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Identification and Characterization of Molecular Abnormalities of 11p Genes in Human Breast Cancer

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We have been investigating the hypothesis that 11p15 harbors one or more breast cancer suppressor genes. We have defined a minimal region of loss of heterozygosity in breast cancer between D11S988 and D11S1318. We have also performed genetic complementation experiments on breast cancer cells using subchromosomal transferable fragments (STF), but the STFs did not prove to be sufficiently stable for these cells. We have therefore developed a novel one-step retrofitting vector containing a mammalian selectable gene that allows us to modify bacterial artificial chromosome (BAC) for genetic complementation experiments, and we have successfully introduced these into tumor cells. We are currently testing these modified BACs on MCF-7 and HuMi breast cancer cells. We have also identified two new candidate genes within the region of LOH, termed TSSC4 and TSSC6. Twelve to 98 breast cancer samples were sequenced over the entire coding sequence of 8 genes within the region of LOH. While no somatic mutations were found, we did observe a germline alteration of one gene with in a patient multiple primary breast cancers. In addition, we found a novel alteration, relaxation of splicing fidelity, affecting multiple genes, in over half of human breast cancers.
Award Number DAMD17-94-J-4308

TITLE: Identification and Characterization of Molecular Abnormalities of 11p Genes in Human Breast Cancer

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REPORT DATE: March 1999

TYPE OF REPORT: Final

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

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INTRODUCTION

We have been investigating the hypothesis that 11p15 contains at least 1 tumor suppressor gene involved in the pathogenesis of breast cancer. The goals of this project are to localize and identify candidate genes for breast cancer within this region. We have taken two complementary but substantially different approaches toward this goal: the use of novel reagents for genetic complementation experiments, in order to suppress tumorigenicity of breast cancer cells in vitro or in vivo; and a more conventional positional cloning approach to identify candidate genes and then analyze them for mutations. While the first approach is more elegant in principle, the second approach has been more successful in identifying candidate genes and we have identified and characterized five novel genes in this region.

In the past year, we have completed an exhaustive analysis of all of the genes we have previously identified, examining the complete coding sequence of each gene. We completed the characterization of genes whose identification was in progress and included them in the mutational analysis. We attempted to better define the critical region of LOH using laser capture microdissection. We also developed a novel more successful strategy for functional genetic complementation, that we are currently applying. Finally, under this project, we discovered and characterized a novel type of genetic alteration in cancer, relaxation of splicing fidelity.

BODY OF REPORT

1. EXPERIMENTAL METHODS

Positional cloning: These efforts were largely completed in the previous year. However, two previously trapped exons were sequenced and this information was used to design PCR primers to perform exon connection experiments, and to screen a cDNA library as described earlier. The sequence of the identified clones was used to identify expressed sequence tags (ESTs). These EST’s were then used to extend sequence to include the entire predicted coding sequence. In this manner, two new genes were identified, as described in the Results.

Conventional isolation of DNA and RNA from tissues for conventional LOH analysis: Breast cancers and their matched normal tissues were obtained from The Johns Hopkins Hospital Pathology Department and the Cooperative Human Tissue Network, and normal fetal tissues were from the University of Washington Fetal Tissue Bank. The tissues were stored at -135°C until use. Breast cancers represented stages 1-4. The tissues were microdissected and pulverized in liquid nitrogen and suspended in TE9 [0.55 M Tris-HCl (pH 9.0), 20 mM EDTA (pH 8.0), and 10 mM NaCl]. Proteinase K (0.2 mg/ml) and 1% SDS were added to lyse the cells and digest the proteins at 50°C for overnight. To isolate RNA, tissues were cut into small pieces and homogenized in 4 ml of RNAzol B (Tel-Test). RNAs were stored at -70°C.

LOH analysis: LOH analysis was performed both by Southern hybridization as described for restriction site polymorphisms, and with Genescan software using an Applied Biosystems 7700 automated sequencer for microsatellite analysis.

Laser capture microdissection: In addition to conventional LOH analysis, laser capture microdissection (LCM) was performed on breast cancers from 16 patients, as described. LCM permitted the essentially complete separation of tumor from normal cells, and was done in
collaboration with Dr. Lance Liotta of the National Cancer Institute. LCM typically yielded DNA and RNA in the 1-3 µg range, compared to 100-300 mg from direct tissue preparation. However LCM tumor samples were homogeneous.

**Mutational analysis:** Breast cancers and their matched normal tissues, obtained from the Johns Hopkins Hospital Pathology Department and the Cooperative Human Tissue Network, were pulverized in liquid nitrogen and suspended in TE9, and digested with proteinase K and SDS as described. RNA was isolated by homogenization in RNAzol B. Sequence analysis was performed on tumor samples using dye-terminator automated sequencing on an Applied Biosystems 7700. Any sequence variation was confirmed through a second PCR and sequencing reaction, and by sequencing in the opposite direction. Sequence variations that withstood this analysis were then investigated by sequencing the paired normal specimen in each case to distinguish polymorphisms from somatic mutation.

**Analysis of RNA splicing fidelity:** RT-PCR was performed as described. However, amplification was done by single round rather than nested PCR.

**Microcell transfer of subchromosomal transferable fragments:** STFs were prepared as described. MCF-7 and HuMi cells were cultured in DMEM/10% fetal calf serum. A9 cells carrying STFs were cultured for 48 hours in cytochalasin B and G148 (400 µg/ml). Microcells were prepared by culturing STF-containing donor cells in Colcemid (0.5 mg/ml) for 48 hours, centrifuging in cytochalasin B (10 µg/ml), and filtering, and then fusing the microcells to recipient tumor cells with polyethylene glycol (PEG) as described.

**Retrofitting of BAC clones for introduction into tumor cells:** In the current year, we developed a novel protocol intended to achieve integration of a mammalian selectable marker gene (neo) into the vector portion of any BAC clone. This modification results in the integration of a G418-selectable marker, 600 bp upstream of the chloramphenicol gene into the BAC vector. This approach involves our development of a novel vector, termed pOOK, that undergoes homologous recombination directly in the bacterial cell containing the BAC of interest, so that the BAC can then simply be purified and transfected into mammalian cells. The vector contains a temperature-sensitive origin of replication, which allows the plasmid to replicate at the permissive temperature, 30 °C. Included in the vector are: GFP, to monitor transfection efficiency; pSV2neo for selection in mammalian cells; recA, which drives the homologous recombination/integration; the tetracycline gene for bacterial selection; and Region A homology (RA) and Region B homology (RB) sequences, for homologous recombination.

Bacteria containing a given BAC are washed in 10% glycerol, transformed with pOOK, and selected on LB agar plates containing neomycin, tetracycline and chloramphenicol overnight at 30 °C. Co-integrate clones are identified by Southern blotting and have two bands, a 1.4 kb and a band greater than 12 kb in size. The 1.4 kb band is only generated when the pOOK vector integrates into the BAC DNA after recombination between the RA and RB sequences. Frequency of obtaining co-integrates is 10 - 40%. Once co-integrates are obtained, subsequent culture requires only neomycin as the selection antibiotic. Retrofitted BACs and controls are then introduced into tumor cells by lipofection according to the manufacturer’s protocol (LTI).
RESULTS

Task 1, LOH Mapping of 11p15: We were able to obtain a sufficient amount of material after microdissection from 80 paired breast cancers and matched normal samples. Given the limited amount of tumor and/or normal tissue after dissection, we focused for LOH study on the following panel of 16 highly informative, well-spaced, and reproducible markers to apply to the analysis of these tumors: D11S932, D11S4188, D11S4149, D11S1331, D11S1758, D11S1323, D11S1760, D11S1330, D11S4124, D11S4187, D11S988, D11S4146, D11S4088, D11S860, D11S922, D11S2071. We observed that 40% of samples showed LOH of one or more markers. Ten tumors also showed retention of heterozygosity of D11S988, but LOH of all informative markers telomeric to it. In addition, one sample showed LOH of D11S860 but retention of heterozygosity of D11S1318, confirming that at least one tumor suppressor gene lies between D11S988 and D11S1318. Unfortunately, despite the large number of analyses (80 x 16), the data obtained could not further delimit the minimum region of LOH.

We therefore performed a related task as part of the basic goal of LOH mapping. Since there were so many samples with LOH, but no further recombination breakpoints further delimiting the tumor suppressor region, we attempted to identify small regions of homozygous deletion with the region of LOH. Through a collaboration with Dr. Lance Liotta, we performed laser capture microdissection (LCM) on 16 paired breast cancers for which matched normal specimens were available. Each sample required about one-half day of repeated laser sampling. In this manner we estimated that 1-3 μg of DNA and/or RNA could be obtained. While this yield is too low for LOH mapping, these samples are ideal for assays for homozygous deletion. This would not be possible using conventional specimens, but the LCM samples are of sufficient purity to allow the identification of homozygous deletions as described 6. We thus performed a PCR using primer sets spaced at approximately 100 kb intervals from throughout the candidate region. Unfortunately, despite this large amount of work, none of the samples showed homozygous deletions.

Task 2, Functional localization of an 11p15 gene in breast cancer: We attempted to suppress tumorigenicity of MCF-7 cells using subchromosomal transferable fragment (STF) 74-2, since earlier we were unable to maintain a suppressed phenotype using STF 74-1-6, even though the same fragment was able to suppress tumorigenicity in previous experiments. Unfortunately we had the same problem with STF 74-2 that we did with STF 74-1-6. Nevertheless, our data and published studies 10 suggest that genetic complementation can be used to identify tumor suppressor genes within this region. We reasoned that the problems that we were having with STF-dependent stable suppression of tumorigenicity were dependent on the size of the transferred genomic fragment, which is very large (several Mb) and is also carried within a mouse chromosome. In addition, these clones must be introduced by microcell transfer, which is a cumbersome technique and also requires exposure of the cells to a variety of noxious agents including polyethylene glycol. We therefore wished to turn to a simpler strategy involving simple lipofection of more manageable inserts, namely BACs, that contain only the gene region of interest without much extraneous genetic material. Such experiments also enable a much greater efficiency compared to microcell transfer. Thus we would be able perform these experiments at earlier passage and more importantly be able to assess growth properties much earlier after gene transfer, reducing the chance of selection of mutated or silenced clones that escaped tumor suppression.

We therefore embarked on a new strategy in the past year using bacterial artificial chromosome (BAC) clones from 11p15. There are now BACs available that cover most of the human genome, usually redundantly, and these are available commercially. It is thus relatively easy to obtain large
genomic clones covering any given genomic region of interest. In particular, we have found BACs spanning all of 11p15. Unfortunately, the commercially available BAC clones lack a mammalian selectable marker gene that would enable their transfer into tumor cells for tumor suppressor experiments. We therefore designed a strategy for introducing the mammalian selectable marker gene into BACs by homologous recombination, directly within the bacteria that are used as a host for commercial transfer of the BACs to our laboratory. This was done by modifying the vector pSV1.RecA of Heintz's lab, described in Nature Biotechnology, by introducing a GFP and neomycin gene from pEGFP.N3 (Clontech). PSV1.RecA vector is designed to introduce knockout vectors into BACs, which are then used as transgenes.

Our approach was to design a novel retrofitting vector, that includes part of pSV1.RecA, but with the neomycin gene integrated into pBeloBAC vector sequence (the BAC vector), in turn inserted into pSV1.RecA. Our vector, termed pOOK, then recombines by homologous recombination with any BAC directly in the bacterial strain, and the integration of neomycin allows selection on subsequent lipofection of the modified BAC into mammalian cells. We have found that the efficiency of retrofitting using our vector is very high (>15%), as identified by Southern hybridization. This strategy is shown schematically in Figure 1, shown on the next page:
**Figure 1. Strategy for Retrofitting Any BAC with a Mammalian Selectable Marker Gene.**

Our novel vector, pOOK, undergoes homologous recombination with the region A homologous region (RA) (shown) or region B (RB). The resulting co-integrate is shown at the bottom. The BAC insert is represented as a dashed line.

To date, 10 separate BACs spanning most of the candidate gene region have been prepared. Because of the previous problems dealing with microcell transfer and the need to develop this retrofitting strategy, we have just begun to introduce these retrofitted BACs into breast cancer cells by the time that our funding under this grant ended. Our initial experiments, however, show a much higher efficiency of transfer to tumor cells than we obtained with the STFs, based on the fraction of cells (>70%) that are positive for GFP after 10 days’ selection in G418. An advantage of the GFP tag is that it confirms that the BAC has not been transcriptionally silenced, which can be determined by fluorescent microscopy without disrupting the cells. We will continue to perform these experiments on MCF-7 and HuMI breast cancer cells in the next few weeks. Hopefully we will be able to identify a growth suppressor activity within the defined region in the very near future.

**Task 3, Identification of Candidate Breast Cancer Genes:** We had previously identified 3 novel genes within the candidate gene region, and in the past year we have completed the
identification of 2 others. These are termed TSSC4 (Tumor Suppressing STF cDNA 4) and TSSC6 (Tumor Suppressing STF cDNA 6). TSSC4 was found to encode a predicted protein of 329 amino acids within a cDNA of 1463bp (Figure 2).

Figure 2. DNA and Predicted Protein Sequence of TSSC4. The first methionine of the open reading frame is at nucleotide 182 and the stop codon is at nucleotide 1171. Arrows indicate the positions of intron-exon boundaries.

The entire coding sequence lies with the second of two exons. A BLAST search indicated the absence of any protein homologous to TSSC4. Motif analysis identified one predicted protein kinase A site at Ser311. TSSC4 also contained 5 predicted protein kinase C phosphorylation sites, at Ser90, Ser129, Ser146, Ser248, and Ser307. Comparison of TSSC4 in genomic sequence indicated that the gene contains 2 exons, and that in the entire coding region is located within the second exon and it is shown in Figure 2.
TSSC6 was analyzed in a similar manner, and comparison with genomic sequence revealed that it contains 9 exons. Interestingly, EST clone 325072 lacks exon 4, causing a shift in the reading frame most likely because of aberrant RNA splicing. The fact that this aberrantly splice product is present within an unselected cDNA library and not generated by PCR, supports our hypothesis that aberrant splicing is a real abnormality of biological significance and certainly does not arise as a PCR artifact, at least in our hands. The predicted protein of TSSC6 is 290 amino acids with a cDNA of 1241bp and is shown in Figure 3.

**Figure 3.** DNA and Predicted Protein Sequence of TSSC6. The first methionine of the open reading frame is at nucleotide 146 and the stop codon is at nucleotide 1018. Arrows indicate the positions of intron-exon boundaries.
A conventional BLAST search did not reveal any homology to known gene sequences. However, a PsiBLAST search revealed that TSSC6 shows weak similarity to the rat TAPAl gene, which belongs to the tetramembrane-spanning protein family. Interestingly, the human TAPAl gene is located 60kb centromeric to TSSC6, suggesting that one of the genes may have arisen in part by duplication followed by considerable sequence divergence. TSSC4 detected a strongly hybridizing 1.6 kb transcript in fetal tissues and a weakly hybridizing fragment in all adult tissues. TSSC6 did not detect a signal on Northern blot. However, it did amplify by RT-PCR from all tissues tested. Both genes were expressed in breast tissue. In work not supported by this grant, we also found that TSSC4 and TSSC6 are not imprinted at any significant level, in at least late gestation and adult tissues. Thus TSSC4 or TSSC6 could serve as a conventional tumor suppressor gene even though it is in the middle of a large imprinted gene domain.

**Task 4, Mutational Analysis of Candidate Breast Tumor Genes:** Over the past year, we performed exhaustive mutational analysis of all of the genes we or others have identified within this region in breast cancer, altogether 8 genes. We have now sequenced in their entirety all of these genes in at least 12 samples and in as many as 98, depending on the size of the gene. While no somatic mutations were identified, we did identify several novel polymorphisms which should be useful to laboratories for both LOH and imprinting studies. The most frequent polymorphisms were: TGGAGGTGCA(A/G)TATTACAACA, at nucleotide 1645 of the GOK gene; CGAGGGGAGACG(C/T)TGGAGAAGCG, at nucleotide 54 of the TSSC3 gene; and CCATGGCCTG(C/G)ATG-TCCAGCG, at nucleotide 1166 of the TSSC5 gene. A fourth sequence variation was found in NUP98, changing Thr at position 87 to Ala, which was not observed in 200 other individuals with other tumors or normal specimens. This represents a change from a polar to nonpolar side chain, which may be significant. We had hoped to obtain a blood sample from the patient, in case this mutation may have arisen as an early developmental abnormality, but the patient refused. However, the fact that the patient developed multiple primary breast tumors suggests that there was a germline predisposition to breast cancer, and she did not have known BRCA1 mutations. NUP98 must remain for now a tantalizing candidate gene.

While somatic mutations were not found, we did identify relaxation of RNA splicing fidelity in half of the breast tumors studied. Thus 8 of 16 tumors showed relaxation of splicing fidelity of at least one of the three genes that was investigated in detail. Interestingly, tumors that showed altered splicing for one genes also frequently showed altered splicing for other genes, suggesting that an underlying defect in the splicing machinery itself may be important in tumor development or progression.

**Task 5, Analysis of LOI in Breast Cancer:** As noted before, this task was deleted previously because the grant was not funded for that work.

**Task 6, Analysis of Genes as Markers of Disease Subtype:** This task involved analysis of disease stage-specific alterations in 11p15 genes in breast cancer. In the course of these studies we have found two genetic changes at appreciable frequency, LOH of 11p15 markers, as well as relaxation of splicing fidelity of multiple genes within 11p15. Analysis of LOH by disease subtype showed that LOH, when present, occurred in both early and late stage disease. Thus, half of the tumors with LOH were of Stage 1 or 2. Similar to the LOH studies, we found that altered splicing occurred in both early and late stage disease. Thus, both abnormalities represent early events in tumorigenesis.

**Task 7, Analysis of the Mechanism of Altered Splicing in Breast Cancer:** This was not approved for additional funding in the last project period, but we have extended the number of samples analyzed at no additional cost to the grant, as described under Task 4.
2. DISCUSSION

Over the last year we have confirmed our localization of a region of LOH of 11p15 that extends from D11S988 to D11S1318. In an effort to further refine this boundary, we performed laser capture microdissection on 16 specimens, in order to purify the tumors to homogeneity. Sufficient material was obtained for analysis for homozygous deletions, which was a reasonable approach given its success in the analysis of other tumor types, but unfortunately none of the specimens showed deletions. In addition, in order to overcome the technical obstacles in performing genetic complementation experiments using subchromosomal transferable fragments, we developed an alternative approach over the past year that allows us to transfer large genomic BAC fragments with high efficiency into tumor cells. This process involved the development of a novel retrofitting vector, which can be used generally on any BAC to test for genetic complementation, where the gene region is known but the gene is not yet identified, or where a large gene needs to be transferred that is not amenable to conventional cDNA transfection. We believe that we will be able to determine whether one or more of the genes we have identified lies within the complementing region over the next few months. Furthermore this vector should be of wide general utility to other cancer researchers, and we have already provided it to other investigators studying breast and other cancers.

We have also identified two novel genes in the past year within the candidate region, TSSC4 and TSSC6. One of these, TSSC6, is homologous to members of the tetramembrane-spanning protein family. Finally, we extended our previous observations of relaxation of splicing fidelity in breast cancer. We found that this is a frequent alteration involving greater than 50% of tumors. It involves multiple genes, and it appears to involve a defect in the splicing machinery itself, since tumors with altered splicing of one gene show altered splicing of other genes. We believe that this alteration will serve to be an important novel mechanism in tumor progression and is a major contribution of the work done under this grant.

CONCLUSIONS

1. The minimal overlapping region of 11p15 LOH in breast cancer extends from D11S988 to D11S1318.
2. No microdeletions were observed in 12 cancers analyzed by laser capture microdissection.
3. A novel strategy of genetic complementation was developed to allow transfer of any BAC into tumor cells. Toward this end, a retrofitting vector was developed that contains a mammalian selectable marker gene and can be introduced at high efficiency into any BAC in a single transfection step.
4. Two novel genes, TSSC4 and TSSC6, were identified within the candidate gene region.
5. No somatic mutations were observed over the complete coding sequence of 8 candidate genes, in 12 to 98 tumors, depending on the gene examined. However, a germline alteration was found in NUP98 that was not present in 200 other samples.
6. Relaxation of RNA splicing fidelity was found in over half of breast cancers, and it appeared to involve a defect in the splicing machinery itself, as tumors with altered splicing of one gene showed similar involvement of multiple genes.
7. Both LOH and relaxation of splicing fidelity are early events in human breast cancer.
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