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13. ABSTRACT : In published studies, we have shown that chromosome 11p15.5 exhibits loss of heterozygosity (LOH) in ~60% of breast tumors, and that there is a significant correlation between 11p LOH, lymphatic invasion and aggressive metastatic disease. Our data suggests that chromosome 11p15.5 harbors a tumor/metastasis suppressor gene. An intriguing candidate gene that we have mapped to the tumor/metastasis suppressor locus on chromosome 11p15.5 is Integrin-linked kinase (ILK). ILK is a newly identified ankyrin-repeat containing serine/threonine kinase that binds to the cytoplasmic domains of both ß1 and the ß3 integrins. 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.

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Pratima Karnik

PI - Signature Date Sep. 18. 2001

Table of Contents

A. INTRODUCTION:

Genetic alterations at the short arm of chromosome 11 are a frequent event in the etiology of cancer. Several childhood tumors demonstrate LOH for 11p including rhabdomyosarcoma (1), adrenocortical carcinoma (2), hepatoblastoma (3), mesoblastic nephroma (4) and Wilms' tumors (5). Recurrent LOH at 11p is also observed in adult tumors including bladder (6), ovarian (7), lung carcinomas (8), testicular cancers (9), hepatocellular carcinomas (10) and breast carcinomas (11,12), suggesting the presence of one or more critical tumor gene(s) involved in several malignancies. We have identified loss of heterozygosity (LOH) of 11p15 and microsatellite instability at a specific marker D11S988 on chromosome 11p15 as late genetic events in mammary tumorigenesis (11). This suggests a crucial role for this region in breast cancer progression. More recently, we have mapped and identified two distinct regions on chromosome 11p15 that are subject to LOH during breast tumor progression and metastasis (12). We have found a significant correlation between loss of heterozygosity at the two chromosomal regions and the clinical and pathological features of the breast tumors. LOH in region 1 correlated with tumors that contain ductal carcinoma in situ synchronous with invasive carcinoma. This suggests that the loss of a critical gene in this region may be responsible for early events in malignancy or invasiveness. LOH at region 2 correlated with clinical parameters which portend a more aggressive tumor and a more ominous outlook for the patient, such as aneuploidy, high S-phase fraction and the presence of metastasis in regional lymph nodes. Our data strongly suggests the presence of a metastasis suppressor gene on chromosome 11p15.5 (12). Winquist et al (13) have shown that LOH for chromosome 11p15 is associated with poor survival after metastasis. The association between 11p LOH, tumor progression and metastasis, that we describe, is analogous to the observations made in other epithelial tumors. For example, LOH at 11p correlated with advanced T stage and nodal involvement in Non-small cell lung carcinoma (14) as well as subclonal progression, hepatic involvement (15), and poor survival in ovarian and breast carcinomas (7,13). Phillips et. al. (16) have shown that micro-cell mediated transfer of a normal human chromosome 11 into the highly metastatic breast cancer cell line MDA-MB-435, had no effect on tumorigenecity in nude mice, but suppressed metastasis to the lung and regional lymph nodes. These studies further support our observation that chromosome 11 harbors a tumor/metastasis suppressor gene.

An intriguing candidate gene that we have mapped to the metastasis suppressor locus on chromosome 11p15.5 is the Integrin-linked kinase (ILK)(12). ILK is a newly identified ankyrin-repeat containing serine/threonine kinase (17), that binds to the cytoplasmic domains of both ß1 and the ß3 integrins. 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 (18). 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 (19). Although *ILK* maps to the commonly deleted chromosome 11p, the potential of this gene to serve as a tumor suppressor has not been established. We therefore analyzed the effect of *ILK* expression on the *in vitro* and *in vivo* tumor growth and invasion of human mammary carcinoma cells.

B. BODY:

Localization of *ILK* to the LOH region on chromosome 11p15.5

The LOH region 2 (Karnik et al., 1998) extends between the markers D11S1760-D11S1331 on chromosomal band 11p15.5 (Figure-1). We constructed a 500 kb genomic contig (Karnik et al, unpublished results) that includes the critical region between D11S1760 and D11S1331. Using a PCR-based screening method, we initially isolated PAC and BAC clones that contained D11S1760 and D11S1331 markers. The order of the genomic clones in the contig was confirmed by mapping of STSs, ESTs, unigene clusters and known genes that were previously mapped to chromosome 11. Eleven novel

transcripts and seven previously reported genes were PCR-mapped to the critical region between D11S1760 and D11S1331. Three of the known genes, Tata box-binding protein-associated protein (*TAF II 30*) (20), Lysosomal pepstatin insensitive protease (*CLN2*) (21) and Integrin -linked kinase (*ILK*) (17) were previously mapped only at the level of cytogenetic resolution. However, with the current mapping data, we have been able to determine the precise genomic locations of these three genes (Figure-1). The map location and its role in multiple signaling pathways makes ILK an attractive candidate tumor suppressor gene.

Loss of *ILK* expression in human breast carcinomas

To determine whether *ILK* has a role in breast cancer progression, mRNA expression in normal and tumor breast epithelial cells was compared by Northern blot hybridization (Figure-2). A single 1.8 kb *ILK* mRNA is highly expressed in all samples of normal breast epithelial cells. Three representative examples are shown in Figure-2 (N1, N7 and N8) In sharp contrast, there is complete loss of *ILK* mRNA expression in 9 out of 15 (~60%) invasive breast tumors and a 2-5 fold down-regulation of *ILK* mRNA in the remaining breast tumors (Figure-2A). Comparison of *ILK* mRNA expression in a panel of well-characterized breast cancer cell lines and in the non-malignant breast epithelial cell line MCF-10A is shown in Figure-2B. *ILK* mRNA expression in MCF-10A is comparable to the expression in normal breast tissue (N7, N8) (Figure-2B). However, there is a 3-5 fold down-regulation of *ILK* mRNA expression in the breast cancer cell lines MCF-7, T47D, ZR75.1, MDA-468, MDA-134, MDA-231 and MDA-435 (Figure-2B).

To further confirm these observations, *ILK* protein expression was also examined using indirect immunofluorescence microscopy in frozen samples of 20 normal and corresponding pathological human breast tissue samples. Figure-3 shows four representative examples. Immunohistochemical staining of normal breast tissue with *ILK*-specific primary antibody and rhodamine labeled secondary antibody shows specific staining of the mammary epithelial cells surrounding the lumen in normal breast tissue from breast cancer patients. *ILK* expression is particularly intense in epithelial cells both within large ducts and within terminal duct lobular units but not in the stromal compartment. Incubation with purified nonspecific rabbit immunoglobulin IgG, did not result in any positive staining of the normal epithelium of the breast (control). The normal breast tissue from four representative patients were positive, (3N, 12N, 6N and 10N) whereas ILK expression was nearly completely lost in the four corresponding infiltrating ductal carcinomas (3T, 12T, 6T, 10T) (Figure-3). These data show that *ILK* production by breast tumor cells correlates inversely with tumorigenecity and metastatic potential.

The *ILK* gene maps to chromosome 11p15.5 a region that displays a high frequency (~60%) of LOH in breast cancer. All breast tumor samples described in Figures 2 and 3 have previously been identified to contain LOH at the 11p15.5. (12). Allelic loss results in the reduction of gene dosage and thus may result in decreased expression. However, as seen in Figure-2, all tumors have LOH for 11p15.5 and yet, only some tumors show complete loss of ILK expression. Therefore, intragenic mutations or epigentic mechanisms might contribute to the biallelic silencing of the ILK gene in breast tumors. We sought to determine if mutations are involved in the dysregulation of the *ILK* gene during the progression of human breast cancer. The *ILK* gene consists of 13 exons (Melchior et al., 2000, GenBank database, GI accession AJ404847). Primers derived from the sequences flanking each exon of *ILK* were used to analyze genomic DNA from 20 invasive breast tumors and matched normal tissue from the same patients. Using PCR-single strand conformation polymorphism (PCR-SSCP), only one of the 20 tumors analyzed showed a band shift in the SSCP assay. Subsequent DNA sequencing confirmed a silent mutation at codon 352 (GCA--->GCG) (data not shown). These results demonstrate that *ILK* mRNA and protein expression is consistently down-regulated during the progression of human breast cancer and this down-regulation does not commonly

involve mutations. Epigenetic mechanisms as a probable cause of *ILK* gene silencing are currently under investigation.

ILK suppresses cell growth in human breast carcinoma cells

The inverse correlation between *ILK* expression and tumorigenecity suggested the hypothesis that elaboration of *ILK* by tumor cells into their environment may exert an inhibitory effect. To test this hypothesis, we transfected the human breast carcinoma cell line MDA-MB-435 with the *ILK* cDNA. This cell line synthesizes very low levels of *ILK* compared to normal mammary epithelial cells (Figure-2B) and can be injected into the mammary fat pad of nude mice to provide an orthotopic model system for human breast cancer tumorigenecity and metastasis. The MDA-MB-435 cells were transfected with a mammalian expression vector pIRES-EGFP containing full length *ILK* cDNA under control of the CMV promoter. A total of four stable clones expressing different levels of *ILK* have been established. Comparison of mRNA expression by Northern blot analyses revealed that the clones TR4 and TR5 expressed slightly higher levels of *ILK* mRNA compared to the clones TR2 and TR3 (Figure 4B). Based on Northern analysis, *ILK* expression in clone TR5 is 2-3 fold higher compared to the expression in the non-malignant breast epithelial cell line MCF-10A and to the expression in normal mammary epithelial cells (Figure-2B) suggesting that ILK is overexpressed in the TR5 clone. The expression of *ILK* in empty vector controls (data not shown) is comparable to untranfected MDA-MB-435 cells (UT). *ILK* protein levels in transfected (TR5) and untransfected cells was determined by indirect immunofluorescence. High levels of *ILK* protein are expressed in the transfected MDA-MB-435 cells (Figure 4Ac) compared to the untransfected control (Figure-4Ab). The *ILK* protein is localized in the cytoplasm. Most strikingly, corresponding to the low levels of *ILK* mRNA (Fig. 2B), the highly metastatic MDA-MB-435 cell line showed very little detectable *ILK* protein (Figure 4Ab).

To determine whether *ILK* overexpression had any effect on the growth properties of the MDA-MB-435 cells, we determined the growth kinetics of the clones TR3 and TR5. *ILK* expression causes the MDA-MB-435 cells to grow to a low saturation density (Figure-5A) and there is substantial growth suppression of the TR5 clone compared to untransfected MDA-MB-435 cells. The growth suppression of the transfectants was *ILK* concentration dependent with TR5 (high expressing clone) growing to a lower saturation density than TR3 (low expressing clone). Furthermore, the growth rate of TR5 was decreased by ~ 40% with a cell doubling time of 96 hours compared to the growth rate of cells transfected with vector alone or untransfected cells which had a doubling time of 48 hours.

The ability of *ILK* to suppress growth could be due to a non-specific lethal effect of protein overproduction. Alternatively, it could be a manifestation of a more specific effect on cell proliferation. To further investigate these possibilities and to establish a link between a functional *ILK* and growth suppression, we tested the growth kinetics of two *ILK* variants. *ILK* contains four ankyrin repeats at the NH2-terminus (22) that participate in protein-protein interactions important for integrin-, growth-factor- and Wnt- mediated signaling. First, a deletion mutant, Δ ANK lacking this domain was constructed. In addition, the residue E359 has been shown to be essential for *ILK* function (22). We therefore constructed an *ILK* point mutant (E359K) in which the highly conserved Glu359 within the *ILK* catalytic domain was substituted with lysine. The growth rates of the stably transfected *ILK* mutant clones Δ ANK and E359K compared to the *ILK* transfectant TR5 are shown in Figure 5B. As discussed above, overexpression of the wild-type *ILK* strongly inhibited growth of the MDA-MB-435 cells. In contrast, both the Δ ANK and E359K mutants lost their capacity to suppress the growth of the MDA-MB-435 cells (Figure-5B) arguing against a non-specific effect of protein overproduction.

Expression of *ILK* in MDA-MB-435 Cells Leads to a G1 Cell Cycle Arrest

The observed growth suppression by *ILK* could be caused by either increased apoptosis or inhibition of cell proliferation. To investigate the mechanisms underlying the growth suppression by *ILK* expression, we studied apoptosis by fluorescence-activated cell sorting (FACS) analysis of Annexin-V stained *ILK* and vector transfectants. There was no increase in the rate of apoptosis in *ILK*-expressing cells compared to vector transfectants (data not shown). Therefore, programmed cell death does not seem to account for the growth suppression of *ILK* transfected cells.

To test for cell cycle regulation by ILK, propidium iodide stained MDA-MB-435 clones were analyzed by flow cytometry. Expression of ILK increased the number of cells in G0/G1 from 64 to 85% (Figure-5C, VT and TR5-ILK) and decreased inversely the number of cells in S and G2/M phase from 26 and 10% to 9 and 5% (Figure-5C, VT and TR5-ILK). In contrast, the cell cycle profiles of the two ILK variants Δ ANK and E359K were very similar to the parental MDA-MB-435 cells. These results indicate that ILK growth suppression results from G1 cell cycle arrest. The accumulation of cells in the G0/G1 phase of the cell cycle suggests arrest predominantly at the G1/S boundary. ILK overexpression does not induce cell death or apoptosis but induces a very pronounced growth arrest with 85% of the cells in G0/G1, a property that is the hallmark of growth/tumor suppressors. Growth suppressor genes play an important role in checkpoint function and loss of genes associated with checkpoint functions seem to have important implications in the development of cancer. The percentage G1 arrest induced by *ILK* is comparable to the effect on cell cycle progression induced by the *p21* cyclin-dependent kinase inhibitor (23).

ILK Suppresses the Invasive Phenotype of Human Breast Carcinoma Cells

The invasiveness of tumor cells represents one of several important properties necessary for the formation of metastases. Cell migration on vitronectin in vitro has been linked to the metastatic capacity of tumor cells in vivo (24,25). To examine the effects of *ILK* expression on breast cancer cell invasion, the ability of vector and *ILK* transfected MDA-MB-435 cells to degrade and invade vitronectin -coated polycarbonate membrane was investigated. As shown in Figure-6A, a significant reduction in invasive potential was noted in the ILK expressing clone TR5 (ILK) compared to vector transfected MDA-MB-435 cells (VT) (Figure-6A). Cell invasion through membranes coated with vitronectin, is decreased by 60% in MDA-MB-435 cells expressing *ILK* compared to vector transfected MDA-MB-435 cells. In contrast, the two ILK variants Δ ANK and E359K have no significant effect on cell invasion under identical conditions (Figure-6A). In fact, there is a slight increase in invasive potential of the variant clones (Δ ANK and E359K), suggesting a dominant-negative effect, perhaps due to inhibition of endogenous ILK in the MDA-MB-435 cells. These results indicate that *ILK* expression abates extracellular matrix invasion of tumor cells *in vitro*, one of the hallmarks of tumorigenecity and transformed cell growth.

Cell adhesion, migration and invasion are controlled by the levels of integrins and by the amount of fibronectin matrix around the cell (18). Because the αvβ3 and α5ß1 integrins have been implicated in the regulation of angiogenesis, tumor cell migration, invasion and metastasis, we speculated that *ILK* might regulate cell migration via alteration of the cellular composition of integrins. Using a panel of specific antibodies against these integrins in flow cytometry analysis, we compared integrin expression patterns in relation to the *ILK* expression status. The results are shown in Figure-6B. The *ILK* transfected cells demonstrated a 22% increase in levels of the growth-suppressing integrin α5ß1 and a 31% decrease in levels of the growth-promoting integrin αvβ3 compared to the control cells. The changes in levels of αvβ3 and α5ß1 expression in *ILK* transfected cells although relatively moderate in comparison to control cells, nonetheless, were highly significant. Collectively, these observations suggest that *ILK* reduces the invasive potential of MDA-

MB-435 cells by altering their integrin profiles, which changes their ability to perceive and interact with their extracellular environment.

ILK suppresses tumor formation and metastasis in nude mice

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 (26). 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 $\pm$ s.d.) at 15 weeks (Fig. 7A 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-7C). 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.

C. CONCLUSIONS:

Once breast cancer has been diagnosed, the most crucial question is whether the cancer is confined to the breast or whether it has already spread to distant sites. Unfortunately, there is no prognostic method to identify cells possessing the metastatic phenotype within a primary tumor population. In primary breast cancer, the axillary lymph node status is still the most important prognostic factor and is used for deciding on adjuvant therapy. However, the prognostic value of the axillary lymph node is not absolute, as 30% of node-negative patients still die within ten years because of recurrent disease and 30% of node-positive patients survive ten years without disease. Therefore, routine axillary lymph node dissection has recently become a matter of debate, and search for other factors to identify patients at high risk of (early) relapse is thus needed. It is hoped that the identification of biochemical or genetic alterations will provide markers that can be applied to these clinical problems.

Loss of tumor/metastasis suppressor genes is an important event during progression of a tumor cell from a non-metastatic to a metastatic phenotype. Thus, knowledge of genetic loci and genes whose loss or inactivation contributes to metastasis development is of critical significance not only for basic knowledge, but also, perhaps, for the eventual design of novel therapeutic approaches and for crucial decisions of treatment and prognosis of the disease. We have reported that chromosome 11p15.5 exhibits loss of heterozygosity (LOH) in ~60% of breast tumors, and that there is a significant correlation between 11p LOH and clinical parameters which portend a more aggressive tumor and a more ominous outlook for the patient, such as aneuploidy, high S-phase fraction and the presence of metastasis in regional lymph nodes. Our data strongly suggest that

chromosome 11p15.5 harbors a metastasis suppressor gene for human breast cancer. An intriguing candidate gene that we have mapped to the metastasis suppressor locus on chromosome 11p15.5 is Integrin-linked kinase (ILK).

Growth inhibitory functions of *ILK*

The present study reveals that expression of *ILK* potently suppresses the growth and tumorigenecity of the human mammary carcinoma cells MDA-MB-435 *in vitro* and *in vivo*. This 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. The demonstration of a growth suppressive function establishes *ILK* as a tumor suppressor gene and directly implicates its loss in processes regulating the growth and maintenance of the malignant phenotype in human breast cancer. Our results strongly suggest that the growth suppression by *ILK* is not due to apoptosis but is mediated by its ability to block cell cycle progression at G1 phase. 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. Our results are consistent with earlier micro-cell mediated chromosome transfer experiments showing that introduction of human chromosome 11 into MDA-MB-435 cells suppressed metastasis in immunocompromised animals (16).

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 cell growth, survival, and differentiation. Like many tumor suppressor genes such as *p53, APC, p16INK4a* and *p21, ILK* arrests tumor cell growth by blocking cell-cycle progression in the G0/G1 phase. Growth suppressor genes play an important role in checkpoint function and silencing of genes associated with checkpoint functions seem to have important implications in the development of cancer. Integrin signals are necessary for cells to traverse the cell division cycle. Progression through the G1 phase of the cell cycle requires the sequential activation of the cyclin-dependent kinases *(Cdk's) Cdk 4/6 and Cdk 2* and the activities of these kinases are regulated by integrins (27). In view of our observation that *ILK* regulates cell-cycle progression at the G1 phase, it is quite probable that the integrin interactions with the *Cdk's* are mediated by *ILK*. *ILK* could interact with specific integrin cytoplasmic domains and couple them to appropriate downstream signaling pathways. This in turn could regulate such functions as coordination of growth factor signals and altering gene expression required for cell proliferation and differentiation.

The interaction of cells with the surrounding extracellular matrix (ECM) affects many aspects of cell behavior, including the migratory properties of cells, their growth, and differentiation (27). Integrins are transmembrane heterodimeric proteins that mediate such interactions. The large extracellular part of both α and ß subunits bind proteins within the ECM. The short cytoplasmic domain of the β integrin subunit anchors the cytoskeleton to the plasma membrane via intermediary adaptor proteins. In *Drosophila* (19), *ILK* has been shown to be a component of the structure linking the cytoskeleton and plasma membrane at sites of integrin-mediated adhesion. The absence of *ILK* function in *Drosophila* causes defects similar to loss of integrin adhesion. Similarly, the downregulation of *ILK* expression in mammary epithelial cells could cause the cells to become more invasive. Indeed, as seen in our present study, *ILK* overexpression in the highly metastatic breast cancer cell line MDA-MB-435 causes the cells to lose their tumorigenecity and metastatic potential. What is the biological significance of *ILK*-mediated regulation of the α5ß1 and αvβ3 integrins? Previous studies have shown that α5ß1 expression is frequently lost during malignant progression, a phenomenon that has been observed in human colonic, mammary and pancreatic cancer (28,29). Expression of the α5ß1 integrin in HT29 human colon carcinoma cells also blocks tumorigenecity in nude mice (30). In contrast, the αvβ3 integrin cooperates with certain growth factors, potentiating their effects on cells and its expression correlates with a role in metastasis. Indeed, expression of integrin αvβ3 is

significantly higher in breast tumors of patients with metastases than in those without metastasis and may have a role in skeletal metastases (31). Therefore, our observation that *ILK* modulates the levels of α5ß1 and αvβ3 integrins is very significant and suggests that *ILK* may reduce the invasive potential of MDA-MB-435 cells by altering their integrin profiles.

Frequent Down-Regulation and Lack of Mutations of the ILK Gene in Breast Carcinoma

We determined by Northern blot and immunohistochemical analysis that most invasive breast carcinomas exhibit complete loss or very low expression of *ILK* mRNA and protein. However, in our present study, we detected no homozygous deletions or intragenic mutations in the *ILK* gene. Thus, it is likely that the *ILK* gene is not a target for mutations in many cancers, and other mechanisms for *ILK* down-regulation should be considered. *ILK* maps to chromosome 11p15.5, a region that exhibits a high frequency (40-60%) of LOH in breast and other adult and childhood tumors (11). It is thought that LOH alone cannot completely suppress *ILK* expression, as many genes can be expressed monoallelically (32,33). Although all breast tumors used in this study were previously described (11) to have LOH at 11p15.5, a small number of breast tumors still express *ILK* suggesting that *ILK* can be expressed monoallelically. Biallelic inactivation of the *ILK* gene could result either from epigenetic inactivation of both parental alleles or from epigenetic modification of one allele and loss of the second allele via mechanisms that result in LOH. Indeed, the *p16/CDKN2* and the *p15INK4B* cell cycle regulator genes are located at a region of high LOH on chromosome 9p21 and individual alleles in neoplasia are selectively silenced by promoter hypermethylation (34,35). While the *Maspin* tumor suppressor gene is biallelically inactivated by aberrant cytosine methylation and heterochromatinization of the promoter (36), the down-regulation of the *KAI1* metastasis gene involves neither mutations nor promoter hypermethylation (37,38). It has been suggested that there is a group of tumor suppressor genes that are unrecognized because the primary mechanism for their silencing is not known. Such genes may affect the cancer cell phenotype by expression changes and have been classified as Class II tumor suppressor genes (39). The molecular basis for the down-regulation of the *ILK* tumor suppressor gene in breast cancer is currently under investigation. The loss of expression that occurs during malignant progression of primary breast tumors suggests that *ILK* has potential value as a prognostic marker. Future studies should test the prognostic value of *ILK* on a larger scale, in order to establish more firmly a correlation between loss of *ILK* expression and progression of breast and other cancers.

The paradoxical effect of *ILK* on tumorigenecity.

Previous studies have shown that *ILK* overexpression results in loss of cell-cell adhesion (40), promotes suppression of anoikis by activation of *PKB/Akt* signaling (22) and oncogenic transformation of the rat intestinal epithelial cells by activation of the *LEF-1/ß-catenin* signaling pathways (41,42). In stark contrast, recent studies in *Drosophila* (19) have shown that *ILK* is required for integrin-mediated adhesion, but not for signaling involving *ß-catenin (armadillo)* or *PKB*. *ILK* mutations in *Drosophila* cause embryonic lethality and defects in muscle attachment, and clones of cells lacking ILK in the adult wing fail to adhere, forming wing blisters. The *ILK* coding sequence is highly conserved in different species (19), suggesting that it has an essential biological function in evolution. Our present data is consistent with the observations made in *Drosophila*. We have shown that transfection of the MDA-MB-435 mammary carcinoma cells with the *ILK* gene reduced the cells' ability to induce tumors and to invade through vitronectin membranes *in vitro*. The down-regulation of *ILK* in metastatic breast cancer cell lines and invasive breast tumors strongly suggests that *ILK* might block uncontrolled cell growth in normal breast tissue and that its absence may be permissive for malignant tumor growth. The negative correlation between *ILK* expression and growth suppression is unexpected when

considered with the current concept that kinases are positively associated with tumorigenesis (for example, c-erB2). However, Lynch et al.(43) have demonstrated that *ILK* is not a typical protein kinase and lacks a DFG motif or a conserved substitute for the catalytic aspartate residue found in other kinases and they and other investigators (44) have failed to detect protein kinase activity in *ILK* immunoprecipitates. Recent evidence (43) strongly suggests that *ILK* does not possess serine-473 kinase activity but functions as an adaptor to recruit either a serine-473 kinase or phosphatase. Mutations in the kinase domain shown to inactivate the kinase activity of human *ILK* do not show any phenotype in *Drosophila* (19), suggesting a kinase independent function for *ILK*. Thus, it is likely that the functions of ILK are more complex than previously envisioned; and the divergent and often paradoxical effects mediated by *ILK* may depend on the particular cell-type, the cell-specific integrins that are activated by a cell, and on whether the adaptor protein *ILK* activates a serine-473 kinase or phosphatase.

In conclusion, we have shown that the loss of *ILK* expression is associated with the acquisition of a malignant breast tumor phenotype and that *ILK* may directly act as a tumor suppressor, presumably by controlling cell division. The absence of the *ILK* tumor suppressor protein, may promote uncoordinated G1 cell cycle progression, allowing cells to bypass the normal signaling processes regulated by growth factors and cell anchorage, leading to tumorigenesis. This novel information regarding the biological effects of *ILK* provides hopeful therapeutic utility for this potent tumor suppressor gene in the management of breast cancer.

D. KEY RESEARCH ACCOMPLISHMENTS:

- Chromosome 11 harbors a breast cancer tumor/metastasis suppressor gene (5,11,12).
- Integrin linked kinase (ILK) is a key candidate gene that maps to this region (11).
- ILK expression is downregulated in breast carcinomas that metastasize
- ILK expression inhibits the *in vitro and in vivo* growth of the metastatic breast cancer cell line MDA-MB-435
- Growth suppression by *ILK* is not due to apoptosis but is mediated by its ability to block cell cycle progression in the G1 phase.
- ILK functions as a tumor suppressor gene in breast cancer (manuscript submitted for publication).

E. REPORTABLE OUTCOMES:

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. 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.

Reportable Outcomes (continued)

Publications resulting from this award:

1. **Karnik P**, Paris M, Williams BRG, Casey G, Crowe J and Chen P. Two Distinct tumor suppressor loci within chromosome 11p15.5 mediate breast tumor progression and metastasis. *Hum. Mol. Gen.* 7: 895-903. 1998

2. **Karnik P**, Chen P, Paris M, Yeger H and Williams BRG. Loss of heterozygosity at chromosome 11p15 in Wilms tumor: identification of two independent regions. *Oncogene* 17: 237-240, 1998
3. Chen P and **Karnik P.** Integrin-Linked Kinase, a tumor suppressor gene for Breast Cancer on chromosome 11p15.5. (Submitted)

Abstracts:

1. **Karnik P**, Chen P, Tidwell N and Shen W-Z. Identification of breast cancer associated genes on chromosome 11. Cancer Genetics and Tumor Suppressor Genes Meeting, Cold Spring Harbor, 1998

2. **Karnik P**, Williams BRG, Casey G, Crowe J and Chen P. Two regions of consistent deletion in breast cancer map within chromosome 11p15.5-p15.4. "Era of Hope", Department of Defense Breast Cancer Research Program Meeting, Washington DC, November 1997.

3. **Karnik P** and Williams BRG. LOH mapping of two distinct tumor suppressor loci on chromosome 11p15 in breast cancer. Oncogenes and Tumor Suppressor genes, Cold Spring Harbor Meetings, NewYork, August 1996.

Funding applied for based on work supported by this award

2000-2003 DOD US Army Medical Research and Material Command grant, "Role of Integrin Linked Kinase in Breast Cancer Metastasis"
PI Pratima Karnik, Total Award: $326,250.

Cell Lines:

MDA-MB-435 cell lines with ectopic expression of wild-type and mutant forms ILK have been developed.

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APPENDIX

FIGURE LEGENDS:

Figure-1: Localization of *ILK* gene to the tumor suppressor region (LOH region 2) on chromosome 11p15.5. A transcript map of the LOH region is schematically represented with the relative location of the polymorphic markers, known genes, unigene clusters and expressed sequence tags (EST's).

Figure-2: Northern blot analysis of *ILK* mRNA expression. (A) Total RNA was isolated from normal breast tissue (N1, N7, N8), and fifteen invasive breast tumors (T1 to T15). B) Total RNA from exponentially growing non-malignant (MCF-10A), non-metastatic (MCF-7, T47D, ZR75.1, MDA-468 and MDA-134) and metastatic (MDA-435, MDA-231) breast cancer cell lines and normal breast tissue (N7, N8) was hybridized with ^{32}P-labeled *ILK* probe. Hybridization with the β-actin probe serves as control.

Figure-3: Immunohistochemical detection of *ILK* expression in normal breast tissues (3N, 12N, 6N, 10N) and corresponding invasive ductal carcinomas (3T, 12T, 6T, 10T). Normal ducts were positive for *ILK* expression whereas the invasive ductal carcinomas expressed little or no *ILK*. Control-Normal tissue minus primary antibody.

Figure-4: MDA-MB-435 cells were transfected with pIRES-EGFP vector containing full length *ILK* cDNA and four stable clones were isolated. (A) Immunohistochemical analysis of *ILK* expression in the MDA-MB-435 cells (b) before and (c) after transfection (stable clone TR5-*ILK*) (a) no primary antibody control. (B) Northern blot analysis of parental (UT) and *ILK* transfected MDA-MB-435 cells. TR2, 3, 4 and 5 represent stable *ILK* expressing clones. mRNA expression was determined by hybridization with ^{32}P-labeled *ILK* probe. β-actin expression serves as control.

Figure-5: Growth effects of wild type and mutant alleles of *ILK* in MDA-MB-435 breast cancer cells. The MDA-MB-435 cells were transfected with either full-length *ILK* cDNA, *ILK* mutant ΔANK, *ILK* mutant E359K or eukaryotic expression vector and stable clones were obtained. (A) Growth rates of two stable *ILK* expressing MDA-MB-435 cell clones (TR3-*ILK* and TR5-*ILK*) that contain full length *ILK* cDNA compared with a stable clone containing empty vector (VT) and untransfected MDA-MB-435 cells (UT). (B) Growth rates of *ILK* mutants ΔANK and E359K compared with the wild type *ILK* expressing clone TR5-*ILK*. The means of three independent experiments are shown. Bars represent SE. (C) Cell-cycle analysis by propidium iodide staining in MDA-MB-435 cells (UT), transfected with empty vector (VT), with full length *ILK* cDNA (TR5-*ILK*) or with the *ILK* mutants ΔANK and E359K. The regions between the vertical lines from left to right represent cells in G0/G1, S and G2/M respectively.

Figure-6: Cell invasion assay of MDA-MB-435 cells transfected with vector (VT), full length *ILK* and its variants (ΔANK, E359K). Cell invasion through vitronectin was analyzed using a modified Boyden chamber. Cells that invaded to the lower surface of the membrane were lyzed and absorbance determined at 560 nm. (B) Flow cytometric analysis of α5β1 and αvβ3 integrins expressed on the surface of *ILK* transfected and parental MDA-MB-435 cells. The relative fluorescence intensity of cells stained with α5β1 and αvβ3 antibodies is represented as percentage of cell shift. Bars represent S.E.

Figure-7: (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

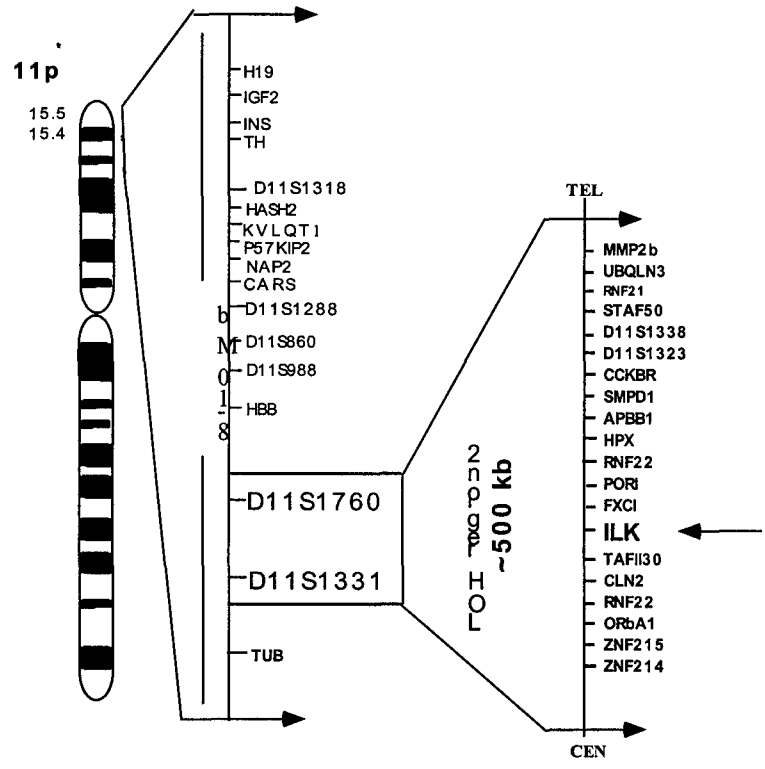

11p
15.5
15.4
H19
IGF2
INS
TH
D11S1318
HASH2
KVLQT1
P57KIP2
NAP2
CARS
D11S1288
D11S860
D11S988
HBB
D11S1760
D11S1331
TUB
bMQ1-8
2mpgx HOL
~500 kb
TEL
MMP2b
UBQLN3
RNF21
STAF50
D11S1338
D11S1323
CCKBR
SMPD1
APBB1
HPX
RNF22
PORI
FXCI
ILK
TAFII30
CLN2
RNF22
ORbA1
ZNF215
ZNF214
CEN

Figure 1

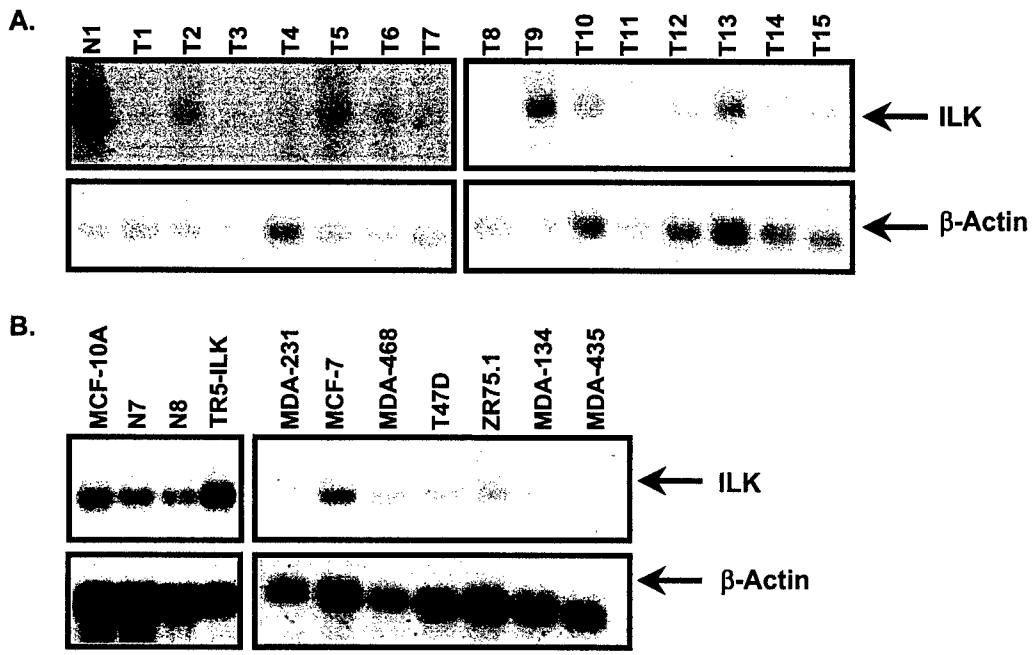

A.
N1
T1
T2
T3
T4
T5
T6
T7
T8
T9
T10
T11
T12
T13
T14
T15
ILK
β-Actin
B.
MCF-10A
N7
N8
TR5-ILK
MDA-231
MCF-7
MDA-468
T47D
ZR75.1
MDA-134
MDA-435
ILK
β-Actin

Figure 2

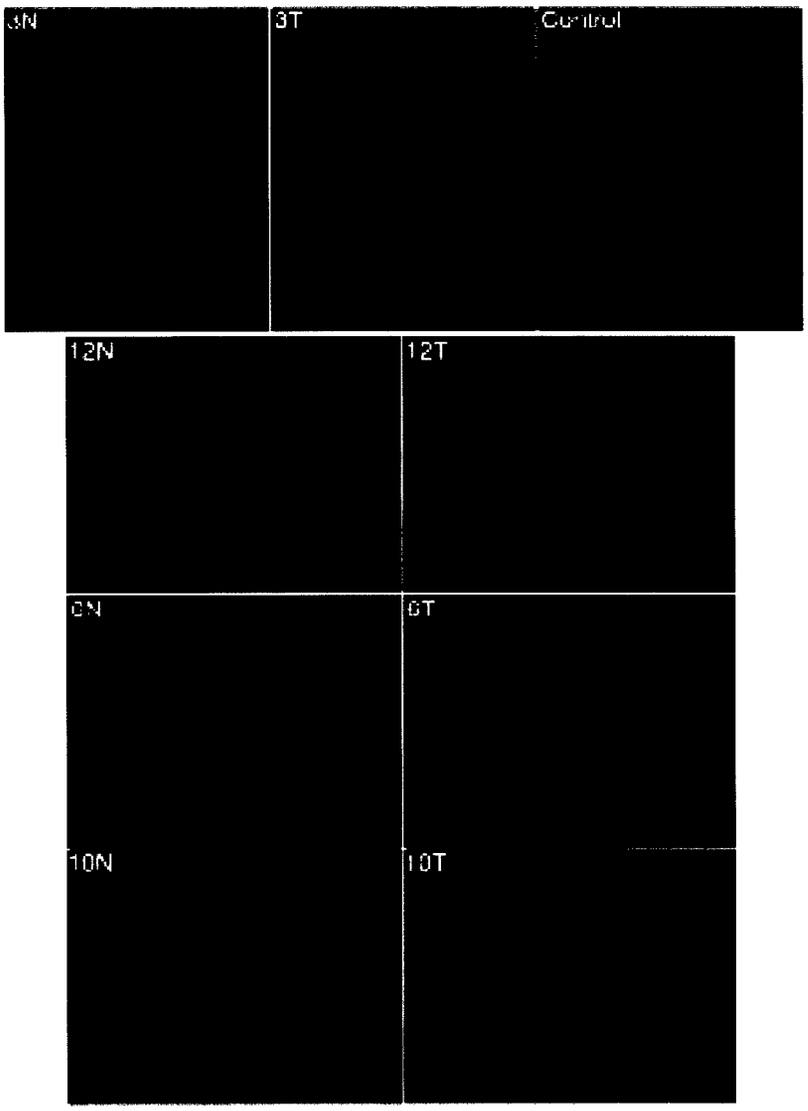
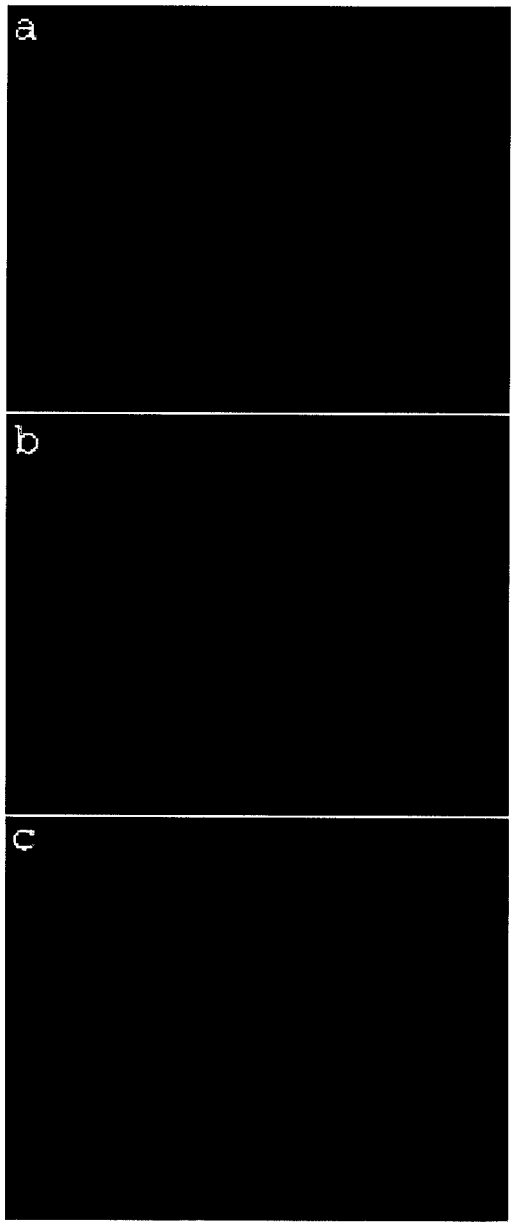


Fig. 3 Immunohistochemical detection of ILK expression in normal breast tissues (3N, 12N, 6N, 10N) and corresponding invasive ductal carcinomas (3T, 12T, 6T, 10T). Normal ducts were positive for ILK expression whereas the invasive ductal carcinomas expressed little or no ILK. Control- Normal tissue minus primary antibody.

A.



B.

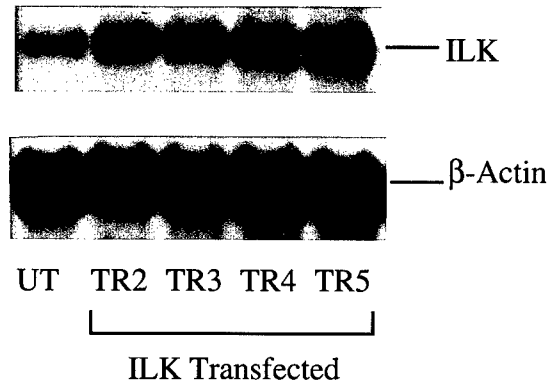


Fig. 4 MDA-MB-435 cells were transfected with pIRES2-EGFP vector containing full length ILK cDNA and four stable clones were isolated. (A) Immunohistochemical analysis of ILK expression in the MDA-MB -435 cells (b) before and (c) after transfection (stable clone TR5-ILK) (a) no primary antibody control. (B) Northern blot analysis of Parental (UT) and ILK transfected MDA-MB-435 cells. TR2,3,4 and 5 represent stable ILK expressing clones. mRNA expression was determined by hybridization with ^{32}p labeled ILK probe. β-Actin serves as control.

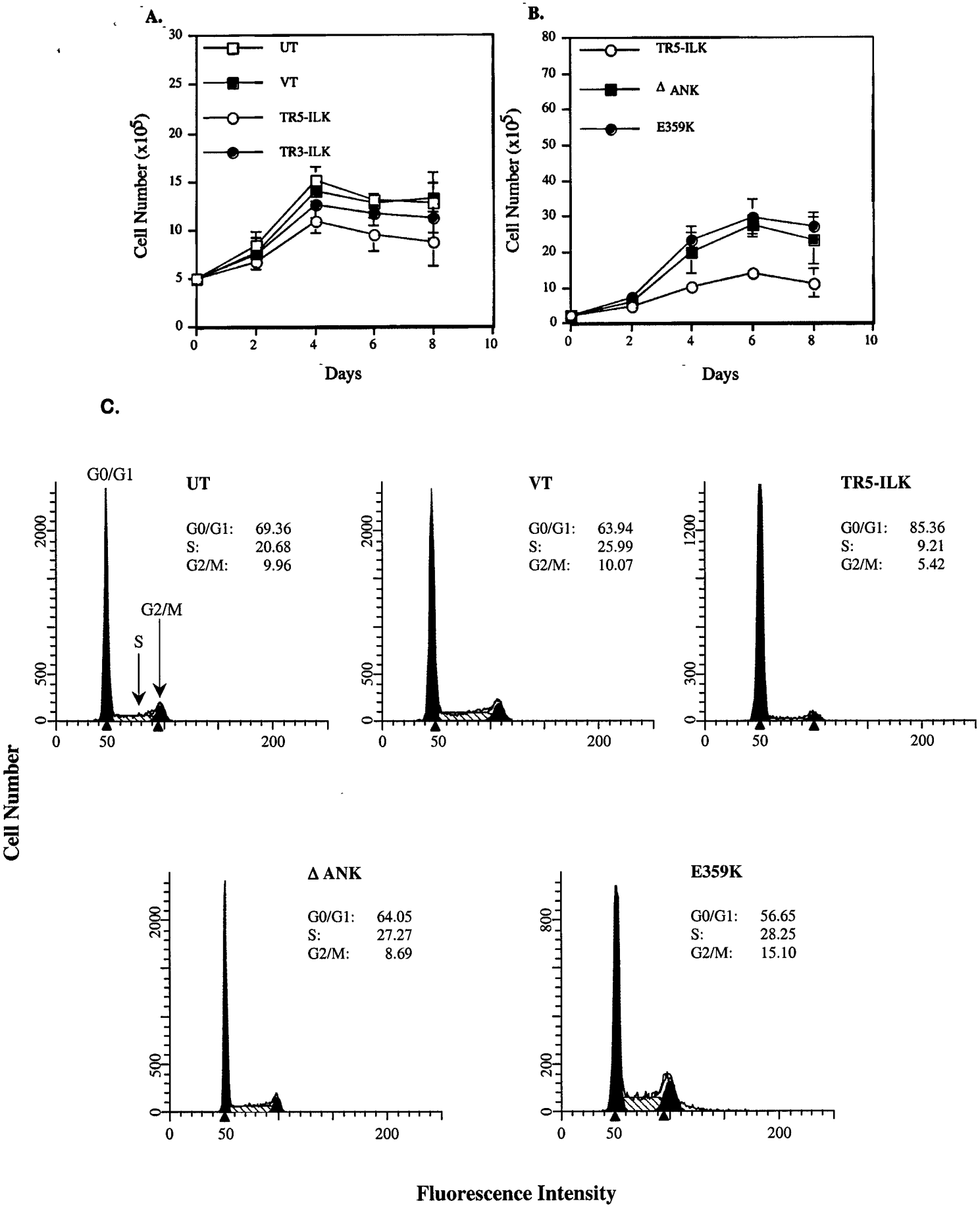

A.
B.
C.
UT
VT
TR5-ILK
TR3-ILK
Δ ANK
E359K
Cell Number (x10^5)
Days
G0/G1
S
G2/M
G0/G1: 69.36
S: 20.68
G2/M: 9.96
G0/G1: 63.94
S: 25.99
G2/M: 10.07
G0/G1: 85.36
S: 9.21
G2/M: 5.42
G0/G1: 64.05
S: 27.27
G2/M: 8.69
G0/G1: 56.65
S: 28.25
G2/M: 15.10
Cell Number
Fluorescence Intensity

Figure 5

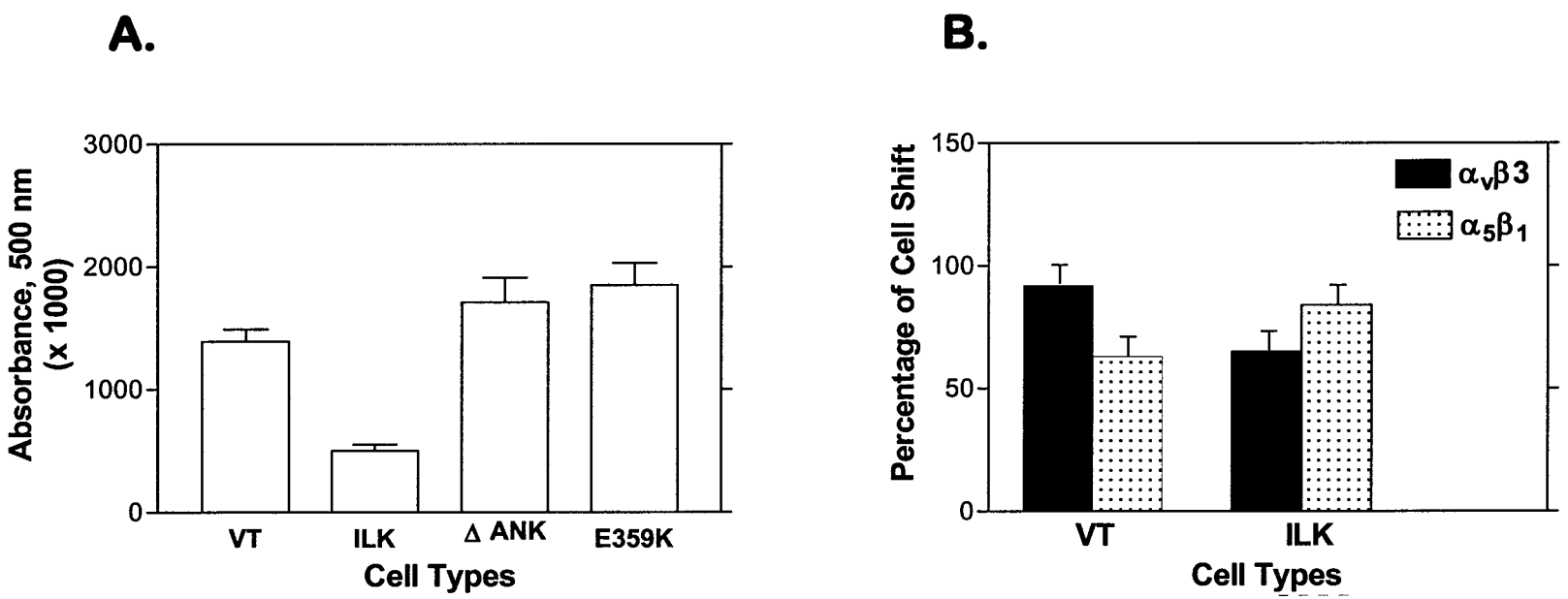
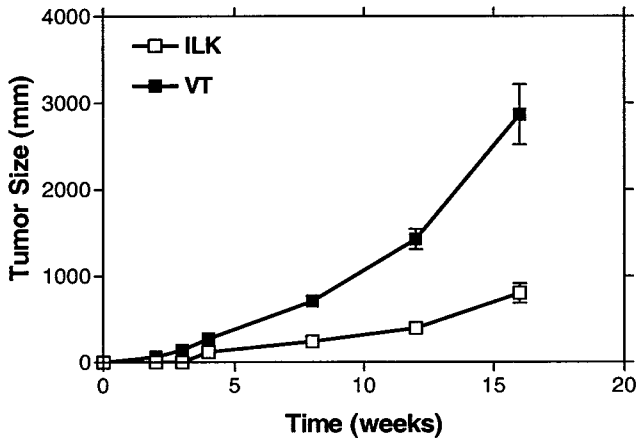
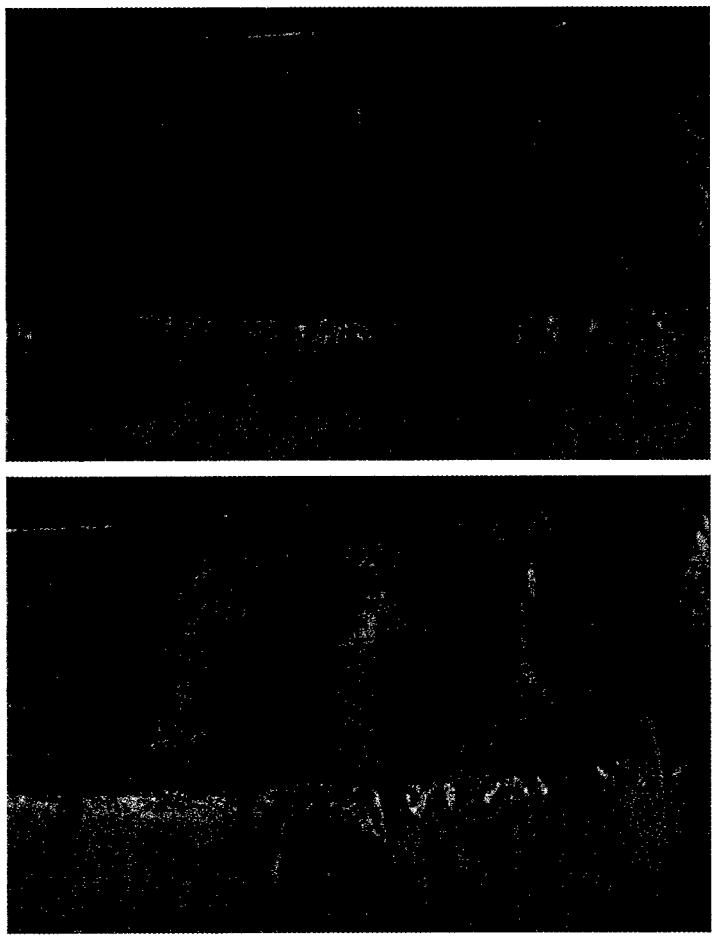

A.
Absorbance, 500 nm (x 1000)
3000
2000
1000
0
VT
ILK
Δ ANK
E359K
Cell Types
B.
Percentage of Cell Shift
150
100
50
0
αvβ3
α5β1
VT
ILK
Cell Types

Figure 6

A.


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

B.



C.

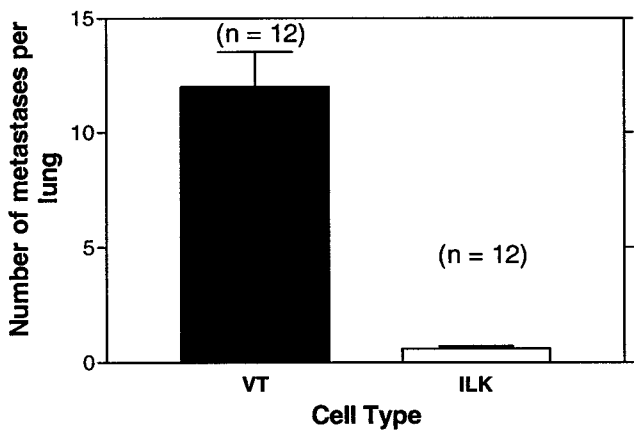

15
10
5
0
(n = 12)
(n = 12)
Number of metastases per lung
VT
ILK
Cell Type

Figure 7

List of Personnel receiving pay from the research effort

Dr. Pratima Karnik- PI
Ping Chen- Senior Research Technologist
Wei-Zhen Shen-Research Technician

Human Molecular Genetics, 1998, Vol. 7, No. 5 895–903

Two distinct tumor suppressor loci within chromosome 11p15 implicated in breast cancer progression and metastasis

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Chromosome 11p15 has attracted considerable attention because of the biological importance of this region to human disease. Apart from being an important tumor suppressor locus showing loss of heterozygosity (LOH) in several adult and childhood cancers, 11p15 has been shown by linkage analysis to harbor the gene(s) for the Beckwith–Wiedemann syndrome. Furthermore, the clustering of known imprinted genes in the 11p15.5 region suggests that the target gene may also be imprinted. However, positional cloning efforts to identify the target genes have been complicated by the large size (~10 Mb) and complexity of LOH at 11p15. Here, we have analyzed 94 matched normal and breast tumor samples using 17 polymorphic markers that map to 11p15.5–15.4. We have defined precisely the location of a breast tumor suppressor gene between the markers *D11S1318* and *D11S4088* (~500 kb) within 11p15.5. LOH at this region occurred in ~35–45% of breast tumors analyzed. In addition, we have fine-mapped a second, critical region of LOH, that spans the markers *D11S1338–D11S1323* (~336 kb) at 11p15.5–p15.4, that is lost in ~55–60% of breast tumors. There is a striking correlation between the loss of the two 11p loci and the clinical and histopathological features of breast tumors. LOH at region 1 correlated significantly ($P = 0.016$) with early events in malignancy and invasiveness. In contrast, the loss of the more proximal region 2, is highly predictive ($P = 0.012$) of aggressive metastatic disease. Thus, two distinct tumor suppressor loci on chromosome 11p15 may contribute to tumor progression and metastasis in breast cancer. The fine mapping of this intriguing chromosomal region should facilitate the cloning of the target genes and provide critical clues to understanding the mechanisms that contribute to the evolution of adult and childhood cancers.

INTRODUCTION

Breast cancer is both genetically and clinically a heterogeneous and progressive disease. The severity of disease may be determined by the accumulation of alterations in multiple genes that regulate cell growth and proliferation. The inactivation of tumor suppressor genes, by a two-hit mechanism involving mutations and loss of heterozygosity (LOH), appears to be a common event in the genetic evolution of breast carcinomas (1). Several chromosome arms, including 1p, 1q, 3p, 11p, 11q, 13q, 16q, 17p, 17q and 18q, have been reported to show moderate (20–40%) to high (>50%) frequencies of LOH in breast tumors (1). This implies that multiple tumor suppressor genes are likely to be involved in the development and progression of breast cancer.

Genetic alterations at the short arm of chromosome 11 are a frequent event in the etiology of cancer (2–17). Several childhood tumors demonstrate LOH for 11p, including rhabdomyosarcoma (7,8), adrenocortical carcinoma (9), hepatoblastoma (10), mesoblastic nephroma (11) and Wilms' tumors (WT) (12). Recurrent LOH at 11p is also observed in adult tumors including bladder (13), ovarian (14), lung carcinomas (15), testicular cancers (16), hepatocellular carcinomas (17) and breast carcinomas (2–6), suggesting the presence of one or more critical tumor suppressor gene(s) involved in several malignancies.

Birch *et al.* (18) have reported an increased risk of breast cancer among mothers of children with embryonal rhabdomyosarcoma, providing genetic evidence for the apparent high-risk association between these two tumor types. The familial association between breast cancer and rhabdomyosarcoma and the other childhood tumors may well be the consequence of alterations in chromosome 11p15. The ability of a tumor suppressor gene(s) on chromosome 11 to re-establish control of the malignant phenotype has been demonstrated by transfer of a normal human chromosome 11 to the breast cancer cell line MDA-MB-435 (19). However,

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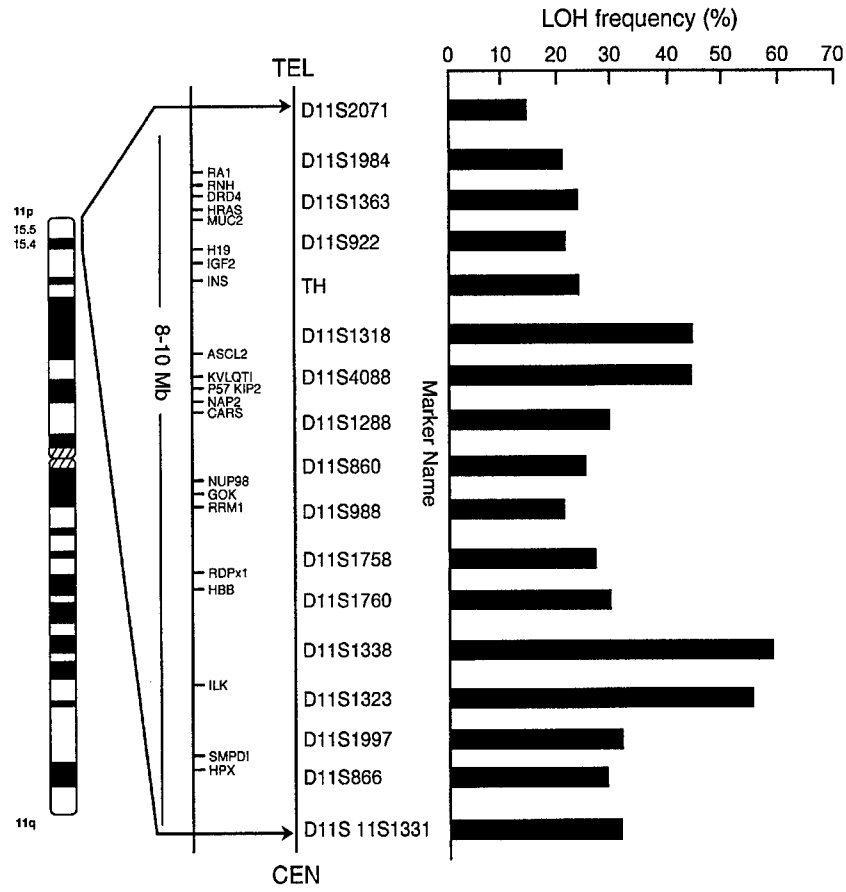

Figure 1. Representation of 11p15.5–15.4 and approximate location of the microsatellite repeats (21,22) and genes that map to this region [sequence map of chromosome 11 (http:// mcdermott.swmed.edu/)]. The histogram shows the percentage of LOH for each of the microsatellites in the informative breast tumor samples studied.

positional cloning efforts to identify the target genes on 11p15 have been complicated by the large size of this region (~10 Mb) and the complexity of LOH at 11p15.

With the goal of identifying the putative tumor suppressor gene(s) on chromosome 11p15, we have refined the minimal regions of LOH in this region, using a high-density marker analysis of 94 informative primary breast tumors and paired normal breast tissue. We have defined precisely and identified two distinct regions of chromosome 11p15.5–p15.4 that frequently are deleted in breast cancer. The association of LOH with clinical and histological parameters reveals the biological role of the putative tumor suppressor genes in the etiology of breast cancer.

RESULTS

Refinement of the tumor suppressor loci on chromosome 11p involved in breast cancer

Fluorescent PCR semi-automated genotyping (5) was used to detect and analyze allelic losses on chromosome 11 using a panel of 17 microsatellite markers. Previous studies have determined that this technique is more rapid and sensitive compared with the classical radioactive method in determining LOH in tumor DNAs (20). To identify the smallest common deleted region on chromosome 11p15 in breast tumors, 94 paired normal–tumor DNAs were assessed for LOH at 17 chromosome 11p15-specific microsatellite markers. These markers encompass the chromosomal sub-regions 11p15.5–11p15.4, estimated to be ~8–10 Mb (21,22) (Fig. 1). The results indicate that the loss of all or part of chromosome 11p is a more common event in human breast cancer than previously appreciated (3,4). LOH occurred in at least one marker on the short arm of chromosome 11 in 56 of 94 (60%) informative tumors. The overall frequency of LOH for each marker varies from 16 to 60%, with two peaks seen at markers *D11S1318* (45%) and *D11S1338* (60%) (Fig. 1). In addition to the 23% LOH at the *D11S988* locus (Fig. 1), there was a high incidence of microsatellite instability (MSI) at this marker as we had described earlier (5). Therefore, the possibility that MSI obscures the accurate determination of LOH at the *D11S988* locus in some of these tumors cannot be ruled out.

Tumors 57, 94, 6 and 24 (Genescans; Fig. 2) are illustrative examples of LOH patterns seen on chromosome 11p15 and provide a critical description of the LOH regions. Interstitial deletions, examples of which are seen in tumors 57, 94, 6 and 24, were observed more commonly than loss of the entire chromosomal arm as seen in tumor 7 (Fig. 3). In some cases, an example of which is seen at the marker *D11S1997* in tumor 24 (Fig. 2), it was observed that the peak for the allele which loses heterozygosity does not change between normal and tumor tissues. Rather, the peak for the other allele increases by several fold in the tumor.

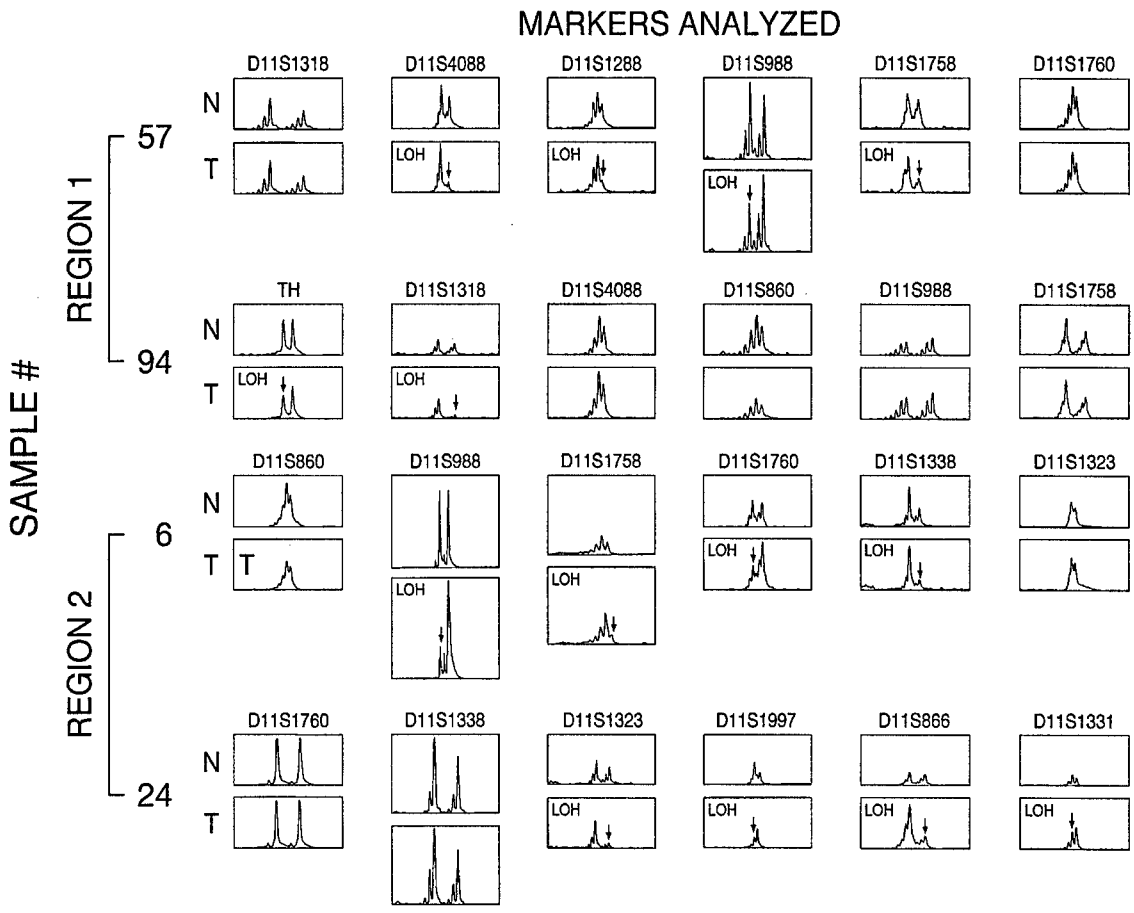

Figure 2. LOH studies of normal (N) and tumor (T) breast cancer pairs. Genescans of samples 57 (*D11S1318*, *D11S4088*, *D11S1288*, *D11S988*, *D11S1758* and *D11S1760*), 94 (*TH*, *D11S1318*, *D11S4088*, *D11S860*, *D11S988* and *D11S1758*), 6 (*D11S860*, *D11S988*, *D11S1758*, *D11S1760*, *D11S1338* and *D11S1323*) and 24 (*D11S1760*, *D11S1338*, *D11S1323*, *D11S1997*, *D11S866* and *D11S1331*) are shown. Arrows represent allelic loss. LOH represents samples that exhibit loss of heterozygosity and was calculated as described in the text.

Since the surrounding markers show LOH, we believe that this allelic imbalance represents LOH and not gene amplification.

The genotypes of the 13 representative breast tumors described in Figure 3, along with other tumors analyzed (data not shown), serve to refine and identify two distinct regions of LOH on 11p15. Region 1 is encompassed by markers *D11S1318* and *D11S4088* and is defined by the LOH break points in tumors 57 and 94. Tumor 57 retained heterozygosity for the markers *D11S2071*, *D11S1984*, *D11S1363*, *D11S922*, *TH* and *D11S1318*, but showed LOH for the markers *D11S4088*, *D11S1288*, *D11S860*, *D11S988* and *D11S1758*. This tumor also retained heterozygosity for all the remaining proximal markers. Tumor 94 showed LOH at markers *D11S2071*, *D11S1984*, *D11S922*, *TH* and *D11S1318*. This tumor was non-informative for the marker *D11S1363* and retained heterozygosity at all the proximal markers. Tumors 94 and 57, therefore, refine the LOH region 1 to a distance of ~500 kb between the markers *D11S1318* and *D11S4088*. This distance was calculated based on the estimation of Reid *et al.* (23) and the sequence map of chromosome 11 (http://mcdermott.swmed.edu/). Importantly, these results narrow the region containing this tumor suppressor gene from 2 Mb reported earlier (3,4) to ~500 kb. Tumors 42, 57 and 94 are examples of tumors that contain interstitial deletions exclusively in region 1 (Fig. 3).

The more centromeric region of LOH (region 2) is defined by breakpoints in the tumors 6 and 24 (Figures 2 and 3). Tumors 6, 24, 35, 45 and 76 are examples of tumors that harbor interstitial deletions in region 2 (Fig. 3). Tumor 6 showed LOH for the markers *D11S988*, *D11S1758*, *D11S1760* and *D11S1338*, but retained heterozygosity at all the markers distal to *D11S988* and at all the markers proximal to *D11S1338*. Tumor 24 was heterozygous for all the markers distal to *D11S1323* but showed LOH at *D11S1323*, *D11S1997*, *D11S866* and *D11S1331*. It is notable that tumors 6 and 24 exhibit LOH at either *D11S1338* or *D11S1323*, while the other locus retains heterozygosity. This clearly indicates that region 2 is within the interval that spans the markers *D11S1338–D11S1323*, a distance of ~336 kb based on the estimate of James *et al.* (22) and the sequence map of chromosome 11 (http://mcdermott.swmed.edu/). The yeast artificial chromosome (YAC) 847a12 that contains the markers *D11S1338*, *D11S1323* and *D11S1997* is ~1.4 Mb in length and is non-chimeric (STS-based map of the human genome; http:// www-genome.wi.mit.edu). We have identified integrin-linked kinase (*p59ILK*)

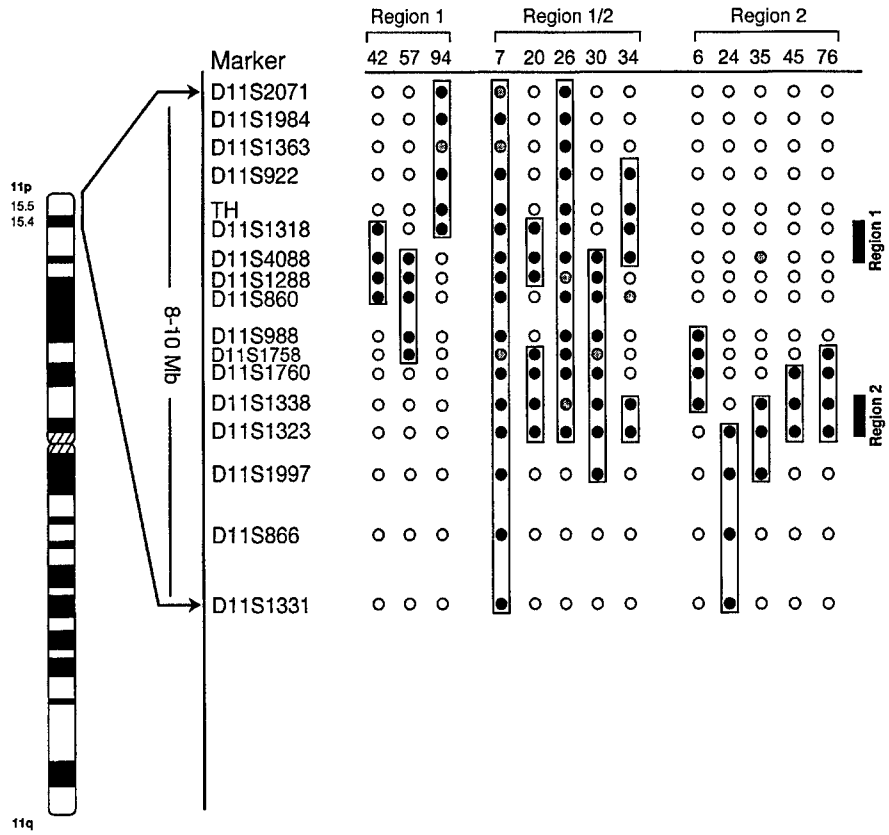

Figure 3. Genotypes of 13 representative tumors and the smallest regions of shared LOH in sporadic breast carcinoma. Tumor numbers are listed across the top, with the markers analyzed to the left. Open circles represent informative samples with no LOH; filled circles represent informative samples with LOH; and stippled circles represent non-informative (homozygous) samples. The maximum area of LOH is boxed for each LOH region in each tumor. The bars to the right represent the extent of the proposed common regions of LOH (regions 1 and 2). Tumors that exhibit LOH at region 1 only, regions 1 and 2, and region 2 only are grouped together.

as a candidate gene for this locus. *p59ILK* previously was mapped to the *CALC–HBBC* region on chromosome 11p15 (24). We have refined the map location of *p59ILK* and placed it on the YAC 847a12. PCR amplification of DNA from the YAC 847a12 with several different *p59ILK* primers produced the expected length fragments. No products were seen from a BAC DNA specific for the marker *D11S1323* or from yeast DNA (data not shown).

A total of five tumors, examples of which were seen in tumors 7, 20, 26, 30 and 34 (Fig. 3), appeared to have lost both of the regions on the chromosome 11p arm. In tumor 7 (Fig. 3), 14 of the 17 markers analyzed showed LOH. This tumor was non-informative for the markers *D11S2071*, *D11S1363* and *D11S1758*. The probability of three or more allelic losses in the same fragment being caused by independent events is small, and a series of LOH in contiguous markers is more likely to be due to deletion of the entire segment. In most instances, however, LOH on 11p15 appeared to be interstitial (e.g. tumors 20, 26, 30 and 34) and, therefore, restricted to relatively small chromosomal regions.

These data attest the presence of two distinct regions of LOH within 11p15.5–15.4. Region 1 lies between markers *D11S1318* and *D11S4088* (~500 kb) and region 2 lies between markers *D11S1338* and *D11S1323* (~336 kb). As described in Figure 3, the two regions were lost in different tumors, although in some tumors both of these regions appeared to be lost due either to interstitial deletions or to the loss of the entire 11p arm.

Negrini *et al.* (4) previously have reported a third LOH region, towards the telomere, between the markers *D11S576* and *D11S1318*. The percentage LOH that we observe for the telomeric markers *D11S2071*, *D11S1984*, *D11S1363* and *D11S922* (16–22%) is consistent with the observations of Negrini *et al.* (4). However, the percentage LOH for these markers is well within the background LOH seen at the remaining 11p markers (Fig. 1). In addition, we did not identify tumors that showed LOH exclusively in the telomeric markers *D11S2071–D11S922*. In our tumor panel, LOH at the distal markers occurred in concert with LOH at region 1. We therefore did not represent the distal region as an independent and third region of LOH.

Correlation between loss of heterozygosity at 11p and pathological features of breast tumors

Conflicting clinical data and clinical correlations of 11p LOH in breast cancer exist in the literature. This study was initiated with those concerns in mind. To examine the role of 11p LOH in breast cancer and to determine if the two regions are involved differentially in predicting the clinical course of this disease, we correlated our LOH data with the various clinical and histological parameters (Table 1).

Table 1. 11p LOH and clinico-pathological features of sporadic breast tumors

Clinical features	LOH in region 1		LOH in region 2		*P*-value
	N	%	*N*	%	
Ductal					
Yes	26	86.7	35	87.5	0.92
No	4	13.3	5	12.5	
If ductal					
In situ and invasive	4	15.4	0	0.0	0.016[a]
Invasive	22	84.6	35	100.0	
Lobular					
Yes	4	13.3	5	12.5	0.92
No	26	86.7	35	87.5	
If lobular					
In situ and invasive	1	25.0	0	0.0	0.24
Invasive	3	75.0	5	100.0	
Ploidy					
Diploid or near diploid	16	66.7	4	16.0	<0.001[a]
Aneuploid	8	33.3	21	84.0	
% S-phase cells					
≤10%	17	68.0	12	46.2	0.12
>10%	8	32.0	14	53.9	
ER/PR status					
ER+/PR+	3	23.1	8	50.0	0.23
ER+/PR–	7	53.9	4	25.0	
ER–/PR–	3	23.1	4	25.0	
Grade					
I–II	10	33.3	9	26.5	0.16
II–III	15	50.0	12	35.3	
III	5	16.7	13	38.2	
Lymphatic invasion					
Yes	4	28.6	20	69.0	0.012[a]
No	10	71.4	9	31.0	

[a]Statistically significant ($P < 0.05$).

All tumors described in Table 1 were infiltrating ductal carcinomas, which account for the largest single category of mammary carcinomas. The histological classification of the tumors, described under clinical features, was based on the WHO classification (25). Tumors were subdivided into two categories: (i) tumors that had lost only region 1 and (ii) tumors that had lost only region 2. Clinical features of breast tumors are summarized as frequencies and percentages, separately for each region. The χ^2 test was used to compare these features between regions 1 and 2. All statistical tests were performed using a 5% level of significance.

A correlation was observed between LOH in region 1 and breast tumors containing ductal carcinoma *in situ* (DCIS) synchronous with invasive carcinoma. Fifteen percent (4/26) of ductal tumors with LOH in region 1 contained breast cancer tissues with synchronous DCIS and invasive carcinoma, while none of the tumors with LOH in region 2 contained a DCIS component ($P = 0.016$). DCIS of the breast is considered a pre-invasive stage of breast cancer and may be a precursor of infiltrating breast cancer (26). Although the number of tumors analyzed is small, the statistically significant association between LOH in region 1 and such tumors suggests the involvement of a target gene in this region with early events in malignancy or invasiveness. The statistical analysis showed a significant association between 11p LOH and tumor ploidy. The majority of tumors (16/24) with region 1 LOH were either diploid or near diploid ($P < 0.001$). In contrast, the majority of tumors with region 2 LOH, were aneuploid ($P < 0.001$).

A trend was also observed between LOH at 11p and S-phase fraction (SPF). Fifty four percent of tumors with LOH in region 2, had a high SPF (>10% of cells in S-phase), compared with only 32% tumors with LOH in region 1. However, due to the small number of tumors in each category, statistical significance could not be established. It has been suggested that abnormal ploidy or elevated SPF identifies patients with shorter survival, and worsened disease-free survival, as well as being associated with poor outcome in locoregional control of the disease (27). The association between LOH at region 2 and tumors with high SPF and abnormal ploidy, that we observe, is therefore very relevant.

A striking correlation was observed between loss of region 2 and lymphatic invasion. Importantly, 69% of patients with 11p LOH in region 2 showed lymphatic invasion, whereas this infiltration was present in only 29% of patients with region 1 LOH. Thus, tumors that had lost region 2 reveal a significantly higher incidence of metastasis to a regional lymph node(s) ($P = 0.012$) than tumors that had lost region 1. Tumors that had lost the entire 11p arm, or had lost both regions, showed the clinicopathological features of tumors that had lost region 2. We also observed the trend that LOH in region 2 occurs more frequently in higher grade (grade III) tumors than LOH in region 1. Thus, LOH at region 2 may be a late event in mammary tumorigenesis, potentially enabling a clone of previously transformed cells to exhibit greater biological aggressiveness.

DISCUSSION

We have identified two distinct regions on chromosome 11p15 that are subject to LOH during breast tumor progression and metastasis. The high frequency of somatic loss of genetic information and the striking clinical correlation observed suggest their role in the pathogenesis of breast cancer.

We have defined precisely and narrowed the location of the putative tumor suppressor gene in region 1 from ~2 Mb (3,4) to ~500 kb. The critical region appears to extend between the markers *D11S1318* and *D11S4088* at 11p15.5. Previous studies (3,4) had only been able to place the putative gene in the larger overlapping area between *TH* and *D11S988* (Fig. 4). Although LOH frequencies for this region are consistent (24–45%, this report; 35%, ref. 3 and 22%, ref. 4) , the peak incidence of LOH in this report is highest at *D11S1318*, ~1 Mb distal to the peak at *D11S860* reported by Winquist *et al.* (3) and Negrini *et al.* (4). This discrepancy may reflect the characteristics of the tumor samples analyzed or a difference in interpretation of the corresponding allelic patterns. LOH involving region 1 coincides with regions implicated in the pathogenesis of rhabdomyosarcoma (7,8), WT (7), ovarian carcinoma (14), stomach adenocarcinoma (28) and with a region conferring tumor suppressor activity

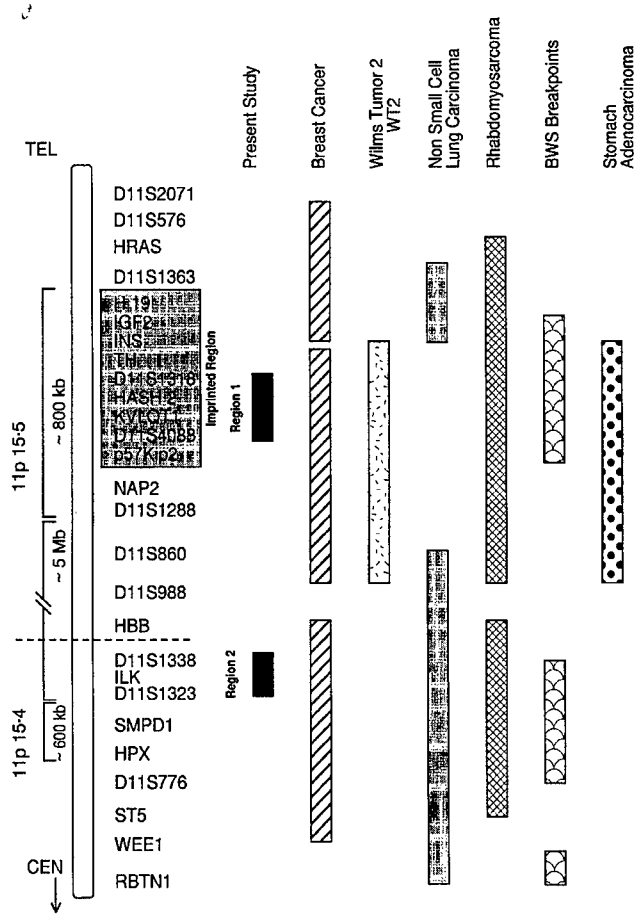

Figure 4. Schematic representation of regions on chromosome 11p15.5–15.4 harboring potential tumor suppressor and/or disease loci described in the present study and by other groups in breast cancer (2–5), Wilms tumor (7), non-small cell lung carcinoma (43), rhabdomyosarcoma (7), Beckwith–Wiedemann syndrome (31) and in stomach adenocarcinoma (28).

previously identified by genetic complementation experiments (29). Reid *et al.* (30) have used a functional assay to localize a 11p15.5 tumor suppressor gene that maps to this region in the G401 cell line. It is interesting to note that the physical map and contig of the *BWSCR1–WT2* region described recently (23) overlaps with our LOH region 1. Inversions and translocations at chromosome band 11p15.5, associated with malignant rhabdoid tumors and Beckwith–Wiedemann syndrome (31), also overlap with both regions of LOH in this study. It remains to be determined whether a single pleiotropic gene or a cluster of tumor suppressor genes play a role in the genesis of different cancers, possibly at different stages of tumor development and progression.

In childhood tumors such as WT and embryonal rhabdomyosarcoma, there is a strong bias toward loss of maternal 11p15 markers (32), suggesting the existence of an imprinted tumor suppressor gene in region 1. Since LOH for 11p15 is a common event in several adult tumors, a similar bias in allele loss could also be expected in the latter. Although the existence of parental bias towards LOH of 11p15 markers has not yet been demonstrated in adult tumors, two genes that map to 11p15, namely human insulin-like growth factor II gene (*IGF2*) and *H19*, are known to be expressed monoallelically in adult tissues (33,34), suggesting that genomic imprinting may be maintained in adult tissues. As illustrated in Figure 4, several genes that map to the LOH region 1 are subject to imprinting. It has been suggested that deregulation of imprinting may play a role during tumorigenesis (35,36). One model proposes that inappropriate methylation (hypermethylation) silences one copy of a tumor suppressor gene (36). This could be due to inappropriate activation of, or mutations in imprintor genes (37). If the first 'hit' represents the non-expression of one of the alleles due to the imprinting process, the second 'hit' may be mutational or may result from loss of all or part of the chromosome carrying the remaining functional tumor suppressor allele, thereby fulfilling Knudson's 'two-hit' hypothesis (38). Hypermethylation as an alternative pathway for tumor suppressor gene inactivation has been demonstrated elegantly for the retinoblastoma (*Rb1*) (39), the von Hippel Landau (*VHL*) syndrome (40), and the p16 tumor suppressor genes (37).

The other mechanism of altered imprinting that may affect tumorigenesis involves a gene activation hypothesis (36) that results in the reactivation of the silent allele due to the relaxation or loss of imprinting (LOI). LOI mutations have been detected at *H19*, *IGF2*, and to a lesser extent at p57KIP2 in *WT* and *BWS* (35). LOI of *IGF2* has been observed in benign and malignant breast tumors, in other adult and childhood tumors, and in a subgroup of patients with BWS (35,41). IGF2 is a potent mitogen with autocrine and paracrine effects, and the biallelic expression and possible increased dosage of the growth factor may explain the somatic overgrowth characteristics found in BWS patients and may play a role in tumor development. Although *IGF2* maps outside the LOH region 1, the presence of novel genes that map to region 1 that may have similar mitogenic effects cannot be ruled out. p57KIP2 (42) is a potential tumor suppressor candidate gene that maps to region 1. However, single strand conformation analysis and direct sequencing of 20 breast tumors (with LOH) and 15 breast tumors (without LOH) failed to reveal mutations in the coding region of this gene (P. Karnik *et al.*, unpublished observations).

The second hot-spot of LOH (region 2) in breast tumors is defined by markers *D11S1338–D11S1323*, and spans a distance of ~336 kb (22). Region 2 is centromeric to the putative *WT2* gene (region 1) and overlaps with LOH regions previously described for breast cancer (2) and non-small cell lung carcinoma (43). Importantly, we have refined this region from 5–10 Mb described earlier (2,43) to ~336 kb, with the highest incidence of LOH at the marker *D11S1338*. Previous studies (2,43), have only analyzed a few markers, sparsely distributed in the region proximal to *HBB*. Our study, therefore, is the first report of a detailed analysis of markers proximal to *HBB* that has considerably refined the boundaries of LOH in region 2.

We observed a significant correlation between LOH at the two chromosomal regions and the clinical and pathological parameters of the breast tumors. LOH in region 1 correlated with tumors that contain ductal carcinoma *in situ* synchronous with invasive carcinoma. This suggests that the loss of a critical gene in this region may be responsible for early events in malignancy or invasiveness. LOH at region 2 correlated with clinical parameters which portend a more aggressive tumor and a more ominous outlook for the patient, such as aneuploidy, high S-phase fraction and the presence of metastasis in regional lymph nodes. The association between 11p LOH, tumor progression and metastasis that we describe is analogous to the observations made

in other epithelial tumors including breast cancer (3,6). For example, LOH at 11p correlated with advanced T stage and nodal involvement in non-small cell lung carcinoma (44) as well as subclonal progression, hepatic involvement (45) and poor survival in ovarian and breast carcinomas (3,46). Phillips *et al.* (19) have shown that micro-cell-mediated transfer of a normal human chromosome 11 into the highly metastatic breast cancer cell line MDA-MB-435 had no effect on tumorigenecity in nude mice, but suppressed metastasis to the lung and regional lymph nodes. This further supports the observation that chromosome 11 harbors a metastasis suppressor gene. The integrin-linked kinase gene (24) has been shown to induce anchorage-independent growth and a tumorigenic phenotype in rodents. We have refined the map location of *p59ILK*, and placed this gene on the YAC 847a12 that spans the markers *D11S1338* and *D11S1323*. Thus, *p59ILK* is a tumor suppressor candidate for region 2.

It is not clear if LOH involving regions 1 and 2 act independently or synergistically in breast tumors. The identification of two subsets of tumors that have lost either region 1 or region 2 suggests that LOH at the two regions occurs independently and perhaps at different time points during breast tumor progression. This is consistent with the possibility that at least two tumor suppressor genes involved in the progression of breast cancer are located on the chromosome 11p15.5–15.4. These genes may function at distinct stages in the development and progression of breast cancer; alternatively, different target genes may be inactivated in different tumors. It is possible that specific subsets of tumors are defined by the particular set of mutations that they contain, which results in the clinical heterogeneity that is frequently seen in breast cancer.

Chromosome 11p15 contains several imprinted genes and two or more tumor suppressor genes. The fine mapping of this intriguing chromosomal region should facilitate the identification of novel genes, the evaluation of candidate genes and the establishment of the mechanisms whereby they contribute to the evolution of adult and childhood cancers.

MATERIALS AND METHODS

Patient materials and preparation of genomic DNA

Primary tumor and adjacent normal breast tissue samples were obtained from 94 randomly selected breast cancer patients undergoing mastectomy at the Cleveland Clinic Foundation (CCF). Samples of these tumors and corresponding non-involved tissue from each patient were collected at the time of surgery, snap-frozen and transferred to –80°C. Clinical and histopathological features of the tumors described in Table 1 were performed by the Pathology Department at CCF and were revealed only after the LOH study had been completed. The breast tumors described in Table 1 were classified according to the WHO classification (25). Tumor grading described in Table 1 was based on the Scarff–Bloom–Richardson method (25). DNA ploidy and S-phase determinations were done using the fine needle aspiration method (5). ER and PR status were done using the Ventana 320 automated immunostandard and the modified labeled streptavidin biotin technique (5).

An initial cryostat section was stained with H&E stain to determine the proportion of contaminating normal tissue, and only DNA purified from specimens thought to be highly enriched in tumor tissue was used for PCR. Generally we use tumor samples that contain <40% contamination of normal cells. In cases where LOH is questionable, where possible, regions containing a high proportion of normal tissue were physically removed from the original block by microdissection followed by DNA isolation. These improvements combined with the automatic quantitation of results using the Genescan Analysis have given us a better indication of LOH in tumor samples. Genomic DNA was isolated from normal and tumor tissue samples as described earlier (5) and quantitated by determining the optical densities at 260 and 280 nm.

Microsatellite polymorphisms and primers

DNA sequences flanking polymorphic microsatellite loci on chromosome 11p15.5 were obtained from the chromosome 11 databases and the Genome Data Base (GDB). Dye-labeled (FAM or HEX; Applied Biosystems) primers were either obtained from Research Genetics (Huntsville, AL) or synthesized as described earlier (5). Only one primer in each pair was fluorescently labeled so that only one DNA strand was detected on the gel. The physical distances between the polymorphic loci were calculated based on the sequence map of chromosome 11 (http://mcdermott.swmed.edu/) and the radiation hybrid map of James *et al.* (22). According to their calculation, 1 CR_{9000} = 50.2 kb.

Polymerase chain reaction (PCR) and analysis of PCR products using Genescan software

PCR of the DNA sequences was performed as described (5). PCR products were analyzed on Seaquate 6% DNA sequencing gels (Garvin, OK) in 1× TBE buffer in a Model 373A automated fluorescent DNA sequencer (Applied Biosystems) which is a four-color detection system. One µl of each PCR reaction was combined with 4 µl of formamide and 0.5 µl of a fluorescent size marker (ROX 350; Applied Biosystems). The gel was run for 6 h at 30 W. During electrophoresis, the fluorescence detected in the laser scanning region was collected and stored using the Genescan Collection software (Applied Biosystems). The fluorescent gel data collected during the run were analyzed automatically by the Genescan Analysis program (Applied Biosystems) at the end of each run. Each fluorescent peak was quantitated in terms of size (in base pairs), peak height and peak area.

LOH analysis with Genescan

Fluorescent technology (5) was used to detect and analyze CA repeat sequences. The ratio of alleles was calculated for each normal and tumor sample and then the tumor ratio was divided by the normal ratio, i.e. T1:T2/N1:N2, where T1 and N1 are the area values of the shorter length allele and T2 and N2 are area values of the longer allele product peak for tumor and normal respectively. We assigned a ratio of 0.70 or less to be indicative of LOH on the basis that tumors containing no normal contaminating cells and showing complete allele loss would theoretically give a ratio of 0.0, but because some tumors in this series contained an estimated 30–40% normal stromal cells (interspersed among the tumor cells), complete allele loss in these tumors would give an allele ratio of only 0.70. At least three independent sets of results were used to confirm LOH in each tumor.

Statistical analysis

Clinical features of breast tumors are summarized as frequencies and percentages, separately for each LOH region. The χ^2 test was

used to compare these features between regions 1 and 2. All statistical tests were performed using a 5% level of significance.

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Loss of heterozygosity at chromosome 11p15 in Wilms tumors: identification of two independent regions

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Loss of heterozygosity (LOH) on the short arm of chromosome 11 is the most frequent genetic alteration in Wilms tumors, indicating that one or more tumor suppressor genes that map to this chromosomal region are involved in the development of the disease. The WT1 gene located on 11p13 has been characterized but mutations in this gene occur in only about 10% of Wilms tumors. A second locus (WT2) at chromosome 11p15 has also been described in Wilms tumors but thus far efforts to clone the WT2 gene(s) have been frustrated by the large size (~10 Mb) of this region. Using a high-density marker LOH analysis of 11p15.5–15.4, we have refined the location of a Wilms tumor suppressor gene between the markers D11S1318–D11S1288 (~800 kb) within 11p15.5. We have also identified a second, novel region of LOH that spans the markers D11S1338–D11S1323 (~336 kb) at 11p15.5–p15.4. Thus a second distinct locus, in addition to the previously defined WT2, on chromosome 11p15.5, appears to play a role in the development of Wilms tumors.

Keywords: Wilms tumor; tumor suppressor genes; chromosome 11p15; loss of heterozygosity

Introduction

Wilms tumor (WT), a childhood kidney cancer which occurs in approximately 1 per 10 000 live births, is responsible for about 400 tumors per year in the USA (Young *et al.*, 1986). The majority of cases are diagnosed by 6 years of age, although rare cases have been documented in adolescents and adults. The inactivation of tumor-suppressor genes, by a two-hit mechanism involving mutations and loss of heterozygosity (LOH), appears to be a common event in the development of Wilms tumors (Knudson and Strong, 1972). Several chromosomal regions, including 1p, 11p13, 11p15 and 16q show consistent genetic changes in tumor tissue (Grundy *et al.*, 1995), suggesting that multiple suppressor genes are likely to be involved in the etiology of the disease. The association of Wilms tumor with aniridia, genito-urinary abnormalities and mental retardation (the WAGR syndrome), and cytogenetically visible alterations of chromosome 11p13 led to the identification of the WT1 locus (Franke *et al.*, 1979). The characterization of homozygous deletions observed in individuals with WAGR and in tumors from sporadic WT patients enabled the isolation of the WT1 gene (Bonetta *et al.*, 1990; Huang *et al.*, 1990; Call *et al.*, 1990; Gessler *et al.*, 1990). Analyses of WT1 mutations in patients with Denys-Drash syndrome (intersex disorders and nephropathy coupled with Wilms tumor), established the WT1 gene as an important tumor suppressor which also played a crucial role in urogenital development (Pelletier *et al.*, 1991). However, extensive mutational analyses of WT1 in sporadic Wilms tumors indicates that mutations occur at a frequency of only about 10% (Varanasi *et al.*, 1994). A second locus on chromosome 11p15 (WT2) shows a high frequency of loss of heterozygosity in Wilms tumors (Coppes *et al.*, 1992). Furthermore, linkage analysis and cytogenetic rearrangements have mapped the gene(s) for Beckwith-Weidemann syndrome (BWS) to 11p15.5 (Ping *et al.*, 1989). BWS is characterized by hemihypertrophy, macroglossia, umbilical hernia, and an increased risk for developing childhood cancers including Wilms tumors (Sotelo-Avila and Gooch, 1976). Functional studies using chromosome mediated gene transfer into the rhabdoid tumor cell line G401 have also been used to map a tumor suppressor locus to 11p15 (Reid *et al.*, 1996). Since positional cloning efforts to identify the target genes on 11p15 are complicated by the large size of this region (~10 Mb) and complexity of LOH, we have refined the mapping of the WT2 locus by performing a detailed LOH analysis of the 11p15.5–11p15.4 region using a high-density marker analysis of 38 informative tumor DNAs. The earlier described WT2 locus, has been refined from 2 Mb (Besnard-Guerin *et al.*, 1996) to ~800 kb. We have also identified a second novel locus of approximately 336 kb, that is proximal to WT2 and is also frequently lost in Wilms tumors. Thus two distinct regions of LOH on chromosome 11p15, appear to play a role in the development of Wilms tumors.

Results

To identify the smallest common deleted region on chromosome 11p15 in Wilms tumors, thirty eight tumor DNAs and matching constitutional DNA samples were assessed for LOH at 15 chromosome 11p15-specific polymorphic loci. These markers encompass the chromosomal sub-regions 11p15.5–11p15.4, estimated to be ~8–10 Mb (James *et al.*, 1994). LOH occurred in at least one marker on the short arm of chromosome 11 in 16 of 38 (43%) informative tumors. The results confirm that interstitial

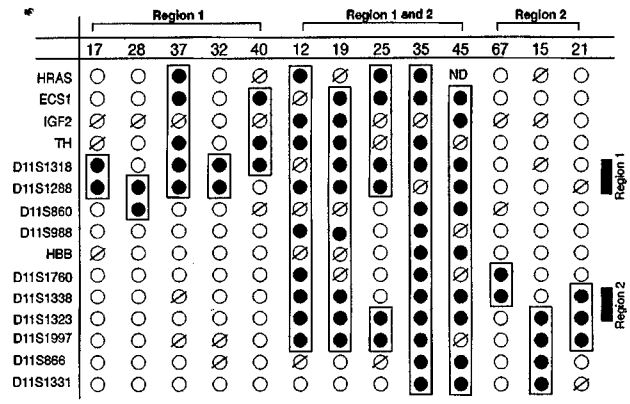

Figure 1 Genotypes of thirteen representative tumors and the smallest regions of shared LOH in Wilms tumors: Tumor numbers are listed across the top, with the markers analysed to the left. Open circles represent informative samples, with no LOH; filled circles represent informative samples with LOH; ND represents not determined and stippled circles represent non-informative (homozygous) samples. The maximum area of LOH is boxed for each LOH region in each tumor. The bars to the right represent the extent of the proposed common regions of LOH (region 1 and region 2). Tumors that exhibit LOH at region 1 only, region 1 and region 2, and region 2 only are grouped together

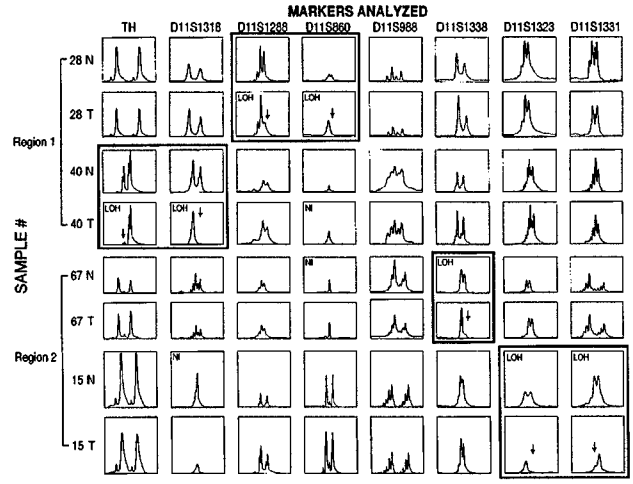

Figure 2 LOH studies of normal (N) and tumor (T) Wilms tumor pairs. Genescans of samples 28 and 40 (region 1) and samples 67 and 15 (region 2) for the markers TH, D11S1318, D11S1288, D11S860, D11S988, D11S1338, D11S1323 and D11S1331 are shown. Arrows represent allelic loss. NI- non informative for the marker. LOH represents samples that exhibit loss of heterozygosity. Samples showing LOH are boxed

The genotypes of the 13 representative Wilms tumors (Figure 1), along with other tumors analysed (data not shown), serve to identify and refine the LOH regions on 11p15. Figure 2 depicts the primary genescan data of the four critical tumors 28, 40, 67 and 15, showing the restricted areas of LOH. Interstitial deletions, examples of which were seen in tumors 17, 28, 37, 32, 40, 25, 67, 15 and 21, (Figure 1) define the LOH breakpoints and provide a critical description of the region. Two regions of LOH at 11p15 can be identified. The telomeric region (region 1) is encompassed by the markers TH and D11S1288 and is defined by the LOH breakpoints in tumors 28 and 40 as identified using Genescan (Figure 2). Tumor 28 retained heterozygosity for HRAS, ECS1, TH and D11S1318, but showed LOH for the markers D11S1288 and D11S860. ECS1, is a novel gene that we have recently mapped to the 11p15 region (Paris *et al.*, in preparation). Tumor 28 was non-informative for the marker IGF2 but retained heterozygosity for the proximal markers D11S988, HBB, D11S1760, D11S1338, D11S1323, D11S1997, D11S866 and D11S1331. Tumor 40 showed LOH at markers ECS1, TH and D11S1318 and was non-informative for the markers HRAS and IGF2 and for the proximal marker D11S860. This tumor was heterozygous for all the remaining markers including D11S1288. Tumors 28 and 40, therefore, refine the LOH Region 1 to a distance of ~800 kb between the markers D11S1318 and D11S1288. Importantly, these results narrow the region containing this tumor suppressor gene from 2 Mb reported earlier (Besnard-Guerin *et al.*, 1996) to ~800 kb.

The more centromeric region of LOH (region 2) is defined by breakpoints in the tumors 67 and 15 (Figures 1 and 2). Tumor 67 was heterozygous for and IGF2. This tumor showed LOH for the markers D11S1760 and D11S1338 and was heterozygous for the remaining proximal markers. Tumor 15 showed LOH at the markers D11S1323, D11S1997, D11S866 and D11S1331. This tumor was non-informative for the markers HRAS, IGF2, D11S1318 and was heterozygous at all the remaining markers. It is notable that tumors 67 and 15 exhibit LOH at either D11S1338 or D11S1323. This suggests that region 2 is within the interval that spans the markers D11S1338–D11S1323 a distance estimated to be ~336 kb (James *et al.*, 1994). We have mapped integrin-linked kinase (p59ILK) on the yeast artificial chromosome (YAC) 847a12 that harbors the markers D11S1338 and D11S1323. PCR amplification of the DNA from the YAC 847a12 with several different p59ILK primers produced the expected length fragments (data not shown). No amplification products were observed from a BAC DNA specific for the marker D11S1323 or from yeast DNA. Thus p59ILK is a candidate tumor suppressor gene for this locus. p59ILK was previously mapped to the CALC-HBBC region on chromosome 11p15 (Hannigan *et al.*, 1997).

The data described indicate the presence of two distinct regions of LOH within 11p15.5–15.4. Five of the tumors in the group studied, examples of which are 12, 19, 25, 35 and 45 (Figure 1), appeared to have lost both of the LOH regions on the chromosome 11p arm. However, only tumors 35 and 45 in this group appeared to have also lost the 11p13 markers (Coppes *et al.*, 1992) suggesting the loss of the entire 11p arm in these tumors.

Discussion

We have identified two distinct regions on chromosome 11p15 that are subject to LOH in Wilms tumors. The

al., 1998). LOH involving region 1 coincides with regions implicated in the pathogenesis of rhabdomyosarcoma (Besnard-Guerin *et al.*, 1996; Sait *et al.*, 1994), breast cancer (Karnik *et al.*, 1998; Winquist *et al.*, 1995), ovarian carcinoma (Viel *et al.*, 1992), stomach adenocarcinoma (Baffa *et al.*, 1996) and with a region conferring tumor suppressor activity previously identified by genetic complementation experiments (Reid *et al.*, 1996; Koi *et al.*, 1993). Inversions and translocations at chromosome band 11p15.5, associated with Beckwith-Wiedemann syndrome and malignant rhabdoid tumors (Sait *et al.*, 1994; Hoovers *et al.*, 1995) overlap with both regions of LOH in this study. Importantly, we have more precisely refined the location of the putative tumor suppressor gene in region 1 from 2 Mb (Besnard-Guerin *et al.*, 1996) to 800 kb. As illustrated in Figure 3, several genes that map to this region are subject to allele-specific imprinting (Reid *et al.*, 1997) thereby raising the possibility that the tumor suppressor gene that maps to 11p15.5 may be imprinted in a tissue-specific manner. Given the size of LOH region 1, it is possible that a single pleiotropic gene rather than a cluster of genes may play a role in the genesis of different cancers, possibly at different stages of tumor development and progression. More extensive analysis and isolation of the target gene that maps to this region will be important to establish whether loss or alteration of the same or different genes is involved in each of these cases. p57KIP2 and NAP2 (Reid *et al.*, 1997) are potential tumor suppressor candidate genes that map to region 1. However, single strand conformation analysis and direct sequencing of Wilms tumors failed to reveal mutations in these genes (Karnik *et al.*, unpublished observations).

In addition to the previously described WT2 locus, we have identified a second region of LOH (region 2) at 11p15 in Wilms tumors. This novel LOH locus in Wilms tumors, is defined by markers D11S1338–D11S1323, which spans a distance of ~336 kb and is centromeric to the putative WT2 gene. We have narrowed this region from 5–10 Mb (Tran and Newsham, 1996; Ali *et al.*, 1987) to ~336 kb, with the highest incidence of LOH, at the marker D11S1338. Integrin-linked kinase (p59ILK) was earlier shown to map to the CALC-HBBC region and was shown to induce anchorage-independent growth and a tumorigenic phenotype in rodents (Hannigan *et al.*, 1997). We have refined the map location of p59ILK, and placed this gene on the yeast artificial chromosome (YAC) 847a12, that harbors the markers D11S1338 and D11S1323. Thus, p59ILK is a potential tumor suppressor candidate for region 2.

These findings suggest the presence of two distinct tumor suppressor loci on chromosome 11p15 that appear to play a role in the development of Wilms tumors. The fine-mapping of these loci should lead to the cloning of the target genes and the establishment of mechanisms that contribute to the development of Wilms tumor and other childhood and adult cancers.

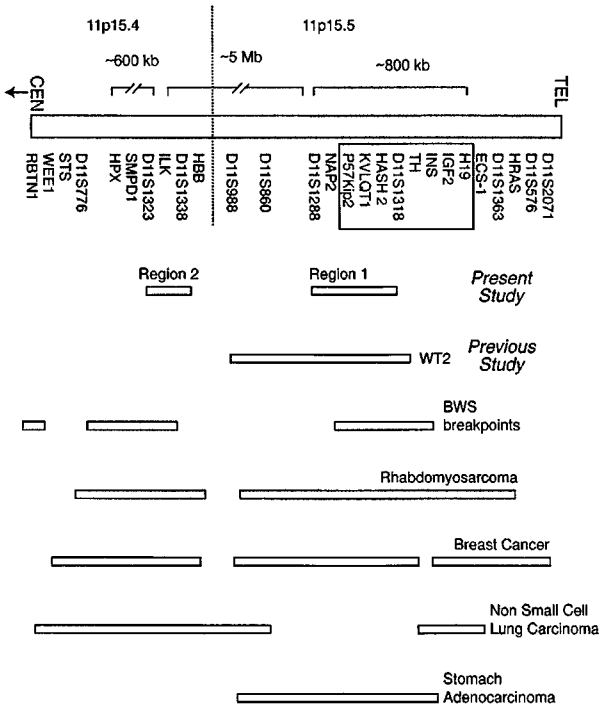

Figure 3 Schematic representation of regions on chromosome 11p15.5–15.4 harboring potential tumor suppressor and/or disease loci described in the present study and by other groups in Wilms tumor, Beckwith-Wiedemann syndrome, Rhabdomyo-

Materials and methods

Patient materials and preparation of genomic DNA

Constitutional and tumor DNA from patients were obtained for 38 Wilms tumor cases as described previously (Coppes *et al.*, 1992). Thirty-three patients had unilateral Wilms tumors and five had bilateral disease. The median age at diagnosis was 3 years (range, 3 months to 14.7 years). There were 18 male and 20 female patients. Constitutional DNA was obtained from peripheral blood leukocytes, lymphoblastoid cell lines, primary skin fibroblast cultures, or normal kidney tissue. Tumor tissues taken for DNA and RNA analyses were directly snap-frozen in liquid nitrogen.

Microsatellite polymorphisms and primers

DNA sequences flanking polymorphic loci on chromosome 11p15.5 were obtained from the Genome Data Base (GDB). The sequence analysis and characterization of the new gene, ECS1 will be described elsewhere (Paris *et al.*, in preparation). Dye labeled (FAM or HEX from Applied Biosystems) primers were either obtained from Research Genetics (Huntsville, Alabama) or synthesized as described earlier (Karnik *et al.*, 1995). One primer in each pair was fluorescently labeled so that only one DNA strand was detected on the gel.

Polymerase Chain Reaction (PCR) and analysis of PCR products using genescan software

PCR of the DNA sequences was performed as described (Karnik *et al.*, 1995). PCR products were analysed on Seaquate 6% DNA sequencing gels (Garvin, OK) in 1×TBE buffer in a Model 373A automated fluorescent DNA sequencer (Applied Biosystems) which is a four color

cent size marker (ROX 350, Applied Biosystems). The gel was run for 6 h at 30 W. During electrophoresis, the fluorescence detected in the laser scanning region was collected and stored using the Genescan Collection software (Applied Biosystems). The fluorescent gel data collected during the run was automatically analysed by the Genescan Analysis program (Applied Biosystems) at the end of each run. Each fluorescent peak was quantitated in terms of size (in base pairs), peak height and peak area.

LOH analysis with Genescan

To detect the presence and extent of LOH in the tumors, we analysed constitutional and tumor DNA from each patient for CA repeat sequences using fluorescent technology (Karnik *et al.*, 1995). In most Wilms tumors, LOH was identified by the complete loss of one allele. The ratio of alleles was calculated for each normal and tumor sample and then the tumor ratio was divided by the normal ratio, i.e. T1 : T2/N1 : N2, where T1 and N1 are the area values of the shorter length allele and T2 and N2 are area values of the longer allele product peak for tumor and normal respectively. At least three independent sets of results were used to confirm LOH in each tumor.

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REPLY TO
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