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13. ABSTRACT *(Maximum 200 Words)*

We are investigating the role of α6β4 integrin expression in the progression of breast cancer. No amplification of the β4 gene was detected using probes derived from BAC clones. Cell lines MDA-MB-231 and BT474 (with high α6β4 expression) showed increased phosphorylation of PI3Kp110 but no change in the phosphorylation of PI3Kp85 upon clustering surface β4. When surface α6 was clustered, MDA-MB-231 and MDA-MB-134 showed increased phosphorylation of FAK, and MCF-7 showed increased phosphorylation of Akt. No changes in phospho-IRS-1, phospho-PDK-1, phospho-Erk1,2, and phospho-Shc were detected upon clustering either surface β4 or α6. We designed an oligonucleotide probe for β4 integrin mRNA and correlated β4 mRNA expression in paraffin sections with β4 protein expression in corresponding frozen sections. This probe will be used to correlate β4 expression in breast cancer specimens with clinical outcome. Sixty fresh invasive breast carcinoma specimens have been collected so far by fine-needle aspiration, surface β4 has been clustered, cytospin preparations have been prepared, and cell lysates have been prepared and frozen for future Western blot analyses. Changes in the phosphorylation of α6β4 signaling intermediates will ultimately be correlated with clinical parameters.

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Introduction:

As the principal cell surface receptors for extracellular matrix proteins, integrins may play an important role in tumor cell invasion and metastasis. Recent reports suggest that the α6β4 integrin, in particular, may be associated with the progression of breast cancer. In this project, we are investigating the hypothesis that: 1) the expression of α6β4 integrin and /or its signaling intermediates is associated with poor prognosis in breast cancer, and 2) that increased α6β4-mediated signaling correlates with poor prognosis in breast cancers that overexpress α6β4.

Body:

During our first year of funding, I believe we have made significant progress towards our overall goal, but we have also encountered some significant obstacles that we have had to address. In this first annual progress report, I will address each task in the original statement of work and discuss progress made, problems encountered, and proposed modifications for the future. I will discuss Task 1 last because of special problems encountered (see below).

Original Task 2. To measure gene copy numbers of the α6 integrin precursor and β4 gene in frozen breast cancer specimens and correlate results with α6 and β4 protein expression and clinical data (months 1-36):

We obtained 2 BAC clones containing the integrin β4 subunit precursor from Research Genetics (clones RP11-474I11 and RP11-552F3). The clones were verified after isolation using PCR primers derived from GenBank sequences (stSG27931 and STS WI07037), and the isolated DNA was labeled by DIG-nick translation. Specificity of probe hybridization was verified by performing FISH on a lymphocyte-derived metaphase spread using each of the labeled probes and a commercially available probe for CEP 17, which recognizes the α satellite DNA band region 17p11.1-q11.1. Figure 1A shows the specificity of one of the β4 probes for the long arm of chromosome 17.

FISH assays using these probes for the β4 subunit precursor gene were performed on multiple cell lines with known α6β4 protein expression. We observed no evidence of β4 gene amplification in breast carcinoma cell lines with known high or low α6β4 protein expression (Figure 1B and 1C)

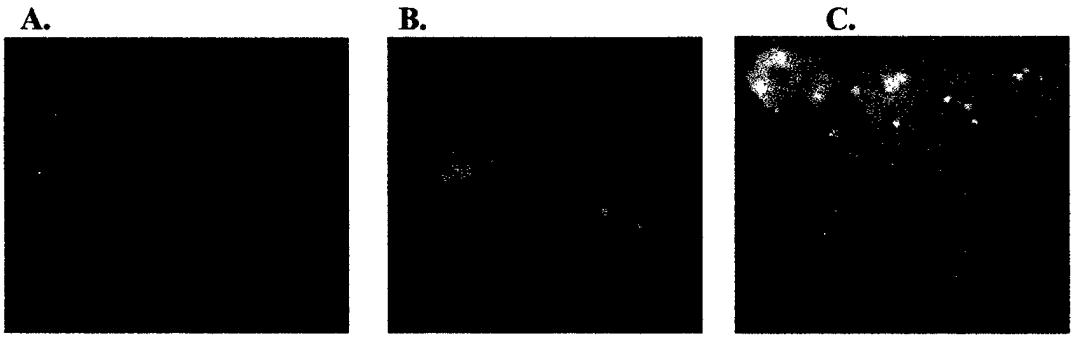

Figure 1. (A) FISH performed on a normal metaphase spread using probes for CEP17 (orange) and the integrin β4 subunit precursor gene (green). The same probes used on breast carcinoma cell lines MDA-MB-231(B) and MCF-7 (C). The cell lines are aneuploid; although individual cells show more than 2 signals for β4, the ratio of β4:CEP17 is not increased.

Since we were not able to observe evidence of β4 gene amplification using 2 different probes for the β4 gene in breast cancer cell lines known to express the α6β4 integrin, I believe at this point that it will not be fruitful to pursue the examination of α6β4 gene amplification in the patient specimens. Since Task 3 is the most innovative and important task in this project, I would like to modify the specific aims to reduce Task 2 to what has been performed as described above and to expand Task 3, as will be discussed in detail below.

Modified Task 2. To measure gene copy numbers of the β4 gene in multiple breast cancer cell lines with known α6β4 integrin expression using probes derived from 2 different BAC clones (months 1-8).

Original Task 3. To evaluate laminin-induced phosphorylation of PI3K, c-erbB-2, Shc, and FAK in 100 fresh breast cancer specimens and correlate findings with clinical data (months 8-36):

a. prospectively acquire and isolate tumor cells from fresh breast cancer specimens, and measure laminin-induced phosphorylation of signaling intermediates (months 8-30)
b. correlate findings with clinical data (months 30-36).

Based on feedback and suggestions that we have received from reviewers, we have modified the technique used to stimulate the α6β4 signaling pathways in fresh tumor specimens. Commercially available laminin preparations contain various laminin isoforms, so using such preparations to stimulate α6β4 would result in stimulation of multiple additional pathways. A more specific way to activate the α6β4 integrin pathway is to cluster the α6β4 surface receptors with antibodies to the β4 subunit. This is accomplished by first performing fine-needle aspiration of fresh tumor cell specimens, then exposing the tumor cell suspensions to either anti-β4 or anti-MHC II (control) on ice for 40 min, followed by anti-IgG at 37°C for 30 min. The last step results in a clustering of cell surface α6β4 integrins, which activates the α6β4 signaling pathway.

So far we have collected and processed 60 fresh invasive breast carcinoma specimens. The specimens have been collected by fine-needle aspiration and processed as described above, and cytospin preparations have been prepared and stored for future immunohistochemical staining. For those specimens with sufficient cellularity, cell lysates have also been prepared and frozen for future Western blot analyses. In our initial in-vitro studies using phosphorylation state-specific antibodies to phospho-FAK, phospho-PDK1, phospho-IRS-1, phospho-Shc, phospho-Erk, and phospho-Akt, **we have not detected changes in the phosphorylation of these signaling intermediates in the β4-positive breast cancer cell lines following clustering of surface β4.** (We have seen increased phosphorylation of FAK and Akt following clustering of α6, but this apppear to be a result of α6β1 stimulation rather than α6β4. See Task 4). Therefore, we have not yet confirmed the best phosphorylation-state specific antibodies to use on the tumor specimens. As we continue to prepare tumor cell suspensions from breast cancer specimens and store them for future analysis, collaborative arrangements have been made with Research Genetics, Inc. to develop an antibody to phospho-β4. If successful, we will try to use this antibody on the cytospins and cell lysates, as well as antibodies to phospho-IRS-1 and phospho-Akt.

I believe Task 3 is the most important and innovative task of this project, since it involves the activation and measurement of α6β4 signaling intermediates in fresh breast cancer specimens. To correlate findings with clinical outcome, it is important to use previously untreated tumor specimens, as we originally proposed. So far, we have collected 60 out of the 100 such specimens we proposed to evaluate. However, I would like to maximize the number of specimens evaluated in this manner. Many of the patients at M.D. Anderson receive neoadjuvant chemotherapy prior to surgery. We could increase the number of specimens available to study α6β4-mediated signaling by including these specimens as well. Although these additional specimens could not be used to correlate α6β4 signaling with clinical outcome (as will be done with those specimens that have not been previously treated), it is also important to simply determine whether the clustering of surface α6β4 integrins in fresh tumor specimens results in changes in the phosphorylation state of signaling intermediates, and whether these changes can be detected using cytospin preparations of tumor cells and/or Western blots of tumor cell lysates. These additional specimens can be used for this purpose. We currently have an IRB-approved protocol to examine α6β4 signal transduction in both untreated and treated breast cancer specimens, and we would like to expand this part of our DOD proposal to include such specimens as well.

Therefore, we would like to modify Task 3 as follows:

Modified Task 3. To evaluate α6β4-mediated phosphorylation of signaling intermediates in fresh breast cancer specimens (months 1-36):

a. prospectively acquire and isolate tumor cells from 100 fresh previously-untreated breast cancer specimens, and measure α6β4-mediated phosphorylation of signaling intermediates (months 1-30)
b. correlate findings with clinical data (months 30-36)
c. prospectively acquire and isolate tumor cells from 100 additional fresh breast cancer specimens previously treated with chemotherapy, and measure α6β4-mediated phosphorylation of signaling intermediates (months 1-30)

Original Task 4. To determine whether α6β1 and/or α6β4 heterodimers are involved in laminin-induced phosphorylation of PI3K, c-erbB-2, Shc, and FAK in breast cancer cell lines (months 9-30).

Using the modified technique described above, we have looked more specifically at the stimulation of α6 integrin pathways by exposing cell suspensions of the tumor cell lines to either anti-α6, anti-β4 or anti-MHC I (control) on ice for 40 min, followed by anti-IgG at 37°C for 30 min. (Function-blocking antibodies, such as those used to block integrin-mediated cell adhesion, cannot be used to block this type of signal transduction assay.)

By Western blot analysis, all of the 7 breast cancer cell lines we have examined (MDA-MB-231, MCF-7, MDA-MB-134, MDA-MB-435, MDA-MB-453, BT 474, and SKBr3) show expression of the α6 integrin subunit. However, only 4 of the 7 breast cancer cell lines (MDA-MB-231, MCF-7, MDA-MB-453, and BT-474) show β4 expression. When surface β4 is clustered in the manner described above, cell lines MDA-MB-231 and BT474 (which have the highest α6β4 expression) show increased phosphorylation of PI3Kp110 (but no change in the phosphorylation of PI3Kp85)(Figure 2). In contrast, cell line MCF-7 (which has low α6β4 expression) shows decreased phosphorylation of PI3Kp110.

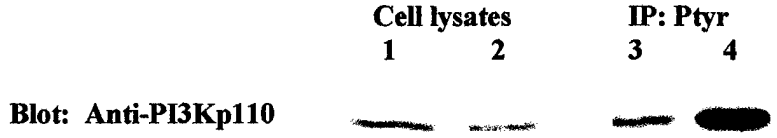

Figure 2. Immunoprecipitation with anti-phosphotyrosine performed on breast carcinoma cell line MDA-MB-231 exposed to anti-MHC I control (lanes 1,3) or anti-β4 (lanes 2,4) on ice for 40 min, followed by anti-IgG at 37°C for 30 min. Whole cell lysates and immunoprecipitation products are blotted with anti-PI3K p110.

When surface α6 is clustered for 30 min, MDA-MB-231, MDA-MB-134, and MDA-MB-435 show increased phosphorylation of PI3Kp110, while decreased phosphorylation of PI3Kp110 is seen for MCF-7 and SKBr3. Under these conditions, MDA-MB-231 and MDA-MB-134 also show increased phosphorylation of FAK.

Interestingly, when surface α6 is clustered for 30 min, the amount of phosphorylated PI3Kp110 associated with the β4 subunit decreases for MB-231 and BT474 but increases for MCF-7. MCF-7 also shows increased phosphorylation of Akt (ser473 and thr308). However, changes in phospho-IRS-1, phospho-PDK-1, phospho-Erk1,2, and phospho-Shc were not detected upon clustering either surface β4 or surface α6.

The above findings suggest that α6 integrin-mediated signaling varies between cell lines expressing α6β1 and α6β4 surface integrins. The two cell lines showing the highest α6β4 expression (MDA-MB-231 and BT474) produce increased phosphorylation of PI3Kp110 when surface β4 is clustered, but the amount of phosphorylated PI3Kp110 associated with β4 in these two cell lines decreases. Therefore, it appears that PI3Kp110 becomes activated and then dissociates from β4 when surface β4 is clustered. In contrast, MCF-7 (which shows low α6β4 expression) produces decreased phosphorylation of PI3Kp110 when surface β4 is clustered, but the amount of phosphorylated PI3Kp110 associated with β4 increases. Therefore, it appears that PI3Kp110 becomes less activated in MCF-7 and then associates with β4 when surface β4 is clustered.

These findings suggest that the presence of α6β4 alone will be insufficient to predict α6β4-mediated signaling in a particular tumor, and that the measurement of particular activated signaling intermediates coupled with the presence of the upstream receptor may yield better predictive or prognostic information.

Original Task 1. To evaluate protein expression of α6, β4, PI3K, c-erbB2, Shc, and FAK in frozen breast cancer specimens and correlate findings with clinical data (months 1-8; 30-36).

We proposed originally that 250 frozen breast cancer specimens from the M.D. Anderson Breast Tumor Bank would be used in Task 1. The director of our tumor bank had given approval prior to our proposal, but we have since been unable to get final approval from the Breast Cancer Steering Committee for the use of these frozen tissues. The principal reason why we initially proposed using frozen tissues was that antibodies to the α6β4 integrin could not be used successfully in formalin-fixed, paraffin-embedded tissues.

We have worked with various antigen retrieval methods to try to get these antibodies to work on paraffin-embeded tissues instead, but without success to date. We therefore sought to determine whether in-situ hybridization could be used successfully on the paraffin-embedded tissues as an alternative to immunohisochemistry to detect the

α6β4 integrin. If so, we could perform Task 1 on archival paraffin-embedded tissues instead of frozen tissues. We think this would actually be more informative than performing the study on the frozen tissues, since we have the paraffin tumor blocks and over 10 years of clinical follow-up information available from a group of 250 patients with previously untreated, axillary node-negative breast cancers. This would be ideal for evaluating the prognostic significance of α6β4 integrin expression.

We therefore designed a 40-base hyperbiotinylated oligonucleotide probe for the β4 integrin subunit for use in an in-situ hybridization (ISH) assay on paraffin-embedded tissues. ISH signal was detected using an alkaline-phosphatase-based protocol and the chromagen Fast Red. We initially tested this probe on two breast cancer cell lines, one known to express the α6β4 integrin (MDA-MB-231), and one known to be negative for α6β4 (MDA-MB-134). Strong cytoplasmic expression of β4 was detected in MDA-MB-231, whereas no expression was detected in MDA-MB-134 (Figure 3). The signal in MDA-MB-231 could be competitively inhibited by excess unlabeled β4 probe, indicating probe specificity.

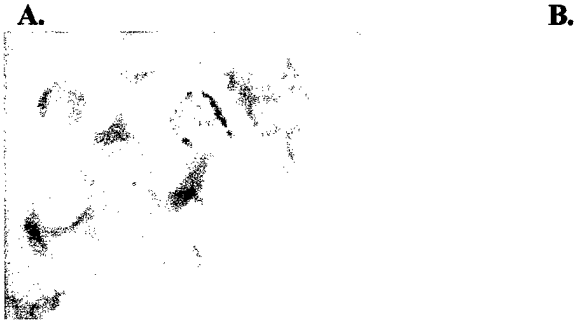

Figure 3. In-situ hybridization for β4 subunit mRNA in breast carcinoma cell lines MDA-MB-231 (A) and MDA-MB-134 (B).

We then performed ISH analysis on 15 cases of formalin-fixed, paraffin-embedded invasive breast carcinoma using the β4 probe and compared results to immunohistochemical staining for β4 protein on corresponding frozen tissue sections. Hybridization with a poly(dT)-20 base oligonucleotide was done as a control to confirm the presence of mRNA in the paraffin tissues. Additional controls included competition with excess unlabeled probe and staining with chromagen alone. ISH detected the presence of β4 mRNA in 6 cases. Positive signal was observed in the cytoplasm of invasive tumor cells (Figure 4). Protein was detected in the corresponding frozen sections in 4 of these 6 cases. In two cases β4 mRNA was detected without corresponding observable protein expression, raising the possibility that splice variants of the β4 subunit may not be recognized by the monoclonal antibody used on the frozen sections. All cases positive for α6β4 integrin protein on frozen sections could be detected by ISH on the paraffin embedded tissues. Therefore, ISH appears to be a valid method for the detection of α6β4 integrin in paraffin-embedded archival material.

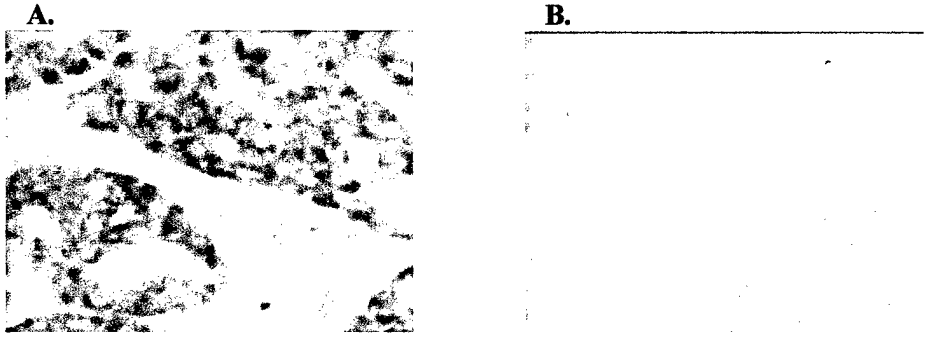

Figure 4. In-situ hybridization for β4 subunit mRNA performed on a formalin-fixed, paraffin-embedded section of invasive breast carcinoma (A), and competitive inhibition of in-situ hybridization using 100-fold excess of unlabeled probe (B).

Since frozen tissues will not be available to test the protein expression of the other proposed signaling intermediates (PI3K, c-erbB-2, Shc, and FAK), we would also like to modify Task 1 to correlate the ISH expression of α6β4 with protein expression of a few of the important prognostic markers known to be detectable on paraffin sections, including estrogen receptor, progesterone receptor, and c-erbB-2.

We therefore propose to modify Task 1 as follows:

Modified Task 1. To evaluate α6β4 expression by in-situ hybridization using archival paraffin-embedded tissue sections from 250 cases of node-negative invasive breast carcinoma and correlate findings with ER, PR, and c-erbB-2 protein expression and clinical follow-up data (months 13-20; 21-28):

a. perform and interpret ISH using a custom 40-base oligonucleotide probe for the β4 integrin subunit on paraffin sections from 250 cases of node-negative invasive breast carcinoma (months 13-20).
b. correlate findings with ER, PR, and c-erbB-2 protein expression and clinical follow-up data (months 21-28).

Key Accomplishments:

- Two different probes for the β4 integrin subunit were made from BAC clones RP11-474I11 and RP11-552F3 and used on several breast cancer cell lines. No amplification of the β4 gene was detected.
- A 40-base hyperbiotinylated oligonucleotide probe for the β4 integrin subunit was designed for use in an in-situ hybridization (ISH) assay, tested on multiple breast cancer cell lines and paraffin-embedded tissue sections of invasive breast cancer, and shown to be specific for β4 integrin subunit mRNA.
- Sixty fresh invasive breast carcinoma specimens have been collected so far by fine-needle aspiration, surface β4 has been clustered, cytospin preparations have been prepared, and cell lysates have been prepared and frozen for future Western blot analyses.
- The expression of α6 and β4 integrin subunits has been characterized in multiple breast cancer cell lines, and changes in the phosphorylation of signaling intermediates following clustering of surface α6 and β4 have been analyzed.

• Reportable Outcomes:

- Abstract: LK Diaz, X Zhou, K Welch, J Roach, A Sahin, R Herbst, MZ Gilcrease. In-Situ Hybridization for Alpha6Beta4 Integrin in Breast Cancer: Correlation with Protein Expression. Annual Meeting of the United States and Canadian Academy of Pathology, February, 2002.
- R21 NIH proposal entitled "Expression of Alpha6Beta4 Splice Variants in Breast Cancer" submitted February, 2002.

Conclusions:

In summary, we have made significant progress towards our goal of evaluating the hypothesis that: 1) the expression of α6β4 integrin and /or its signaling intermediates is associated with poor prognosis in breast cancer, and 2) that increased α6β4-mediated signaling correlates with poor prognosis in breast cancers that overexpress α6β4. We found that the β4 gene does not appear to be amplified in breast cancers that overexpress the α6β4 integrin, but we designed a probe for β4 mRNA that will be useful in evaluating α6β4 expression in formalin-fixed, paraffin-embedded tissues. Sixty fresh invasive breast carcinoma specimens have been collected so far by fine-needle aspiration, surface β4 has been clustered, cytospin preparations have been prepared, and cell lysates have been prepared and frozen for future Western blot analyses. The expression of α6 and β4 integrin subunits has been characterized in multiple breast cancer cell lines, and changes in the phosphorylation of signaling intermediates following clustering of surface α6 and β4 have been analyzed.

We encountered several difficulties that require modification of the proposed statement of work, but overall we feel that these are improvements over the original proposal.

The proposed modified statement of work is as follows:

Task 1. To evaluate α6β4 expression by in-situ hybridization using archival paraffin-embedded tissue sections from 250 cases of node-negative invasive breast carcinoma and correlate findings with ER, PR, and c-erbB-2 protein expression and clinical follow-up data (months 13-20; 21-28):

a. perform and interpret ISH using a custom 40-base oligonucleotide probe for the β4 integrin subunit on paraffin sections from 250 cases of node-negative invasive breast carcinoma (months 13-20).
b. correlate findings with ER, PR, and c-erbB-2 protein expression and clinical follow-up data (months 21-28).

Task 2. To measure gene copy numbers of the β4 gene in multiple breast cancer cell lines with known α6β4 integrin expression using probes derived from 2 different BAC clones (months 1-8).

Task 3. To evaluate α6β4-mediated phosphorylation of signaling intermediates in fresh breast cancer specimens (months 1-36):

a. prospectively acquire and isolate tumor cells from 100 fresh previously-untreated breast cancer specimens, and measure α6β4-mediated phosphorylation of signaling intermediates (months 1-30).
b. correlate findings with clinical data (months 30-36).
c. prospectively acquire and isolate tumor cells from 100 additional fresh breast cancer specimens previously treated with chemotherapy, and measure α6β4-mediated phosphorylation of signaling intermediates (months 1-30).

Task 4. To characterize α6β4-mediated signaling pathways in multiple breast cancer cell lines (to assist in determining the best phosphorylation-state specific antibodies to use on the clinical specimens in Task 3)(months 1-20).

Data obtained from these studies should not only help to confirm the prognostic significance of α6β4 integrin expression in breast cancer but also determine whether increased phosphorylation of particular signaling intermediates yields greater prognostic significance than α6β4 expression alone. If so, α6β4 and its signaling intermediates may be important therapeutic targets for breast cancer in the future.