AD_____

Award Number: DAMD17-00-1-0205

TITLE: A Novel Role for Integrin-Linked Kinase in Breast Cancer

PRINCIPAL INVESTIGATOR: Pratima Karnik, Ph.D.

CONTRACTING ORGANIZATION: The Cleveland Clinic Foundation
Cleveland, Ohio 44195

REPORT DATE: September 2002

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2002	Annual (1 Sep 01 -31 Aug 02)

4. TITLE AND SUBTITLE
A Novel Role for Integrin-Linked Kinase in Breast Cancer

5. FUNDING NUMBERS
DAMD17-00-1-0205

6. AUTHOR(S)
Pratima Karnik, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Cleveland Clinic Foundation
Cleveland, Ohio 44195

E-MAIL: karnikp@ccf.org

8. PERFORMING ORGANIZATION REPORT NUMBER

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

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

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

Allelic loss at the short arm of chromosome 11 is one of the most common and potent events in the progression and metastasis of breast cancer. Here, we present evidence that the Integrin-Linked Kinase (*ILK*) gene maps to the commonly deleted chromosome 11p15.5 and suppresses malignant growth of human breast cancer cells both *in vitro* and *in vivo*. *ILK* is expressed in normal breast tissue but not in metastatic breast cancer cell lines or in advanced breast cancers. Transfection of wild-type *ILK* into the MDA-MB-435 mammary carcinoma cells potently suppressed their growth and invasiveness *in vitro*, and reduced the cells' ability to induce tumors and metastasize in athymic mice. Conversely, expression of the ankyrin repeat or catalytic domain mutants of *ILK* failed to suppress the growth of these cells. Growth suppression by *ILK* is not due to apoptosis but is mediated by its ability to block cell cycle progression in the G1 phase. These findings directly demonstrate that *ILK* deficiency facilitates neoplastic growth and suggest a novel role for the *ILK* gene in tumor suppression.

14. SUBJECT TERMS
ILK, gene transfer, breast cancer, nude mouse assay

15. NUMBER OF PAGES
12

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

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

__NA_ Where copyrighted material is quoted, permission has been obtained to use such material.

_NA__ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

_NA__ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

__X_In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

__ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

. In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

__ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Pratima Karnik
PI - Signature

Date September 12, 2002

Table of Contents

A. INTRODUCTION

Genetic alterations that occur in breast cancer are believed to be of importance for initiation as well as progression of the disease. These genetic alterations lead to the loss or activation of a number of critical genes, such as those involved in cell proliferation, differentiation, apoptosis, and genetic stability. The genetic abnormalities most frequently observed in breast tumors are amplification of proto-oncogenes (*MYC*, *ERBB2* and *CCND1*), mutations of *TP53*, and loss of heterozygosity (LOH) on chromosomes 3p, 6q, 7q, 8p, 9p, 11, 13q, 17, 18q and 22q (1, 2). Metastatic phenotypes have been linked to such genes as *NME1* (17q), *CDH1* (16q), *BRMS1* (11q), and *KISS1* (1q) (1, 3-5). LOH analyses have defined regions of deletion associated with metastasis on chromosomes 3p21, 15q14, 16q22 and 11p15 (2, 6)

Frequent genetic alterations on chromosome 11p15 suggest a crucial role for this region in breast (6, 7) and other adult (8-12) and childhood cancers (13-17). More recently, we have mapped two distinct regions on chromosome 11p15.5 that are subject to LOH during breast tumor progression and metastasis (6). LOH at region 1 correlated with tumors that contain ductal carcinoma *in situ* suggesting that the loss of a critical gene in this region may be responsible for early events in malignancy. LOH at region 2 correlated with a more aggressive tumor and an ominous outlook for the patient, such as aneuploidy, high S-phase fraction and the presence of metastasis in regional lymph nodes. Although considerable advances have been made in the fine-mapping of chromosome 11p15.5, the tumor suppressor gene(s) encoded by this region have evaded identification.

Integrin-linked kinase (*ILK*) is an intriguing serine/threonine kinase that has been implicated in integrin-, growth-factor- and Wnt-signaling pathways (18). It binds to the cytoplasmic domains of β1 and β3 integrins and mediates the down-stream signaling events in integrin function (19). Interactions between integrins and their ligands are involved in the regulation of many cellular functions, including embryonic development, cell proliferation, tumor growth and the ability to metastasize (20). In *Drosophila*, the absence of *ILK* function causes defects similar to loss of integrin adhesion and *ILK* mutations cause embryonic lethality and defects in muscle attachment (21). Although *ILK* maps to the commonly deleted chromosome 11p, the potential of this gene in tumor suppression has not been established. We have therefore analyzed the effect of *ILK* expression on the *in vivo* tumor growth and invasion of human mammary carcinoma cells.

B. BODY:

1. Results:

ILK suppresses tumor formation and metastasis in nude mice

In the last progress report, we reported that we have transfected the ILK gene into the metastatic breast cancer cell line MDA-MB-435 and have isolated four different clones that express different levels of ILK mRNA and protein. We are now testing these cells using a nude mouse metastatic model.

The most stringent experimental test of neoplastic behavior is the ability of injected cells to form tumors in nude mice. Yet not all of the cellular growth properties commonly associated with the cellular state *in vitro* are required for neoplastic growth *in vivo* and vice versa. Therefore, loss of tumorigenecity under expression of *ILK in vivo* would be a critical test to substantiate the growth suppressor function of *ILK*. The mammary carcinoma cell line MDA-MB-435 forms tumors at the site of orthotopic injection, metastasizes in nude mice and closely resembles the course of human breast cancer (22). To investigate whether *ILK* expression affected tumor formation in nude mice, two different *ILK* transfectant clones (TR5-ILK and TR3-ILK) and two vector controls were inoculated into the subaxillary mammary fat pads of 4-6 week old athymic nude mice. Tumors were measured weekly thereafter to assess the growth rate. All MDA-MB-435 vector transfectants were already palpable 7 days after injection. Subsequently, the tumors of vector transfectants grew steadily attaining mean volumes of 3.0 cm^3 (mean ± s.d.) at 15 weeks (Fig. 1A and B). In contrast, only 2 of 12 mice injected with ILK transfectants

developed tumors. The tumor growth of ILK transfectants was significantly slower than than that of control transfectants ($P < 0.005$, Fisher variance analysis). At sacrifice, (15 weeks) the *ILK* tumors reached a mean volume of only 0.45 cm^3 (mean $\pm$ s.d.) which was significantly smaller than control tumors ($P < 0.001$, Student's *t*-test). Vector transfected MDA-MB-435 cells developed an average of 12-24 lung metastases per mouse (Figure-1C). Additional tumor masses were present in central venous blood vessels, the diaphragm, and lymph nodes of vector transfectants (data not shown). In contrast, with the ILK transfectants, only one of the two animals that developed tumors showed a single metastatic colony in the lung. The presence of additional microscopic metastases in random lung sections was not observed by H&E staining (data not shown). These results clearly demonstrate that the expression of *ILK* in human MDA-MB-435 breast carcinoma cells significantly suppresses tumorigenecity and metastatic ability in athymic nude mice.

2. Methods:

Tumorigenecity and Metastasis Assays

Cells (10^6) were injected into the subaxillary mammary fat pads of 4-6 week-old female athymic nude mice Ncr nu/nu (10-12 mice/group; Taconic Labs, Germantown, NY) as described (52). Mice were maintained under the guidelines of NIH and the Cleveland Clinic Foundation. All protocols were approved and monitered by the the Institutional Animal Care and Use Committee. Food and water were provided *ad libitum*. Tumors were monitered weekly after inoculation. When the mean tumor diameter reached 1.0-1.3 cm, primary tumors were surgically removed under Ketaset-Rompun anesthetic. Mice were then maintained for an additional 4 weeks to allow further growth of lung metastases. After euthanasia, all organs were checked for metastases.

C. CONCLUSIONS:

In previous reports, we have provided evidence that ILK expression is down-regulated in primary breast tumors and in cell lines derived from metastatic breast tumors. We have shown that ILK overexpression inhibits the growth of the highly metastatic breast cancer cell line MDA-MB-435. In addition, ILK overexpression stimulates the levels of the growth suppressing integrin $\alpha5\beta1$ and inhibits the levels of $\alpha v\beta3$, a growth promoting integrin. These innovative studies suggest a novel role for ILK in the etiology of breast cancer. Functional studies in animal models were therefore undertaken to establish ILK as a metastasis suppressor gene. These studies are part of this year's report.

The present study reveals that expression of *ILK* potently suppresses *in vivo* tumorigenecity of the human mammary carcinoma cells. The MDA-MB-435 cells are a model for deficient *ILK* protein expression and transfection of the *ILK* gene is designed to restore this deficiency. As shown in the last year's report, the growth suppression activity requires a functional *ILK* protein, since expression of wild-type *ILK*, but not the ankyrin repeat or the catalytic domain mutants, resulted in growth suppression of MDA-MB-435 cells. These results suggest a possible role for *ILK* in the suppression of tumor growth and metastasis and directly implicate its loss in processes regulating the malignant phenotype in human breast cancer. *ILK* seems to play a dual role in the MDA-MB-435 model system. First, it regulates cell-cycle progression at the G1/S boundary and second, it modulates the levels of integrins, transmembrane receptors that have been shown to regulate growth, differentiation and invasiveness of cells. During this process, the neoplastic cells cease to proliferate and lose their ability to migrate through vitronectin membranes and to induce tumor growth and metastasis in nude mice (Figure-1).

D. KEY RESEARCH ACCOMPLISHMENTS:

- Chromosome 11 harbors a breast cancer metastasis suppressor gene
- Integrin linked kinase (ILK) is a key candidate gene that maps to this region
- ILK expression is downregulated in breast carcinomas that metastasize
- ILK expression inhibits the growth of the metastatic breast cancer cell line MDA-MB-435 both in vitro and in vivo.

These data suggest that ILK functions as a metastasis suppressor gene in breast cancer

E. REPORTABLE OUTCOMES:

- These results are being prepared as a manuscript for publication.

F. REFERENCES:

1. Driouch, K; Briffod, M; Bieche, I; Champeme, M.H; and Lidereau, R. Location of several putative genes possibly involved in human breast cancer progression. Cancer Res. *58*: 2081-2086, 1998.
2. Bieche, I. and Lidereau, R., Genetic alterations in breast cancer. Genes Chromosomes Cancer *14*: 227-251, 1995.
3. Siitonen, S.M; Kononen,J.T; Helin, H.J; Rantala, I.S; Holli, K.A. and Isola, J.J. Reduced E-cadherin expression is associated with invasiveness and unfavorable prognosis in breast cancer. Am. J. Clin. Pathol . *105*: 394-402, (1996).
4. Seraj, M.J; Samant,R.S; Verderame, M.F. and Welch, D.R. Functional evidence for a novel human breast carcinoma metastasis suppressor, BRMS1, encoded at chromosome 11q13. Cancer Res. *60*: 2764-2769, 2000.
5. Lee, J.H; Miele, M.E; Hicks, D.J; Phillips, K.K; Trent, J.M; Weissman, B.E. and Welch, D.R. *KISS-1,* a novel human malignant melanoma metastasis-suppressor gene. J. Natl. Cancer Inst. *88*: 1731-1737, 1996.
6. Karnik, P; Paris, M; Williams, B.R.G; Casey, G, Crowe, J and Chen P. Two distinct tumor suppressor loci within chromosome 11p15 implicated in breast cancer progression and metastasis. Human Mol Genet *7*: 895-903, 1998.
7. Karnik, P., Plummer, S., Casey, G., Myles, J., Tubbs, R., Crowe, J. and Williams, B.R.G. Microsatellite instability at a single locus (D11S988) on chromosome 11p15.5 as a late event in mammary tumorigenesis. Human Mol Genet *4*: 1889-1894, 1995.
8. Fearon, E.R., Feinberg, A.P., Hamilton, S.H. and Vogelstein, B. Loss of genes on the short arm of chromosome 11 in bladder cancer., Nature *318*: 377-380, 1985.
9. Viel, A., Giannini, F., Tumiotto, L., Sopracordevole, F., Visetin, M.C. and Biocchi, M. Chromosomal localisation of two putative 11p oncosuppressor genes involved in human ovarian tumours. Br. J. Cancer *66*: 1030-1036, 1992.
10. Bepler, G. and Garcia-Blanco, M.A. Three tumor-suppressor regions on chromosome 11p identified by high-resolution deletion mapping in human non-small cell lung cancer. Proc. Natl. Acad. Sci., USA *91*: 5513-5517, 1994.
11. Lothe, R.A., Fossa, S.D., Stenwig, A.E., Nakamura, Y., White, R. and Borresen, A.L. and Brogger, A. Loss of 3p or 11p alleles is associated with testicular cancer tumors. Genomics *5*: 134-138, 1989.
12. Wang, H.P. and Rogler, C.E. Deletions in human chromosome arms 11p and 13q in primary hepatocellular carcinomas. Cytogenet.Cell Genet. *48*: 72-78, 1988.
13. Karnik, P; Chen, P; Paris, M; Yeger, H. and Williams, B.R. Loss of heterozygosity at chromosome 11p15 in Wilms tumors: identification of two independent regions. Oncogene *17*: 237-240, 1998.
14. Besnard-Guerin, C., Newsham, I., Winquist, R. and Cavenee, W.K. A common loss of heterozygosity in Wilms tumor and embryonal rhabdomyosarcoma distal to the D11S988 locus on chromosome 11p15.5. Hum. Genet., *97*: 163-170, 1996.
15. Henry, I., Grandjouan, S., Couillin, P., Barichard, F., Huerre-Jeanpierre, C., Glaser, T., Philip, T., Lenoir, G., Chaussain, J.L. and Junien, C. Tumor specific loss of 11p15.5 alleles in del 11p13 Wilms tumor and in familial adrenocortical carcinoma. Proc. Natl. Acad. Sci. *86*: 3247-3251, 1989.
16. Koufos, A., Hansen, M.F., Copeland, N.G., Jenkins, N.A., Lampkin, B.C. and Cavenee, W.K. Loss of heterozygosity in three embryonal tumours suggests a common pathogenetic mechanism. Nature *316*: 330-334, 1985.

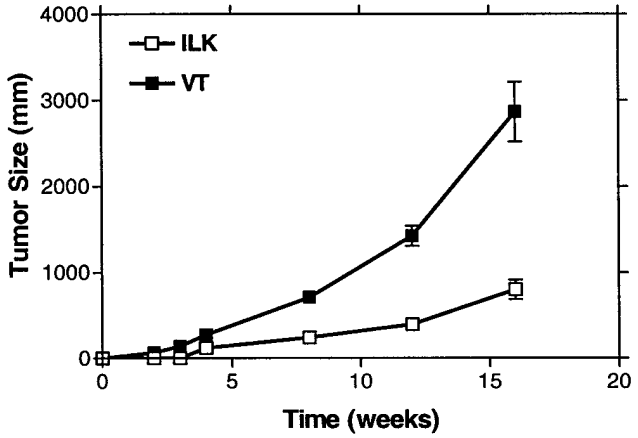
17. Sotel-Avila, D. and Gooch, W.M. III. Neoplasms associated with the Beckwith-Wiedemann Syndrome. Perspect. Pediatr. Pathol. *3*: 255-272, 1976.
18. Dedhar, S; Williams, B. and Hannigan, G., Integrin-Linked Kinase (ILK): a regulator of integrin and growth-factor signalling., Trends in Cell Biol. *9*: 319-323. 1999
19. Hannigan, G.E; Leung-Hagesteijn, C; Fitz-Gibbon, L; Coppolino, M.G; Radeva, G; Filmus, J; Bell, J.C. and Dedhar , S. Regulation of cell adhesion and anchorage-dependent growth by a new beta 1-integrin-linked protein kinase. Nature *379*: 91-96, 1996.
20. Hynes R.O. Integrins: versatility, modulation, and signaling in cell adhesion. Cell *69*: 11-25, 1992.
21. Zervas, C.G; Gregory, S.L and Brown, N.H. Drosophila Integrin-linked Kinase Is Required at Sites of Integrin Adhesiom to Link the Cytoskeleton to the Plasma Membrane. J. Cell Biol. *152*: 1007-1018, 2001.
22. Price, J.E; Polyzos, A; Zhang, R.D. & Daniels, L.M. Tumorigenecity and metastasis of human breast carcinoma cell lines in nude mice. Cancer Res. *50*: 717-721, 1990.

FIGURE LEGEND:

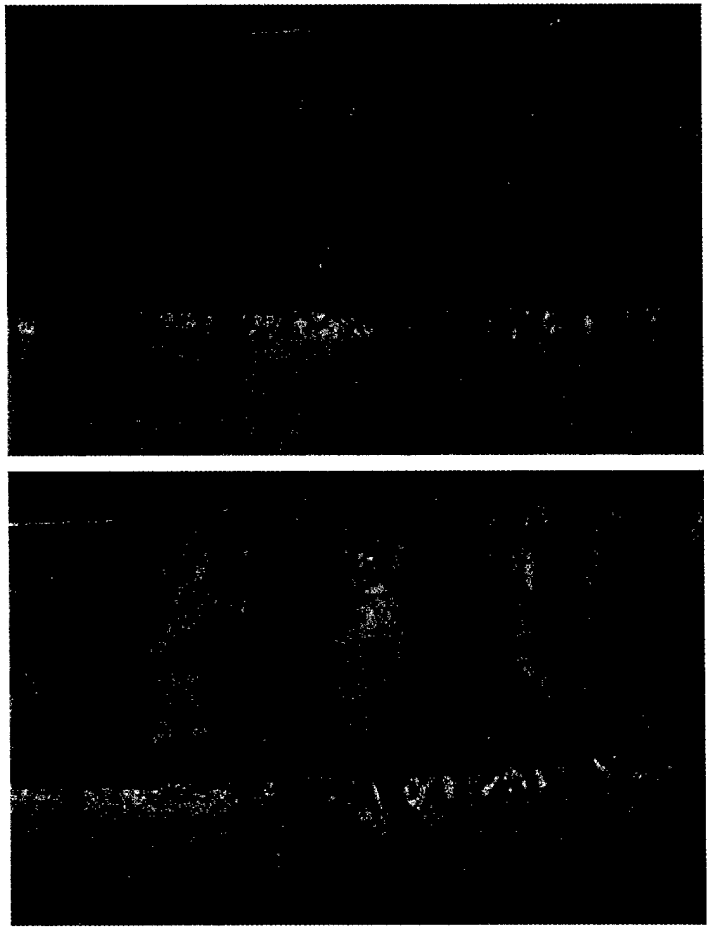
Figure-1: (A) In vivo tumor growth of *ILK* transfected (-□-) and vector transfected (-■-) MDA-MB-435 cells in mammary fat pads of athymic nude mice. Each point represents the mean ± SE of tumors. (B) Five x 10^5 cells of *ILK* transfected (top panel) or vector transfected (bottom panel) MDA-MB-435 cells were injected s.c. into the mammary fat pad area below the nipple. Tumors were allowed to grow for 15 weeks at which time the mice were photographed and sacrificed. (C) Lung colony formation in athymic nude mice injected with vector transfected (VT) or *ILK* transfected *(ILK)* MDA-MB-435 cells. Bars represent S.E.

APPENDIX

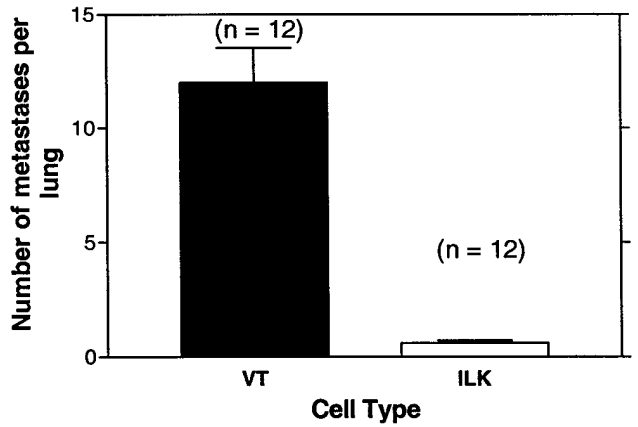
A.


4000
3000
2000
1000
0
ILK
VT
Tumor Size (mm)
0
5
10
15
20
Time (weeks)

B.



C.


15
10
5
0
(n = 12)
(n = 12)
Number of metastases per lung
VT
ILK
Cell Type