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The cell cycle inhibitor p27kip1 has been implicated in a wide array of human cancers, including breast cancer. The overall goal of this research is to understand the role of p27kip1 as a tumor suppressor by studying the interaction of p27 with other genes, particularly p130, a member of the Retinoblastoma protein family, in multi-step cancer. We have used insertional mutagenesis in p27-null mice to create a model of p27-associated lymphomas and have identified myc genes and several candidate loci as p27-collaborating gene activations. We have also bred p27/p130 double null mice to study the interaction of these two genes in tumor suppression. We have also developed an experimental strategy to study the role of p27-loss in mammary carcinogenesis in mice. Finally we have analyzed p130 expression more than 100 primary human breast cancers. Understanding these genetic pathways may lead to mechanistic insights into the tumor suppressor function of p27kip1, as well as new therapeutic approaches to p27kip-associated cancers.

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The p27Kip1 Tumor Suppressor and Multi-Step Tumorigenesis
Principal Investigator: Bruce E Clurman

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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 (reviewed in Clurman and Porter, 1998). 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 (Fero et al, 1998). 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.

The goal of this task is to use insertional mutagenesis to identify genes that cooperate with p27-loss in tumorigenesis. Initially we have used the Moloney murine leukemia virus (M-MuLV) to study lymphomagenesis because it is a potent carcinogen in most strain backgrounds, and because many insertion sites have been previously identified. Approximately 100 newborn mice from p27+/- X p27+/- crosses were infected i.p. with MuLV. In this initial study, the latency of lymphomas induced by M-MuLV was strongly dependent upon p27-genotype, and the p27 null animals developed lymphomas and died within a remarkably narrow window approximately 20 weeks after infection (Appendix 1). Thus like radiation and chemical carcinogens, p27-null mice develop retroviral-associated neoplasms at an increased rate.

We have mapped and cloned many of the integrated viruses in almost 50 lymphomas and found that these tumors contain between 2-10 independent integrations each. We are using several concurrent strategies to identify the genes activated by these viruses are described below. Because many MuLV integration sites are already known, in the first approach we are analyzing these tumors directly with probes corresponding to known sites. Myc genes are the most commonly found sites of integration in retroviral lymphomagenesis in rodents and birds. Recently, c-myc has been implicated in p27 regulation; overexpression of c-myc leads to p27 inactivation, and it has been suggested that inactivation of p27 is a critical means through which c-myc leads to neoplasia. We

thus screened a group of p27-null tumors with a c-myc probe, and found that c-myc rearrangements occur more frequently within p27-null lymphomas than p27 wild type lymphomas, suggesting that myc overexpression and p27-loss may collaborate in multi-step tumorigenesis. This continued selection for c-myc activation in tumors lacking p27 indicates that downregulation of p27 is not *the* critical function by which c-myc activation leads to tumorigenesis, although it remains possible that p27 down-regulation is one of many mechanisms through which myc deregulation leads to cancer

In the second approach we are directly cloning and sequencing the cellular DNA that flanks integrated viruses. We have now cloned more than 30 tumor-specific junction fragments using an inverse PCR strategy that allows us to clone cellular sequences adjacent to viral sequences using primers derived only from viral sequence. We are now using these clones to comprehensively screen all of the lymphomas to determine if integrations represent common integration sites in independent tumors. Because viral integrations occur randomly, the identification of a common integration site in multiple independent tumors indicates a strong biologic selection for the integration, and these loci are likely to harbor relevant insertionally activated genes.

In the third approach we are analyzing tumor RNA to identify transcripts overexpressed as a result of insertional activation. Some genes will be activated by promoter insertion and can be directly identified because they contain viral sequences. Most M-MuLV insertions activate genes by enhancer, rather than promoter insertion and cannot be identified with viral probes. We will use a genomic approach to identify these transcriptionally activated genes in tumors from p27-null and wild type animals. In this approach, oligonucleotide chips containing a large number of known genes are screened with tumor-derived cellular RNA to survey levels of gene expression.

This project has progressed more rapidly than anticipated and is thus significantly ahead of the proposed statement of work. In accordance with the original reviews of this proposal suggesting that task 1 is too comprehensive for this proposal and should be funded under a different mechanism, we have applied for and now obtained independent funding for the remainder of the work in task 1. We thus propose to focus on the expanded task 2 and task 3 described below for the remainder of the funding period, and remove the remainder of task 1 from the proposal. Accordingly, in a separate request to our Grants Officer, we will be requesting approval to reduce Dr. Clurman's effort to reflect these changes, and to increase the effort of Dr. Kemp's, Porter's and Robert's groups.

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

The goal of this Task is to create mouse models to study the interaction of p27 and p130 in tumor suppression, and the role of p27 in breast carcinogenesis. 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.

We have completed the first study to determine if p27 -/- mice are predisposed to mammary tumor development. p27+/- mice were crossed 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 adenosquamous carcinomas. This experiment was hindered by the unexpected early development of pituitary tumors in DMBA treated *p27-/-* mice. Nevertheless, results were strongly suggestive of a breast tumor predisposition in both *p27-/-* and +/- mice. These results provide ample justification for further tests of the hypothesis.

We propose the following approach to circumvent the above problem. We will cross p27 deficient mice to transgenic mice that express mutant p53 in the mammary epithelial tissue and which develop mammary tumors spontaneously. This approach avoids any carcinogen treatment. These mice express a mutant p53 172^{R-H} transgene under the whey acidic protein (WAP) promoter and develop mammary adenocarinomas (Li et al., 1998). We will generate p27-/- p53 172^{R-H} compound mutant mice and observed them for mammary tumor development. If tumor development is accelerated in the *p27-/- p53* mutant mice relative to p53 mutant mice alone, this would provide direct evidence that *p27* is a breast tumor suppressor gene. This experiment will necessitate a larger breeding program than originally noted in the grant budget.

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 task, 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.

To date, we have analyses the data from 119 cases using the Santa Cruz Biotechnologies, Santa Cruz, CA p130 antibody. We used a combination of staining intensity and percent cells with nuclear positive stain to establish high and low categories. The relationship of p130 expression and other patient and tumor characteristics is presented in Appendix 2. In this small group of women, high p130 expression correlates with high proliferative rate (by Ki-67). Tumors with high p130 were also more likely to show overexpression of c-erbB-2, be PR negative and cyclin E positive but these associations did not reach statistical significance. As of the April 1999 follow-up of mortality, p130 was not associated with survival in this small group of women.

Over the next year, we will continue to test breast tumors and correlate our findings with updated clinical follow-up as it becomes available.

Key Research Accomplishments

- Determination that p27-null mice exhibit accelerated lymphomagenesis in response to MuLV infection.
- Identification of the c-myc and n-myc genes as possible collaborators in p27-associated tumorigenesis.
- Development of methods to clone and identify MuLV insertion sites.
- Cloning of several common integration sites in p27-null lymphomas.
- Breeding of p27/p130 double null mice.
- Completion of pilot studies of DMBA administration to p27 -/- and +/- mice and observation of affects on mammary epithelium/carcinogenesis.
- Development of experimental plan to study p27 function in mammary carcinogenesis that will not cause excessive death from pituitary tumors.
- Optimization of p130 immunostaining and p130 staining of 119 cases of primary human breast cancer.

Reportable Outcomes

Abstracts. Hwang., H, Randel, E., Fero, M., Roberts, J., and Clurman, B. 1999 Stage-Specific Gene Activations in P27-null Lymphomas. Abstract Presentation-American Society of Hematology Annual Meeting, New Orleans, La.

Alternate funding applied for based on work supported by this grant. Based on the data obtained in Task 1, Dr. Clurman applied for and has received funding for a full analysis and expansion of the project described in Task 1 through the National Institutes of Health. This alternate funding was recommended by the initial reviewers of this DOD proposal due to the large scope of Task 1.

Conclusions

The work completed in task 1 has validated the experimental strategy of using retroviral insertional mutagenesis to identify genes that cooperate with p27 loss in multi-step cancer. We have developed and adapted methods that allow rapid and large-scale analysis of viral insertion sites. We have also 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 have also cloned several novel insertion sites in p27-associated tumors which may represent additional p27-complementing oncogenes.

The work completed thus far under task 2 has also justified the experimental strategy outlined in our proposal and we have found that p27-null animals may exhibit increased mammary carcinogenesis after DMBA administration. However, we have found that the experimental approach as originally designed resulted in an unacceptable death rate due to pituitary cancers. We have thus adopted a modified approach to address

this problem and will apply this strategy to study the role of p27 and p130 in breast carcinogenesis.

It is too early too conclude if p130 expression will be associated with survival in women with breast cancer, but the preliminary analysis detailed in Table 1 suggests that this may not be the case. We will continue to analyze these specimens as described in our research proposal.

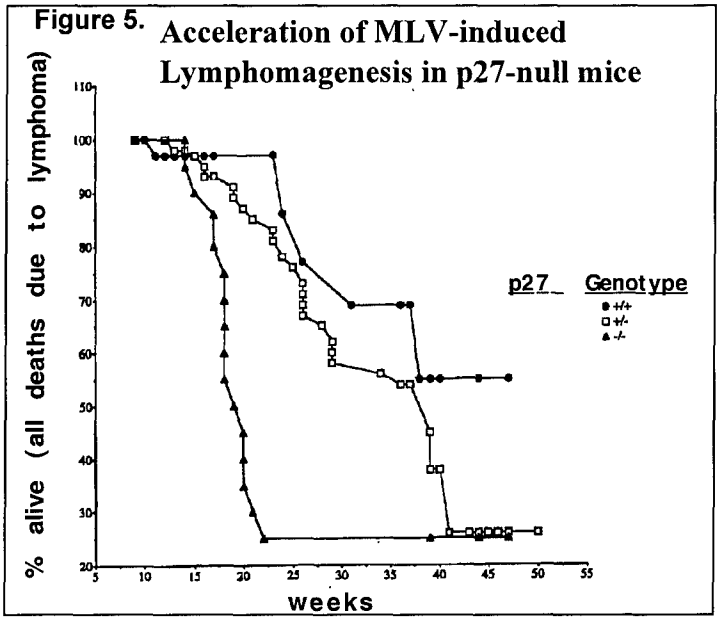
References

Clurman, B. and P. Porter. 1998. New Insights into the Tumor Suppressor Function of P27kip1. Proc. Natl. Acad. Sci. (USA), 95:15158-15160.

Fero, M., Randall, E., Gurley, K., Roberts, J. & Kemp, C. (1998) The murine gene p27kip1 is haplo-insufficient for tumor suppression *Nature* **396,** 177-180.

Li, B., Murphy, K., Laucrica, R., Kittrell, F., Medina, D., and Rosen, J. 1998. A transgenic mouse model for mammary carcinogenesis. Oncogene 16: 997-1007.

Appendix 1: Survival Curve of p27 +/+, +/-, and -/- mice after MuLV infection



Appendix 2. Comparison of tumor characteristics to p130

Characteristic	P130		
	Negative/Low	Intermediate/High	P*
Nodal status			
Negative	27 (50.9)	33 (58.9)	
Positive	26 (49.1)	23 (41.1)	.402
Vital status – 5/99			
Alive	44 (80.0)	49 (84.5)	
Dead	11 (20.0)	9 (15.5)	.533
Stage			
Local	28 (50.9)	33 (56.9)	
Regional/Distant	27 (49.1)	25 (43.1)	.523
ER			
Positive	34 (61.8)	38 (65.5)	
Negative	21 (38.2)	20 (34.5)	.683
PR			
Positive	41 (74.6)	35 (60.3)	
Negative	14 (25.5)	23 (39.7)	.108
Ki-67			
Low	43 (78.2)	31 (53.5)	
High	12 (21.8)	27 (46.6)	.006
c-erbB-2			
Negative	35 (63.6)	27 (46.6)	
Positive	20 (36.4)	31 (53.5)	.068
p53			
Negative	39 (70.9)	35 (60.3)	
Positive	16 (29.1)	23 (39.7)	.238
BCL-2			
High	28 (50.9)	27 (46.6)	
Low	27 (49.1)	31 (53.5)	.643
Cyclin E			
Low	46 (83.6)	42 (72.4)	
High	9 (16.4)	16 (27.6)	.151
p27			
High	29 (52.7)	33 (56.9)	
Low	26 (47.3)	25 (43.1)	.656

*Pearson chi square