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GRANT NO: DAMD17-93-J-3005

TITLE: Insulin-Like Growth Factor II as a Prognostic Indicator in Breast Cancer

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CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057

REPORT DATE: January 1996

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 1996	Final (15 Dec 92 - 14 Dec 95)

4. TITLE AND SUBTITLE
Insulin-Like Growth Factor II as a Prognostic Indicator in Breast Cancer

5. FUNDING NUMBERS
DAMD17-93-J-3005

6. AUTHOR(S)
Kevin J. Cullen, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20057

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The objective of this proposal is to determine the prognostic role of expression of Insulin-Like Growth factor II in breast cancer. IGF-II is a potent mitogen for breast tumor epithelium, and is expressed in the stroma of invasive bresat cancers. In this study, we analyzed expression of IGF-II mRNA and protein in two separate series of patients with invasive breast cancer, and compared the results with other clinical parameters, prognostic indicators and patient outcome.

IGF-II mRNA and protein expression were easily detected in the majority of the 193 cases that were informative and had complete clinical follow up. While analysis of the cases from the two data sets individually showed that IGF-II expression was associated with clinical outcome depending on hormone receptor status. However, IGF-II expression by itself was not a prognostic indicator, and the relationship with hormone receptor status was lost when the separate data sets were analyzed together.

We were unable to prove our initial hypothesis that IGF-II expression would be associated with poor prognosis in invasive breast cancer.

14. SUBJECT TERMS
breast cancer

15. NUMBER OF PAGES
21

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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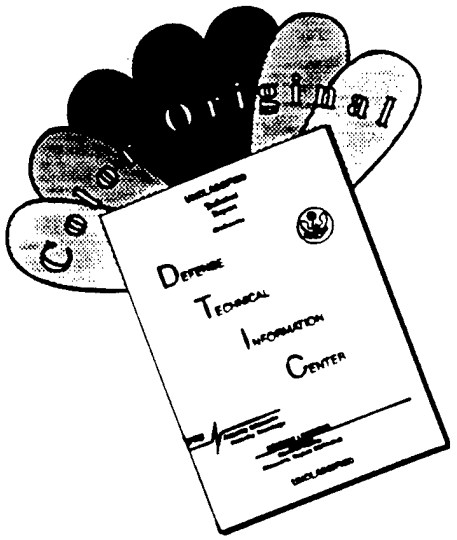

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PI - Signature Date

Kevin J. Cullen, M.D.
Grant DAMD17-93-J-3005
Final Report

Table of Contents

Introduction	Pages 5-7
Body	Pages 7-18
References	Pages 19-21

Insulin-like growth factor II as a prognostic variable in breast cancer.
Kevin J. Cullen, PI
Final Report

Introduction

The objective of this project is to examine the role of Insulin-like growth factor II (IGF-II) in interactions between stromal and epithelial cells in human breast cancer. The fundamental hypothesis for this work is that IGF-II produced by breast tumor stroma is an essential and active participant in the process of malignant tumor progression. While the coordinated growth of stromal and epithelial elements is necessary for tumor survival, the specifics of the growth enhancing interactions between these cell types is not clearly defined. We hope that these studies will provide a better understanding of the growth promoting relationships between breast tumor stromal and epithelial cells and may help to identify new therapeutic targets for more rational and effective breast cancer treatment.

In the last decade, a large body of experimental evidence has emerged regarding the importance of peptide growth factors in the regulation of breast cancer and other malignancies.[1,2] Among the various families of peptide growth factors, the insulin-like growth factors, IGF-I and IGF-II, are part of a group of ligands, receptors and binding proteins which have a significant role in normal development and growth. IGF-I mediates the effects of growth hormone, while IGF-II appears to be an important fetal growth factor with unknown function in adult life.[3,4,5]

Pre-clinical data

Several groups, including our own, have previously shown that IGF-I and IGF-II are potent mitogens for breast cancer epithelial cells. [6,7,8,9] We have shown that while surgical breast tumor specimens express both IGF-I and IGF-II on an mRNA level.[8,10] However, studies of cultured breast tumor epithelial cells as well as *in situ* hybridization studies suggested that the IGF expression was primarily originating in the stroma of the breast rather than in tumor epithelium. IGF-II message appeared to originate in tumor stroma, while IGF-I message arose from stroma in normal breast.[8,10,11]

Since these data suggested that tumor IGF production originates in stroma rather than epithelium, we examined primary cultures of breast fibroblasts derived from benign and malignant lesions for expression of various growth factors, including IGF-I and IGF-II.[12] The results are summarized on Table 1. The most significant finding from this study is that the majority of fibroblasts derived from malignant lesions expressed IGF-II, while the majority of fibroblasts from benign lesions expressed IGF-I. No tumor specific differences in gene expression were observed with any of the other growth factors examined. This further supports the notion of a paracrine growth promoting role

Fibroblast	IGF I	IGF II	PDGF A	PDGF B	TGF α	TGF ß1	basic FGF	FGF 5	Source
191	+	-	+	-	-	+	+	+	Red. Mammo.
365	+	-	+	-	-	+	+	+	Red. Mammo.
429	+	-	ND	ND	ND	ND	ND	ND	Red. Mammo.
446	ND	-	ND	ND	ND	ND	ND	ND	Red. Mammo.
879	+	-	ND	ND	ND	ND	+	+	Fibroadenoma
987	+	-	ND	ND	ND	ND	+	ND	Fibroadenoma
999	-	-	+	-	ND	ND	ND	ND	Fibroadenoma
1034	+	-	+	-	-	+	+	+	Fibroadenoma
1097	+	+	ND	ND	ND	ND	+	ND	Red. Mammo.
197	ND	ND	ND	ND	ND	ND	+	+	Tumor
406	+	-	ND	ND	ND	ND	+	+	Tumor
559	-	+	+	-	-	+	+	+	Tumor
788	-	+	+	-	-	+	ND	ND	Tumor
906	-	+	+	-	-	+	+	ND	Tumor
926	-	-	ND	ND	-	+	+	+	Tumor
971	-	+	ND	ND	ND	ND	ND	ND	Tumor
974	-	-	+	-	-	+	+	+	Tumor
977	-	+	+	-	-	+	ND	ND	Tumor
995	-	-	+	-	-	+	+	+	Tumor

Table 1. Growth factor mRNA expression by breast fibroblast cell lines.
Key: IGF-I = Insulin-like growth factor I; IGF-II = Insulin-like growth factor II; PDGF A = Platelet derived growth factor A chain; PDGF B = Platelet derived growth factor B chain; TGF α - Transforming growth factor alpha; TGF-ß1 = Transforming growth factor beta 1; basic FGF = basic Fibroblast Growth Factor; FGF-5 = Fibroblast growth factor 5; ND = not done.

for the insulin-like growth factors in breast lesions, and suggests that IGF-II may be the more important growth promoter in malignant lesions.

Since our results suggested that IGF-II might be serving as a paracrine growth stimulant in breast cancer, we asked whether overexpression of IGF-II by a breast tumor epithelial cell line could confer phenotypic changes associated with malignant progression in breast cancer, such as loss of estrogen dependence for growth. We infected a well differentiated, estrogen-dependent breast tumor epithelial cell line (MCF-7) with a retroviral vector containing the coding sequence for the IGF-II pre-prohormone along with a constitutive CMV promoter sequence. MCF-7 cells do not normally express IGF-I or IGF-II.[7]

All IGF-II overexpressing clones showed marked phenotypic changes associated with malignant progression i.e. growth on plastic in the complete absence of estrogen, cloning in soft agar without estrogen. Wild-type MCF-7 cells and control cells infected with an irrelevant DNA sequence showed none of these properties.[13]

Finally, we have developed co-culture models which demonstrate that soluble factors produced by tumor derived fibroblasts are markedly mitogenic for breast tumor epithelial cells, and that these mitogenic effects can be inhibited by an antibody which blocks the IGF-I receptor,[14] implying stromal IGF-II is the source of the mitogenic signal.

The present study

Recent work in other centers has suggested that biochemical markers such as c-erb-b2 and cathepsin D can be significant independent prognostic indicators in breast cancer[15,16] with great potential clinical utility. In light of the laboratory data discussed above, it is therefore reasonable to ask if the same applies to IGF-II.

Body

Materials and Methods

IGF-II *in situ hybridization*

The *in situ* hybridization protocol was modified from the procedure of Bondy, *et al.*[17]

IGF-II Riboprobe : The IGF-II cDNA was kindly provided by Dr.Graeme Bell (Howard Hughes Institute, Chicago, IL). An 833 base pair *PstI* fragment was sub cloned into a pGEM4 vector (Promega,Madison,WI). The vector was linearized with *RSAI*, providing a template for an antisense riboprobe protecting a 336 base pair mRNA fragment. Labeled antisense RNA was transcribed using T7 polymerase according to the manufacturer(Promega). Labeled sense RNA transcribed using SP6 polymerase was utilized for the negative control. Probes were double labelled with ^{35}S-UTP and ^{35}S-CTP(Amersham- Arlington Heights, IL) to increase specific activity.

In-Situ Hybridization : 5 micron sections were cut from paraffin blocks and mounted on sialinized slides. The sections were deparaffinized and rehydrated through a progressive ethanol series. The sections were digested with 1 mcg/ml proteinase K in 100 mM TRIS-50 mM EDTA for 30 minutes a 37 C. After washing in DEPC ddH2O, the sections were acetylated for 10 minutes at room temperature(RT) in fresh acetic anhydride, diluted 1:400 in 0.1 triethanolamine, pH:8.0. The sections were then dehydrated in an ascending ethanol serie and air dried for 1 hour. The tissues were hybridized with 3X10^{7}cpm/ml labeled antisense RNA in 50% formamide, 10% dextran sulfate, 50mM Tris pH:8, 2.5mM EDTA, 2.5% Denhardt's, 0.2 M NaCl, and 250mcg/ml yeast tRNA. The slides were coversliped and placed in humid chamber sealed with paraffin at 55°C overnight. After hybridization, the slides were placed in a shaking bath

in 4X SSC at room temperature until the coverslips floated off. The slides were then washed in four changes of 4X SSC, five minutes each. Next, the tissues were placed in 50% formamide for 10 minutes at 60°C. The slides were then dipped in 2XSSC, with 2ml 1M DTT, followed by incubation with RNase A 20 mcg/ml in 0.5M NaCl, Tris 10 mM pH8, EDTA 1mM, 2ml DTT. The final washes consisted of decreasing concentrations of SSC (2X, 1X, 0.5X for 5 minutes each at RT, 0.1X for 15 minutes at 50°C and 0.1X to cool). Sections were dehydrated through an ascending ethanol series and the air dried for 1 hour. The sections sections were then exposed to Hyper film-beta max (Amersham)for 3 days to check the completeness of washing. If further washing was necessary the sections were rehydrated and rewashed. The sections were then exposed to NTB-2 emulsion (Eastman Kodak,Rochester,NY) for three weeks. The emulsion was developed with D19 solution for 4 minutes at 15°C. Developing was stopped with in a solution of 200 ml ddH2O with 1.33 ml glacial acetic acid, and the slides were fixed in 30% Sodium Thiosulfate for 3 minutes. The slides were washed and placed in ddH2O for 10 minutes. The sections were then stained with hematoxylin and eosin, dehydrated, and coverslipped.

A time course study of the hybridization reaction showed maximum signal after overnight incubation at 55°C. Background signal increased significantly after this period without increase in specific hybridization signal. Riboprobe synthesized in the sense direction was used as a negative control in all assays. All control samples were noted to have some IGF-II mRNA detected in the smooth muscle walls of blood vessels. This served as an intrasample control. Any breast tumor specimens which did not show some specific signal in blood vessel walls was excluded from subsequent analysis. For each sample, 10 high power fields within the tumor were analyzed, and specific clusters of silver grains were counted, corresponding to a cell which expressed IGF-II mRNA. Based on these counts, the tumors were divided into tumors were divided in four groups based on the level of IGF-II mRNA signal: 0 (absent), + (low), ++ (intermediate) and +++ (high). For some of the subsequent statistical analysis, the negative and + scores were grouped together, as were the ++ and +++ scores.

IGF II Immunohistochemistry

Anti IGF-II antibody: We used a polyclonal rabbit antibody raised against intact human recombinant IGF-II peptide, generously provided by Dr. Judith Heisserman (Lilly Research Labs, Eli Lilly Corporation, Indianapolis, IN). Antibody specificty was confirmed by ELISA, wich showed less than 1O% cross reactivity with recombinant IGF-I and no reactivity with recombinant epidermal growth factor (EGF). Additionally, the antibody was affinity purified using intact recombinant peptide. Affinity purified antibody showed identical staining and reactivity in the ELISA as that obtained with polyclonal antibody.

Immunohistochemical staining: Paraffin sections were deparaffinized and rehydrated

through progressive ethanol series. The slides were washed in PBS and incubated with normal goat serum for 20 minutes at room temperature. The primary IGF-II antibody was diluted 1:2000 in PBS containig 1% BSA and 1% sodium azide and incubated on the tissue sections at 4^0C overnight. The slides were then rinsed twice in PBS for 3 min. each wash. The reaction was visualized with Biogenix multilink system (Biogenix - San Ramon, CA). The streptavidin/alkaline phosphatase linked secondary antibody was incubated for 20 minutes at room temperature. The labelled secondary antibody was detected with the chromagen fast red.

Results

To date, we have processed slightly over 300 surgical specimens for both IGF-II *in situ* hybridization and immunohistochemistry. 60 of these cases were discarded because of lack of positive signal detection by control in *situ* hybridization and or immunohistochemistry, indicating loss of detectable antigen or mRNA. The remaining 240 cases include a separate series of 80 cases from a collaboration with Dr. Claudio Giani, of the University of Pisa - Pisa, Italy. The remaining 160 cases were from Georgetown University. All cases have been pathologically scored. Complete clinical comparative data are available for all the cases from the University of Pisa, and for 113 of the cases from Georgetown University.

IGF-II *in situ* and immunohistochemistry scores for the informative cases analyzed so far are as follows:

IGF-II Score	***In situ* hybridization**	**Immunohistochemistry**
Negative	53 (23%)	39 (16%)
Low (+)	77 (33%)	56 (23%)
Moderate (++)	68 (29%)	103 (43%)
High (+++)	36 (15%)	42 (18%)
Total	234	240

Table 2. IGF-II *in situ* hybridization and immunohistochemistry scores for 240 consecutive breast cancer specimens. The distribution of both scores is similar, although the average score for immunohistochemistry is somewhat higher than for in situ hybridization, possibly reflecting a greater sensitivity for that technique.

For the purposes of this presentation, the two data sets from the University of Pisa and from Georgetown University were analyzed separately.

University of Pisa series - 80 cases

Nuclear Grade and lymph node involvement

The distribution of nuclear grade was as following: 18(22.8%) grade 1, 39 (49.4%) grade 2, 22 (27.8%) grade 3. Axillary lymph node involvement was found in 47 cases (59.5%)

ER and PR status

Thirty two (40.5%) out of 79 tumors were ER, PR positive (ER+PR+), 17 (21.5%) ER+PR-, 7 (8.9%) ER-PR+ and 23 (29.1%) ER-PR-. The nuclear immunostaining was located only in malignant epithelial cells (Fig.1). No ER or PR staining presence was seen in the stroma of the tumor.

Proliferating activity

High proliferating activity (> 10% immunoreactive tumor cells) was found in 24 of 77 cases (31.1%).

ras, c-erb-B2, p53

Thirty six (46.7%) of 77 tumors were positive for p21 protein, 20 (25.8%) for p185 and 12 (15.6%) for p53.

IGF-II in situ

Two tumors had no significant (0)IGF-II and 35 had low(+) IGF-II mRNA expression, 20 and 18 showed moderate(++) or high(+++) IGF-II mRNA content, respectively. Thus, the 0, + group included 37(49.4%) cases and the ++, +++ group comprised 38 cases (50.6%). The IGF-II mRNA signal was generally located in the stromal component of the tumor, usually tightly adjacent to the malignant epithelial cells. (Fig.2) Furthermore, higher IGF-II mRNA expression was present in the loose stroma rather than in dense (established) stroma.

IGF-II Immunohistochemistry

IGF-II protein was expressed in 57 out of 75 cases (76%) Fourteen(24.5%) showed slight (+), 31(54.3%) moderate(++) and 12 (21%) high (+++) IGF-II protein

expression. The pattern of protein expression detected by immunohistochemistry generally correlated well with IGF-II mRNA expression. Protein staining easily identified in the smooth muscle component of blood vessel walls. Inside the tumor, IGF-II protein was usually detected in stromal fibroblasts and in small blood vessels.(Fig.4D, Fig 4E)

IGF-II protein staining of tumor epithelial cells was seen in only three cases. In the most dramatic case, intense IGF-II staining was seen in cytoplasmic granules within the tumor (Fig 4H). IGF-II mRNA signal was strongly positive in that case, but was confined to stroma and vessels adjacent to the tumor epithelium. (Fig 4G). Interestingly, that patient had an equally dramatic clinical course, dying of disease within 6 months of diagnosis.

In two additional cases, a similar epithelial staining was observed, but only in scattered cells.

Outside the tumors, IGF-II protein was typically expressed in a linear pattern in myoepithelium surrounding both lobules and terminal ducts.(Fig.4C) Interestingly, in lobular hyperplasia a loss of linear IGF-II staining was detected with evidence of discontinuous staining pattern. Generally, IGF-II protein expression correlated well with mRNA expression with a concordance of 57/75 (76%). Only 1 out of 57 tumors with IGF-II immunostaining showed no IGF-II mRNA expresion.

Clinical correlation study

IGF-II in situ vs menopausal status : no statistical relation was found between IGF-II mRNA and menopausal status.

IGF-II in situ vs node involvement and tumor size: no relationship was found between IGF-II mRNA content and node status or number of metastatic nodes. Similarly no relationship was present with tumor size.

IGF-II in situ vs nuclear grade : no relationship was found between IGF-II expression and nuclear grades of the tumors.

IGF-II in situ vs ER and PR . Higher IGF-II mRNA was detected in ER+PR+ tumors (67.8%) than in the others ER/PR classes ($p<0.05$) (Tab.1). When ER and PR were separately examined, no relationship was found between ER and IGF-II expression. Opposite results were obtained for PR: twenty-four out of 35 (68.6%) PR+ tumors had high IGF-II expression and 11(31.4%) low or absent IGF-II mRNA content. In contrast, most tumors which expressed undetectable or low IGF-II levels (65%) were also PR-. The relationship between PR status and IGF-II expression was significant ($p<0.01$) (Tab.2). Furthermore, IGF-II mRNA content was directly related with both percentage values ($p=0.006$) and staining intensity ($p=:0.003$) of PR positive cells.

ER and PR vs stromal proliferation : Twenty-two of 36 (61.1%) tumors with marked stromal proliferation were ER+PR+. In contrast, ER+PR+ was detected in 10 out of 43 (23.2%) tumors with faint stromal proliferation. The relationship between ER, PR status and desmoplasia were significant ($p<0.01$). The separate analysis of ER and PR showed that this correlation was present only for PR ($p<0.01$). In fact, 28 out of 36 (77.8%) specimens with marked stromal proliferation were PR+ and 8 (22.2%) were PR-. Interestingly, the relation between PR and stromal proliferation was independent of IGF-II mRNA expression as shown by multi variate analysis. Finally, no relationship was found between desmoplasia and ER status.

IGF-II vs proliferating activity:: IGF-II mRNA expression was not related with proliferating activity assessed by Ki 67

IGF-II vs oncogene protein products: p53, p21 and p185 were not related to IGF-II mRNA expression

IGF-II and clinical outcome : Generally, IGF-II mRNA expression did not relate to patient's survival. However, the patients whose tumors had high IGF-II mRNA content showed a very poor prognosis in absence of ER or PR.(Figure 2) The relation between IGF-II expression and outcome in estrogen receptor patients was part of a Cox proportional hazards model analysis. The model included lymph node status, ER, IGF-II, p53 and the interaction between ER and IGF-II. Even after adjusting for p53 and lymph nodes, the effect of ER, IGF-2 and its interaction were each a significant addition to the model. ($p=0.03$). Stated another way, among IGF-II positive patients, ER negativity was associated with a relative risk of relapse of 9.8. (95% confidence interval 1.9-50). Among IGF-II negative patients, ER had no effect (RR=0.8. 95% confidence interval 0.3-2.5).

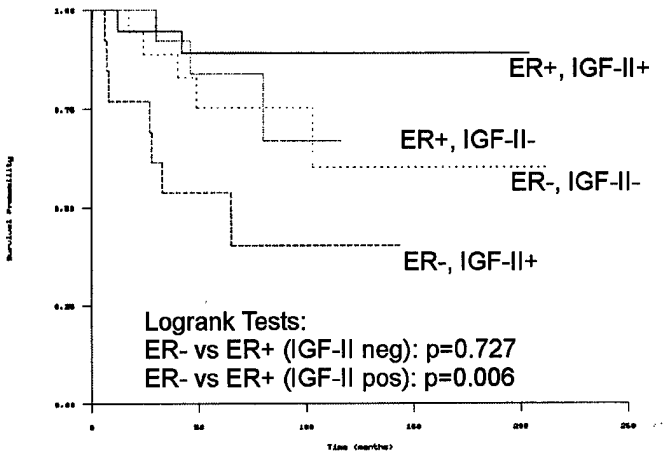


Figure 1. Overall survival in patients with infiltrating ductal carcinoma as a function of IGF-II expression. (University of Pisa patients) In this series of 75 patients with complete follow up data and informative slides, IGF-II

expression is associated with poor survival in the subset of patients who are estrogen receptor negative. When IGF-II positive, ER negative patients (bottom line) are compared with IGF-II positive, ER positive patients (top line), survival is cut nearly in half. (p=0.006).

Georgetown University series - 113 cases

Among the 113 informative cases from Georgetown University, the distribution of IGF-II scores determined by in situ hybridization was similar to that seen in the series from the University of Pisa. 2 cases (2%) showed absent IGF-II expression. 48 cases (42%) had low IGF-II message, 36 cases (33%) had intermediate expression and 27 cases (24%) had high IGF-II message. In this data set the low and absent expressors grouped together accounted for 44% of cases, while the intermediate and high expressors together accounted for the remaining 56%.

No significant correlation was seen between IGF-II message level and age, tumor size, lymph node status, menopausal status, or s-phase fraction. As with the Pisa data set, there was a significant statistical correlation between IGF-II expression and estrogen receptor. However, the results in the two data sets were (suprisingly) virtually opposite. In the Georgetown patients, IGF-II expression was associated with a good prognosis, especially in the subset of ER- patients. This is the opposite of what was seen in the Pisa patients, where the same group of patients fared poorly.

The Kaplan-Meier curves for the Georgetown patients are shown in figure 2.

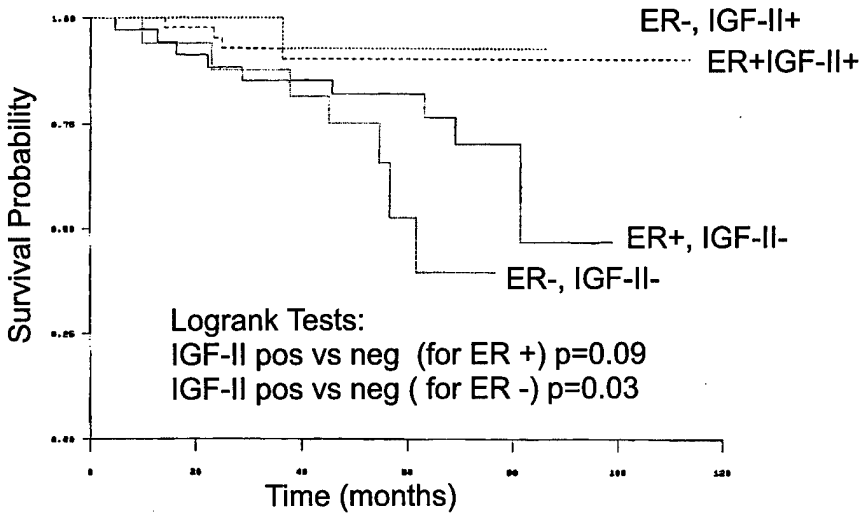


Figure 2. Overall survival in patients with infiltrating ductal carcinoma as a function of IGF-II expression. (Georgetown University patients) In this series of 113 patients with complete follow up data and informative slides,

IGF-II expression is associated with favorable survival in the subset of patients who are estrogen receptor negative. When IGF-II negative, ER negative patients (bottom line) are compared with IGF-II positive, ER negative patients (top line), survival is cut more than in half. (p=0.03).

The Kaplan-Meier results for the Georgetown patients are summarized below.

Situ: 0-4=neg/low, >4=high

Time to Relapse by IGF-II and ER:	Log-rank test:	Time to Relapse by IGF-II and PR:	Log-rank test:
Overall	p=0.75	Overall	p=0.11
Among ER positive	p=0.44	Among PR positive	p=0.14
Among ER negative	p=0.72	Among PR-negative	p=0.11
Overall Survival by IGF-II and ER:	Log-rank test:	Overall Survival by IGF-II and PR:	Log-rank test:
Overall	p=0.40	Overall	p=0.02
Among ER positive	p=0.26	Among PR positive	p=0.02
Among ER negative	p=0.47	Among PR negative	p=0.03

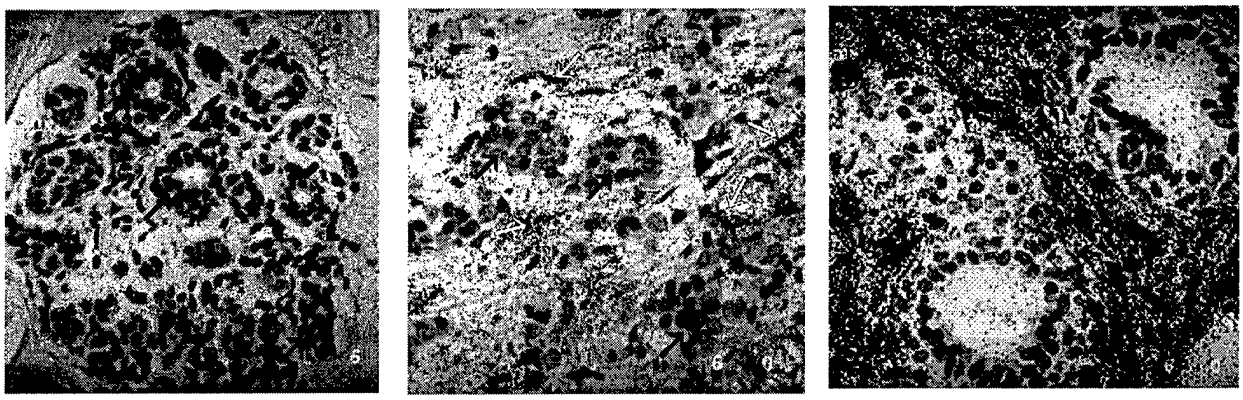
IHC: 0-3=neg/low, >3=high

Time to Relapse by IGF-II and ER:	Log-rank test:	Time to Relapse by IGF-II and PR:	Log-rank test:
Overall	p=0.26	Overall	p=0.009
Among ER positive	p=0.10	Among PR positive	p=0.84
Among ER negative	p=0.32	Among PR negative	p=0.004
Overall Survival by IGF-II and ER:	Log-rank test:	Overall Survival by IGF-II and PR:	Log-rank test:
Overall	p=0.01	Overall	p=0.0003
Among ER positive	p=0.09	Among PR positive	p=0.58
Among ER negative	p=0.03	Among PR negative	p=0.0007

Table 3. Kaplan-Meier summary for the interaction between ER/PR and IGF-II expression in the Georgetown University series.

IGF-II expression in tumor subtypes

As part of this study, we were interested in examining the effect of IGF-II expression on survival in patients with infiltrating ductal carcinoma. However, early on in the study, we noted that high levels of stromal IGF-II expression was noted in several histologic subtypes of breast cancer, particularly typical and atypical hyperplasia.



Normal Terminal Lobule | Infiltrating Cancer | Atypical Hyperplasia

Same Specimen

Figure 3. *In situ* hybridization demonstrating IGF-II mRNA expression in breast cancer and in atypical ductal hyperplasia in a patient with breast cancer. The two left panels are photographed from a single tumor section. The left hand panel shows an area of normal breast. There is no significant IGF-II message. The middle shows an area of infiltrating ductal carcinoma adjacent to the area of normal breast. Silver grains indicating IGF-II expression are seen throughout the stromal component (white arrows) of the cancer, but not in the tumor epithelium (black arrows). The right hand panel shows an area of atypical ductal hyperplasia in a separate case. Intense IGF-II mRNA expression in the stroma is indicated by the abundant silver grains. Ductal epithelium does not express IGF-II. Immunohistochemistry in each case showed a pattern and intensity of IGF-II protein staining very similar to the results seen here (not shown).

The finding shown in the right hand panel of figure 1 that IGF-II is markedly overexpressed in the stroma of atypical hyperplasia is striking. This has been a reproducible finding in over 8 cases of atypical ductal hyperplasia examined so far. All of these cases were in the setting of patients with known invasive carcinoma.

Several studies have demonstrated that atypical hyperplasia is associated with a 4-5 fold increased risk in the subsequent development of breast cancer.[18] In the setting of a positive family history, atypical hyperplasia indicates a breast cancer risk that is 8-10 fold above background.[19] It remains controversial whether atypical hyperplasia is simply a marker of increased risk for breast cancer, or whether it represents a non-obligate precursor of breast cancer. [20]

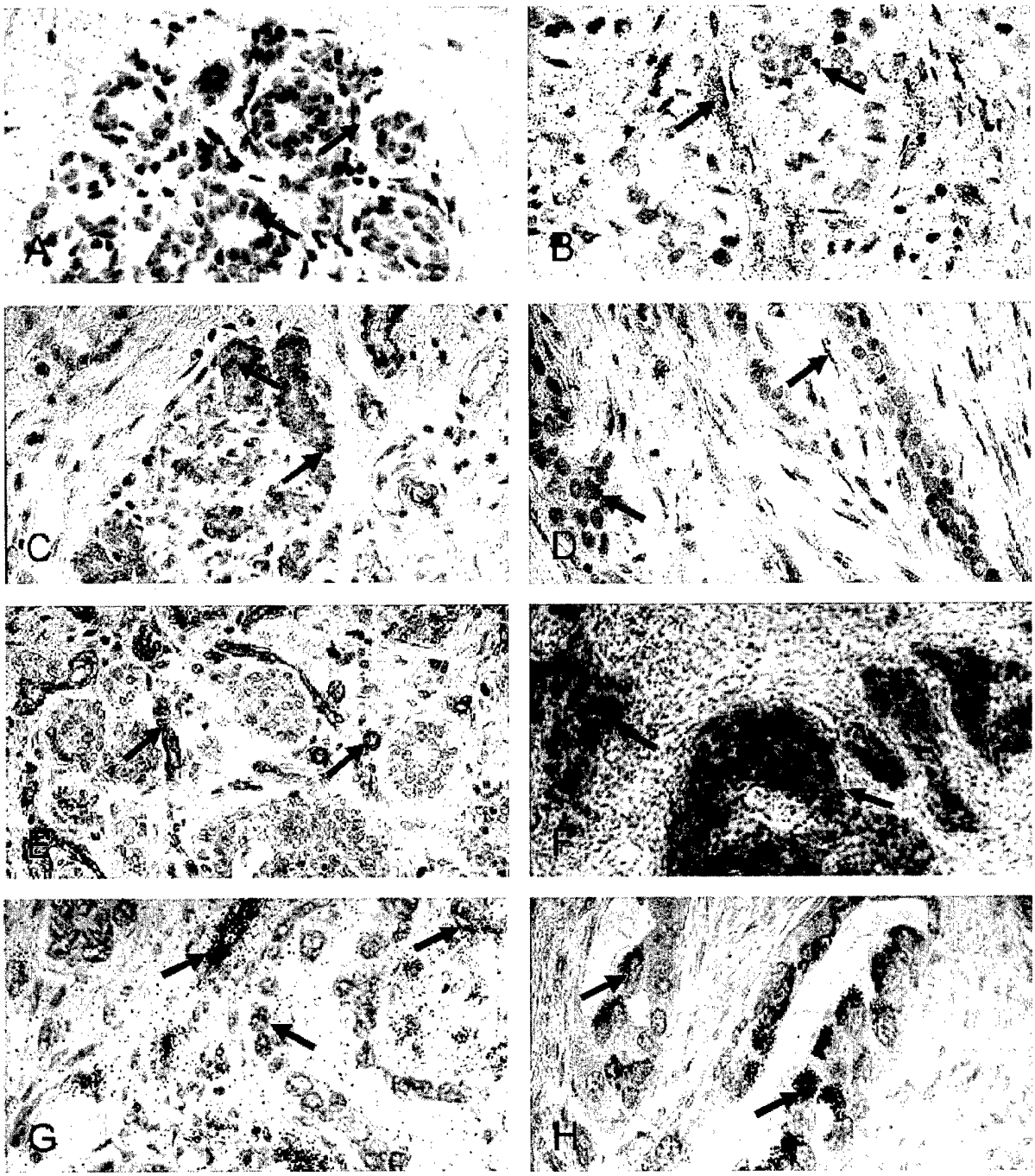


Figure 4. IGF-II mRNA and protein expression in normal and malignant breast tissue. A. IGF-II *in situ* hybridization in a terminal lobule of normal breast. No significant mRNA signal is detected either in breast epithelium (black arrow) or stroma (red arrow). B. IGF-II *in situ* hybridization in infiltrating ductal breast cancer. Abundant silver grains are seen in areas of stroma, indicating IGF-II mRNA expression by these cells, (red arrow) but not over tumor epithelial cells (black arrow). C. IGF-II immunohistochemistry in a normal breast terminal lobule. Positive staining (red chromogen)is seen in myoepithelial cells (red arrow) immediately adjacent to lobular epithelial cells (black arrow). D. IGF-II immunohistochemistry in infiltrating ductal carcinoma. Abundant stromal staining is seen (red arrow) with little appreciable staining of tumor epithelium (black arrow). E. IGF-II immunohistochemistry in infiltrating ductal carcinoma. Stromal staining for IGF-II is easily evident, largely in the walls of blood vessels (red arrows). Tumor epithelium is unstained. F. IGF-II *in situ* hybridization in a comedo carcinoma. In this case, intense IGF-II signal is seen in much of the tumor epithlium. (Black arrows). However, in this case no IGF-II protein was detected by immunohistochemistry in epithlium or stroma (not shown). G. IGF-II *in situ* hybridization in an aggressive infiltrating carcinoma. Abundant IGF-II mRNA is detected in tumor stroma. H. IGF-II immunohistochemistry in the case shown in panel G. Intense deposition of IGF-II immunoreactive material is seen in cytoplasmic granules within tumor epitheium.

CONCLUSION

The role of IGF-II in the epithelial-stromal interactions in breast cancer has been the focus of several groups. Conditioned medium from breast cancer fibroblast cultures has been found to exert a marked stimulatory effect on MCF-7 cells[21]. Transformed fibroblasts are able to increase the growth of several tumor cell types both *in vitro* and *in vivo*[22] Several groups, including our own,[2,8] have demonstrated that IGF-II, as well as IGF-I, have a clearly defined mitogenic effects on breast tumor epithelium. In previously published work,[12] we have also shown that IGF-II mRNA and protein are generally expressed by the fibroblasts cultured from breast malignancies while IGF-I mRNA is expressed in fibroblasts cultured from normal breast. Treatment of breast tumor fibroblasts with PDGF, known to be expressed in tumor epithelium,[23] results in an increase in IGF message. All these experiments suggest the presence of a cooperative paracrine pathway between stromal end epithelial cells in breast cancer mediated at least in part by the synthesis and the secretion of growth factors.

In agreement with a previous IGF-II *in situ* hybridization report[11] on a small number of samples, our results confirm that IGF-II is commonly expressed in the stroma of breast tumors. In this study, we have also deomonstrated a direct relationship between IGF-II mRNA content and the amount of stroma present within the tumor. Further, we found that IGF-II mRNA was usually detected in loose rather than hyaline (dense)stroma.

IGF-II expression was generally highest in close proximity to tumor epithelial cells, suggesting a paracrine role of IGF-II in the regulation of epthelial growth and tumor progression.

IGF-II is widely expressed by immature organogenic cells during fetal development.[24,25] Fibroblasts isolated from patients with hereditary breast cancer have been found to display fetal characteristics in culture.[26] Skin fibroblasts from patients with a number of malignancies, including breast cancer, have been shown to have a fetal pattern of motility in collagen gels[27], while skin fibroblasts from normal individuals do not. Various authors have demonstrated phenotypic differences between fibroblasts from tumor or skin of cancer patients compared with normals[28,29,30]. Sappino *et al*[31] reported expression of *alpha* smooth muscle actin in fibroblasts derived from breast tumors; generally this protein is not present in fibroblasts from normal breast tissue.

Taken together, these data suggest that the stromal cells associated with malignant epithelial cells have a more fetal phenotype and IGF-II expression may be considered a marker of this phenotype.

Our data indicate that IGF-II expression by tumor epithelium occurs rarely, while IGF-II expression in tumor stroma is common. Previous data from studies of cultured tumor epithelial cells agrees that IGF-II expression is seen in a relatively small minority of well characterized breast cancer cell lines. T47D cells as well as some subclones of MCF-7 cells have been shown to express IGF-II.[2,32]

Previously published data from our laboratory and others show that when IGF-II null MCF-7 cells are infected with a retroviral IGF-II expression vector, they acquired

phenotypic changes associated with malignant progression[33], losing all requirements for estrogen or other growth factors.

Interestingly, the epithelial cells and stroma of normal lobules showed little or no IGF-II mRNA expression. However, immunohistochemistry showed consistent IGF-II staining in the myoepithelial cell layer tightly adjacent to normal lobular epithlium. It is possible that this IGF-II protein is bound to receptors on or in the myoepithelial cells, having arrived there from the local circulation.

Three tumors showed IGF-II immunostaining in malignant epithelial cells while IGF-II mRNA expression in thee tumors was completely confined to stromal cells. In these cases the IGF-II protein synthetized by local stromal fibroblasts is bound to tumor epithelium. The ability to detect IGF-II protein in tumor epithelium by immunohistochemistry may reflect the ability of the epithelial cell to degrade the protein in lysosomes once it binds receptor(s) at the cell surface. This is another balance mechanism which may ultimately play a significant role in determining the biologic effect that the paracrine IGF-II is able to exert on the epithelial cell.

Our data indicate that IGF-II mRNA and protein are expressed in the stroma of the majority of breast cancers. Correlative analysis of the first 80 cases from the University of Pisa indicates that IGF-II mRNA may confer a poor prognosis in estrogen receptor negative patients. However, we saw nearly an opposite relationship in the 115 cases from Georgetown University. Together, the opposing results essential cancel each other.

The reasons for this disparity are not evident at the present time. We have re-analyzed all the data carefully to assure that a simple statistical error in the computer coding system did not account for the discrepency in our results. Likewise, we have reviewed the scoring of the cases from the Pisa and Georgetown Series, and do not find a systematic error that would explain the difference.

Likewise, there is no obvious biological explanation for the difference in the data produced from the two patient sets.

Taken together, the results from the two groups do not permit us to conclude at the present time that there is a significant prognostic role for IGF-II in breast cancer. We have not so far been able to confirm the original hypothesis in this proposal.

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