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Original Article

Cullin1 is up-regulated and associated with poor patients' survival in hepatocellular carcinoma

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Abstract: Cullin1 (Cul1) is a scaffold protein of the ubiquitin E3 ligase Skp1/Cullin1/Rbx1/F-box protein complex, which ubiquitinates a broad range of proteins involved in cell-cycle progression, signal transduction, and transcription. To investigate the role of Cul1 in the development of hepatocellular carcinoma (HCC), we evaluated the Cul1 expression by immunohistochemistry using a tissue microarray (TMA) containing 90 cases HCC tissues and paired adjacent non-cancerous tissues. We analyzed the correlation between Cul1 expression and clinicopathologic variables and patients survival using two independent HCC cohorts TMA. Our data showed that Cul1 expression was apparently increased in HCC tissues compared with paired adjacent non-tumor tissues. We also demonstrated that Cul1 staining was significantly correlated with tumor size, histology grade and TNM stage. Furthermore, we showed a strong correlation between high Cul1 expression and worse 5-year overall and disease-specific survival rates in HCC patients. Finally, univariate and multivariate Cox proportional hazards regression analysis investigated that high Cul1 expression was a strong independent prognostic indicator of HCC. Our data indicated that Cul1 may be an important prognosis marker for human HCC.

Keywords: Cullin1, hepatocellular carcinoma, prognostic, tissue microarray, immunohistochemistry

Introduction

Hepatocellular carcinoma (HCC), a cancer with extremely poor prognosis, is one of the most common and aggressive solid organ tumors in many countries, particularly Asian and African countries [1]. Despite significant advances in diagnostic and therapeutic strategies, there has been little improvement in overall survival over the past few decades, owing mainly to intra-hepatic and distant tumor metastasis [2]. Therefore, further elucidation of the molecular and cellular processes involved in HCC metastasis in order to develop reliable biomarkers to predict poor patient outcome would be of significant clinical value.

Cullins are a family of hydrophobic proteins providing a scaffold for ubiquitin ligases (E3). Cullin1 (Cul1) is an essential scaffold of the Skp1-Cul1-F-box protein (SCF) E3 ubiquitin ligase complex [3], which mediates the ubiquitination of proteins involved in cell cycle progression, signal transduction and transcription [4]. Previously, we demonstrated that Cul1 regulates cell proliferation, cell-cycle control, migration, invasion, and associates with patients' prognostic in gastric cancer and breast cancer [5, 6]. Recently, studies also have revealed that Cul1 expression is elevated in the cancer tissues and associated with the poor prognosis of tumors, including melanoma [7], lung cancer [8] and glioma [9]. However, the role of Cul1 in HCC progression is still unclear.

To investigate if Cul1 is elevated in human HCC tissues, we used a tissue microarray (TMA) containing 90 cases HCC tissues and paired adjacent non-cancerous tissues. Moreover, we analyzed the correlation between Cul1 expression and clinicopathologic variables and patients survival using two independent HCC cohorts TMA.

Materials and methods

Patient specimens

Two independent HCC cohorts TMAs were utilized in this study. The training cohort TMA was

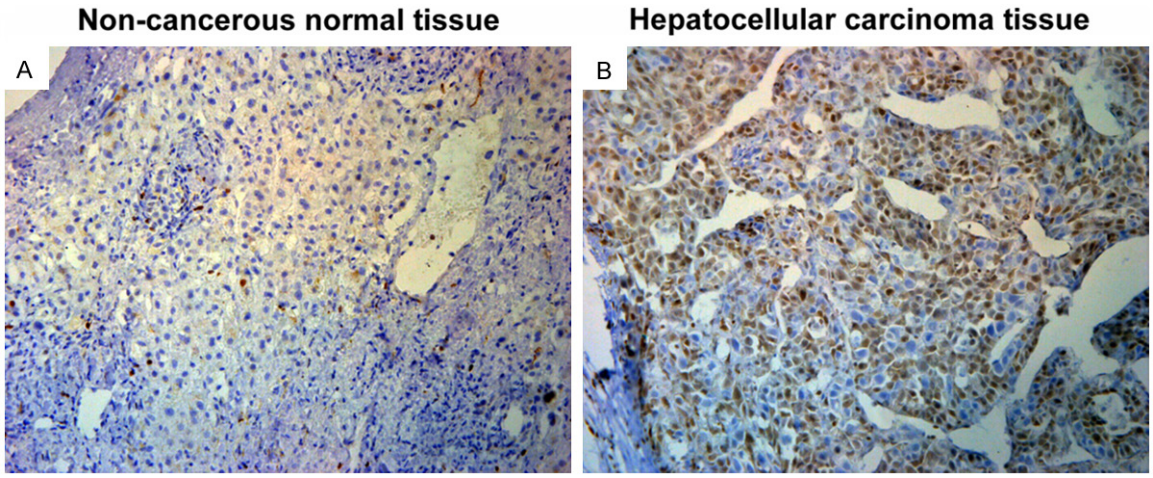

Figure 1. Representative images of Cul1 immunohistochemical staining in human HCC tissues and adjacent non-cancerous tissues. A. Negative staining of adjacent non-cancerous tissues. B. Positive staining of human HCC tissues. Magnification ×400.

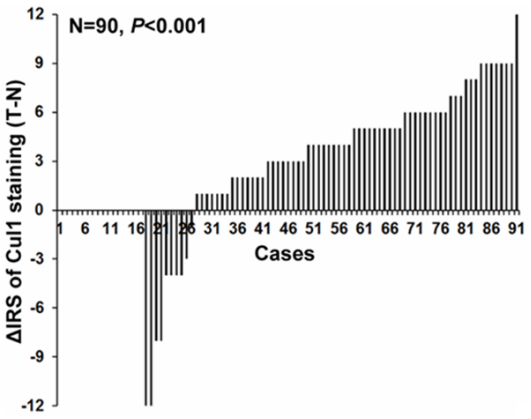

Figure 2. Expression of Cul1 is increased in HCC tissues. The distribution of the difference in Cul1 staining ($\Delta IRS=IRS_T-IRS_N$). Immunoreactivity score (IRS) of Cul1 staining was available from 90 pairs of tissues; *P* values were calculated with the Wilcoxon test. Cul1 expression was higher in tumor tissues (T) compared with paired adjacent non-tumor tissues (N).

purchased from Shanghai Xinchao Biotechnology (Shanghai, China). Pathologic grades of tumors were defined according to the WHO criteria as follows: ninety cases of HCC tissues and paired non-cancerous tissues (Grade I, II, III and IV). The array dot diameter was 1.5 mm, and each dot represented a tissue spot from one individual specimen that was selected and pathologically confirmed.

The validation cohort TMA consisted of 320 HCC surgical cases was purchased from Shanxi Chaoying Biotechnology (Xi'an, Shanxi, China). Five-year clinical follow-up results were available for 290 patients. The patients' clinicopathologic information including age at diagnosis, sex, tumor size, histology grade and TNM stage was obtained from these two companies. Ethical approval was obtained from the Children's Hospital of Xuzhou Research Ethics Committee.

Immunohistochemistry

Immunohistochemistry was performed as described before [10]. According to the streptavidin-peroxidase (Sp) method using a standard Sp Kit (Zhongshan biotech, Beijing, China). TMA slides were dewaxed at 60°C for 20 minutes followed by two 10-minute washes with xylene and then rehydrated with graded ethanol and distilled water. Endogenous peroxidases were inhibited by 3% H_2O_2 for 30 minutes. Antigen retrieval was performed in a microwave oven with 10 mM citrate buffer (pH 6.0) at 95°C for 30 minutes. After 30-minute blocking with 5% normal goat serum, the sections were incubated with monoclonal mouse anti-Cul1 antibody (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The slides were then incubated for 1 hour with a biotin-labeled secondary antibody, followed by avidin-peroxidase reagent and 3, 3'-diaminobenzidine (DAB; Zhongshan biotech, Beijing, China) substrate. After hematoxylin counterstain and Dehydration, the sections were sealed with cover slips. Negative controls were performed by Phosphate buffered saline (PBS) replaced Cul1

Table 1. Relationship between Cul1 staining and clinicopathological characteristics of the individuals in two cohorts of HCC patients

Variables	Training cohort (90 cases)			Validation cohort (320 cases)		
	Low (%)	High (%)	*P**	Low (%)	High (%)	*P**
Age						
≤48 years	21 (47.7)	23 (42.3)	0.816	61 (40.7)	89 (59.3)	0.755
>48 years	20 (43.5)	26 (46.5)		67 (39.4)	103 (60.6)	
Gender						
Male	36 (45.6)	43 (44.4)	0.849	101 (38.3)	163 (61.7)	0.638
Female	5 (45.5)	6 (44.5)		27 (48.2)	29 (51.8)	
Tumor size						
≤5 cm	26 (56.5)	20 (43.5)	0.016	86 (56.2)	67 (43.8)	0.001
>5 cm	15 (34.1)	29 (63.9)		42 (25.1)	125 (74.9)	
Histology grade						
I	26 (56.5)	20 (43.5)	0.008	81 (45.5)	97 (54.5)	0.011
II	12 (44.4)	15 (55.6)		35 (38.5)	56 (61.5)	
III	3 (17.6)	14 (82.4)		12 (23.5)	39 (76.5)	
TNM stage						
I-II	34 (58.6)	24 (41.4)	0.001	94 (50.0)	94 (50.0)	0.002
III-IV	7 (21.9)	25 (78.1)		34 (25.8)	98 (74.2)	

*Two sided Fisher's exact tests.

antibody during the primary antibody incubation.

Evaluation of immunostaining

Positive Cul1 immunostaining is defined as cytoplasmic with or without nuclear staining. We grade it according to both the intensity and percentage of cells with positive staining. The immunoreactivity was assessed by 2 pathologists simultaneously, and a consensus was reached for each core. The staining intensity of Cul1 proteins involved in our study was scored 0 to 3 (0=negative; 1=weak; 2=moderate; 3=strong). The percentage of protein-positive stained cells was also scored into 4 categories: 1 (0-25%), 2 (26%-50%), 3 (51%-75%), and 4 (76%-100%). The level of relevant proteins staining was evaluated by immunoreactive score (IRS) [11], which is calculated by multiplying the scores of staining intensity and the percentage of positive cells. Based on the IRS, staining pattern was defined as negative (IRS: 0), weak (IRS: 1-3), moderate (IRS: 4-6), and strong (IRS: 8-12).

Statistical analysis

For TMA, statistical analysis was performed with SPSS 20.0 software (SPSS, Inc, Chicago, IL). Differences in IRS for Cul1 staining in primary tumors and their paired adjacent normal tissues were assessed by the Wilcoxon test (grouped). The association between Cul1 staining and the clinicopathologic parameters of the HCC patients, including age at diagnosis, sex, tumor size, histology grade and TNM stage, was evaluated by two sided Fisher's exact tests. The Kaplan-Meier method and log-rank test were used to evaluate the correlation between Cul1 expression and patient survival. Cox regression model was used for multivariate analysis. Differences were considered significant when $P<0.05$.

Results

Cul1 expression is increased in human HCC

In order to determine whether Cul1 expression is changed in human HCC. Immunohistochemistry staining was utilized in TMA slides to evaluate the Cul1 expression in HCC tissues and paired adjacent non-cancerous tissues (**Figure 1**). In training cohort TMA slides containing 90 cases HCC tissues with paired adjacent non-cancerous tissues, we observed that a significantly higher expression of Cul1 in tumor tissues compared with paired adjacent non-tumor tissues ($P<0.001$, **Figure 2**).

Increased Cul1 expression correlates with clinicopathological parameters

The clinicopathologic characteristics of the training cohort and the validation cohort of HCC biopsies were summarized in **Table 1**. Samples with IRS 0-3 and IRS 4-12 were classified as low and high expression of Cul1. As shown in **Table 1**, two sided Fisher's exact analysis revealed that Cul1 expression in the carcinoma tissues of the training cohort conspicuously correlated with some clinicopathological features, such as tumor size ($P=0.016$), histology grade ($P=0.008$), and TNM stage ($P=0.001$). These findings were confirmed in the validation

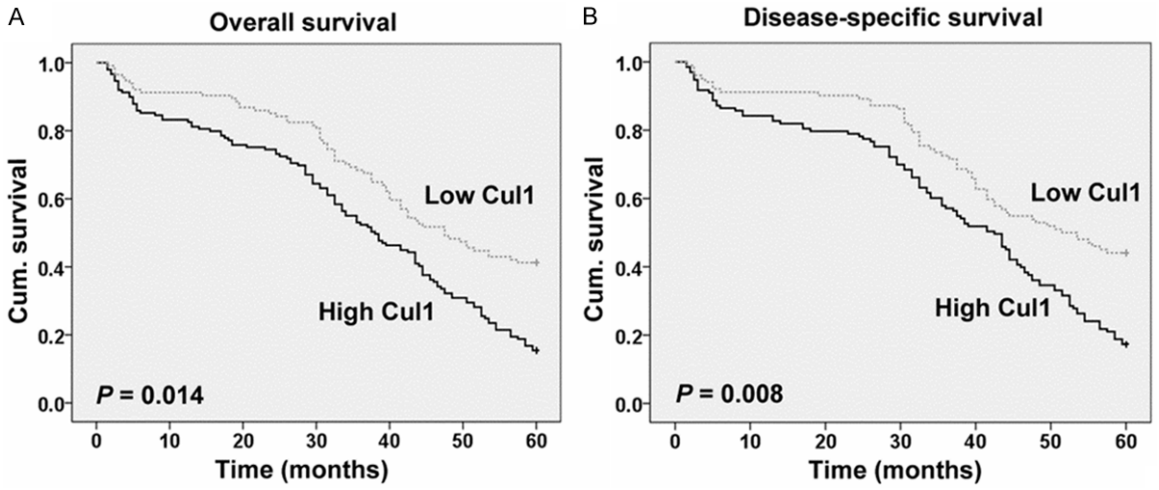

Figure 3. Kaplan-Meier survival analyses of HCC patients. A. High Cul1 expression correlates with a poorer 5-year overall survival (*P*=0.014, log-rank test). B. High Cul1 expression correlates with a poorer 5-year disease-specific survival (*P*=0.008, log-rank test). Cum, cumulative.

Table 2. Univariate Cox proportional regression analysis on 5-year overall and disease-specific survival of 290 HCC patients

Variable*	Overall survival			Disease-specific survival		
	Hazard ratio	95% CI†	*P**	Hazard ratio	95% CI†	*P**
Cul1						
Low	1.000		0.009	1.000		0.008
High	1.940	0.949-2.839		2.004	1.217-3.475	
Age						
≤48 years	1.000		0.705	1.000		0.877
>48 years	1.084	0.713-1.647		1.040	0.633-1.747	
Gender						
Male	1.000		0.186	1.000		0.178
Female	1.430	0.847-2.425		1.461	0.946-2.852	
Tumor size						
≤5 cm	1.000		0.009	1.000		0.010
>5 cm	2.743	1.690-4.431		2.740	1.601-4.559	
Histology grade						
I	1.000		0.003	1.000		0.002
II-III	2.413	1.464-3.975		2.460	1.208-3.995	
TNM stage						
I-II	1.000		0.000	1.000		0.000
III-IV	4.414	2.731-7.096		4.465	2.678-7.470	

*P values are from Log-rank test. †CI: confidence interval.

cohort of HCC patients (**Table 1**). However, we did not find significant correlation between Cul1 expression with other clinicopathologic features in both training cohort and validation cohort, including age and gender.

Increased Cul1 expression correlates with poor patient survival

To further study whether increased Cul1 staining in HCC patients correlates with a worse prognosis, Kaplan-Meier survival curves were constructed using 5-year overall or disease-specific cumulative survival to compare the patients with high Cul1 staining to those with low Cul1 staining (n=290, follow-up time, 60 months). Our data revealed that high Cul1 staining correlated with both worse overall and disease-specific survival in HCC (*P*=0.014 and *P*=0.008, respectively, log-rank test; **Figure 3**). The 5-year overall cumulative survival rate dropped from 41.2% in patients with low Cul1 expression to 15.4% in those with high Cul1 expression, and the 5-year disease-specific cumulative survival rate dropped from 44.1% in patients with low Cul1 expression to 17.3% in those with high Cul1 expression.

Table 3. Multivariate Cox regression analysis on 5-year overall and disease-specific survival of 290 HCC patients

Variable*	Overall survival			Disease-specific survival		
	Hazard ratio	95% CI†	*P*	Hazard ratio	95% CI	*P*
Cul1	1.310	0.975-1.500	0.013	1.327	1.032-1.708	0.008
Age	0.894	0.733-1.092	0.272	0.943	0.745-1.192	0.622
Gender	0.998	0.779-1.254	0.923	1.201	0.912-1.583	0.193
Tumor size	1.471	1.192-1.815	0.000	1.675	1.313-2.138	0.000
Histology grade	1.695	1.190-2.416	0.003	2.291	1.511-3.475	0.000
TNM stage	2.815	2.185-3.660	0.000	2.618	0.880-5.338	0.000

*Coding of variables: Cul1 was coded as 1 (low), and 2 (high). Age was coded as 1 (≤48 years), and 2 (>48 years). Gender was coded as 1 (male), and 2 (female). Tumor size was coded as 1 (≤5 cm), and 2 (>5 cm). Histology grade was coded as 1 (I), and 2 (II-III). TNM stage was coded as 1 (I-II), and 2 (III-IV). †CI: confidence interval.

Cul1 serves as an independent molecular prognostic indicator for HCC

Moreover, we examined whether Cul1 expression was an independent prognostic factor for HCC. We performed a univariate Cox regression analysis including Cul1 expression, age, gender, tumor size, histology grade and TNM stage to study the effects of Cul1 on patient survival in HCC. The univariate Cox regression analysis showed that Cul1 expression was an independent prognostic marker for HCC patients overall survival (hazard ratio, 1.940; 95% CI, 0.949-2.839; *P*=0.009; **Table 2**), and disease-specific survival (hazard ratio, 2.004; 95% CI, 1.217-3.475; *P*=0.008; **Table 2**). In multivariate Cox regression analysis, we found that Cul1 expression was also an independent prognostic marker for 5-year overall survival (hazard ratio, 1.310; 95% CI, 0.975-1.500; *P*=0.013; **Table 3**) and disease-specific survival (hazard ratio, 1.327; 95% CI, 1.032-1.708; *P*=0.008; **Table 3**).

Discussion

Many recent studies have demonstrated that Cul1 overexpression is associated with various malignant tumors, like gastric cancer [5], breast cancer [6], non-small cell lung cancer [8], malignant melanoma [7] and glioma [9]. In particular, high Cullin1 expression was significantly correlated with worse survival in gastric carcinoma [5], breast cancer [6] and non-small cell lung cancer patients [8]. However, the Cul1 expression status and its correlation with the clinicopathological features in HCC have never been investigated.

In the present study, two independent HCC cohorts TMA were studied. Our data showed that Cul1 expression was apparently increased in HCC tissues compared with paired adjacent non-tumor tissues (**Figure 2**). We also demonstrated that high Cul1 staining was significantly correlated with tumor size, histology grade and TNM stage (**Table 1**). And Kaplan-Meier survival analysis revealed that high Cul1 staining correlated with both worse overall and disease-specific survival in HCC patients (**Figure 3**). Moreover, univariate and multivariate Cox proportional hazards regression analysis investigated that high Cul1 expression was a strong independent prognostic indicator of HCC (**Tables 2**, **3**).

In these two independent HCC cohorts TMA, we both found that high Cul1 expression was positively correlated with much larger tumor size, suggesting that increased Cul1 expression was involved in HCC tumor growth. Studies have reported that during cell-cycle regulatory processes, cyclin-CDK complexes positively drive progression of the cell cycle, whereas by binding to and inactivating cyclin-CDKs, Cip/Kip proteins, including p21 and p27, negatively regulate progression through the cell cycle [12-14]. The SCF E3 ubiquitin ligase complex is involved in the proteasomal degradation of numerous proteins regulating cell cycle progression and cumulative evidences show the alterations in the ubiquitylation of cell cycle regulators in the aetiology of many human malignancies [4, 15, 16]. Previous results indicated that Cul1 positively regulated the expression of Cyclin A, Cyclin D and Cyclin E, whereas negatively associated with the p21 and p27 expression in gastric cancer [5], breast cancer [6], melanoma

[17] and glioma [9]. So we propose that Cul1 may regulate HCC tumor growth by the same pathway.

Our TMA results also showed that patients with high Cul1 expression had a worse histology grade and TNM stage. These results suggested that Cul1 expression was involved in HCC invasion and metastasis. We previous reported that Cul1 regulated cell migration and invasion via TIMP-MMP pathway in breast cancer and glioma [6, 9]. And a number of studies have shown that MMP-2 and MMP-9 proteolytically degrade ECM components to allow tumor cells metastasis [18], whereas TIMP has the inhibitory role against MMP to suppress the cancer cell metastasis [19, 20]. Here, we think Cul1 may also positively regulated HCC migration and invasion through the TIMP-MMP pathway, which finally results in the HCC lymph node metastasis and distance metastasis.

In conclusion, our findings clearly indicated that Cul1 is involved in the progression of HCC and high Cul1 expression is associated with poor prognosis in HCC patients. These results suggested that Cul1 may serve as a molecular prognostic marker and potential therapeutic target for this aggressive disease.

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Disclosure of conflict of interest

None.

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References

[1] Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D. Global cancer statistics. CA Cancer J Clin 2011; 61: 69-90.

[2] Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Häussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J; SHARP Investigators Study Group. Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 2008; 359: 378-390.

[3] Zheng N, Schulman BA, Song L, Miller JJ, Jeffrey PD, Wang P, Chu C, Koepp DM, Elledge SJ, Pagano M, Conaway RC, Conaway JW, Harper JW and Pavletich NP. Structure of the Cul1-Rbx1-Skp1-F boxSkp2 SCF ubiquitin ligase complex. Nature 2002; 416: 703-709.

[4] Nakayama KI and Nakayama K. Ubiquitin ligases: cell-cycle control and cancer. Nat Rev Cancer 2006; 6: 369-381.

[5] Bai J, Zhou Y, Chen G, Zeng J, Ding J, Tan Y, Zhou J and Li G. Overexpression of Cullin1 is associated with poor prognosis of patients with gastric cancer. Hum Pathol 2011; 42: 375-383.

[6] Bai J, Yong HM, Chen FF, Mei PJ, Liu H, Li C, Pan ZQ, Wu YP and Zheng JN. Cullin1 is a novel marker of poor prognosis and a potential therapeutic target in human breast cancer. Ann Oncol 2013; 24: 2016-2022.

[7] Chen G, Cheng Y, Martinka M and Li G. Cul1 expression is increased in early stages of human melanoma. Pigment Cell Melanoma Res 2010; 23: 572-574.

[8] Xu M, Yang X, Zhao J, Zhang J, Zhang S, Huang H, Liu Y and Liu J. High expression of Cullin1 indicates poor prognosis for NSCLC patients. Pathol Res Pract 2014; 210: 397-401.

[9] Fan YC, Zhu YS, Mei PJ, Sun SG, Zhang H, Chen HF, Chen C and Miao FA. Cullin1 regulates proliferation, migration and invasion of glioma cells. Med Oncol 2014; 31: 227.

[10] Bai J, Yong HM, Chen FF, Song WB, Li C, Liu H and Zheng JN. RUNX3 is a prognostic marker and potential therapeutic target in human breast cancer. J Cancer Res Clin Oncol 2013; 139: 1813-1823.

[11] Remmele W and Stegner HE. [Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue]. Pathologe 1987; 8: 138-140.

[12] Denicourt C and Dowdy SF. Cip/Kip proteins: more than just CDKs inhibitors. Genes Dev 2004; 18: 851-855.

[13] Guardavaccaro D and Pagano M. Stabilizers and destabilizers controlling cell cycle oscillators. Mol Cell 2006; 22: 1-4.

[14] Salon C, Brambilla E, Brambilla C, Lantuejoul S, Gazzeri S and Eymin B. Altered pattern of Cul-1 protein expression and neddylation in human lung tumours: relationships with

CAND1 and cyclin E protein levels. J Pathol 2007; 213: 303-310.
[15] Johansson P, Jeffery J, Al-Ejeh F, Schulz RB, Callen DF, Kumar R and Khanna KK. SCF-FBXO31 E3 ligase targets DNA replication factor Cdt1 for proteolysis in the G2 phase of cell cycle to prevent re-replication. J Biol Chem 2014; 289: 18514-18525.
[16] Sarikas A, Hartmann T and Pan ZQ. The cullin protein family. Genome Biol 2011; 12: 220.
[17] Chen G and Li G. Increased Cul1 expression promotes melanoma cell proliferation through regulating p27 expression. Int J Oncol 2010; 37: 1339-1344.
[18] Gupta GP and Massague J. Cancer metastasis: building a framework. Cell 2006; 127: 679-695.
[19] Chaudhary AK, Pandya S, Ghosh K and Nadkarni A. Matrix metalloproteinase and its drug targets therapy in solid and hematological malignancies: an overview. Mutat Res 2013; 753: 7-23.
[20] Libra M, Scalisi A, Vella N, Clementi S, Sorio R, Stivala F, Spandidos DA and Mazzarino C. Uterine cervical carcinoma: role of matrix metalloproteinases (review). Int J Oncol 2009; 34: 897-903.