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TITLE: Investigation of Alpha6 Integrins and Their Signaling Intermediates as Prognostic Markers for Breast Cancer

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13. ABSTRACT (Maximum 200 Words)

Our goal was to evaluate the expression of α6β4 integrin in breast carcinoma and to test whether 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. We designed a probe for β4 mRNA useful in evaluating α6β4 expression in formalin-fixed, paraffin-embedded tissues, and showed that β4 mRNA expression appears not to be a prognostic factor in node-negative invasive breast carcinoma. Adhesion-independent cross-linking of α6β4 was found to be associated with phosphorylation of nonmuscle myosin II heavy chain, which may affect actin-myosin filament organization. We showed by immunofluorescence microscopy that cell-surface α6β4 clustering can be demonstrated following antibody-mediated cross-linking of the integrin not only in cell lines but also in fine-needle aspirates of breast carcinoma specimens. Although we could not demonstrate clinical significance in this study, further refinements are needed in the techniques to induce and detect integrin clustering in carcinoma specimens in order to determine whether detection of integrin clustering as a surrogate of integrin function will ultimately prove to be useful clinically.

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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:

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).

Completed, as reported in the previous annual report.

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).

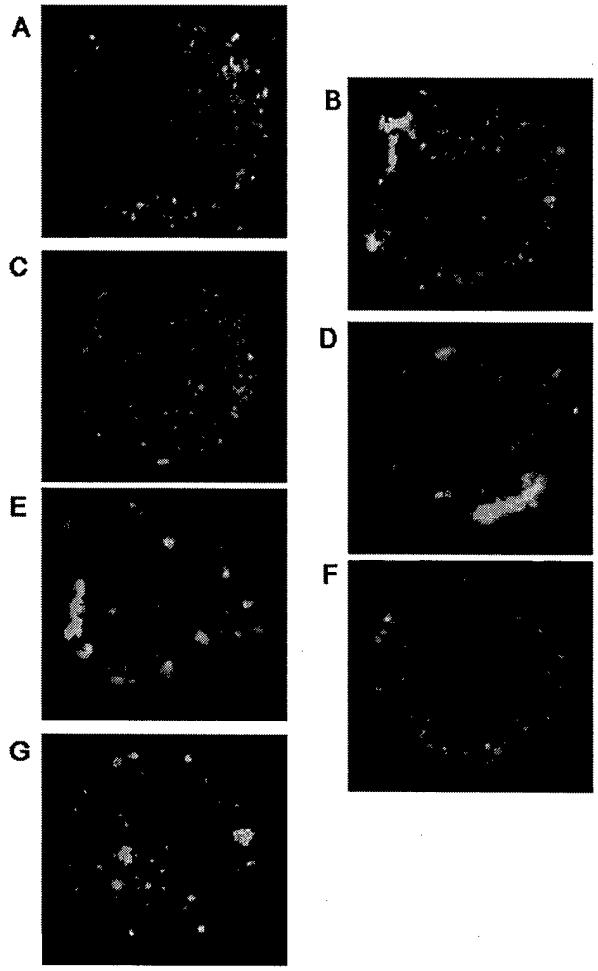
Completed, as reported in the first annual summary.

Because new findings obtained from Task 4 were used to complete Task 3, we will report Task 4 first:

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).

In our first annual report, we indicated that our in-vitro studies failed to show changes in the phosphorylation of FAK, PDK1, IRS-1, Shc, Erk, or Akt after cross-linking cell-surface α6β4 integrin. In our second annual report, we showed that nonmuscle myosin II heavy chain (NMMHC) undergoes increased serine phosphorylation after cross-linking cell-surface α6β4, and that this increase was not seen in the presence of the PI3K inhibitor LY294002. We do not yet have a phosphorylation-state specific antibody to test whether there is increased phosphorylation of NMMHC in the fine-needle aspirates of the clinical specimens we have collected. However, we were able to demonstrate cell-surface α6β4 clustering by immunofluorescence in the MDA-MB-231 cell line following cross-linking of cell-surface α6β4, and we showed that this process requires PI3K activity. We hypothesized that detection of α6β4 clustering by immunofluorescence might serve as a technique for measuring an intact α6β4 signaling pathway in fine-needle aspirates of breast carcinoma specimens.

Cell-surface α6β4 was cross-linked on MDA-MB-231 cells in suspension by treating cells with a monoclonal antibody to the β4 integrin subunit at 4°C, followed by anti-IgG at 37°C. Immunofluorescence microscopy revealed that adhesion-independent cross-linking of α6β4 resulted in the formation of prominent α6β4 clusters (Figure 1A and 1B). Clustering of α6β4 was significantly blocked when cross-linking was performed in the presence of PI3K inhibitors LY294002 and wortmannin (Figure 1C and 1F). In contrast, no significant inhibition of clustering was observed with protein kinase C inhibitor GF109203X (Figure 1D), rapamycin (Figure 1E), or heparin (Figure 1G).



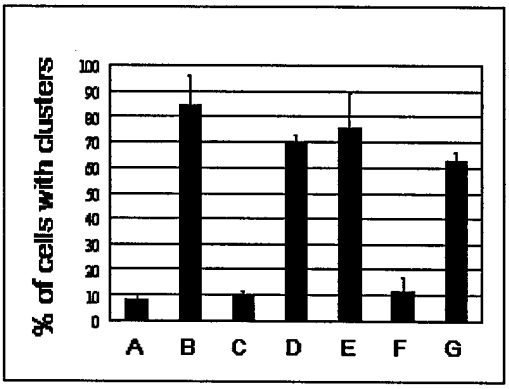


Figure 1. Immunofluorescence microscopy performed on cells treated with anti-MHC I control (A) or anti-β4 (B-G) on ice for 40 min, followed by anti-IgG in suspension at 37°C for 30 min without inhibitors (A,B) or with LY294002 (C), GF109203X (D), rapamycin (E), wortmannin (F), or heparin (G). Representative images show predominant β4 integrin distribution (clustered vs. dispersed). Table shows mean values of percent of cells with clusters ± standard deviation for either three or four experiments performed in triplicate. At least 100 cells were evaluated in each group. Statistically significant differences were observed between A and B ($p<0.001$), B and C ($p<0.001$), and B and F ($p=0.001$).

LY294002, although highly specific for PI3K, is also known to inhibit casein kinase 2 (CK2). CK2 is known to phosphorylate nonmuscle myosin II heavy chain (NMMHC-II), which appears to play a role in "capping" of some cell-surface antigens. Integrin clustering may share some features of antigen capping, so it is important to exclude involvement of CK2. CK2 is extremely sensitive to inhibition by heparin. Lack of inhibition of integrin clustering by GF109203X or heparin indicates that neither PKC nor CK2 are required for integrin clustering. Inhibition of integrin clustering by both LY294002 and wortmannin and lack of inhibition by GF109203X or heparin effectively demonstrates that **adhesion-independent clustering of α6β4 requires PI3K activity**. Future work in our laboratory will focus on delineating the mechanism of PI3K-dependent α6β4 clustering.

Because α6β4 clustering is a PI3K-mediated process, detection of α6β4 clustering by immunofluorescence may serve as a technique for measuring an intact α6β4 signaling pathway.

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).

Out of our goal of 200 specimens, we have collected and processed 171. Due to some loss of cellularity and/or viability upon completing the washing steps, only 72 specimens had enough cellularity to divide into two groups for treatment. Cytospins preparations were made of those with insufficient cellularity for treatment, to be banked for future studies. Those 72 specimens with sufficient cellularity were treated with either anti-β4 or anti-MHC I (control) on ice for 40 min, followed by anti-IgG at 37°C for 30 min. Forty-nine of the 72 treated specimens had sufficient cellularity following treatment to evaluate for by immunofluorescence.

Most specimens had some degree of cell-surface β4 expression, ranging from minimal fluorescence of weak intensity to strong diffuse staining. Immunofluorescence, therefore, appears to be more sensitive than either immunohistochemical staining or in-situ hybridization for β4 mRNA. Nineteen out of 49 specimens (39%) underwent some degree of clustering after cross-linking cell-surface α6β4. No clear differences were observed after cross-linking α6β4 on some specimens (Figure 2, A,B). Occasional cells from some specimens had distinct clusters (C,D), and many to most cells from other specimens had multiple prominent clusters (E,F) after cross-linking α6β4.

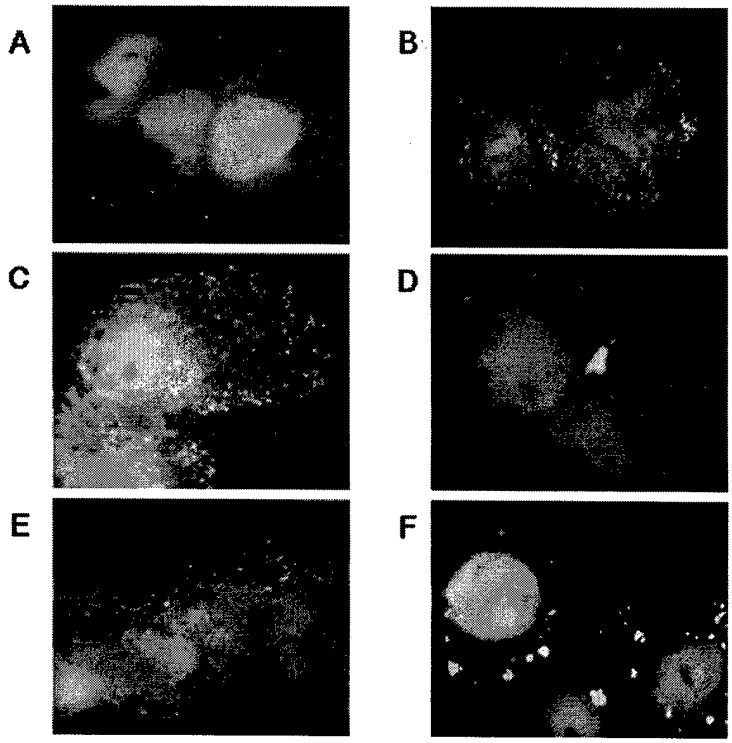


Figure 2. Immunofluorescence microscopy performed on fine-needle aspirates of three different invasive breast carcinoma specimens (A,B, specimen 1; C,D, specimen 2; E,F, specimen 3) treated with anti-MHC I control (A,C,E) or anti-β4 (B,D,F) on ice for 40 min, followed by anti-IgG in suspension at 37°C for 30 min. Representative images show predominant β4 integrin distribution (dispersed vs. clustered) and DAPI-stained nuclei.

The lymph node status of the 49 patients whose tumors were evaluated by immunofluorescence was reviewed to determine whether any correlation could be found between cell-surface α6β4 clustering and the presence of lymph node metastases. Twenty-three of the patients (48%) had lymph node metastases at the time of pathologic staging. For those patients whose tumors showed α6β4 clustering, 7 (39%) had lymph node metastases, compared to 16 (53%) with lymph node metastases whose tumors did not show α6β4 clustering. For the number of patients evaluated, this difference was not statistically significant.

Refinements of this technique are clearly needed, as less than one third of the specimens collected were actually able to be evaluated by immunofluorescence after treating the specimens with our current technique. However, the good news is that α6β4 clustering can be induced and detected in some breast carcinoma specimens. Because α6β4 clustering is a PI3K-mediated process that represents an early step in some α6β4-mediated cell processes, our results demonstrate that a functional integrin signaling pathway can be detected in some breast carcinoma specimens. This pioneering study may help to shed light on how we can effectively measure cell-surface receptor function in carcinoma specimens in the future.

There is a great need to develop assays to measure functional signaling pathways in cancer specimens. The ability to detect functional α6β4 in breast carcinoma specimens may be particularly relevant, as the α6β4 integrin or its downstream signaling effectors could be important therapeutic targets for a subset of breast carcinomas that currently lack targeted therapies. In our future work, we will try to refine the technique for cross-linking α6β4 to make it more feasible to perform on low-cellularity fine-needle aspiration specimens. Out of the 72 specimens treated, only 29 had sufficient remaining cellularity to prepare cell lysates following immunofluorescence analysis. These lysates will remain frozen and may prove to be useful in our future work as we further delineate the pathway of α6β4 clustering.

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 (reported in first annual summary).
- 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 (reported in first annual summary).
- The oligonucleotide probe was used to evaluate the prognostic significance of β4 integrin subunit mRNA expression in a patient cohort with node-negative invasive ductal carcinoma of the breast. Expression of β4 mRNA was shown not to be a prognostic factor in this patient cohort, but the association of β4 mRNA expression with tumor size suggests that this integrin may nevertheless play a role in tumor progression, as suggested by in-vitro studies (reported in second annual summary).
- Cross-linking α6β4 on breast carcinoma cells in suspension was shown to result in the PI3K-dependent phosphorylation of nonmuscle myosin II heavy chain.
- Cross-linking α6β4 on breast carcinoma cell line MDA-MB-231 was shown to result in prominent α6β4 cluster formation by immunofluorescence, and antibody-induced clustering of α6β4 was shown to be an active process mediated by PI3K.
- Antibody-induced α6β4 cluster formation in breast carcinoma specimens was demonstrated by immunofluorescence. Cluster formation by immunofluorescence may serve as a surrogate for functional α6β4 integrin.

• Reportable Outcomes:

- LK Diaz, X Zhou, K Welch, J Roach, A Sahin, R Herbst, MZ Gilcrease. In-situ hybridization for α6β4 integrin in breast cancer: correlation with protein expression. Mod Pathol 15(1):33A, 2002

- Diaz LK, Cristofanilli M, Zhou X, Welch K, Smith TL, Sneige N, Sahin A, Gilcrease MZ. In-situ hybridization for β4 integrin subunit in node-negative invasive carcinoma of the breast. Modern Pathol 16(1):110, 2003
- Gilcrease MZ, Zhou X, Welch K. Alpha6beta4 integrin mediates PI3K-dependent phosphorylation of nonmuscle myosin II heavy chain in breast carcinoma cells in vitro. Breast Cancer Res Treat 82(Suppl. 1):382, 2003
- Diaz LK, Zhou X, Welch K, Sahin AA, Gilcrease MZ. Chromogenic in-situ hybridization for alpha6beta4 integrin in breast cancer: correlation with protein expression. J Mol Diagn 6(1):10-15, 2004
- Preliminary data from this study resulted in funding from the Susan G. Komen Breast Cancer Foundation to evaluate alpha6beta4 integrin as a prognostic marker in node-positive breast carcinoma (PI: MZ Gilcrease, BCTR02-2043, 5/1/2003 through 4/30/2005, $249,996).

Conclusions:

In summary, our goal was to evaluate 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. We designed a probe for β4 mRNA useful in evaluating α6β4 expression in formalin-fixed, paraffin-embedded tissues, and we showed that β4 mRNA expression appears not to be a prognostic factor in node-negative invasive breast carcinoma. However, an association between β4 mRNA expression and tumor size suggests that α6β4 may nevertheless play a role in tumor progression.

Signal transduction by α6β4 integrin was studied in breast carcinoma cell lines. Although we found no changes in the phosphorylation of FAK, PDK1, IRS-1, Shc, Erk, or Akt after cross-linking cell-surface α6β4 on cells in suspension, we did find α6β4 cross-linking to be associated with phosphorylation of nonmuscle myosin II heavy chain. This may affect actin-myosin filament organization and could play a role in tumor cell motility. We showed by immunofluorescence that cell-surface α6β4 clustering can be demonstrated following antibody-mediated cross-linking of the integrin not only in cell lines but also in fine-needle aspirates of breast carcinoma specimens. Clustering of α6β4 in breast carcinoma specimens could represent a surrogate marker of α6β4 integrin function. We could not demonstrate any correlation between functional α6β4 integrin, as assessed by antibody-induced α6β4 clustering in fine-needle aspirates of breast carcinoma specimens, and the presence of lymph node metastases at the time of pathologic staging. Further refinements are needed in the techniques to induce and detect integrin clustering in carcinoma specimens in order to determine whether integrin clustering as a surrogate of integrin function will ultimately prove to be useful clinically.