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Original article

The expression of human epididymis protein 4 and cyclin-dependent kinase inhibitor p27Kip1 in human ovarian carcinoma

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Background: Cyclin-dependent kinase inhibitor $p27^{Kip1}$ is a new class of tumor suppressor in a dosage dependent manner to control cell cycle progression. Human epididymis protein 4 (HE4) is a potentially useful biomarker for ovarian carcinoma, comparable with cancer antigen 125 in identifying women with ovarian cancer, both localized and advanced. However, the prognostic significance of $p27^{Kip1}$ and HE4 in ovarian cancer is unclear.
Objective: Investigate the expression of $p27^{Kip1}$ and HE4 in the progression of human ovarian cancer.
Material and method: Immunohistochemical analysis was performed on formalin-fixed paraffin sections of 61 specimens. The association between HE4 and $p27^{Kip1}$expression and clinicopathological features was analyzed using χ^2-test. For analysis of survival data, Kaplan-Meier curves were constructed, and the log-rank test was performed.
Results: The expression of $p27^{Kip1}$ negatively related with HE4 expression, but it correlated significantly with lymph nodes. On the other hand, HE4 expression correlated significantly with disease stage and lymph nodes. Kaplan-Meier analysis of the survival curves of $p27^{Kip1}$ and HE4 revealed a highly significant separation between low *vs.* high expressers in ovarian carcinoma.
Conclusion: Expression of HE4 was up-regulated significantly in human ovarian carcinoma. Overexpression of HE4 might be responsible for oncogenesis and development of ovarian carcinoma. HE4 and $p27^{Kip1}$ may be of prognostic significance in human ovarian cancer.

Keywords: HE4 (WFDC2), human ovarian cancer; $p27^{Kip1}$, immunohistochemistry, prognosis

Ovarian cancer is a major cause of cancer deaths among women. It is potentially curable if diagnosed when localized [1], but most ovarian cancers are diagnosed at an advanced stage when the survival rate is 20% despite aggressive surgery and chemotherapy [1]. No definitive biomarkers have been identified for early detection of ovarian cancer. The known biomarker is cancer antigen 125 (CA125) that is produced in ovarian cancer and benign conditions such as endometriosis, pregnancy, and liver disease [2].

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Human epididymis protein 4 (HE4) is a new biomarker for ovarian cancer. It is a member of the Whey AcidicProtein (WAP) domain family of proteins [3, 4]. This gene family includes the antiproteinases, secretory leukocyte protease inhibitor (SLPI) and elafin. SLPI and elafin protect against proteolytic enzymes that are released by inflammatory cells [4].

Recently, Ingegerd et al. [5] performed a blind study on sera from patients with ovarian carcinoma (late or early) or who have benign ovarian disease with sera from matched healthy controls. They also determined the CA125 levels in the sera. Their findings indicate that HE4 is a potentially useful biomarker for ovarian carcinoma, comparable with CA125, in distinguishing women with ovarian cancer, both localized and advanced, from healthy women. Thus,

HE4 may be better than CA125 in distinguishing patients with malignant ovarian disease from those with benign ovarian disease at high specificity.

Cyclin-dependent kinase inhibitor $p27^{Kip1}$ is a new class of tumor suppressor in a dosage dependent manner to control cell cycle progression [6-8]. Recently, decreased expression of $p27^{Kip1}$ has been frequently detected in human cancers [9-11], including ovarian carcinoma [12]. These studies indicated that $p27^{Kip1}$ protein levels might be associated with the development of human cancers. They appear as an important marker of cancer progression. Dysregulation of cell cycle control is implicated in the pathogenesis of most human cancers, including ovarian cancer.

The reduced expression of $p27^{Kip1}$ is reported to correlate with tumor progression and poor patient survival [9, 13]. Thus, $p27^{Kip1}$ may participate in tumor suppression by inhibiting abnormal cell-cycle progression.

Although $p27^{Kip1}$ is considered as a classic inhibited carcinoma gene, HE4 is a latest biomarker for ovarian cancer. Investigation of the two proteins in ovarian cancer may provide the biomarker for ovarian cancer, and may offer clue for detecting ovarian cancer at an early stage, and intervention target for therapy. However, the expression of HE4, $p27^{Kip1}$ and correlation of HE4 and $p27^{Kip1}$ in ovarian cancer has not been identified yet.

In the present study, we examined 61 primary heptocellular carcinoma (HCC) immuno-histochemically and determined the correlation between the levels of these proteins, and various clinical and pathological features including prognosis.

Materials and methods

This study was approved by the Ethics Committee of Nanjing Medical University. Ovarian carcinoma tissues were obtained from 61 patients at Wuxi Hospital for Maternal and Child Health Care. Informed consent was obtained from all patients.

The patients' ages ranged from 23 to 71 years (mean: 53.5 years). Twenty-six patients were positive for lymph node, 30 were positive for ascites. None of the patients received post-operative adjuvant therapy.

Tissue samples were processed immediately after surgical removal. For histological examination, all tumorous and surrounding non-tumorous tissue portions were fixed in formalin and embedded in paraffin. Histological grades were classified into three grades: well differentiated tumors (grade 1, n = 22), moderately differentiated tumors (grade 2, n = 21), and poorly differentiated tumors (grade 3, n = 18). The follow-up time was five years for 61 patients, ranging from one to 60 months.

Immunohistochemistry (IHC)

Tissue sections were cut in 4 □m thickness, placed on APES (3-Triethoxysilylpropylamine) pretreated slides, deparaffinized, rehydrated through graded alcohol, and quenched in 3% hydrogen peroxide. Antigen retrieval was performed by microwave heating at high power (750 W) in 10 mM sodium citrate buffer (pH 6.0) for three cycles of five minutes each. After blocking with normal serum for one hour at room temperature, the sections were incubated overnight at 4□C with anti-human HE4 mouse monoclonal antibody (diluted 1:100; Sigma, St. Louis, USA), anti-$p27^{Kip1}$ mouse monoclonal antibody (diluted 1:80; invitrogen). Negative control slides were processed in parallel using a nonspecific immunoglobulin IgG (Sigma, St. Louis, USA) at the same concentration as the primary antibody. All slides were processed using the peroxidase-antiperoxidase method (Dako, Hamburg, Germany). Diaminobenzidine was used as the final chromogen, and Gill's hematoxylin was used for counterstaining.

For assessment of HE4 and $p27^{Kip1}$, five high-power fields in each specimen were randomly selected, and nuclear staining was examined under high power magnification. More than 500 cells were counted to determine the mean percent, which represented the percentage of immunostained cells relative to the total number of cells [14]. In half of the samples, staining was repeated twice to avoid possible technical errors, but similar results were obtained in these samples.

Statistical analysis

Statistical analysis was performed using the Stat View 5.0 software package. The association between HE4 and $p27^{Kip1}$ expression and clinicopathological features was analyzed using χ^2-test. For analysis of survival data, Kaplan-Meier curves were constructed, and the log-rank test was performed. Multivariate analysis was performed using Cox's proportional hazards model. A p-value less 0.05 was considered statistically significant.

Results

HE4 and $p27^{Kip1}$ expression in ovarian cancer

The expression of HE4 and $p27^{Kip1}$ by IHC were

detected in 61 ovarian cancer samples. **Figure 1** shows immunochemical images to indicate HE4 and p27^{Kip1} expression in ovarian cancer. Interestingly, low expression of p27^{Kip1} was well correlated with high HE4 in the same ovarian carcinoma specimen, while high expression of p27^{Kip1} was correlated with low HE4 in the same ovarian carcinoma specimen.

Correlation of HE4 and p27^{Kip1} expression with clinicopathologic variables

The results of 61 ovarian carcinomas by IHC are summarized in **Table 1**. The mean percents of p27^{Kip1} and HE4 were 38.1%±15.1% and 27.5%±19.1%, respectively. Based on their mean percents, patients were divided into two groups: high p27^{Kip1} expressers (>38.1%) and low p27^{Kip1} expressers (27.5%) by p27^{Kip1}. Similarly, patients were divided into two groups: high HE4 expressers (>27.5%) and low HE4 expressers (<27.5%) by HE4. Interestingly, p27^{Kip1} expression correlated significantly with lymph node (p <0.05), but there was no relationship between p27^{Kip1} expression and other prognostic factors (tumor stage, Grade, residual disease and ascites). On the other hand, HE4 expression correlated significantly with disease stage and lymph node (p <0.05). No significant correlation was found between HE4 expression and other clinicopathologic variables.

Relationship between p27^{Kip1} and HE4 expression

In most ovarian cancer specimens, the proportion of p27^{Kip1}-positive tumor cells was greater than the proportion of HE4-positive tumor cells. p27^{Kip1} expression *vs*. HE4 expression are plotted in **Figure 2.** An inverse correlation between p27^{Kip1} expression and HE4 expression was observed.

Prognostic significance of p27^{Kip1}expression and HE4 expression

Survival status and clinicopathological parameters in 61 ovarian cancer specimens are summarized in **Table 2**. Concerning survival, only 6 out of 24 (25.0%) patients in the high-expresser group died of disease *vs.* 24 out of 37 (64.9%) in the low-expresser group of p27^{Kip1} expression. On the other hand, 17 out of 25 (68.0%) patients in the high-expresser group died of disease *vs.* 13 out of 36 (36.1%) in the low-expresser group of HE4 expression. When all variables were compared separately to survival status, only lymph node (p <0.05), p27^{Kip1} (p <0.05), and HE4 (p <0.05) significantly influenced the survival.

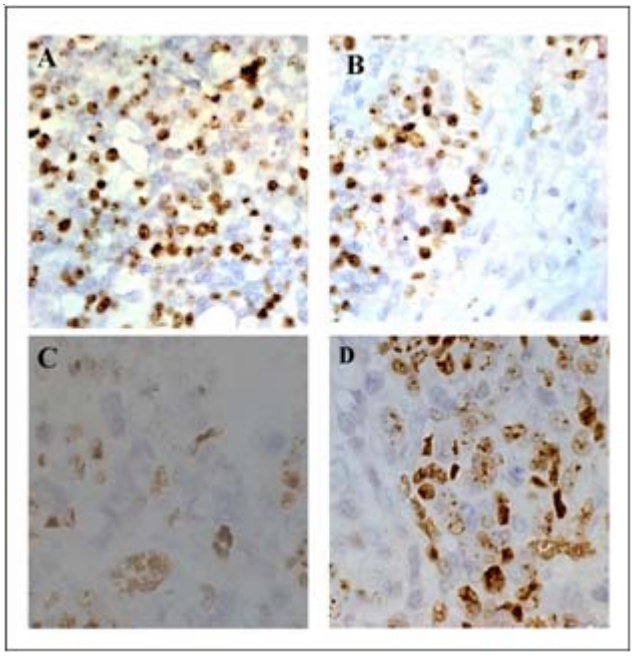

Figure 1. The expression of HE4 and p27^{Kip1} in ovarian cancer by immunochemistry. **A**: high expression of HE4, **B**: low expression of p27^{Kip1}, **C**: low expression of HE4, **D**: high expression of p27^{Kip1} (Scan Probe x400).

Table 1. HE4 0 P27^{Kip1} expression and clinic-pathological parameters in 61 ovarian cancer specimens. Statistical analyses were performed by the Pearson χ^2-test.

Parameters	Number	P27^{Kip1} Low expression (≤38.1%)	P27^{Kip1} High expression (>38.1%)	P27^{Kip1} P-value	HE4 Low expression (≤27.5%)	HE4 High expression (>27.5%)	HE4 P-value
Age (year)							
≤50	34	20	14	0.742	20	14	0.973
>50	27	17	10		16	11	
IFGO disease stage							
I, II	35	18	17	0.087	28	7	0.001*
III, IV	26	19	7		8	18	
Grade							
G1	22	15	7	0.661	14	8	0.647
G2	21	12	9		13	8	
G3	18	10	8		9	9	
Lymph node							
Negative	35	27	8	0.002*	16	19	0.014*
Positive	26	10	16		20	6	
Residual disease							
≤2 cm	31	21	10	0.858	17	14	0.500
>2 cm	30	16	14		19	11	
Ascites							
Absent	31	18	13	0.674	17	14	0.500
Present	30	19	11		19	11	

IFGO=International Federation of Gynecology and Obstertrics. *statistically significant ($p < 0.05$), compaired with other variables.

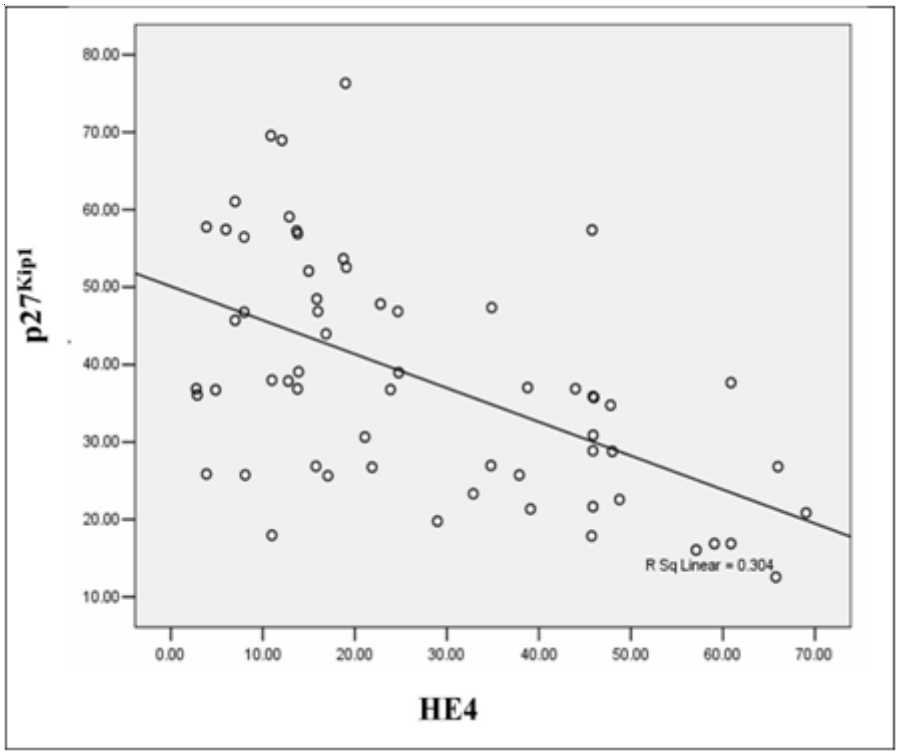

Figure 2. Relationship between p27^{Kip1} and HE4 expression in ovarian cancer. Scatterplot of p27^{Kip1} *vs.* HE4 with regression line shows a correlation of the two cell cycle regulators using Spearman's correlation coefficient ($p > 0.05$).

Table 2. Survival status and clinicopathological parameters in 61 ovarian cancer specimens. Statistical analyses were performed by the Pearson χ^2-test.

	Number	Survival status		P-value
		Alive	Dead	
Age (year)				
≤50	34	18	16	0.710
>50	27	13	14	
IFGO disease stage				
I, II	35	19	16	0.531
III, IV	26	12	14	
Grade				
G1	22	11	11	0.985
G2	21	11	10	
G3	18	9	9	
Lymph node				
Negative	35	13	22	0.013*
Positive	26	18	8	
Residual disease				
≤2 cm	31	17	14	0.500
>2 cm	30	19	11	
Ascite				
Absent	31	16	15	0.900
Present	30	15	15	
p27Kip1				
Low expression	37	13	24	0.002*
High expression	24	18	6	
HE4				
Low expression	36	23	13	0.014*
High expression	25	8	17	

IFGO=International Federation of Gynecology and Obstertrics. *statistically significant ($p < 0.05$) compaired with other variables.

Figure 3 shows the Kaplan-Meier survival curves of low *vs.* high expressers of $p27^{Kip1}$ and HE4. It is noted that low *vs.* high expressers of $p27^{Kip1}$ and HE4 are separated significantly. In univariate analysis, the Kaplan-Meier survival curves did not show any significant relationship between FIGO disease stage (p=0.545, log-rank test) or other potential prognostic factors ($p > 0.05$, log-rank test) and survival.

A multivariate Cox proportional hazard model was constructed, including age, FIGO disease stage, Grade, lymph node metastasis, residual disease, ascites, $p27^{Kip1}$, and HE4 expression. **Table 3** shows contribution of various potential prognostic factors to survival. We note that $p27^{Kip1}$ was the strongest independent predictor of survival ($p < 0.05$), the second predictor being HE4 and lymph node.

Discussion

Ovarian cancer has a high mortality rate in spite of significant advances in therapeutic strategies. Early diagnosis is the most effective means of reducing the high mortality rate associated with almost all histological types of ovarian epithelial carcinoma [1]. In the present study, an additional significant molecular distinction between $p27^{Kip1}$ and HE4 in ovarian cancer demonstrated a significantly higher expression of HE4 proteins that are critical for the ovarian cancer.

$P27^{Kip1}$, a negative regulator of the cell cycle, is a new class of tumor suppressor [15]. Reduced expression of $p27^{Kip1}$ is frequently detected in human cancers and correlate with carcinogenesis and poor survival [9]. Since $p27^{Kip1}$ inhibits cyclin-dependent kinases in a dose-dependent manner to control cell cycle progression [16], it is conceivable that decreased expression of $p27^{Kip1}$ may result in abnormal cell proliferation in these tumors. In the present study, $p27^{Kip1}$ expression correlated significantly with lymph node metastasis, but there was no relationship between

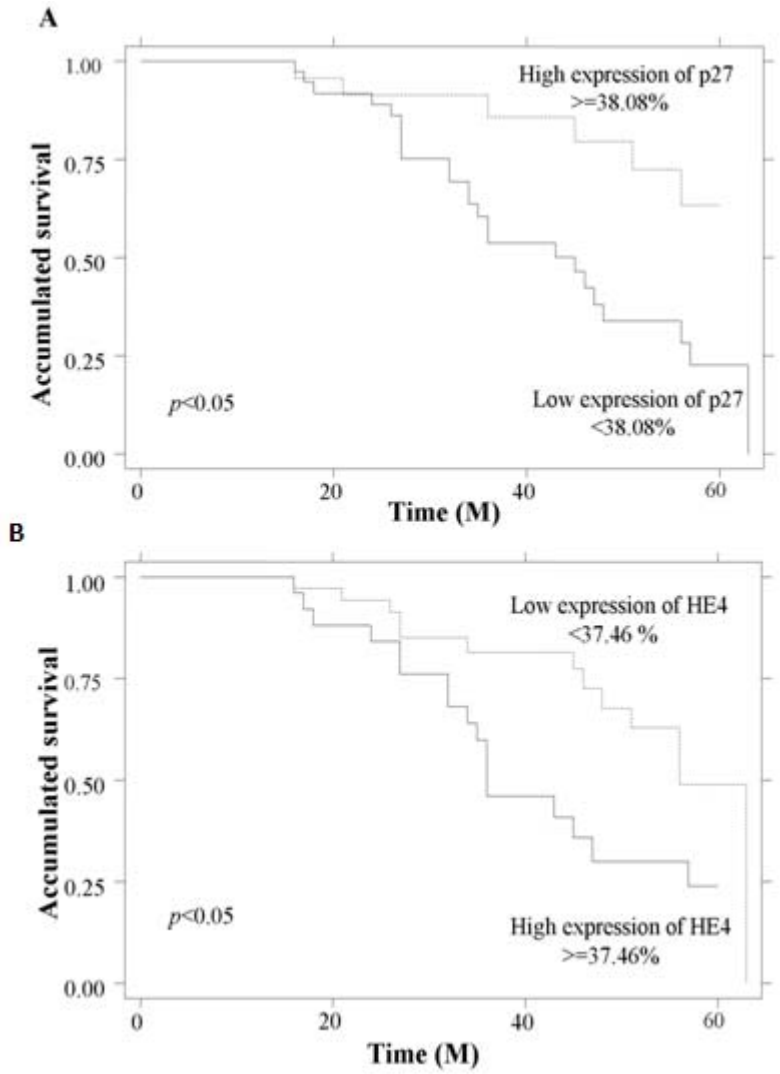

Figure 3. Accumulated survival curves according to $p27^{Kip1}$ (**A**) and HE4 (**B**)

Table 3. Contribution of various potential prognostic factors to survival by Cox regression analysis in 61 ovarian cancer specimens. Statistical analyses were performed by the Pearson χ^2-test.

	Hazard ratio	95% confidence interval	P-value
Age (year)	1.214	0.585-2.518	0.603
IFGO disease stage	1.254	0.604-2.604	0.545
Grade	0.925	0.587-1.459	0.737
Lymph node	0.389	0.172-0.882	0.017*
Residual disease	0.576	0.272-1.221	0.144
Ascite	0.893	0.430-3.773	1.854
$p27^{Kip1}$	0.311	0.126-0.767	0.005*
HE4	2.383	1.131-5.021	0.021*

*statistically significant ($p < 0.05$), compared with other variables.

p27Kip1 expression and other prognostic factors such as tumor stage, Grade, and residual disease and ascites.

Recently, Bingle et al. [17] reported that the HE4 gene is also expressed in some normal tissues, undergoing complex alternative splicing to yield multiple protein isoforms. This implies that HE4 proteins are not tumor specific, but they may still have sufficient levels of tumor selectivity to serve as putative biomarkers for ovarian carcinoma. Therefore, Bingle et al. generated mono-antibodies (MAbs) to HE4 epitopes using a method that has been successfully applied in other systems [18]. Constructions were made that encoded fusion proteins incorporating the HE4 gene fused to a gene encoding IgFc domains from (IgG) mouse or humans. Mice were immunized with HE4 fusion protein that had a mouse Ig tail, after which hybridomas were generated and their supernatants screened against the human HE4 fusion protein. Two MAbs were derived, 2H5 and 3D8, which recognize different HE4 epitopes. These findings indicate that HE4 is a potentially useful biomarker for ovarian carcinoma, comparable with CA125 in distinguishing women with ovarian cancer, both localized and advanced, from healthy women. HE4 may be better than CA125 in distinguishing patients with malignant ovarian disease from those with benign ovarian disease at high specificity. In our experiment, HE4 expression correlated significantly with disease stage and lymph node. No significant correlation was found between HE4 expression and clinicopathologic variables. In addition, there was no correlation between the expression of HE4 and clinicopathologic variables in ovarian carcinoma.

The present analysis of the relationship between HE4 and p27Kip1 suggested that low expression of p27Kip1 (B) was correlated with high HE4 in the same ovarian carcinoma specimen while high expression of p27Kip1 was correlated with low HE4 in the same ovarian carcinoma specimen. An inverse correlation between p27Kip1 expression and HE4 expression was observed as shown in **Figure 2.** Although our obtained p-value of the correlation was greater than 0.05, the potential relationship between p27Kip1 and HE4 is worthy of further study.

Cyclin-dependent kinase inhibitor p27Kip1 p27Kip1 is frequently down-regulated in human cancers. Anish et al. [19] reported the loss of p27Kip1expression correlated with poor prognosis in several tumor types, including breast and prostate cancer. Three studies support an independent prognostic role of p27Kip1 in determining the clinical outcome of patients with epithelial ovarian cancer (EOC), including serous cancer. A further study of 185 patients reported a trend (which did not reach significance) towards reduced overall survival (OS) in a small subgroup of patients whose ovarian cancers completely lacked p27Kip1 expression. In our study, we found that reduced p27Kip1 expression was a marker of poor clinical outcome (shorter OS and progression-free interval) in univariate analysis. However, this did not retain significance as an independent prognostic factor when adjusted for other clinicopathological and molecular variables of disease outcome.

p27Kip1 is maximal in quiescence, and mitogen-stimulated p27 Kip1 loss during G_0-to-S phase progression leads to cyclin E-Cdk2 activation. Several mechanisms regulate p27 Kip1 levels to control cell proliferation. p27 Kip1 translation is maximal in G_0 and falls abruptly with G_0 exit and during G_1 progression [20]. At least, four pathways regulate p27 Kip1 degradation. In late G_1 through S and into M phase, p27 Kip1 is degraded by the SCF Skp2 ubiquitin ligase, whose interaction with p27 Kip1 is activated by cyclin E-Cdk2-dependent p27 Kip1 phosphorylation at T187 [21]. In early G_1, T187-independent p27 Kip1 proteolysis occurs by both Skp2-dependent [22] and Skp2-independent [23]. In early G_1, p27 Kip1 phosphorylation at S10 promotes p27 Kip1-CRM1 binding and nuclear export [24]. Proteolysis of cytoplasmic p27 Kip1 in G1 involves KPC1 ubiquitin ligase [25]. Rapid S10-independent p27 Kip1 proteolysis may also occur in the nucleus in early G_1 [24]. In quiescent cells, p27 Kip1 proteolysis requires an intact p27 Kip1-cyclin-Cdk binding motif [26].

According to Kamura et al. [27], the degradation of p27 is regulated by two distinct mechanisms, translocation-coupled cytoplasmic ubiquitination by KPC (Kip1 ubiquitinationpromoting complex) at G1 phase and nuclear ubiquitination by Skp2 at S and G2 phases. The nuclear export of p27 by CRM1 (a carrier protein for nuclear export) [28] appears to be necessary for KPC-mediated proteolysis [27]. Since the expression p27 Kip1 is regulated by its phosphorylation, our results suggested an inverse correlation between p27Kip1 expression and HE4 expression. Phosphorylation of p27Kip1 may be regulated by HE4, but the specific mechanism requires further research.

Acknowledgments

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