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Metastatic Progression of Breast Cancer by Allelic Loss on Chromosome 18q21

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The molecular basis of a pro-oncogenic role of TGFβ in promoting advanced metastatic cancer remains unclear. The TGFβ levels are increased locally and systemically in advanced breast tumors particularly at the leading edges and in metastasis. Our studies provide direct evidence for the association between the inactivation of SMAD4 gene localized to chromosome 18q and upregulation of pro-angiogenic metastatic factors such as angiopoietin and VEGF in breast cancer. Additionally, our preliminary data also provides evidence for the synergistic activation of pro-angiogenic metastatic effects by TGFβ in the presence of defective SMAD4 in breast cancer. We therefore hypothesize that normal Smad4 mediated events are required to maintain suppression of metastasis and disabling these events is a major step towards the development of metastatic malignant breast cancer. We are in the process of the identification and characterization of the mediator and effector genes that regulate metastatic progression of breast cancer upon inactivation of the Smad4 mediated events.
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ANNUAL REPORT OF THE USAMRMC FUNDED ACTIVITY
Title of the grant: Metastatic progression of breast cancer by allelic loss on chromosome 18q21.

1. Introduction/ Project Overview/ Scientific Progress and future directions:
   An association has been established between the high frequency of deletion of chromosome 18q21, where the SMAD4 gene is localized, with advanced stages of cancers (1). These observations have received added credence from a recent report that suggested up-regulation of metastasis mediator genes such as VEGF and down-regulation of metastatic suppressor genes such as TSP1 in cell lines with SMAD4 deletions or mutation (2). Our preliminary data very strongly supports these observations and provides the first line of evidence in breast cancer. Additionally, the lack of inactivation of Smad4 at a high frequency in breast tumors prompted us to hypothesize that even though the SMAD4 gene may not be a direct frequent target for mutational inactivation, the other unidentified target genes in the Smad4 mediated events could potentially be of greater importance in breast cancer due to differences in tissue specificity (1).

   Our survey of the various Smad genes has provided the first clues in identifying the Smad8 gene as an important target for loss of expression in nearly 30% of breast cancers which we believe is a significant finding as even the most celebrated tumor marker, HER/neu gene amplification, also occurs in about 20%-30% breast cancer cases (3). We found that the inactivation of the Smad8 gene leading to loss of its expression is mediated by epigenetic DNA methylation (3). It still remains to be determined whether Smad8 inactivation could also be an alternate target for Smad2 or Smad4 inactivation.

   Furthermore, our investigations of the consequences of Smad4 inactivation revealed that that lack of Smad4 could favor angiogenesis/ metastasis, which is further enhanced by TGFβ and hypoxia. We are continuing to characterize the cell culture models so that we could undertake comprehensive analysis of the mediator and effector genes, which regulate metastatic progression of breast cancer upon inactivation of the Smad4 signaling pathway.

2. Modified tasks, summary of findings and future directions:

   We are making steady progress in increasing the understanding of the implications of the direct or indirect inactivation of SMAD4 localized to chromosome 18q in metastatic breast cancer.

Task 1. Determination and identification of genetic and epigenetic alterations in known and novel SMADs as potential target genes and the elucidation of their implications to metastatic breast cancer.

   With the uses of a novel technique known as TEGD (targeted expressed gene display), we identified that loss of SMAD8 gene expression could play a major role in the genesis of breast cancer. Our studies provided the first direct clues for epigenetic silencing of SMAD8 expression due to promoter DNA hypermethylation in breast cancer (3). We have already constructed expression vectors for the SMAD8 gene. We are in the process of generating defective derivatives of the wildtype SMAD8 gene as well as planning to undertake siRNA based experiments to dissect the consequences of the SMAD8 defect in breast cancer. One of the other goals will be to raise monoclonal antibodies against Smad8 to develop potential diagnostic tests. We are currently seeking financial support to take this project forward in a full fledged manner.
Task 2. Identification and elucidation of the roles of alternate target genes involved in the Smad4 signaling pathway.

We have successfully established isogenic cell culture model systems that are proficient and deficient in Smad4 expression. Our data strongly support that the Smad4 defect favor angiogenesis/metastasis with significant enhancement in the presence of TGFβ under hypoxic conditions consistent with advanced cancer. Bioinformatics tools will be exploited to construct network of interacting pathways/events using the various genes identified from the characterization of the model cell lines.

Task 3. Evaluation of candidate target genes.

This task remains unmodified and would begin once we have identified legitimate mediator and effector genes in Tasks 1 & 2. The putative target gene(s) will be evaluated for genetic and epigenetic alterations causing inactivation and/or deregulation in tumors.

Body: Procedures and progress report:

A.

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Smad4/DPC4 69kDa

B.

C.

Figure 1. Isolation and initial characterization of Smad4 stable cell lines. A. Western blotting was used to screen for stable cell lines that constitutively express Smad4 and corresponding isogenic controls that have integrated the empty vector. Lanes 1-5 correspond to derivatives of HCT116 Smad4-/- stably transfected with TGFβ-RII receptor and empty
vector (1&2) or pCMV-Smad4 (3-5). Lanes 6-9 are BR05(Smad4"") stably transfected with an empty vector (6&8) or TGFβ-RII receptor (7) or pCMV-Smad4 (9). Please note that the clone in lane 5 is a false positive. Although the original HCT116/− is defective for Smad4, BR05(Smad4"") constitutively expresses defective forms of Smad4. B. RPA analysis was performed using fifteen μg each of the indicated RNA samples with the RiboQuant multi-probe template set (HAngio-1, BD-PharMingen, San Diego, CA) to detect the indicated mRNAs. L32 and GAPDH were included in each template set as internal controls. C. Western blotting was used to screen for Smad4 and VEGF using β-actin as internal control in original HCT116 Smad4/−, HCT116 Smad4++ and the indicated derivative stable cell lines that either constitutively express or not express Smad4.

The stable cell lines that are proficient and deficient for Smad4 expression showed that over-expression of Smad4 could suppress the expression of pro-angiogenic/ pro-metastatic effectors such as VEGF (Figure 1). Furthermore, we also examined the potential synergistic effects of hypoxia and/or TGFβ itself under the SMAD4 deficient and proficient conditions (Figure 2). The results from these studies strongly support our hypothesis that the hypoxic conditions and/or the presence of TGFβ had no effect on the levels of VEGF in the presence of abundant levels of Smad4 expression while there was a synergistic enhancement in the levels of VEGF when the cells were defective for Smad4 expression (Figure 2).

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Figure 2. The effect of hypoxia and or TGFβ on VEGF expression in SMAD4 proficient and deficient cells.

The HCT116 Smad4/− cell line stably transfected with TGFβ-RII receptor and either empty vector (C2) or pCMV-Smad4 (T9) were evaluated for VEGF expression in the presence or absence of TGFβ and/or hypoxia condition (95%N2) by RNase Protection Assay.
Figure 3. TGF-beta1 and hypoxia enhances the transcriptional activation of VEGF in SMAD4 defective cells.
The MDAMB 468 Smad4-/- cell line stably transfected with either empty vector (2,3) or pCMV-Smad4 (7,9), and transiently transfected with the VEGF reporter and Renilla luciferase internal control reporter plasmid. Luciferase activity was measured in cell lysates and plotted as the average with standard deviation for triplicate determinations. VEGF reporter activity was measured in normoxia (green bars) and hypoxia (yellow bars) culture conditions respectively in the indicated Smad4 deficient (2,3) and proficient (7,9) clones.

Figure 4. The effects of SMAD4 status, TGFβ and hypoxia on cell migration.
The indicated cells were plated into the transwell insert with 8.0 um of polycarbonate membrane of a 6-well plate at a density of 2 x 10^5 cells/well and cultured 18hrs. After incubation, the cells on the insert were fixed, stained with 0.2% crystal violet, cells on the top of the membrane were photographed and removed with the help of a cotton swab. The cells which have already migrated into the membrane and are not removed by cotton swab were photographed from the bottom of the membrane of the insert. Three fields per insert were selected randomly for photography and all the experiments were independently performed at least twice.

We have further confirmed these observations at the level of gene regulation by performing reporter assays. We chose stable transfectants of the breast cancer cell line MDAMB468 that are either proficient or deficient for SMAD4 to evaluate the role of Smad4 in the regulation of a pro-angiogenic/pro-metastatic marker gene VEGF in the presence/absence of TGFβ as well as normoxic/hypoxic conditions that mimic the advanced tumors (Figure 3). The hypoxic conditions and/or the presence of TGFβ had little or no effect on the levels of VEGF in the presence of abundant levels of Smad4 expression while there was a synergistic enhancement in the levels of VEGF when the cells were defective for Smad4 expression (Figure 2).

After successfully establishing the regulation of a marker pro-angiogenic and/or pro-metastatic factor, VEGF in favor of increased propensity for angiogenesis/metastasis in Smad4 defective cells, we also found that the defect in Smad4 enabled these cells to exhibit increased motility and migration with synergistic enhancement under hypoxic conditions and in the presence of TGFβ (Figures 4).

In summary, our preliminary data strongly support that Smad4 is a primary suppressor of angiogenesis and/or metastasis and the presence of SMAD4 gene or signaling defect could be the major switch in the conversion of benign tumors to malignancy during multi-step progression of cancer.

We are planning to extend these studies to not only confirm this phenomenon with other candidate genes but also identify a wide spectrum of other critical genes important for the metastatic progression of breast cancer using the microarray (Affymetrix) technology.

Once legitimate metastasis mediator and effector gene(s) are identified, evaluation of the status of the candidate gene(s) for inactivation/activation in metastatic breast cancer will commence as described in the original proposal (Task 3).

4. Key research accomplishments:

Our study provide the first direct evidence that 30% of the breast cancers exhibit loss of Smad8 expression and makes it as one of the highly valued markers similar to Her/neu. Our studies also provide the first direct evidence that the silencing of gene expression via DNA hypermethylation of the Smad8 gene could be an important event in breast cancer progression and metastasis. Therefore, Smad8 has the potential to become a key target for the development of diagnostic, prognostic and therapeutic strategies to combat breast cancer.

We have also identified/generated appropriate tumor cell lines as well as experimentally developed derivative test and control cell lines as model systems to identify and isolate the metastatic breast cancer mediator and effector genes involved in the Smad4 signaling pathway.

5. Conclusions:

(1) The loss of SMAD8 expression in breast cancers is primarily mediated by gene silencing due to epigenetic DNA methylation of regulatory regions.
(2) A combination of SMAD4 inactivation, high levels of TGFβ and hypoxic conditions could favor angiogenesis/metastasis.
(3) The identification of target gene(s) that disable Smad4 or Smad8 signaling to promote breast cancer could potentially provide not only novel and valuable diagnostic and prognostic tumor markers but also key arsenals to combat breast cancer.

6. References:

7. Scientific presentation/publications/patent relevant to this grant:

Presentations by Dr. Sam Thiagalingam:
The SMAD connection to cancer, Genetics and Genomics Friday Seminar, Boston University School of Medicine, January 23, 2004
The SMAD connection to cancer, TASME 2004 Conference, Quelph, Canada, July 3, 2004

Publications:

Patent:
1. Method of determining gene expression-Targeted Expressed Gene Display – Filed provisional application (BU03-16; March 25, 2003); Boston University.