AD_____

Award Number: DAMD17-00-1-0511

TITLE: Use p27KIP1 Degradation for Breast Cancer Diagnosis and Therapy

PRINCIPAL INVESTIGATOR: Hui Zhang, Ph.D.

CONTRACTING ORGANIZATION: Yale University
New Haven, Connecticut 06520

REPORT DATE: July 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Annual (1 Jul 00 - 30 Jun 01)

4. TITLE AND SUBTITLE

Use p27KIP1 Degradation for Breast Cancer Diagnosis and Therapy

5. FUNDING NUMBERS

DAMD17-00-1-0511

6. AUTHOR(S)

Hui Zhang, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Yale University
New Haven, Connecticut 06520
email -

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

It is well established that p27(KIP1), a CDK inhibitor, serves as an excellent prognostic marker for breast cancers. Loss or low levels of p27 are closely associated with poor prognosis in breast cancer patients. We have identified a ubiquitin E3 ligase complex,SCF(SKP2), that binds and targets p27 for ubiquitin-dependent proteolysis. We found that SKP2, the rate limiting component of SCF(SKP2) complex, is highly induced in many transformed cells. We proposed to determine whether there is an inverse relationship between the levels of p27 and SKP2 in breast cancer cells and tumor samples. In the past year, we have affinity purified antibodies against the components of SCF(SKP2) including anti-SKP2, SKP1, CUL-1, and p27 antibodies. The affinity purified antibodies were used to stain breast tumor samples. We also began to characterize the determinants on phospho-p27 peptide that mediate interaction between p27 and SKP2. Mutant peptides were made that contain altered amino acid residues around phosphorylated threonine187 in p27 to determine whether they are involved in SKP2 binding. They were used to determine the interaction between SKP2 and p27. We are testing whether silencing of SKP2 inhibits breast cancer cell growth by promoting p27 accumulation.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer	11
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction:

Many lines of evidence suggest that $p27^{KIP1}$, a CDK inhibitor, serves as an excellent and a novel prognostic marker for breast cancers. Loss or low levels of p27 are closely associated with poor prognosis in breast cancer patients. The inverse relationship between the levels of p27 and tumor malignancy has also been observed in prostate, lung, gastric and colorectal cancers. p27 is a major cell cycle regulator for the G1 progression towards the S-phase. The levels of p27 are primarily regulated by its protein stability during cell cycle progression. Phosphorylation on Thr187 of p27 is required for its ubiquitin-dependent proteolysis. Consistently, abnormally enhanced p27 degradation has been found to associate with aggressively growing tumors. Our proposal is based on our novel findings that a ubiquitin E3 ligase complex, SCF^{SKP2} (SKP1, CUL-1, F-box/SKP2), specifically binds to the Thr187-phosphorylated p27 and targets it for ubiquitin-dependent proteolysis. We also found that SKP2, the SCF component that specifically binds to the phosphorylated p27, is highly induced in many transformed cells and expression of SKP2 induces p27 degradation in vivo. Our discovery thus identifies SCF^{SKP2} as a unique and novel p27 regulatory complex that is likely to be altered in breast cancers. We propose to determine whether SCF^{SKP2} is induced in breast cancers and whether such an alteration correlates with low levels of p27 associated with poor survival. We are devising novel strategies to target the SCF^{SKP2}-mediated proteolytic degradation of p27 for breast cancer therapy. Our investigations should reveal novel insights into the regulation of p27 levels in breast cancers and also identify the SCF^{SKP2} complex as a potentially novel prognostic marker as well as a novel therapeutic target for breast cancer therapy.

Body:

Objective 1:

We have proposed to determine the relationship between SCF^{SKP2} and p27 in primary breast cancers. We are using specific rabbit polyclonal antibodies against SKP2, p27 and other components of SCF^{SKP2} complex to determine whether there is an inverse relationship between the levels of p27 and SKP2 using immunohistochemistry in breast cancer samples. So far, we have used the rabbit antiserum to direct stain the breast cancer samples but high background was observed. To overcome this problem, we have affinity purified some of the antisera against SKP2, p27 and other components of SCF^{SKP2} complex, CUL-1, and SKP1 to determine whether these procedures help to reduce the background. The work is in progress.

Objective 2:

We also proposed to characterize the determinants on p27 and SKP2 for their interaction. We have made mutant p27 peptides which contain altered amino acid residues around phospho-threonine187 in p27 which mediates SKP2 interaction. We found that proline188 is essential for this interaction. We are trying to determine the domain and the determinants on SKP2 that mediate SKP2 interation with p27. We have made various deletion mutants of SKP2 on its region immediate downstream of the F-box and seven leucine-rich repeats, which appear to be responsible for p27 binding. These mutant proteins and wildtype SKP2 were in vitro transcribed and translated. They were used for the in vitro binding to phosphorylated p27 peptide. These studies are underway. However, the binding affinity of these proteins synthesized in the programmed rabbit reticulocyte lysates was not very high as compared with the HeLa cell lysates . It was recently found that CKS1/p9, a protein we found previously to complex with SKP1/SKP2/cyclin A/CDK2 (1), helps SKP2 to bind to p27 (2, 3). We have made GST-CKS1 protein and are examining whether the inclusion of CKS1/p9 helps the binding of SKP2 and its mutant proteins to p27.

Objective 3:

We try to directly deliver the p27 peptide or SKP2 peptide into breast cancer cells to see whether they induce p27 accumulation and inhibit breast cancer cell proliferation. These experiments are underway since we need to map the shortest peptides first (see above) to design the inhibitory peptides. However, recently it has been shown that the expression of many proteins can be silenced using the double-stranded, short RNA

oligonucleotides (21 nucleotide in length). We are currently using this method to silence the expression of SKP2 in breast cancer cells to determine whether it causes p27 accumulation and inhibits cell proliferation.

Key research achievements:

We have affinity purified the antibodies for the immunohistochemistry on the breast cancer samples. We have made various mutants of SKP2 to map its binding sites for p27.

Reportable outcomes:

Works are still in progress. No reportable outcomes yet for publication.

Conclusions:

We have found that proline188 in p27 is important for p27 interaction with SKP2. This suggests that SKP2 binding to p27 is highly specific and this interaction is not just dependent on the phosphorylated threonine 187. This finding will be incorporated into the consideration in designing the peptide inhibitor for breast cancer cells. Other works are in progress to eliminate the background for immunohistochemical analysis in breast cancer samples and to map the interaction motifs for p27 and SKP2 interaction.

References:

1. Zhang, H., Kobayashi, R., Galaktionov, K. & Beach, D. (1995) p19Skp1 and p45Skp2 are essential elements of the cyclin A-CDK2 S phase kinase *Cell* **82,** 915-25.

2. Ganoth, D., Bornstein, G., Ko, T. K., Larsen, B., Tyers, M., Pagano, M. & Hershko, A. (2001) The cell-cycle regulatory protein Cks1 is required for SCF(Skp2)-mediated ubiquitinylation of p27 *Nat Cell Biol* **3,** 321-4.

3. Spruck, C., Strohmaier, H., Watson, M., Smith, A. P., Ryan, A., Krek, W. & Reed, S. I. (2001) A CDK-Independent Function of Mammalian Cks1. Targeting of SCF(Skp2) to the CDK Inhibitor p27(Kip1) *Mol Cell* **7,** 639-650.

List of personnel in this project:

Hui Zhang, Ph. D., Principal Investigator

Eun-Hee Shim, Ph. D., Postdoctoral Associate

Sophia Ryzhikov, Technician