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ORIGINAL ARTICLE

Oncogenic Role of Skp2 and p27^{Kip1} in Intraductal Proliferative Lesions of the Breast[△]

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Key words: breast cancer; intraductal proliferative lesions; p27^{Kip1}; S-phase kinase-associated protein 2

Objective To investigate whether the connection of p27^{Kip1} to S-phase kinase-associated protein 2 (Skp2) plays an oncogenic role in intraductal proliferative lesions of the breast.

Methods Here we investigated the mechanism involved in association of Skp2's degradation of p27^{Kip1} with the breast carcinogenesis by immunohistochemical method through detection of Skp2 and p27^{Kip1} protein levels in 120 paraffin-embedded tissues of intraductal proliferative lesions including usual ductal hyperplasia (UDH, *n*=30), atypical ductal hyperplasia (*n*=30), flat epithelial atypia (FEA, *n*=30), and ductal carcinoma *in situ* (DCIS, *n*=30). Moreover, the expression status of Skp2 and p27^{Kip1} in 30 cases of the normal breast paraffin-embedded tissues were explored.

Results The DCIS group was with the highest Skp2 level and the lowest p27^{Kip1} level, and the UDH group was with the lowest Skp2 level and the highest p27^{Kip1} level. Both Skp2 and p27^{Kip1} levels in the DCIS group were significantly different from those in the UDH group (all *P*<0.01). The levels of Skp2 and p27^{Kip1} in the FEA group were significantly different from both the DCIS and UDH groups (all *P*<0.05). p27^{Kip1} was negatively correlated with Skp2 in both the UDH group (*r*=-0.629, *P*=0.026) and DCIS group (*r*=-0.893, *P*=0.000).

Conclusion Overexpression of Skp2 might be the mechanism underlying p27^{Kip1} over degradation.

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INTRADUCTAL proliferative lesions, as a traditional concept, were updated by the WHO in 2003 and flat epithelial atypia (FEA) was brought into this concept as one subtype. Intraductal proliferative lesions consist of cytologically and architecturally diverse lesions, typically originated from the terminal duct-lobular unit and confined to the mammary duct-lobular system. The four subtypes of intraductal proliferative lesions are as follows: usual ductal hyperplasia (UDH), FEA, atypical ductal hyperplasia (ADH), and ductal carcinoma *in situ* (DCIS). These subtypes are associated with an increased risk, albeit of greatly different magnitudes, for the subsequent genesis and progression of carcinoma. Moreover, the development of most breast cancer follows the pattern: UDH→ADH→DCIS→invasive breast cancer. Particularly, FEA is characterized by a few layers of mildly atypical luminal epithelial cells. Invasive ductal breast cancers are also found in FEA in some researches, albeit at a lower concentration.¹ Therefore, FEA is probably a kind of pre-cancerous lesion and there might be some implication between FEA and carcinogenesis.

S-phase kinase associated protein 2 (Skp2) belongs to ubiquitin-proteasome system (UPS) that plays vital roles in regulating many biological processes by controlling the timely turn-over of proteins. UPS includes three types of enzymes for exerting UPS function *via* a cascade of enzymatic reactions. The three enzymes are ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3).² The initial step is to add ubiquitin to E1 and subsequently transfer of it to the E2 enzyme. The ubiquitin-charged E2 then interacts with E3 and transfers the ubiquitin onto the substrate, leading to the formation of a mono-ubiquitin or polyubiquitin chain due to the consecutive addition of ubiquitin moieties to target proteins. It is evident that the specificity of substrate selection depends on the E3, leading to substrate degradation by the 26S proteasome.³ It is known that Skp2 is one of the components of the Skp1-Cullin1-F-box (SCF) E3 ligase complex. The SCF complexes comprise a family of multi-subunit E3 ubiquitin ligases that target selected proteins for destruction by the 26S proteasome degradation. It is clear that Skp2 is an F-box protein of the SCF complex. Because Skp2 is originally discovered with Skp1 associated with the S-phase kinase Cdk2/cyclin A, it was named as Skp2.⁴ Among the many F-box proteins, Skp2 has been well-characterized and it has been shown to be involved in the ubiquitin-mediated degradation of several key regulators of mammalian G1 progression in cell cycle, including p27^{Kip1}, a dosage-dependent tumor suppressor.⁵ Therefore, Skp2 might execute oncogenic function through

down-regulation of p27^{Kip1}, and lead mitosis through G1/S checkpoint without limit, subsequent boundless division growth, and final formation of malignant clone.⁶ In addition to p27^{Kip1}, specific substrates of Skp2 have also been identified which include P57Kip2, Tob1, and FOXO1.⁷⁻⁹ These substrates are involved in many cellular processes such as cell cycle regulation, proliferation, differentiation, apoptosis, and survival. Without a doubt, Skp2 has important functions in the regulation of these cellular processes due to degradation of its substrates, most of which are tumor suppressor proteins. Because Skp2 is responsible for the degradation of the above-mentioned tumor suppressor proteins, Skp2 is thought to function as oncoprotein. Many studies have shown that over expression of Skp2 was observed in a variety of human cancers, including prostate cancer,¹⁰ melanoma,¹¹ nasopharyngeal carcinoma,¹² and pancreatic cancer.¹³

Therefore, the purpose of the present study was to examine the role of Skp2 and p27^{Kip1} in breast carcinoma genesis and the essence of FEA as one subtype of intraductal proliferative lesions.

MATERIALS AND METHODS

Patient and tissue selection

One hundred and twenty female patients (median age, 49; range 24-71 years) with intraductal proliferative hyperplasia diagnosed and treated at Tianjin Tumor Hospital between January, 2004 and December, 2006 were enrolled in this study including 30 DCIS, 30 ADH, 30 FEA, and 30 UDH. In addition, 30 cases with histologically normal lobules of mammary glands were selected as control, whose age ranged from 25 to 75 years old.

Histological sections were reviewed in a double-blind manner by 2 senior pathologists. The diagnostic criteria of four subtypes of intraductal proliferative lesions were according to the latest WHO classification from Tavassoli's series.¹⁴ Adenosis, invasive carcinoma, and metastasis were all excluded. Those patients after chemotherapy as well as operative treatment were also excluded.

Immunohistochemistry

Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded tissue sections. Sections (4 μm) were deparaffinized with xylene and rehydrated in a series of ethanols. For epitope retrieval, slides were heated in 1 mmol/L EDTA buffer (pH 8.0), in wet autoclave at 210°C until the steam injection issued from snifting valve, and incubated at 130°C for 90 seconds. After cooling, slides were treated for 10 minutes with 3% H₂O₂ in order to

block endogenous peroxidase.

Slides were incubated with anti-Skp2 (1:50, Zymed Inc., San Francisco, CA, USA) and anti-p27^{Kip1} monoclonal antibodies (1:75, Sigma-Aldrich Company, Germany) at 4°C overnight. Following the protocol of the manufacturer, then the slides were incubated with polymer helper at 37°C for 20 minutes and polyperoxidase-anti-mouse/rabbit IgG for 30 minutes. Finally antigen-antibody complex was colored by the DAB kit.

The used anti-Skp2 monoclonal antibody can not distinguish Skp2 from the F-box protein Skp2B. Skp2B has little if any influence on the degradation of p27, but in contrast to Skp2 (which is strictly localized in the nucleus), Skp2B is localized only in the cytoplasm.¹⁵ Because of the restriction of our analysis to expression of nuclear Skp2, the results reflect only levels of Skp2, and not those of Skp2B.

Evaluation of immunohistochemical staining

Scoring of immunohistochemical slides was done according to the percentage of cells exhibiting nuclear staining. Percentage of breast tissues expressing p27^{Kip1} and Skp2 were determined by observing 10 high-power fields. Average percentage of positive cells at 10 fields of vision under high power was regarded as final positive expression percentage. Those epithelial cells were considered as positive staining for p27^{Kip1} and Skp2 when nuclear staining was present.

We used a cut-off of 40% for p27^{Kip1} and 10% for Skp2, which was the cut-off used in the previous studies.^{16, 17} Cases with 1%-40% nuclear p27^{Kip1} expression were regarded as +, 41%-70% as ++, 71%-100% as +++, 0% as -. Cases with 1%-10% nuclear Skp2 expression were considered as +, 11%-55% as ++, 56%-100% as +++, 0% as -. When stained, cells exhibited similar intensity of nuclear staining of Skp2 or p27^{Kip1} regardless of the percentage of cells stained, and therefore the intensity of staining was not included in the score.

Statistical analysis

Statistical analyses were performed using SPSS 11.0 statistical software package. Paired comparisons of Skp2 level and p27^{Kip1} expression among four subtypes were performed with *Kruskal-Wallis* rank test (*K-W* test). *Spearman* rank correlation was used to judge the relationship of Skp2 with p27^{Kip1}. *P* value less than 0.05 was considered statistically significant.

RESULTS

Typical representative immunohistochemical staining

pictures of Skp2 and p27^{Kip1} in four subtypes of intraductal proliferative lesions are shown by Fig. 1.

Skp2 level was increased along with elevated lesions grade in four subtypes of intraductal proliferative lesions

Significant difference in Skp2 level existed between normal control and intraductal proliferative lesions ($\chi^2=44.150$, $P=0.000$). Skp2 level was increased along with elevated lesion grade ($P<0.05$). There was no significant difference in Skp2 level between the FEA and ADH groups ($P>0.05$). The paired comparison results of Skp2 among four subtypes and normal control are shown in Table 1.

p27^{Kip1} expression decreased in four subtypes of intraductal proliferative lesions

As shown in Table 2, various levels of nuclear p27^{Kip1} staining were observed in four subtypes. It was found that p27^{Kip1} level of the normal control group was significantly higher than that of four subtypes in whole ($\chi^2=35.430$, $P=0.000$). p27^{Kip1} level was decreased in turn of UDH, ADH or FEA, and DCIS.

Skp2 level was inversely related with p27^{Kip1} level in the DCIS as well as UDH groups

In the UDH group, those cases with positive p27^{Kip1} added up to 24 (24/30), and those with positive Skp2 were 10 (10/30). In the DCIS group, the total number of cases with positive Skp2 and positive p27^{Kip1} was 29 (29/30) and 2 (2/30), respectively. p27^{Kip1} was negatively correlated with Skp2 in both the UDH group ($r=-0.629$, $P=0.026$) and DCIS group ($r=-0.893$, $P=0.000$). However, no significant correlation of p27^{Kip1} and Skp2 was found in the ADH ($r=0.215$, $P=0.098$) or FEA group ($r=0.199$, $P=0.128$).

DISCUSSION

It was observed in our study that Skp2 level was increased along with elevated lesions grade in four subtypes of intraductal proliferative lesions, however, p27^{Kip1} level was decreased in four subtypes. The results indicated that elevation of Skp2 and reduction of p27^{Kip1} might be involved in breast carcinogenesis. It is possible that elevated Skp2 levels in breast lesions are not causative of the hyper proliferation but simply reflect increased cell cycle entry in these lesions driven by a different mechanism.

Cell cycle progression is regulated by a wide variety of external factors, including cyclin dependant kinase inhibitor (CKI), such as p16, p18, p19, p21, p27, p57, and promoter factors, such as cyclins and cyclins dependent kinase.

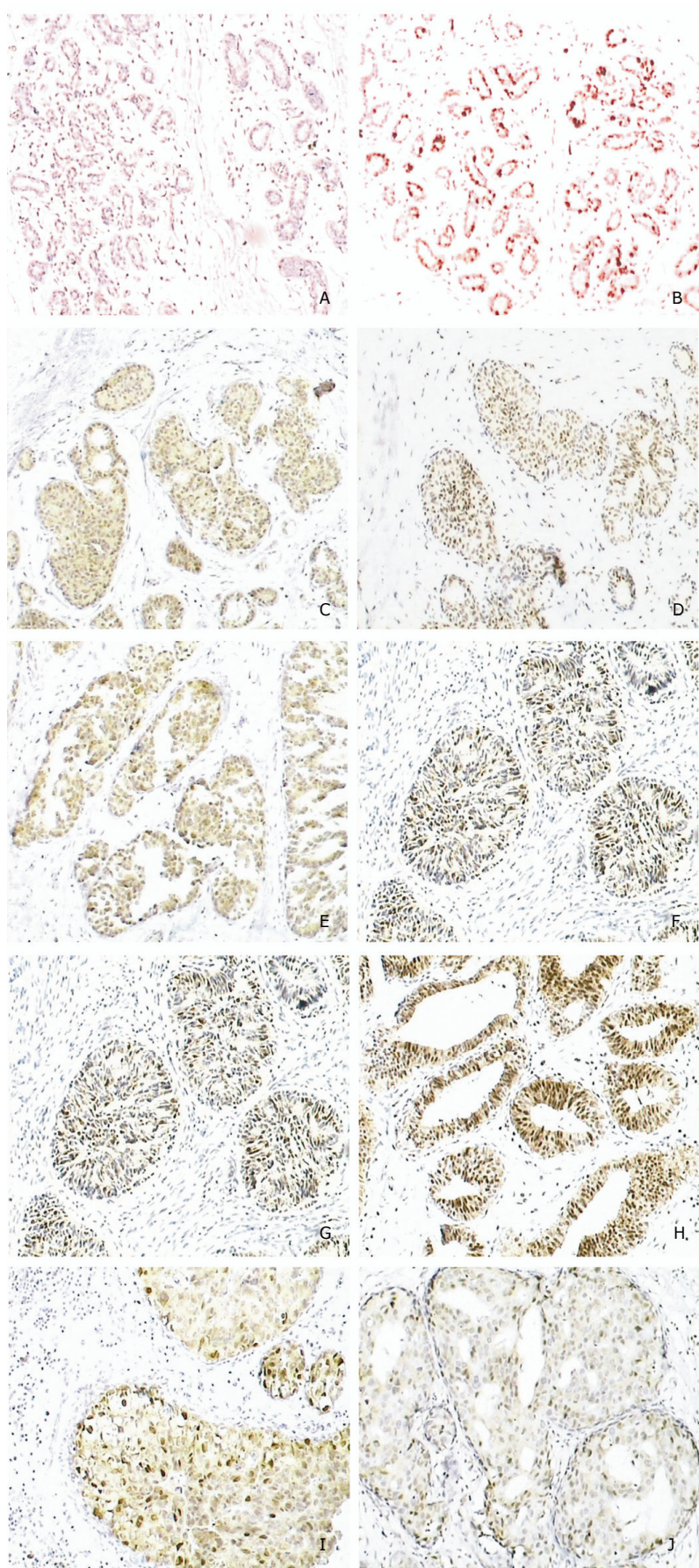


Figure 1. Representative staining images of immunohistochemical staining for Skp2 and p27^{Kip1} in normal control and four subtypes of intraductal proliferative lesions. Envision $\times 100$

A. Negative nuclear Skp2 expression in normal lobules of mammary gland.

B. Positive nuclear p27^{Kip1} expression in normal lobules of mammary gland.

C. Locally distributed positive Skp2 staining in UDH breast tissue.

D. Diffusely distributed positive p27^{Kip1} in UDH breast tissue.

E. Mild staining of Skp2 in ADH breast tissue.

F. Mild staining of p27^{Kip1} in ADH breast tissue.

G. In sections with FEA, cells show powerful nuclear-positive Skp2.

H. Powerful nuclear-positive p27^{Kip1} in FEA breast tissue.

I. DCIS breast tissue exhibits strong nuclear Skp2 staining.

J. Weak nuclear p27^{Kip1} staining in DCIS breast tissue.

Skp2: S-phase kinase-associated protein 2; UDH: usual ductal hyperplasia; ADH: atypical ductal hyperplasia; FEA: flat epithelial atypia; DCIS: ductal carcinoma *in situ*.

Table 1. Paired comparison of Skp2 level among four subtypes of intraductal proliferative lesions and normal control ($n=30$)

Groups	Skp2 level			
	-	+	++	+++
Normal control group	29	1	0	0
UDH group	20	9	1	0
FEA group ^{***}	11	2	8	9
ADH group [#]	11	0	12	7
DCIS group ^{**}	1	0	11	18

[#] $P<0.05$ compared with the DCIS group; ^{*} $P<0.05$, ^{**} $P<0.01$ compared with the UDH group.

Table 2. Paired comparison of p27^{Kip1} level among four groups of intraductal proliferative lesions and normal control ($n=30$)

Groups	p27 ^{Kip1} level			
	-	+	++	+++
Normal control group	5	6	10	9
UDH group	6	3	8	13
FEA group ^{**}	15	2	10	3
ADH group [#]	17	1	8	4
DCIS group ^{##}	28	1	1	0

[#] $P<0.05$, ^{##} $P<0.01$ compared with the UDH group; ^{*} $P<0.05$ compared with the DCIS group.

In recent years, the ubiquitin-mediated proteolysis pathway has been considered as one of carcinogenesis mechanisms.^{18,19} Skp2 is one subunit of ubiquitin protease pathway which plays an important role in degradation of cell cycle regulators. Narayan *et al*²⁰ found that level of Skp2 protein in gastric cancer was higher than that of atypical hyperplasia, while p27^{Kip1} level in cancer was lower than that in atypical hyperplasia. In addition, it was also found that over expression of Skp2 was connected with carcinogenesis of cervical neoplasm, non-small lung cancer, and cutaneous tumor.²¹⁻²³ Above researches suggested that Skp2 and p27^{Kip1} might play important roles in carcinogenesis.

The data from our experiments demonstrated that Skp2 expression was inversely related with p27^{Kip1} levels in the DCIS and UDH groups. Skp2 has been shown to regulate cellular proliferation by targeting several cell cycle-regulated proteins for ubiquitination and degradation, including cyclin-dependent kinase inhibitor p27^{Kip1}. p27^{Kip1} is also one of substrates in ubiquitin protease pathway. The degradation of p27^{Kip1} by Skp2 promotes smooth shift of G1/S phase during cell cycle. Consequently, appropriate

protein level of Skp2 and p27^{Kip1} is essential for normal cell cycle.²⁴ Certainly, increase of Skp2 and over degradation of p27^{Kip1} induce loss of inhibition of G1/S, unlimited mitosis, immature differentiation, and genesis of tumor.²⁵ However, Miranda-Carboni *et al*²⁶ showed us an interesting result by Wnt10b-expressing mammary tumors that the degradation of p27^{Kip1} in this model appears independent of Skp2 and correlates with induction of a functional CUL4A-E3 ligase complex.

In addition, we found that Skp2 protein level of the ADH group was significantly higher than that of the UDH group ($P<0.05$), suggesting that high level of Skp2 might be associated with atypical ductal hyperplasia. Arbin *et al*²⁷ also found the prostate epitheliums of Skp2 transgenic mouse got atypical hyperplasia.

FEA with distinctive morphological feature has been recently recognized by pathologists.²⁸ Many debates on nature of FEA exist since it has been recognized. Some researchers held the opinion that FEA was a type of precancerosis like ADH.²⁹ Ductal Intraepithelial Neoplasm (DIN) grading system which was presented by the WHO Work Group in 2003 agreed with this opinion. Our results showed none of significant differences was observed between the FEA and ADH groups in the expression levels of both Skp2 and p27^{Kip1} proteins ($P=1.000$), but levels of Skp2 and p27^{Kip1} in the FEA group was significantly different from both the DCIS and UDH groups (all $P<0.05$). It suggested that FEA might be a precancerous lesion in a way. The conclusion was similar to Lee and his colleague.³⁰ In their study they observed that FEA was more frequently seen with ADH, indicating that FEA was associated with malignant tumors.

In conclusion, the findings of our study demonstrated that alteration of Skp2 and p27^{Kip1} levels in intraductal proliferative lesions of the breast would contribute to atypical hyperplasia and genesis of breast cancer, and FEA might be a kind of precancerous lesion. It is worth mentioning that elevated Skp2 levels in the breast lesions are not direct causative of the hyper proliferation but simply reflect increased cell cycle entry in these lesions driven by a different mechanism.

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REFERENCES

1. Sudarshan M, Meguerditchian AN, Mesurrolle B, et al. Flat epithelial atypia of the breast: characteristics and be-

- haviors. *Am J Surg* 2011; 201:245-50.
2. Weissman AM, Shabek N, Ciechanover A. The predator becomes the prey: regulating the ubiquitin system by ubiquitylation and degradation. *Nat Rev Mol Cell Biol* 2011; 12:605-20.
3. Frescas D, Pagano M. Deregulated proteolysis by the F-box proteins SKP2 and beta-TrCP: tipping the scales of cancer. *Nat Rev Cancer* 2008; 8:438-49.
4. Nakayama KI, Nakayama K. Ubiquitin ligases: cell-cycle control and cancer. *Nat Rev Cancer* 2006; 6:369-81.
5. Huang HC, Way TD, Lin CL, et al. EGCG stabilizes p27^{Kip1} in E2-stimulated MCF-7 cells through down-regulation of the Skp2 protein. *Endocrinology* 2008; 149:5972-83.
6. Shi P, Zhang Y, Tong X, et al. Dihydrotestosterone induces p27 degradation *via* direct binding with SKP2 in ovarian and breast cancer. *Int J Mol Med* 2011; 28:109-14.
7. Kamura T, Hara T, Kotoshiba S, et al. Degradation of p57Kip2 mediated by SCFSkp2-dependent ubiquitylation. *Proc Natl Acad Sci USA* 2003; 100:10231-6.
8. Hiramatsu Y, Kitagawa K, Suzuki T, et al. Degradation of Tob1 mediated by SCFSkp2-dependent ubiquitination. *Cancer Res* 2006; 66:8477-83.
9. Huang H, Regan KM, Wang F, et al. Skp2 inhibits FOXO1 in tumor suppression through ubiquitin-mediated degradation. *Proc Natl Acad Sci USA* 2005; 102:1649-54.
10. Wang Z, Gao D, Fukushima H, et al. Skp2: a novel potential therapeutic target for prostate cancer. *Biochim Biophys Acta* 2011a; 1825:11-7.
11. Rose AE, Wang G, Hanniford D, et al. Clinical relevance of SKP2 alterations in metastatic melanoma. *Pigment Cell Melanoma Res* 2011; 24:197-206.
12. Xu HM, Liang Y, Chen Q, et al. Correlation of Skp2 overexpression to prognosis of patients with nasopharyngeal carcinoma from South China. *Chin J Cancer* 2011; 30:204-12.
13. Schuler S, Diersch S, Hamacher R, et al. SKP2 confers resistance of pancreatic cancer cells towards TRAIL-induced apoptosis. *Int J Oncol* 2011; 38:219-25.
14. Tavassoli FA, Devilee P. Intraductal proliferative lesions. The WHO classification of tumors of the breast and female genital organs. Lyon: International Agency for Research on Cancer Press, 2003. p. 10-112.
15. Radke S, Pirkmaier A, Germain D. Differential expression of the F-box proteins Skp2 and Skp2B in breast cancer. *Oncogene* 2005; 24:3448-58.
16. Slotky M, Shapira M, Ben-Izhak O, et al. The expression of the ubiquitin ligase subunit Cks1 in human breast cancer. *Breast Cancer Res* 2005; 7:737-44.
17. Hsu JD, Kao SH, Ou TT, et al. Gallic acid induces G2/M phase arrest of breast cancer cell MCF-7 through stabilization of p27^(Kip1) attributed to disruption of p27^(Kip1)/Skp2 complex. *J Agric Food Chem* 2011; 59:1996-2003.
18. Hu D, Liu W, Wu G, et al. Nuclear translocation of Skp2 facilitates its destruction in response to TGFβ signaling. *Cell Cycle* 2011; 10:285-92.
19. Ma XM, Yu H, Huai N. Peroxisome proliferator-activated receptor-gamma is essential in the pathogenesis of gastric carcinoma. *World J Gastroenterol* 2009; 15:3874-83.
20. Narayan G, Murty VV. Integrative genomic approaches in cervical cancer: implications for molecular pathogenesis. *Future Oncol* 2010; 6:1643-52.
21. Havens CG, Walter JC. Mechanism of CRL4 (Cdt2), a PCNA-dependent E3 ubiquitin ligase. *Genes Dev* 2011; 25:1568-82.
22. Chen M, Gutierrez GJ, Ronai ZA. Ubiquitin-recognition protein Ufd1 couples the endoplasmic reticulum (ER) stress response to cell cycle control. *Proc Natl Acad Sci USA* 2011; 108:9119-24.
23. Su Y, Wang F, Qi H, et al. Small interfering RNA targeting of S phase kinase-interacting protein 2 inhibits cell proliferation of pterygium fibroblasts. *Mol Vis* 2011; 17:247-56.
24. olota VG, Tzelepi VN, Leotsinidis M, et al. Histologic-type specific role of cell cycle regulators in non-small cell lung carcinoma. *J Surg Res* 2010; 164:256-65.
25. Chen G, Cheng Y, Zhang Z, et al. Cytoplasmic Skp2 expression is increased in human melanoma and correlated with patient survival. *PLoS One* 2011; 6:17578.
26. Miranda-Carboni GA, Krum SA, Yee K, et al. A functional link between Wnt signaling and SKP2-independent p27 turnover in mammary tumors. *Genes Dev* 2008; 22:3121-34.
27. Arbin AA, Greco M, Yao JL, et al. Skp2 over expression is associated with loss of BRCA2 protein in human prostate cancer. *Am J Pathol* 2011; 178:2367-76.
28. Leibl S, Regitnig P, Moinfar F. Flat epithelial atypia (DIN 1a, atypical columnar change): an underdiagnosed entity very frequently coexisting with lobular neoplasia. *Histopathology* 2007; 50:859-65.
29. Darvishian F, Singh B, Simsir A, et al. Atypia on breast core needle biopsies: reproducibility and significance. *Ann Clin Lab Sci* 2009; 39:270-6.
30. Lee TY, Macintosh RF, Rayson D, et al. Flat epithelial atypia on breast needle core biopsy: a retrospective study with clinical-pathological correlation. *Breast J* 2010; 16:377-83.