DNA-Based Assay for EPHB6 Expression in Breast Carcinoma Cells as a Potential Diagnostic Test for Detecting Tumor Cells in Circulation

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Abstract. *The early detection of breast cancer is critical for improved treatment and better management of the disease. The dissemination of tumor cells into the blood stream is known to occur early in tumor progression and these circulating tumor cells (CTCs) may be detectable before the occurrence of tumor metastasis. Methylation-specific polymerase chain reaction (MSP) can be exploited for detecting CTCs on the basis of differential methylation of numerous gene promoters in normal and carcinoma cells. In this study, we describe the relationship between loss of Ephrin receptor B6 (EPHB6) expression and the aggressiveness of breast carcinoma cell lines (BCCLs). The loss of EPHB6 expression in more aggressive BCCLs is regulated in a methylation-dependent manner. We demonstrate the ability of an EPHB6 MSP to distinguish between methylated and unmethylated EPHB6 promoters, and to predict expression of the EPHB6 transcript and protein. The sensitivity of MSP was related to the volume of blood processed for DNA isolation. As few as 50 tumor cells in 5 ml blood were detectable with a high efficiency. However, the detection of 10 tumor cells/5 ml was not as efficient. On the other hand, 5 tumor cells or 100 pg of free DNA in 200 μl of blood was also easily detectable. Our results suggest that MSP could be applied to detect even a single cell in 1 ml of blood by employing appropriate modifications. The EPHB6 MSP has clinical implications for the prognosis and/or diagnosis of breast and other cancer types including neuroblastoma, melanoma, and non-small cell lung carcinoma wherein EPHB6 expression is lost in more aggressive forms of the disease.*

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The accumulation of gain- or loss-of-function mutations is important for tumor progression and metastasis. In addition, epigenetic alterations are known to play a causative role in this progression. These epigenetic changes involve temporal or cell-specific transcriptional regulation of genes by methylation of CpG dinucleotides found within promoter sequences. There are many cases of hypermethylation and hypomethylation of gene promoters leading to transcriptional silencing of tumor suppressor or metastasis suppressor genes and overexpression of tumor promoting genes, respectively (1-4). Changes in the methylation status of specific gene promoters are important diagnostic and prognostic indicators of cancer and these genes may be exploited as therapeutic targets (2, 5-13).

The accumulation of mutations and epigenetic changes can lead to the dissemination of cells from a tumor into the peripheral blood prior to metastasis. Circulating tumor cells (CTCs) are present in patients with all major carcinomas but not in healthy individuals or individuals with nonmalignant forms of these tumors (14). The detection of CTCs can have a major impact on the prognosis and diagnosis of cancer (15-17). Many techniques exist to detect CTCs (18-24).

In addition to CTCs, it has been shown that circulating tumor DNA, in particular methylated DNA, also has the potential to be used for the detection of tumors (25-28). It is becoming clear that hypermethylated DNA can be detected in the blood of individuals with cancer (29-31). The identification and characterization of differentially methylated DNA markers are important for detecting free methylated tumor DNA or methylated DNA from CTCs. The methylation patterns of genes and gene promoters can be mapped using a sodium bisulfite sequencing protocol that relies on the chemical modification of cytosines, but not methylated cytosines (32). These maps can be used to design specific primers for differential amplification of either a methylated or an unmethylated promoter after it has been treated with sodium bisulfite. Such polymerase chain reaction (PCR)-based detection methods are robust, sensitive, and lend themselves to automation (6, 7, 9, 12, 33).

The Ephrin (EPH) family of receptors, with 14 members identified to date (34), contains several genes known to be

involved in the progression of different types of cancer including breast cancer (35-37). The potential usefulness of EPH receptors for cancer prognosis and/or diagnosis is evident from their altered expressions in melanomas, neuroblastomas, non-small cell lung carcinomas and breast carcinoma (38-44). There are reports demonstrating methylation-dependent transcriptional regulation of EPHB6 in neuroblastoma (45) and breast carcinoma (46), EPHA3 in hematopoietic tumor cells (47), and EPHA7, EPHB2 and EPHB4 in colorectal cancer (48-50). To our knowledge there is no clinically tested DNA-based assay to predict ephrin receptor expression in cancer cells using promoter methylation status. Previously, we described a methylation-specific PCR (MSP) assay capable of distinguishing methylated and unmethylated *EPHB6* promoters in breast cancer cells. In this study, we have systematically determined the sensitivity of this assay and confirmed its potential utility to detect a limited number of CTCs and a limited amount of free methylated tumor DNA in the background of a large number of normal cells. Given that the expression of this gene can be predicted by MSP in an extremely sensitive manner, and that the expression of this gene is an indicator of tumor phenotype, we suggest MSP of EPHB6 as a potential diagnostic and prognostic test of breast cancer.

Materials and Methods

Cell culture. A variety of human breast cancer cell lines such as MCF-7, MDA-MB-231, MDA-MB-435, BT549, and Hs578T (51) were cultured at 37˚C in the presence of 7% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Gibco Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Thermo Scientific HyClone, Logan, UT, USA), 2.0 mM L-glutamine (Gibco), 25 units/ml penicillin (Gibco), and 25 μg/ml streptomycin (Gibco).

Blood collection. Random blood samples: Blood from ten random individuals (male and female) known to be free of breast cancer were obtained from the Blood Bank Collection.

Blood samples for cell/DNA-mixing experiments: Blood samples were collected using Vacutainers™ containing heparin (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and stored at 4˚C until use. Immediately following collection, the blood samples were heated to 56˚C for 30 minutes to inactivate the complement system (52).

Mixture of human blood with tumor cells. In order to investigate the sensitivity of the proposed diagnostic test for detecting tumor cells in circulation, we created an experimental sample of blood containing breast tumor cells. An aliquot (5 ml) of blood was mixed with 200 μl suspension of a known number of cells from breast cancer cell line, MDA-MB-231, in phosphate-buffered saline (PBS). The tumor cells were subsequently isolated from the above mixture to determine the minimum number of tumor cells that can be reliably separated from blood and confirmed by the methylation pattern of EPHB6 promoter sequence.

Isolation of tumor cells from blood. A mixture of 5 ml of blood and 200 μl suspension of MDA-MB-231 cells in PBS was layered onto an equal volume of Histopaque (Sigma, St Louis, MO, USA) and spun at 400 xg for 30 minutes at room temperature. The red blood cells were left on the bottom of the tube and the remainder of the sample was moved to a conical tube. The cells were washed by mixing with 10 ml of PBS and centrifuging at 2,500 xg for 10 minutes at room temperature. This procedure was repeated, samples were spun at 2,500 xg, the supernatant was removed, and the pellet (containing the mononuclear breast tumor cells) was suspended in 200 μl of PBS and processed for genomic DNA isolation as described below in the DNA isolation protocol.

Alternative method for isolating tumor cells without separating the mixture of cells. A fixed amount of human blood was mixed with different numbers of tumor cells and the entire mixture was processed for isolating the genomic DNA as described below in the DNA isolation protocol. This method eliminated the need for separating the tumor cells before processing them for DNA isolation. The rationale and details are presented in the results section.

Mixture of human blood with externally added tumor cell DNA. The samples of human blood containing tumor cell DNA were simulated by adding different amounts of genomic DNA fromMDA-MB-231 cells to a fixed amount (200 μl) of human blood. The mixture was then processed to isolate total genomic DNA as described below.

DNA isolation. DNA was isolated from the random blood samples using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendation.

For the isolation of DNA from: i) cells, ii) blood samples containing free MDA-MB-231 DNA, iii) blood samples containing MDA-MB-231 cells, and iv) purified mononuclear cells from 5 ml of blood, the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) was used. The elution step was performed using 110 μl of elution buffer to recover a final volume of approximately 100 μl.

Bisulfite conversion of genomic DNA. Purified DNA samples were treated with sodium bisulfite using the EZ DNA Methylation-Gold kit (Zymo Research, Orange, CA, USA) according to the manufacturer's recommendations. The converted DNA was eluted from the supplied column with 11 μl of water to recover a final volume of approximately 9 μl. The converted DNA corresponding to the *EPHB6* promoter region was cloned and sequenced to determine methylated CpG dinucleotide as previously described (46).

MSP. Bisulfite-converted DNA (3 μl) was subjected to MSP in 10 μl reactions using the HotStarTaq kit (Qiagen). Reaction mixtures contained 400 μM dNTPs, 6 pmol each of a forward and reverse primer (46) and 0.25 units *HotStarTaq™* polymerase in supplied buffer. First, the reactions were incubated at 95˚C for 15 minutes to activate the enzyme. Subsequently, 45 cycles of a three temperature PCR were performed using the following conditions: 94˚C for 30 seconds, 60˚C for 30 seconds, and 72˚C for 30 seconds. Following the 45th cycle, a final extension was performed by incubating the samples at 72˚C for 10 minutes.

Results

Promoter methylation has predictive value for EPHB6 expression. We have previously shown differential expression of EPHB6 in a panel of breast carcinoma cell lines with varying phenotypes and in breast tumor RNA isolated from

Table I. *Methylated CpG dinucleotides around the EPHB6 transcriptional start site in breast cell lines.*

Cell line	Number of methylated CpG dinucleotides per clone* (Mean±standard deviation)
MCF-10A	0±0
MCF-7	0±0
MDA-MB-435	38.9±4.6
MDA-MB-231	35.6±2.8
BT549	41.6±5.3
Hs578T	0±0

*54 CpG dinucleotides are present between nucleotides –285 and +115 relative to the *EPHB6* transcriptional start site.

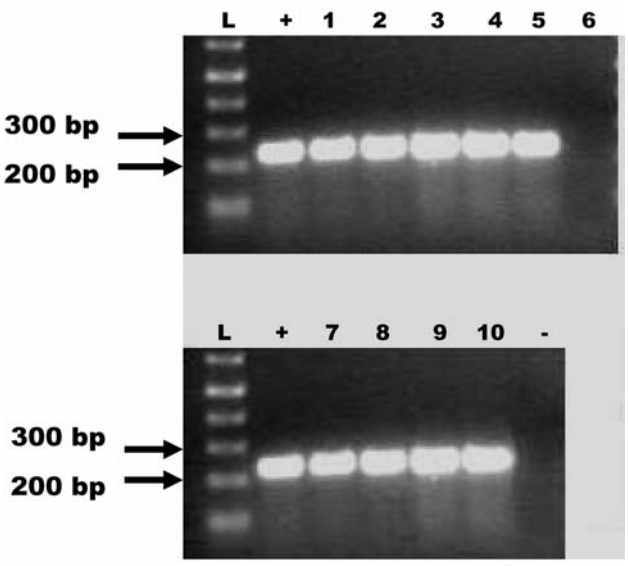

Figure 1. *MSP using primers recognizing unmethylated EPHB6 promoter. DNA from ten random blood samples (labeled 1-10) obtained from the Blood Bank Collection were purified, bisulfite treated, and used as templates in the EPHB6 MSP assay as described in Materials and Methods. PCR products were separated on 1.5% agarose gel and the ethidium bromide-stained gel was photographed. Positive control (+) is MCF-7 bisulfite-treated DNA. The PCR shown contained primers recognizing unmethylated EPHB6 promoters; no amplification was seen in the reactions containing primers recognizing methylated EPHB6 promoters. The lane marked as L contains a DNA ladder. The size of the expected PCR product is 248 bp.*

breast cancer patients (43, 44, 46). Overall, *EPHB6* expression was lost in the more invasive breast carcinoma cell lines and a sample of metastatic breast tumor. The transcription of this gene was shown to be regulated in a methylation-dependent manner, and differences in the methylation status in the *EPHB6* promoter are detectable using MSP (46). In order to characterize the relationship between methylation status of the *EPHB6* promoter and transcript abundance, we have expanded our analysis to include additional invasive breast cancer cell lines. As shown in Table I, methylated CpG dinucleotides are present in a stretch of 400 basepairs of *EPHB6* promoter in invasive breast cancer cell lines MDA-MB-231, MDA-MB-435, and BT549. The *EPHB6* transcript and protein are not detectable in these cell lines. The number of methylated cytosines listed in the Table are an average of 10 independent clones of bisulfite converted genomic DNA from each cell line. There are 54 CpG dinucleotides within the *EPHB6* promoter region between nucleotide positions –285 and +115 relative to the transcriptional start site. A majority (66%-77%) of CpG dinucleotides within the *EPHB6* promoter are methylated in cell lines with no detectable *EPHB6* expression. On the other hand, *EPHB6* transcript and protein are not expressed and *EPHB6* promoter is not methylated in MCF-10A, MCF-7 and Hs578T cell lines.

MSP detects methylated EPHB6 promoter. The MSP primers were originally designed to regions of the promoter that were shown to be methylated in 10 clones of bisulfate-treated genomic DNA from MDA-MB-231 cells (46) and unmethylated in 10 clones of each sample of bisulfate-treated genomic DNA from MCF-10A and MCF-7 cells. As described in our earlier study and confirmed by the observations with the additional cell lines presented here (Table II), we demonstrate a correlation between *EPHB6* expression and promoter methylation. These results demonstrate methylation of the promoter and silencing of *EPHB6* transcription in the majority of invasive breast carcinoma cell lines studied here.

EPHB6 MSP has potential clinical application. In order for the *EPHB6* MSP to be considered a potential diagnostic or prognostic tool for detecting circulating breast cancer cells with a methylated *EPHB6* promoter, the methylation status of the *EPHB6* promoter in blood samples from normal individuals was characterized. DNA from 10 individuals known to have no breast cancer was obtained and processed for the *EPHB6* MSP. The results presented in Figure 1 indicate that only unmethylated *EPHB6* promoter was detectable in this experiment. The primers recognizing methylated *EPHB6* promoters yielded no amplification. These results demonstrate that *EPHB6* promoter in mononuclear cells in the blood of normal individuals is unmethylated, and thus confirm the feasibility of using MSP of *EPHB6* promoter for CTCs.

EPHB6 MSP is robust, specific and sensitive. The specificity of this assay was determined in several ways. First, the DNA from mixtures of one million MCF-7 cells containing varying numbers of MDA-MB-231 cells was subjected to *EPHB6*

Table II. *Methylation status of the CpG* dinucleotides in EPHB6 promoter***.

Cell line	CpG #1	CpG #2	CpG #3	CpG #4	CpG #5	CpG #6	CpG #7	CpG #8	CpG #9	CpG #10	CpG #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%
MDA-MB-435	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
BT549	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Hs578T	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

*The forward MSP primer contains CpG dinucleotides 1-6 and the reverse MSP primer has 7-11 CpG dinucleotides. **Data for MCF-10A, MCF-7 and MDA-MB-231 cells have been described previously (46).

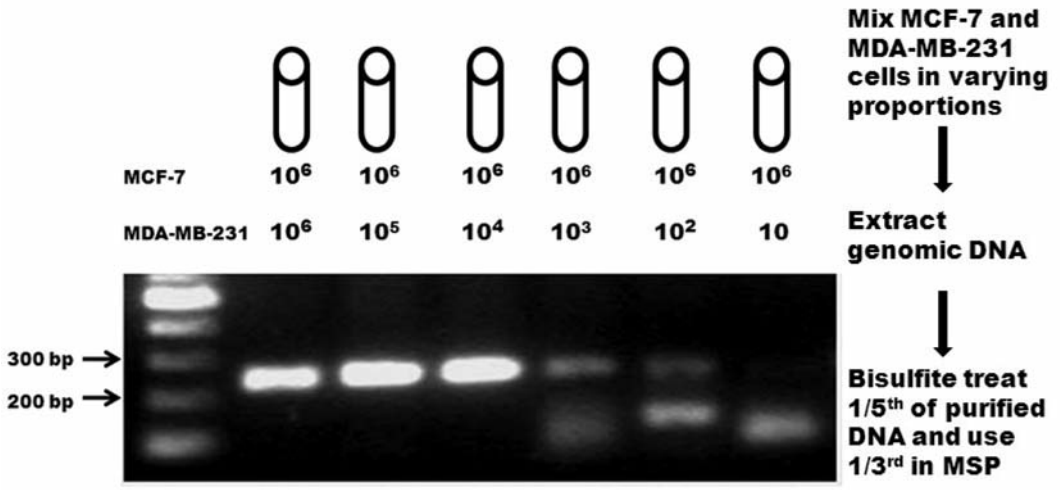

Figure 2. *MSP, using primers recognizing methylated EPHB6 promoter, with bisulfite-treated DNA from mixtures of MCF-7 and MDA-MB-231 cells. DNA was purified from mixtures of MCF-7 and MDA-MB-231 cells, bisulfite treated and used as template for the EPHB6 MSP. The PCR products were separated on 1.5% agarose gels. The size of the expected PCR product is 248 bp.*

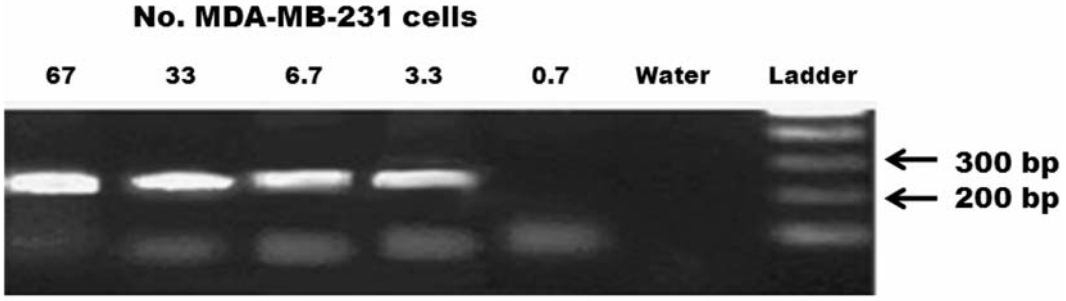

Figure 3. *EPHB6 MSP for detecting as few as 50 MDA-MB-231 cells in 5 ml of blood. DNA was purified and bisulfite-treated from mixtures of MDA-MB-231 cells and 5 ml of whole blood as described. The amount of DNA added to the MSP reaction was only 1/15th of the total DNA purified from the samples. The size of the expected PCR product is 248 bp.*

MSP following bisulfite conversion. As shown in Figure 2, a product of the expected size was readily detectable in samples containing as few as 100 MDA-MB-231 cells in a background of one million MCF-7 cells ($1:10^4$). To evaluate the possibility of the *EPHB6* MSP detecting CTCs in blood samples, different numbers of MDA-MB-231 cells were added to 5 ml of blood and the mixture was processed for the detection of the methylated EPHB6 promoter. As shown in Figure 3, as few as 50 MDA-MB-231 cells per 5 ml of blood can be detected using this assay. The detection of 10 cells per

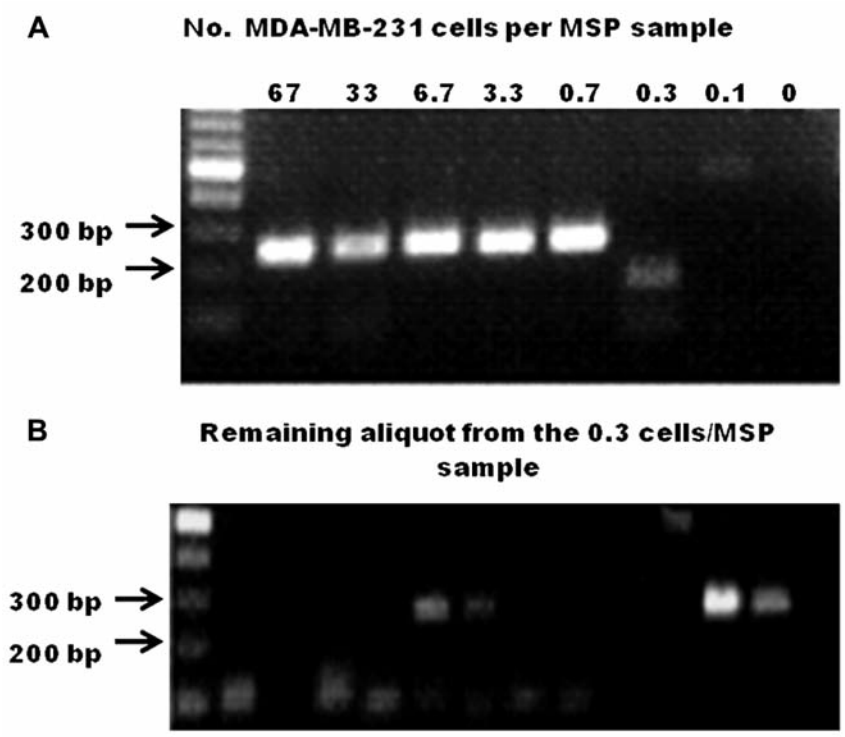

Figure 4. *EPHB6 MSP for detecting 5 MDA-MB-231 cells in 200 µl of blood. DNA from mixtures of MDA-MB-231 cells and 200 µl of blood was purified and treated with sodium bisulfite as described. The number of tumor cells used in each reaction is listed. A: Amplification is seen for samples containing 0.7 or more cells. B: The remaining aliquot of DNA from 0.3 cell sample was divided into four parts, bisulfite treated and subjected to three amplification reactions per sample. The size of the expected PCR product is 248 bp.*

5 ml of blood was not successful in the first trial. However, when the assay was repeated two additional times, detection of the methylated *EPHB6* promoter from 10 cells in 5 ml of blood was achieved. The failure of the first detection assay is attributed to less than optimum recovery of mononuclear cells during the isolation procedure.

Since the mononuclear cell isolation procedure appeared suboptimal, we decided to evaluate the sensitivity of MSP by eliminating the cell isolation/enrichment step as described below. Different numbers of MDA-MB-231 cells were added to 200 µl of blood and DNA from the entire mixture was purified and used for the MSP assay. For these analyses, 1/5th of the purified DNA was bisulfite treated and 1/3rd of the treated DNA was used in a single tube for MSP. Therefore, each reaction contains 1/15th of the initial sample containing 200 µl of blood and MDA-MB-231 cells. As shown in Figure 4A, the methylated promoter from 10 cells per 200 µl of blood was detected in the first trial. Since only a small amount of the DNA isolated from the mixture containing 5 cells was used in the first trial (Figure 4A), the remainder of the sample was processed in the same manner. Figure 4B indicates that the methylated *EPHB6* promoter was detected in 4/12 samples processed, meaning that as few as 5 MDA-MB-231 cells can be detected when mixed with 200 µl of blood.

In order to characterize the sensitivity of the *EPHB6* MSP in detecting free DNA, the following experiment was performed. Different amounts of MDA-MB-231 DNA were added to 200 µl of blood and the samples processed for detection of methylated *EPHB6* promoter. As above, only 1/15th of the initial sample containing blood and MDA-MB-231 DNA was processed in a single tube for MSP. The initial trial was capable of detecting as little as 1 ng free MDA-MB-231 DNA (Figure 5A). When the remaining DNA from the sample containing 100 pg of MDA-MB-231 DNA was processed, methylated *EPHB6* promoter was detected in 4/12 samples, indicating that this assay is at least capable of detecting 100 pg of free DNA from cells with a methylated *EPHB6* promoter in 200 µl of blood (Figure 5B).

Discussion

We demonstrate here the confirmation of our initial findings that the expression of EPHB6 can be predicted in breast carcinoma cell lines using MSP (46). The EPHB6 promoter is hypermethylated in three invasive breast carcinoma cell lines lacking EPHB6 protein and its transcript. Furthermore, the promoter region can be amplified from bisulfate-treated genomic DNA using a set of primers designed to amplify

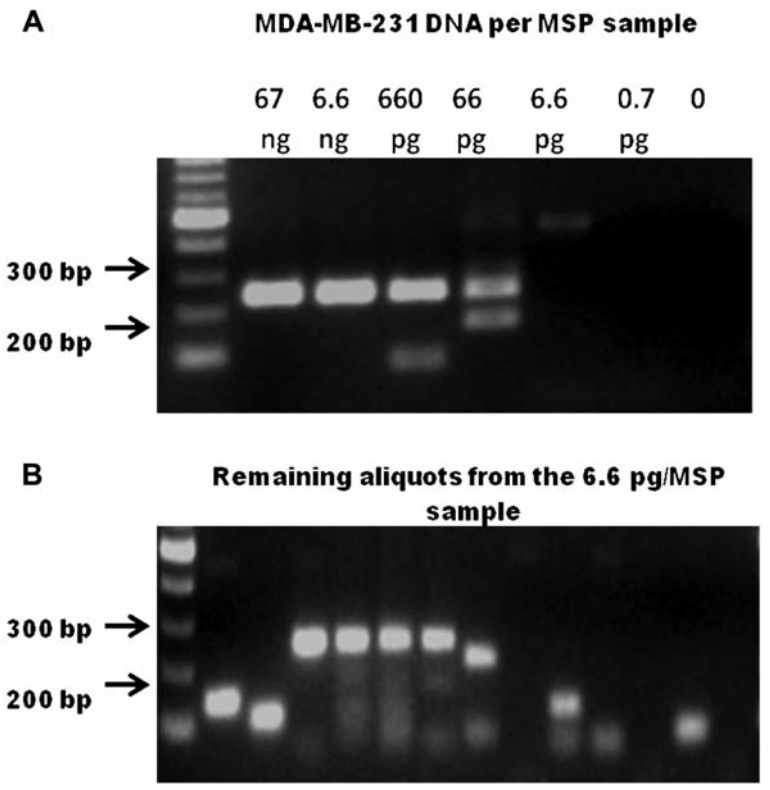

Figure 5. *EPHB6 MSP for detecting 6.6 pg of MDA-MB-231 DNA per reaction. DNA from mixtures of MDA-MB-231 DNA and 200 μl of blood was purified and treated with sodium bisulfate as described. The amount of tumor DNA used in each MSP reaction is listed. A: Amplification is seen in all samples containing 66 ng or more MDA-MB-231 DNA per reaction. B: The remaining aliquot from the 6.6 pg DNA sample was divided into four parts, bisulfite treated and subjected to three amplification reactions per sample. The size of the expected PCR product is 248 bp.*

both methylated and unmethylated regions of this promoter. This amplified genomic region can then be checked for the presence of the methylated regions by using MSP primers.

The expression status of EPHB6 can be harnessed toward potential diagnostic/prognostic prediction of tumor phenotype based on the following observations. *EPHB6* transcript is known to be indicative of less severe forms of neuroblastoma, melanoma, and non-small cell lung carcinoma (39-42), and its presence in breast carcinoma cell lines and tumor specimens generally indicates a less advanced case of this disease (43, 44). It has also been recognized that tumors frequently shed cells and DNA into the blood stream. The number of CTCs is often an indicator of tumor size, aggressiveness and likely response to treatment (15-16). The detection of these CTCs is a growing area of interest and can be an accurate indicator of disease phenotype in breast cancer (17), as well as an indicator of response to treatment (41). Recently, it has been shown that free tumor DNA is a useful marker of tumor phenotype (25-27, 29). The absence of hypermethylated *EPHB6* promoter in mononuclear blood cells has thus indicated the feasibility of detecting the presence of methylated *EPHB6* promoter in epithelial cells or tumor DNA in the circulation.

MSP (7) is a common technique which can be useful for the detection of CTCs. MSP analysis of DNA isolated from epithelial and mononuclear cells, separated by gradient centrifugation, led to the detection of 50 cancer cells in 5 ml of blood. It was capable of detecting 10 cells in 5 ml of blood with lesser efficiency. The suboptimal detection of 10 or <10 cells was attributed to the lack of efficiency of gradient separation. Notwithstanding its limitations, the detection of the methylated *EPHB6* promoter from such a small number of tumor cells in a large background of blood mononuclear cells is quite promising. Since the limitation in this experiment is likely the purification of mononuclear cells from blood, we demonstrated better sensitivity of the *EPHB6* MSP by preparing DNA from whole blood without separating the epithelial and mononuclear cells. A recent study showed that during chemotherapy, the presence of >6 CTCs correlated to a worse prognosis of patients with metastatic breast cancer (53). MSP could serve as a useful prognostic or diagnostic tool for detecting such a small number of CTCs. We have shown that the *EPHB6* MSP described here is capable of: i) detecting 100 cells with methylated *EPHB6* promoter in a background of 10^6 cells, ii) detecting fewer than 5 such cells

when mixed with 200 µl of blood, iii) detecting as little as 100 pg of DNA containing methylated DNA when mixed with 200 µl of blood, and iv) capable of detecting as few as 10 cells in 5 ml of blood. The sensitivity of this assay could be improved by incorporating an immunomagnetic enrichment (54) step for selective isolation of epithelial cells from blood, thus allowing potential detection of a single cell.

Although breast cancer markers on disseminated tumor cells have been characterized (55, 56), the number of clinically useful markers is not adequate. Given that metastasis occurs in a significant number of cases of localized and node-negative breast cancer and shedding of breast cancer cells is an early event during tumorigenesis, it would be advantageous to develop additional markers capable of predicting the invasive and metastatic potential of CTCs. Our observations indicating a correlation between *EPHB6* expression and invasiveness of breast carcinoma cell lines clearly make *EPHB6* a potential predictor of invasiveness. The sensitivity of MSP for detecting promoter methylation as described here provides the basis for commencing a large-scale investigation to establish a correspondence between specific breast tumor phenotype(s) and *EPHB6* promoter methylation.

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