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13. ABSTRACT ***(Maximum 200 Words)***

EGFRvIII is a tumor specific, ligand-independent, constitutively active variant of the epidermal growth factor receptor. Its expression has been detected in gliomas and various other human malignancies. In order to evaluate the importance of EGFRvIII expression in human breast cancer, we examined the frequency of EGFRvIII protein expression immunohistochemically in paraffin embedded specimens from 109 patients with breast cancer. With a specific anti-EGFRvIII antibody, 67% (73 of 109) of primary breast carcinomas and 16% of DCIS (2/12) expressed EGFRvIII. No EGFRvIII expression was detected in normal breast tissue and benign tumors. We also observed 56% (413 of 564) in a breast cancer tissue microarray. These results confirm our pilot studies and suggested that expression of EGFRvIII may play a crucial role in breast cancer progression.

To delineate the biological significance of EGFRvIII in human breast cancer, we expressed EGFRvIII in MCF-7 human breast cancer cells. Expression of EGFRvIII in MCF-7 produced a constitutively activated EGFRvIII receptor. These MCF-7/EGFRvIII transfectants exhibited approximately a three-fold increase in colony formation in 1% serum with no significant effect observed at higher percentages of serum. Collectively, these results provide the first evidence that EGFRvIII could play a pivotal role in human breast cancer progression.

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Carem K. Tang 7/25/00

PI - Signature Date

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PART 1: **P.I. Careen Tang**

INTRODUCTION

Statements of rationale: The epidermal growth factor receptor (EGFR) is a promising target for various antineoplastic agents. However, this receptor is also expressed in normal tissue. Reports have demonstrated that spontaneous rearrangements within the EGF receptor gene (EGFRvIII) do arise in primary human glioblastoma tumors. This EGFRvIII receptor is also frequently expressed in other human cancers, but was never found in normal tissue. Our pilot studies showed that EGFRvIII receptor is expressed in 62% (31 of 50) of primary breast carcinomas. Furthermore, EGFRvIII was present in 100% (11 of 11) of lymph node metastatic cells selected from EGFRvIII postive primary breast cancer patients. These data suggested for the first time that EGFRVIII expression correlates with lymphatic invasion. The understanding of the function and biology of EGFRvIII will have important implications for the prognosis and treatment of breast cancer. Little is known about the expression or the clinical significance of EGFRvIII in the diagnosis and prognosis of human breast cancer. It is important to define the role of EGFRvIII receptor and their biological significance human breast cancer. We will expend our pilot study to determine the EGFRvIII expression by immunohistochemical analysis and correlate its expression with clinicopathological prognostic factors in human breast cancer. These molecular epidemiological data will enable us to provide important new information for the prognosis and treatment of breast cancer. We will transfect EGFRvIII cDNA into several human breast cancer cell lines at different malignant stages. We will characterize these transfectants to elucidate whether overexpression of EGFRvIII will induce tumorigenicity and metastasis potential in breast cancer cells. **These studies will provided valuable insights in the role of EGFRvIII in breast cancer.**

Background: The epidermal growth factor receptor (EGFR/ErbB) family is a group of tyrosine kinases, frequently overexpressed in a variety of carcinomas (1-3). This class I subfamily is comprised of four members: EGFR (4), *HER2*/p185^{erbB-2}/neu (5), *HER3*/p160$^{erbB\ 3}$(6, 7), and *HER4*/p180 $^{erbB-4}$ (8). High levels of EGFR and HER-2 have been found in 30-40% of breast carcinomas. Expression of these proto-oncogene proteins inversely correlated with estrogen receptor expression and appears to confer a worse prognosis (9). However, since normal EGFR may be targeted by these anti-EGFR agents, a more appropriate strategy might be to target an alteration within the receptor. Several reports have documented spontaneous rearrangements within the EGF receptor gene in primary human glioblastoma tumors (10). Three different types of mutants result from these rearrangements (11). The most common of these rearrangements is the Type III EGF deletion-mutant receptor (EGFRvIII), which involves a deletion of exons 2-7, (cDNA nucleotide 275-1075), presumably through alternative splicing or rearrangements (11-13). Overexpression of this mutant EGF receptor in NIH3T3 cells results in transformed morphology, enhanced growth, and tumorigenicity in athymic mice (12). Crosslinking experiments with EDAC demonstrate that the mutant EGF receptor is dimerized in the absence of ligand. EGFRvIII has not been detected in normal adult tissues (14, 15). Recent reports demonstrated that the EGFRvIII is also frequently detected in other human cancers (15). Our preliminary results detected 62% (31 of 50) of primary breast cancer specimens expressing EGFRvIII. Furthermore, EGFRVIII expression was found in 100% (11 of 11) of the lymph node metastatic cells selected from EGFRvIII positive patients. This phenomenon is the first evidence that EGFRvIII expression can be correlated with lymphatic invasion. However, the tumorigenicity and metastatic potential of EGFRvIII in breast cancer cells

has not yet been explored. The understanding of the function and biology of EGFRvIII, will have important implications in the prognosis and treatment of breast cancer. 1) Does EGFRvIII expression play a role in human breast cancer progression? 2) Does EGFRvIII play a role in promoting the metastasis of primary breast cancer cells to distant sites? We will address these questions in AIM 1 of this proposal in order to examine the role of EGFRvIII in breast cancer progression and its metastatic potential.

In this report, we will discuses our research progress during the last 12 months (7/1/99-6/30/00).

PART 2:

BODY

AIM 1. To determine the expression of EGFRvIII and correlation with clinicopathological prognostic factors in human breast cancer specimens:.

Rationale: In our preliminary study, we demonstrated that EGFRvIII expressed in 62% of human primary breast carcinomas and 100% of lymph-node metastatic breast cancer. To investigate whether any correlation might exist between the expression of EGFRvIII and several clinicopathological and /or biological characteristics that have been shown as important prognostic factors in breast cancer, including axillary-lymph-node involvement, ER and progesterone-receptor (PgR) status, histologic and nuclear grading. To achieve this goal, we will perform immunohistochemical analysis with large sample size to examine the expression of EGFRvIII and to analyze its implication for clinical outcome.

Progress Results:

A) Characterization of specific EGFRvIII antibody.

In our pilot study, we have used a well characterized polyclonal antibody (a gift from Dr. Albert Wong). We now obtained a specific EGFRvIII monoclonal antibody (Ab-18). We used wild-type EGFR cells or EGFRvIII transfected NIH 3T3, as well as MDA-MB-468 cells which expresses high levels of endogenous EGFRvIII. Figure 1 illustrated this EGFRvIII (Ab-18) only react with EGFRvIII and do not react with the wild-type EGFR (See Illustration Figure 1, page 11).

B) Determination of EGFRvIII expression by immunohistochemirey and cerrelation with clinipathological prognostic factors in human breast cancer specimens.

We have expanded our pilot studies and confirm our findings with large sample size to analysis and determine whether any correlation between expression of EGFRvIII and other clinicopathological prognostic indicators in human breast cancer.

My collaborator, Dr. Perter Watson at University of Manitoba NCIC-Manitoba Breast Tumor Bank, has provided us with the paraffin embedded breast specimens for this study. We examined the frequency of EGFRvIII protein expression immunohistochemically in paraffin embedded specimens from 181 patients with breast cancer. With a specific anti-EGFRvIII antibody, 62% (103 of 165) of

primary breast carcinomas and 38% of DCIS (10/26) expressed EGFRvIII. No EGFRvIII expression was detected in normal breast tissue and benign tumors. The following tables summarizes the results **(not published results).**

Table 1. Summary of immunohistochemical analysis results in paraffin embedded breast tissues.

Expression of EGFRvIII # of positive/total # of evaluated	
Benign	0/10 (0%)
DCIS	10/26 (38%)
Invasive Carcinomas	72/97 (74%)

We also collaborate with Dr. Olli Kallioniemi (NIH), who has kindly provided us breast cancer tissue microarray, which allow us to examine 564 different cases of primary breast cancer in a single slide under uniform immunohistochemical conditions **(not published results).**

Table II. Summary of Breast Tissue Array Results

Receptor	% of expression (Total 564 cases)
EGFRvIII	55.7% (314 of 564); 17.5 % (99 of 564) (+) 26.8% (152 of 564) (-)

Figure 2. illustrates the immunohistochemical analysis on a tissue microarray (See Illustration Figure 2, page 12).

These results further confirmed and verified our findings that EGFRvIII plays an important role in breast cancer progression.

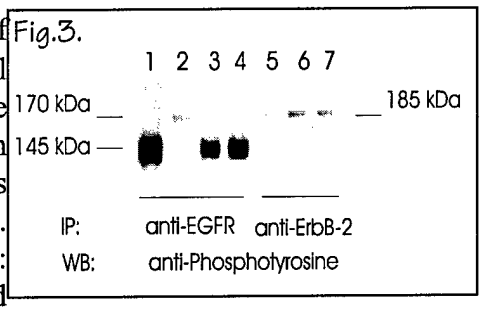
AIM 2: To test the hypothesis that over-expression of deletion-mutant EGFRvIII is capable of increasing the malignancy of breast cancer.

In order to assess the biological significance of EGFRvIII in human breast cancer, we expressed EGFRvIII in MCF-7 cells. Stably transfected MCF-7/ EGFRvIII cells were selected. The expression of EGFRvIII was evaluated by FACS analysis (Data not shown). The biological effects of EGFRvIII in MCF-7 cells were evaluated as follow.

A) Ligand-independent constitutive autophosphorylated EGFRvIII in EGFRvIII transfected MCF-7 cells

We first evaluated the activation of EGFRvIII in MCF-7/EGFRvIII transfected cells. Autophosphorylation of EGFRvIII was assessed by immunoprecipitation with an anti-EGFRvIII specific antibody and Western blot with an anti-phosphotyrosine specific antibody. Figure 3 shows that EGFRvIII was constitutively activated in a ligand-independent manner in MCF-7/EGFRvIII cells. In addition, ErbB-2 phosphorylation was enhanced in EGFRvIII transfected MCF-7 cells. These results indicated that EGFRvIII could activate ErbB-2 kinase activity.

Figure 3. Expressing EGFRvIII induces autophosphorylation of EGFRvIII and ErbB-2 in MCF-7 cells. 500 μg of whole cell lysates was immunoprecipitated with anti-EGFR antibody (Lane 1-4) or ErbB-2 antibody (Lane 5-7) and subsequently with Western blotting with an antiphosphotyrosine antibody. Bands were visualized using a chemiluminescence detection system.. Lane 1: EGFRvIII transfected NIH 3T3 cells as control. Lane 2: MCF-7 wild-type cells. Lane 3 and 4 are EGFRvIII transfected MCF-7 cells. Lane 5: MCF-7 wild-type cells. Lane 6 and Lane 7 are MCF-7/EGFRvIII transfectants.



B) Effects of EGFRvIII on human breast cancer cell growth in vitro.

We next evaluated the influence of EGFRvIII on the cellular growth rate in MCF-7/EGFRvIII cells by anchorage-dependent as well as anchorage-independent growth assays. There was no significant growth effect in medium containing 10% FBS (data not shown). However, at 1% FBS, overexpression of EGFRvIII resulted in a significant induction of proliferation in an anchorage-dependent growth assay and three-fold induction of colony formation in an anchorage-independent growth assay (Figure 4). Induction of colony formation was independent of threshold colony size. Moreover, EGF-like ligands increased proliferation substantially (Figure 5). These data suggest that EGFRvIII could play an important role in breast cancer progression. Constitutively autophosphorylated EGFRvIII may contribute to enhanced proliferation of MCF-7/EGFRvIII cells. Expression of EGFRvIII increases the spectrum and potency of ligand-mediated proliferation *in vitro*.

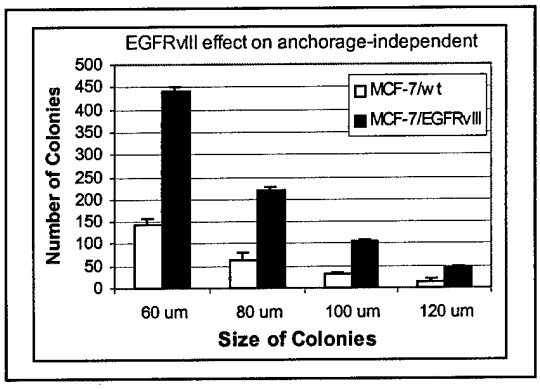


Figure 4. Growth effects of EGFRvIII on MCF-7 cells. Expression of the EGFRvIII in MCF-7 cells induces colony formation, independent of colony size. Anchorage-independent growth assays: A bottom layer of 0.1 ml IMEM containing 0.6% agar and 10% FCS was prepared in 35mm tissue culture dishes. After the bottom layer solidified, cells (10,000 per dish) were added in a 0.8 ml top layer, containing 0.4% Bacto Agar, and 5% FCS. All samples were prepared in triplicate. The cells were incubated for approximately 12 days at 37°C. Colonies larger than 60μm, 80μm, 100μm, and 120μm were counted by a cell colony counter.

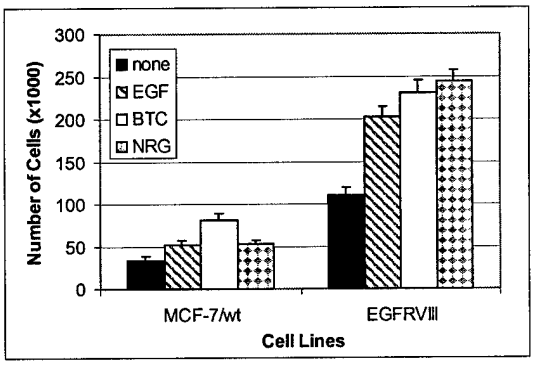


Figure 5. Expression of EGFRvIII in MCF-7 cells increases the spectrum and potency of EGF-like ligands-mediated proliferation in an anchorage-dependent assay. Cells were plated on 24 well plates in IMEM with 1% of FBS and untreated (control) or treated with 100 ng/ml of EGF, BTC or neuregulin (NRG). Cells were then counted on day 2, 4 and 7. The histogram represents the results from day 7. All samples were prepared triplicates.

Conclusion: These results provide the first evidence that EGFRvIII play a pivotal role in human breast cancer progression.

Future Directions:

1) We will continue to analyze the IHC data to correlate it with clinicopathological prognostic factors.
2) To evaluate the tumorigenicity of MCF-7/EGFRvIII *in vivo*.
3) To generate the specific ribozyme targeted to EGFRvIII and determine the EGFRvIII ribozyme intracellular efficacy and efficiency in 32D cell model system.

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APPENDICES

ILLUSTRATION

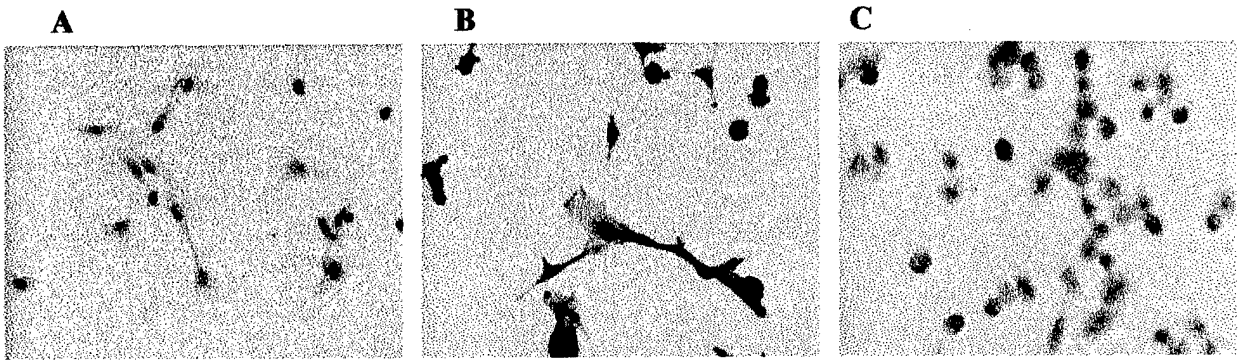

Figure 1. Illustration of EGFRvIII antibody specificity by immunohistochemical analysis with anti-EGFRvIII antibody and counterstained with hematoxylin for viewing negative stained cells (Blue). A) EGFRvIII antibody does not recognize wild-type EGFR in EGFR tansfected NIH3T3 cells. B) EGFRvIII antibody only recognize EGFRvIII in EGFRvIII transfected NIH3T3 cells (Brown). C) EGFRvIII antibody does not recognize endogenous wild-type EGFR in MDA-MB-468, which expresses high levels of EGFR.

APPENDICES

ILLUSTRATION

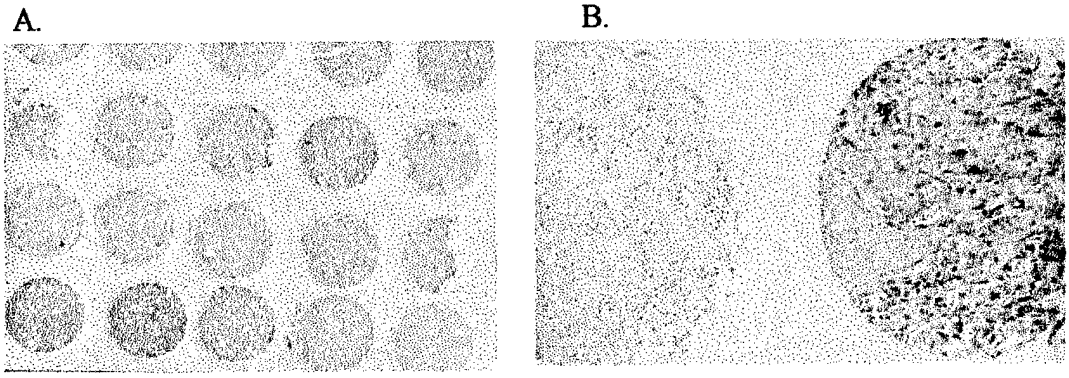

Figure 2 Illustrates the immunohistochemical analysis on a tissue microarray with anti-EGFRvIII antibody and counterstained with hematoxylin for viewing negative stained cells (Blue). EGFRvIII positive stained cells shows in brown color. A and B illustrate the breast carcinoma tissue microarray under different magnification.