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GRANT NUMBER DAMD17-94-J-4357

TITLE: p53 Mutation Profiles in Premenopausal Versus Postmenopausal Breast Cancer in a Stationary Population

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REPORT DATE: September 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1996	Annual (1 Sep 95 - 31 Aug 96)

4. TITLE AND SUBTITLE p53 Mutation Profiles in Premenopausal Versus Postmenopausal Breast Cancer in a Stationary Population

5. FUNDING NUMBERS

DAMD17-94-J-4357

6. AUTHOR(S)

BaoFa Yu, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Salk Institute for Biological Studies
La Jolla, California 92037-1099

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Abstract: One major clinical problem is how to predict which patients will relapse and metastasize. Recently, Dr. Yu has found that loss of the p16 gene is directly associated with the loss of cancer cell adhesion, the first step for metastasis. We aim to test our hypothesis that 1) the inactivation of p16 gene is higher in the metastatic cancers than non-metastatic cancers; 2) the adhesion strength of $p16^+$ cancer cells is higher than that of $p16^-$ cancer cells; 3) the incidence of metastasis *in vivo* is higher in $p16^-$ cancer cells than $p16^+$ cancer cells and 4) transfection of wildtype p16 cDNA can reduce metastasis *in vivo*. This study will help us to elucidate whether the p16 gene plays a role in the metastasis of cancers, and to screen and mark cancer metastasis potential, which will be beneficial in the early detection, prevention, and gene therapy of cancer metastasis.

14. SUBJECT TERMS Humans, Anatomical Samples, p53, Mutations, Loh, Breast Cancer, Cancer Molecular Epidemiology

15. NUMBER OF PAGES 21

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Baofa Yu 10-20-96

PI - Signature Date

(4) Table of contents:

(5) Introduction:

(6) Body:

(7) Conclusion:

(8) References:

Introduction: We noted that there have been remarkable changes in breast cancer (BC) treatment within the last decade. Greater and greater numbers of BC patients receive chemotherapy after the primary surgery. At the time of diagnosis and primary surgery, about 90% of all patients are free of metastasis, while 5 years later almost 50% of the patients will relapse (3, 4, 5, 6). Tumor metastasis is a crucial course in the failure of BC therapy. Bone and bone marrow are the most common sites for BC metastasis (1). The major problem in the prediction of which patients will relapse and how to decide what percentage of patients (50%) should receive chemotherapy, which is so extremely toxic to the immune system that it may shorten the lives of patients whose cancer may not have the potential to metastasize. So far there are no accurate tools to screen and predict the tumor metastasis potential, and it is critical to develop a cell-molecular biology tool to help the oncologist in deciding which patients should receive chemotherapy. Many gene alterations have been detected in BC, including amplification of oncogenes and inactivation of tumor suppressor genes (1,2).

Because my mentor, Dr. Sara Sukumar, was leaving the Salk Institute to take a position at Johns Hopkins University, I sought out a laboratory in which to continue research on breast tumors associated with the tumor suppressor gene such as p53 and p16. For this reason, I transferred to the laboratory of Dr. Paul Song, who developed a method for measuring the cell adhesion strength by micropipetter which related with cancer metastasis. Also his laboratory is working on the relationship between tumor supressor gene expression (p53) and cell adhesion strength of Be-13 cell line.

I have taken advatange of this technique to develop the possible strategies for predicting which patients will relapse and how to decide what percentage of patients (50%) should receive chemotherapy. It was reported that inactivation of the p16 gene is an important event in all common cancers (9) and p16 gene is related to the cell invasion and metastasis of melanoma cells (7). Also it appears that inactivation of the p16 gene is not a critical genetic step in the formation of primary BC (8, 9). Of course, the process of metastasis involves a series of elements and factors, but the molecular changes of the tumor itself are more crucial than the matrix of the tumor. In this proposal, we emphasize the study of p16 gene expression associated with cancer cell adhesion and metastasis.

<u>p16 (CDK4I) gene in human cancer and human cancer cell lines:</u> Tumorigenesis involves positive regulators of the transformed state (oncogenes) as well as negative regulators (tumor suppressor genes:TS).

Controlling tumor growth might involve the direct regulation of the cell cycle. Indeed, several oncogenes and TS genes were found to participate directly in the cell cycle. For instance, one of the cyclins (E or A), a class of protein that promotes DNA replication and mitosis, has been implicated as an oncogene (10), and the retinoblastoma (RB) TS gene is a substrate of the cyclin-dependent protein kinases (CDKs) (10). In April of 1994, the p16 (CDK4I) gene, which encodes a cell cycle regulator protein, grabbed much of the limelight in cancer genetics when this gene was reported to be involved in almost half of all human tumors. Alexander Kamb reported that the p16 gene was deleted in half of 290 human tumor cell lines (10). The cultures were derived from a dozen different tumor types, including lung, breast, brain, bone, skin, bladder, kidney, ovary, and lymphocyte. Tsutomu Nobori reported that p16 gene is frequently deleted in a variety of tumor cell lines including 46 human tumor cell lines, p16 gene was missing in 60% of the melanoma cells, 87% of the gliomas and 36% of the non-small-cell lung cancers. In both reports, however, they did not examine tissue taken directly from primary tumors (11). Paul Cairns and David Sidransky looked for p16 gene mutation in 75 primary human tumors that had lost a single copy of the 9p21 chromosome region containing p16 (12). They found that the percentage with p16 mutation is 20-30% in pancreatic cancer, 5-40% in esophagus carcinoma and 5-10% in head and neck cancers (12), They found that 3 of 5 osteosarcoma cell lines have homozygous deletions (11) and 2 out 5 BC cell lines have homozygous deletion of the p16 gene but no mutation was found in any of the primary BC (8).

DNA Methylation as another mechanism of inactivative of p16 gene: J. Herman. (9) has reported that another way of inactivation of p16 gene involves loss of transcription associated with *de novo* methylation of a 5' CpG island of CDKN2/p16 in cell lines of BC (33%), prostate cancer (60%), renal cancer (23%), and colon cancer (92%). And primary BC (31%) and colon cancer (40%) display *de novo* methylation of this CpG island (9). This kind of inactivation is not common in a few tumor types such as pancreatic carcinoma (14) and familial melanoma (13), inactivation may not involve point mutation of one allele or loss of the other allele. In contrast to other TS genes, the two most important mechanisms of losing p16 gene function are homozygous deletion and loss of transcription associated with hypermethylation of the 5' CpG island region.

Roles of p16 gene in invasion and metastasis of cancers: Jon A. Reed reported that loss of p16 expression was detected in 44% of metastasic lesions and 43% of primary invasive melanomas. In contrast, expression of p16 gene was detected in all of no-invasion melanomas. His results

indicate that loss of p16 expression is not important for initiation in malignant melanoma but is potentially related more to invasiveness and metastasis of melanomas (7), and it may be the same situation for BC, which needs to be in the lower cell adhesion for cancer cells detached from the primary tumor. Xu L. reported that no mutation of p16 gene was observed in any of the primary BC and mutation of p16 gene may not be a crucial step in the formation of primary BC (8), and loss of p16 gene function may be more related to invasiveness and metastasis of BC(no reports yet). Dr. Yu did not find any mutations in 4 osteosarcoma cells (US-20, RD and Saos-2) by PCR-SSCP analysis. It suggests that the mutation of p16 gene may not be critical in the formation of the osteosarcoma (unpublished data). There are no reports about the function of p16 gene in the metastatic BC and BC cell lines by this gene inactivation including deletion, mutation or DNA methylation. Studies on the inactivation of p16 gene including deletion, mutation and DNA methylation in primary BC with and without metastasis will lead to an understanding of the molecular basis of metastasis of human BC and the differences at the molecular level between BC with and without metastasis.

Body:

Preliminary results related to this project: Dr. BaoFa Yu, PI in this project, has found that deletion of p16 gene in BC cell line (MDA-MB-231, MCF-7 and Hs578t) resulted in lower cell adhesion strength (0.7, 1.23 and 2.0 mdynes) while BC cell lines (MDA-MB-435 and 468) with normal p16 gene have higher cell adhesion strengths (3.78 and 6.21 mdynes). These results clearly indicate that deletion of p16 gene is associated with loss of cell adhesion of BC (Fig.1 and 5). By transfector (BTX, San Diego, CA), Anti-p16 protein and albumin monocolonal antibodies were micro-injected into normal p16 gene BC cell lines (MDA-MB-435 and 468) at 50 mg/ml concentration. It resulted in a decrease of cell AS in both BC cell lines with anti-p16 antibody microinjection (4.36 mdyne for MDA-MB-435, 4.93 mdyne for MDA-MB-436) compared with anti-albumin antibody microinjection (7.67 mdyne for MDA-MB-435, 9.13 mdyne for MDA-MB-436) (Fig. 2). It also resulted in a three-fold decrease of the adhesion cell numbers in both BC cell lines with p16 protein antibody microinjection compared with albumin antibody microinjection. (Table 1). The results indicated that p16 antibody can bind and inhibit the function of p16 protein. If it increases the concentration of anti-p16, it may result in a futher decrease of cell adhesion strength and number of adhesion cells.The data suggest that p16 protein can upregulate cell adhesion strength to fibronectin by an unknown pathway. In another experiment, we transfected PCMV-p16 gene and pMV-7-p16 gene (wild type p16 gene cDNA) into

MDA-MB-231 BC cells. It was found that expression of p16 gene increased cell adhesion strength. By Western blotting and RNA protection assay, over expression of p16 gene was confirmed in the pCMV-p16-MDA-MB-231 and pMV-7-p16-MDA-MB-231cells, but not in MDA-MB-231 control cells, while adhesion strength is significantly higher in pCMV-p16 MDA-MB-231 and pMV-7-p16-MDA-MB-231 cells than MDA-MB-231 cells (Fig.3).

Dr. Yu also tried to find the relationship between p16 gene methylation and cancer cell adhesion. By PCR-based methylation assay, we found that BC cell line ZY75-1 contained both methylated p16 alleles, which resulted in low cell adhesion strength (Fig. 4). After treating this cell line ZR75-1 with the demethylating agent: 5-deoxyazacytidine (1mM for 3 days), the cell adhesion strength of ZR75-1 was increased to four fold (Fig. 4) along with detectable p16 mRNA expression as same as reports. The cell adhesion strength of T47D and MDA-MB-231 were increased only a little after same treatment (Fig. 4). This indicated that aberrant DNA methylation is essential for maintaining transcriptional silencing and DNA demethylation will release the transcriptional silencing for p16 gene expression, which can upregulate cell adhesion strength. Dr. Yu's preliminary results are very promising and have encouraged him, in collaboration with his coworkers, to study cell adhesion and metastasis of human BC with and without metastasis. He has collected 40 primary BC paraffin embedded tissues without metastasis and 40 with metastasis. Also he will collect fresh BC samples (about 100 to 200 samples) from the UCSD hospital. He will collect primary BC tissue debris from biopsy. The objective of this project is to understand the molecular basis of metastasis in human BCs and to determine the role of the p16 gene in the metastasis of BCs. This study also aims to find the molecular difference between BC with and without metastasis. Attention is being focused on the TS gene p16 (CDK4I), which appears to play a role in tumor metastasis and is useful as a molecular tool for the screening of human BC. In addition, more human BC tumor tissues or debris will be collected by biopsy or operation throughout the research period. It is expected that hard work and rigorous technique will produce significant results.

Hypothesis/Purpose: We hypothesize that 1) the frequency of p16 gene inactivation (mutation, deletion and DNA methylation) is higher in BC patients with metastasis than in BC patients without metastasis; 2) cell adhesion strength is higher in the normal p16 gene BC cells than in the inactivated p16 gene BC cells; 3) primary BC with p16 gene inactivation and lower cell adhesion strength have a higher metastasis potential than those with normal p16 gene and higher cell adhesion strength; 4) incidence of metastasis *in vivo* is higher in the inactivated p16 gene BC cell lines

than in the normal p16 gene BC cell lines; and 5) transfection of pCMV-p16 and pMV-7-p16 cDNA into p16 gene negative BC cells will reduce the metastasis potential *in vivo,* and generation of recombinant p16 adenovirus is an alternative way for this research. The profiles from this study will give us some clues to elucidate the function of p16 gene in the metastasis of BC. They will allow us to confirm that p16 gene expression is upregulating the cell adhesion strength by an unknown pathway, which is important in tumor metastasis; and the studies *in vivo* will further confirm our hypothesis that the p16 gene plays a crucial role in BC metastasis. Finally, we can use the cell adhesion strength and p16 expression as cell-molecular biology tools to screen and predict the BC metastasis potential for the early detection and prevention of BC metastasis through its potential use in gene therapy.

Technical Objective: The objective of this project is to understand the role of p16 gene in the metastasis of BC (rather than the breast tumorigenesis) and to determine whether it is useful as a molecular biology tool for screening of BC metastasis potential. To establish the feasibility of this study, we obtained 40 samples of BC with metastasis and 40 without metastasis. In order to compare the molecular events of the TS gene p16 and roles of p16 in cancer cell adhesion, we will do the following:

A 1. Extract DNA and RNA from blocks of BC and fresh BC tissues.

2. PCR-based Methylation Assay and PCR-SSCP analysis of exons 1 and 2 of the tumor suppressor gene p16 to compare the difference in molecular organization between metastasic BC and non-metastasic BC. We will determine if inactivation occurs by one of several possible mechanisms such as DNA methylation, gene rearrangement, loss of heterozygosity and point mutations.

3. Sequence exons 1 and 2 of the p16 gene (which are mutant by PCR-SSCP analysis) in order to search for "hot spots" of mutations in the p16 gene.

B. 1. Preparation of slides by section of each BC samples.

2. Immunostaining each slide using anti-p16 antibodies to check the expression of p16 gene. It will be found whether over expression of p16 gene occurs in BC with or without metastasis.

C. 1. Preparation and selection of pCMV and pMV-7 construct with insert of p16 cDNA and p16 adenovirus.

2. Transfection of the p16 deletion BC cell lines (MDA-MB-231, MCF-7 and Hs578s) with pCMV-p16 cDNA, pMV-7-p16 or p16 gene adenovirus.

3. Examine the expression of p16 gene in the transfected cells by Western blotting and RNA protection assay.

D. 1. We will culture the primary cells from fresh BC tissues.

2. RT-PCR and Southern blotting analysis of p16 status in these fresh BC tissues in order to know whether there is p16 gene deletion or DNA methylation.

3. By a micro pipette single cell manipulation system, cell adhesion strength will be measured from 100 single cells of each cell line at different time courses for comparison of adhesion strength between p16 gene normal and abnormal human BC cells. Finally, we can confirm the relationship between cell adhesion and expression of the p16 gene.

E. 1. Preparation of $p16^+$ and $p16^+/RB^-$ $p16^-$ by transfection by pCMV-p16 cDNA, pMV-7-p16 cDNA or p16 adenovirus and $p16^-/RB^-$ human BC cells in the amount of $1x10^4$-$1x10^5$ for each BC cell line.

2. Intracardiac inoculation of human BC cells into nude (knock out p16 gene transgenic mouse) mouse in order to compare the incidence of bone (or lung) metastasis between those BC cell lines with p16 status, and effects of p16 gene on the reduction of metastasis.

Methods:

PCR and RT-PCR procedure: This was previously performed on DNA extracted from BC tissues (or debris) and the paraffin embedded section using a 3-day extraction procedure utilizing detergents and enzyme digestion. The following modifications have resulted in DNA of better quality. DNA is extracted with xylene from 8-10 micro paraffin sections of BC and the pellet is re-extracted twice with 95% ethanol (16). In addition, DNA and RNA will be extracted from human BC cells and cell lines (15). RNA was reverse transcribed using random hexamers.DNA or cDNA was amplified using primers specific for the p16/CDKN2 gene or the GAPDH gene which was used as a control. Polymerase chain reaction is performed as previously described (17). This procedure also can be used for checking the p16 status (deletion) and expression of the p16 gene.

Analysis of mutations in the p16 gene: The vast majority of mutations in the p16 gene in BC have been located in exon 1 and 2. The SSCP approach we have been using with DNA from both tissue and paraffin-embedded sections is essentially. Using intron-sequence primers for exon 1 sense: 5'-GGGGAGCAGCATGGAGCCG-3' and antisense: 5'-AGTCGCCCGCCATCCCCT-3' produce a 204 bp product; exon 2 sense: 5'-CTACACAAGCTTCCTTTCCG-3' and antisense: 5'-CTCAGATCATCAGTCCTCCAC-3' produce a 395 bp product; exons 1-2 of the p16 are amplified in two PCR reactions in which one of the four dNTPs is radiolabeled.

Sequencing of the p16 gene: The PCR is performed under standard conditions in 100 ml. The amplified products containing exons 1 and 2, respectively, are cloned into the TA 1000 cloning vector (Invitrogen, San Diego, CA). T7 or M13 primers are used to sequence the 5' end of exon 1 and 2. The primers used for SSCP analysis could be used for DNA sequencing as well.

Southern blotting and hybridization analysis: 10 mg of DNA from BC cells was digested with ECOR I overnight, run on a 1% argarose gel at 24 V overnight, transfer it ovenight into nitrocellulose membrane. The nitrocellulose was baked at 80^0C for 32 hrs. Preparation of the p16 or RB probe, perform prehybridization and hybidization for examining status of p16 and RB genes.

Analysis of expression p16 in paraffined BC tissue: Sections of BC blocks are deparaffinized and rehydrated. The sections for fresh BC tissues do not need this step. The sections are incubated at room temperature with methanol containing 3% hydrogen peroxide for 15 minutes to inhibit endogenous peroxidase, normal rabbit serum 1/20 for 20 min, monoclonal antibodies (or polyclonal AB) against p16 protein for 60 min, rabbit anti-mouse antibodies 1/200 for 30 min, and diaminobenzidine. Between each step, the sections were washed with phosphate-buffered saline (PBS). Finally, they were counterstained with hematoxylin. These sections are examined under the microscope in order to determine the expression of the p16. Also the cell culture is prepared in multichamber slides for all BC cell lines, and immunostaining will be performed as with paraffin sections using anti-p16 antibodies.

PCR-based Methylation Assay: A PCR assay relying on the inability of some restriction enzymes to cut methylated sequences (21) to be used to analyze the methylation status of the first and second exons of the p16 genes. For exon 1 of p16 gene, the sites examined were: one *Fnu*DII, one *Sac*II, one *Hpa* II and two *Cfo* I; for exon 2 of p16 gene, the sites examined were: one *Sma* I, four *Hpa* II and six *Cfo* I. 1 mg DNA was digested for 2 hours, with 10 units of enzyme/mg of DNA, taking 50 ng of digested DNA as template for PCR amplification with primers flanking the restriction sites. The primers for exon 1 of p16 were 5'-AGC CTT CGG CTG ACT GGC TGG-3' (sense) and 5'-CTG GAT CGG CCT CCG ACC GTA-3' (antisense), under the following condition:94^0 for 3 min, 30 cycles of 94^0C for 1 min, 55^0C for 30 s, and 72^0C for 40 s, followed by incubation at 72^0C for 5 min. Primers for exon 2 of p16 gene were 5'-CTG CTT GGC GGT GAG GGG G-3' (sense) and 5'-CCT CAC CTG AGG GAC CTT C-3' (antisense); condition were the same as exon 1 except for an annealing temperature of 57^0C. The PCR products

were resolved on 2% agarose gels, transferred to a nylon membrane, and hybridized as for Southern blotting (22).

Plasmid constructions and stable p16 cDNA transfection: pCMV-p16 and pMV-7 -p16 gene were constructed by Wei Jiang at the Salk Institute. A recombinant ad5CMV-Lacz adenovirus DNA was digested with restriction enzyme *Xba* I and Cla I, and the 32-kb partial adenovirus DNA fragment was purified in a 0.3% agarose gel. The DNA fragment and pAD-p16 plasmid DNA were contransfected into 293 cells by $CaPO_4$-mediated transfection. The cells were maintained in this medium until the onset of cytopathic effect. The newly generated recombinant adenovirus containing p16 was identified by PCR analysis. All of BC cell lines were cultured in 10% FBS DMEM medium. Cells were transfected with pCMV-p16 or pMV-7-p16 using the lipofectin method. After 12 days of selection in culture medium containing 0.5mg/ml G418 (GIBCO), colonies were pooled and maintained in the presence of 0.2 mg/ml of G418. Also the cell lines were infected by the addition of viral solutions to cell monolayers and incubation at room temperature for 30 min with brief agitation every 5 min.which was followed by the addition of culture medium and the return of the infected cells to the 37°C incubator.

Preparation of primary cell cultures: Human BC tissue debris requires special handling and processing techniques to obtain satisfactory cell cultures for this study. We obtain the BC tissue debris in a small amount (5 to 10 ml) of sterile saline solution or phosphate-buffered saline (PBS) in a suitable container to transfer the sample to the laboratory. The tissue debris is placed in the tube and centrifuged for 5 min at 800 rpm and the supernatant is discarded. The cells and small tissue pieces are suspended in 5 ml of 10% CFS DMEM medium containing insulin and glutathione at a final concentration of 5 and 10 mg/ml, respectively. The suspension is incubated at 37°C. The medium in the flasks is renewed on alternate days. The culture is examined daily after the fourth day. When the colonies are large enough and are actively proliferating, the cells are harvested. Cultures are usually ready within 7 to 10 days.

Measurement of adhesion strength: The system used for the measurement of adhesion strength is similar to that previously described (18, 19). Micropipettes with an internal radius of 2.5 to 3.3 mm are prepared (Model P-87, Sutter Instrument Company, Novato, CA), and then mounted on a hydraulic micromanipulator with the wide end of the pipette connected to a pressure regulating system (20). The adhesion characteristics are measured under direct microscopic observation and

recorded on a video recording system. The micro pipette is manipulated to aspirate a small portion of the cell body, which is attached to the fibronectin substrate, by using a small aspiration pressure. The force (product of aspiration pressure and cross-section area of pipette tip) required to separate the fibroblast cell is measured by stepped increments of aspiration pressure in the holding pipette, and the pressure is allowed to equilibrate. Then the micropippette is pulled away (2-4 mm/s rate) from the adhesion area by micromanipulation. The minimum force required to detach the cell from the substrate is defined as the adhesion strength.
Metastasis test *in vivo*:: 5-7 weeks after intracardiac injection of 10^4-10^5 BC cells into mice, mice are sacrificed by gas CO_2, and the main organs (lung, bone, liver, brain, intestine, stomach and lymph nodes) are collected, sectioned, and examined by a pathologist at UCSD in order to find metastasic tumors.

Conclusions:

About 90% of Breast cancer(BC) patients are free of metastasis at primary surgery but 50% of those patients will relapse and metastasize in 5 years. Tumor metastasis is one of the major causes of the failure of breast cancer (BC) therapy and over 90% of BC patients die of bone or lung metastasis rather than the primary tumor. A major clinical problem is how to predict which patients will relapse and metastasize, and how to decide which patients (50%) should receive chemotherapy, which is very toxic to the immunesystem and may shorten the patient's survival time. The metastasis of BC involves a series of consecutive attachment and detachment events. The process is initiated by disaggregation of invasive cells from the primary tumor, a step that requires losing cancer cell adhesion. p16 gene inactivation, which includes deletion, mutation and DNA methylation, has been reported in many human cancers and human cancer cell lines, including BC. It is also reported that inactivation of the p16 gene is not necessary to initiate cancer, but is related to the ability to metastasize in malignant melanoma. There are no similar reports on BC and BC cell lines. The objectives of this proposal are (1) to study the frequency of p16 gene inactivation between BC patients with and without metastasis; (2) to compare whether adhesion strength of cancer cells is higher in normal p16 gene human BC cells than in inactivated p16 gene human BC cells, using a single-cell micropippetter technique; (3) to test whether the incidence of metastasis *in vivo* is higher in the inactivated p16 gene human BC cells than in normal p16 gene human BC cells, and if transfection of the wild type p16 gene cDNA will reduce the incidence of metastasis. The results from this study may give us some clues to elucidate the roles of the p16 gene in metastasis in BC patients; whether the loss of

p16 gene expression is associated with the adhesion strength, which is an important step for tumor metastasis; and the metastasis study *in vivo* will further confirm our hypothesis of whether or not the p16 gene plays a crucial role in the metastasis of BC. Finally, we can use the adhesion strength assay by micropipetter and the p16 gene expression as cell-molecular biology tools to screen and predict the BC metastasis potential for the earlier detection and prevention of BC metastasis and gene therapy.

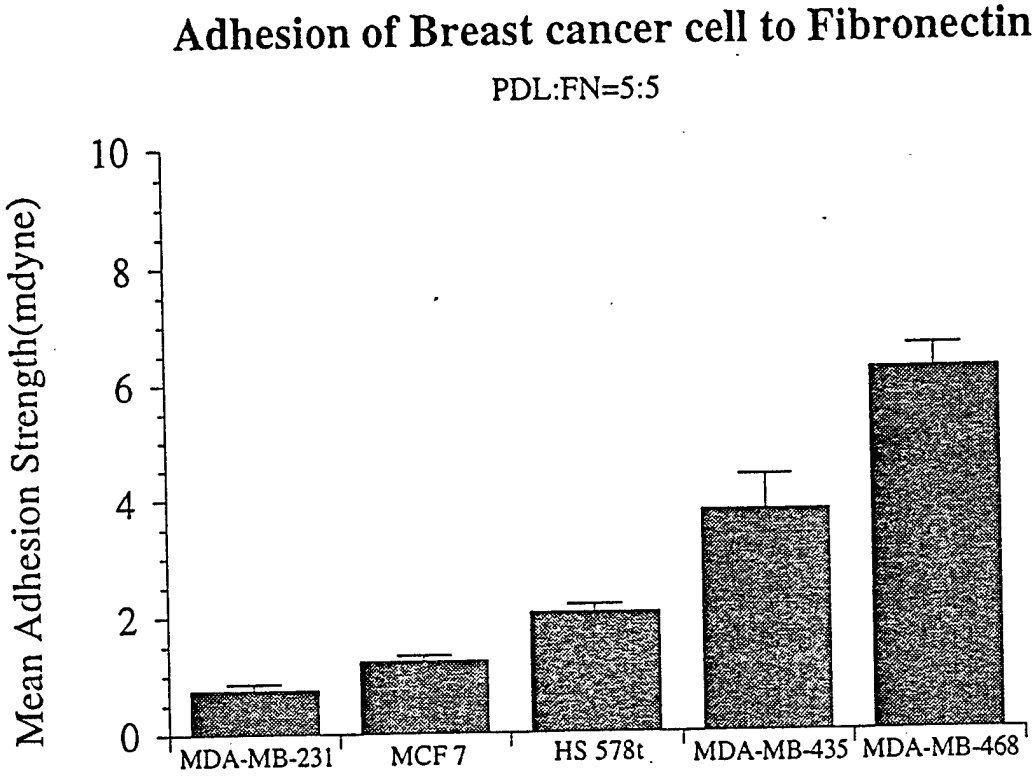

Fig. 1. Deletion of p16 gene in BC cell line (MDA-MB-231, MCF-7 and Hs578t) resulted in lower cell adhesion strength (0.7, 1.23 and 2.0 mdynes) while BC cell lines (MDA-MB-435 and 468) with normal p16 gene have higher cell adhesion strengths (3.78 and 6.21 mdynes); p16 gene status was checked by PRC Reaction.

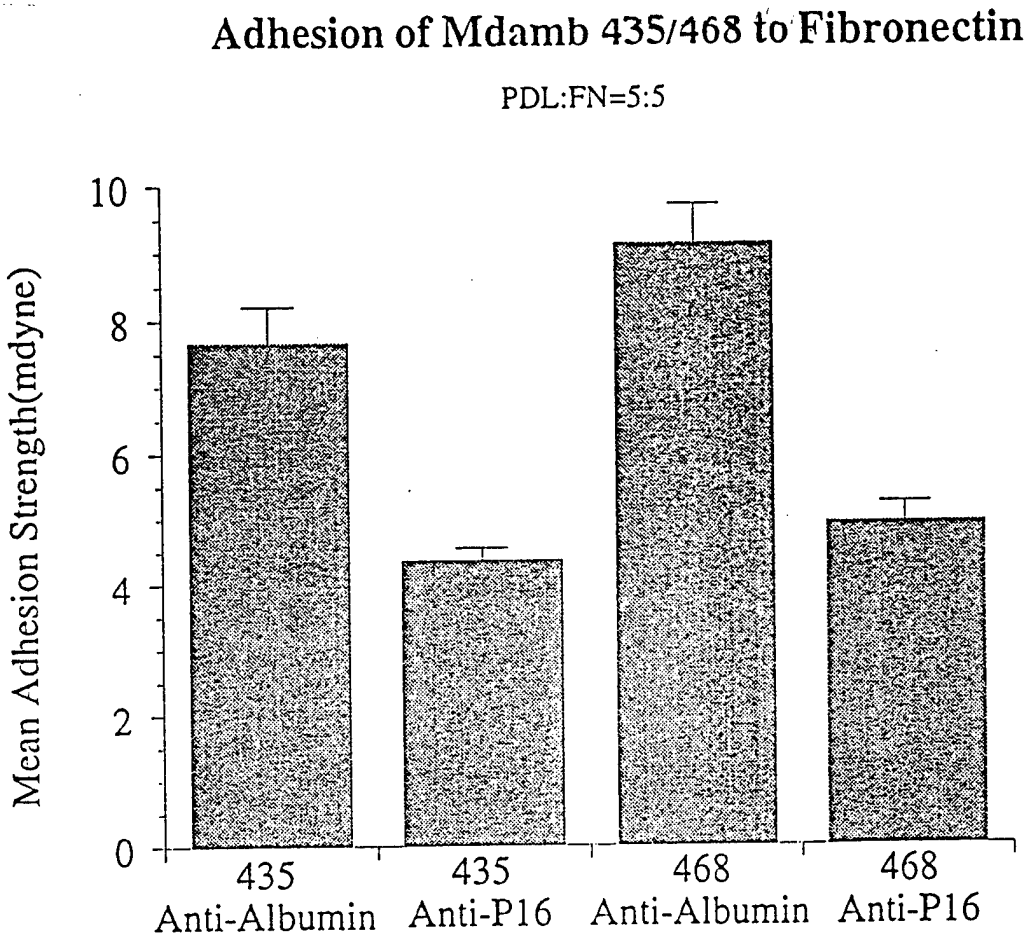

Fig. 2. By transfector (BTX, San Diego, CA), Anti-p16 protein and albumin monocolonal antibodies were micro-injected into normal p16 gene BC cell lines (MDA-MB-435 and 468) at 50 mg/ml concentration. It resulted in a decrease of cell AS in both BC cell lines with anti-p16 antibody microinjection (4.36 mdyne for MDA-MB-435, 4.93 mdyne for MDA-MB-436) compared with anti-albumin antibody microinjection (7.67 mdyne for MDA-MB-435, 9.13 mdyne for MDA-MB-436).

Table 1: No. of Adhesion Cell of Breast Cancer with Anti-p16 Blocking

Type of Cells	Time (min)	Anti-p6	Anti-albumin	Control
MDA-MB-435	15	80	230	250
	60	100	310	300
MDA-MB-468	15	100	400	300
	60	240	500	600

Table 1. By transfector (BTX, San Diego, CA), p16 protein and albumin monocolonal antibodies were micro-injected into normal p16 gene BC cell lines (MDA-MB-435 and 468) at 50 mg/ml concentration. These cells were incubated at 37^0C for 15 and 60 minutes, and washed three times by the wash buffer in the flow chambers. Then stain these cells and count the cells left on the slides under microscope.

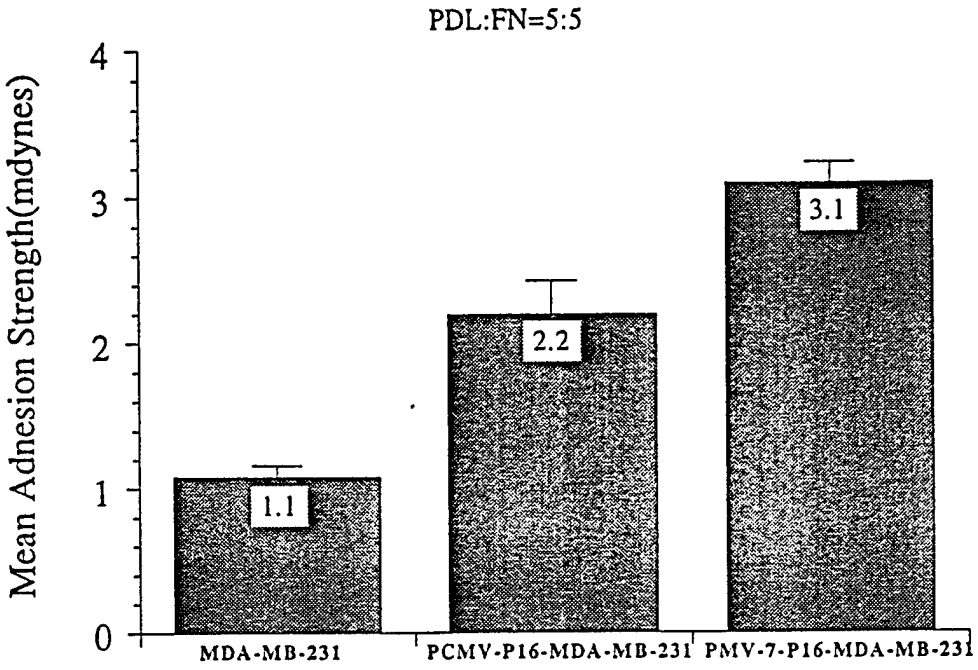

Fig. 3. We transfected PCMV-p16 gene and pMV-7-p16 gene (wild type p16 gene cDNA) into MDA-MB-231 BC cells. It was found that expression of p16 gene increased cell adhesion strength. By Western blotting and RNA protection assay, over expression of p16 gene was confirmed in the pCMV-p16 MDA-MB-231 cells and pMV-7-p16 gene MDA-MB-231 cells, but not in MDA-MB-231 control cells, while adhesion strength is significantly higher in PCMV-p16 MDA-MB-231 cells than MDA-MB-231 cells.

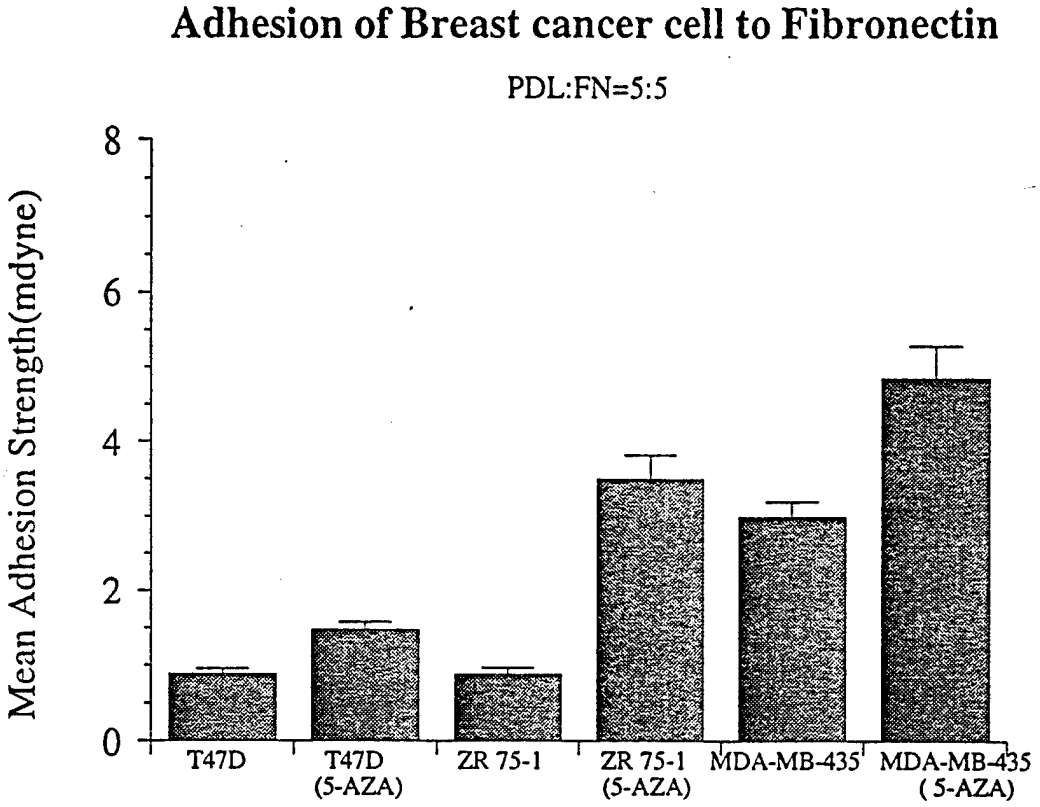

Fig. 4. BC cell line ZY75-1 contained both methylated p16 alleles, which resulted in low cell adhesion strength.. After treating this cell line ZR75-1 with the demethylating agent: 5-deoxyazacytidine (1μM for 3 days), the cell adhesion strength of ZR75-1 was increased to four fold, along with detectable p16 mRNA expression as same as reports.

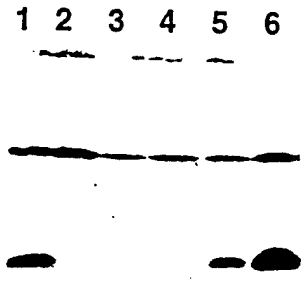

Fig. 5. Westen Bloting were performed by anti-p16 monoclony antibody for these breast cancer cell lines:1: MDA-MB-435; 2: MDA-MB-231; 3: Hs578t; 4:MCF 7; 5: ZR 75-1; 6: MDA-MB-468. #1 and 6 show that p16 protein was full expressed; #2, 3 and 4 did not show any band of p16 gene expression; # 5 show that this cell line has partial expression of p16 gene.

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