CANCER GENOMICS & PROTEOMICS *1*: 339-344 (2004)

Keratinocyte Growth Factor-Mediated Pattern of Gene Expression in Breast Cancer Cells

XIAO-PING ZANG[1], MEGAN L. LERNER[2,3], SON V. DO[2], DANIEL J. BRACKETT[2,3] and J. THOMAS PENTO[1]

Departments of [1]Pharmaceutical Science, College of Pharmacy and
[2]Surgery, University of Oklahoma, Health Sciences Center and
[3]VA Medical Center, Oklahoma City, Oklahoma 73190, U.S.A.

Abstract. *Background: Breast cancer metastasis is associated with the motility and invasiveness of breast cancer cells. In a previous study we reported the motility enhancement effect of keratinocyte growth factor (KGF) on breast cancer cells. This study established and characterized the influence of KGF on breast cancer cell motility and determined that KGF-induced motility was observed only in estrogen receptor-positive breast cancer cells. The objective of the present study was to identify genes involved in the KGF motility response in human breast cancer cells. Materials and Methods: Using cDNA expression assays, we compared the expression of mRNA in control and KGF-treated MCF-7 breast cancer cells. Scatter plots and cluster analysis of gene expression were used to determine KGF-mediated gene expression patterns. Results: It was determined that over 100 genes were up- or down-regulated from 3-100 fold at 1h following KGF treatment. We identified up-regulated and down-regulated target genes that are associated with some aspect of tumor progression, proliferation or metastasis. Conclusion: Knowledge of specific genes and patterns of gene regulation associated with KGF-enhanced cell motility may provide important new information concerning the mechanisms involved in tumor metastasis. In addition, these genes and/or protein products may serve as novel therapeutic targets or biomarkers of metastatic progression. The pattern gene of expression observed in this study provides new information on the molecular signature associated with the motility and metastatic progression of breast cancer.*

An important characteristic of tumor malignancy and the major cause of morbidity and mortality in cancer patients is the ability of tumor cells to metastasize to distant sites in the body. Metastatic dissemination of tumor cells is associated with highly motile behavior (1). Stromal tissue surrounding the tumor cells produces growth factors and cytokines which are known to enhance tumor cell proliferation and progression to a metastatic phenotype (2).

KGF, originally isolated from human embryonic lung fibroblasts (3), is a member of the fibroblast growth factor family (also designated FGF-7) and is present in stromal tissue of the breast and other tissue (4). Although not produced by epithelial tissue, KGF stimulates DNA synthesis, proliferation and migration of epithelial cells in the breast and other tissue (5). These target epithelial cells contain high affinity KGF receptors (KGFR) (6). *In situ* hybridization studies in mammary tissue provide further evidence that KGF is a mesenchymally-derived mediator of epithelial cell proliferation and migration (7). Consistent with this concept, KGFR gene up-regulation, as determined by RT-PCR, was observed in human primary breast tumor specimens (8). This suggests that KGF-mediated stimulation of primary breast cancer cells may be one of the early events in the metastatic progression of this disease.

In previous studies, we demonstrated that KGF produced a rapid enhancement of the motility of ER ~ positive human breast cancer cells (9) and that treatment with antiestrogens, heparin or a specific KGFR fragment inhibited KGF-induced effects (9,10). The objective of the present study was to identify genes involved in the KGF-motility response in ER-positive MCF-7 human breast cancer cells. If KGF is an important early signal involved in breast cancer metastasis, then KGF-regulated genes, KGFR and the associated tyrosine kinase signaling pathway may be important as biomarkers or as therapeutic targets for the inhibition of breast cancer progression to a metastatic phenotype.

Materials and Methods

Cell culture methods and KGF treatment. MCF-7 human breast cancer cells, obtained from the Michigan Cancer Foundation, USA, were maintained as monolayer cultures in RPMI 1640 media

Correspondence to: J. Thomas Pento, Ph.D., Department of Pharmaceutical Sciences, College of Pharmacy, University of Oklahoma Health Sciences Center, 1110 N. Stonewall Ave., Oklahoma City, OK 73117, USA. Tel: (405) 271-6593, Fax: (405) 271-7505, e-mail: tom-pento@ouhsc.edu

Key Words: KGF, cDNA microarray, breast cancer, cell motility, metastasis.

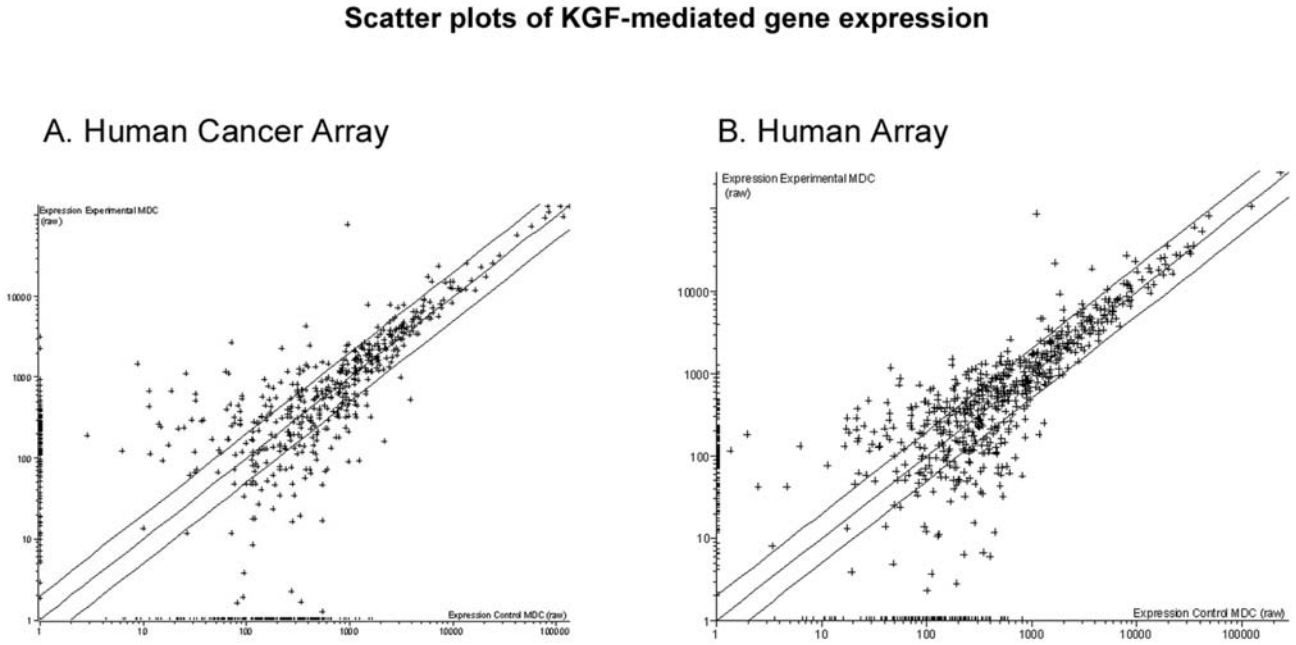

Figure 1. *The vertical axis represents the fold up-regulation of the genes on the KGF treatment array and the horizontal axis represents the fold up-regulation of the genes on the control array. The diagonal lines represent equal expression ± 5% in both the KGF treatment and control arrays. Each point represents the expression of an individual gene on the expression array.*

(without phenol red) as previously described (9-10). Media was filter-sterilized and stored at 4˚C prior to use. Cancer cells were treated with recombinant human KGF (Pharmingen, San Diego, CA, USA) at a final concentration of 50 ng/ml in the evaluation of KGF effects on cell motility and mRNA expression. Control cultures were treated in the same manner with empty vehicle. Approximately 18 h prior to KGF treatment, the cells were trypsinized, seeded in T-25 flasks at a confluency of approximately 70%, and allowed to attach overnight in RPMI media. On the day of the experiment, the cells were washed with RPMI, and KGF was added to the media one hour prior to motility determination and mRNA extraction. Cell motility was determined by time-lapse videomicroscopy as previously described (9). This KGF treatment protocol was used since we had previously observed optimal KGF-induced cell motility under these experimental conditions (9).

cDNA microarray methods. One hour following KGF treatment, mRNA was extracted using the Atlas™ Pure Total RNA Labeling System (Clontech, Palo Alto, CA, USA). Two to 5 μg RNA prepared from each of the treatment samples was used to synthesize probe mixtures by reverse transcribing each RNA population using the cDNA Synthesis Primer Mix (gene specific oligonucleotide primers and MMLV reverse transcriptase) provided in the kit and α-^{32}P dATP at 50˚C for 25 min. The labelled probes were purified with column chromotography to remove unincorporated isotope resulting in a total of 2-10 x 10^6 counts per minute . The purified labelled cDNA was then used to probe the target sequences immobilized on the nylon membranes. An equal amount of ^{32}P activity from a control and an experimental sample was then individually hybridized overnight at 68˚C to each of two identical array membranes. This study employed both the Clontech Atlas Human 1.2 array and the Human Cancer 1.2 nylon array (Clontech). Both cDNA expression arrays contain 1200 genes with a 400 gene overlap. Thus, approximately 2000 genes were examined in this study.

After hybridization, membranes were washed under stringent conditions (2X SSC, 1% SDS followed by 0.1X SSC, 0.5% SDS) at 68˚C and then exposed to a phosphorimaging screen (Molecular Dynamics, Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). An image was generated using the Storm Phosphorimaging System (Molecular Dynamics, Amersham Pharmacia Biotech Inc.). The samples of RNA to be compared were always processed, hybridized and imaged simultaneously using arrays from a single lot, quality-controlled by the manufacturer. Nonspecific hybridization was minimal in each array and similar across all membranes. The nonspecific hybridization signal was subtracted from the signal of each of the target genes on the membrane. Housekeeping genes were used as positive controls and hybridization to the negative controls was never observed in these studies. The expression levels of the genes on each array was analyzed and compared using Array Vision Software (Imaging Research, Inc., St. Catharines, Ontario, Canada). This software was used to evaluate the absolute difference in pixel value and the fold increase or decrease in signal in experimental (KGF-treated) compared to control arrays. The experiments were repeated using the same two Clonetech arrays. The expression data from each experiment was compared and analyzed using GeneSpring software (Silicon Genetics, Redwood, CA, USA).

Table I. *Up-regulated in response to KGF.*

Growth factor	Oncogenes	Binding proteins	Cytokines	Proteases	Signal transduction mediators	Transcription factors
IGF	c-jun	cadherin 1	interleukin 1	MMP 1	PI 3-kinase	transcription factor SP1
PDGF	c-fos	cadherin 2	interleukin 3	MMP 2	FAK	transcription factor 101
TGF-B	c-myc	cadherin 5	interleukin 5	MMP 3	STAT-1	transcription factor 103
TGF-B receptor II	Ras-related protein	cadherin 6	interleukin 6	MMP 7	Grb-2	transcription initiation factor
VEGF receptor		cadherin 8	interleukin 14	MMP 8	tyrosine kinase receptor	transcription activator SNF-2
FGF a		cadherin 11	interleukin 15	MMP 12	MAP kinases	
FGF b		cadherin 12	interferon gamma	MMP15	Wnt 2B	
FGF 6		intergrin 2	interferon receptor	MMP 16	Wnt 5A	
FGF 8		integrin 4		TIMP 2	Wnt 8B	
FGF receptor		integrin 5		TIMP 4		
KGF receptor		integrin 6		uPA		
		integrin 8		uPA receptor		
		delta catenin				
		fibronectin				

Table II. *Down-regulated in response to KGF.*

Death receptor-associated proteins	DNA damage signaling/ repair proteins	Oncogenes and tumor suppressor proteins	Apoptosis-associated proteins
DAP 6	G22P 1	MARK 3	BIRC 3
TNFSF 4	PMS 1	DLG 3	DAP 3
TNFSF 7	PMS 2	GRB 10	DNASE1L1
	RAD21	ING 1	GADD45A
	TDG	KSR	GSTT 2
	ABL1	PRKRI	SIAH 1
			BAD

Results

The scatter plots from the Human and Human Cancer arrays, presented in Figure 1, demonstrate that, in both arrays, the majority of the 1200 genes on each array were not significantly altered by KGF treatment and fall within the 95% confidence limit. On the Human Array, 133 genes were up-regulated 3-fold or greater while, on the Human Cancer Array, 113 genes were found to be up-regulated by 3-fold or greater. A list of genes that were up-regulated by KGF treatment and associated with cancer, progression or metastasis, listed by functional family, is presented in Table I. A list of genes down-regulated by KGF treatment and associated with inhibition of cancer, progression or metastasis, listed by functional family, is presented in Table II. The expression of each gene which was significantly altered by KGF treatment was compared between the replicate experiments and found to be within a confidence limit of 85%, with few exceptions.

Discussion

The metastatic dissemination of tumor cells to secondary distant sites has been correlated with highly motile behavior (11) and tumor cell motility and metastasis is regulated by growth factors and cytokines (12,13). It is also known that stromal tissue surrounding the tumor cells produces factors which, in some instances, enhance tumor cell proliferation and progression to a metastatic phenotype (14). Further, it has been shown that the ductal epithelium of adult mammary tissue is very sensitive to KGF. Systemic administration of KGF in mice and rats for 5 days produced massive mammary ductal hyperplasia, which is known to be characteristic of premalignant breast lesions leading to neoplasia (15,16). Similarly, Kitsberg and Leder (17) observed that female mice, with a constitutively up-regulated KGF transgene, developed mammary epithelial hyperplasia and eventually all animals developed metastatic mammary carcinomas. We have previously reported that KGF produced a rapid increase in the motility of ER-positive breast cancer cells, which persisted for up to 48 hours (9). Thus, the purpose of the present study was to identify genes whose expression is altered or regulated by KGF using cDNA expression arrays.

It has been shown that genes in a functional category tend to be expressed together (18). Thus, in our analysis of the array data, we focused on functional categories of genes and especially those functional categories known to be involved in tumor cell motility and metastasis. Further, it has been reported that cluster profiling of tumor samples from cancer patients identified a molecular signature that is predictive of metastatic development (19).

The pattern of genes which were up-regulated by KGF treatment is largely associated with a phenotype of tumor progression, metastasis and resistance to apoptosis. Increased expression of genes such as growth factors, cytokines, oncogenes, proteases and signaling pathways would suggest a cellular phenotype which is becoming more aggressive and malignant. Accordingly, in the present study, KGF treatment increased the expression of the KGF receptor, the IGF receptor and the TGF-β receptor, which are also known to enhance breast cancer cell migration and are associated with cancer progression and metastasis (12,13,20,21). Similarly, expression array studies done with breast tumor samples indicate that genes involved in cell cycle, motility and signal transduction are associated with a signature of poor clinical prognosis (19). Interestingly, genes associated with both the receptor tyrosine kinase and Wnt signal transduction pathways were up-regulated, and both pathways have been associated with breast cancer progression (19, 22).

Cluster analysis of genes that were up-regulated in response to KGF to the same extent as KGFR (within a 95% confidence limit) identified the following genes: FGF-β (another member of the FGF family of growth factors), MMP 9 and 12, BRCA2 and p53. Interestingly, these genes are biomarkers for breast cancer and/or its progression (23). Warnberg *et al*. (24) found that the current clinical biomarkers ER, PR and c-erbB2 were associated with tumor grade, but did not indicate metastatic phenotype, so that it is important to identify better biomarkers of metastatic progression. An advantage of using a cancer cell line to identify potential biomarkers or therapeutic targets is the greater uniformity in gene expression when compared to variation between tumor samples within or between tumors in a single patient and between tumor samples from different cancer patients (25).

A consideration of the pattern of genes which were found to be down-regulated, such as death receptor-associated proteins, death kinases, DNA damage signaling/repair proteins, tumor suppressor proteins and apoptosis-associated proteins, defines a tumor cell phenotype which would promote tumor progression, malignancy and metastasis. The picture which emerges from the pattern of gene expression observed in response to KGF treatment in this study is a phenotype of malignancy in which mediators of cell growth and metastatic progression are up-regulated, while mediators of cell regulation, genomic repair and cell apoptosis are suppressed.

These results support the concept that KGF is an early signal which is involved in the coordination of metastatic progression. The initiation of metastatic progression may involve an increased release of KGF from breast stromal tissue, an up-regulation of KGFR or associated signal transduction pathways in developing breast cancer tissue or some combination of these events. Thus, therapeutic approaches that would inhibit KGF, KGFR and/or KGFR-mediated signaling pathways may effectively inhibit the progression of breast cancer to a more malignant and metastatic phenotype. Further, the genes and/or gene products found to be altered significantly by KGF in this study may serve as early biomarkers of tumor progression and metastasis.

Acknowledgements

This study was supported in part by grants from NIH/NCI (CA-89740) and DOD (DAMD17-01-1-0591) and the Presbyterian Health Foundation, USA.

References

1 Nicholson GL: Cancer metastasis - tumor cell and host organ properties important in colonization of specific secondary sites. Biochem Biophys Acta *948*: 175-224, 1988.

2 Peehl DM and Rubin JS: Keratinocyte growth factor: an androgen-regulated mediator of stromal epithelial interactions in the prostate. World J Urol *13*: 312-317, 1995.

3 Rubin JS, Osada H, Finch PW *et al*: Purification and characterization of a newly identified growth factor specific for epithelial cells. Proc Natl Acad Sci *86*: 802-806, 1989.
4 Rubin JS, Bottaro DP, Chedid M *et al*: Keratinocyte growth factor as a cytokine that mediates mesenchymal-epithelial interaction. In: Goldberg ID and Rosen EM, (eds). Epithelial-Mesenchymal Interactions in Cancer: Verlag Basel 191-214, 1995.
5 Aaronson SA, Bottaro DP, Miki T *et al*: Keratinocyte growth factor. A fibroblast growth factor family member with unusual target cell specificity. Ann NY Acad Sci *638*: 62-77, 1991.
6 Bottaro DP, Rubin JS, Ron D *et al*: Characterization of the receptor for keratinocyte growth factor. Evidence for multiple fibroblast growth factor receptors. J Biol Chem *265*: 12767-12770, 1990.
7 Mason IJ, Fuller-Pace F, Smith R *et al*: FGF-7 (keratinocyte growth factor) expression during mouse development suggests roles in myogenesis, forebrain regionalization and epithelial-mesenchymal interactions. Mech Dev *45*: 15-30, 1994.
8 Koos RD, Banks PK, Inkster SE *et al*: Detection of aromatase and keratinocyte growth factor expression in breast tumors using reverse transcription-polymerase chain reaction. J Steroid Biochem Mol Biol *45*: 217-225, 1993.
9 Zang XP and Pento JT: Keratinocyte growth factor-induced motility of breast cancer cells. Clin Eptl Metastasis *18*: 573-580, 2001.
10 Zang XP, Nguyen TN and Pento JT: Specific and non-specific KGF inhibition of KGF-induced breast cancer cell motility. Anticancer Res *22*: 2539-2546, 2002.
11 Partin AW, Isaacs JT, Treiger B *et al*: Early cell motility changes associated with an increase in metastatic ability in rat prostatic cancer cells transfected with the v-Harvey-ras oncogene. Cancer Res *48*: 6050-6053, 1989.
12 Kim H and Juller WJ: The role of the epidermal growth factor receptor family in mammary tumorigenesis and metastasis. Exp Cell Res *253*: 78-87, 1999.
13 Yu H and Rohan T: Role of the insulin-like growth factor family in cancer development and progression. J Natl Cancer Inst *92*: 1472-1489, 2000.
14 Peehl DM and Rubin JS: Keratinocyte growth factor: an androgen-regulated mediator of stromal epithelial interactions in the prostate. World J Urol *13*: 312-317, 1995.
15 Yi ES, Bedova AA, Lee H *et al*: Keratinocyte growth causes cystic dilation of mammary glands of mice. Am J Pathol *145*: 1015-1022, 1994.
16 Ulich TR, Yi ES, Cardiff R *et al*: Keratinocyte growth factor is a growth factor for mammary epithelium *in vivo*. Am J Pathol *144*: 862-868, 1994.
17 Kitsberg DI and Leder P: Keratinocyte growth factor induces mammary is a growth factor for mammary epithelium *in vivo*. Oncogene *13*: 2507-2515, 1996.
18 Eisen MB, Spellman PT, Brown PO *et al*: Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci *95*: 14863-14868, 1998.
19 van't Veer LJ, Dai H, van de Vijver MJ *et al*: Gene expression profiling predicts clinical outcome of breast cancer. Nature *415*: 530-536, 2002.
20 Welch DR, Fabra A and Makajima M: Transforming growth factor β stimulates mammary adenocarcinoma cell invasion and metastic potential. Cell Biol *87*: 7678-7682, 1990.
21 Tong GM, Rajah TT and Pento JT: The differential influence of EGF- and IGF-1 and TGF-β on the invasiveness of human breast cancer cells. In Vitro Cell Dev Biol *36*: 493-94, 2000.
22 Howe LR and Brown AM: Wnt signaling and breast cancer. Proc Natl Acad Sci *100*: 15853-58, 2003.
23 Ross JS, Linette GP, Stec J *et al*: Breast cancer biomarkers and molecular medicine. Expert Rev Mol Diagn *2*: 169-88, 2004.
24 Warnberg F, Nordgren H, Bergkvist L *et al*: Tumour markers in breast carcinoma correlate with grade rather than with invasiveness. Br J Cancer *85*: 869-874, 2001.
25 Ma XJ, Salunga R, Tuggle JT *et al*: Gene expression profiles of human breast cancer progression. Proc Natl Acad Sci *100*: 5974-5979, 2003.

Received July 18, 2004
Accepted July 27, 2004