APPLICATION FOR SURF GRANT

ACTIVATION OF SRC FAMILY KINASES IN BREAST TUMOR TISSUES

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1. History and Definition of Project:

The long term goal of the proposed research is to develop a simple, reliable clinical test that can determine which breast cancer patients will be unlikely to have a recurrence of their cancer with surgical removal of the tumor alone and which patients truly need additional treatments. This would reduce the number of patients who must be exposed to the discomfort, toxicity and expense of unnecessary treatment. It would also assure that patients who are likely to experience recurrence could begin additional hormonal treatment or chemotherapy without delay.

Clinical studies have demonstrated that a percentage of breast cancer patients benefit from hormonal and chemotherapy after surgical removal of their tumor even though the cancer had not spread to the lymph nodes (McGuire et al., 1992; Devita 1989). However, approximately 70% of all breast cancer patients in whom cancer has not spread to lymph nodes will survive more than ten years without these additional treatments (McGuire et al., 1992). There are many factors involved in deciding prognosis and prediction of treatment response of breast cancer (Berns 1998; Ferno 1998; Ravaioli et al, 1998, 1996, 1993; Migliaccio et al, 1998; Umetikita et al, 1998, Voogd et al; 1998; Allard et al., 1997; Weidner et al., 1997; Valavaaea 1997; Castoria et al., 1996; Dhingra et al., 1996; Arnold et al., 1995; Denton et al., 1992). Preliminary studies from other laboratories suggest tht an active form of a protein called Src kinase is frequently found in breast tumors of patients with a poor prognosis who tend to have recurrence of their cancer (Ottenhoff-Kaliff et al., 1992). A Src family kinase protein is an enzyme that adds a phosphate group to the amino acid tyrosine on another protein. This addition of a phosphate group changes the shape of the protein thus inducing a change in its activity (Williams et al., 1997; Chedin, et al., 1997; Alexandropoulos et al., 1996; Cooper et al., 1993; Sladowski et al., 1986). The procedures currently used to detect Src and other related kinases are not useful for large scale screening of tumors. Our laboratory has developed antibodies and peptides that bind selectively to active, cancerous Src (Roussel et al., 1991; Roussel et al., 1990; Roussel, 1990). They do not bind to inactive, non-cancerous Src.

I have used this antibody in several experimental procedures and have demonstrated that it specifically binds to active, cancerous Src and not to inactive, non-cancerous Src. We can detect the presence of activated Src by binding it with a specific antibody that is visualized using various techniques to be discussed later. These experiments have also shown that the protein to which the antibody binds weighs approximately 60 kDa (kilodaltons- a measure of molecular weight). This is the molecular weight of the Src protein.

Hypothesis #1: The presence of active Src will be able to predict the outcome of patients with breast cancer that has not yet spread to the lymph nodes.

Objective #1: To detect the active form of Src in fresh-frozen and paraffin-embedded tumors.

Objective #2: Develop a simple, reliable screening test using paraffin-embedded tumor sections.

Objective #3: To determine if the presence of active Src correlates with more aggressive tumors and poor prognosis.

2. Methodology

To test this hypothesis and reach my objectives I will be using immunohistochemistry procedures where paraffin-embedded tumor sections are fixed to a glass slide. During this research I will be using the selective antibodies and peptides to treat slides and then stain them to determine if active Src is present in the tumor sections. Dr. Gary York of the Pathology Department of Concord Hospital will provide paraffin-embedded tumor sections to us. Our immunohistochemistry results will be checked and confirmed by Dr. York. We may also receive fresh-frozen breast tumors from Dr. York as well as Dr. David Novis of the Pathology Department of Wentworth-Douglass Hospital and Dr. Julia Bruce of the Pathology Department of Frisbie Memorial Hospital. These fresh tumors will be tested using immunohistochemistry as well as immunoprecipitation, western blot analysis and ECL detection. These procedures are described below. We will then statistically analyze our results.

Method #1

Immunohistochemistry:

Immunohistochemistry is a procedure that I will use to treat both paraffin-embedded tumor sections and quick-frozen fresh tumor sections. The slides are treated with specific antibodies and peptides. Staining the slides shows the presence of active Src.

For Paraffin Embedded Tumor Sections:

A. Paraffin is removed from the glass slides to make the tumor cells available for antibody treatment.
B. The slides will then be treated with the primary antibody that binds to the active Src.
C. Normal non-cancerous cells will be used as a negative control.
D. Phosphorylated and non-phosphorylated peptides will be used to determine the specificity of the antibody.
E. After washing the slides they will be treated with the secondary antibody. The slides will then be stained with the Vectastain ABC Elite Kit to show which tumors contain the active Src protein.
F. Various antibody dilutions will be tested to determine the conditions that give the most specific staining.

For Fresh Tumor Sections:

The same procedure will be used except there is no paraffin to be removed.

Expected Results for Method 1 for Immunohistochemistry:

- If the primary antibody binds specifically to active, cancerous Src proteins in both fresh tumors and paraffin embedded tumor sections then we expect to see apparent specific staining in cells known to contain active Src.
- If the antibody is specific, then there should be no staining of normal non-cancerous cells used as a negative control.
- It is anticipated that the phosphorylated peptide will block staining by binding to the antibody's binding site. The non-phosphorylated peptide is not expected to block antibody binding.

Immunoprecipitation and Western Blot Analysis of Fresh Tumors:

A. Immunoprecipitation (IP) is a process that separates tumor proteins from other cellular material.

- The tumor will be pulverized and homogenized. This part of the procedure will be performed by Dr. Andy Laudano in a glove bag and a biohazard hood.
- The homogenate will be treated under denaturing conditions to precipitate the proteins.

B. The proteins will be analyzed by Western Blot Analysis- a procedure that determines the weight of the protein that is bound to the antibody.

- The protein sample will be run through a 9% polyacrylamide gel by electrophoresis to separate the proteins by weight.
- The proteins from the gel will be transferred to a nitrocellulose membrane.
- The membrane will be treated with the primary antibody that specifically binds to active Src.
- After being washed, the membrane will be treated with a secondary antibody that binds to the primary antibody and contains an enzyme that produces light when exposed to detection chemicals.

C. The membrane will be treated with Enhanced chemiluminescence (ECL) detection reagents. This procedure causes the luminescence of the protein bands that are then exposed on film.

Expected Results for Method 1 with Immunoprecipitation and Western Blot:

- If fresh-frozen tumors containing active Src are immunoprecipitated and analyzed by Western blot, then we would expect to see a distinct band present at 60 kDa representing the presence of active Src. We would expect this band to be absent when the sample is treated with the phosphorylated peptide and present when treated with the non-phosphorylated peptide.

<u>**Method #2**</u>

Using the immunohistochemistry techniques I will vary the types of solvents, incubation times and antibody dilutions in order to optimize the conditions for a simple, reliable screening test.

Expected Results for Method 2:

- If conditions are optimized for a screening test, then we expect the staining to be very clear and specific in cells known to contain active, cancerous Src and no or minimal staining in normal non-cancerous cells. If the level of Src present can be quantified, then we expect to see a gradation of staining consistent with the level of Src present.

Method #3

A. We will use anonymously coded hospital records to see if a poor patient outcome correlates with the presence of active Src.

- All tumor samples and slides will be anonymously coded so that the identity of the patient is not revealed to us. Patient outcome will only be known after the testing is done.
- The patient outcome data for the fresh tumor samples will not be available for some time so correlation of these samples will not be possible until a number of years have passed.
- Many of the paraffin embedded samples from the hospitals will be historical samples that will have patient outcome data available. These data sets will be statistically analyzed using the Chi-squared Test.

Expected Results for Method 3:

- If a positive test result for active Src correlates with more aggressive tumors that have a poor patient outcome when compared with those tumors having a negative test result, then we expect that the chi-squared test will show that $p<.05$.

3. Significance:

The outcome of this research will be very significant if we can demonstrate that a simple clinical test can be used to screen tumor samples routinely for the presence of activated Src. If the presence of active Src correlates with more aggressive tumors it will be possible to make more effective treatment decisions. Reducing the amount of unnecessary medical treatments for patients who are unlikely to have a recurrence would certainly be worthwhile since these treatments can be painful, toxic and expensive for the patient. Also if patients who are likely to have a recurrence of the cancer are treated immediately with additional hormonal or chemotherapy there may be a more positive outcome for them. Both of these situations represent a benefit to patients, doctors and insurance companies, as the treatment will be more cost effective and medically justified.

4. Outcome:

This research experience will certainly benefit both my educational and career goals. I have already worked for two years in the Chemistry Department at UNH doing undergraduate research. I am currently working in the Biochemistry Department at UNH doing undergraduate research. This is however the first opportunity that I have had to be involved in the whole process of research from start to finish. I have read related articles, learned necessary laboratory skills and been involved in the conception and genesis of this research. I have participated in the process of forming collaborations with several Pathology Departments as well as working to obtain administrative approval for this research. I feel that I have already benefited from this preparatory work. I look forward to carrying out this research, as my career goal is to continue to work in the field of cancer research after graduation. I am also looking forward to presenting my work in a seminar as well as by publication.

5. Location:

This research will be conducted in Rudman Hall, Room 334 at UNH. Human Breast tumor samples will be obtained from Concord Hospital, Wentworth-Douglas Hospital and Frisbie Memorial Hospital.

6. My Role:

Dr. Andy Laudano, my faculty advisor, will perform the pulverization and homogenization of the fresh tumors in a glove bag in a biohazard hood. I am preparing a protocol to submit for approval from Biohazard Safety. Dr. Laudano will also be available for expert consultation throughout this research study. Dr. Gary York will provide expert consultation and confirmation of our immunohistochemistry results. My role in this research will be to perform immunoprecipitations, western blots, ECL detection and immunohistochemistry. I will also be responsible for some of the coordination of receiving tumor samples and data from the hospitals.

My initial work in this field has been to determine the specificity of the antibody produced in our lab. I have performed an immunoprecipitation of a breast tumor grown in a knockout mouse. I then analyzed the protein binding of the antibody with a Western blot and ECL detection. I have also performed several other Western blot analyses with MCF-7 cell known to contain active Src, normal non-cancerous cells, and various combinations of phosphorylated and on-phosphorylated peptides.

7. Timetable:

Week 1: Optimal conditions for immunohistochemistry will be defined utilizing cultured cancer cells known to express active Src. Normal non-cancerous cells will be used as negative controls.

Week 2: The specificity of the antibodies for blocking the antibody-binding site with the phosphorylated peptide will optimize immunohistochemistry on cultured cells used for immunization. The nonphosphorylated peptide will also be tested for blocking activity.

Week 3-5: Immunohistochemistry will be performed on fresh and paraffin-embedded tumor sections.

Weeks 6-8: Immunoprecipitation and Western blot analysis of fresh frozen tumors.

Week 9: Statistical analysis of data.

8. Bibliography:

Alesandropoulos, K. and Baltimore, D. (1996). Coordinate activation of c-Src by SH3-and SH2-binding Sites on a novel p130Cas-related protein, Si Genes Dev. *10*, 1341-1355.

Allard, P., Atfi, A., Landry, F., Chapdelaine, A. and Chevalier, S. (1997). Estradiol activates p60src, p53/56lyn and renatured p 50/55 protein tyrosine kinases in the dog prostate. Mol. Cell Endocrinol. *126*, 25-34.

Allred, D.C., Harvey, J.M., Bernardo, M., and Clark, G.M. (1998). Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod. Pathol. *11*, 155-168.

Arnold, S.F., Vorheikina, D.P., and Notides, A.C. (1995). Phosphorylation of tyrosine 537 on the human estrogen receptor is required for binding to an estrogen response element. J. Biol. Chem. *270*, 30205-30212.

Belshes, A.P., Haskell, M.D., and Parsons, S.J. (1997). Role of c-Src tyrosine kinase in Ecf-induced mitogenesis. Front. Biosci. *2*:d501-18, d501-d518.

Berns, E.M., van S., I., Klijn, J.G. and Foekens, J.A. (1998). Predictive value of SRC-1 for tamoxifen response of recurrent breast cancer. Breast Cancer Res. Treat. *48*, 87-91.

Bertheau, P., Steinberg, S.M., and Merino, M.J. (1998). C-erbB-2, p53, and nm23 gene product expression in breast cancer in young women: immunohistochemical analysis and clinicopathologic correlation. Hum.Pathol. *29*, 323-329.

Castoria, G., Migliaccio, A., Bilancio, A., Pagano, M., Abbondanza, C., and Auricchio, F. (1996). A 67kDa non-hormone binding estraiol receptor is present in human mammary cancers. Int. J. Cancer, *65*, 574-583.

Chedin, M. Filhol, O., Duminy, D., Bolla, M., Benistant, C., Rioche, S., Chambaz, E.M., and Cochet, C. (1997). Characterization of two different cytoplasmic protein tyrosine kinases from human breast cancer, Carninogenisis *18*, 1463-1472.

Cooper, J.A., and Howell, B. (1993). The when and how of Src regulation. Cell *73*, 1051-1054.

Denton, R.R., Koszewshi, N.J., and Notides, A.C. (1992). Estrogen receptor phosphorylation. Hormonal dependence and consequence on specific DNA binding. J. Biol.Chem. *267*, 7263-7268.

Devita, V.T. (1989). Breast cancer therapy: exercising all our options. N.Engl.J. Med. *320*: 527-529.

Dhingra, K. and Hortobagyi, G.N. (1996). Critical evaluation of prognostic factors., Semin.Oncol. *23*, 436-445.

Gasparini, G. Toi, M. Gion, M. Verderio, P., Dittade, R., Hanatani, M., Matsubara, I., Vinante, O., Bonoldi, E., Boracchi, P., C., Suzuki, H., and Tominga, T. (1997). Prognostic significance of vascular endothelial growth factor protein in node-negative breast carcinoma. J.Natl. Cancer Inst. *89*, 139-147.

Ferno, M. (1998). Prognostic factors in breast cancer: a brief review. Anticancer Res. *18*, 2167-2171.

McGuire, W.L., A.K. Tandon, D.C. Allred, G.C. Chamnes, P.M. and G.M. Clark (1992). Prognosis and retreatment decisions in patients with breast cancer without axillary node involvement. Cancer Supplement *70*:1775-1781.

Migliaccio, A., Pagano, M. and Auricchio, F. (1993). Immediate and transient stimulation of protein tyrosine phosphorylation by estradiol in MCF-7 cells. Onceogene *8*, 2183-2191.

Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E., and Auricchio, F. (1996). Tyrosine kinase/p21 ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells, EMBO J. *15*, 1292-1300.

Ottenhoff-Kalff, A.E., Rijksen, G., van Beurden, E.A., Hennipman, A., Michels, A.A., and Staal, G.E. (1992). Characterization of protein tyrosine kinases from human breast cancer: involvement of the c-src oncogene product. Cancer Res. *52*, 4773-4778.

Oude, W.P., Ottenhoff-Kalf, A.E., Vendrig, M.P., van Beurden, E.A., Staal, G.E., and Rijksen, G. (1994). Functional interaction between epidermal growth factor receptor and c-src kinase activity. FEBS Lett. *352*, 296-300.

Ravaioli, A., Bagli, L., Zucchini, A., and Monti, F. (1998). Prognosis and prediction of response in breast cancer: the current role of the main biologic markers. Cell Prolif. *31*, 113-126.

Roussel, R., Coull, J., Laudano A. (1991). Selective binding of activated pp60c-src by an immobilized phosphopeptide modeled on the carboxyl terminus pf pp60c-src. Proc. Natl. Acad. Sci. USA *89*:11637-11641.

Sadowski I., Stone, J.C., and Pawson, T. (1986). A non-catalytic domain conserved among cytoplasmic protein-tyrosine kinases modifies the kinase function and transforming activity of Fujinami sarcoma virus P130gag-fps. Mol.Cell. Biol. *6*, 4396-4408.

Strobl, J.S., Wonderlin, W.F., and Flyn, D.C. (1995). Mitogenic signal transduction in human breast cancer cells. Gen.Pharmacol. *26*, 1643-1649.

Umekita, Y., Sagara, Y., and Yoshida, H. (1998) Estrogen receptor mutations and changes in estrogen receptor and progesterone receptor protein expression in metastatic or recurrent breast cancer. Jpn.J.Cancer Res. *89*, 27-32.

Valavaara, R. (1997). Reliability of estrogen receptors in predicting response to antiestrogens. Oncology (Huntingt.) *11*, 14-18.

Voogd, A.C., Crommelin, M.A. Repelaer, v.D.O., MC, T.N.-P., Vreugdenhil, G., and Coedbergh, J.W. (1998). [The importance of steroid receptors for the prognosis and hormone treatment of breast cancer patients: a retrospective study in Southeastern Neherlands]. Ned. Tijdschr.Geneeskd. *142*, 1772-1778.

Weidner, N., Cady, B., and Goodson, W.H. (1997). Pathologic prgnostic factors for patients with breast carcinoma . Which factors are important. Surg.Oncol.Clin.N.Am. *6*, 415-462.

Williams, J.C., Weijland, A., Gonfloni, S., Thompson, A., Courtneidge, S.A., Superti-Furga, G., and Wierenga, R.K. (1997). The 2.35 A Crystal Structure of the inactivated form of chicken Src: a dynamic molecule with multiple regulatory interactions. J.Mol. Biol. *274*, 757-775.