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Abstract:
While the aberrant overexpression of HER family receptors is known to be involved in breast cancer, the complex nature of the genetic events that trigger the development of breast cancer remain to be identified. We have developed, refined and applied a retrovirus-based gene transfer assay to identify activated oncogenes in breast cancer. Progress during the past year has involved: (1) the use of breast carcinoma cell lines for generation of retrovirus expression libraries; (2) development of better functional screens for activated oncogenes, and (3) evaluation of protocols to improve the rescue of cDNAs from the transformed cell populations. We have generated several retrovirus-based cDNA expression libraries that represent genes expressed in noninvasive (MCF-7 and T47D) and invasive (MDA-MB4682 and BT549) human breast cancer cell lines. These libraries have been introduced into Rat-1 rodent fibroblasts and RIE-1 rat intestinal epithelial cells and transforming activity was then assayed for. Two isolates from our analyses encode the Raf-1 serine/threonine kinase and the fibroblast growth factor receptor 2 (FGFR2). In the final year of support, we have characterized the transforming activity of FGFR2 and evaluated possible signaling mechanisms by which it causes transformation. We find that pharmacologic inhibitors of MEK1/2 potently block FGFR2 transforming activity.

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Introduction

Cancer is a multi-step genetic process involving mutation of oncogenes and tumor suppressor genes (1). With a few notable exceptions (e.g., HER2) the genetic determinants which contribute to the development of breast cancer remain unknown. Conventional techniques using genomic DNA for identifying oncogenes in human tumor cells are cumbersome, inefficient and have met with limited success. While these studies identified the importance of the ras and neu oncogene in human cancers, they also suggested that cancer arises from the involvement of only a handful of oncogenes (2). Moreover, for practical reasons they have relied on the transformation of fibroblast cell lines to identify oncogenes in epithelial cell-derived tumors. Thus, it seems likely that many oncogenes present in epithelial cell-derived breast carcinomas may not produce an obviously transformed phenotype in fibroblast cells. These limitations argue that oncogenes and tumor suppressor genes that contribute to transformation in human breast cancer remain to be detected. The development of retrovirus vector-based cDNA libraries overcomes the key obstacles to expression cloning of oncogenes contributing to the transformation of epithelial cells (3,4). Thus, we propose to generate retroviral cDNA libraries from human breast tumor cells and screen them for DNA sequences which contribute to the transformation of breast epithelial cells. We believe that this approach will lead to the successful identification of new genetic markers for breast cancer and identify novel targets for the rational design of anti-cancer drugs against breast cancer.

Body

The goal of our proposed studies was to identify novel oncogenes involved in breast cancer development. We chose a functional screen that allowed the identification of such genes by biological activity, rather than merely aberrant expression, in breast carcinoma cells. The approach we employed utilized retrovirus expression libraries that represented genes expressed in breast carcinoma cell lines. A significant component of our work during the funding period involved initial efforts to use patient-derived breast cancer tissue rather than cell lines. Unfortunately, the sample sizes we were able to obtain did not allow us to generate expression libraries with sufficient complexity, such that we would not be able to effectively screen the majority of genes expressed in these breast cancer samples. Therefore, we switch our efforts to use human breast carcinoma cell lines.

Another facet of our previous analyses was the further refinement of the retrovirus screen, with regards to the isolation of transforming genes from the transformed cell populations that arose from our screen. We found that the PCR-based approaches to isolate these sequences, from the genomic DNA of transformed cells, was very inadequate and provided the most significant technical limitation of our screen. Thus, while our screens identified many transformed cell isolates, our analyses were limited to those genes that we were able to successfully rescue from cells. We were able to isolate two transforming genes, Raf-1 and fibroblast growth factor receptor 2 (FGFR2), in our analyses. The first gene, Raf-1, is an intriguing one in light of the recent studies that have identified mutationally activated B-Raf, a related protein, in a variety of human cancers. The second one, FGFR2, is an interesting gene, in light of the fact that mutated forms of FGFR2 have been described, but these are involved in developmental abnormalities, rather than cancer. Various FGFR family proteins, as well as their ligands, have been implicated
in oncogenesis, with limited documented evidence for its involvement in breast cancer (5). However, to date, little is known regarding their mechanism of transformation. Therefore, in the final year of support, we have further characterized the transforming activity of FGFR2 in model cell systems, and begun an assessment of the signaling pathways that may be utilized by FGFR2 to cause transformation. The main goal of our studies is to validate FGFR2 as an important mediator of breast cancer development, with the long term goal of establishing its promise as a target for drug discovery for anti-breast cancer drugs.

**FGFR2 is a transforming gene expressed in human breast carcinoma cells.** Our initial screen employed Rat-1 fibroblasts and RIE-1 rat intestinal epithelial cells. Our analyses identified FGFR2 in both our Rat-1 and RIE-1 screens (Figure 1). When we sequenced the breast tumor-derived cDNA sequence, we did not find any mutations in the sequence. However, there exists many splice variants of FGFR2, and while all sequences of our isolated FGFR2 can be found in the database, the specific variant we identified was not deposited in GenBank sequences.

We isolated the FGFR2 sequence and subcloned it into several different expression vectors. We first verified that the isolated gene was a transforming sequence. We found that retrovirus infection of Rat-1 and RIE-1 showed very potent transforming activity.

![Image of cellular transformation](image.jpg)

**Figure 1.** RIE-1 cells and Rat-1 fibroblasts were infected with PCTV3 or PCTV3-FGFR2 and selected in growth medium supplemented with hygromycin. Drug-resistant colonies were pooled and replated for a secondary focus formation assay. Cells were allowed to grow to confluence for 21 days before transformed foci were photographed (left panel). The cells were fixed and stained with 0.4% crystal violet (right panel).

We also determined if FGFR2 caused transformation of NIH 3T3 cells. Since the vast majority of studies of oncogene function utilize NIH 3T3 cells, we wanted to be able to compare the potency and mechanism of transformation of FGFR2 with other oncogenes. FGFR2 showed very potent focus-forming activity in NIH 3T3 cells (data not shown). Thus, despite lacking any structural mutations, we found that FGFR2 was a very potent oncogene.

**FGFR2 transforming activity is blocked by inhibitors of MEK and PI3K.** Little is known regarding the signaling pathways activated by FGFR2 and consequently, how FGFR2 causes transformation is not known. Perhaps the two most important signaling pathways utilized by oncogenes to promote cellular transformation are the Raf-MEK-ERK mitogen-activated protein kinase cascade and the phosphatidylinositol 3-kinase (PI3K)-Akt serine/threonine protein...
kinase cascade. For example, both pathways are activated by the Ras oncogene and inhibition of either pathway effectively blocks Ras transformation. The importance of the Raf>MEK>ERK cascade in oncogenesis is reflected by the fact that inhibitors of both Raf and MEK have been evaluated in phase II clinical trials as target-based anti-cancer drugs (6,7).

To address a possible role for these two signaling pathways in FGFR2, we first determined if FGFR2-transformed cells could form colonies in soft agar. We wanted to determine this, since this transformation assay is the most commonly used one to evaluate the ability of compounds to block cancer cell growth. We found that FGFR2-transformed cells readily formed colonies in soft agar (Figure 2).

Next, we evaluated the ability of a pharmacologic inhibitor of MEK1/2 (U0126), to block ERK1/2 activation, to block the growth of FGFR2-transformed cells in soft agar. We found that U0126 treatment essentially abolished soft agar growth (Figure 2). Then we determined if treatment with the LY294002 PI3K inhibitor could block the growth of FGFR2-transformed cells in soft agar. We found a partial block in colony formation (Figure 2). Thus, both ERK and PI3K activity are important for FGFR2 transforming activity.

**Figure 2.** RIE-1 cells expressing either pCTV3 or pCTV3-FGFR2 were plated in 0.4% soft agar. The cells were incubated for 2 weeks in the absence or presence of 10 μM LY294002 (PI3K inhibitor) or 30 μM U0126 (MEK inhibitor). Cells were photographed about 3 weeks after plating (left panel). Colonies per dish were counted under a phase contrast microscope after 2 weeks after plating. Duplicate plates were counted (right panel).

**ERK and Akt activities are not upregulated in FGFR2-transformed cells.** Our observation that inhibition of MEK activation of ERK, or PI3K activation of Akt, blocked FGFR2 transformation of RIE-1 cells suggested that FGFR2 causes hyperactivation of these two signaling pathways. Therefore, we evaluated this possibility. For these analyses, we used Ras-transformed RIE-1 cells, where Ras causes activation of ERK, but in contrast to other cell types, does NOT cause upregulation of Akt. We used phosphospecific antibodies to recognize the phosphorylated and activated forms of ERK1/2 and Akt (Figures 3 and 4). Surprisingly, neither ERK nor Akt activity were upregulated in FGFR2-transformed RIE-1 cells. However, these results are preliminary, and the level of ERK and Akt activity could not be verified convincingly in our positive controls. Therefore, further analyses need to be done to address the role of ERK or Akt hyperactivation in FGFR2-transformed cells.
**Figure 3.** RIE-1 cells and Rat-1 fibroblasts stably expressing PCTV3, PCTV3-HRas61L or PCTV3-FGFR2 were starved of serum for 24 h. The cells were lysed and assayed for Akt activation by immunoblot analyses using antibodies that recognize the activated phosphorylated form of Akt. Blots were also analyzed with anti-Akt antibodies.

**Figure 4.** RIE-1 cells and Rat-1 fibroblasts stably expressing PCTV3, PCTV3-HRas61L or PCTV3-FGFR2 were starved of serum for 24 h. The cells were lysed and assayed for ERK1/2 activation by immunoblot analyses using antibodies that recognize phosphorylated /activated form of ERK1/2. Blots were also analyzed with anti-ERK1/2 antibodies for total ERK expression.

**Key Research Accomplishments**

- Generation of retrovirus cDNA expression libraries representing genes expressed in human breast carcinoma cell lines – in contrast to our libraries made from mRNA derived from patient breast cancer samples, these should be complete and represent the entire repertoire of genes expressed in each tumor cell line.
- Adaptation and application of Rat-1 and RIE-1 cells lines as biological screens for transforming genes expressed in breast cancer cells – additionally, we are developing and characterizing the HMEC immortalized cells as recipients for our library screens. Ultimately, we feel that these cells may provide the most relevant cell type for our screens.
- Refinement of PCR-based techniques to isolate transforming sequences from transformed cell populations – this technical breakthrough allows us to overcome the rate-limiting step in our library screens. We are currently evaluating another retrovirus vector system for these studies. This system will allow the isolation of inserted retroviruses as replicating plasmids in bacterial cells. This will eliminate the need for
PCR-based isolation techniques to isolate our transforming sequences.

- Identification of Raf-1 and FGFR2 as transforming genes expressed in breast carcinoma cells – as both of these genes are already implicated in growth regulation, these validate the usefulness of the screen for detection of novel transforming activity.
- Established that our isolated FGFR2 gene is a novel variant and exhibits potent transforming activity in Rat-1 rat fibroblasts, RIE-1 rat epithelial cells, and NIH 3T3 mouse fibroblasts. Future studies will evaluate FGFR2 transforming activity in mammary epithelial cells.
- Determined that FGFR2 transforming activity can be blocked by pharmacologic inhibitors of MEK1/2 and PI3K. However, currently we have not found ERK or Akt activation in FGFR2-transformed cells. Therefore, it is unclear whether FGFR2 causes hyperactivation of these signaling cascades. Future studies will evaluate a possible role for PAK4 serine/threonine kinase activation, and STAT activation, in FGFR2 transforming activity.

**Reportable Outcomes**

* Poster presentation at Era of Hope Meeting, Orlando, FL 2002

**Conclusions**

Our identification of FGFR2 verifies that a function screen, coupled with retrovirus expression libraries representing genes expressed in breast cancer cells, can be an effective and powerful approach to identify novel oncogenes involved in breast cancer development. Much of our earlier work involved technical development and refinement, as well as trouble-shooting, the retrovirus screening approaches. We are now ready to expand our screening efforts to identify other oncogenes. As described above, we have developed another retrovirus vector system for these studies. We have generated two breast cancer cell line libraries using this vector system, have identified a large number of transformed cell populations, and we are beginning to isolate the transforming sequences.

Additionally, we will continue our analyses of FGFR2 signaling and transformation. First, is FGFR2 overexpressed in breast carcinomas? If so, will downregulation of FGFR2 expression, using RNAi approaches, inhibit the growth of these tumor cells? What signaling pathways are crucial for FGFR2 transformation? Will MEK inhibitors be useful for blocking the growth of FGFR2-transformed breast epithelial cells? In summary, with the work supported by this grant, we have been able to take our project to a level where other sources of research funding may now be available.

**References**


Appendices - None