Skp2 Overexpression Is a Prognostic Factor in Patients with Ovarian Adenocarcinoma[1]

Kazushi Shigemasa,[2] Lijun Gu, Timothy J. O'Brien, and Koso Ohama

Department of Obstetrics and Gynecology, Hiroshima University Graduate School of Biomedical Sciences, Hiroshima 734-8551, Japan [K. S., L. G., K. O.], and Departments of Biochemistry and Molecular Biology and Obstetrics and Gynecology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205-7199 [T. J. O.]

ABSTRACT

Purpose: The purpose of this study was to examine Skp2 expression in epithelial ovarian tumors and to identify the association of Skp2 expression levels with patient survival.

Experimental Design: Skp2 protein expression was examined by immunohistochemistry in 134 epithelial ovarian tumors [20 adenomas, 23 low malignant potential (LMP) tumors, and 91 adenocarcinomas]. Results of immunostaining were correlated with clinicopathological variables and overall survival. Skp2 mRNA expression was examined by semiquantitative PCR in 32 ovarian adenocarcinomas and in 3 ovarian cancer cell lines.

Results: Skp2 expression was detected in neither ovarian adenomas nor LMP tumors. In contrast, Skp2 expression was detected in 47.3% (43 of 91) of adenocarcinomas. Positive Skp2 expression was detected significantly more often in adenocarcinomas than in LMP tumors ($P < 0.0001$) or in adenomas ($P < 0.0001$). A significantly higher detection rate of Skp2 expression was observed in advanced-stage diseases compared with early-stage diseases ($P = 0.010$). Log-rank testing showed that Skp2 overexpression was significantly correlated with poor patient survival ($P = 0.0035$). Older age ($P = 0.0026$), advanced clinical stage ($P < 0.0001$), and high histological grades of the tumors ($P = 0.0018$) were also significantly associated with poor prognoses. In multivariate analysis, Skp2 overexpression ($P = 0.0069$) and clinical stage ($P < 0.0001$) remained significantly associated with overall survival, whereas age and histological grade lost their significance. Considerable levels of Skp2 mRNA expression were detected in all ovarian adenocarcinomas examined by semiquantitative PCR.

Conclusions: Skp2 expression might play an important role in the development and progression of ovarian adenocarcinomas, and Skp2 overexpression is an independent prognostic marker of ovarian adenocarcinoma patients.

INTRODUCTION

In recent years, many of the genes involved in cell cycle control have been identified and cloned, and the products of these genes have been described in the literature (1, 2). It is now evident that the progression of cells through the cell cycle is regulated by both positive signals (CDKs)[3] and negative signals (CDK inhibitors). Furthermore, tumor cells have typically acquired damage to genes that directly regulate their cell cycle.

Ovarian cancer is the most lethal of the gynecological malignancies. Although important progress continues to be made in the understanding and treatment of ovarian cancer, the 5-year survival rate for all ovarian cancer patients has remained at less than 50% for the past 30 years (3). As is the case with other common human carcinomas, a series of multiple alterations in cell cycle control-related gene products are believed to be involved in ovarian cancer pathogenesis. It is therefore important to clarify the cell cycle control mechanisms involved in the development and progression of ovarian cancer.

Skp1-Cullin-F-box protein (SCF) complexes comprise a large family of ubiquitin ligases that contain several constant subunits (Cullin-1, Skp1, and ROC1) and a variable subunit called an F-box protein (4). Each F-box protein binds a specific subset of protein substrates and thus promotes their ligation to ubiquitin and subsequent degradation (4–6). Skp2, an F-box protein necessary for DNA replication, was originally identified as a protein that interacts with the cyclin A-CDK2 complex (7). In the normal cell cycle, levels of Skp2 are low in G_0-G_1 and increase in S phase (7). It is also reported that expression of Skp2 is required for entry of cells into S phase and that the level of Skp2 is greatly increased in many transformed cells (7). In addition, Skp2-deficient mice grow more slowly and have smaller organs than littermate controls, suggesting the importance of Skp2 in positively regulating cell proliferation (8). Recently, it has been reported that p27 is specifically recognized and targeted for ubiquitination by Skp2 (9–11). Skp2 is required for the ubiquitination and subsequent degradation of p27 both *in vivo* and *in vitro*. Skp2 only binds to and allows ubiquitination of p27 when the latter is phosphorylated on Thr[187] by CDK2. In quiescent cells, levels of p27 are high, but in response to mitogenic stimuli, levels of cyclin E, cyclin A1, and Skp2

[3] The abbreviations used are: CDK, cyclin-dependent kinase; LMP, low malignant potential; TBS, Tris-buffered saline.

increase, resulting in the Thr^{187} phosphorylation of p27 and its subsequent ubiquitin-mediated degradation.

p27, a member of the cip1/kip family of CDK inhibitors, binds to a wide variety of cyclin-CDK complexes including CDK2 and CDK4, inhibits kinase activity, and blocks the cell cycle. p27 regulates the progression from G_1 to S phase and results in G_1 arrest (12). Therefore, p27 is thought to be important in maintaining cellular homeostasis and stability. Decreased expression of the p27 protein is associated with a broad range of human malignancies, including breast (13–15), ovarian (16–18), gastric (19), colorectal (20), prostate (21), and endometrial (22) cancer, suggesting that it plays an important role in human cancer pathogenesis. These findings suggest that the enhanced p27 degradation observed in many aggressive human tumors might be attributable to increased levels of Skp2. However, the possibility of abnormal Skp2 expression in ovarian tumor cells has not been assessed.

The aim of the present study was to determine the potential role of Skp2 in ovarian cancer development and/or progression. Skp2 protein expression in epithelial ovarian tumors was investigated immunohistochemically. The results were analyzed in terms of tumor type, clinical stage, histological grade, and histological type. The prognostic significance of the Skp2 expression levels in ovarian cancer patients was also analyzed. Immunohistochemical Skp2 expression status was compared with p27 expression status in the selected tissue samples. Finally, Skp2 mRNA expression levels were examined by semiquantitative PCR in selected ovarian cancer samples and ovarian cancer cell lines.

MATERIALS AND METHODS

Tissue Samples and Clinical and Pathological Data. For immunohistochemical analysis, epithelial ovarian tumor tissue samples were collected from 134 patients who were treated surgically between 1988 and 1999. None of the patients had received any chemotherapy before surgery. The patients were diagnosed histologically and received follow-up care in the Department of Obstetrics and Gynecology at Hiroshima University Hospital. Informed consent was obtained from each subject according to institutional guidelines. The histological subtype of each ovarian tumor sample was diagnosed according to accepted criteria (23). The primary pathological diagnosis was adenocarcinoma for 91 patients (39 serous, 19 mucinous, 18 endometrioid, and 15 clear cell adenocarcinomas), LMP tumor for 23 patients (8 serous and 15 mucinous LMP tumors), and adenoma for 20 patients (10 serous and 10 mucinous adenomas). The ages of the adenocarcinoma patients ranged from 23–80 years, with an average age of 51.2 years. All adenocarcinoma patients underwent complete surgical staging to exclude the presence of occult metastatic disease. Clinical staging was determined in accordance with the criteria of the International Federation of Gynecology and Obstetrics (FIGO) staging system (24). The initially diagnosed disease was stage I for 34 patients, stage II for 12 patients, stage III for 40 patients, and stage IV for 5 patients. Most of the patients with stage Ic, stage II, stage III, or stage IV ovarian cancer had received cisplatin-containing chemotherapy after surgery. The mean follow-up time was 52.0 months (range, 2.7–159.9 months). Tumor sam-

ples were fixed in 10% neutral-buffered formalin and then embedded in paraffin.

For semiquantitative PCR analysis, fresh surgical specimens of 32 ovarian adenocarcinomas were collected. Of 32 cases, 17 cases showed positive Skp2 immunostaining on paraffin-embedded samples, and 15 cases showed negative Skp2 immunostaining on paraffin-embedded samples. Informed consent was obtained from each subject according to institutional guidelines. The specimens were obtained immediately after the surgical procedure and cut in half. One half of each specimen was processed for histological examination to determine the percentage of tumor cells, which was never lower than 80%, whereas the other half was used for mRNA preparation. Tissues were frozen in liquid nitrogen and stored at $-80°C$ before mRNA isolation. Three ovarian cancer cell lines [OVK18 (TKG0323; Cancer Cell Repository, Research Institute for Tuberculosis and Cancer, Tohoku University), MCAS (JCRB0240; Japanese Cancer Research Resources Bank), and TYK-nu (JCRB0234.0; Japanese Cancer Research Resources Bank)] were included in the semiquantitative PCR analysis.

Immunohistochemical Examination. Formalin-fixed and paraffin-embedded sections (4-μm thick) were cut and mounted on aminopropyltriethoxysilane-treated slides. Slides were routinely deparaffinized with xylene and rehydrated with ethanol washes. Nonenzymatic antigen retrieval was performed by microwave heat treatment for 3×7 min in a 0.01 M sodium citrate buffer (pH 6.0). Endogenous peroxidase and nonspecific background staining were blocked by incubating slides with methanol containing 0.3% H_2O_2 for 30 min. Slides were washed with TBS for 15 min and then incubated with anti-Skp2 rabbit polyclonal antibody (SC-7164; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 37°C. The working dilution of the primary antibody was 1:50. Sections were rinsed with TBS for 15 min and then incubated with ENVISION+, rabbit/horseradish peroxidase (DAKO Corp., Carpinteria, CA) for 45 min. After being washed with TBS for 15 min, the final products were visualized by using the 3-amino-9-ethylcarbazole substrate system (DAKO Corp.), and sections were counterstained with Mayer's hematoxylin for 20 s before mounting. Both positive and negative controls were used for each section; a breast carcinoma was used as a positive control according to the manufacturer's instructions (catalogue number 18-0307; Zymed Laboratories Inc., San Francisco, CA). Normal rabbit serum IgG (Vector Laboratories, Burlingame, CA) was used in place of the primary antibody as a negative control. All experiments were performed in duplicate. The percentage of nuclear-positive tumor cells was scored as follows. When no nuclear-positive tumor cell stain could be identified, or when focally distributed nuclear-positive tumor cells reached up to 5%, the staining was considered negative. When 5–25% of the tumor cells showed positive nuclear staining, staining was categorized as partial positive. When more than 25% of the tumor cells showed positive nuclear staining, staining was considered diffuse positive. All cases were scored independently by two of the authors (K. S. and L. G.) without knowledge of patient status. A Kaplan-Meier survival curve of ovarian cancer patients was categorized according to Skp2 protein expression status. In addition, immunohistochemical expression of p27 was examined in selected

Table 1 Immunohistochemical expression of Skp2 in epithelial ovarian tumors

| | | Skp2 expression (%) | | |
	N	Negative	Partial positive[a]	Diffuse positive[b]
Adenoma	20	20 (100)[c]	0 (0)	0 (0)
LMP tumor	23	23 (100)[d]	0 (0)	0 (0)
Adenocarcinoma	91	48 (52.7)[c,d]	25 (27.5)	18 (19.8)

[a] Partial positive, 5–25% positive tumor cell staining.
[b] Diffuse positive, more than 25% positive tumor cell staining.
[c] Significant; adenoma *versus* adenocarcinoma: $P < 0.0001$ (Fisher's exact test).
[d] Significant; LMP tumor *versus* adenocarcinoma: $P < 0.0001$ (Fisher's exact test).

Table 2 Skp2 expression in relation to age, clinical stage, histological type, and histological grade in ovarian adenocarcinomas

	N	Skp2-positive expression (%)[a]
Adenocarcinoma	91	43 (47.3)
Age (yr)		
<50	41	16 (39.0)
>51	50	27 (54.0)
Clinical stage		
Stage I	34	10 (29.4)[b]
Stage II/III/IV	57	33 (57.9)[b]
Histological type		
Serous	39	23 (59.0)[c]
Mucinous	19	5 (26.3)[c]
Endometrioid	18	8 (44.4)
Clear cell	15	7 (46.7)
Histological grade		
Grade 1	56	27 (48.2)
Grade 2/3	35	16 (45.7)

[a] Skp2 expression positive, more than 5% positive tumor cell staining including partial positive and diffuse positive.
[b] Significant; stage I *versus* stage II/III/IV: $P = 0.010$ (Fisher's exact test).
[c] Significant; serous *versus* mucinous adenocarcinoma: $P = 0.019$ (Fisher's exact test).

ovarian tumor samples to compare the expression status between Skp2 and p27 using previously described methods (18).

Semiquantitative PCR. Extraction of mRNA from ovarian tissue specimens and cDNA synthesis were carried out according to previously described methods (25–28). mRNA was isolated using a RiboSep mRNA isolation kit (Becton Dickinson Labware, Bedford, MA). The amount of mRNA recovered was measured by UV spectrophotometer. cDNA was synthesized with 2.0 μg of mRNA by random hexamer priming using the Advantage RT-for-PCR kit (Clontech, Palo Alto, CA). The efficiency of cDNA synthesis was estimated using glyceraldehyde-3-phosphate dehydrogenase amplimers (Clontech) as a positive control.

Skp2 mRNA expression levels were examined using a semiquantitative PCR technique performed according to a previously described method, with some modifications (25–28). The Skp2 sense primer sequence was 5′-CGTGTACAGCACATGGACCT-3′, and the antisense primer sequence was 5′-GGGCAAATTCAGAGAATCCA-3′. β-Tubulin DNA amplification was used as an internal PCR control. The β-tubulin sense primer was 5′-TGCATTGACAACGAGGC-3′, and the antisense primer was 5′-CTGTCTTGACATTGTTG-3′. The predicted sizes of the amplified genes were 199 bp for Skp2 and 454 bp for β-tubulin. The PCR reaction mixture consisted of cDNA derived from 50 ng of mRNA, 5 pmol of sense and antisense primers, 200 μmol of deoxynucleotide triphosphates, and 0.625 units of Taq DNA polymerase with reaction buffer (Takara Shuzo Corp., Kyoto, Japan) in a final volume of 25 μl. Thirty cycles of PCR were carried out in a thermal cycler. Each cycle included 30 s of denaturation at 94°C, 30 s of primer annealing at 57°C, and 30 s of extension at 72°C. A tube containing all of the ingredients except the templates was included in all runs as a negative reaction control. The PCR products were separated on 2.0% agarose gels, and the density of each PCR product was determined using a Printgraph-Densitograph system (ATTO Corp., Tokyo, Japan). In the present study, we used the expression ratio Skp2:β-tubulin as measured by densitometry to evaluate gene expression. The results were expressed as mean ± SD.

Statistical Analysis. For statistical analysis, the χ^2 test of significance and Fisher's exact test were used to analyze the distribution of Skp2 expression in the cases according to their clinicopathological characteristics. Statistically significant dif-

ferences in overall survival rates were determined using the log-rank test. Overall survival time was defined as the period between the time of initial surgery and the time of death. Survival times of patients who were still alive were noted along with the date of the last follow-up appointment. For multivariate analyses, we used the Cox proportional hazards model. The unpaired *t* test was used to assess the differences of the mean value of the expression ratio Skp2:β-tubulin between groups. All *P*s quoted are two sided. Significance was defined as $P < 0.05$. The Statview 5 program (Abacus Concepts, Inc., Berkeley, CA) was used for statistical analysis.

RESULTS

Immunohistochemical Analysis of Skp2 Expression. Immunohistochemical expression of Skp2 was examined in 134 epithelial ovarian tumors (20 adenomas, 23 LMP tumors, and 91 adenocarcinomas). The immunohistochemical expression patterns of Skp2 in epithelial ovarian tumors are summarized in Table 1. All 20 adenoma tissues showed no Skp2 protein expression in tumor cells. In addition, Skp2 expression was detected in none of the 23 LMP tumor tissues. Skp2 protein expression was found in 43 of 91 (47.3%) adenocarcinomas. Positive Skp2 expression including partial positives and diffuse positives was significantly higher in the adenocarcinoma samples than in either LMP tumors or adenomas (adenocarcinoma *versus* LMP tumor, $P < 0.0001$; adenocarcinoma *versus* adenoma, $P < 0.0001$, Fisher's exact test). Positive Skp2 expression in relation to age, clinical stage, histological type, and histological grade in ovarian adenocarcinoma samples is shown in Table 2. Skp2 expression was observed more frequently in serous adenocarcinomas than in mucinous adenocarcinomas ($P = 0.019$, Fisher's exact test). Skp2 expression correlated with advanced clinical stage (stage I *versus* stage II/III/IV, $P = 0.010$, Fisher's exact test). Finally, no significant correlation
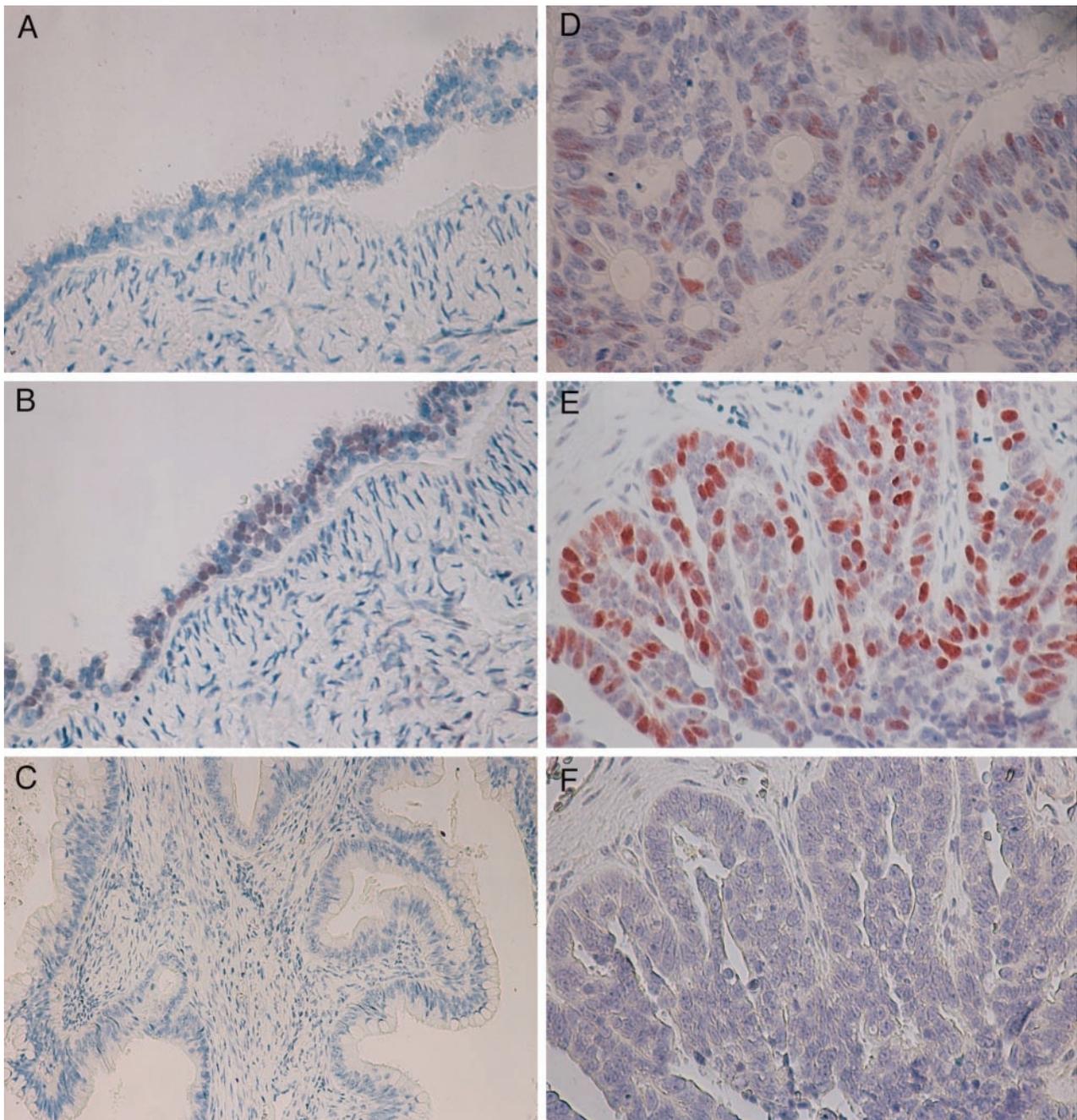
Fig. 1 Immunohistochemistry. Skp2 expression is not detected in serous adenoma cells (*A*, ×100), whereas p27 expression is observed in the same tumor cells (*B*, ×100). Mucinous LMP tumor cells are negative for Skp2 staining (*C*, ×50). Nuclear Skp2 staining is observed in mucinous adenocarcinoma cells (*D*, ×100). Positive staining of Skp2 is detected in serous adenocarcinoma cells (*E*, ×100), but no positive staining of p27 is observed in the same tumor cells (*F*, ×100).

was found between positive Skp2 expression and histological grade.

Fig. 1 shows representative results of immunohistochemistry experiments. Skp2 expression was not detected in serous adenoma cells (Fig. 1*A*), whereas p27 expression was observed in these same tumor cells (Fig. 1*B*). Mucinous LMP tumor cells were negative for Skp2 staining (Fig. 1*C*). In contrast, nuclear

Skp2 staining was observed in mucinous adenocarcinoma cells (Fig. 1*D*). Positive Skp2 staining was detected in serous adenocarcinoma cells (Fig. 1*E*), but no positive staining of p27 was observed in these tumor cells (Fig. 1*F*).

Survival Analysis of Skp2 Expression in Patients with Ovarian Adenocarcinoma. Univariate analysis of age (<50 years *versus* >51 years), clinical stage (stage I *versus* stage

Table 3 Univariate analysis of age, clinical stage, histological type, histological grade, and Skp2 expression status for overall survival in ovarian adenocarcinoma patients

Factors	P^a
Age (<50 yrs *vs.* >51 yrs)	0.0026
Clinical stage (stage I *vs.* stage II/III/IV)	<0.0001
Histological grade (grade 1 *vs.* grade 2/3)	0.0018
Skp2 expression[b]	
Negative *vs.* partial positive *vs.* diffuse positive	0.0102
Negative *vs.* partial or diffuse positive	0.0365
Negative or partial positive *vs.* diffuse positive	0.0035

[a] Log-rank test.
[b] Skp2 expression partial positive, 5–25% positive tumor cell staining; diffuse positive, more than 25% positive tumor cell staining.

Fig. 2 Log-rank testing showed that Skp2 overexpression is significantly correlated with poor prognosis in ovarian adenocarcinoma patients. *A,* negative *versus* partial positive *versus* diffuse positive expression of Skp2, $P = 0.0102$. *B,* negative *versus* partial or diffuse positive expression of Skp2, $P = 0.0365$. *C,* negative or partial positive *versus* diffuse positive expression of Skp2, $P = 0.0035$.

II/III/IV), histological grade (grade 1 *versus* grade 2/3), and Skp2 expression status for overall survival in ovarian adenocarcinoma patients is summarized in Table 3. Log-rank testing revealed that older age, advanced clinical stage, and high histological grade were significantly correlated with poor patient survival (age, $P = 0.0026$; stage, $P < 0.0001$; grade, $P = 0.0018$). When the ovarian adenocarcinoma patients were categorized along a Kaplan-Meier survival curve according to negative *versus* partial positive *versus* diffuse positive expression of Skp2, a statistically significant association between Skp2 expression levels and patient survival was observed ($P = 0.0102$; Table 3; Fig. 2*A*). When patients were categorized according to negative *versus* partial or diffuse positive expression of Skp2, positive Skp2 expression was significantly correlated with poor patient survival ($P = 0.0365$; Table 3; Fig. 2*B*). When patients were categorized according to negative or partial positive *versus* diffuse positive expression of Skp2, the most significant association was found between diffuse positive Skp2 expression and survival ($P = 0.0035$; Table 3; Fig. 2*C*). Associations between diffuse positive Skp2 expression and overall survival in each subgroup of ovarian adenocarcinomas are summarized in Table 4. A statistically significant association between diffuse positive expression of Skp2 and patient survival was observed in patients with advanced-stage disease (stage II/III/IV; $P = 0.0038$), whereas no association was found in patients with early-stage disease (stage I). With regard to histological types, diffuse positive Skp2 expression was significantly correlated with patient survival in patients with serous adenocarcinomas ($P = 0.0189$), whereas no correlation was found in patients with nonserous subtypes, including mucinous, endometrioid, and clear cell adenocarcinomas. Finally, a statistically significant association was found in patients with low histological grade disease, as well as in patients with high histological grade disease (grade 1, $P = 00094$; grade 2/3, $P = 0.0149$).

Multivariate analysis was performed using representative parameters from Table 3. Age (<50 years *versus* >51 years), clinical stage (stage I *versus* stage II/III/IV), histological grade (grade 1 *versus* grade 2/3), and Skp2 expression status (negative or partial positive *versus* diffuse positive) were selected as covariables (Table 5). Clinical stage and Skp2 expression were identified as significant and independent variables (stage, $P < 0.0001$; Skp2 expression, $P = 0.0069$). Diffuse positive Skp2 expression yielded a hazard ratio of 2.736, with a 95% confi-
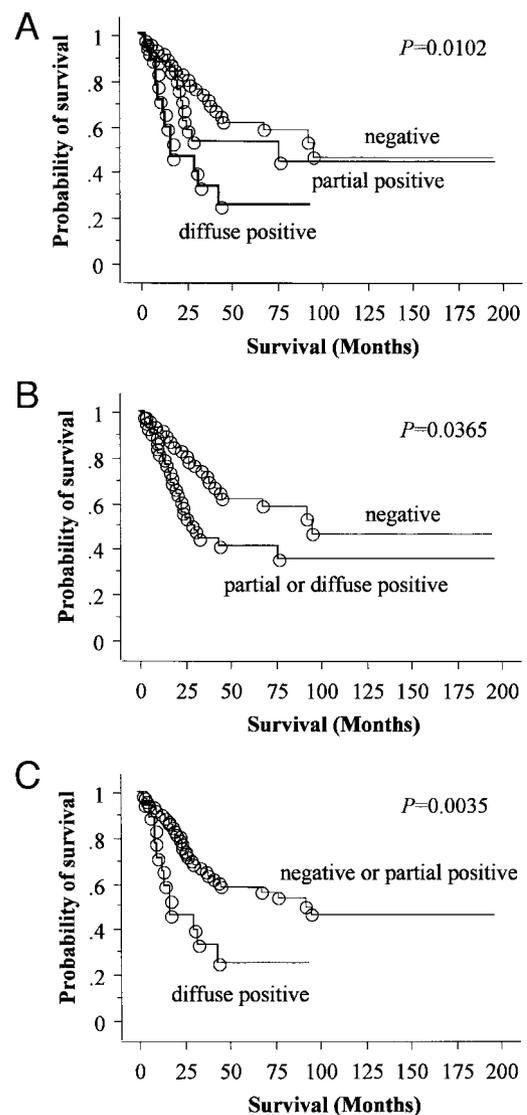
dence interval ranging from 1.317 to 5.680. No other variables including age and histological grade were significantly associated with survival according to the multivariate analysis.

Semiquantitative PCR Analysis for Skp2 mRNA Expression. Semiquantitative PCR analysis was performed to investigate Skp2 mRNA expression in 32 ovarian adenocarcinomas and 3 ovarian cancer cell lines. In a preliminary study, the linearity of the PCR amplification was confirmed. Fig. 3 shows an example of the semiquantitative PCR results using Skp2 primers coamplified with the internal control β-tubulin primers. Consistent levels of Skp2 mRNA expression were observed in all three ovarian cancer cell lines (OVK18, MCAS, and TYK-nu). In all 32 ovarian adenocarcinoma cases exam-

Table 4 Association between diffuse positive Skp2 expression and
overall survival in each subgroup of ovarian adenocarcinomas[a]

	N	P[b]
Adenocarcinoma	91	0.0035
Clinical stage		
Stage I	34	0.2368
Stage II/III/IV	57	0.0038
Histological type		
Serous	39	0.0189
Nonserous	52	0.1527
Histological grade		
Grade 1	56	0.0094
Grade 2/3	35	0.0149

[a] A Kaplan-Meier survival curve of ovarian cancer patients was
categorized as negative, partial positive *vs.* diffuse positive Skp2 expression.
[b] Log-rank test.

Table 5 Multivariate analysis of survival of individual parameters in
ovarian adenocarcinoma patients

Factors	RR[a]	95% CI[b]	P
Age (<50 yrs *vs.* >51 yrs)	1.304	0.637–2.669	0.4683
Clinical stage (stage I *vs.* stage II/III/IV)	10.395	3.487–30.989	<0.0001
Histologic grade (grade I *vs.* grade 2/3)	1.686	0.912–3.119	0.0957
Skp2 expression (negative or partial *vs.* diffuse)[c]	2.736	1.317–5.680	0.0069

[a] RR, risk ratio.
[b] CI, confidence interval.
[c] Skp2 expression was categorized as negative, partial positive *vs.*
diffuse positive.

Fig. 3 Semiquantitative PCR. *A*, Skp2 mRNA expression was detected in ovarian cancer cell lines OVK18, MCAS, and TYK-nu. *B*, consistent levels of Skp2 expression coamplified with β-tubulin were detected in all ovarian adenocarcinoma cases examined (*Lane 1*, 100-bp marker; *Lanes 2, 3, 5, 8,* and *9,* serous adenocarcinomas; *Lane 4*, endometrioid adenocarcinoma; *Lane 6*, mucinous adenocarcinoma; *Lane 7*, clear cell adenocarcinoma; *Skp2 IHC*, Skp2 immunohistochemistry results; *P*, positive Skp2 staining; *N*, negative Skp2 staining).

ined, consistent levels of PCR products for Skp2 were also detected, confirming expression of Skp2 at the transcriptional level. The expression ratio (mean ± SD) was determined for samples with positive Skp2 immunostaining (1.269 ± 0.236) and samples with no Skp2 immunostaining (1.184 ± 0.230). Interestingly, there was no significant relationship between the Skp2 mRNA and protein expression levels.

DISCUSSION

This study supports a growing body of literature demonstrating that Skp2 expression may contribute to the development and progression of human cancers. Gstaiger *et al.* (29) reported that Skp2 cooperates with H-Ras[G12V] to malignantly transform primary rodent fibroblasts, as scored by both colony formation in soft agar and tumor formation in nude mice. Latres *et al.* (30) also demonstrated that Skp2 cooperates with activated N-Ras in tumorigenesis of T-cell lymphoma in a transgenic mice model. Furthermore, Nelsen *et al.* (31) have reported that cotransfection of Skp2 and cyclin E synergistically promoted cell cycle progression in cultured primary hepatocytes in the absence of mitogen or in the presence of growth inhibitors. They further showed that transfection of hepatocytes *in vivo* with cyclin E and Skp2 promoted abundant hepatocyte replication and hyperplasia of the liver. These findings suggest that Skp2 plays an important role in human oncogenesis. Previous studies have already documented overexpression of Skp2 in several malig-
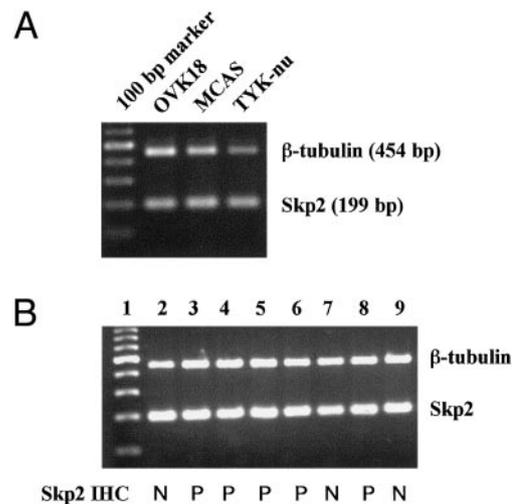
nant tumors, including hepatocellular carcinoma (32), colorectal carcinoma (33), oral squamous cell carcinoma (29, 34), and lymphoma (30). In this study, we found that Skp2 protein was expressed in 47.3% of ovarian adenocarcinoma cases, whereas it was not expressed in either ovarian adenoma or LMP tumor cells. In addition, Skp2 expression significantly correlated with advanced clinical stage, suggesting that Skp2 expression might play an important role in epithelial ovarian cancer development and progression. The most striking association found in the present study was a significant correlation between increased Skp2 protein expression levels and poor prognosis in ovarian adenocarcinoma patients, according to both univariate and multivariate analysis. This is the first reported evidence that Skp2 is associated with aggressive clinical behavior of ovarian adenocarcinoma and that Skp2 is an independent prognostic marker for ovarian adenocarcinoma patients. When we analyzed the associations between diffuse positive Skp2 expression and overall survival in each subgroup of ovarian adenocarcinoma patients, diffuse positive Skp2 expression was significantly correlated with patient survival in patients with advanced-stage disease and in patients with serous adenocarcinomas. Kudo *et al.* (34) observed previously that high expression of Skp2 correlates with a poor prognosis in oral squamous cell carcinoma patients. Therefore, evaluation of Skp2 overexpression could be applicable to the clinical management of ovarian cancer patients, especially those with advanced clinical stage and serous subtype, allowing identification of patients with poor prognoses.

It has been reported that increased Skp2 expression levels are associated with cyclin A overexpression (32) and with reduced p27 expression (29, 30, 33, 34). Skp2 appears to act in tumorigenesis by ubiquitination and subsequent degradation of p27 (9–11). It is well known that reduced p27 expression is

frequently found in various human cancers and is correlated with poor survival of cancer patients (13–22). In fact, Masciullo *et al.* (17) have reported a frequent loss of immunohistochemical p27 expression and a significant correlation between the presence of p27 and a longer time to progression in epithelial ovarian cancer. We also reported previously that the underexpression of p27 caused by posttranslational mechanisms might contribute to the development and progression of disease and might result in a poor prognosis in patients with serous ovarian cancers (18). The present study demonstrates p27 expression in Skp2-negative serous adenoma cells and loss of p27 expression in Skp2-positive serous adenocarcinoma cells in selected ovarian tumor tissues. These findings suggest that increased Skp2 expression may have a causative role in decreasing p27 expression in epithelial ovarian tumors.

The present results showed that the Skp2 mRNA expression levels detected by semiquantitative PCR were similar, regardless of the immunohistochemical Skp2 protein expression data. Although the mechanism of Skp2 protein regulation in cancer tissues is unknown, our results may support the notion that Skp2 protein levels are regulated mainly at the posttranslational level instead of the transcriptional level and that a posttranslational regulation of Skp2 protein could play an important role in ovarian cancer development and progression.

Although our observations will have to be confirmed in a larger number of ovarian cancer patients, the present study demonstrates that Skp2 expression might play an important role in the development and progression of ovarian adenocarcinomas and that Skp2 overexpression is an independent prognostic factor in patients with ovarian adenocarcinoma. Although the molecular mechanisms underlying increased Skp2 expression levels in many cancer cells have not been elucidated, the Skp2-p27 pathway may represent a novel molecular target for human cancer prevention or treatment. Zhang *et al.* (7) have reported that the abolition of Skp2 function by using antisense oligonucleotides or antibodies prevents transformed cells from entering into S phase. The results presented here argue for the use of Skp2 inhibitors and/or Skp2-regulatory sequences as therapeutic agents for the treatment and prevention of ovarian cancers.

REFERENCES

1. MacLachlan, T. K., Sang, N., and Giordano, A. Cyclins, cyclin-dependent kinases and cdk inhibitors: implications in cell cycle control and cancer. Crit. Rev. Eukaryotic Gene Expression, *5:* 127–156, 1995.

2. Sherr, C. J. Cancer cell cycle. Science (Wash. DC), *274:* 1672–1677, 1994.

3. Parker, S. L., Tong, T., Bolden, S., and Wingo, P. A. Cancer statistics. CA Cancer J. Clin., *46:* 5–27, 1996.

4. Deshaies, R. J. SCF and Cullin/Ring H2-based ubiquitin ligases. Annu. Rev. Cell Dev. Biol., *15:* 435–467, 1999.

5. Patton, E. E., Willems, A. R., and Tyers, M. Combinatorial control in ubiquitin-dependent proteolysis: don't Skp the F-box hypothesis. Trends Genet., *14:* 236–243, 1998.

6. Kipreos, E. T., and Pagano, M. The F-box protein family. Genome Biol., *1:* 3002.1–3002.7, 2000.

7. Zhang, H., Kobayashi, R., Galaktionov, K., and Beach, D. p19^{Skp1} and p45^{Skp2} are essential elements of the cyclin A-CDK2 S phase kinase. Cell, *82:* 915–925, 1995.

8. Nakayama, K., Nagahama, H., Minamishima, Y. A., Matsumoto, M., Nakamichi, I., Kitagawa, K., Shirane, M., Tsunematsu, R., Tsukiyama, T., Ishida, N., Kitagawa, M., Nakayama, K., and Hatakeyama, S. Targeted disruption of Skp2 results in accumulation of cyclin E and p27^{Kip1}, polyploidy and centrosome overduplication. EMBO J., *19:* 2069–2081, 2000.

9. Carrano, A. C., Eytan, E., Hershko, A., and Pagano, M. SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat. Cell Biol., *1:* 193–199, 1999.

10. Sutterluty, H., Chatelain, E., Marti, A., Wirbelauer, C., Senften, M., Muller, U., and Krek, W. p45^{SKP2} promotes p27^{Kip1} degradation and induces S phase in quiescent cells. Nat. Cell Biol., *1:* 207–214, 1999.

11. Tsvetkov, L. M., Yeh, K. H., Lee, S. J., Sun, H., and Zhang, H. p27^{Kip1} ubiquitination and degradation is regulated by the SCF^{Skp2} complex through phosphorylated Thr187 in p27. Curr. Biol., *9:* 661–664, 1999.

12. Reynisdottir, I., Polyak, K., Iavarone, A., and Massague, J. Kip/Cip and Ink4 cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-β. Genes Dev., *9:* 1831–1845, 1995.

13. Porter, P. L., Malone, K. E., Heagerty, P. J., Alexander, G. M., Gatti, L. A., Firpo, E. J., Daling, J. R., and Roberts, J. M. Expression of cell-cycle regulators p27^{Kip1} and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. Nat. Med., *3:* 222–225, 1997.

14. Catzavelos, C., Bhattacharya, N., Ung, Y. C., Wilson, J. A., Roncari, L., Sandhu, C., Shaw, P., Yeger, H., Morava-Protzner, I., Kapusta, L., Franssen, E., Pritchard, K. I., and Slingerland, J. M. Decreased levels of the cell-cycle inhibitor p27^{Kip1} protein: prognostic implications in primary breast cancer. Nat. Med., *3:* 227–230, 1997.

15. Wu, J., Shen, Z. Z., Lu, J. S., Jiang, M., Han, Q. X., Fontana, J. A., Barsky, S. H., and Shao, Z. M. Prognostic role of p27^{Kip1} and apoptosis in human breast cancer. Br. J. Cancer, *79:* 1572–1578, 1999.

16. Sui, L., Tokuda, M., Ohno, M., Hatase, O., and Hando, T. The concurrent expression of p27 (kip1) and cyclin D1 in epithelial ovarian tumors. Gynecol. Oncol., *73:* 202–209, 1999.

17. Masciullo, V., Sgambato, A., Pacilio, C., Pucci, B., Ferrandina, G., Palazzo, J., Carbone, A., Cittadini, A., Mancuso, S., Scambia, G., and Giordano, A. Frequent loss of expression of the cyclin-dependent kinase inhibitor p27 in epithelial ovarian cancer. Cancer Res., *59:* 3790–3794, 1999.

18. Shigemasa, K., Shiroyama, Y., Sawasaki, T., Fujii, T., Nagai, N., Parmley, T. H., O'Brien, T. J., and Ohama, K. Underexpression of cyclin-dependent kinase inhibitor p27 is associated with poor prognosis in serous ovarian carcinomas. Int. J. Oncol., *18:* 953–958, 2001.

19. Mori, M., Mimori, K., Shiraishi, T., Tanaka, S., Ueo, H., Sugimachi, K., and Akiyoshi, T. p27 expression and gastric carcinoma. Nat. Med., *3:* 593, 1997.

20. Loda, M., Cukor, B., Tam, S. W., Lavin, P., Fiorentino, M., Draetta, G. F., Jessup, J. M., and Pagano, M. Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. Nat. Med., *3:* 231–234, 1997.

21. Guo, Y., Sklar, G. N., Borkowski, A., and Kyprianou, N. Loss of the cyclin-dependent kinase inhibitor p27^{Kip1} protein in human prostate cancer correlates with tumor grade. Clin. Cancer Res., *3:* 2269–2274, 1997.

22. Bamberger, A. M., Riethdorf, L., Milde, L. K., Bamberger, C. M., Thuneke, I., Erdmann, I., Schulte, H. M., and Loning, T. Strongly reduced expression of the cell cycle inhibitor p27 in endometrial neoplasia. Virchows Arch., *434:* 423–428, 1999.

23. Russel, P. Surface epithelial-stromal tumors of the ovary. *In*: R. J. Kurman (ed.), Blaustein's Pathology of the Female Genital Tract, pp. 705–782. New York: Springer-Verlag, 1995.

24. International Federation of Gynecology and Obstetrics (FIGO) Committee on Gynecologic Oncology, Benedet, J. L., Bender, H., Jones, H., III, Ngan, H. Y. S., and Pecorelli, S. FIGO staging classifications and clinical practice guidelines in the management of gynecological cancers. Int. J. Gynecol. Obstet., *70:* 209–262, 2000.

25. Shigemasa, K., Hu, C., West, C. M., Clarke, J., Parham, G. P., Parmley, T. H., Korourian, S., Baker, V. V., and O'Brien, T. J. p16 overexpression: a potential early indicator of transformation in ovarian carcinoma. J. Soc. Gynecol. Invest., *4:* 95–102, 1997.

26. Shigemasa, K., Hu, C., West, C. M., Moon, S. H., Parham, G. P., Parmley, T. H., Korourian, S., Baker, V. V., and O'Brien, T. J. p21: a monitor of p53 dysfunction in ovarian neoplasia. Int. J. Gynecol. Cancer, *7:* 296–303, 1997.

27. Shigemasa, K., Tanimoto, H., Parham, G. P., Parmley, T. H., Ohama, K., and O'Brien, T. J. Cyclin D1 overexpression and p53 mutation status in epithelial ovarian cancer. J. Soc. Gynecol. Invest., *6:* 102–108, 1999.

28. Shigemasa, K., Underwood, L. J., Beard, J., Tanimoto, H., Ohama, K., Parmley, T. H., and O'Brien, T. J. Overexpression of testisin, a serine protease expressed by testicular germ cells, in epithelial ovarian tumor cells. J. Soc. Gynecol. Invest., *7:* 358–362, 2000.

29. Gstaiger, M., Jordam, R., Lim, M., Catzavelos, C., Mestan, J., Slingerland, J., and Krek, W. Skp2 is oncogenic and overexpressed in human cancers. Proc. Natl. Acad. Sci. USA, *98:* 5043–5048, 2001.

30. Latres, E., Chiarle, R., Schulman, B. A., Pavletich, N. P., Pellicer, A., Inghirami, G., and Pagano, M. Role of the F-box protein Skp2 in lymphomagenesis. Proc. Natl. Acad. Sci. USA, *98:* 2515–2520, 2001.

31. Nelsen, C. J., Hansen, L. K., Rickheim, D. G., Chen, C., Stanley, M. W., Krek, W., and Albrecht, J. H. Induction of hepatocyte proliferation and liver hyperplasia by the targeted expression of cyclin E and skp2. Oncogene, *20:* 1825–1831, 2001.

32. Chao, Y., Shih, Y-L., Chiu, J-H., Chau, G-Y., Lui, W-Y., Yang, W. K., Lee, S-D., and Huang, T-S. Overexpression of cyclin A but not Skp 2 correlates with the tumor relapse of human hepatocellular carcinoma. Cancer Res., *58:* 985–900, 1998.

33. Hershko, D., Bornstein, G., Ben-Izhak, O., Carrano, A., Pagano, M., Krausz, M. M., and Hershko, A. Inverse relation between levels of p27[Kip1] and of its ubiquitin ligase subunit Skp2 in colorectal carcinomas. Cancer (Phila.), *91:* 1745–1751, 2001.

34. Kudo, Y., Kitajima, S., Sato, S., Miyauchi, M., Ogawa, I., and Takata, T. High expression of S-phase kinase-interacting protein 2, human F-box protein, correlates with poor prognosis in oral squamous cell carcinomas. Cancer Res., *61:* 7044–7047, 2001.

Clinical Cancer Research

Skp2 Overexpression Is a Prognostic Factor in Patients with Ovarian Adenocarcinoma

Kazushi Shigemasa, Lijun Gu, Timothy J. O'Brien, et al.

Updated version	Access the most recent version of this article at: http://clincancerres.aacrjournals.org/content/9/5/1756

Cited articles	This article cites 31 articles, 8 of which you can access for free at: http://clincancerres.aacrjournals.org/content/9/5/1756.full#ref-list-1
Citing articles	This article has been cited by 7 HighWire-hosted articles. Access the articles at: http://clincancerres.aacrjournals.org/content/9/5/1756.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/9/5/1756. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.