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Candidate proto-oncogenes and tumor suppressor genes were previously isolated in a gene expression study involving breast cancer. The present research focus on the characterization of genetic alterations in a number of these candidate proto-oncogenes and tumor suppressor genes to understand how they may be contributing to mammary carcinogenesis. In particular, the candidate proto-oncogene CC-41/LAF-4, which has a cell-type specific expression, was found to be highly expressed in a third of breast tumors. A possibility remained that high CC-41/LAF-4 expression resulted from infiltrating inflammatory cells, which express the gene at high levels. Histology review and *in situ* hybridization demonstrate that CC-41/LAF-4 high expression detected in some breast tumors was tumor cell-specific. This result indicates that CC-41/LAF-4 overexpression may be an important molecular genetic event during mammary tumorigenesis. Another gene isolated from the gene expression analysis was hypothesized to be a candidate tumor suppressor gene. Although presenilin-2 maps to a chromosomal region affected by LOH, analysis of presenilin-2 entire coding region has failed to detect any mutation in 60 different breast tumors. Recent development in the lab with other cancers suggests that presenilin-2 mutational analysis should target breast cancers characterized by an early age of onset. This work is currently being pursued.

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PI - Signature

June 30/99
Date

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INTRODUCTION

The main objective of this research focus on characterizing putative proto-oncogenes and tumor suppressor genes which were identified by differential display PCR in an analysis of gene expression in breast cancer. Three different genes – 2 candidate proto-oncogenes and 1 candidate tumor suppressor gene – were selected for further characterization because preliminary experiments indicated a potential involvement of these genes in mammary carcinogenesis. In particular, the candidate proto-oncogene CC-41/LAF-4, which has been reported to be expressed almost exclusively in cells of the hemopoetic lineage, was found to be over-expressed in approximately a third of breast cancers examined. CC-41/LAF-4 overexpression may be a consequent of an acquired genetic alteration because in a number of tumor-normal pairs the level of CC-41/LAF-4 transcript was greatly higher in the breast tumor in comparison to the corresponding normal breast tissue. A similar result was observed with the novel gene CC-24 in a study of gene expression in tumor-normal pairs. The chromosomal mapping of CC-24 to a region frequently amplified in cancers further supports the hypothesis that CC-24 is a novel proto-oncogene transcriptionally deregulated in breast cancers. The third gene, CC-12/presenilin-2, was hypothesized to be a candidate tumor suppressor gene because it maps to a chromosomal region on 1q for which loss of heterozygosity has been reported, and its expression was reduced in some breast tumors. Moreover, the observation that the level of CC-12/presenilin-2 transcript in cell lines elevated with increasing culture confluence suggested that the gene might have a role in contact inhibition or cell cycle regulation. In the research supported by the USMRMC, the applicant proposed to further characterize the genetic alterations associated with the above genes, and to determine how alterations in these genes can potentially result in progression of breast cancer. The goal of the research is to add to a growing description of genes targeted by genetic alterations during mammary carcinogenesis, which will ultimately improved current understanding, detection, and treatment of breast cancer.

PROGRESS SUMMARY

A: Academic Training

The applicant has successfully completed three core coursed which are required for the degree of Philosophical Doctorate (Ph.D.) by the Graduate Department of Molecular and Medical Genetics of the University of Toronto. The courses are described below:

(i) Protein Structure and Folding:

This course provided the applicant with a basic understanding of the principles governing the folding of polypeptides into functional structures, and a comprehensive description of technology available for analyzing protein properties. The course involved a combination of lectures and practical tutorials based on available computer software. Course evaluation included assignments, a final written examination, and a research proposal.

(ii) Cancer Biology:

This course provided the applicant with a basic understanding of biochemical and genetic regulation of biological functions that are often compromised in cancer. The course was consisted of a series of lectures, and the evaluation was based on a formal research proposal.

(iii) Fundamentals of Human Genetics:

This course provided the applicant with the knowledge of the genetic tools and approaches that have been used for the purpose of cloning and characterizing genes associated with human diseases. The course involved a series of presentations and group discussions on different topics in human genetics. Evaluation was based on the presentations and participation.

B: Research Progress:

In view of the suggestions made by the reviewers, much of the work has focused mainly on only CC-41/LAF-4 and CC-12/presenilin-2.

(i) CC-41/LAF-4:

Since the expression of LAF-4 has been demonstrated to be specific to cells of the hemapoietic lineage (Ma and Staudt, Blood 87: 734, 1996), it was necessary to confirm that LAF-4 overexpression in breast tumors was not due to infiltrating leukocytes. To address this issue, the histologies of a series of breast tumors with available LAF-4 expression data were examined for the degree of infiltrating leukocytes. There was no correlation between the extent of infiltrating leukocytes and LAF-4 transcript level, indicating that the detected LAF-4 overexpression was likely tumor cell-specific. To further confirm this conclusion, a LAF-4 specific riboprobe was synthesized for the purpose of *in situ* hybridization. The specificity of the probe was demonstrated by the detection on a Northern blot of a transcript of the expected size only in the breast cancer cell line (T47D) previously shown to express LAF-4 by RT-PCR. When the riboprobe was used for the purpose of *in situ* hybridization, positive staining was clearly seen in the tumor cells and not in histologically adjacent normal cells. This result

convincingly confirm the conclusion that that the overexpression of LAF-4 detected in some breast tumors is not due to infiltrating leukocytes, but rather is an aberrant property of the tumor cells themselves.

To further confirm the overexpression of LAF-4 in some breast tumors, additional LAF-4 specific primers have been obtained to be used in quantitative RT-PCR. These primers target a region of LAF-4 coding sequence different from that of the primers described in the initial research proposal. The conditions for quantitative RT-PCR using these new LAF-4 primers have been established. Both sets of LAF-4 primers will be used in all subsequent quantitative RT-PCR analysis. The expression of LAF-4 in an additional cohort of breast tumors is in progress. As well, for a number of the breast tumors which has been demonstrated to express LAF-4, the histologic stage at which LAF-4 overexpression first occurs will be determined. For this purpose, regions of fresh frozen breast tumors representing invasive cancer and ductal carcinoma *in situ* (DCIS) have been microdissected. RNA from the microdissected cells will be processed for RT-PCR analysis using a protocol we have recently developed (To *et al.*, American J. Pathology 153: 47, 1998).

In order to determine the effect of LAF-4 overexpression, it was proposed that LAF-4 be overexpressed in a cell line in which LAF-4 expression was not detected. To this end it was necessary to clone LAF-4 coding region into an expression vector (pcDNA3.1). LAF-4 specific primers were designed to enable PCR amplification of the gene's entire coding region using cDNA synthesized from total RNA extracted from the breast cancer cell line T47D. It is appropriate to obtain LAF-4 from the cell line T47D since it remains a possibility that the LAF-4 transcript overexpressed in some breast cancers has structural differences in comparison to the published sequence. However, PCR amplification of LAF-4 entire coding region from T47D cDNA has proven to be difficult. The primers encompassing LAF-4 coding region appears to be of good quality because when additional internal primers that would give smaller PCR products were used with these external primers, the appropriate PCR product was efficiently obtained. It is likely the encountered problem is with the length of LAF-4 coding region and the low overall level of LAF-4 transcript in T47D. To circumvent this problem, polyA RNA is currently be purified from T47D total RNA pool in order to enrich for the LAF-4 transcript. As well, Taq polymerase capable of supporting long range amplification is being purchased. Alternatively, the LAF-4 coding region can be amplified as smaller subfragments using internal primers, and the subfragments ligated together at the end. Unique restriction sites within LAF-4 coding region have been identified to facilitate the ligation of LAF-4 subfragments, and appropriate internal primers have already been designed. In a set of preliminary PCR amplification it appears that LAF-4 sub-fragments are obtainable.

(ii) CC-12/Presenilin-2:

Using a combination of single strand conformational polymorphism (SSCP) analysis and DNA sequencing, the entire coding region of presenilin-2 has been studied for the presence of sequence alterations in a cohort of 60 breast tumors. The analysis employed both DNA and cDNA as template for PCR amplification. The coding region of presenilin-2 was divided into 9 different PCR fragments for the initial SSCP analysis. All aberrantly migrated patterns on the SSCP gels were further analyzed by direct DNA sequencing. Although a

number of sequence polymorphisms were detected in both the exonic and intronic sequence of presenilin-2, no alterations which would result in amino acid substitution or structural disruption in the presenilin-2 protein was detected. This result suggest that although presenilin-2 maps to a chromosomal region for which LOH has been detected in breast cancer, alterations affecting presenilin-2 protein are not selected for during mammary carcinogenesis. However, the analysis does not rule out the possibility for molecular genetic alterations in the regulatory sequence, namely the promoter, of presenilin-2.

To further analyze the expression of presenilin-2 in breast tumors, new primers specific to presenilin-2 have been designed for the purpose of quantitative RT-PCR analysis. In contrast to the original RT-PCR primers, which targeted the non-coding region of presenilin-2 cDNA, the new primers target sequences within the coding region of presenilin-2. The conditions for quantitative RT-PCR analysis using these new primers have already been established. The analysis of expression of presenilin-2 in a larger cohort of breast tumors is in progress. Upon completion of this analysis, a group of tumors with reduced presenilin-2 expression will be selected for quantitative DNA PCR analysis to determine whether allelic imbalance, (namely, LOH/deletion), of the presenilin-2 genomic region underlies the reduction in expression.

To explore the role of presenilin-2 in senescence, it was proposed that the expression of presenilin-2 be analyzed in normal mammary epithelial cell lines that have undergone senescence. This is underway as we are in the process of obtaining additional normal mammary epithelial cell lines from Dr. Martha Stampfer. These cell lines will allow to grow for 3-4 passages after which time they will senesce and stop to proliferate.

Given the recent reports of the involvement of presenilins in the processing of β-catenin, we hypothesized that presenlin-2 may be a target for mutations in colon cancers, in which β-catenin has been shown to be frequently deregulated. Only one small region of presenilin-2 has been analyzed for mutations in colorectal tumors. Two novel DNA sequence alterations in this region of presenilin-2 have been identified in colorectal tumors that are distinct from those described for Alzheimer disease. Both alterations resulted in amino acid substitutions. Using a well-published assay for presenilins that involved *C. elegans*, both alterations have been shown to compromise the function of presenilin-2 protein. Interestingly, these alterations were only found in a number of colon cancer cases with an early age of onset, but not in sporadic colon cancers, which are characterized by a later age of onset. This suggests that alterations in presenilin-2 may predispose individuals to an early onset of cancer, and may explain the lack of mutations in the 60 breast cancers we have analyzed. These 60 breast cancers are axiallary node negative, which are not likely to be characterized by early onset. Currently, we are searching our database for cases of early onset breast cancer. Presenilin-2 mutational analysis in early onset breast cancer cases will be performed.

APPENDIX

A. Research Accomplishments:

- Detection of LAF-4 overexpression in some breast cancer cases.
- LAF-4 overexpression is not due to infiltrating lymphocytes, but is rather tumor cell specific as demonstrated by a review of histologies and *in situ* hybridization.
- Although presenilin-2 maps to a chromosomal region with LOH in breast cancer, no mutations in presenilin-2 in 60 breast cancers were detected.
- Two functional mutations in presenilin-2 were detected in young colon cancer cases, and suggest that breast cancer cases with an early age of onset should be examined for presenilin-2 mutations.

B. Reportable Outcomes:

(i) Abstracts and Presentations:

1. To M.D., Beatty, B.G., Scherer, S. W., Tsui, L.-C., and Andrulis, I. L. Identification of Putative Proto-oncogenes in Breast Cancer. Poster presentation at the 1998 Samuel Lunenfeld Research Institute Annual Research Retreat. Maple Lake, Ontario, Canada. September 27-29, 1998. (Abstract enclosed)

2. Minh D. To, Timothy, G. Doyle, Robert Gryfe, Mark Redston, Steve Gallinger, and Irene Andrulis. Potential Involvement of Presenilin 2 in Tumorigenesis. Poster presentation at Keystone Symposia: The Molecular Basis of Cancer. Taos, New Mexico, USA. March 15-21, 1999. (Abstract enclosed).

3. Minh D. To. Presenilin-2 Potential Involvement in Tumorigenesis. Samuel Lunenfeld Research Institute Seminar Series. May 25, 1999.

4. Susan J. Done, Minh D. To, and Irene L. Andrulis. Overexpression of LAF-4, a Putative Proto-oncogene in Breast Cancer. Poster presentation at the 1999 Reasons for Hope Breast Cancer Research Conference. Toronto, Ontario, Canada. June 10-12, 1999. (Abstract enclosed).

(ii) Funding:

Based on this work the proposal entitled "Characterization of LAF-4, a Putative Proto-oncogene Involved in the Development of Breast Cancer," (Proposal Number BC980946) was submitted to the USAMRMC under the IDEA category. The proposal has been accepted for funding by the USAMRMC (Funding No. DAM17-99-1-9366).

Identification of Putative Proto-oncogenes in Breast Cancer. To, M.D., Beatty, B.G., Scherer,S.W., Tsui,L.-C. and Andrulis, I.L. Department of Molecular & Medical Genetics, University of Toronto, Ontario Cancer Institute, Princess Margaret Hospital, Department of Genetics, YAC core Facility, Hospital for Sick Children, Samuel Lunenfeld Research Institute, Mt Sinai Hospital, Toronto, Ontario, Canada M5G 1X5.

Deregulation in expression of cancer genes occurs commonly during the pathogenesis of breast carcinoma. In an effort to identify genes with a potential role in mammary tumorigenesis, we used mRNA differential display for analysis of gene expression in breast carcinomas. We describe two putative proto-oncogenes, CC-24 and Laf-4, that were isolated from the analysis. CC-24 is a novel cDNA fragment that maps to 12q13, a chromosomal region amplified in a number of cancers. The expression of CC-24 was observed to be upregulated in a number of breast tumors in comparison to normal mammary tissues. Another cDNA fragment isolated from the analysis displayed a perfect sequence identity to the recently cloned Laf-4 gene. Laf-4 is highly homologous to AF-4, a gene involved in the t(4;11) translocation in acute lymphoblastic leukemia. In agreement with the reported lymphoid-specific expression of Laf-4, RT-PCR analysis showed a lack of Laf-4 expression in normal mammary epithelial cell lines. Similarly, there was no significant LAF-4 expresion in 34/45 (75%) breast carcinomas. However, in 11/45 (25%) of breast carcinomas, we detected a significant level of the Laf-4 transcript. These results suggest that Laf-4 may represent a putative proto-oncogene that is transcriptionally activated in some breast carcinomas.

Potential Involvement of Presenilin 2 in Tumorigenesis

Minh D. To, Timothy G. Doyle, Robert Gryfe, Mark Redston, Steve Gallinger, and Irene L. Andrulis. Dept. of Molecular and Medical Genetics, University of Toronto; Integrated Program of Cellular, Molecular, and Biophysical Studies, Columbia University, New York, NY, USA; Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, ON, M5G 1X5, Canada.

Presenilins encode multi-transmembrane proteins, and are functionally mutated in Alzheimer disease. Several studies suggest that presenilins may also have a role in cancer-related pathways. Work in *C. elegans* demonstrated the involvement of presenilins in lin-12/Notch signaling. Work in mammalian cells indicates that presenilins play a role in apoptosis, potentially acting through a pathway involving trimeric G-proteins. A recent finding that presenilin expression may be regulated by the *p53* tumor suppressor gene during *p53*-induced apoptosis further support the potential involvement of presenilins in tumorigenesis.

We initially isolated presenilin 2 (PS-2) in an analysis of gene expression in breast tumors using differential display. PS-2 maps to a chromosomal region targeted by LOH/deletion, suggesting that its loss may be involved in tumorigenic progression. Indeed, we observed low PS-2 mRNA levels in a number of breast tumors. Furthermore, we found the level of PS-2 mRNA to be induced with increasing cell density, suggesting that PS-2 may have a role in cell cycle regulation. Mutational analysis of PS-2 in breast tumors did not detect any mutation. However, given the semi-repetitive nature of a short stretch of the PS-2 nucleotide sequence, we hypothesized that this region may be more susceptible to mutations in cancers with impaired DNA repair. In a selected cohort of colorectal tumors, two different nucleotide sequence changes within the vicinity of this region were detected. Both nucleotide changes are present in the patients' normal tissue DNA, and lead to amino acid substitutions at codons distinct from the Alzheimer disease-associated mutations. In an *in vivo* assay utilizing *C. elegans*, both alterations were found to compromise the function of the PS-2 protein. We are continuing to characterize the function of PS-2 in an effort to determine how the detected alterations may be contributing to tumorigenesis.

Supported by grants from CBCRI (MR and ILA) and fellowship from USAMRMC (MDT).

OVEREXPRESSION OF LAF-4, A PUTATIVE PROTO-ONCOGENE, IN BREAST CANCER

Susan J.Done MB BChir[1], Minh D. To MSc[2], and Irene L. Andrulis PhD[1,2].
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Abstract

It is unlikely that all of the genes involved in the pathogenesis of breast cancer have been identified. We have used differential display PCR in a panel of breast cancers to detect novel or previously undescribed genes. One of the genes we identified was LAF-4, a candidate proto-oncogene which has high amino acid homology to AF-4, the gene involved in the t(4:11) translocation in acute lymphoblastic leukemia. To investigate the role of LAF-4 in breast cancer we have studied its expression levels in normal and tumor tissues.

We expanded our initial differential display analysis by examining LAF-4 mRNA expression in a larger cohort of 45 breast tumors using quantitative RT-PCR. In 11 of the cases, high level of LAF-4 mRNA was observed, whereas the expression was barely detectable in the remaining 34 cases. We also examined LAF-4 expression in 7 different tumor-normal pairs. In two cases, LAF-4 mRNA levels were clearly elevated in the tumor in comparison to the corresponding normal surrounding tissue.

To examine whether the observed LAF-4 expression originated from the tumor cells rather than infiltrating inflammatory cells we reviewed the histology of the breast cancer cases. We found no correlation between the level of LAF-4 mRNA expression and the proportion of infiltrating leucocytes. Furthermore, using in situ hybridization, we demonstrated the presence of LAF-4 mRNA within mammary carcinoma cells. To confirm the expression levels in tumor cells we are currently analyzing LAF-4 mRNA expression in microdissected breast carcinomas. We are also using microdissection to determine the histologic stage at which expression becomes elevated.

We have found LAF-4 expression levels are elevated in some breast cancers. LAF-4 protein has transcriptional transactivation potential, hence its aberrant expression can have far reaching implications because it can affect the expression of many downstream target genes.