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TITLE: AKT1 - A New Marker for Tamoxifen Resistance in ER-Dependent Breast Cancer

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AKT1 - A New Marker for Tamoxifen Resistance in ER-Dependent Breast Cancer

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While the significance of the serine/threonine protein kinase AKT expression and/or activity in human breast cancer has become increasingly evident, consistent alterations of a specific isoform have not been well documented. A specific isoform of AKT may be preferentially activated or activated proteins may have different substrate preferences, providing a therapeutic opportunity to target a particular isoform. The primary endpoint is to compare responsiveness to tamoxifen in ER-α-positive, ErbB2 low tumors with high AKT1 activity versus no AKT1 activity. 7 tumors each of 1) ER-α-positive, ErbB2 high; 2) ER-α-positive, ErbB2 low; 3) ER-α-positive, no ErbB2; 4) ER-negative, ErbB2 high; 5) ER-negative, ErbB2 low; and 6) ER-negative, no ErbB2 will be tested for AKT (AKT1, AKT2, AKT3), ErbB (EGFR, ErbB2, ErbB3, and ErbB4) and ER-α expression and activity. We have received 26 tumors and their surrounding normal tissue. Paraffin sections were prepared from most of these tissues and the sections were immunostained with total anti-Akt, phosphospecific anti-Akt, anti-progesterone receptor, total and phosphospecific antibody against ErbB2. We are in the process of scoring of these paraffin sections. In addition, we prepared cell lysates and RNA from the tumors and normal tissues for further analyses.
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INTRODUCTION

The significance of AKT expression and/or activity in human cancer has become increasingly evident. However, consistent alterations in overexpression and/or activity of a specific AKT isoform in human breast tumors have not been well documented. A specific isoform of AKT may be preferentially activated (1-5) or activated proteins may have different substrate preferences, providing a therapeutic opportunity to target a particular isoform. Our in vitro data show that the growth factors EGF, IGF-I, and HRG-β1, as well as estradiol, can activate the PI3/K/Akt pathway in ER-positive breast cancer cells. The effects of estradiol and HRG-β1 are mediated by membrane ER-α and by the ErbB2 but not by the EGFR signaling pathway. Moreover, estradiol and growth factors can induce anchorage-dependent and independent proliferation and membrane ruffling that can be inhibited by antiestrogens, selective ErbB2 inhibitors and by either PI 3-K inhibitors or dominant negative Akt mutants. In contrast, Akt exerts estrogen-like effects on cell growth, membrane ruffling, and ER-α regulation and tamoxifen cannot fully abolish its effect. Taken together, these data suggest that estradiol, EGF, or HRG-β1 interact with membrane ER-α and a heterodimer with ErbB2, leading to tyrosine phosphorylation. This results in activation of the PI 3-K and AKT1. AKT1, in turn, may interact with nuclear ER-α, altering its expression and activity, as well as cell proliferation and response to tamoxifen. Therefore, this proposal will determine ErbB (EGFR, ErbB2, ErbB3, and ErbB4), AKT (AKT1, AKT2, and AKT3), and ER-α expression and activity in 42 breast tumors, their surrounding normal tissue, and stroma. The primary endpoint is to compare responsiveness to tamoxifen in ER-positive, ErbB2 normal (0.1-0.19 pg/cell) tumors with high AKT1 activity versus no AKT1 activity.

BODY:

Task 1: Selection of frozen tumors (including adjacent control tissue) and paraffin sections

Since the Tissue Shared Resource of the Lombardi Cancer Center could not provide us with more than 4 frozen tumor tissues, we contacted the National Disease Research Interchange (NDRI) and we are still in the process of selection of frozen tumors (including adjacent control tissue) and paraffin sections for gathering the 7 samples in each of the following 6 groups of tumors based on ER-α and ErbB2 levels:

- Group 1: ER-positive, ErbB2 normal (0.1-0.19 pg/cell)
- Group 2: ER-positive, ErbB2 overexpressed (>0.2 pg/cell)
- Group 3: ER-positive, ErbB2 negative (<0.09 pg/cell)
- Group 4: ER-negative, ErbB2 normal
- Group 5: ER-negative, ErbB2 overexpressed
- Group 6: ER-negative, ErbB2 negative

We are also requesting follow-up information (tamoxifen responsiveness) as well as traditional prognostic markers (tumor stage, tumor type, nodal status, differentiation, etc.) (See attached Table with Tumor Characteristics).

So far, we have received 26 frozen tumors and 26 adjacent normal tissues and 18 paraffin sections of tumors and 18 paraffin sections of normal adjacent tissue.
Task 2: Measurement of the expression of ErbB receptors

a) Lysates from 26 frozen tumors and 26 adjacent normal tissue were prepared
b) Protein expression for ErbB2 was analyzed in sections from 8 tumors and 8 adjacent normal tissue using immunocytochemistry and anti-ErbB2 antibodies (see results in Table 2). The rest of 10 paraffin sections were sent to the Histopathology and Tissue Shared Resources of the LCC for slide preparation and immunostaining.
In addition, we are testing EGFR, ErbB3, and ErbB4 antibodies for Western blot as well as immunohistochemistry, as well as phosphospecific antibodies to determine the activity of ErbBs in all the tumors.

Task 3: Determination of the expression of AKT1, AKT2, AKT3 in frozen tumor tissue

a) We designed the primers for RT-PCR and ordered them
b) We are currently testing the primers by RT-PCR
c) We detected protein expression for total Akt by immunostaining of paraffin sections of 8 tumors with total anti-Akt antibody. Scores as intensity of staining were assigned - this reflects level of expression low (1+) to high (3+). Also percent of tumour positivity (entire, partial or localized) were noted.

Task 4: Measurement of the activity of Akt

a) Immunostaining of paraffin sections of 8 tumors and their adjacent normal tissues was performed using phosphospecific anti-Akt (P-Akt) antibody to detect whether activation of Akt is derived from tumor cells or stromal tissue.

KEY RESEARCH ACCOMPLISHMENTS, REPORTABLE OUTCOMES, and CONCLUSIONS

- We are still selecting tissues for the 6 tumor groups. So far we have 4 tumors in group 6, 3 tumors in group 4, 2 tumors in group 3, and 1 tumor in group 2. We expect to collect all tumors by the summer of 2005
- We are still gathering follow-up information about: age of the patient, tumor histology, ER, PR, ErbB2 status of the tumor, as well as tamoxifen response.
- We prepared lysates from 26 frozen tumor and 26 adjacent normal tissue samples and are in the process of analyzing ErbB receptors, AKT isoforms expression and activity and AKT isoform mRNA
- We have analyzed 8 tumors and 8 adjacent normal tissues by immunohistochemistry for ErbB2, PR, Akt and phosphospecific Akt (Akt activity) for localization and staining intensity
- A very good correlation was observed between the ErbB2 and progesterone receptor PgR immunohistochemical score that was obtained from NDRI and the measured score in the sample of 8 tumors
Akt staining occurs in ductal areas of tumors and in cell groups surrounded by lymphocytes and inflammatory cells. Its expression is higher in the cytoplasm than in the nucleus. Adjacent normal tissue can also express Akt, but the staining intensity is lower.

Akt activity is usually correlated with Akt expression in both tumor and normal surrounding tissue. In normal tissue, hyperplastic ducts and scattered end units are also positive for active Akt. Nuclear staining is higher for active Akt when compared to expressed Akt.

REFERENCES:


APPENDICES:

1) Table 1: Tumor Characteristics

2) Table 2: Immunohistochemical Analysis of 8 Tumors (CA) and Matched Adjacent Normal Tissue (N or NAJ) for Akt, P-Akt, PgR, and ErbB2
<table>
<thead>
<tr>
<th>#</th>
<th>Tumor Type</th>
<th>Lymph Nodes</th>
<th>Age</th>
<th>ER</th>
<th>PR</th>
<th>ErbB2</th>
<th>Other</th>
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<td>+</td>
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<td>3</td>
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<td>72</td>
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<td>12</td>
<td>infiltr. duct. adenocarc. with lymph &amp; pagetoid, gr. 2</td>
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<td>73</td>
<td>+</td>
<td>+</td>
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<td>13</td>
<td>high gr. (nucl. 3) in situ ductal carc. w 2 foci of microinv.</td>
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<td>invasive ductal carc. in fibromuscular tissue, gr. 2-3</td>
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<td>26</td>
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<td>17/17</td>
<td>80</td>
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</table>
### Table 2: Immunohistochemical Analysis of 8 Tumors (CA) and Matched Adjacent Normal Tissue (N or NAJ) for Akt, P-Akt, PgR, and ErbB2

<table>
<thead>
<tr>
<th>Specimen ID #</th>
<th>Description</th>
<th>Act</th>
<th>P-Akt</th>
<th>PgR</th>
<th>ErbB2</th>
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<tr>
<td>12746-05</td>
<td>L. Breast CA</td>
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<td>12746-07</td>
<td>L. Breast N</td>
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<tr>
<td>12748-05</td>
<td>R. Breast T</td>
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<td>Breast CA</td>
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<td>Breast NAJ</td>
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<td>13485-11</td>
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<tr>
<td>13549-04</td>
<td>L. Breast T</td>
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</table>

**Notes:**
- Akt: Entire specimen staining positive 2+ cytoplasm and 2+ nucleus.
- P-Akt: Entire specimen - carcinoma staining positive 3+ cytoplasm and 2+ nucleus.
- PgR: Variable: 1. hyperplastic ducts all cells positive. 2. End units scattered positive cells. 3. In situ showed many cells positive. 4. Cystic acinar the lining cells were negative. Nuclear.
- ErbB2: Entire specimen staining positive 3+ cytoplasm and 2+ nucleus.

**Specific Tumor Characteristics:**

1. **L. Breast CA**
   - Has some normal - same as #4 above i.e. low intensity staining mostly cytoplasm and also nucleus.
   - Akt: Tumor and normal cytoplasm 2+.
   - P-Akt: Entire specimen - carcinoma staining positive 3+ cytoplasm and 2+ nucleus.
   - PgR: Negative nuclear staining. Note distribution follows Akt pattern, with negative staining in in cells perimeter.
   - ErbB2: Negative.

2. **R. Breast T**
   - Invasive carcinoma 1+ cytoplasm.
   - PgR: Collagen only - no breast lobule or duct in sample.

3. **Breast NAJ**
   - Includes 1. ducts with hyperplasia - negative. 2. Area of cystic ducts with duct containing within the lobule.
   - Akt: All sites stain cytoplasm 2+ nucleus 2+.
   - P-Akt: Variable: 1. hyperplastic ducts all cells positive. 2. End units scattered positive cells. 3. In situ showed many cells positive. 4. Cystic acinar the lining cells were negative. Nuclear.
   - PgR: Variable: Generally 1+. 1. hyperplastic ducts low level staining cytoplasm predominantly rare membrane. 2. End units some low positive cells. Cytoplasm, very weak staining. Others negative. 3. Hyperplastic acinar units positive cytoplasm weak and also membrane staining, moderate. Cystic dilated acini variable negative predominantly, some weak positive cytoplasm.

4. **Breast CA**
   - Cytoplasm 1+; nuclei negative.
   - Akt: Not done.
   - P-Akt: Ducts and end units expression in cytoplasm 1-2+; nucleus 1+.
   - PgR: Entire tumor positive. Cytoplasm and membrane. High level of expression 3+.
   - ErbB2: Negative.

5. **L. Breast N**
   - Ducts negative. Some end units 1+ cytoplasm. Some only individual cells positive.
   - Akt: Not done.
   - P-Akt: Not done.
   - PgR: Not done.
   - ErbB2: Normal breast lobule unit cytoplasm 1+.

6. **L. Breast T**
   - Negative.
   - Akt: Ducts and end units expression in cytoplasm 1-2+; nucleus 1+.
   - P-Akt: Almost entirely positive nuclear, also cytoplasm (variable). Some neoplastic cells negative.
   - PgR: Negative. However a few localized ductal elements within tumor showed very weak staining including membrane and cytoplasm.

7. **L. Breast**
   - Not done.
   - Akt: Not done.
   - P-Akt: Not done.
   - PgR: End units most cells positive.
   - ErbB2: End units most cells positive. Ducts some cells positive.

8. **L. Breast T**
   - Not done.
   - Akt: Not done.
   - P-Akt: Not done.
   - PgR: Not done.
   - ErbB2: Most of tumor 50% low level of expression 1+. Some areas negative. 2. End units also positive very weak staining, cytoplasm 1+.