Clinical and biological impact of cyclin-dependent kinase subunit 2 in esophageal squamous cell carcinoma

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Abstract. Cyclin-dependent kinase subunit 2 (CKS2) is a cyclin-dependent kinase subunit (CKS) family member that participates in cell cycle regulation. Few studies have investigated its involvement in esophageal squamous cell carcinoma (ESCC). The aim of the present study was to assess the clinical significance of CKS2 in ESCC. We used immunohistochemistry to study the clinicopathologic significance of CKS2 protein expression in 121 patients with ESCC. Using real-time reverse transcriptase-polymerase chain reaction (RT-PCR), we examined the expression of *CKS2* mRNA in tumors and the corresponding normal esophageal tissues that were obtained from 62 patients. Finally, siRNA-mediated attenuation of CKS2 expression was examined *in vitro*. CKS2 protein expression was significantly correlated with depth of tumor invasion, clinical stage, lymphatic invasion and distant metastasis (p=0.033, 0.028, 0.041 and 0.009, respectively). *CKS2* mRNA expression was higher in cancer tissue than in corresponding normal tissue (p<0.001). Patients with positive-CKS2 protein expression had a poorer five year survival frequency than patients who did not express CKS2 protein (p=0.025). *In vitro*, siRNA-mediated suppression of CKS2 slowed the growth rate of ESCC cells compared to control cells (p<0.001). The evaluation of CKS2 expression is useful for predicting the cause of malignant tumors and the prognosis of patients with ESSC.

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Abbreviations: ESCC, esophageal squamous cell carcinoma; CKS, cyclin-dependent kinase subunit; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcriptase-polymerase chain reaction

Key words: cyclin-dependent kinase subunit 2, esophageal squamous cell carcinoma, prognosis, biomarker

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive cancers of the gastrointestinal tract. It frequently progresses to invasion and metastasizes to the lymph nodes and other organs, and once metastatic, prognosis is poor (1,2). Although the biological factors affecting the malignant potential of ESCC have been identified, the molecular mechanisms underlying its progression have not been completely elucidated. Finding a cure for this intractable malignancy depends on the identification of genetic and molecular markers of malignancy potential, that may serve as specific treatment targets. However, the regulation of complex processes over multiple events precludes the identification of practical markers for carcinogenesis, tumor progression and metastasis.

Cyclin-dependent kinase subunit (CKS) proteins are small (9-kDa) cyclin-dependent kinase (Cdk)-interacting proteins that are expressed in all eukaryotic lineages. Those proteins include the highly conserved paralogs CKS1 and CKS2 in mammals (3). Both CKS1 and CKS2 consist of 79 amino acids and they possess 81% homology. The structural basis for the CKS-Cdk interaction is well understood, as the three dimensional configuration of the heterodimeric complex has been determined by X-ray diffraction crystallography (4). In addition, genetic analysis of CKS protein function in mammals is quite advanced. CKS1 is a specific co-factor that is necessary for the degradation and ubiquitination of p27 by SCF^{Skp2}. Human CKS1 binds to Skp2 and increases the binding of threonine 187-phosphorylated p27 to Skp2 (5,6). On the other hand, Cks2 is essential for meiosis. This phenotype results

due to Cks1 not being expressed in meiotic cells, leaving them completely deficient for CKS protein expression (7).

CKS proteins are essential for the G2- to M-phase cell cycle transition, as they are vital for efficient transcription of a number of crucial cell cycle control genes (8). Cell cycle phase transitions are regulated by cyclin-dependent protein kinases (cdks) and their cyclin binding partners.

Cell cycle regulatory failure is associated with the development or the progression of cancer (9,10). In addition, CKS proteins have been found frequently overexpressed in a broad spectrum of other human malignancies (11-14). However, the precise molecular rules of CKS proteins remain largely unknown in this context. In addition, the mechanistic link between CKS protein overexpression and oncogenesis remains unknown. To elucidate the roles of CKS2 proteins and to determine whether CKS2 expression may be a prognostic marker in ESCC, we investigated CKS2 expression in 121 patients and analyzed the significance.

Materials and methods

Esophageal cancer cell lines. The human esophageal cancer cell line KYSE70 was obtained from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan). It was maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and antibiotics at 37˚C in a 5% humidified CO_2 atmosphere.

Tissue sampling. In order to verify the presence of CKS2 proteins in esophageal cancer tissues, we initiated immunohistochemical studies of tissue microarray from surgical specimens from 121 patients with ESSC (109 men and 12 women). These patients had undergone esophagectomy with lymph node dissection between 1987 and 1998 at Kagoshima University Hospital, Japan. The median age of the patients was 65 years (range, 47-87 years). Postoperative follow-up data were obtained from all patients, with a median follow-up period of 24 months (range, 1-181 months).

To verify the presence of *CKS2* mRNA in esophageal cancer tissues, we conducted studies of surgical specimens from 62 esophageal cancer patients (58 men and 4 women) who underwent esophagectomy with lymph node dissection between 1998 and 2005 at Kagoshima University Hospital and the Medical Institute of Bioregulation Hospital, Kyushu University, Japan. The median age of the patients was 65 years (range, 47-87 years). Postoperative follow-up data were obtained from all patients, with a median follow-up period of 17 months (range, 1-75 months).

All samples of tissues were collected from patients after informed consent had been obtained in accordance with the institutional guidelines of our hospital. Using the tumor node metastasis classification of the International Union Against Cancer (15), all of the M1 tumors exhibited distant lymph node metastases.

Immunohistochemical study of CKS2 expression. The tissue microarray was cut into 3-micron-thick sections, that were mounted on glass slides. Immunohistochemical staining was carried out using the avidin-biotin-peroxidase complex method (Vectastatin Elite ABC kit; Vector, Burlingame, CA, USA), following the manufacturer's instructions. Briefly, the immunostaining was performed manually at room temperature. The sections were deparaffinized in xylene and dehydrated in ethanol; endogenous peroxidase activity was blocked by incubating sections for 10 min in 3% hydrogen peroxide in methanol. Then, the sections were autoclaved in citrate buffer (0.01 mol/l, pH 6.5) at 121˚C for 15 min to reveal the antigen. After cooling, the sections were pre-incubated in 1% BSA for 20 min. Next, sections were incubated with anti-CKS2 mouse monoclonal antibody (1:50, CKS2; LifeSpan Biosciences, Inc., Seattle, WA, USA) for 60 min. After rinsing with phosphate-buffered saline (PBS) for 15 min, the sections were incubated with secondary antibody for 20 min and washed again. After washing with PBS for 10 min, sections were incubated with avidin-biotin complex for 30 min and washed again, and reactions were visualized using diaminobenzidine tetrahydrochloride for 2 min. All samples were lightly counterstained with hematoxylin for 1 min. No antigen retrieval was performed. Positive and negative controls were used for each section.

Evaluation of immunohistochemistry was independently performed by two investigators. Positive expression of CKS2 was defined as detectable immunoreaction in nuclear regions of >20% of the cancer cells. To evaluate expression of CKS2, 10 fields (within the tumor and at the invasive front) were selected and expression in 1,000 tumor cells (100 cells/field) was evaluated using high-power (x200) microscopy.

The negative controls consisted of sections treated with the same protocol but with PBS instead of the primary antibody. The human esophageal cancer cell line KYSE150 was used as a positive control.

Quantitative reverse transcription-PCR. Sixty-two paired malignant and normal specimens of esophageal mucosa were homogenized and total RNA was extracted according to the manufacturer's instructions (16). Complementary DNA (cDNA) was immediately synthesized from the extracted RNA. The oligonucleotide primers for *CKS2* were: sense primer, 5'-TGTCTGAAGAGGAGTGGAGGA-3' and antisense primer, 5'-CATGCACAGGTATGGATGAAA-3'. The length of the amplified fragment was 241 bp. We used glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control. The primers for GAPDH were: sense primer, 5'-TTGGTATCGTGGAAGGACTCA-3' and antisense primer, 5'-TGTCATCATATTTGGCAGGTT-3'. The lengths of the amplicons were 249 bp. Real-time monitoring of PCR reactions was carried out using the LightCycler System (Roche Applied Science, Indianapolis, IN, USA) and SYBR-Green I dye (Roche Diagnostics) (17). Monitoring was performed according to the manufacturer's instructions.

We determined the levels of *CKS2* and *GAPDH* mRNA expression by comparing results to cDNA from the human esophageal cancer cell line KYSE150. After proportional baseline adjustment, the fit point method was employed to determine the cycle in which the log-linear signal was distinguished from the baseline, and that cycle number (threshold cycle) was used as a crossing-point value. The standard curve was produced by measuring the crossing-point of each standard value (4-fold serially diluted cDNAs of KYSE150) and

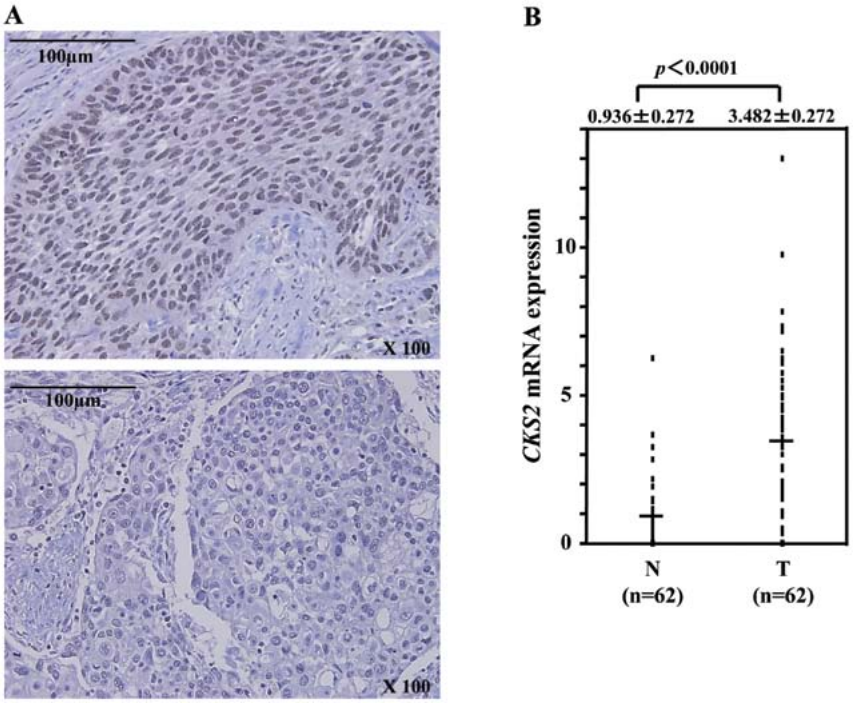

Figure 1. The expression of CKS2 protein and *CKS2* mRNA in ESCC. (A) Immunohistochemical identification of CKS2 protein in ESCC at x100 original magnification. The upper image represents a CKS2 high expression case. Most positively stained cells were associated with the tumor and the expression of CKS2 protein was restricted to the nuclei. The lower image shows a low expression case. (B) *CKS2* mRNA expression in ESCC patients as assessed by real-time quantitative PCR (n=62). Horizontal lines indicate the mean value of each group (N, non-cancerous tissue; T, cancerous tissue). CKS2, cyclin-dependent kinase subunit 2; ESCC, esophageal squamous cell carcinoma.

plotting them against the logarithmic value of the concentrations. Standard curve samples were included in each run. The concentrations of all samples were then calculated by plotting their crossing-points against the standard curve.

Calculated concentrations of all samples were relative to the concentration of the cDNA of KYSE150, and the amount of the target molecule was then divided by the amount of the endogenous reference (*GAPDH*) to obtain normalized CKS2 expression values (17). Each assay was performed three times to verify the results, and the mean mRNA expression value was used for subsequent analysis.

CKS2 RNA interference. *CKS2*-specific siRNA (Stealth™ siRNA duplex oligoribonucleotides) and negative control RNAi (Stealth™ Negative Control siRNA duplex oligoribonucleotides) were purchased from Invitrogen. Logarithmically growing cells (KYSE70) were seeded at either $1.0x10^5$ or $2.0x10^3$ cells/well in a final volume of 2 ml or 100 μl in 6- or 96-well flat bottom microplates, respectively. The cells were cultured overnight for adherence. RNAi oligomer was diluted with OPTI-MEM I reduced serum medium (Invitrogen Corp.) and incubated for 5 min at room temperature. The diluted RNAi oligomer was mixed with diluted Lipofectamine™ RNAiMAX (Invitrogen Corp.). The RNAi-Lipofectamine™ RNAiMAX complexes were added to each well at a final concentration of 30 pmol/ml. The cells were incubated for 5 h followed by replacement of the media. The assays were performed after 48 h incubation.

In vitro proliferation assays. Proliferation was determined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche Diagnostics Corp., GmbH). Logarithmically growing cells were seeded at $5.0x10^3$ cells/well in flat-bottomed 96-well microtiter plates in a final volume of 100 μl culture medium/well, and incubated in a humidified atmosphere (37˚C and 5% CO_2). MTT labeling reagent (10 μl at a final concentration 0.5 mg/ml) was then added to each well. The microtiter plate was incubated for 4 h in a humidified atmosphere, after which solubilization solution (100 μl) was added to each well. The plate was then incubated overnight in a humidified atmosphere. Once complete solubilization of the purple formazan crystals was confirmed, the absorbance of the samples was measured using a Model 550 Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA), at a wavelength of 570 nm corrected to 655 nm. Each independent experiment was performed in triplicate.

Statistical analysis. The statistical analysis of group differences was performed using the χ^2 test, the Student's t-test and the repeated-ANOVA test. Overall survival curves were plotted according to the Kaplan-Meier method, with the Wilcoxon test applied for comparisons and $p<0.05$ was considered statistically significant. Variables with a value of $p<0.05$ by univariate analysis were used in subsequent multivariate analyses based on Cox's proportional hazards model. All statistical analyses were performed using JMP™ for Windows (version 5.0.1; SAS Institute Inc., Cary, NC, USA).

Results

Clinicopathologic significance of the expression of CKS2 protein and CKS2 mRNA in ESCC. CKS2 protein

Table I. Relationship between CKS2 mRNA, CKS2 protein and clinicopathological factors.

		CKS2 protein expression			CKS2 mRNA expression			
Factors	Total	Negative (n=69) no. (%)	Positive (n=52) no. (%)	P-value	Total	Negative (n=31) no. (%)	Positive (n=31) no. (%)	P-value
Gender								
Male	109	61 (56.0)	48 (44.0)	0.472	58	28 (48.3)	30 (51.7)	0.291
Female	12	8 (66.7)	4 (33.3)		4	3 (75.0)	1 (25.0)	
Tumor location								
Upper	18	10 (55.6)	8 (44.4)	0.957	8	4 (50.0)	4 (50.0)	0.593
Middle	57	32 (56.1)	25 (43.9)		32	12 (37.5)	20 (62.5)	
Lower	46	27 (58.7)	19 (41.3)		22	10 (45.6)	12 (54.4)	
Histology								
Well	51	26 (51.0)	25 (49.0)	0.345	14	8 (57.1)	6 (42.9)	0.593
Moderate	53	31 (58.5)	22 (41.5)		34	15 (44.1)	19 (55.9)	
Poor	17	12 (70.6)	5 (29.4)		14	8 (57.1)	6 (42.9)	
pT								
T1,2	48	33 (68.8)	15 (31.2)	**0.033**	32	20 (62.5)	12 (37.5)	**0.041**
T3,4	73	36 (49.3)	37 (50.7)		30	11 (36.7)	19 (63.3)	
pN								
N0	52	33 (63.5)	19 (36.50)	0.213	18	12.0 (66.7)	6 (33.30)	0.091
N1	69	36 (52.2)	33 (47.80)		44	19.0 (43.2)	25 (56.80)	
pM								
M0	87	56 (64.4)	31 (35.60)	**0.009**	59	29.0 (49.2)	30 (50.80)	0.091
M1	34	13 (38.2)	21 (61.80)		3	2.0 (66.7)	1 (33.30)	
p-Stage								
I, II	58	39 (67.2)	19 (32.80)	**0.028**	28	17.0 (60.7)	11 (39.30)	0.125
III, IV	63	30 (47.6)	33 (52.40)		34	14.0 (41.2)	20 (58.80)	
Lymphatic invasion								
Negative	45	31 (68.9)	14 (31.1)	**0.041**	19	14 (73.7)	5 (26.3)	**0.012**
Positive	76	38 (50)	38 (50)		43	17 (39.5)	26 (60.5)	
Vascular invasion								
Negative	92	53 (57.6)	39 (42.4)	0.817	16	10 (62.5)	6 (37.5)	0.240
Positive	29	16 (55.2)	13 (44.8)		46	21 (45.7)	25 (54.3)	

CKS2, cyclin-dependent kinase subunit 2.

was expressed and distributed in the nuclei of cancer cells (Fig. 1A). Positive expression of CKS2 protein was observed in 52 patients (43.0%). CKS2 protein expression was significantly associated with the following clinicopathologic parameters: depth of tumor invasion (category T of TNM classification), distant lymph node metastasis, stage and lymphatic invasion (Table I).

In the mRNA analysis, 58/62 patients (93.5%) showed higher expression levels of *CKS2* mRNA in cancerous tissues than in non-cancerous tissues by real-time quantitative reverse transcription-PCR. The mean expression level of *CKS2* mRNA in tumor tissues was 3.482±0.272 (means ± SD), which was significantly higher than the value obtained from the corresponding normal tissues (0.936±0.272, p<0.0001) (Fig. 1B). The patients with values below the median expression level in tumor tissues were assigned to the low expression group (n=31), whereas those with values above the median were assigned to the high expression group (n=31). *CKS2* mRNA expression was significantly associated with the depth of tumor invasion (category T of TNM classification) and the incidence of lymphatic invasion (Table I).

Relationships between CKS2 protein expression, mRNA expression and prognosis. The 5-year overall survival rates of patients with high *CKS2* mRNA levels and those with low CKS2 mRNA levels were 42.5 and 53.9%, respectively. The CKS2 high expression group tended to have a poorer prognosis than the low expression group; however, the survival difference between these two groups was not statistically significant (p=0.550) (Fig. 2A). The 5-year overall survival

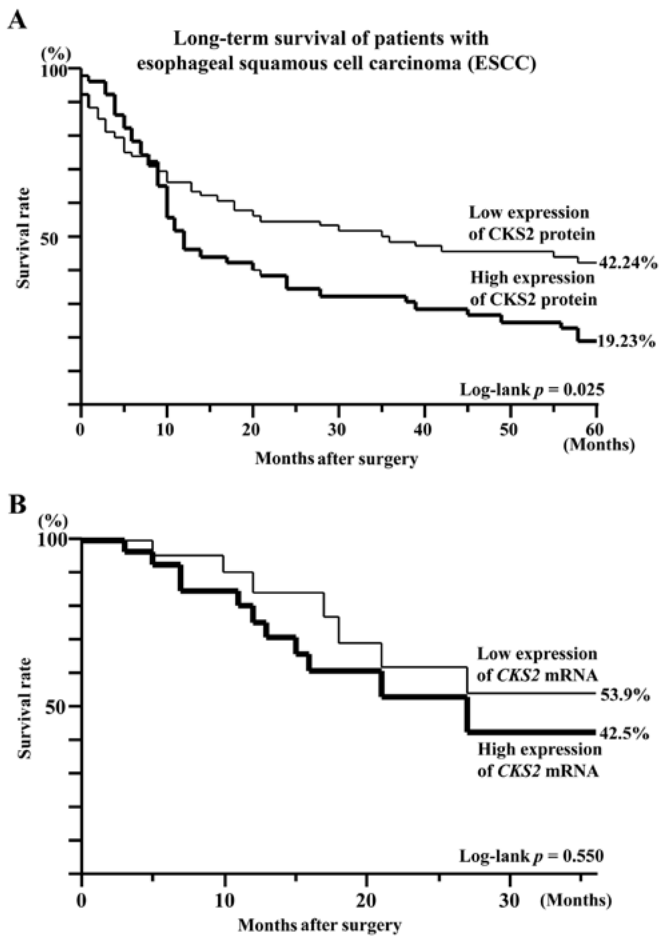

Figure 2. Long-term survival of patients with ESCC. (A) Survival of patient groups with high and low levels of CKS2 protein expression in cancerous tissue. Patients in the high CKS2 protein group had a significantly poorer prognosis than those in the low CKS2 expression group (p=0.025). (B) Survival of patient groups with high and low levels of *CKS2* mRNA expression in malignant tissue. Patients in the *CKS2* mRNA expression group tended to have poorer prognosis than those in the low *CKS2* mRNA expression group. The survival difference between these two groups was not statistically significant (p=0.550). ESCC, esophageal squamous cell carcinoma; CKS2, cyclin-dependent kinase subunit 2.

rates of patients with high CKS2 protein levels and those with low CKS2 protein levels were 19.23 and 42.24%, respectively. The 5-year survival rate was significantly lower in patients with positive-CKS2 protein expression than in those with negative-CKS2 protein expression (p=0.035; Fig. 2B).

Univariate and multivariate analyses of survival. Table II shows univariate and multivariate analyses of factors related to patient prognosis according to CKS2 protein expression. Multivariate regression analysis indicated that the depth of invasion and lymph node metastasis were independent prognostic factors, but that lymphatic invasion and CKS2 protein expression were not independent prognostic factors.

Effect of CKS2 gene silencing. KYSE70 cells normally express mRNA at a high level. Suppression of *CKS2* mRNA was confirmed by real-time quantitative PCR (Fig. 3A), with subsequent reduction in the proliferation rate of KYSE70 cells (p<0.001) (Fig. 3B).

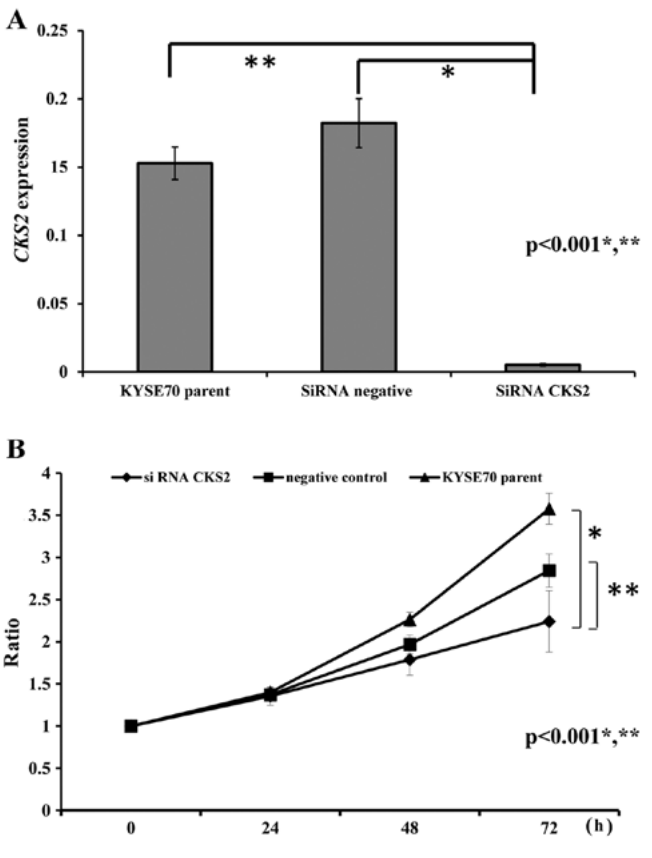

Figure 3. (A) CKS2 expression is suppressed by CKS2 specific-siRNA in KYSE170 cells. Forty-eight hours after siRNA addition, CKS2 expression was measured by real-time quantitative PCR. (B) Effect of CKS2 suppression on KYSE170 cell proliferation as assessed by the MTT assay. CKS2-suppressed cells were less proliferative than control cells (p<0.01). CKS2, cyclin-dependent kinase subunit 2.

Discussion

We previously analyzed genes related to lymph node metastasis in ESCC. Using laser microdissection techniques and cDNA and oligo nucleotide microarray, several genes were identified simultaneously in comparisons of lymph node-positive and -negative primary tumors or primary tumors and lymph node metastasis. CKS2 was among the genes of interest that were identified (18,19). In the present study, CKS2 expression was associated with lymphatic invasion and distant metastasis. This finding is consistent with our previous findings as a category of distant metastasis contains distant lymph node metastasis which arises frequently in ESCC (15). Moreover, in gastric cancer, we previously showed that CKS2 mRNA expression was associated with lymph node metastasis (20). Li *et al* reported that CKS2 was overexpressed in cancer tissues compared to non-cancer tissues of the colon. A similar observation was made in cancers with liver metastasis compared to those without liver metastasis by means of genome-wide cDNA microarray (21). This result suggests that CKS2 also plays an important role in lymph node metastasis as well as hematogenous metastasis. In addition, the expression of CKS2 (both mRNA and protein) correlated with the depth of tumor invasion. Kawakami *et al* and Chen *et al* demonstrated that bladder cancer of the invasive type has higher expression of

Table II. Univariate and multivariate analysis of clinicopathological factors affecting overall survival rate.

Variables	N	5-year survival rate (%)	Univariate analysis P-value	Multivariate analysis Relative risk (CI)	Multivariate analysis P-value
Tumor depth					
pT1,2	48	50.3	**<0.0001**	1.680	**0.0001**
pT3,4	73	18.4		(1.297-2.224)	
p-Stage					
I, II	48	58.8	**<0.0001**	0.876	0.238
III, IV	73	7.9		(0.702-1.092)	
Lymph node metastasis					
Negative	52	55.4	**<0.0001**	1.639	**0.0001**
Positive	69	14.0		(1.274-2.139)	
Lymphatic invasion					
Negative	45	47.6	**<0.0001**		
Positive	76	22.9			
Venous invasion					
Negative	92	39.1	**0.002**	1.168	0.217
Positive	29	10.3		(0.910-1.483)	
Distant metastasis					
Negative	87	42.5	**<0.0001**		
Positive	34	23.5			
CKS2 protein expression					
Negative	69	42.4	**0.025**	1.146	0.228
Positive	52	19.2		(0.918-1.431)	

N, number of patients; CI, confidence interval; CKS2, cyclin-dependent kinase subunit 2.

CKS2 than that of the superficial type (22,23). The previous reports are in line with our recent study.

Our previous study of gastric cancer revealed that CKS2 mRNA expression was an independent prognostic factor (20). In the present study of ESCC, CKS2 protein expression correlated well with depth of invasion or lymph node metastasis and was predicted to be a prognostic factor, although it was not independent in multivariate analysis. However, this is the first study reporting a correlation between CKS2 expression and prognosis in a large scale study of ESCC. Further study of CKS2 expression is required to better understand the clinical and prognostic significance in ESCC.

In the patients studied here, *CKS2* mRNA was overexpressed in ESCC tissues compared to normal esophageal tissues. Similar results have been reported in the analyses of esophagus (24), colon (21), cervical cancers (12), malignant melanomas (25) and human gliomas (26,27). Moreover, we analyzed both the expression of mRNA and protein. While the trend of expression of mRNA was similar to that of protein, differences were apparent. We hypothesized that the reason for this difference is post transcriptional regulation including epigenetic changes such as DNA methylation and histone modification (28). For instance, microRNAs function throughout post-transcriptional gene silencing by modulating the translation of mRNAs into proteins (29) and there are recent reports on epigenetic regulation by non-coding RNAs (30). Indeed, Lv *et al* reported that miR26a targets and represses CKS2 expression in papillary thyroid carcinoma (31).

The suppression of CKS2 expression by siRNA reduced cellular proliferation (Fig. 3). Several reports have demonstrated that the inhibition of CKS2 decreases cell proliferation and increases caspase 3 and Bax expression at the protein level concerning apoptosis (13,20,26). In ESCC, CKS2 may play an important role by inhibiting apoptosis as observed in other cancers. Liberal *et al* showed that overexpression of CKS2 in malignancy overrode the intra-S-phase checkpoint that blocks aberrant DNA replication in response to stress (32). These results may explain why inhibition of CKS2 increased the proliferation of cancer cells in our *in vitro* study.

In conclusion, we demonstrated that the expression of CKS2 in ESCC was elevated relative to levels in normal tissue, and that CKS2 overexpression is associated with the depth of tumor invasion, lymphatic invasion, clinical stage, distant metastasis and poor prognosis. Therefore, the expression profile of CKS2 may contribute to the creation of a new clinical classification predicting an aggressive tumor phenotype. The evaluation of CKS2 expression may be useful for predicting the malignant properties and prognosis of patients with ESSC.

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