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PRINCIPAL INVESTIGATOR: Lalitha Nagarajan, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030

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Lalitha Nagarajan, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030

E-Mail: inagarajan@notes.mdacc.tmc.edu

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In the second year, we have refined the chromosomal location as well as characterized the protein product of the novel homeo box gene MIXL. In searching for a more global instability

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Lalita Nagarsenker 7/25/01

PI - Signature Date

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INTRODUCTION

The overall goals of this proposal are to isolate target oncogenes, tumor suppressor genes and regions of chromosomal instability by the use of a long interspersed nuclear element based PCR assay. In the second year of this award we exploited the near completion of the human genome sequencing with bioinformatic tools to achieve our goals.

We targeted both known and novel genomic loci for potential candidate genes. The first region was the c-Met oncogene, because of its association with poor prognosis in node negative breast cancer (1). The second line of investigations on a novel sequence specific single stranded DNA binding protein (SSDPs) stemmed from our parallel studies on human leukemia with poor prognosis. Originally isolated in the chicken as a gene whose product binds pyrimidine rich mirror repeats elements (2), the existence of three members that map regions of chromosomal instability was unknown until our studies uncovered this novel family. In the last quarter of the second year, our investigations centered on characterization of the protein products for the human *MIXL* gene reported last year and the SSDP2 gene product in breast cancer cell lines. Our progress with these promising candidate genes is likely to yield biologically and clinically relevant reagents to diagnose breast cancer with enhanced sensitivity.

Body

The first part of Year 2 of the proposal was to focus on tasks v and vi of Specific Aim 1 which were to Develop PCR for minimal material and correlate data from tasks (i-v) of specific aim 1 with stage and grade to determine consistent quantitative or qualitative patterns can be discerned. The remaining part was to complete specific Aim 2 that was to localize PCR rescued fragments to chromosomal regions and then identify whether these loci are targets of allelic imbalance in breast cancer.

With the near completion of the human genome sequencing studies under this specific aim were re-focused and approached in a cost and time effective manner by utilizing bioinformatic tools. In addition, we also expanded the scope of the proposed studies by initiating experiments on the protein products of potentially important candidate genes. Thus the current report comprises of two parts: (1) Chromosomal localization of regions of instability and identification of candidate genes and (2) Development of antibodies and characterization of expression of potential tumor promoting and tumor suppressor genes in breast cancer cell lines.

1. Chromosomal localization of regions of instability and identification of candidate genes:

1.1 Chromosome 7q31.1 locus:

A search of the human genome database for novel LINE rich segments showed a high LINE content on chromosome 7q31.1 The *Met* oncogene encoding a tyrosine kinase receptor, and a marker for poor prognosis in breast cancer (1) localizes to this region. Therefore, we searched the genomic sequences in the public domain for putative polymorphic sequences. A Bacterial Artificial Chromosome 354 L07 had a > 60% repetitive sequences and an unusually high number of L1Hs elements. We developed three novel markers from these sequences designated ca1, ca2 and gata (fig.1)

In collaboration with Dr. Nour Sneige in the Dept. of Pathology, we dissected 25 breast tumors and isolated normal and tumor DNAs. We developed PCR conditions to detect allelic imbalance for ca1, ca2, and gata in minimal patient material. These markers along with 7 other highly polymorphic markers (designated D7S***) were used in allelo typing studies of 25-breast tumor and matched control DNAs. 13 out of 25 tumors examined showed allelic imbalance. As shown in Fig.1 we could identify two critical regions, one flanked by the C- MET protooncogene and second a more centromeric region between the loci *D7S471* and *D7S1817*.

All of the 25 breast tumors used to screen for allelic imbalance of 7q31.1 showed lymph node involvement. We will interrogate the clinical correlation of both the previously identified chromosome 1q41-42 locus as well as additional loci characterized here once we have conditions optimized to detect the candidate gene expression by immuno staining.

1.2 A novel family of sequence specific single stranded DNA binding proteins localize to regions of allelic imbalance:

Another novel family of evolutionarily conserved genes, which localize to regions of genomic instability, were identified in our laboratory as part of our studies on human leukemia. The three members of this family encode a putative sequence specific single stranded DNA binding activity (SSDPs1-3). More importantly these map to regions of deletions in breast cancer identified by several studies (3-5). SSDP1 maps to chromosome 1p32, SSDP2 to chromosome 5q13.3 and SSDP3 to chromosome 19p12 (Fig.2).

In addition, we also examined the genomic organization of SSDP2, which localizes near a chromosomal break point in the breast cancer cell line SKBR3, as well as a human leukemia cell line ML3 (Fig.3). The gene is encoded by 17 exons and there are two large introns (> 100kbp), 1 and 4 with several copies of L1 Hs elements. Interestingly SSDP1 and 3 also have identical genomic organizations with 17 exons although the intron sizes are smaller.

In order to, determine whether the expression pattern of the SSDP genes in altered in cancer we initiated preliminary screens with the SSDP2 gene. Relative RT PCR in a variety of malignant cell lines including six mammary cell lines is shown in Fig.4. In contrast to normal tissues and some malignant cell lines (Weri), SKBR3, MDA- MB453, MDA- MB435, MDA-MB468, SKBr-3 and MCF 7) as well as the SV40 immortalized mammary epithelial cell line MCF10A appear to express low SSDP2 transcripts.

2. Development of antibodies, characterization in breast cancer cell line models

A corollary of our hypothesis is that altered expression, either enhanced expression in the case of oncogenes or loss of expression in the case of putative tumor suppressor genes at the site of chromosomal instability, contribute towards the phenotypic evolution of breast cancer cells, we developed highly specific antibodies against peptide epitopes of both MIXL and SSDP2 gene products. Two different

epitopes were identified and affinity purified antisera were generated. The specificity of the antibodies was verified in cell lines induced to over express under transient transfection conditions. Thus the antibodies were evaluated stringently and then utilized in immunoblotting experiments with human breast cancer cell lines.

2.1 MIXL2 protein is expressed in immortalized and malignant mammary cell lines:

As stated in last year's report, expression of MIXL appears to be highly restricted by RTPCR and Northern blotting analyses. This experimental evidence is confirmed by Bioinformatic analyses of cDNA sequences in the public domain. To date the dBEST Database of more than 10000 entries from over 30 tissues, contains only 4 sequences for the MIXL2 gene. These are from a infiltrating ductal carcinoma in situ, germ cell tumor, normal pre B lymphoid cells and the highly metastatic fibrosarcoma cell line HT1080. Interestingly, all the breast cancer cell lines (SKBR3, MDA- MB435, MDA-MB453, MDA-MB468 and MCF 7) and the SV40 immortalized mammary epithelial cell line MCF10A express the MIXL protein as shown in Fig. 5. This is in contrast to other malignancies including hematopoietic cancer where we detect a highly restricted expression. Thus aberrant MIXL expression might confer a proliferative/ survival advantage to breast cancer cells.

2.1 SSDP2 protein is not expressed in immortalized and malignant mammary cell lines:

The putative sequence specific single stranded DNA binding proteins in themselves may be involved in genomic stability as they are postulated to bind single stranded sequences that loop out when mirror repeat elements in the DNA assume a triple helical confirmation. Los of expression of these elements that reside at regions of genomic instability and deletion may confer a survival/ loss of ability to repair DNA damage advantage to malignant cells. As shown in Fig.6 none of the breast cancer cell lines as well as the immortalized breast epithelial expresses the SSDP2 protein. This is in contrast to certainT lymphoid cell lines, which express abundant SSDP2 protein. Our future studies will elucidate the mechanism by which loss of SSDP2 expression confers survival advantage in cancer.

Key Research Accomplishments:

- Characterization of the 7q31.1 loci flanking the *MET* protooncogene
- Identification of a novel family of sequence specific single stranded DNA binding protein (SSDPs1-3) and localization to human genome
- Development a PCR assay to detect expression of SSDPs 1-3
- Development of MIXL specific antibodies
- Detection of MIXL expression in breast cancer cells in culture
- Development of antibodies to SSDP2
- Lack of SSDP2 expression in breast cancer

(8) Reportable outcomes:

Manuscripts in preparation:

1. Hejlik DP and Nagarajan L. Retro transposition and recombination of LINE-1 Elements as a mechanism of chromosomal deletion and genomic instability (Manuscript being revised for resubmission)

2. Guo W., Chan A., Liang H., Etkin L.D., and Nagarajan L. A human Mix.1 like gene shows functional conservation with the Xenopus homolog. (Manuscript to be submitted by Aug. 01)

3. Guo W., Liang H., Ma J., and Nagarajan L., Regions of instability in breast cancer-role of repetitive elements.

Development of FISH and immunohistochemical staining probes:

We have characterized a BAC probes from chromosomes 1p32, 5q13.3, 7q31.1 and 19p13.2 that can be used in Fluorescence in situ hybridization.

We have developed highly specific antibodies to MIXL2 and SSDP2, which can be used in immuno histochemical staining.

Informatics:

The full length MIXL 2 sequence has been submitted to Genbank.

Full length cDNA and exon specific genomic sequences for SSDPs 1-3 have been submitted to Genbank

Funding applied for based on work supported by this award:

Some of the results obtained in the present award period contributed to the development of an RO1 proposal to NIH entitled "SSDP2 gene pathway in Myeloid Neoplasm", submitted Feb.1, 01.

Conclusions:

1) In breast caner, there are two critical regions of allelic imbalance centromeric of the c-Met oncogene. Whether imbalances of these intervals are selected for or result from an overall genomic instability remains to be investigated.

2) The sequence specific single stranded DNA binding proteins are encoded by a novel gene family. All three members localize to regions of chromosomal deletions in breast cancer.

3) A novel paired type- homeodomain protein Mixl2 is expressed in immortalized as well as transformed mammary epithelial cell lines.

In summary, we have delineated 5 distinct regions of instability that harbor potential target genes.

REFERENCES

1. Camp RL, Rimm EB, Rimm DL Met expression is associated with poor outcome in patients with axillary lymph node negative breast carcinoma. Cancer 86:2259-65,1999.

2. Bayarsaihan D, Soto RJ, Lukens LN. Cloning and characterization of a novel sequence-specific single-stranded-DNA-binding protein Biochem J . 331:447-52,1998.

3. Millikan RC, Ingles SA, Diep AT, Xue S, Zhou N, Florentine BD, Sparkes RS, Haile RW. Linkage analysis and loss of heterozygosity for chromosome arm 1p in familial breast cancer. Genes Chromosomes Cancer 25:354-61,1999.

4. Richard F, Pacyna-Gengelbach M, Schl]uns K, Fleige B, Winzer KJ, Szymas J, Dietel M, Petersen I, Schwendel A. Patterns of chromosomal imbalances in invasive breast cancer. Int J Cancer 89:305-10,2000.

5. Oesterreich S, Allred DC, Mohsin SK, Zhang Q, Wong H, Lee AV, Osborne CK, O'Connell P. High rates of loss of heterozygosity chromosome 19p13 in human breast cancer.British J Cancer 84:1-6. 2001.

Fig.1 Delineation of Critical Regions of Allelic Imbalances on Chromosome 7q

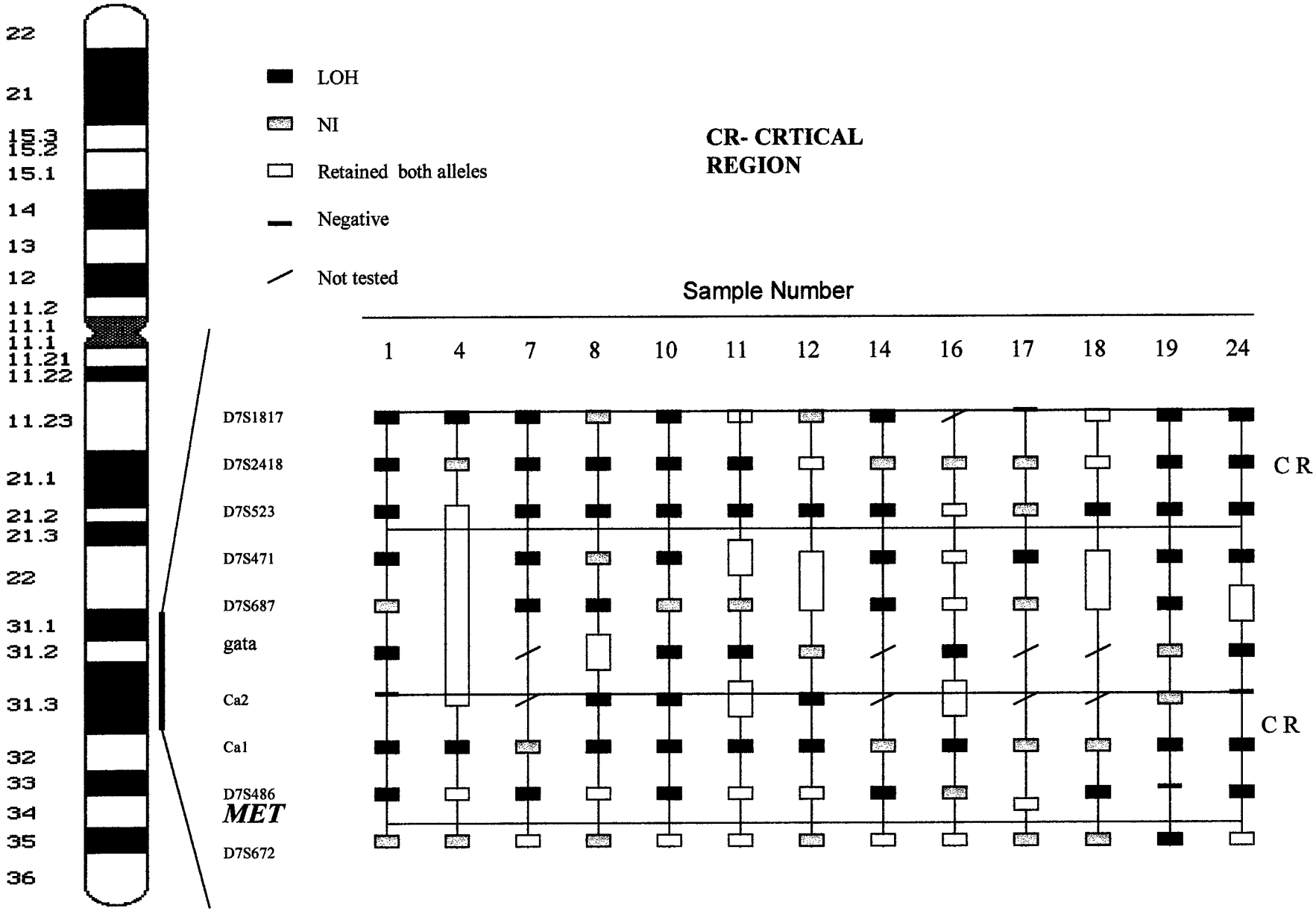


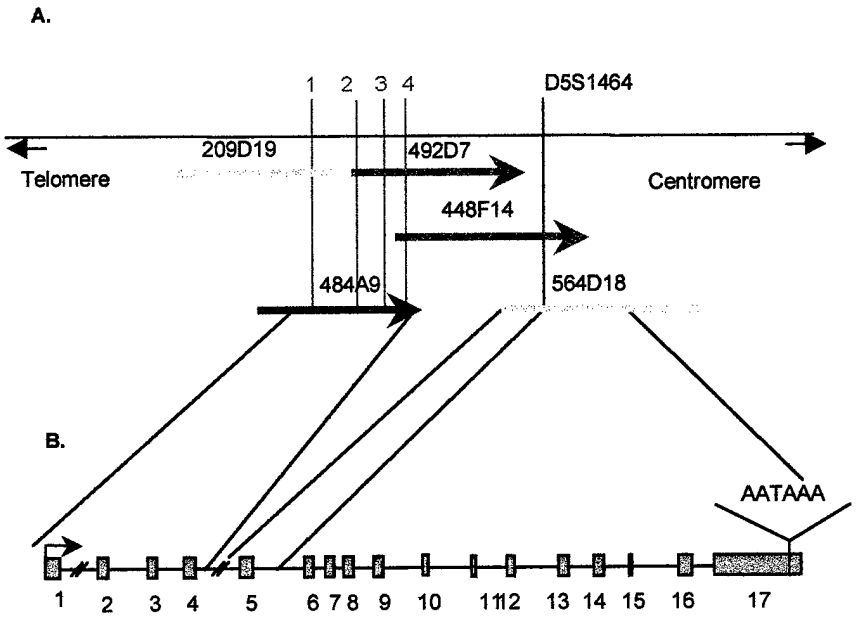
Fig. 2 Predicted open reading frames of SSDPs1-3.

```
1                    1       ↓          2          50↓
SSDP1  MFAKGK..GS AVPSDGQARE KLALYVYEYL LHVGAQKSAQ TFLSEIRWEK
SSDP2  MYGKGKSNSS AVPSDSQARE KLALYVYEYL LHVGAQKSAQ TFLSEIRWEK
SSDP3  MYAKG.GKGS AVPSDSQARE KLALYVYEYL LHIGAQKSAQ TFLSEIRWEK

       51      3        ↓                 4          ↓   100
SSDP1  NITLGEPPGF LHSWWCVFWD LYCAAPERRD TCEHSSEAKA FHDYSAAAAP
SSDP2  NITLGEPPGF LHSWWCVFWD LYCAAPERRE TCEHSSEAKA FHDYSAAAAP
SSDP3  NITLGEPPGF LHSWWCVFWD LYCAAPDRRE ACEHSGEAKA FQDYSAAAAP

       101         5              ↓            6      ↓    150
SSDP1  SPVLGNIPPN DGMPGGPIPP GFFQPFMSPR YAGGPRPPIR MGNQPPGGVP
SSDP2  SPVLGNIPPG DGMPVGPVPP GFFQPFMSPR YPGGPRPPLR IPNQALGGVP
SSDP3  SPVMGSMAPG DTMAAGSMAA GFFQPFMSPR FPGGPRPTLR MPSQPPAGLP

       151         7     ↓             8             ↓    9 200
SSDP1  GTQPLLPNSM DPT.RQQGHP NMGGSMQRMN PPRGMGPMGP GPQNYGSGMR
SSDP2  GSQPLLPSGM DPT.RQQGHP NMGGPMQRMT PPRGMVPL.. GPQNYGGAMR
SSDP3  GSQPLLPGAM EPSPRAQGHP SMGGPMQRVT PPRGMASV.. GPQSYGGGMR

       201           ↓         10     ↓      11    ↓   250
SSDP1  PPPNSL.GPA MPGINMGPGA GRPWPNPNSA NSIPYSSSSP GTYVGPPGGG
SSDP2  PPLNALGGPG MPGMNMGPGG GRPWPNPTNA NSIPYSSASP GNYVGPPGGG
SSDP3  PPPNSLAGPG LPAMNMGPGV RGPWASP.SG NSIPYSSSSP GSYTGPPGGG

       251 12      ↓                13     ↓        14 300
SSDP1  GPPGTPIMPS PADSTNSSDN IYTMINPVPP GGSRSNFPMG PGSDGPMGGM
SSDP2  GPPGTPIMPS PADSTNSGDN MYTLMNAVPP GPNRPNFPMG PGSDGPMGGL
SSDP3  GPPGTPIMPS PGDSTNSSEN MYTIMNPIGQ GAGRANFPLG PGPEGPMAAM

       301           ↓     15 ↓         16                350
SSDP1  GGMEPHHMNG SLGSGDIDGL PKNSPNNISG ISNPPGTPRD DGEL..GGNF
SSDP2  GGMESHHMNG SLGSGDMDSI SKNSPNNMS. LSNQPGTPRD DGEM..GGNF
SSDP3  SAMEPHHVNG SLGSGDMDGL PKSSPGAVAG LSNAPGTPRD DGEMAAAGTF

       351     ↓    17  368
SSDP1  LHSFQNDNYS PSMTMSV*
SSDP2  LNPFQSESYS PSMTMSV*
SSDP3  LHPFPSESYS PGMTMSV*
```

Arrows denote intron- exon boundaries

Conserved amino acid residues are bolded

Fig.3 Genomic Organization of SSDP2



Panel A shows the genomic BAC clones and panel B shows individual exons. The large, L1Hs rich introns of unknown sizes are denoted by slashes.

Fig 4. SSDP2 Transcript Levels are Low in Breast Cancer Cell Lines

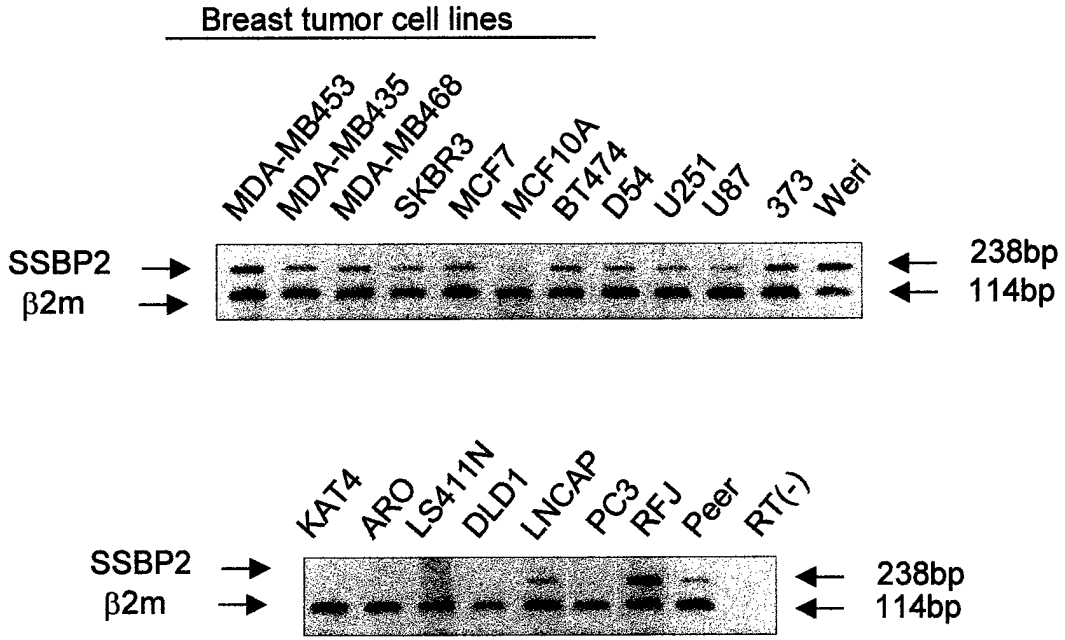


Fig. 5 MIXL Protein is expressed in Breast Cancer Cell lines

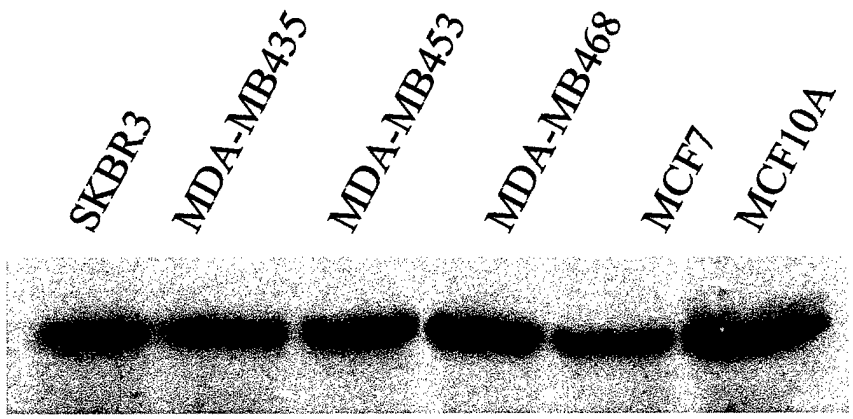
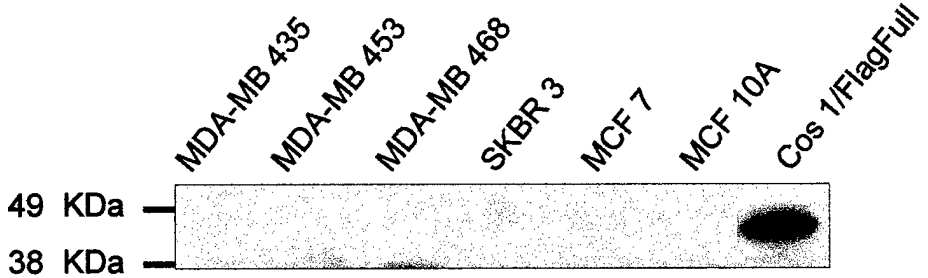


Fig. 6 Absence Of SSDP2 Protein Expression in Breast Cancer Cell Lines



Cos1 cell line transfected with Flag epitope tagged SSDP2 was used as control