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13. ABSTRACT *(Maximum 200 words)*

This proposal seeks to evaluate the frequency of *FGF8* overexpression in mammary carcinogenesis. We examined the frequency of *FGF8* expression in human breast cancer cell lines and fresh-frozen pathological specimens by reverse transcription-polymerase chain reaction (RT-PCR) methods. Total RNA was prepared from human breast cancer cell lines and pathological specimens and converted into cDNA by reverse transcription. The resulting cDNAs were analyzed by PCR and gel electrophoresis. We used primers specific to human *FGF8* that did not amplify mouse *Fgf8*. We observed expression of *FGF8* in 8 of 24 human breast cancer cell lines, 2 of 16 lymph nodes containing metastatic breast cancer, and 10/30 primary human breast cancer specimens. Under the conditions employed, normal mammary tissue and lymph nodes do not have detectable expression of *FGF8*. Attempts to examine archival paraffin-embedded tissue for FGF-8 protein by immunohistochemical methods was not successful because of technical problems with the immunological reagents used. In summary, approximately 30% of human breast cancers overexpress *FGF8*. As MMTV-FGF8b transgenic mice have a mammary cancer phenotype, the observed overexpression of *FGF8* likely contributes to the pathogenesis of cancer in those overexpressed cases.

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Introduction:

Overexpression of FGF-8 has been identified as a causative factor in mouse mammary tumors, using transgenic mouse models. The purpose of this work was to identify whether overexpression of FGF-8 occurred in human breast cancer, and if it occurred, what the prevalence of overexpression of FGF-8 in human breast cancer. Both RT-PCR analysis of frozen tumor specimens and immunohistochemical analysis of paraffin-embedded specimens were examined.

Body:

***Task 1.* RT-PCR analysis of FGF-8 expression in human breast cancer samples of primary and metastatic (lymph node) human breast cancer (months 1-12)**

We prepared total RNA by guanidinium isothiocyanate/acid-phenol methods [Chomczynski, 1987 #191]. Total RNA was converted to cDNA by reverse transcription with oligo (dT) primers, and the resulting cDNAs were analyzed for *FGF8* expression by PCR. We designed PCR primers based on the sequence of the human gene (1). The forward primer is in exon 2 and the reverse primer is in exon 3. These primers are separated by almost 1 kilobase in the human *FGF8* gene, and hence do not amplify genomic DNA under the PCR conditions employed. Additionally, these primers are specific for human *FGF8* cDNA, and do not amplify mouse *Fgf8* cDNA under the conditions employed. These primers will lead to the amplification of a 243-bp fragment from any of the human cDNAs encoding a FGF-8 isoform. Southern blotting of the resulting gels, with a human *FGF8* exon 2-containing probe (BK 1.0, (1)) will confirm that the observed 243-bp fragment is due to amplification of the *FGF8* cDNA from the cell lines. These methods were successful in amplifying *FGF8* cDNA derived from RNA from some human breast cancer cell lines (Fig. 1). RNA from normal mammary gland did not express *FGF8* (Fig. 1). The primers used were specific for human *FGF8*, since they did not amplify mouse *Fgf8*, which is expressed in E10.5 day mouse embryos (2). RNA in all samples were intact and able to be amplified by PCR primers specific for rat, mouse or human GAPDH (Fig. 1). We have observed *FGF8* expression by RT-PCR analysis in 8 of 24 human breast cancer cell lines examined to date.

Since some human breast cancer cell lines express *FGF8*, we extended our analyses to primary human breast cancers. Pathologic specimens were obtained and total RNA prepared and analyzed as above for *FGF8* expression. The human breast cancer material was identified by unique patient numbers, and the identity of the patients was confidential. Human breast cancers of infiltrating ductal and infiltrating lobular histology were analyzed to determine if *FGF8* expression correlated with a particular histology of breast cancer. Using these techniques, we have determined that 2 of 16 lymph node samples containing metastatic breast cancer express *FGF8*, while normal lymph nodes do not (Fig. 2). Additionally, 10 of 30 primary breast cancer

specimens expressed *FGF8* by RT-PCR analyses. Both ductal (9/26) and lobular (1/4) histology specimens were found to express *FGF8*. These results demonstrate that approximately 30% of primary breast cancer specimens express *FGF8*. Similar results have been published recently by others (3).

Task 2. Immunohistochemical analysis of FGF-8 expression in human breast cancer samples (months 1-12)

Since there are limited frozen tissue resources available, we sought to examine fixed, paraffin-embedded, pathological sections by immunohistochemical methods. We have a rabbit anti-FGF-8 polyclonal antibody that recognizes all FGF-8 isoforms (Fig. 3), and has been used in immunohistochemical staining of fixed cell lines (Fig. 4) (4). ABC detection kits with biotinylated secondary antibody (anti-rabbit IgG) and avidin-linked horseradish peroxidase were used to detect a diaminobenzidine tetrachloride substrate. Despite the ability of these immunological reagents to detect FGF-8 in western blots of human breast cancer cell lines (Fig. 5), we were unable to find appropriate conditions that allowed specific FGF-8 detection in paraffin-embedded tissues. Variables manipulated included the concentration of the primary rabbit anti-FGF-8 polyclonal antisera (0.01-0.5 μg/ml), affinity purification of the primary antisera, the concentration of the secondary antibody (1:1000-1:20,000 dilutions), and the use of different manufacturer's kits (Vector Labs, Burlington, CA; and Santa Cruz Biotechnology, Inc., Santa Cruz, CA). At higher concentrations of antisera, the negative controls were positive. At lower concentrations, the positive controls were negative. We also purchased a monoclonal anti-FGF8 antibody from R&D Systems ands attempted to perform the experiments, with the same results. In conclusion, we were unable to generate any conclusive results regarding FGF-8 expression in archival paraffin-embedded breast cancer specimens.

Key Research Accomplishments:

- Although a small sample size studied, overexpression of FGF-8 is relatively common finding in human breast cancer (approximately 30%)

Reportable Outcomes:

Abstracts:

1. MacArthur CA, Daphna-Iken D, Lawshé A, Ornitz DM: FGF-8 Expression In Breast Oncogenesis. Gordon Research Conference on Peptide Growth Factors, 1998.

Funding Applied for Based on Work Supported by this Award:

1. USAMRMC CDA for 1999, entitled, "Mammary Cancer Progression in MMTV-FGF8b Transgenic Mice."

2. NIH RO1 for 1999, entitled, "FGF8-induced mammary tumorigenesis."

Conclusions:

Results of our work and published results from England (3) demonstrate that overexpression of FGF8 is found in 30% of human breast cancer. Overexpression of FGF8 in human breast cancer is more common than loss of function mutations in BRCA1 or BRCA2, and is as common as overexpression of the her2/neu/erbB2/EGFR2 receptor. Given the frequency of FGF8 overexpression in human breast cancer, further effort should be placed on developing immunological reagents to FGF8 that will adequately assess the archival breast cancer specimens, allowing confirmation of our findings in a larger study.

If a larger study demonstrates that FGF8 overexpression is commonly seen in human breast cancer specimens, then efforts to disrupt the signaling pathways that FGF8 invokes would be warranted. The use of an existing MMTV-FGF8b transgenic mouse line that has a mammary carcinoma phenotype would be useful to identify potential contributing factors in a presumably multistep tumorigenic pathway driven by FGF8 overexpression.

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1. Gemel, J., Gorry, M., Ehrlich, G. D., and MacArthur, C. A. Structure and sequence of human *FGF8*. Genomics, 35: 253-257, 1996.

2. Heikinheimo, M., Lawshé, A., Shackleford, G. M., Wilson, D. B., and MacArthur, C. A. *Fgf-8* expression in the post-gastrulation mouse suggests roles in the development of the face, limbs and central nervous system. Mech. Dev., 48: 129-138, 1994.

3. Marsh, S. K., Bansal, G. S., Zammit, C., Barnard, R., Coope, R., Roberts-Clarke, D., Gomm, J. J., Coombes, R. C., and Johnston, C. L. Increased expression of fibroblast growth factor 8 in human breast cancer. Oncogene, 18: 1053-1060, 1999.

4. MacArthur, C. A., Lawshé, A., Shankar, D. B., Heikinheimo, M., and Shackleford, G. M. FGF-8 isoforms differ in NIH 3T3 cell transforming potential. Cell Growth Diff., 6: 817-825, 1995.

Appendices:

Figures:

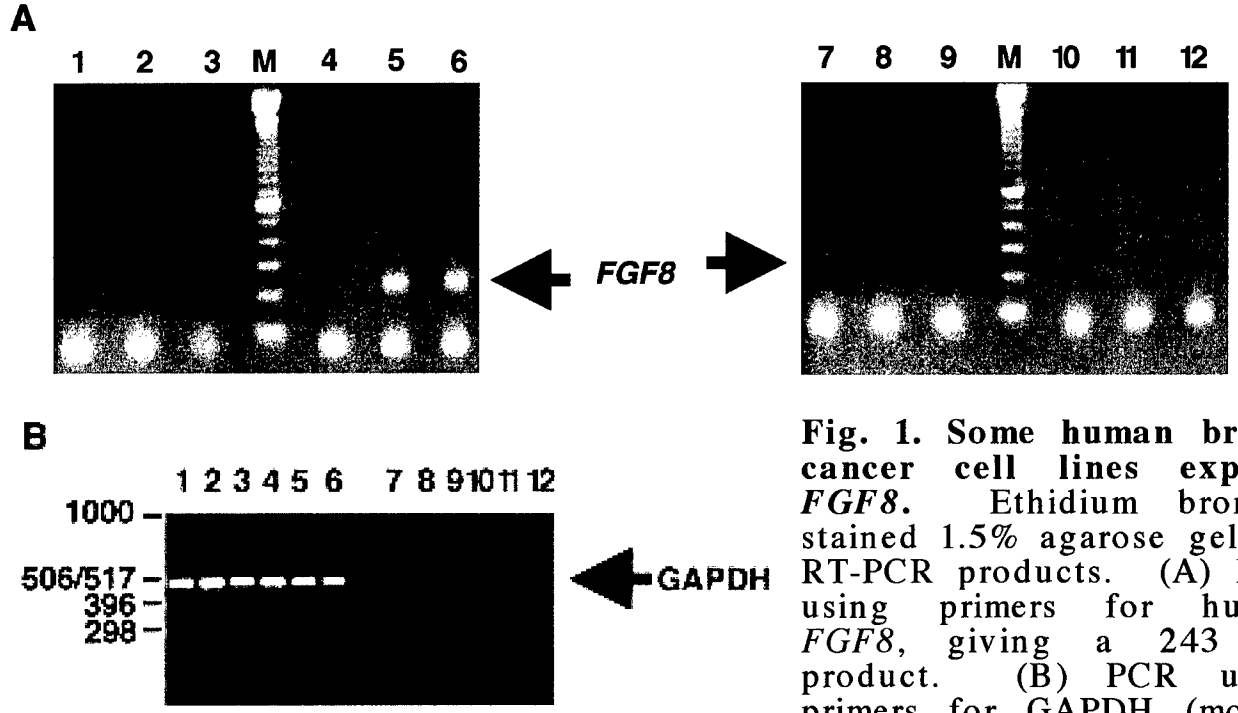

Fig. 1. Some human breast cancer cell lines express *FGF8*. Ethidium bromide stained 1.5% agarose gels of RT-PCR products. (A) PCR using primers for human *FGF8*, giving a 243 bp product. (B) PCR using primers for GAPDH (mouse, rat, and human, Clontech), giving a 450 bp product. Lanes: M, 100 bp ladder (Gibco-BRL); 1, E10.5 mouse embryo cDNA; 2, normal human mammary gland cDNA; 3, ATCC HTB-20 (BT-474) cDNA; 4, ATCC HTB-30 (SK-BR-3) cDNA; 5, ATCC HTB-122 (BT-549) cDNA; 6, ATCC HTB-126 (HS 578T) cDNA; 7, E10.5 mouse embryo no RT control; 8, normal human mammary gland no RT control; 9, ATCC HTB-20 (BT-474) no RT control; 10, ATCC HTB-30 (SK-BR-3) no RT control; 11, ATCC HTB-122 (BT-549) no RT control; 12, ATCC HTB-126 (HS 578T) no RT control. The *FGF8* PCR conditions are 95°C for 3 minutes, then 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. The PCR conditions for GAPDH are 95°C for 3 minutes, then 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 60 seconds. The *FGF8* PCR primers are specific for human and **not** mouse cDNA, as indicated by the failure of the primers to amplify cDNA from E10.5 mouse embryos (Lane 1). Arrows indicate the location of the *FGF8* (A) and GAPDH (B) amplified fragments.

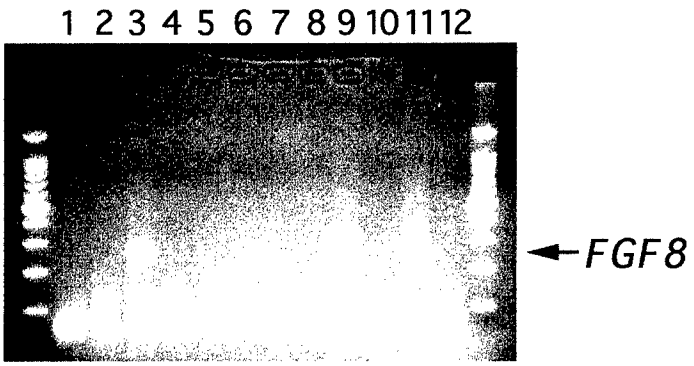

Fig. 2. *FGF8* expressed in some metastatic human breast cancers. Ethidium bromide stained 1.5% agarose gels of RT-PCR products. RNA was prepared from axillary lymph nodes containing metastatic human breast cancer (lanes 2-9), axillary lymph node not containing metastatic cancer (lane 1), normal human mammary tissue (lane 12), or from the HTB-126 human breast cancer cell line (lane 11). The RNA was analyzed by RT-PCR as in Fig. 5. The outer two lanes contain 100 bp ladder marker fragments. The 243 bp *FGF8* fragment is indicated by an arrow at right.

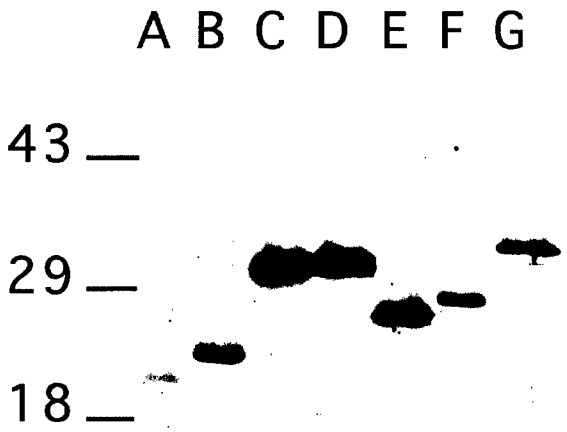

Fig. 3. Polyclonal antibody recognizes all recombinant FGF-8 isoforms. Western blot of recombinant c-His_6-recombinant FGF-8 isoforms. SDS-PAGE in 12% polyacrylamide gel, followed by electrophoretic transfer and incubation with rabbit anti-FGF-8 primary antibody. Secondary antibody is donkey anti-rabbit IgG, and image generated by ECL (Amersham). Letters refer to specific FGF-8 isoforms (5), and numbers at left indicate molecular weight standards in kilodaltons.

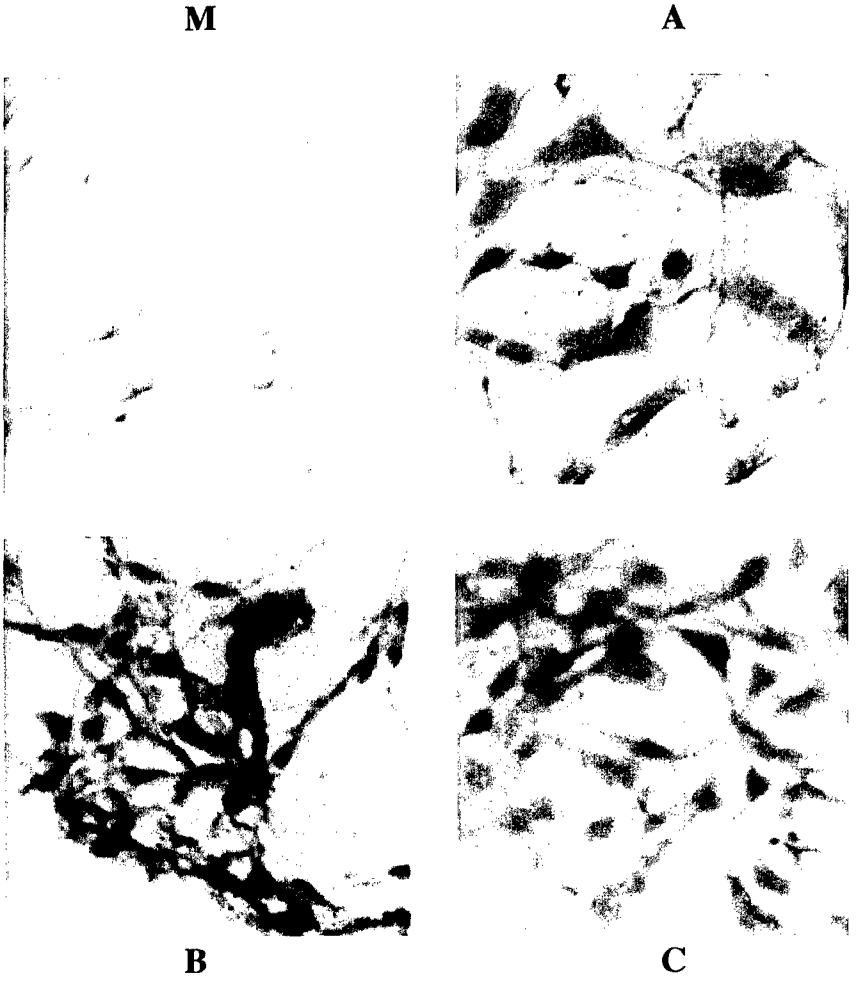

Fig. 4. Polyclonal anti-FGF-8 recognizes protein produced by transfected NIH 3T3 cells. Cells were transfected in a stable fashion with vector only (M), or vector containing FGF-8a (A), FGF-8b (B), or FGF-8c (C) cDNA (4). The cells were fixed in 4% paraformaldehyde. The primary antibody was the anti-FGF-8 used in the Western blot of Fig. 6, and the secondary antibody was an avidin-conjugated donkey anti-rabbit IgG. Detection was with biotin-conjugated-horseradish peroxidase activity on a diaminobenzidine substrate.

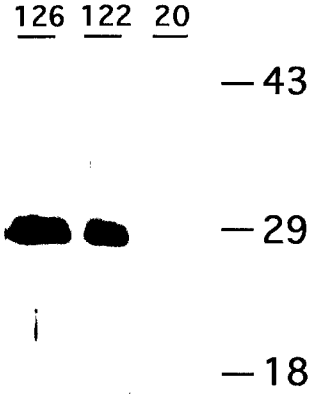

Fig. 5. Polyclonal anti-FGF-8 recognizes FGF8b isoform from HTB-126 and HTB-122 human breast cancer cell lines. Conditioned media from cells grown to confluence were concentrated by heparin-agarose chromatography and subjected to 10% SDS-PAGE, followed by western blotting with anti-FGF8 antisera as the primary antibody. A horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody was used with ECL detection.

Abstract Reprint

FGF-8 Expression In Breast Oncogenesis. Craig A. MacArthur *, Dorit Daphna-Iken*, Avril Lawshé*, and David M. Ornitz+. Departments of Pediatrics and Pathology*, and Molecular Biology and Pharmacology+, Washington University School of Medicine, St. Louis, MO 63110. Supported by NIH-R01-CA-70106 (CAM) and USAMRMC DAMD17-98-1-8264 (CAM).

Prior studies have identified murine *Fgf8* as a likely proto-oncogene in mouse mammary tumorigenesis. We now report on the generation of MMTV-FGF-8b transgenic mice that express the FGF-8b isoform from a minigene driven by the mouse mammary tumor virus long terminal repeat. Male and female MMTV-FGF-8b mice are viable and fertile. RNA for FGF-8b is detected in RNA from mammary gland and salivary gland tissues of transgenic mice by northern blot analysis. Nearly 80% of the transgenic females have breast tumors by 12 months of age, while non-transgenic littermates have not developed any tumors. These results demonstrate that FGF-8b production in the mammary glands contributes to oncogenesis.

We analyzed *FGF8* expression in human breast cancer cell lines by reverse transcription-polymerase chain reaction (RT-PCR) techniques. *FGF8* was not detected in RNA from normal human mammary tissues, but 8 of 23 human breast cancer cell lines and 2/16 breast cancer specimens tested expressed *FGF8*. These results indicate that *FGF8* is expressed in some human breast cancers, and suggest that *FGF8* may be play a role in some cases of human mammary oncogenesis.

Final Report Data:

Abstract: see above

Personnel: Craig A. MacArthur, M.D., Ph.D.

Avril Lawshé