μM dNTPs, 400 nM each primer (B62159bs-F and B63364bs-R), 2.5 U enzyme and 2 μl bisulfite-treated DNA. The second reaction was performed under the same conditions with different primers (B62568bs-F and B63338bs-R) and 1 μl of primary PCR product used as template. These PCR products were column purified (Qiagen), cloned into pGemT easy (Promega), and at least 10 clones were sequenced per cell line to determine methylation status.

Fig. 7 shows that methylation is correlated with EphB6 transcript levels in the BCCL tested. No methylation between −285 and +115 of the EphB6 TSS was seen in cell lines that expressed the EphB6 transcript, while MDA-MB-231 cells have a high percentage of CpG dinucleotides methylated in this region. There are 54 CG dinucleotides between −285 and +115 of the EphB6 promoter. The sequencing of 10 or more clones from each cell line revealed that 66% of the CGs are methylated in MDA-MB-231 cells. There is no methylation seen in the other cell lines tested (MCF-10A, MCF-7) within the same region. The analysis of an additional 26 CG dinucleotides between bases +115 and +338 of the TSS indicated a slightly higher methylation in MCF-7 cells as compared to MDA-MB-231 cells (68% for MCF-7 versus only 65% for MDA-MB-231). MCF-10A cells again show no methylation within this expanded region. Since MCF-7 has a lower expression level of EphB6 than MCF-10A but higher than MDA-MB-231, we considered the methylation of this region as being responsible for this intermediate expression. The methylation sensitivity of EphB6 expression in MCF7 cells was further confirmed by treating these cells with Aza (data not shown). It is worth noting here that immunocytochemistry experiments with an antibody specific to EphB6 shows strong protein expression in MCF-10A and MCF-7 cells but no detectable signal in MDA-MB-231 (data not shown), suggesting that: (i) EphB6 mRNA in MCF-7 cells is translated into protein, and (ii) transcript levels correspond to the presence or absence of EphB6 protein in these cells.

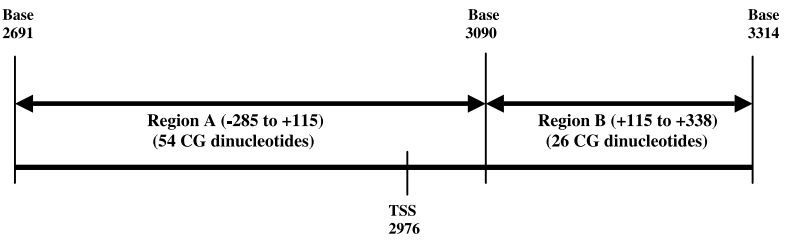
A methylation specific PCR assay to determine methylation status of EphB6 promoter in BCCL

Several studies suggest that mRNA and/or protein levels of EphB6 are useful in the diagnosis or prognosis of certain types of cancer. We have proposed that EphB6 may be involved in or correlated with the invasiveness of breast cancer. Since the methylation status of the EphB6 promoter appears to be correlated to the expression of EphB6 mRNA, we have developed an assay to determine whether or not specific residues within this region are methylated. We have identified numerous methylated CGs in the cell line that does not express the EphB6 mRNA and the same CGs are never methylated in the cell lines that do contain this transcript (Fig. 8). These regions were used to design primer pairs that could distinguish between methylated and non-methylated sequences (Fig. 8). This assay was verified on the BCCL used in this study and the results are presented in Fig. 9. Initially, a PCR product was made using a nested PCR strategy with two primer pairs specific to bisulfite-treated DNA in the region surrounding the EphB6 promoter (Materials and methods and Fig. 9A). The results of this nested PCR experiment are presented in Fig. 9B. The nested PCR will amplify a 1179 bp fragment in all cell lines regardless of methylation status since there are no CG dinucleotides within the sequences used to design primers. The PCR products were diluted 1:1000 and used as templates in parallel PCRs using internal sequences as primers. These parallel PCRs contained primers that in one reaction will generate a product only if the region was methylated (primers MSP F/R-M) and in the other reaction will amplify only if the region is unmethylated (primers MSP F/R-U). The MSP assay can reliably distinguish EphB6 expressing cells from non-expressing cells (Fig. 9C).

Discussion

We have demonstrated here a correlation between promoter methylation and silencing of EphB6 gene expression in MDA-MB-231 cells that represent an invasive breast carcinoma cell line. The authenticity of the promoter's dependence on methylation was confirmed by the following lines of evidence: (i) The ability of an approximately 400 base pair sequence upstream of EphB6 transcriptional start site to stimulate the expression of luciferase gene, (ii) the

A



EphB6 promoter NCBI Accession # AY280502

B

	Region A	Region B
Cell Line	% Methylation between -285 and +115 of B6 TSS (54 CGs)	% Methylation between +115 and +338 of B6 TSS (26 CGs)
MCF-10A	**0%**	**0%**
MCF-7	**0%**	**68%**
MDA-MB-231	**66%**	**65%**

Fig. 7. Percentage of methylated CG dinucleotides in various breast carcinoma cell lines as determined by sodium bisulfite sequencing. (A) A schematic of the EphB6 promoter region used for determining the methylation status in BCCL relative to the TSS. (B) Percent methylation of CG dinucleotides in EphB6 promoter as determined by sodium bisulfite sequencing. Ten clones were sequenced for each cell line.

A

Base 2801of AY280502

CGCGTCTCCCCCGCGCGCCG ---//---CGGCGATCTCGACGCGGG

CG # 1 2 3 4 5 6 7 8 9 10 11

Base 3049 of AY280502

B

Cell Line	CG #1	CG #2	CG #3	CG #4	CG #5	CG #6	CG #7	CG #8	CG #9	CG #10	CG #11
MCF-10A	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
MCF-7	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
MDA-MB-231	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

C

Sequence	**2801-2820 of AY280502 (MSP Primer name)**	**Complement of 3049-3032 of AY280502**
Unconverted	CGCGTCTCCCCCGCGCGCCG	CCCGCGTCGAGAT CGCCG
Converted in methylated cell lines	CGCGTTTTTTTCGCGCGTCG (**MSP 1F M**)	CCCGCGTCGAAAT CGCCG (**MSP 1R M**)
Converted in unmethylated cell lines	TCTGTTTTTTTTGTGTGTTG (**MSP 1F U**)	CCCACATCAAAAT CACCA (**MSP 1R U**)

Fig. 8. A detailed analysis of EphB6 promoter methylation status in breast carcinoma cell lines and methylation specific PCR primer design. (A) A diagram of the sequences between bases 2801–2820 and 3049–3032 of AY280502 with CG dinucleotides numbered. (B) The percent methylation of the listed CGs. Ten clones were sequenced for each cell line at the regions indicated. (C) The sequence of these regions following conversion is different in cell lines with methylation versus cell lines without methylation and these sequences were used to design primers for methylation specific PCR.

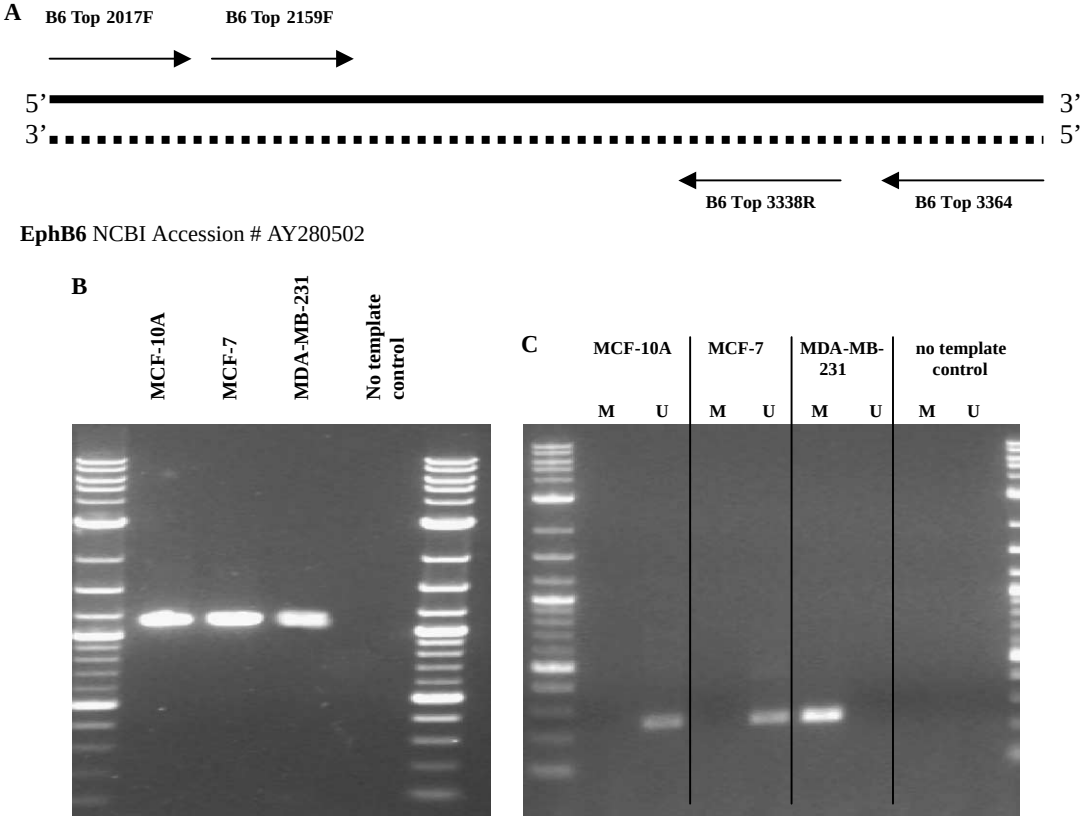


Fig. 9. MSP assay for predicting EphB6 expression in BCCL. (A) A schematic showing primers used for generating a PCR product by nested PCR approach. (B) The secondary PCR products were run on a 1.5% agarose gel. (C) The secondary PCR products were purified and diluted 1:1000, and used in parallel PCRs with primers that will amplify either methylated sequences (M) or unmethylated sequences (U). The amplified products were run on a 1.5% agarose gel and visualized and photographed using Gel Doc (Bio-Rad) imaging software.

abolition of luciferase expression following methylation of luciferase gene construct, (iii) the restoration of EphB6 gene expression in MDA-MB-231 cells after treatment with Aza. Our results support other observations describing methylation-dependent down-regulation of tumor suppressors and metastasis suppressors in cancer cells [37–42]. The

loss of EphB6 expression in MDA-MB-231 cells seems significant in light of other reports demonstrating EphB6 as a prognostic indicator in neuroblastoma and a diagnostic marker of metastatic melanoma [29,30,32].

The comparison of promoter regions in MCF-10A, MCF-7 and MDA-MB-231 cells has allowed us to identify specifically methylated CpG dinucleotides in these cells. The promoter region has sites for well-characterized transcription factors such as SP1 and p300 as determined by TFSearch software (www.cbrc.jp/research/dp/TFSEARCH.html). The binding of many transcription factors is known to be affected in methylated promoters [43–45], thus suggesting the loss of SP1 or p300 binding to EphB6 promoter in MDA-MB-231 cells. The decrease in EphB6 expression in MCF-7 cells as compared to MCF-10A cells may be attributed to methylation of CpG dinucleotides downstream of transcriptional start site. The involvement of downstream CpGs in transcription regulation is confirmed by treating MCF-7 cells with Aza resulting in increased levels of EphB6 transcript. We predict that these CpG dinucleotides are potential binding sites for activator proteins and methylation of these sites prevents the recruitment of activators. We conclude that binding of SP1 and/or p300 as well as some activator protein(s) is both sensitive to promoter methylation, and differential patterns of methylation result in the loss of basal transcription apparatus in MDA-MB-231 cells and inability of the activator to join the basal factors in MCF-7 cells. Although our results do not allow us to pinpoint the role of individual CpGs in promoter function, they do underscore differences in promoter methylation patterns of normal breast cells, non-invasive and invasive breast carcinoma cells. Such differences have important ramifications for breast cancer diagnosis and prognosis.

The detection of methylated CpG regions in the genome has been accomplished by MSP and it has been applied to identify methylated promoters or genes in a variety of human cancers [36–42]. The analysis of EphB6 promoter has allowed us to employ MSP for distinguishing normal breast cells, non-invasive and invasive breast carcinoma cells. These observations thus indicate the potential of EphB6 to supplement the paucity of tumor and invasion markers for breast cancer.

In conclusion, we have demonstrated the potential of EphB6 to serve as a breast cancer biomarker based on the differential methylation of its promoter in normal and cancer cells. The importance of DNA based breast cancer markers is underscored by the fact that: (i) they are amenable for detection by MSP, (ii) MSP is a robust and sensitive protocol [46,47], and (iii) it can potentially be applied to detect cancer cells present in circulation.

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