The p27Kipl Tumor Suppressor and Multi-Step Tumorigenesis

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The most common genetic alterations found in tumor cells are mutations in genes that regulate cell proliferation. We have recently discovered that p27Kipl, a protein that negatively regulates cell division, is a tumor suppressor gene and that its level of expression in human breast cancer is a powerful predictor of tumor aggression and patient mortality.

This research proposal is designed to understand the role of p27 in tumorigenesis by identifying proteins that cooperate with p27 to suppress tumor formation. We have identified c-myc as a p27-complementing oncogene in a mouse lymphoma model and are now examining possible synergy between p27-loss and c-myc in human breast cancers. We are also establishing a mouse model of p27-associated breast cancer by crossing p27-null animals with mice expressing an MMTV-Wnt-1 transgene. Finally, we are examining the cooperative effects of p27 and p130, a protein related to the Retinoblastoma protein, in suppressing tumor formation. We are using a combination of mouse models and immunostaining primary human breast cancer specimens to assess the p27/p130 interaction.

Breast Cancer, Cell cycle, tumor suppressor

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Introduction

Mutations in genes that regulate the cell cycle are the most common genetic changes in cancer cells. One gene that has been intensely studied is p27kip1, a member of the Cip/Kip family of cyclin-dependent kinase inhibitors. Decreased p27 expression is found in a wide variety of human neoplasms and is associated with poor patient outcome in many cancers, including carcinomas of the breast, colon, lung and prostate, and lymphoma. Although these studies of p27 expression in primary tumors have correlated low p27 expression with poor prognosis, they do not demonstrate that p27 loss is a causal step in tumorigenesis. Studies in p27 knockout mice, however, have directly implicated p27 as a dosage-dependent tumor suppressor. Mice with targeted p27 deletions develop pituitary adenomas with high penetrance, and they are hypersensitive to radiation and chemical carcinogenesis. Furthermore, tumor suppression by p27 in mice is haplo-insufficient (e.g. the tumor incidence in p27 heterozygotes is intermediate to that of p27-null or wild type animals), suggesting that the decreased levels of p27 expression observed in human cancers may have similar consequences. Despite its well-studied function as an inhibitor of cyclin-dependent kinases the mechanism of tumor suppression by p27 is not clearly understood. The goal of this research to investigate the role of p27 in tumorigenesis by identifying proteins that cooperate with p27 in multi-step tumorigenesis. We are specifically investigating the cooperativity between p27 and p130, a member of the Retinoblastoma protein family, in tumor suppression.

Body

Task 1: Identification of genes that cooperate with p27 in multistep tumorigenesis. As detailed in our previous report, the retroviral work originally described in Task 1 is now being supported by a separate funding mechanism. However, work that was proposed in this task identifying c-myc as a possible p27-collaborating oncogene is now being performed as part of Task 3 as described below.

Task 2: Determination of Tumor Susceptibility in p27, p130 -/- double knockout mice.

We have bred p27 -/- mice to p130 -/- mice through two generations to obtain p27 -/- p130 -/- compound mutant mice. Through 12 months of age the double mutant mice appear phenotypically normal and have not developed tumors. We will observe this cohort of mice for tumor development through 2 years of age. We are continuing to breed p27 -/- p130 -/- compound mutant mice for this study as well as for treatment with the carcinogen ENU.

As described in last year’s report, we have completed the first study to determine if p27 -/- mice are predisposed to mammary tumor development. p27 +/- mice were intercrossed to generate p27 +/-, +/-, and -/- experimental animals. Beginning at 6 weeks of age these mice were treated with the carcinogen DMBA by oral gavage (1 mg weekly for six doses). 100% of the treated p27 -/- mice developed lethal tumors by 25 weeks of age. The most common cause of death was large pituitary tumors, leading to compression of the brain. Interestingly, 75% of these mice also had extensive mammary gland hyperplasia at the time of sacrifice, indicative of the early stages of mammary tumor development. 39% of p27 +/- and 25% of wild type mice developed tumors of the mammary gland at longer latency, by 35-40 weeks of age. The tumors...
were classified as mammary adenocarcinomas and adeno-squamous carcinomas. We have performed both Western blot and immunohistochemical analysis of p27 expression in breast tumors from wild type mice. The results show very high levels of nuclear p27 expression. This high level of p27 expression is suggestive of a tumor suppressing role for p27 in breast cancer and provides further justification for testing the effects of genetic deletion of p27 on murine breast cancer. The DMBA experiment was hindered by the unexpected early development of pituitary tumors in DMBA treated p27/-/ mice. Nevertheless, results were strongly suggestive of a predisposition to breast cancer in both p27/-/ and +/- mice.

To circumvent the unexpected early pituitary development in DMBA treated p27 deficient mice, we have elected to cross p27 deficient mice to transgenic mice that express the Wnt-1 oncogene in the mammary epithelial tissue. MMTV-Wnt-1 mice develop mammary tumors spontaneously and this model has been used to investigate the biology, genetics and therapeutic response of breast cancer. Further, this approach avoids any carcinogen treatment. We will generate Wnt-1, Wnt-1 p27+/+ and Wnt-1 p27/-/ mice and compare the rate and degree of malignant progression of breast carcinomas between p27 genotypes. We currently have over 150 such mice in an active breeding program. Thus far, six Wnt-1 p27+/- female mice have developed breast tumors compared to two Wnt-1 female mice. We have not yet generated any Wnt-1 p27/-/ mice as this take an extra round of breeding. We expect to generate all necessary mice during the next 3 months and will monitor all mice for tumor development as outlined in the original grant application. We have bred p27 +/- mice to p130 +/- mice through two generations to obtain p27-/- p130-/- compound mutant mice. The litters have been small, necessitating further rounds of breeding. Through 6 months of age the double mutant mice appear phenotypically normal and have not developed tumors. We will observe this cohort of mice for tumor development through 2 years of age. We are continuing to breed p27-/- p130-/- compound mutant mice for this study as well as for treatment with the carcinogen ENU.

**Task 3. Determination of p130 protein levels in primary human breast cancers, and determination of relative risk in patients with tumors expressing various levels of p130 and p27 proteins.**

In this specific aim, we proposed to assess p130 and p27 expression in 600 paraffin-embedded primary breast tumor tissue samples collected as part of a population-based study of young women using immunocytochemical assays to determine the association between p130 and p27 expression in breast cancer and the association of p130 and/or p27 expression with relative risk of subsequent breast cancer or death.

The analysis of p130 as a marker of prognosis in breast cancer showed that p130 alone does not provide prognostic information in this set of women. We have analyzed expression of p27 and p130 in combination and found an increased risk of mortality when either p130 or p27 is absent or expressed at low levels (Appendix 1). However this does not yet reach statistical significance. Over the next year, we will continue to test breast tumors and correlate our findings with updated clinical follow-up as it becomes available.

One of the accomplishments in Task 1 was the identification of c-myc and n-myc genes as possible collaborators in p27-associated tumorigenesis. Amplification of the c-myc gene is reported in 5-50% of breast cancers. Amplification may be underestimated due to the dilutional effects of the stromal and inflammatory cell populations in molecular analysis. In the last year of the grant, we intend to evaluate amplification of c-myc in tumor cells that have been flow...
cytometrically cell sorted using a bivariate cytokeratin and DNA content method. We will then evaluate the clinical significance of combined c-myc amplification and loss of p27 in breast cancer.

Key Research Accomplishments

- Identification of the c-myc and n-myc genes as possible collaborators in p27-associated tumorigenesis
- Completion of studies of DMBA administration to p27 −/− and +/− mice and observation of affects on mammary epithelium/carcinogenesis
- Determined that loss of p27 accelerates clonal expansion of initiated cells in a skin tumor model (Philipp et al., 1999).
- Determined that loss of p27 function does not functionally substitute for activating mutations in the Hras oncogene. P27 is antagonistic to signaling from mutant Hras as loss of p27 leads to accelerated growth of tumors with Hras mutations (1).
- Examination of the prognostic significance of p130 expression alone and in combination with p130 expression in human breast cancer specimens.

Reportable Outcomes

None

Conclusions

In work completed under original Task 1, we have identified c-myc as a candidate p27-complementating oncogene that will now be tested in a variety of transformation assays. This finding has important implications for the relationship between c-myc activation and p27 function in tumor cells. We are now examining the relationship between p27 and c-myc expression in human breast cancer as described above.

The work completed thus far under task 2 has strongly suggested that p27-deficiency is associated with increased breast carcinogenesis. Because of the high frequency of lethal pituitary cancers in these animals we have adopted a modified approach to address this problem consisting of crossing these animals with MMTV-Wnt-1 animals. This breeding program is now well underway. In related work, we have also determined that reduction of p27 cooperates with activating mutations in Hras during epithelial carcinogenesis and that loss of p27 increases clonal expansion of initiated cells (1). We have also mapped the chromosomal location of p27 in the mouse (2). This information will assist with the design and analysis of ongoing breast carcinogenesis experiments.

The work completed in Task 3 thus far indicates that p130 alone may not be a useful prognostic marker in breast cancer, but that the combination of p130 and p27 expression data may be more informative. These studies will continue over the next year.
References


Appendix 1. Comparison of overall survival associated with combined p130/p27 expression

<table>
<thead>
<tr>
<th>P130/p27</th>
<th>Alive</th>
<th>Dead</th>
<th>HR*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hi/Hi</td>
<td>34</td>
<td>5 (12.8)</td>
<td>1.0</td>
</tr>
<tr>
<td>Lo/Hi</td>
<td>17</td>
<td>6 (26.1)</td>
<td>3.1 (0.9-11.4)</td>
</tr>
<tr>
<td>Hi/Lo</td>
<td>23</td>
<td>5 (17.9)</td>
<td>2.3 (0.6-8.7)</td>
</tr>
<tr>
<td>Lo/Lo</td>
<td>19</td>
<td>4 (17.4)</td>
<td>1.8 (0.5-7.2)</td>
</tr>
</tbody>
</table>

*HR adjusted for age, diagnosis year and stage