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Identification of Tumor Suppressor Genes in Breast Cancer by Insertional Mutagenesis and Functional Inactivation

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The development and progression of cancer result from multiple genetic changes accumulated in the cells. The identification of tumor suppressor genes inactivated and proto-oncogenes activated in mammary epithelial cells is essential to understand the genetic basis of breast cancer and is a prerequisite for development of strategies for prevention, diagnosis, and treatment. In breast cancer, loss of heterozygosity (LOH) was detected frequently on chromosome 17 and other chromosomes, suggesting unrecognized tumor suppressor genes. We are applying the novel retroviral-tagging strategy to identify the genes using chromosome 17-suppressed (independent of p53 and BRCA1) breast cancer cell lines. In contrast to the parental tumorigenic cell line CAL51, the suppressed sublines CAL/17-3 and CAL/17-5 display retard growth in flasks, no growth in soft-agar culture and athymic nude mice. In this annual report, we present preparation of biological materials including cell culture, isolation of DNA and RNA, construction of cDNA library and packaging retrovirus particles. In order to provide antisense and mutant proteins to inhibit activities of tumor suppressor genes, poly-(A) RNA was isolated from the suppressed subline CAL17-3 and used to construct the library. We are now in the process to package cDNA library into retrovirus particles for transduction.
DATE: January 19, 1999

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For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

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INTRODUCTION

Breast cancer is the most common malignancy in Western women, affecting up to one in 10 women during their lifetime. The large majority (approximately 90%) of breast carcinomas is sporadic despite of identification of BRCA1 and BRCA2 that are known to involve in familial cases. The development of breast cancer is believed to result from multiple genetic changes accumulating in mammary epithelial cells. In searching for the genetic changes, increasing interest has been focused on tumor suppressor genes involved in control of normal mammary epithelial cells from tumorigenic transformation.

While many investigators have observed LOH in breast cancers on chromosomes 1p, 1q, 3p, 6q, 7q, 11p, 13q, 16q, 17p, 17q, and 18q at the high frequencies variable from 20-60%, chromosome 17 is one of the most frequent carriers of LOH (1-19). On 17p, two distinct regions, 17p13.1 (containing p53) and 17p13.3, have shown LOH with frequencies ranging from 30-60% and 60-70%, respectively (10-11, 20-26). On 17q, three regions of frequent LOH have been identified. 17q21 LOH (13, 26-30) contains BRCA1 (31). LOH at 17q11.1-q12 was detected as frequently as 79% in sporadic breast cancer (32). The third LOH is telomeric to BRCA1 (26, 29). These studies demonstrate that, in addition to p53, BRCA1, BRCA2, and others, chromosome 17 and other chromosomes with the high frequent areas of LOH harbor unrecognized tumor suppressor genes involved in the control of the normal growth of mammary epithelial cells.

Direct evidence supporting the existence of additional breast cancer suppressor genes comes from introduction of a neo-tagged chromosome 17 into breast cancer cell lines by microcell-mediated chromosome transfer that demonstrated suppression of tumorigenicity (33-37). Casey et al. was the first to provide biological evidence that in vitro growth of the breast cancer cell line MCF 7 (carrying wild-type p53) was suppressed by the introduction of a neo-tagged chromosome 17 (33). A very similar result was independently reported by Negrini et al. (34). In addition, anchorage-independent growth, cell growth rate on plastic plates, and tumorigenicity in athymic nude mice of the mammary carcinoma cell line R30 were suppressed by introduction of chromosome 17. Wild-type p53 was not involved in this suppression (35). Furthermore, only the long arm of the transferred chromosome 17 was capable of suppressing the tumorigenicity of the p53-mutant breast cancer cell line MDA-MD-231 (34). Finally, Theile et al. demonstrated that suppression of tumorigenicity of the breast cancer cell line CAL51 by an introduced chromosome 17 did not require transfer of p53 or BRCA1 (37). Thus, additional tumor suppressor genes on chromosome 17 have yet to be identified.

To facilitate identification of tumor suppressor genes we have developed a novel strategy to reverse monochromosome-mediated tumor suppression by retroviral insertional mutagenesis and/or functional inactivation mediated by expressed cDNA fragments (38). Insertional mutagenesis disrupts tumor suppressor genes (e.g., APC for familial adenomatous polyposis [39] and p53 in osteosarcoma [40]) and has been used as a powerful tool to identify various genes including Fli-1, p53, erb-B, and myc (41-43), vin-1/cyclin D2 (44), Tiam-1 (45), bcar-1, and CRL-1 (46-47). Furthermore, it is known that retroviral insertion could activate proto-oncogene. Finally, functional inactivation mediated by expressed cDNA fragments has been achieved by knockout of gene function.
(48) and by methods designed to identify genetic suppressor elements by antisense cDNA or dominant negative mutant proteins (49-51). Combining all components of these established approaches into a single system of retroviral-vector-cDNA, we have developed a novel strategy for identification of genetic loci and tumor suppressor genes (38).

“Suppression and reversion of suppression” are the two basic aspects of this novel strategy. “Suppression” refers to the suppression of tumorigenic phenotypic features including anchorage-independent growth, focus formation in plastic culture, rapid cell population doubling time, and tumor formation in athymic nude mice by introduction of a neo-tagged monochromosome into a cancer cell line via a microcell mediated chromosome transfer. “Reversion of suppression” means the reversion to the tumorigenic phenotype induced by insertional mutagenesis (proviral tagging) and/or functional inactivation of the suppressor gene(s) by antisense or dominant negative mutant proteins following the transduction of a retrovirus expression vector-carried cDNA library into the monochromosome suppressed cells.

The hypothesis underlying this approach is the following. (1) The phenotypic reversion can derive from inactivation of tumor suppressor genes. The suppression related genetic locus or loci on the introduced chromosome provide targets to insertional mutagenesis. In addition, derived from cancer cell lines, the suppressed sublines may carry many mutated genetic loci that leave functional counterparts being “haploid” targets to insertional mutagenesis. Furthermore, the suppressor gene products can be targets to functional inactivation by antisense and mutant proteins. The poly(A)
\(^{+}\)-RNA from the suppressed cancer cell line is used to construct cDNA library and then re-introduced to the suppressed cell line to induce the reversion, reasoning that cDNA fragments of the suppressor genes would be present in the library providing antisense and mutant proteins to inhibit activities of the suppressor genes. It is well known that the phenotypic reversion can also come from activation of proto-oncogenes. (2) The successfully transduced tumorigenic cells can be positively selected in soft agar culture following co-selection for the drug-resistance genes on both the suppressive chromosome and the retroviral vector. (3) The cDNA and genomic sequences tagged to the vectors and involved in the tumorigenic reversion can be readily isolated by PCR-based techniques. Based on this hypothesis we have successfully generated a serial retroviral-tagged revertant cell sublines identified important genetic loci (38) and possibly a tumor suppressor gene (unpublished data), using the chromosome-6 suppressed melanoma cell line UACC903(+6).

We are now applying the same strategy to identify unrecognized tumor suppressor gene(s) from a chromosome-17 suppressed breast cancer cell sublines. This study is using the tumorigenic cell line CAL51 and the chromosome-17 suppressed cell sublines CAL/17-1, CAL/17-3, and CAL17-5 (37). The parental CAL51 cell line demonstrates rapid population doubling time, focus formation in plastic culture, anchorage-independent growth, and rapid formation of subcutaneous tumors in athymic nude mice. All four of these readily detectable phenotypic features are suppressed in the chromosome 17 containing cell sublines (37). The central goal of this project is to identify breast cancer suppressor genes. The specific aims include (1) use of the chromosome-17 suppressed breast cancer cell sublines CAL/17-1, CAL17-3 and CAL17-5 (37) to generate the anchorage-independent revertants by transduction of a retroviral expression vector-carried
cDNA library and (2) use of the anchorage-independent revertants to identify previously unrecognized suppressor genes in breast cancer. Table 1 is the timetable from our original proposal.

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**BODY**

**MATERIALS, METHODS, AND PROCEDURES**

**Cell Culture.** The parental breast cancer cell line CAL51 and the chromosome-17 suppressed cell sublines CAL/17-1, CAL/17-3, and CAL/17-5 (37) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL penicillin G sodium, and 100 µg/mL streptomycin sulfate. Six hundred µg/mL of G418 were added in culture of the chromosome 17-containing cells to select for the introduced human normal chromosome 17 tagged with psv2neo. NIH3T3, Bosc23 cells (52) and Bing (63) cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin G sodium, and 100 µg/mL streptomycin sulfate. Ten% of newborn calf serum was used for culture of GP+envAM12 cells (54). Eight mM of L-histidinol dihydrochloride (hisD^R^) was used for cells containing retrovirus vectors. All media, serum, and antibiotics were from Gibco BRL with exceptions where indicated. *Escherichia coli* strain DH5α cells (GIBCO, BRL) containing plasmid pLM2 (38) and strain Supercompetent cells (Catalogue no. 230140; Stratagene) containing a cDNA library were cultured in Luria-Bertani medium with 100 µg of ampicillin per ml.
Techniques of the Molecular Cloning. Genomic DNA and total RNA were isolated by standard methods (55). Poly(A)^+ RNA was extracted from total RNA using the FastTrack 2.0 mRNA Isolation Kit according to manufacturer's instructions (Catalog nos. K1593-02, K1590-03; Invitrogen). The cDNA library was synthesized from poly(A)^+ RNA using Universal Riboclonie cDNA Synthesis System (Catalog no. C4360; Promega) according to the manufacturer's instruction. Briefly, first strand synthesis was driven by Avian Myeloblastiosis Virus (AMV) reverse transcriptase and random hexameric primers, followed directly by second strand replacement synthesis using Rnase H and DNA polymerase I. After treatment with T4 DNA polymerase to flush the ends, the double-stranded cDNA molecules were prepared for cloning by size fractionation and the addition of EcoRI adaptors. The resulting cDNA samples were cloned into pLM2 plasmid vectors. Plasmid DNA was isolated using alkaline lysis methods (56).

Packaging of Retrovirus Particles. Virus particles containing retroviral vectors were packaged from pLM2 plasmids, using the retrovirus-packaging cell lines Bosc-23 (ectropic) and GP+envAM12 (amphotropic) by methods essentially as described (52, 54). Briefly, 20 ug of plasmid DNA was used to transfect approximately 1 x 10^6 Bosc-23 cells by Ca^{++} coprecipitation using the CellPhect Transfection Kit (Pharmacia Biotech). Serial 10-fold dilution of the supernatant from the transfected cells was used to transduce NIH3T3 cells for the determination of an initial titer of retrovirus particles by histidinol resistance [8 mM L-histidinol dihydrochloride (Sigma Chemical Co.)]. The titer of the packaged retrovirus was approximately 1 x 10^5 colony forming units per ml. To generate a high titer of amphotropic retrovirus particles, 10 ml of supernatant from transfected Bosc-23 cells were used to transduce aliquots of 1 x 10^6 GP+envAM12 cells in the presence of 6 ug Polybrene per ml (54). Transduced GP+envAM12 cells were selected for with L-histidinol. A serial 10-fold dilution of supernatant from his^r cells was exposed to NIH3T3 cells to determine the titer of amphotropic retrovirus particles (1 x 10^6). The method to package retrovirus particles using Bing cell line (52, 53) was the same as above.

RESULTS AND ASSUMPTIONS

Isolation of genomic DNA, total RNA, and poly(A)^+RNA from the parental breast cancer cell line CAL51 and the chromosome-17 suppressed cell sublines CAL/17-1, CAL/17-3 and CAL/17-5. The genomic DNA have been isolated from the cancer cells and the suppressed cells for the future Southern analysis of restriction fragment length polymorphism in comparison to those of the revertant cell sublines to be generated. Total RNA have been isolated from three cell lines including CAL51, CAL/17-3, and CAL/17-5 for the future Northern analysis in comparison to RNA from the revertant cell sublines to be generated. Poly(A)+RNA was isolated from the chromosome 17 suppressed cell subline CAL/17-3 for construction of the cDNA library described below. We failed to isolate both genomic DNA and total RNA from the suppressed cell subline CAL/17-1.
Construction of random-primed and orientation-independent cDNA library in retroviral expression vector pLM2. Poly(A)$^+$-RNA was isolated from the chromosome 17 suppressed cell subline CAL/17-3 and used to synthesize the cDNA library. The reason for using the suppressed cell subline as the RNA source is that the gene(s) involved in the chromosome-17 mediated tumor suppression would be present in the RNA. Thus, the resulting cDNA library could provide possible antisense fragments and mutant proteins for inhibition of activities of suppressor gene products associated to the chromosome-17 mediated suppression.

4 ug poly(A)$^+$-RNA from CAL/17-3 cell line was used to synthesize the cDNA library. Figure 1 represents the synthesized cDNA samples and controls (1.2 kb kanamycin poly(A)$^+$-RNA). The majority of the double-stranded cDNA fragments range from 0.3 kb to 1.5 kb. After removing the fragments less than 0.4 kb by size fractionation and addition of EcoRI adapters, 0.5 ug cDNA preparations were cloned into EcoRI site of pLM2 vectors. This library will be used for both packaging retrovirus particles and isolation of cDNA clones after identification of the retroviral tagged and reversion-related sequences.

pLM2 is the plasmid vector carrying his$^+$ retroviral expression vector. Upon introduction into human cells, the cDNA inserts in the retroviral expression vector can be transcribed from the CMV promoter-enhancer and the sense-oriented cDNA can also be translated from the introduced translational start codon (ATG) and to a stop codon (43). The stop codon can be from the cDNA inserts or on the vector (TGA) in all three reading frames. Therefore, the expression of possible antisense fragments or mutant proteins in the chromosome 17 suppressed cell sublines is expected to knockout activities of the suppressor gene products.

Figure 1. The radioactive labeled cDNA library and controls demonstrate the size ranges of the library synthesized from the CAL/17-3 mRNA. Lanes: 1. A sample of the first strand cDNA library; 2. The first strand cDNA control synthesized from 1.2 kb kanamycin mRNA; 3. A sample of the double stranded cDNA library; 4. The double stranded cDNA control synthesized from 1.2 kb kanamycin mRNA. The sizes of the molecular markers are indicated in the left of the picture in kilobases.
Packaging of Retrovirus Particles. The pLM2 vectors was packaged into retrovirus particles using the packaging cell lines BOSC-23 (ecotropic; Ref. 52) and GP+envAM12 (amphotropic; Ref. 54). The initial titer of the retrovirus particles packaged by BOSC-23 cells was approximately 1 x 10^5 cu per ml determined by the number of NIH3T3 colonies resistant to L-histidinol. Aliquots of 10 ml of supernatant (1 x 10^6 cu) were used to transduce aliquots of 1 x 10^6 GP+envAM12 cells to produce supernatant containing a high titer of amphotropic retrovirus particles (1 x 10^6 cu/ml) to transduce human cells. These particles are for control experiments. We have also packaged retrovirus particles using Bing cell line. Bing cell line is the amphotropic counterpart to the Bosc23 ecotropic envelop-expression packaging line (52, 53).

DISCUSSION AND RECOMMENDATIONS

In the initial research year, we have successfully prepared biological materials for the downstream work although we moved the laboratory from the National Human Genome Research Institute at the National Institutes of Health to Institute of Molecular and Human Genetics and Lombardi Cancer Center at Georgetown University Medical Center. The genomic DNA and total RNA were isolated from the parental breast cancer cell line CAL51 and the chromosome 17 suppressed cell sublines CAL/17-3 and CAL/17-5. The poly(A)+-RNA isolated from CAL/17-3 was used to synthesize cDNA library that was constructed into the retroviral expression vector pLM2. We have packaged retrovirus particles from the pLM2 plasmid vectors. The retrovirus particles without cDNA inserts will be used as the controls for the transduction experiments. Using amphotropic retrovirus packaging cell line Bing to package the particles reduces the two-step packaging (first in Bosc23 cell line and then in GP+envAM12 cell line) into one-step (only in Bing cell line) for saving both time and money without detrimental to our research.

In our original proposal, we planned to construct a random-primed and normalized cDNA library with 200-600 bp inserts. We constructed the random-primed cDNA library and omitted the normalization step. One reason is that we can narrow down to the candidate tumor suppressor genes without the normalization. This view came out from our identification of tumor suppressor gene(s) using the chromosome 6 suppressed melanoma cell line UACC903(+6) and the retroviral transduced tumorigenic revertant cell subline SRS3 (unpublished data). In addition, a total of $298,962 was awarded for a 3-year period to the NIH. Because this grant was originally made to a federal facility no indirect costs were included in the award. After moving from the National Institutes of Health to Georgetown University Medical Center the funding level for this project was cut off 20% for the institution indirect cost. Thus, it is necessary for us to omit few experiments to make sure that the overall project succeeds.

The progress of our research in the first year was not as fast as we would like to. The delay was mainly due to moving and setting up the laboratory, recruiting and training researchers. For this very reason, I have requested no cost extension for this award (please see a copy of the letter in Appendix). In addition, we had a difficult time to culture the chromosome 17 suppressed cell subline CAL/17-1. CAL/17-1 was the cell
subline we had proposed to use in the project. However, in our hand, the CAL/17-1 cell line was nearly no dividing in the Petri-dishes after thawing from the liquid nitrogen storage culture. After repeated failure to culture CAL/17-1 cell subline, we changed to use both CAL/17-3 and CAL/17-5 cell sublines. These two sublines turned out to be healthy and their growth is significantly slower than the parental tumorigenic cell line CAL51. We do not know what caused the CAL/17-1 cell line to stop growing. We know CAL/17-1 was a clone from the 8th passage after introduction of chromosome 17, CAL/17-3 from the 6th passage, and CAL/17-5 from the 10th passage (personnel communication with Dr. Micheal Theile). This difference should not be the reason for the problem. We simply changed the cell lines and moved on because the selection for tumorigenic revertant cells will be conducted in soft-agar culture, where CAL/17-3 and CAL/17-5 like CAL/17-1 do not grow (they are anchorage-dependent) and can be used instead.

CONCLUSIONS

In conclusion, we have successfully prepared biological materials including:

(1) Isolation of genomic DNA and total RNA from the parental breast cancer cell line CAL51 and the chromosome 17 suppressed cell sublines CAL/17-3 and CAL/17-5.

(2) Isolation of poly(A)+-RNA from CAL/17-3, synthesis and construction of cDNA library into the retroviral expression vector pLM2 for current packaging of retrovirus particles and later identification of the retroviral tagged and reversion-related sequences.

(3) Packaging of retrovirus particles from the pLM2 plasmid vectors for the coming transduction experiments.

REFERENCES


APPENDICES

Attached please see the copies of documents regarding:

(1) No cost extension of the grant number DAMD17-97-1-7236.

(2) Grant Transfer from NIH.
September 14, 1998

Patricia Modrow, Ph.D.
Office of the Deputy Chief of
  Staff for Information Management
US Army Medical Research and Materiel Command
Department of the Army
504 Scott Street
Fort Detrick, Maryland 21702-5012

RE: No Cost Extension of Grant Number DAMD17-97-1-7236

Dear Dr. Modrow,

I am writing to request for one-year no-cost extension of the grant number DAMD17-97-1-7236, titled “Identification of Tumor Suppressor Genes in Breast Cancer by Insertional Mutagenesis and Functional Inactivation”. I am requesting the extension for the following reason. In the last year, I became a faculty (Assistant Professor) in the Institute for Molecular and Human Genetics, and Lombardi Cancer Center (LCC) at Georgetown University Medical Center (GUMC). I moved to GUMC from the National Human Genome Research Institute (NHGRI) on August 1, 1997. After the move, the grant was successfully transferred from NHGRI to LCC due to cooperative efforts among your organization, NHGRI, and LCC. However, because of establishment and furnishing of the laboratory, recruitment and significant training of personnel, and advancement of research technology, our research has been delayed. Thus, I am requesting one-year no-cost extension of the grant to ensure the completion of the research project.

We are greatly honored by receiving supports for our research from organizations like yours. The Institute for Molecular and Human Genetics, Lombardi Cancer Center, and the NHGRI provide us with direct and collaborative supports, resulting in our several scientific presentations in the State and the International conferences. For an example, I was given a chance to orally present our research during Symposium on Cancer Genetics and Tumor Suppressor Genes at Cold Spring Harbor Laboratory on August 19-23, 1998 and received good response. The funding provided by your organization will tremendously accelerate our breast cancer research.
It is worth mentioning that in the last fiscal year we spent > 6 months to set up the laboratory and recruit new researchers. We are working in an area of advanced cancer genetic fields. It took significant time to train my crew fitting in the advanced technologies. Fortunately, they all work very hard. We are now focusing on carrying out experiments. In short, we are devoted to, competent for, and working hard at fighting against human cancers.

Again, the funding (DAMD17-97-1-7236) from your organization is essential and adds the effective strength to this laboratory to work toward understanding, preventing, and eradicating human breast cancers. In order to ensure the completion of the research project, I am requesting one-year no-cost extension of the funding (total cost of $298,962 for the 3-year period beginning August 25, 1997) from August 25, 1997 - August 24, 2000 to August 25, 1998 - August 24, 2001.

If you have any questions, please feel free to contact me at phone number: 202-687-6056.

I deeply appreciate your support.

Sincerely yours,

Yan A. Su, M.D., Ph.D.
Assistant Professor
Institute for Molecular and Human Genetics
Lombardi Cancer Center
Georgetown University Medical Center

Cc: Ms. Judy Pawlus
    Office of the Deputy Chief of
    Staff for Information Management
    USAMRMC

Dr. Jeffrey Trent, Ph.D.
    Director. NHGRI
    NIH

Dr. Marc Lippman, M.D.
    Director. IMHG & LCC

Ms. Karen Huff, M.S.
    Research Administrator. IMHG & LCC

Mr. Lenny Fraser.
    Grants and Contracts Officer
    GUMC
MEMORANDUM

TO: Greg Raymond
    Acting Chief Financial Officer

THROUGH: Arthur Raines, Ph.D.
    Acting Associate Dean for Research Operations

FROM: Marc E. Lippman, M.D.
    Director

DATE: November 5, 1997

SUBJECT: Grant Transfer From NIH

Dr. Yan Su joined the Lombardi Cancer Center and the Institute for Molecular and Human Genetics in August of this year and now we are in the process of transferring his U.S. Army grant from his previous employer, the National Center for Human Genome Research at NIH. A total of $298,962 was awarded for a 3-year period to the NIH. Because this grant was originally made to a federal facility no indirect costs were included in the award. Therefore, the purpose of this memo is to respectfully request a reduced indirect cost rate for this grant.

We realize that in our present financial climate it would be irresponsible to request a waiver of indirect costs. We are seeking a reduced indirect cost rate of 20% because any indirect costs charged to the grant would reduce the resources available for the research project. Utilizing an indirect cost rate of 20%, the first year funding of $112,000 would be reduced to $89,600 of available funding. We feel anything higher would be severely detrimental to the research Dr. Su has committed to conduct.

Please respond to Lennox Fraser, the Grants and Contracts officer responsible for the transfer of this grant. If further clarification or information is required please contact Karen Huff at 687-3039.

Kathy Russell
Associate Director for
Planning and Administration
Lombardi Internal Budget Review

MEL/kh
cc: Lennox Fraser
    Yan Su, Ph.D.
    Karen Huff
    Susan Myers