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14. ABSTRACT The aim of this project is to characterize the functional significance of p57Kip2, one of Cyclin-dependent kinases inhibitors (CKI) of the INK4 family, in prostate proliferation, differentiation, tumorigenesis, and progression. In the present study, we have investigated the expression of p57Kip2 in human prostate cancer cases by immunohistochemistry. The average p57Kip2 labeling index in noncancerous lesions was 47.47%. However, the labeling index significantly decreased ($p < 0.001$) in PIN (10.21%) and carcinoma (2.85%) lesions. When virus-mediated overexpression of p57Kip2 in prostate cancer cells (LNCaP), significantly suppressed the cells' motility, potential for invasion, arrested the cell cycles at G0/G1 stage, and induced apoptosis. Furthermore, when the LNCaP cells stable transfected by p57Kip2 expression vector were recombined with rat urogenital mesenchyme (rUGM) and subsequently grafted into a male athymic mouse host using tissue recombinant techniques, the LNCaP tumors transformed into well differentiated squamous tumors and showed increased keratin synthesis or no tumor formation in athymic mice. In addition, the p57Kip2 knockout mouse prostate developed significantly hyperproliferation and hyperplasia at two and four month after grafted underneath the renal capsules of athymic immunodeficient mice. These results suggest that decreased expression of p57Kip2 occurs frequently in human prostate cancer even early in PIN lesion and p57Kip2 overexpression contributes to the downregulation of cell proliferation. Thus, p57Kip2 is an important gene in prostate cancer tumorigenesis and progression.					
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Table of Contents

Cover.....	1
SF 298.....	2
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	6
Reportable Outcomes.....	10
Conclusions.....	10
References.....	10
Appendices.....	10

Statement of Work

Investigating the Role of p57^{Kip2} in Prostate Cancer

Task 1. Characterize the p57^{Kip2} expression in the human prostate cancer. (months 1-6):

1. Determine the levels of expression of p57^{Kip2} in human prostate cancer by Immunohistochemical staining. (months 1-3).
2. Confirm the expression pattern of p57^{Kip2} in human prostate cancer by *in situ* hybridization and/or RT-PCR. (months 3-6).
3. Statistical analysis. (months 5-6).

Task 2. Determine the *in vivo* functional role of p57^{Kip2} in prostate development and tumorigenesis using tissue recombination techniques. (months 6-12):

1. Rescue and tissue recombination will be performed using different genotypes [p57^{Kip2} (-/-), p57^{Kip2} (+/-), p57^{Kip2} (+/+)] of urogenital tissues from p57^{Kip2} knockout mice. Rescued tissues will be harvest at 4-6 weeks for histological and molecular biological analysis. Epithelial cells from rescued tissues will be collected and grafted underneath the renal capsules of nude mice host with rat urogenital mesenchyme (rUGM) using tissue recombinant techniques. Tissue samples will be collected at 1, 2 and 3 month after grafting. Ten mice per group are required. (months 6-12)
2. Characterize grafted rescued and recombined prostate epithelial tissues. (months 6-12).
 - a. Histological characterization.
 - b. Western blot and Immunohistochemical analysis
 - c. Proliferation and apoptosis quantification.
 - d. Hormonal dependence.

Task 3. Investigate the functional role of p57^{Kip2} in prostate cancer cellular processes. (months 12-24):

1. Construct a p57^{Kip2} expression vector using tetracycline-inducible system and establish stable prostate cancer cell lines of LNCaP, PC-3 which produce tetracycline repressor protein for investigation. (months 12-15)
2. Investigate the functional role of p57^{Kip2} in cellular processes using the p57^{Kip2} expression vector transfected/infected into prostate cancer cell lines. (months 12-24)
 - a. Cell cycle and Apoptosis analysis (Flow Cytometric analysis)
 - b. Proliferation (BrdU labeling, MTT assay)
 - c. Characterize the functional role and mechanism of p57^{Kip2} in prostate cancer cellular processes (Western Blot, Immunoprecipitation, and CDKs activity assay etc.)
3. Recombine Tet-inducible p57^{Kip2} stable transfected prostate cancer cells (using LNCaP cells) with rUGM cells to perform tissue recombinant experiments. The p57^{Kip2} stable transfected cells recombinants will be grafted underneath the renal capsules of male athymic mice. Either Dox will be added at 1 month after establishing the LNCaP tumors or controls will remain untreated. Recombinant tissues will be collected at 1, 2 and 3 month after grafting. However, depending upon the rate of tumor growth and histological changes, we will decide to turn on and turn off the p57^{Kip2} expression at a given time point to look at short term and long term changes compared to controls. Ten mice per group are required. (months 12-24)
 - a. Histological characterization.
 - b. Western blot and Immunohistochemical analysis
 - c. Proliferation and apoptosis quantification.

Introduction:

Aberrations in the normal cycling of a cell lead to uncontrolled proliferation and can result in the development of cancer. p57^{Kip2}, one of Cyclin-dependent kinases inhibitors (CKI) of the INK4 family, is located on human chromosome 11p15.5, a region implicated in sporadic cancers. Because of its location, biochemical activities, and imprinting status, p57^{Kip2} has been considered a candidate tumor suppressor gene. However, the mechanism by which p57^{Kip2} exerts its modulatory functions in prostate differentiation and tumorigenesis/progression is not yet fully understood.

The goal of this project is to characterize the functional significance of p57^{Kip2} in prostate proliferation, differentiation, tumorigenesis, and progression. The central hypothesis of this proposal is that altered expression of p57^{Kip2} is important in development and/or progression of prostate adenocarcinoma.

To understand the role of p57^{Kip2} in prostate cancer, we have investigated the expression of p57^{Kip2} in 42 human prostate cancer cases by immunohistochemistry. The average p57^{Kip2} labeling index in noncancerous lesions was 47.47%. However, the labeling index significantly decreased ($p < 0.001$) in PIN (10.21%) and carcinoma (2.85%) lesions. To further understanding the role of p57^{Kip2} on the prostate cancer progression, we investigated the effects of p57^{Kip2} on the prostate cancer cells *in vitro*. When virus-mediated overexpression of p57^{Kip2} in prostate cancer cells (LNCaP), significantly suppressed the cells' motility, potential for invasion, arrested the cell cycles at G0/G1 stage, and induced apoptosis. Furthermore, when the LNCaP cells stable transfected by p57^{Kip2} expression vector were recombined with rUGM and subsequently grafted into a male athymic mouse host using tissue recombinant techniques, the LNCaP tumors transformed into well-differentiated squamous tumors and showed increased keratin synthesis or no tumor formation in nude mice. To further understanding the role of p57^{Kip2} on the prostate development and cancer progression, the urogenital tissues microdissected from E16.5 – E18.5 days of p57^{Kip2} knockout mice were grafted underneath the renal capsules of 6-7 week old male athymic immunodeficient mice. The p57^{Kip2} knockout prostate tissue showed significantly hyperproliferation and hyperplasia at two and four month after rescue.

These results suggest that decreased expression of p57^{Kip2} occurs frequently in human prostate cancer even early in PIN lesion and p57^{Kip2} overexpression contributes to the downregulation of prostate cancer cell proliferation. Downregulation of p57^{Kip2} induces prostate hyperproliferation and hyperplasia in mouse. Thus, p57^{Kip2} is an important gene in prostate cancer tumorigenesis and progression.

Key research accomplishments

Task 1. Characterize the p57^{Kip2} expression in the human prostate cancer.

- We have investigated the expression of p57^{Kip2} in 42 human prostate cancer cases by immunohistochemistry (Fig. 1).
- Two pathologists from Vanderbilt University Medical Center graded and counted all specimens in blinded fashion. Cells were counted as positive for p57^{Kip2} when immunoreactivity is clearly observed in their nuclei. We counted positive cells for p57^{Kip2} by monitoring at least 200 cells for noncancerous, PIN and carcinoma lesions in multiple regions of the same sample.
- The results (labeling index) were analyzed using Newman-Keuls test (q-test), with significance defined as $P < 0.05$ (Fig. 2).

Fig.1.

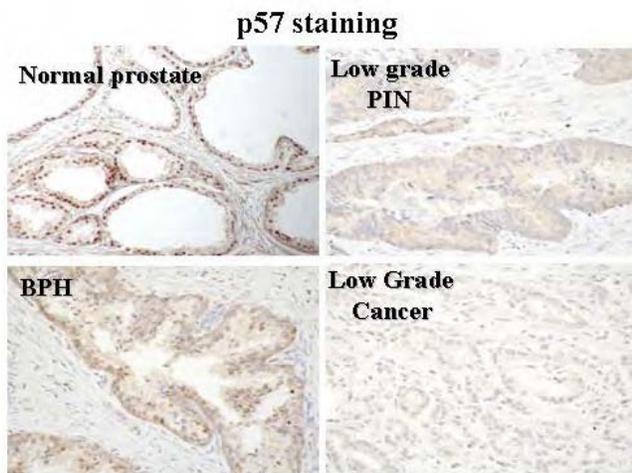


Fig.2.

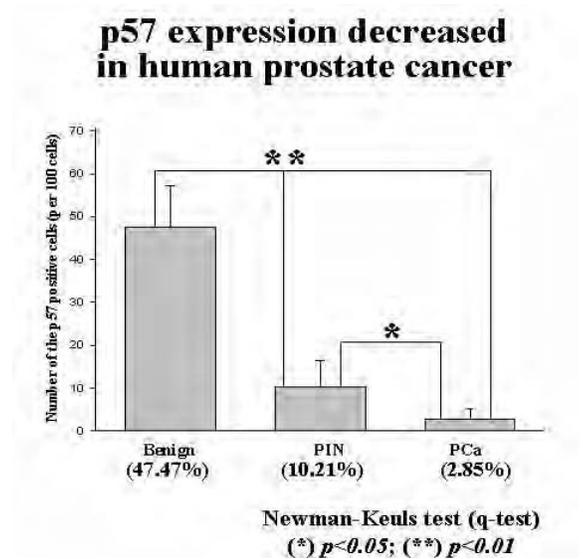


Fig. 1, 2: Immunohistochemical detection of p57^{Kip2} expression in human prostate cancer (Fig. 1). The average p57^{Kip2} labeling index in noncancerous lesions (BPH) was 47.47%. However, the labeling index significantly decreased ($p < 0.001$) (Newman-Keuls q-test) in PIN (10.21%) and carcinoma (2.85%) lesions (Fig. 2).

- Expression pattern of p57^{Kip2} in human prostate cancer was further confirmed by RT-PCR using human prostate cancer tissues (Fig. 3).

Fig. 3.

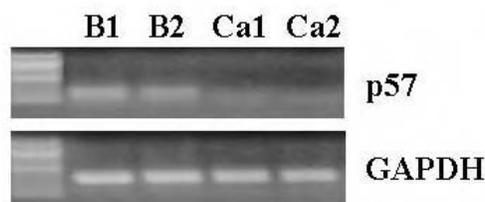


Fig. 3. RT-PCR detection of p57^{Kip2} expression in human prostate cancer. p57^{Kip2} expression in human prostate cancer (Ca 1 and Ca 2) is significantly less or undetectable, compared with human benign prostate tissues (B1 and B2).

Task 2. Determine the *in vivo* functional role of p57^{Kip2} in prostate development and tumorigenesis using tissue recombination techniques.

- To understanding the role of p57^{Kip2} on the prostate development and cancer progression, the urogenital tissues microdissected from E16.5 – E18.5 days of p57^{Kip2} knockout mice were grafted underneath the renal capsules of 6-7 week old male athymic immunodeficient mice. Histological analysis of p57^{Kip2} knockout prostate tissue showed significantly hyperproliferation and hyperplasia at two and four month after rescue (**Fig. 4**). Although no typical PIN or carcinoma lesions were found, in some areas, we still can see that multiple epithelial layers, elongated nuclear, non-uniformed staining in epithelial cells, events that are features of early PIN lesion in mouse prostate (see arrows in **Fig. 4-D**).

Fig. 4.

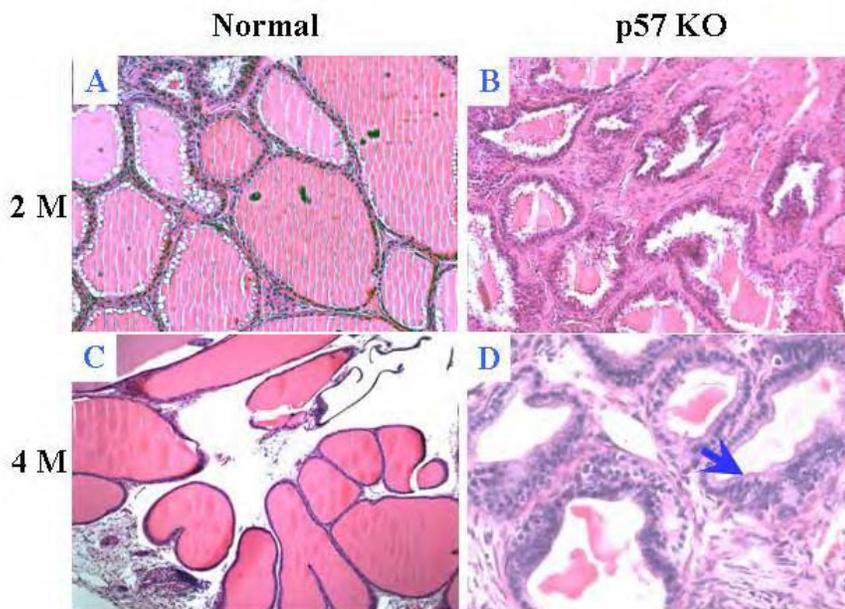


Fig. 4: Determine the functional role of p57^{Kip2} in prostate development and tumorigenesis *in vivo*. The urogenital tissues microdissected from E16.5 – E18.5 days of p57^{Kip2} knockout mice were grafted underneath the renal capsules of 6-7 week old male athymic immunodeficient mice. Histological analysis performed at two and four month after grafting.

Task 3. Investigate the functional role of p57^{Kip2} in prostate cancer cellular processes.

- We have constructed a p57^{Kip2} expression vector using tetracycline-inducible system.
- TetR expressing LNCaP cells were kindly provided by Susan Logan (NYU School of Medicine at VAMC).
- We have established Tet-inducible p57^{Kip2} stable transfected prostate cancer cell line using TetR expressing LNCaP cells (**Fig. 5**).

Fig. 5.

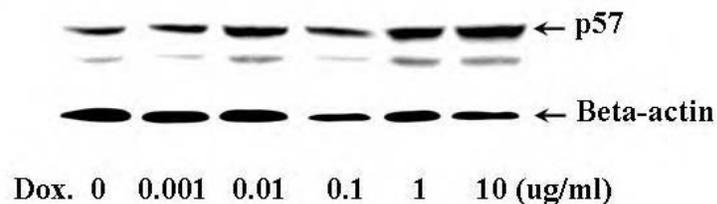


Fig. 3: Establishing the Tet-inducible p57^{Kip2} stable transfected prostate cancer cells. We constructed a p57^{Kip2} expression vector using tetracycline-inducible system and infected that to the TetR expressing LNCaP cells. Established LNCaP-p57 cells response to the Doxycycline dose dependently.

- We have investigated the functional role of p57^{Kip2} in cell motility and invasion by Transwell Invasion Assay (**Fig. 6**). In addition, we have investigated the affect of p57^{Kip2} on cell cycles and apoptosis by Flow Cytometric Analysis (**Fig.7**).

Fig. 6.

p57 expression decreased the motility of LNCaP cells (24 hours)

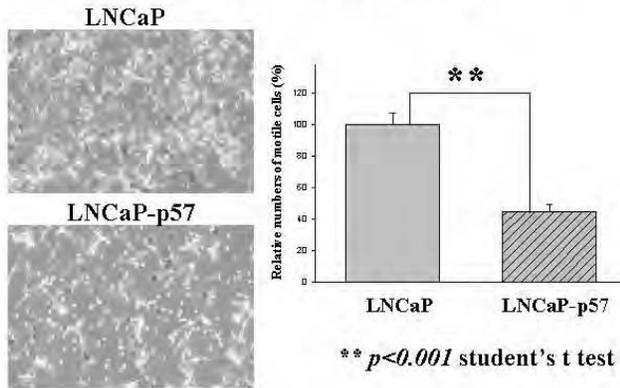


Fig. 7.

p57 expression induced apoptosis and arrested the cell cycle at G0/G1 stage in LNCaP cells

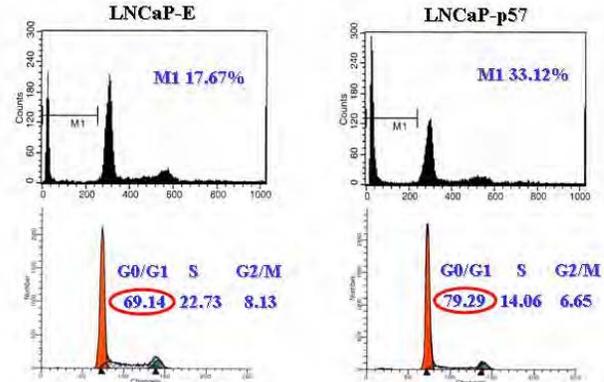


Fig. 6, 7: Transwell Invasion Assay and Flow Cytometric Analysis investigated the functional role of p57^{Kip2} in prostate cancer cellular processes. Overexpression of p57^{Kip2} reduced the motility and invasion of LNCaP cells *in vitro* (Fig. 6), but also arrested the cell cycles at G0/G1 stage, and induced apoptosis (Fig. 7). These results suggest that p57^{Kip2} not only effect on the cell cycles and proliferation but also involve to the tumor metastasis in prostate cancer.

- We have characterized that p57^{Kip2} arrested the cell cycles at G0/G1 stage, and induced apoptosis by affect on the Rb pathway through CDK4/Cyclin D and CDK2 complexes (**Fig. 8**).

Fig. 8.

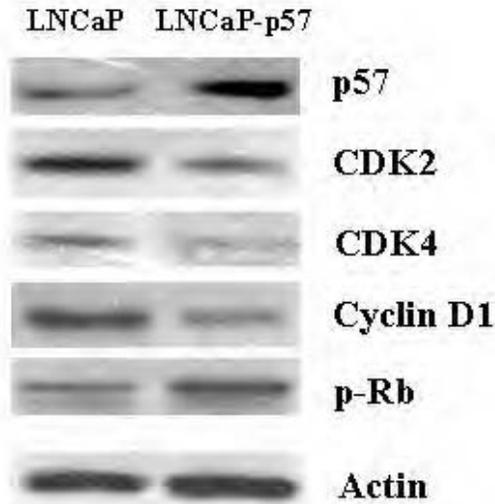


Fig. 8. The functional role and mechanism of $p57^{Kip2}$ in prostate cancer cellular processes were investigated by Western Blot. Overexpression of $p57^{Kip2}$ in LNCaP prostate cancer cells increased RB protein expression and decreased the CDK2, CDK4 and Cyclin D1 protein expression. These results suggest that $p57^{Kip2}$ arrested the cell cycles at G0/G1 stage, and induced apoptosis (see Fig. 7) by affect on the RB pathway through CDK4/Cyclin D and CDK2 complexes.

- We have recombine $p57^{Kip2}$ stable transfected prostate cancer cells (LNCaP-p57) with rUGM cells to perform tissue recombinant experiments. Histological examination and molecular biological evaluation of tissue samples have been performed by H&E staining and immunohistochemistry staining (Fig. 7).

Fig. 7.

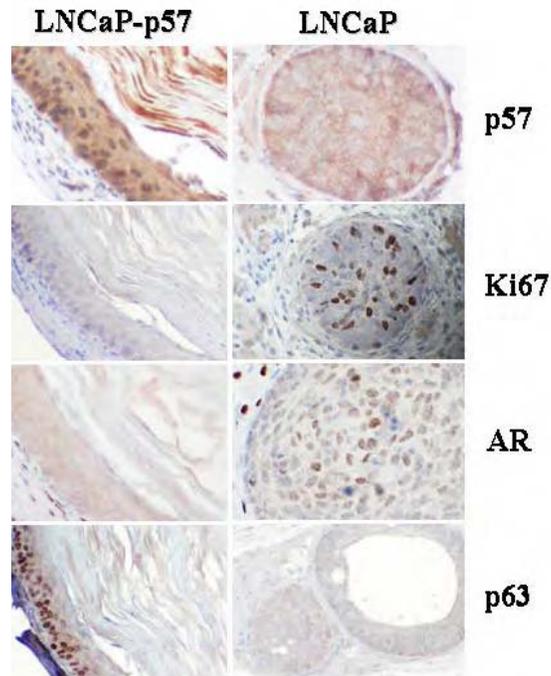


Fig. 7: When the LNCaP cells with an integrated $p57^{Kip2}$ expression vector were recombined with rUGM and grafted underneath the renal capsules of a male athymic mouse host (2 months), the LNCaP tumors transformed to well differentiated squamous tumors and showed increased keratin synthesis (Fig. 7).

These results suggest that p57^{Kip2} not only involve to the proliferation, but also involve to the differentiation of LNCaP cells.

Interestingly, the increased expression of p57^{Kip2} induced the p63 expression in LNCaP cells. It has been reported that p63 is involved with the tumor metastasis in lung cancer. We will do a further study to conform and understand the relationship between the p57^{Kip2} and p63 in prostate cancer progression.

However, when we combined another p57^{Kip2} stable transfected LNCaP cell line (which expresses higher level of p57^{Kip2}, compared that with the first p57^{Kip2} integrated LNCaP cell line) with rUGM and grafted under the renal capsules of the male nude mice, there were no detectable LNCaP tumors formed until two months after grafting (0/5).

Reportable Outcomes

Publications

p57^{Kip2} is down-regulated in prostate cancer and overexpression inhibits proliferation and tumorigenesis of prostate cancer cells.

Renjie Jin, Yongqing Wang, Mingfang Ao, Simon W. Hayward and Robert J. Matusik. (in preparation).

Abstracts

Renjie Jin, Yongqing Wang, Mingfang Ao, Simon W. Hayward and Robert J. Matusik. p57^{Kip2} is down-regulated in prostate cancer and overexpression inhibits proliferation and tumorigenesis of prostate cancer cells. *Society for Basic Urologic Research Fall 2004 Meeting*, Savannah, GA, December 2004.

Renjie Jin, Yongqing Wang, Mingfang Ao, Simon W. Hayward and Robert J. Matusik. p57^{Kip2} is down-regulated in prostate cancer and overexpression inhibits proliferation and tumorigenesis of prostate cancer cells. *Host-Tumor Interactions Program & Department of Cancer Biology 4th Annual Joint Retreat* November 2004. (**2nd Place Award in Oral Presentation**).

Conclusions

These results suggest that decreased expression of p57^{Kip2} occurs frequently in human prostate cancer even early in PIN lesion and p57^{Kip2} overexpression contributes to the downregulation of cell proliferation and tumorigenesis and progression. Thus, p57^{Kip2} is an important gene in the early stages in the development of prostate cancer and progression of the disease.

References

N/A

Appendices

CKI: Cyclin-dependent kinases inhibitor.

CDK: Cyclin-dependent kinases.

Dox: Doxycycline

TetR: Tetracycline Repressor

RT-PCR: Reverse Transcription- Polymerase Chain Reaction

rUGM: rat urogenital mesenchyme.

PIN: prostatic intraepithelial neoplasia.