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13. ABSTRACT (Maximum 200 Words)

We are investigating the role of α6β4 integrin expression in the progression of breast cancer. Using an oligonucleotide probe for β4 integrin mRNA, we evaluated β4 mRNA expression in a cohort of patients with node-negative invasive breast carcinoma. We found no association between β4 mRNA expression and 5-year or 10-year disease-free or disease-specific survival. However, we did observe a correlation between β4 mRNA expression and tumor size, suggesting that α6β4 integrin may nevertheless play a role in tumor progression. One hundred and forty-four 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. We evaluated α6β4-mediated signaling in breast carcinoma cell lines, and we observed that clustering of surface β4 results in the PI3K-dependent phosphorylation of nonmuscle myosin II heavy chain. This may affect actin-myosin filament organization. If changes in actin-myosin filament organization are observed in breast carcinoma cell lines following α6β4 clustering, effects on actin-myosin filament organization will be evaluated subsequently in the breast carcinoma cytospin preparations and 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 $\alpha6\beta4$ 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 $\alpha6\beta4$ integrin and /or its signaling intermediates is associated with poor prognosis in breast cancer, and 2) that increased $\alpha6\beta4$-mediated signaling correlates with poor prognosis in breast cancers that overexpress $\alpha6\beta4$.

Body:

Task 1. To evaluate $\alpha6\beta4$ 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 $\beta4$ 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).

Of the proposed 250 cases of node-negative invasive breast carcinoma with long clinical follow-up, we were able to obtain appropriate paraffin blocks with adequate tumor for analysis for 200 cases. Poly dT(20) oligonucleotides were used to verify the integrity of mRNA in each of the tissue sections. Two cases showed an absence of poly dT(20) staining and were excluded. For the remaining 198 cases, surgeries were performed from July, 1978 through October, 1995. The median follow-up was 15 years. Twenty-four per cent had lumpectomy with axillary dissection, and 76% had modified radical mastectomy. There were 171 T1, 78 T2, and 3 T3 tumors. The median tumor size was 2.0 cm (range 0.6 cm to 7.0 cm). The median patient age was 58 years (range 28 to 83 years). Seventy-nine per cent of patients were postmenopausal, and 21% were premenopausal. Eighty-two per cent of patients were white, 7.5% Hispanic, 7.5% black, and 3% oriental. Although 41% of patients received adjuvant radiation therapy, none received adjuvant tamoxifen or chemotherapy.

Ninety-one per cent of the tumors were invasive ductal carcinomas, 6% were invasive lobular carcinomas, and 3% were other types. Seventy per cent of tumors were ER positive, 32% showed HER-2/neu overexpression (2+ or 3+), and 16% showed strong HER-2/neu overexpression (3+).

No correlation was observed between disease-free or disease-specific survival and patient age, nuclear grade, tumor type, ER/PR status, and HER-2/neu overexpression. However, tumor size was shown to correlate with decreased 5-year and 10-year disease-free ($p=0.04$) and disease-specific ($p=0.01$) survival.

In-situ hybridization was performed using a 40-base hyperbiotinylated oligonucleotide probe for $\beta4$ integrin subunit mRNA in an alkaline phosphatase-based assay. (We reported in the previous annual summary that this probe was both sensitive and specific for $\beta4$ mRNA.) Staining was scored on a scale of 0 to 2+ based on staining intensity. Twenty-nine per cent of tumors had a score of 0, 46 were 1+, and 25% were 2+. No correlation was found between $\beta4$ mRNA expression and patient age, ER/PR status, HER-2/neu overexpression, and nuclear grade. Beta4 mRNA expression was found to correlate with tumor size ($p=0.01$). However, no correlation was found between $\beta4$ mRNA expression and 5-year or 10-year disease-free or disease-specific survival. Moreover, when the largest subtype of tumors (invasive ductal carcinoma) was analyzed separately, there was still no correlation between $\beta4$ mRNA expression and 5-year or 10-year disease-free or disease-specific survival. Therefore, $\beta4$ mRNA expression appears not to be an adverse prognostic factor in this patient cohort. Nevertheless, the correlation observed between $\beta4$ mRNA expression and tumor size suggests that $\alpha6\beta4$ integrin may still play an important role in tumor progression.

Task 2. To measure gene copy numbers of the $\beta4$ gene in multiple breast cancer cell lines with known $\alpha6\beta4$ integrin expression using probes derived from 2 different BAC clones (months 1-8).

Completed, as reported in the first annual summary.

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).

So far we have collected and processed 144 fresh invasive breast carcinoma specimens (108 previously untreated, 36 previously treated with chemotherapy). The specimens have been collected by fine-needle aspiration, and tumor cells suspensions have been exposed to either anti-β4 or anti-MHC I (control) on ice for 40 min, followed by anti-IgG at 37°C for 30 min. Cytospin preparations have been prepared and stored for future staining, and for those specimens with sufficient cellularity, cell lysates have been prepared and frozen for future Western blot analyses.

In the last 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 following clustering of the β4 integrin subunit as described. Collaborative arrangements were made with Research Genetics, Inc. to develop an antibody to phospho-β4, but to date they have not been successful in developing this phosphorylation state-specific antibody. However, as stated below in Task #4, we have recently discovered that nonmuscle myosin II heavy chain undergoes phosphorylation after clustering β4 in a breast carcinoma cell line (see below). Because phosphorylation of this molecule likely plays a role in actin-myosin filament organization, we will be examining changes in actin-mysoin organization following β4 clustering in vitro, and we plan ultimately to evaluate our specimen cytospin preparations by immunoflourescence to correlate actin-myosin alterations with clinical outcome following clustering of surface α6β4 integrin.

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 previous annual report, we indicated that the two cell lines showing the highest α6β4 integrin expression (MDA-MB-231 and BT474) had increased PI3Kp110α in anti-phosphotyrosine immunoprecipitates. We have shown that this is not a result of increased phosphorylation of PI3Kp110α but, rather, probably indicates a translocation of the catalytic subunit of PI3K to the phosphotyrosine fraction, perhaps reflecting increased association with membrane complexes. However, we did not detect increased phosphorylation of Akt. In an effort to determine what may be downstream of PI3K after clustering surface α6β4, we examined the Coomassie stain of anti-phosphotyrosine immunoprecipitation products following clustering of α6β4, and we noted an increase in a 200 kD protein in the phosphotyrosine immunoprecipitates of the β4-clustered cells.

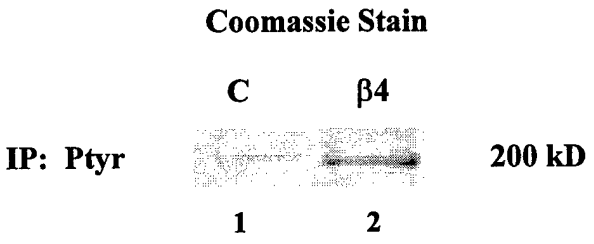

Figure 1. MDA-MB-231 exposed to anti-MHC I control (lane 1) or anti-beta4 integrin subunit (lane 2) at 4°C for 40 min, followed by anti-IgG at 37°C for 30 min.

We expected that this would be the β4 integrin subunit, but we could not demonstrate this by Western blotting with anti-β4. We therefore excised the band and had it analyzed by mass spectroscopy in our core proteomics laboratory under Dr. Ryuji Kobayashi, and it was show to be nonmuscle myosin II heavy chain, isoform A. Because nonmuscle myosin II heavy chain undergoes phosphorylation of serine and threonine residues rather than tyrosine residues, we assumed the increased nonmuscle myosin II heavy chain in the phosphotyrosine fraction following clustering of β4 reflected increased association with other phosphotyrosine-containing proteins. Indeed, immunoprecipitation with anti-nonmuscle myosin II heavy chain following clustering of β4 and blotting with anti-phosphotyrosine showed no increased phosphotyrosine, but blotting with anti-phoshoserine did show increased phosphorylation of nonmuscle myosin II heavy chain on serine. This increase was not seen in the presence of the PI3K inhibitor LY294002, indicating that β4-mediated phosphorylation of nonmuscle myosin II heavy chain is PI3K dependent.

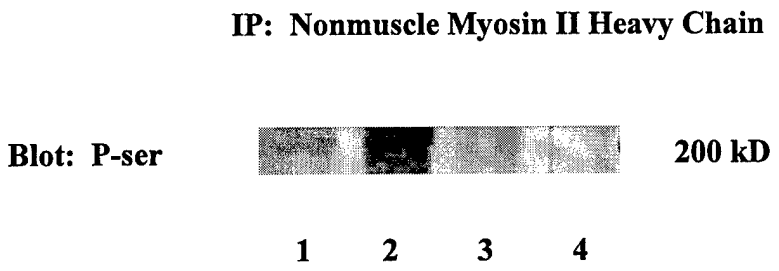

Figure 2. MDA-MB-231 exposed to anti-MHC I control (lanes 1 and 3) or anti-beta4 integrin subunit (lanes 2 and 4) at 4°C for 40 min, followed by anti-IgG at 37°C for 30 min without pretreatment (lanes 1 and 2) or with pretreatment (lanes 3 and 4) with LY294002.

Phosphorylation of nonmuslce myosin II heavy chain in amoeba and slime molds is thought to cause myosin filament disassembly. It will be interesting to see whether it has significant effects on actin-myosin filament assembly or disassembly in breast carcinoma cells. Such effects could be important in α6β4-mediated motility and tumor cell invasion.

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.
- One hundred and forty-four fresh invasive breast carcinoma specimens (eighty-three additional specimens since first annual summary) 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.

- α6β4 integrin-mediated signaling has been studied in breast carcinoma cell lines, and α6β4 clustering of breast carcinoma cells in suspension has been shown to result in the PI3K-dependent phosphorylation of nonmuscle myosin II heavy chain.

- **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.
  - Abstract: LK Diaz, M Cristofanilli, X Zhou, K Welch, TL Smith, N Sneige, A Sahin, MZ Gilcrease. In-Situ Hybridization for Beta4 Integrin Subunit in Node-Negative Invasive Carcinoma of the Breast. Annual Meeting of the United States and Canadian Academy of Pathology, March, 2003.
  - Preliminary data from this study resulted in recent 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, 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 have found so far 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.

One hundred and forty-four 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. Alpha6beta4 integrin-mediated signaling has been studied in breast carcinoma cell lines, and α6β4 clustering of breast carcinoma cells in suspension has been shown to result in the PI3K-dependent phosphorylation of nonmuscle myosin II heavy chain. This may affect actin-myosin filament organization. If changes in actin-myosin filament organization are observed in breast carcinoma cell lines following α6β4 integrin-clustering, effects on actin-myosin filament organization will be evaluated subsequently in the breast carcinoma cytospin preparations and correlated with clinical parameters.