Cyclin-Dependent Kinase 1 Gene Expression Is Associated with Poor Prognosis in Gastric Carcinoma

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ABSTRACT

Purpose: **A low expression level of cyclin-dependent kinase (Cdk) inhibitor p27 is associated with high aggressiveness and poor prognosis of various carcinomas. Human *Cdk subunit 1* (*Cks1*), as well as *S-phase kinase-associated protein 2* (*Skp2*), is an essential and specific factor in the p27 proteolysis by SCF^{Skp2} ubiquitin ligase. The purpose of this study is to clarify the clinical significance of Cks1 expression and the relationship between Cks1 and p27 expression in gastric carcinomas.**

Experimental Design: **We measured Cks1 expression using quantitative reverse transcription-PCR in 76 human gastric carcinomas and p27 expression using immunohistochemistry in 28 cases. Moreover, we established Cks1- and/or Skp2-transfected gastric carcinoma cell lines and assessed the relationship between Cks1, Skp2, and p27 expression using quantitative reverse transcription-PCR and Western blot analysis.**

Results: **Cks1 high expression was correlated with poor prognosis ($P < 0.05$) and Cks1 expression was inversely correlated with the expression level of p27 protein in gastric carcinomas ($P < 0.05$). Using combined Skp2 data [T-a. Masuda, Cancer Res., *62:* 3819–3825, 2002], 88.9% of the Cks1/Skp2 double-high cases expressed a low level of p27 protein and showed the poorest prognosis ($P < 0.05$). Western blot analysis showed that Cks1/Skp2-cotransfected cells expressed a much lower level of p27 protein than the controls.**

Conclusions: **These findings indicate that Cks1, as well as Skp2, regulates the expression level of p27 protein in gastric carcinomas. Cks1 could play an important role in gastric carcinoma progression and would be a novel target for the treatment of gastric carcinomas as well as a strong prognostic marker.**

INTRODUCTION

Many basic and clinical studies have indicated that low levels of p27 are associated with high aggressiveness and poor prognosis in a large variety of malignant tumors (1). We previously reported that p27 expression status was an independent prognostic factor for patients with gastric carcinomas (2). p27 is an inhibitor of the protein kinases Cdk2[4]/cyclin E and Cdk2/cyclin A, which drive cells from the G_1 to S phase in the cell cycle (1), and is mainly regulated in ubiquitin-mediated proteolysis with SCF-type ubiquitin ligase complex (3).

It is widely known that genetic or epigenetic changes in the *p27* gene are rare in carcinomas (4). One main mechanism responsible for the decreased level of p27 protein in carcinomas is the increased expression of SCF^{Skp2}, especially Skp2 (5–9), which is a substrate-specific receptor of p27 (10). We also reported that Skp2 expression was correlated inversely with p27 expression and could be a prognostic factor in gastric carcinomas, and furthermore, Skp2 can modulate the malignant phenotype of gastric carcinomas possibly via p27 proteolysis (11). However, the reduced expression of p27 seems to be not necessarily caused by Skp2 overexpression in carcinomas (6, 9, 11).

Recently, human Cks1 has been identified as an essential and specific cofactor in the ubiquitination and degradation of *p27* by SCF^{Skp2} (12, 13). Cks proteins were originally identified as subunits that interacted tightly with cyclin-Cdk complexes (14, 15). Human Cks1 binds to Skp2 and greatly increases the binding of threonine 187-phosphorylated p27 to Skp2 (12). We hypothesized that Cks1 might be involved in p27 degradation in gastric carcinoma cases with low expression levels of both Skp2 and p27.

We therefore examined the relationship between Cks1 and p27 expression *in vivo* and clarified the clinical significance of Cks1 expression in human gastric carcinomas. We next established Cks1- or Skp2-transfected and Cks1/Skp2-cotransfected human gastric carcinoma cell lines and examined the relationship between Cks1, Skp2, and p27 expression *in vitro*.

MATERIALS AND METHODS

Clinical Samples. Fresh surgical specimens were obtained from 76 patients with both primary gastric carcinoma tissues (T), and their paired adjacent normal gastric mucosa (N) after written informed consent was obtained. The patients had

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[4] The abbreviations used are: Cdk, cyclin-dependent kinase; RT-PCR, reverse transcription-polymerase chain reaction; Cks1, Cdk subunit 1; SCF, Skp1-Cullin-F-box protein; Skp, S-phase kinase associated protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

undergone surgery at the Department of Molecular and Surgical Oncology, Medical Institute of Bioregulation, Kyushu University (Beppu, Japan) from 1993 to 2000. None of these patients received preoperative treatment such as radiation or chemotherapy. Of the 76 patients, 50 received postoperative therapy (*i.e.*, chemotherapy). Data concerning patient outcome, including overall survival and development of metastasis, were available for all 76 patients, and the observation period ranged from 3 to 77 months (the median follow-up period was 36.6 months). Of the 76 patients, 30 died of gastric carcinoma. Paraffin blocks were available from 28 cases and were analyzed by immunohistochemistry. All 76 cases were analyzed with quantitative RT-PCR.

Cell Culture. The two different human gastric carcinoma cell lines, AZ521 and MKN28, were obtained from Cell Resource Center for Biomedical Research Institute of Develop-

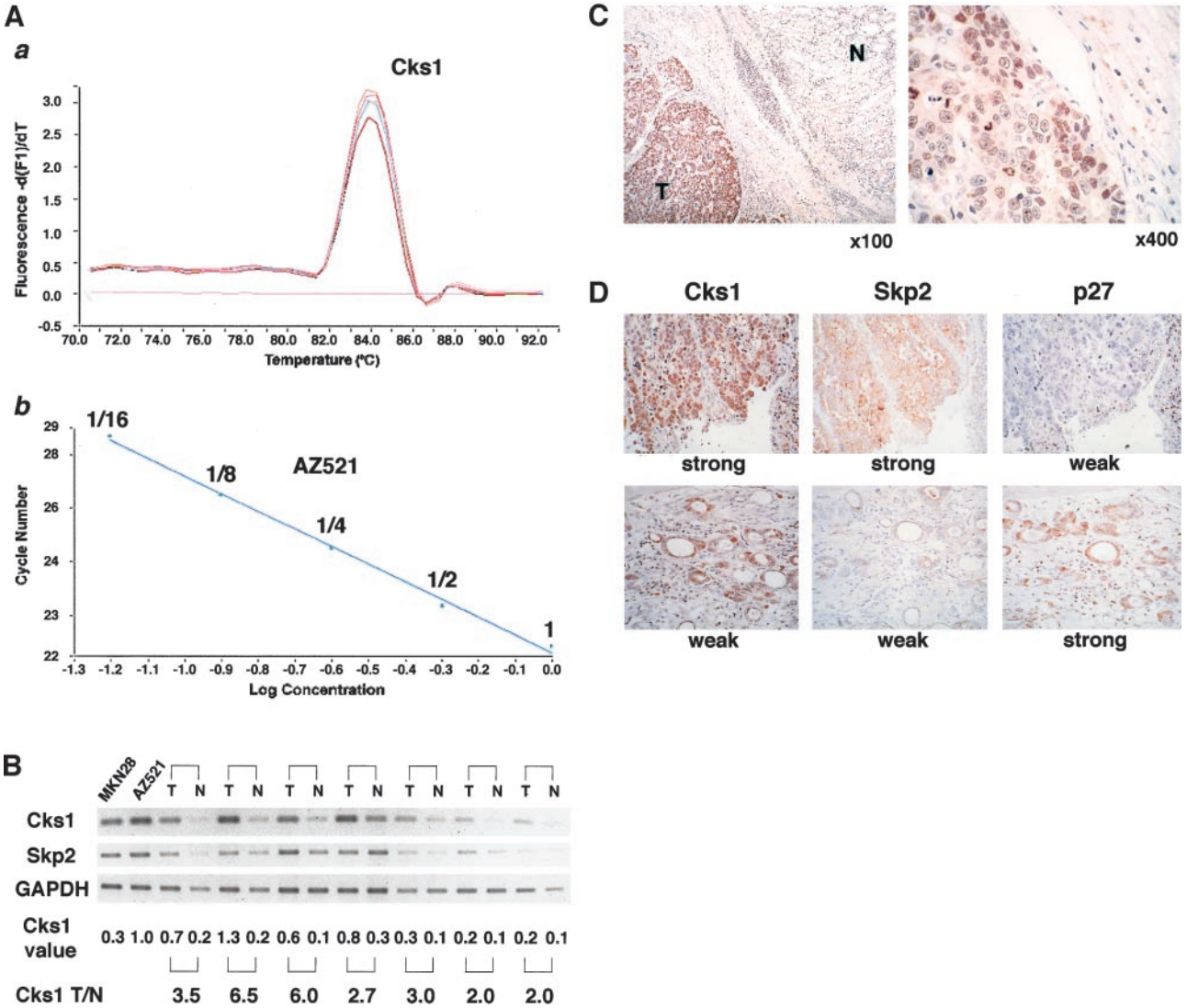

Fig. 1 Cks1, Skp2, and p27 expression in human gastric carcinomas. *A*, quantification of Cks1 mRNA expression by quantitative RT-PCR analysis with LightCycler. *a*, melting curves for PCR products after Cks1 amplification with gastric carcinoma cell line, AZ521. AZ521 is a positive control for this study. Only one sharp peak for each sample was observed. This indicates that only one specific product was amplified. Similar results were obtained when PCR products of GAPDH were analyzed. *b*, quantitative RT-PCR standard curve for Cks1. The standard curve with AZ521 was obtained from cycle number plotted against the log of copy number. Similar results were obtained when PCR products of GAPDH were analyzed. *B*, seven representative cases of quantitative RT-PCR analysis for Cks1 and Skp2 mRNA expression in human gastric carcinomas. The Cks1 value is an expression level of its mRNA normalized by GAPDH. For visualization, PCR products were separated by electrophoresis on a 2.0% agarose gel and stained with ethidium bromide. MKN28 and AZ521, gastric carcinoma cell lines; T, gastric carcinoma tissue; N, normal tissue; GAPDH, internal control. *C*, Cks1 immunostaining in gastric carcinoma tissues. T, gastric carcinoma tissue; N, normal tissue. Original magnification: left, ×100; right, ×400. *D*, the relationship between Cks1/Skp2 and p27 expression in gastric carcinoma tissues. *Top:* case 1, strong Cks1 and Skp2 immunostaining and weak p27 immunostaining. *Bottom:* case 2, weak Cks1 and Skp2 immunostaining and strong p27 immunostaining. Original magnification: ×200.

ment, Aging and Cancer, Tohoku University, and maintained in RPMI 1640 supplemented with 10% fetal bovine serum at 37°C in a 5% humidified CO_2 atmosphere.

Antibodies. Rabbit polyclonal antibody to Cks1 and mouse monoclonal antibodies to Skp2 and p27 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), Zymed Laboratories (San Francisco, CA), and Transduction Laboratories (Lexington, KY), respectively.

Quantitative RT-PCR Analysis. Frozen tissue specimens or cultured cell lines in a state of subconfluency were homogenized, the total RNA was extracted using the modified acid-guanidine-phenol-chloroform method, and reverse transcriptase reaction was performed. The PCR amplification for quantification of Cks1, Skp2, p27, and GAPDH mRNA in tissues and cell lines was performed in the LightCycler using the LightCycler-FastStart DNA Master Sybr Green I kit (Roche Diagnostics). The amplification conditions of 40 cycles consisted of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and elongation at 72°C for 20 s. After the amplification, the products were subjected to a temperature gradient from 68°C to 95°C at 0.2°C/s with continuous fluorescence monitoring to produce a melting curve of the products. After proportional background adjustment, the fit point method was used to determine the cycle in which the log-linear signal was distinguished from the background and that cycle number was used as a crossing-point value. The standard curve was produced by measuring the crossing point of each standard value (2-fold serially diluted cDNAs of AZ521) and plotting them against the logarithmic value of concentrations. The concentrations of each sample were then calculated by setting their crossing points to the standard curve. The expression levels were normalized by GAPDH. We classified the cases into two groups; a Cks1 high expression group (T/N $<$ 1; $n = 41$) and a Cks1 low expression group (T/N $\leqq$ 1; $n = 35$). The expression level of Skp2 mRNA was classified into two groups; a high group ($n = 50$) and a low group ($n = 26$) according to the criteria described previously (11). The following primers were used (all 5′ to 3′ direction): Cks1 (179 bp) sense primer AGCAAACCGAGCGATCATGTC and antisense primer CCCATCCCTGACTCTGCTGAA; Skp2 (292 bp) sense primer GCTGCTAAAGGTCTCTGGTGT and antisense primer AGGCTTAGATTCTGCAACTTG; p27 (225 bp) sense primer ACGGGAGCCCTAGCCTGGAGC and antisense primer TGCCCTTCTCCACCTCTTGCC; and GAPDH (249 bp) sense primer TTGGTATCGTGGAAGGACTCA and antisense primer TGTCATCATATTTGGCAGGTT.

Table 1 Correlation between Cks1 and p27 expression in gastric carcinomas[a]

A. Relationship between Cks1 mRNA and p27 protein expression

	Cks1 mRNA	
p27 protein	Low	High
Low	3	12
High	8	5
Low group ratio (%)	27.3	70.6

B. Relationship between Cks1/Skp2 mRNA and p27 protein expression

	Cks1 and Skp2	Cks1 or Skp2	Cks1 and Skp2
p27 protein	Double-low	High	Double-high
Low	1	6	8
High	6	6	1
Low group ratio (%)	14.3	50	88.9

[a] $P < 0.05$, Fisher's exact test.

Table 2 Cks1 mRNA expression and clinicopathological factors in gastric carcinomas

Factors	Cks1 Low ($n = 35$)	Cks1 High ($n = 41$)	P[a]
Age (yr)	67.4 ± 2.0	63.5 ± 1.6	N.S.[b]
Sex			N.S.
Female	14	13	
Male	21	28	
Histology			N.S.
Differentiated type	17	22	
Undifferentiated type	18	19	
Serosal invasion			N.S.
Absent	22	25	
Present	13	16	
Lymph node metastasis			N.S.
Absent	17	11	
Present	18	30	
Lymphatic invasion			N.S.
Absent	14	11	
Present	21	30	
Vascular invasion			N.S.
Absent	29	33	
Present	6	8	
Postoperative therapy			N.S.
Absent	15	11	
Present	20	30	
Skp2 mRNA expression[c]			N.S.
Low	13	13	
High	22	28	

[a] Correlation was analyzed by Fisher's exact test.
[b] N.S., not significant.
[c] Data from our previous study (11).

Immunohistochemistry. Immunohistochemical studies of Cks1, Skp2 and p27 were performed on specimens available from 28 gastric carcinoma cases using the avidin-biotin-peroxidase method (LSAB2 kit; Dako, Kyoto, Japan) on formalin-fixed, paraffin-embedded tissues. All sections were counterstained with hematoxylin. The primary antibodies against Cks1, Skp2, and p27 were used at dilutions of 1:50, 1:500, and 1:1000, respectively.

p27 scores were measured by observing 1000 cancer cells in at least five high-power fields and were classified as high (staining in $>$50% of cells) or low (staining in $\leqq$50% of cells) as described previously (2). The scoring was done independently by two observers (T. M. and Y. Y.).

Western Blot Analysis. Total protein was extracted from samples with radioimmunoprecipitation assay buffer. Aliquots of total protein were applied to 15% acrylamide gradient gels. After electrophoresis, the samples were electroblotted onto a polyvinylidene membrane (Immobilon; Millipore, Inc., Bed-

ford, MA) at 0.5 A for 30 min at 4°C. Cks1, Skp2, and p27 were detected using the antibodies at dilutions of 1:500, 1:2000, and 1:2500, respectively. The blots were developed with horseradish peroxidase-linked antirabbit or antimouse immunoglobulin (Promega, Inc., Madison, WI). Signals were detected using Supersignal (Pierce, Inc., Rockford, IL).

Transfection Assays. We previously established stably Skp2-transfected gastric carcinoma cell lines (parent cell lines: AZ521 and MKN28; Ref. 11). Human Cks1 cDNA was generated by RT-PCR and subcloned into a pcDNA3.1Hygro+ expression vector (Invitrogen, Carlsbad, CA) and then transfected into the Skp2-transfected cells and the mock cells using Lipofectamine (Life Technologies, Inc., Tokyo, Japan). Mock vector-transfected clones of each cell line were used for the control.

Statistical Analysis. Associations between the variables were tested by the Fisher's exact test. Survival curves were drawn according to the Kaplan-Meier method, and the survival analysis was carried out by the Mantel-Cox test when two curves were being compared or the Tarone-Ware test when three curves were being compared. Multivariate analysis to determine the patient prognosis was performed with Cox regression analysis with the forward stepwise model. All differences were deemed significant at the level of $P < 0.05$. The histological type of gastric carcinomas was classified on the basis of the criteria set by the Japanese Society for Cancer of the Stomach (16).

RESULTS

Cks1 Expression in Gastric Carcinoma Tissues. The gastric carcinoma tissue (T) and normal mucosa (N) showed variable levels of Cks1 and Skp2 mRNA signals by quantitative RT-PCR (Fig. 1, *A* and *B*). This analysis revealed that the expression of Cks1 mRNA (Cks1 value) was greater in T (0.1–1.3; median, 0.2) than in N (0.0–1.0; median, 0.1) in 41 of the 76 cases (53.9%). No correlation in mRNA expression level was found between Cks1 and Skp2 in these cases (data not shown). Cks1 T/N ranged from 0.1 to 5.8 (median, 1.1).

Immunohistochemical analysis revealed that Cks1 was predominantly expressed in the gastric carcinoma cells (Fig. 1*C*).

Correlation between Cks1 and p27 Expression in Gastric Carcinomas. As shown in Table 1*A*, 12 (70.6%) of 17 Cks1 high cases showed a low expression level of p27 protein and 8 (72.7%) of 11 Cks1 low cases showed a high expression level of p27 protein. Thus, Cks1 mRNA expression was inversely correlated with the expression level of p27 protein in gastric carcinomas ($P < 0.05$). Next, we classified these 28 cases into three groups: Cks1/Skp2 double-high group ($n = 9$); Cks1 high and Skp2 low or Cks1 low and Skp2 high group ($n = 12$); and Cks1/Skp2 double-low group ($n = 7$), and examined the correlation with the expression level of p27 protein. As shown in Table 1*B*, 8 (88.9%) of 9 in the Cks1/Skp2 double-high group showed low expression level of p27 protein, and 6 (85.7%) of 7 in the Cks1/Skp2 double-low group showed a high expression level of p27 protein.

In gastric carcinoma tissues, cells positive for Cks1 and Skp2 showed no or very low p27 protein expression and *vice versa*, implying that there was an inverse relationship between the expression profiles of Cks1, Skp2 and p27 proteins (Fig. 1*D*).

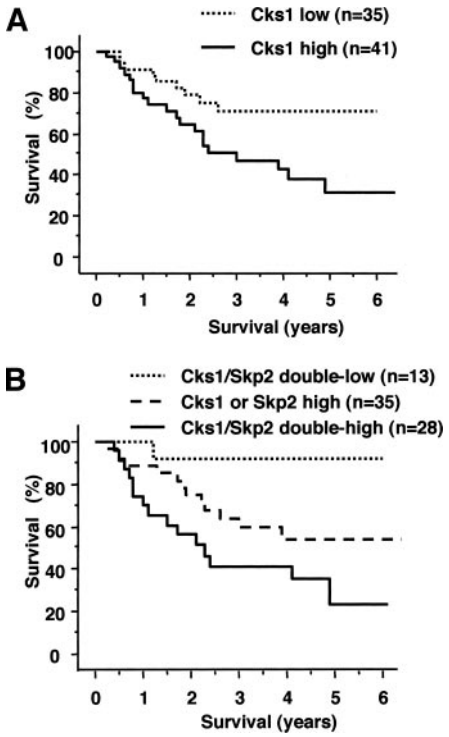

Fig. 2 Overall survival of patients with gastric carcinomas according to the Cks1 or Cks1/Skp2 mRNA expression. Skp2 data are quoted from our previous data (11). *A*, Cks1 mRNA high expression group ($n = 41$). Cks1 mRNA low expression group ($n = 35$). $P = 0.018$; Mantel-Cox test. *B*, these 76 cases with gastric carcinoma were classified into three groups: Cks1/Skp2 double-low group ($n = 13$); Cks1 high and Skp2 low or Cks1 low and Skp2 high group ($n = 35$); and Cks1/Skp2 double-high group ($n = 28$). $P = 0.013$; Tarone-Ware test.

Clinical Significance of Cks1 Gene Expression in Gastric Carcinomas. No significant difference was found between the high and low Cks1 expression groups regarding Skp2 expression and clinicopathological factors such as age, sex, histology, serosal invasion, lymph node metastasis, lymphatic or vascular invasion, and postoperative therapy (Table 2). As shown in Fig. 2*A*, the Cks1 mRNA high expression group showed a significantly poorer prognosis than the low expression group ($P = 0.018$). On multivariate analysis for prognosis, Cks1 mRNA expression, Skp2 mRNA expression, lymph node metastasis, lymphatic involvement, and serosal invasion, which were found to be prognostic factors on univariate analysis, were included for the parameters. This analysis demonstrated that Cks1 mRNA expression was an independent prognostic factor (Table 3). Next, the 76 cases were classified into three groups: Cks1/Skp2 double-low group ($n = 13$); Cks1 or Skp2 high group ($n = 35$); and Cks1/Skp2 double-high group ($n = 28$), and survival analysis was performed (Fig. 2*B*). The Cks1/Skp2 double-high group showed the poorest prognosis, and the Cks1/Skp2 double-low group showed the best prognosis among the three groups ($P = 0.013$).

Table 3 Multivariate analysis on prognosis of the patients with the gastric carcinoma

	Regression coefficient	SE	Odds ratio (95% confidence interval)	*P*
Lymph node metastasis	1.976	0.772	7.212 (1.589–32.724)	0.01
Lymphatic involvement	1.699	1.051	5.466 (0.697–42.896)	0.106
Serosal invasion	0.965	0.463	2.625 (1.059–6.504)	0.037
Cks1 mRNA expression	1.215	0.432	3.369 (1.445–7.856)	0.005
Skp2 mRNA expression	1.162	0.519	3.198 (1.156–8.846)	0.025

Relationship between Cks1/Skp2 and p27 Expression in Cks1- or Skp2-Transfected or Cks1/Skp2-Cotransfected Gastric Carcinoma Cells. We transfected Cks1 cDNA into the two different Skp2-transfected gastric carcinoma cell lines, and the expression levels of Cks1 and Skp2 were analyzed by both quantitative RT-PCR and Western blot (Fig. 3). The level of p27 mRNA was not altered by Cks1 or/and Skp2 transfection. Skp2-transfected cell lines showed a lower level of expression of p27 protein than the controls, and Cks1/Skp2-cotransfected cell lines showed a markedly lower expression level of p27 protein than the controls. Treatment with proteasome inhibitor (MG132) blocked p27 degradation in Cks1- or/and Skp2-transfected cell lines (data not shown).

DISCUSSION

In the present clinical study of gastric carcinomas, Cks1 was overexpressed in gastric carcinoma cells, and its expression was inversely correlated with the expression level of p27 protein, and high Cks1 mRNA expression was significantly correlated with poor prognosis. These findings suggest that Cks1, in addition to Skp2 (11), is strongly related to the expression level of p27 in gastric carcinomas. Cks1 could be a potential prognostic factor for gastric carcinomas.

It was worth noting that the Cks1/Skp2 double-high expression group showed a low expression level of p27 protein in 8 (88.9%) of 9 cases, and the Cks1/Skp2 double-low expression group showed a high expression level of p27 protein in 6 (85.7%) of 7 cases. The Cks1/Skp2 double-high group showed the poorest prognosis, and the Cks1/Skp2 double-low group showed the best prognosis among the three groups. In the *in vitro* study, the Cks1/Skp2-cotransfected gastric carcinoma cells showed a markedly lower expression level of p27 protein than the controls. These findings indicate that both Cks1 and Skp2 overexpression more strongly promote the proteolysis of p27 than either Cks1 or Skp2 overexpression. Thus, Cks1 and Skp2 may cooperate in regulating the expression level of p27 protein in gastric carcinomas. These clinical and *in vitro* data suggest that, in most gastric carcinomas, the expression level of p27 is mainly regulated by the expression of Cks1 and Skp2 through ubiquitin-mediated proteolysis. To clarify the correlation between the Cks1/Skp2 expression and p27 proteolysis, additional studies using more gastric carcinoma tissues will be required.

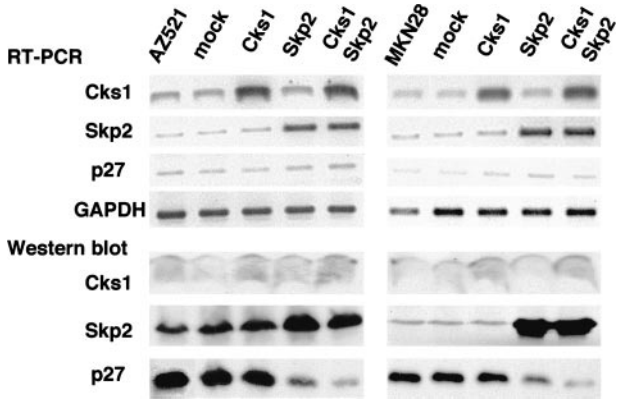

Fig. 3 The relationship between Cks1/Skp2 and p27 expression *in vitro*. RT-PCR and Western blot analysis of Cks1- or/and Skp2-transfected human gastric carcinoma cell lines. AZ521 and MKN28; parent cell lines, mock; two mocks (for Cks1 and Skp2) transfectant, Cks1; mock (for Skp2) and Cks1 transfectant, Skp2; Skp2 and mock (for Cks1) transfectant, Cks1Skp2; Cks1 and Skp2 cotransfectant. Western blot analysis: Cks1; 9 kDa, Skp2; 45 kDa, p27; 27 kDa.

It is unclear how Cks1 participates in the p27 ubiquitination by SCF^{Skp2}. However, one possibility is that Cks1 associates with Skp2 and probably confers an allosteric change in Skp2, resulting in increasing its affinity for the phosphorylated p27 and consequently promoting p27 proteolysis (17, 18).

The mechanism of Cks1 and Skp2 overexpression in gastric carcinoma cells is uncertain. Recently, it was reported that the genomic locus of the Skp2 gene was amplified and the DNA copy number was closely associated with Skp2 expression in small cell lung carcinoma (8). On the other hand, gene amplification of human chromosome 1q21, where the human Cks1 gene is located, has been reported in human epithelial malignancies (19, 20). Cks1 and Skp2 overexpression in carcinomas may be attributable to this gene amplification. We are now examining the genomic alterations of Cks1 and Skp2 and will clarify the mechanism of Cks1 and Skp2 overexpression in gastric carcinomas.

In conclusion, Cks1 expression was correlated inversely with the expression level of p27 protein and could be an independent prognostic factor in gastric carcinomas. Furthermore, Cks1 cooperates with Skp2 to strongly degrade p27 protein, and Cks1/Skp2 coexpression was correlated inversely with the expression level of p27 protein in gastric carcinomas. These findings strongly suggest that Cks1, in addition to Skp2, could play an important role in gastric carcinoma progression and would be a novel molecular target for the treatment of gastric carcinomas, as well as a strong prognostic marker.

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