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# Skp2 is an independent prognosticator of gallbladder carcinoma among p27$^{Kip1}$-interacting cell cycle regulators: an immunohistochemical study of 62 cases by tissue microarray

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**Despite improvement in surgical techniques, prognosis of gallbladder carcinoma remains poor. It is desirable to identify prognostic biomarkers to aid in the development of targeted therapeutic strategies. Two SCF$^{Skp2}$ ubiquitin ligase-related proteins, Skp2 and cyclin-dependent kinase subunit 1 (Cks1), are involved in post-transcriptional degradation of p27$^{Kip1}$ tumor suppressor, which inhibits both cdk2/cyclin E and cdk2/cyclin A complexes and thus prevents transition to the S phase. However, the prognostic utility of p27$^{Kip1}$-interacting cell cycle regulators has not been systematically assessed in gallbladder carcinoma. Immunohistochemistry was performed for p27$^{Kip1}$, Skp2, Cks1, cyclin E, cyclin A, and Ki-67 in tissue microarrays of 62 gallbladder carcinomas with follow-up. The data were correlated with clinicopathological features and overall survival (OS). The cumulative OS rate for all 62 cases was 42.9% at 3 years. Aberrant labeling indices (LIs) of p27$^{Kip1}$ ($<20\%$), cyclin E ($\geq 5\%$), cyclin A ($\geq 5\%$), Cks1 ($\geq 40\%$), and Skp2 ($\geq 10\%$) were identified in 29, 58, 66, 21, and 57% of gallbladder carcinomas, respectively. By log-rank tests, downregulation of p27$^{Kip1}$ ($P=0.0319$) and high LIs of Skp2 ($P=0.0006$), Cks1 ($P=0.0460$), cyclin E ($P=0.0070$), and Ki-67 ($P=0.0037$) were predictive of inferior OS. Furthermore, the combined expression status of Skp2 and Ki-67 robustly defined three prognostically different groups ($P=0.0001$). In multivariate comparison, Skp2 overexpression represented the strongest independent adverse prognosticator ($P=0.004$, risk ratio (RR): 5.538), followed by Ki-67 LI $\geq 50\%$ ($P=0.016$, RR: 3.254) and American Joint Committee on Cancer stages II–IV ($P=0.013$, RR: 3.163). In conclusion, aberrations of p27$^{Kip1}$-interacting cell cycle regulators are common in gallbladder carcinomas. Skp2 overexpression is highly representative of biological aggressiveness and independently associated with poor OS, suggesting that it is a promising novel target for therapeutic intervention in aggressive cases. The combined assessment of Skp2 and Ki-67 LIs effectively risk-stratifies gallbladder carcinomas with different prognosis, which is worth being prospectively validated in future study.**
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Despite improvement in surgical techniques, prognosis of gallbladder carcinoma generally remains poor.[1,2] Among various clinicopathologic parameters, tumour, node, metastasis (TNM) stage is indisputably accepted as an adverse prognosticator in patients with gallbladder carcinoma.[1–3] The effective therapy to cure gallbladder carcinoma is

complete surgical resection at the early stage, whereas most patients present with advanced disease and have few therapeutic options.[1,2] In this context, the survival rate is as high as 85–100% at 5 years for lesions limited to the muscle layer.[2] Nevertheless, even after extensive surgery, a considerable number of patients with stage II–IV gallbladder carcinoma develop distant or intra-abominal metastases that are resistant to conventional chemotherapy.[2]

Sequential morphological alterations from precancerous dysplasia or adenoma to carcinoma have been regarded as models implicated in tumorigenesis of gallbladder carcinoma.[4] However, owing to the rarity of gallbladder carcinoma, the molecular mechanisms underlying its initiation and progression are not fully elucidated. During multistep carcinogenesis, deregulation of multiple cell-cycle regulators represents a common mode of genetic alterations that promote progression in aggressive tumor subsets, thereby conferring an adverse impact on patient survival.[5] Prior studies have characterized genetic aberrations of K-ras oncogene and p53 tumor-suppressor gene as early events in evolution of gallbladder carcinoma, whereas these alterations are not associated with tumor progression and prognosis.[6,7] Accordingly, identification of prognostic biomarkers by better elucidating the molecular basis of gallbladder carcinoma may provide a useful insight that aid in the development of novel targeted therapeutic strategies.

Physiologically, cyclin E levels determine the time point when cells enter S phase,[8,9] and its critical oncogenic role is also shown by its overexpression and profound prognostic impact in breast, ovarian, and non-small cell pulmonary carcinomas, etc.[10–15] Cyclin A is not only required for DNA replication during S-phase progression but also active in the initiation of mitosis.[15,16] Increased cyclin A level has been linked to accelerated cell proliferation and associated with shorter survival in several types of cancers.[15–18] $P27^{Kip1}$ is a new tumor suppressor that specifically inhibits the activity of both cyclin E/CDK2 complex in G1/S transition and cyclin A/CDK2 complex in S phase to control cell cycle progression.[9,13] Reduced expression of $p27^{Kip1}$ protein was recently found correlated with tumor progression and prognosis in gallbladder carcinoma.[19,20] However, downregulation of $p27^{Kip1}$ mRNA is rarely observed in human malignancies.[9] Instead, it becomes apparent that aberrant loss of $p27^{Kip1}$ protein in malignant diseases results mainly from enhanced ubiquitin-mediated proteolysis regulated by Skp2, a member of the F-box protein family, which targets $Thr^{187}$-phosphorylated $p27^{Kip1}$ for degradation.[21–25] More recently, human cyclin-dependent kinase subunit 1 (Cks1) has been identified as a cofactor in the ubiquitination and degradation of $p27^{Kip1}$, which increases the affinity of $SCF^{SKP2}$ complex to phosphorylated $p27^{Kip1}$.[26] Furthermore, the prognostic values of Skp2 and Cks1 proteins have been separately substantiated in colorectal, gastric, and head and neck carcinomas, etc.[21,27–29] Nevertheless, Skp2 or Cks1 did not correlate with low $p27^{Kip1}$ in some malignancies, implying that additional factors may be implicated in $p27^{Kip1}$ degradation.[22,30,31]

To date, very few studies have systematically addressed the prognostic utility of $p27^{Kip1}$-interacting cell cycle regulators in gallbladder carcinoma. By using tissue microarray (TMA), we therefore aimed at analyzing the immunohistochemical expression patterns, associations with clinicopathologic factors and proliferative index, and prognostic implications of cyclin E, cyclin A, $p27^{Kip1}$, Skp2, and Cks1 proteins in 62 cases of gallbladder carcinoma with follow-up information.

## Materials and methods

### Patients and Tumor Material

Ninety-eight cases of gallbladder carcinoma undergoing primary surgery between 1994 and 2004 were retrieved from the databases of two tertiary medical centers in Southern Taiwan. Of these, 62 cases with available paraffin blocks and follow-up were retrospectively classified for histological types and tumor grading according to the latest World Health Organization classification.[1] Other histopathological features evaluated included the presence or absence of spontaneous tumor necrosis and vascular invasion. In addition, pathological staging was determined based on the 6th edition of American Joint Committee on Cancer (AJCC) system.[32] Institutional review boards of the participating hospitals approved retrospective clinical data collection and procurement of archival tissues. Surgical procedures were cholecystectomy in 54 patients and extended operation in the remaining 10 patients. The former procedure comprised single cholecystectomy or cholecystectomy with resection of less than 2 cm depth of the liver bed, whereas extended operation included resection of adjacent organs in addition to the gallbladder. The median period of follow-up was 29 months (range, 1–186) for all 62 patients. For 28 survivors, the minimal and median durations of follow-up were 6 and 31 months, respectively.

### Construction of TMA Blocks and Immunohistochemistry

Recut hematoxylin and eosin-stained sections were examined to define representative tumor and non-neoplastic tissues. Corresponding paraffin blocks were then precisely aligned with the marked slides. To circumvent the problem of tissue heterogeneity, six tissue cylinders (0.6 mm in diameter) for each gallbladder carcinoma specimen were punched using a precision instrument (Beecher Instruments,

Silver Spring, MD, USA) and arrayed into two recipient blocks. Nonneoplastic normal tissues adjacent to gallbladder carcinoma and seven gallbladder adenomas were punched in parallel for comparison. In addition, we also arrayed three each carcinomas of the colorectum, lung, and breast for the purposes of orientation and external positive controls in each TMA block. The procedures of immunohistochemical studies were performed as described previously.[22] In brief, sections of TMA blocks were cut onto an adhesive-coated glass slide system (instrumedicus, Hackensack, NJ, USA) at 3 $\mu$m thickness. The slides were incubated with primary antibodies targeting Skp2 (2C8D9, 1:100, Zymed), Cks1 (4G12G7, 1:250, Zymed), $p27^{Kip1}$ (1B4, 1:20, Novocastra), cyclin A (6E6, 1:50, Novocastra), cyclin E (13A3, 1:40, Novocastra), and Ki-67 (MIB-1, 1:100, DAKO). Primary antibodies were detected using the ChemMate DAKO EnVision kit (DAKO, K5001). The slides were incubated with the secondary antibody for 30 min and developed with 3,3-diaminobenzidine for 5 min. Incubation without the primary antibody was used as a negative control.

### Assessment of Immunohistochemical Staining

One pathologist (CFL) blinded to follow-up data independently evaluated the TMA slides. The percentage of tumor cells with definite moderate to intense nuclear immunoreactivity was recorded. Only cases containing two or more preserved tissue cores with tumor cellularity $\geq$50% were scored, and the median of scores from multiple cores in the same patient was adopted as the labeling index (LI) for each marker. By testing a series of different values (see Statistical Analyses), the cutoffs of LIs to define overexpression or downregulation of proteins were determined as follows: (1) Skp2 overexpression if $\geq$10% of tumor nuclei stained, (2) downregulation of $p27^{Kip1}$ if $<$20% of tumor nuclei stained, (3) Cks1 overexpression if $\geq$40% of tumor nuclei stained, (4) cyclin A overexpression if $\geq$5% of tumor nuclei stained, (5) cyclin E overexpression if $\geq$5% of tumor nuclei stained, and (6) high Ki-67 index if $\geq$50% of tumor nuclei stained.

### Statistical Analyses

Statistical analyses were performed using the SPSS 10 software package. The associations among clinicopathologic factors and expression of protein markers were evaluated using the $\chi^2$ test or Fisher's exact test as appropriate. The end point analyzed was overall survival (OS), which was calculated from the date of operation until death or last follow-up appointment. A series of cutoff values were tested for continuous variables, such as age and LIs of markers. The cutoffs giving the best *P*-values were adopted to construct Kaplan–Meier curves and compare prognostic differences by univariate log-rank test. A multivariate model was performed using stepwise forward Cox proportional hazards regression, including parameters with univariate $P<0.1$. For all analyses, two-sided tests of significance were used with $P<0.05$ considered significant.

## Results

### Clinicopathological Findings and Follow-up

Salient clinical and pathological data are summarized in Table 1. There was an apparent female predilection in the study cohort of 62 patients, consisting of 22 males and 40 females. The patient age at presentation ranged from 48 to 89 years (median, 68; mean 67.5), with 27 patients (43.6%) aged $\geq$70 years. The histologic types were 47 tubular adenocarcinomas, nine invasive papillary adenocarcinomas, three adenosquamous carcinomas, two undifferentiated carcinomas, and one clear cell carcinoma, which were further classified into 35 grade 1 (Figure 1a), 18 grade 2, seven grade 3 (Figure 1b), and two grade 4 lesions. The T stages of 62 gallbladder carcinomas were T1 in 14 cases, T2 in 21 cases, and T3 in 27 cases, and 33, 27, and two cases were categorized as AJCC stage I, II, and IV, respectively. Of nine papillary adenocarcinomas, the invasive components ranged from lamina propria (T1a) to perimuscular connective tissue (T2) in depth. Vascular invasion was present in 24 cases, and 26 cases displayed tumor necrosis. At last

**Table 1** Clinicopathologic features of 62 gallbladder carcinomas

| *Parameters* | | *No. of cases (percentage)* |
|---|---|---|
| Sex | Male | 22 (35.48) |
| | Female | 40 (64.52) |
| Age (years) (mean: 67.45, median: 68, range 48–89 ) | $\leq$60 | 17 (27.42) |
| | 61–70 | 18 (29.03) |
| | 71–80 | 20 (32.26) |
| | 81–90 | 7 (11.29) |
| Primary tumor (T) | T1 | 14 (22.58) |
| | T2 | 21 (33.87) |
| | T3 | 27 (43.55) |
| Stage | I | 33 (53.23) |
| | II | 27 (43.55) |
| | III | 0 (0) |
| | IV | 2 (3.23) |
| Histologic type | Papillary | 9 |
| | Tubular | 47 |
| | Other | Undifferentiated (2); clear cell (1); Adenosquamous (3) |
| Histologic grade | Grade 1 | 35 (56.45) |
| | Grade 2 | 18 (29.03) |
| | Grade 3 | 7 (11.29) |
| | Grade 4 | 2 (3.23) |
| Vascular invasion | Present | 24 (38.71) |
| | Absent | 38 (61.29) |
| Tumor necrosis | Present | 26 (41.94) |
| | Absent | 36 (58.06) |

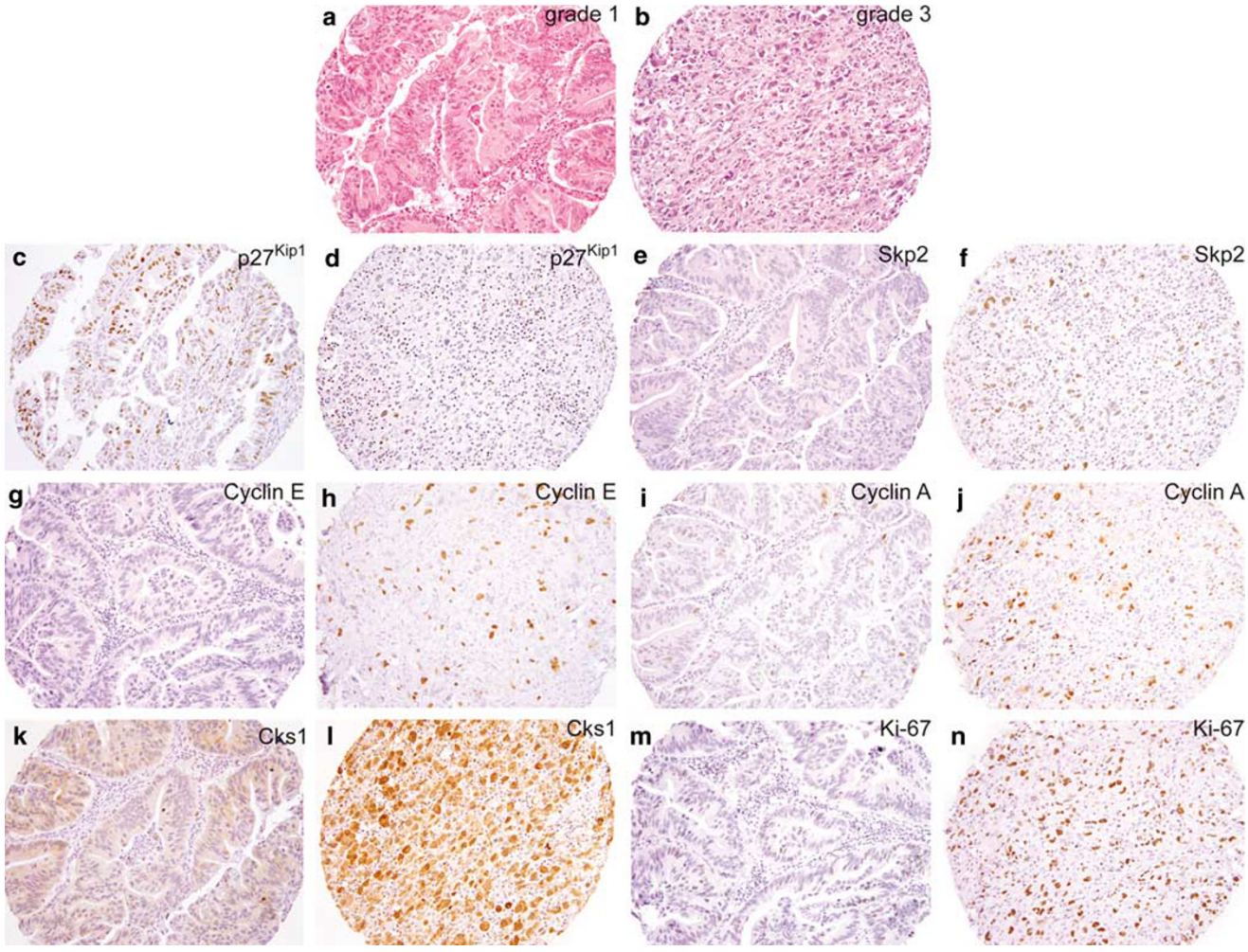


**Figure 1** Histologic features and immunohistochemical expression of $p27^{Kip1}$, Skp2, cyclin E, cyclin A, Cks1, and Ki-67 in grade 1 (**a**) and grade 3 (**b**) gallbladder carcinomas. (Note that the minor tubular component was found in the whole section but not in the illustrated tissue core of this grade 3 lesion.) $P27^{Kip1}$ expression was preserved in grade 1 gallbladder carcinomas (**c**) but lost in grade 3 lesions (**d**). Expression levels of Skp2 (**e**, **f**), cyclin E (**g**, **h**), cyclin A (**i**, **j**), Cks1 (**k**, **l**), and Ki-67 (**m**, **n**) are lower in grade 1 gallbladder carcinomas but significantly overexpressed in grade 3 lesions.

follow-up, 25 patients were alive without evidence of disease, three were alive with relapsed disease, 26 patients died of gallbladder carcinoma, and eight died of unrelated causes.

### Profiling of Immunohistochemical Expression and Correlations with Clinicopathologic Variables and Proliferative Index

TMA-based immunohistochemical data were interpretable and scored for cyclin E, cyclin A, $p27^{Kip1}$, Skp2, Cks1, and Ki-67 in 50, 56, 59, 56, 57, and 55 cases of gallbladder carcinoma, respectively. By using the cutoffs of LIs described in Materials and methods, the normal nonneoplastic epithelial cells and all seven gallbladder adenomas did not show aberrant expression of cyclin E, $p27^{Kip1}$ and Cks1, whereas Skp2 and cyclin A were overexpressed in one adenoma each. The expression of $p27^{Kip1}$ displayed a wide variation in LI from 0 to 100% (median, 35%; Figure 1c and d), and downregulation of $p27^{Kip1}$ was identified in 17 cases (29%, Figure 1d). The LI of Skp2 ranged from 0 to 70% of the carcinoma cells (median, 11%; Figure 1e and f), and distinct overexpression of Skp2 was identified in 32 cases (57%, Figure 1f). In addition, cyclin E (median, 8%, range, 0–85%; Figure 1g and h), cyclin A (median, 8.5%, range, 0–32%; Figure 1i and j), Cks1 (median, 17%, range, 0–100%; Figure 1k and l), and Ki-67 (median, 50%, range, 10–85%; Figure 1m and n) were overexpressed in 29 (58%, Figure 1h), 37 (66%, Figure 1j), 12 (21%, Figure 1l), and 28 (51%, Figure 1n) cases of gallbladder carcinoma, respectively.

The associations between expression status of various immunohistochemical markers and clinicopathological variables are listed in Table 2. Cyclin E and Skp2 were preferentially overexpressed in females ($P = 0.012$) and males ($P = 0.044$), respec-

**Table 2** Associations between immunohistochemical markers and clinicopathologic factors

| | *p27$^{kip1}$* | | | *Cyclin E* | | | *Cyclin A* | | |
|---|---|---|---|---|---|---|---|---|---|
| | *<20%* | *≥20%* | P | *<5%* | *≥5%* | P | *<5%* | *≥5%* | P |
| *Age* | | | | | | | | | |
| <70 years | 6 | 26 | 0.063 | 11 | 15 | 0.963 | 10 | 19 | 0.928 |
| ≥70 years | 11 | 16 | | 10 | 14 | | 9 | 18 | |
| *Sex* | | | | | | | | | |
| Male | 4 | 16 | 0.284 | 18 | 15 | 0.012* | 15 | 22 | 0.145 |
| Female | 13 | 26 | | 3 | 14 | | 4 | 15 | |
| *Histologic type* | | | | | | | | | |
| Tubular | 15 | 30 | 0.310 | 18 | 19 | 0.108 | 17 | 25 | 0.106 |
| Others | 2 | 12 | | 3 | 10 | | 2 | 12 | |
| *Grade* | | | | | | | | | |
| 1 | 8 | 25 | 0.382 | 18 | 12 | 0.002* | 15 | 15 | 0.006* |
| 2+3+4 | 9 | 17 | | 3 | 17 | | 4 | 22 | |
| *Vascular invasion* | | | | | | | | | |
| Absent | 9 | 26 | 0.526 | 17 | 14 | 0.019* | 15 | 18 | 0.029* |
| Present | 8 | 16 | | 4 | 15 | | 4 | 19 | |
| *Necrosis* | | | | | | | | | |
| Absent | 11 | 22 | 0.388 | 16 | 11 | 0.007* | 16 | 15 | 0.002* |
| Present | 6 | 20 | | 5 | 18 | | 3 | 22 | |
| *T stage* | | | | | | | | | |
| T1+2 | 8 | 24 | 0.481 | 14 | 15 | 0.291 | 12 | 19 | 0.400 |
| T3+4 | 9 | 18 | | 7 | 14 | | 7 | 18 | |
| *AJCC stage* | | | | | | | | | |
| I | 8 | 22 | 0.711 | 14 | 13 | 0.126 | 12 | 17 | 0.222 |
| II+III+IV | 9 | 20 | | 7 | 16 | | 7 | 20 | |
| *Ki-67* | | | | | | | | | |
| <50% | 9 | 18 | 0.496 | 14 | 7 | 0.0001* | 13 | 11 | 0.002* |
| ≥50% | 7 | 21 | | 4 | 21 | | 4 | 24 | |
| | *Cks*1 | | | *Skp2* | | | *Ki-67* | | |
| | *<40%* | *≥40%* | P | *<10%* | *≥10%* | P | *<50%* | *≥50%* | P |
| *Age* | | | | | | | | | |
| <70 years | 26 | 5 | 0.319 | 14 | 16 | 0.536 | 18 | 12 | 0.076 |
| ≥70 years/o | 19 | 7 | | 10 | 16 | | 9 | 16 | |
| *Sex* | | | | | | | | | |
| Male | 14 | 5 | 0.509 | 5 | 15 | 0.044* | 8 | 12 | 0.308 |
| Female | 31 | 7 | | 19 | 17 | | 19 | 16 | |
| *Histologic type* | | | | | | | | | |
| Tubular | 34 | 9 | 1.000 | 20 | 23 | 0.315 | 23 | 18 | 0.075 |
| Others | 11 | 3 | | 4 | 9 | | 4 | 10 | |
| *Grade* | | | | | | | | | |
| 1 | 29 | 3 | 0.014* | 19 | 14 | 0.008* | 19 | 11 | 0.021* |
| 2+3+4 | 16 | 9 | | 5 | 18 | | 8 | 17 | |
| *Vascular invasion* | | | | | | | | | |
| Absent | 28 | 6 | 0.517 | 20 | 14 | 0.003* | 22 | 11 | 0.001* |
| Present | 17 | 6 | | 4 | 18 | | 5 | 17 | |
| *Necrosis* | | | | | | | | | |
| Absent | 29 | 2 | 0.003* | 18 | 16 | 0.058 | 18 | 12 | 0.076 |
| Present | 16 | 10 | | 6 | 16 | | 9 | 16 | |
| *T stage* | | | | | | | | | |
| T1+2 | 23 | 7 | 0.656 | 17 | 14 | 0.044* | 14 | 14 | 0.891 |
| T3+4 | 22 | 5 | | 7 | 18 | | 13 | 14 | |

**Table 2** Continued

| | *Cks*1 | | | *Skp2* | | | *Ki-67* | | |
|---|---|---|---|---|---|---|---|---|---|
| | *<40%* | *≥40%* | P | *<10%* | *≥10%* | P | *<50%* | *≥50%* | P |
| *AJCC stage* | | | | | | | | | |
| I | 22 | 6 | 0.945 | 16 | 14 | 0.089 | 14 | 12 | 0.504 |
| II+III+IV | 23 | 6 | | 8 | 18 | | 13 | 16 | |
| *Ki-67* | | | | | | | | | |
| <50% | 26 | 1 | 0.002* | 16 | 9 | 0.004* | | | |
| ≥50% | 17 | 10 | | 6 | 19 | | | | |

*Statistically significant.

tively, whereas $p27^{Kip1}$ downregulation ($P=0.063$) only reached a trend toward significance in older patients. As compared to grade 1 gallbladder carcinomas, the expression levels of cyclin E ($P=0.002$), cyclin A ($P=0.006$), Skp2 ($P=0.008$), Cks1 ($P=0.014$), and Ki-67 ($P=0.021$) were significantly higher in grade 2–4 lesions. Vascular invasion was significantly associated with higher LIs of Ki-67 ($P=0.001$), Skp2 ($P=0.003$), cyclin E ($P=0.019$), and cyclin A ($P=0.029$). In addition, the latter two markers ($P=0.007$ for cyclin E; $P=0.002$ for cyclin A), together with Cks1 ($P=0.003$), were also significantly overexpressed in gallbladder carcinomas with tumor necrosis. Skp2 represented the only marker significantly associated with primary T stage ($P=0.044$) but marginally related to AJCC stage ($P=0.089$). Furthermore, we also found that gallbladder carcinomas overexpressing cyclin E ($P=0.0001$), cyclin A ($P=0.002$), Cks1 ($P=0.002$), and skp2 ($P=0.004$) had higher proliferative activity as determined by Ki-67 LI. Nevertheless, this association with cell proliferation did not hold true in gallbladder carcinomas with $p27^{Kip1}$ downregulation. Neither Skp2 ($P=0.394$) nor Cks1 ($P=0.477$) LIs was inversely related to $p27^{Kip1}$ expression level. In addition, none of the markers tested was associated with the histologic type.

### Survival Analyses

Correlations of clinicopathological and immunohistochemical factors with OS are shown in Table 3 and Figure 2a–j. The cumulative 3-year rate of OS for all 62 patients was 42.9%. In univariate analyses, the following parameters were significantly predictive of inferior OS, including age ≥70 years ($P=0.0111$), vascular invasion ($P=0.0046$, Figure 2a), $T_{3+4}$ stages ($P=0.0055$, Figure 2b), AJCC stages II–IV ($P=0.0092$, Figure 2c), $p27^{Kip1}$ downregulation ($P=0.0319$, Figure 2d), and higher LIs of Skp2 ($P=0.0006$, Figure 2e), cyclin E ($P=0.007$, Figure 2f), Cks1 ($P=0.046$, Figure 2g), and Ki-67 ($P=0.0037$, Figure 2h). However, higher histologic grades ($P=0.0957$) and cyclin A overexpression ($P=0.0751$, Figure 2i) only marginally correlated with adverse outcomes.

In multivariate comparison (Table 4), Skp2 overexpression ($P=0.004$, risk ratio (RR), 5.538) represented the strongest independent negative prognosticator of OS, followed by Ki-67 LI ≥50% ($P=0.016$, RR, 3.254) and advanced AJCC stage ($P=0.013$, RR, 3.163). However, other factors lost statistical significance. The 3-year rates of OS were 20.3% for cases with Skp2 overexpression and 23.5% for those with $p27^{Kip1}$ downregulation. In contrast, the rates increased to 67.7 and 51.3% for those without deregulation of Skp2 and $p27^{Kip1}$, respectively.

Moreover, we further found that the combined expression status of Skp2 and Ki-67 was robust in identifying three prognostic groups with a balanced case distribution and significantly different survival outcomes. The OS rates were 0, 38.1, and 64% at 3 years for gallbladder carcinomas overexpressing both ($n=19$), either one ($n=15$), and none ($n=15$) of Skp2 and Ki-67, respectively ($P=0.0001$, Figure 2j).

## Discussion

The management of advanced gallbladder carcinoma is highly challenging, and accurate risk stratification may assist in selection of candidate patients who will benefit from tailored targeted therapy.[2] It is therefore highly desirable to search effective prognostic biomarkers that are responsible for promoting tumor progression of gallbladder carcinoma, apart from AJCC staging.[2,5] Recently, components of ubiquitin-mediated proteolytic pathway have become attractive targets in anticancer drug development, because they are frequently altered in human cancers.[33] Except for $p27^{Kip}$, expression levels of other markers in the present series were all positively related to tumor cell proliferation, as determined by the Ki-67 LI (Table 2). Furthermore, high expression in most markers, eg cyclin E, cyclin A, Cks1, and Skp2, was more frequently observed in a subset of gallbladder carcinomas characterized by higher grade, more advanced T stages, and/or presence of tumor necrosis or vascular invasion. These findings suggested that the synergistic interaction of cumulative abnormalities in this pathway

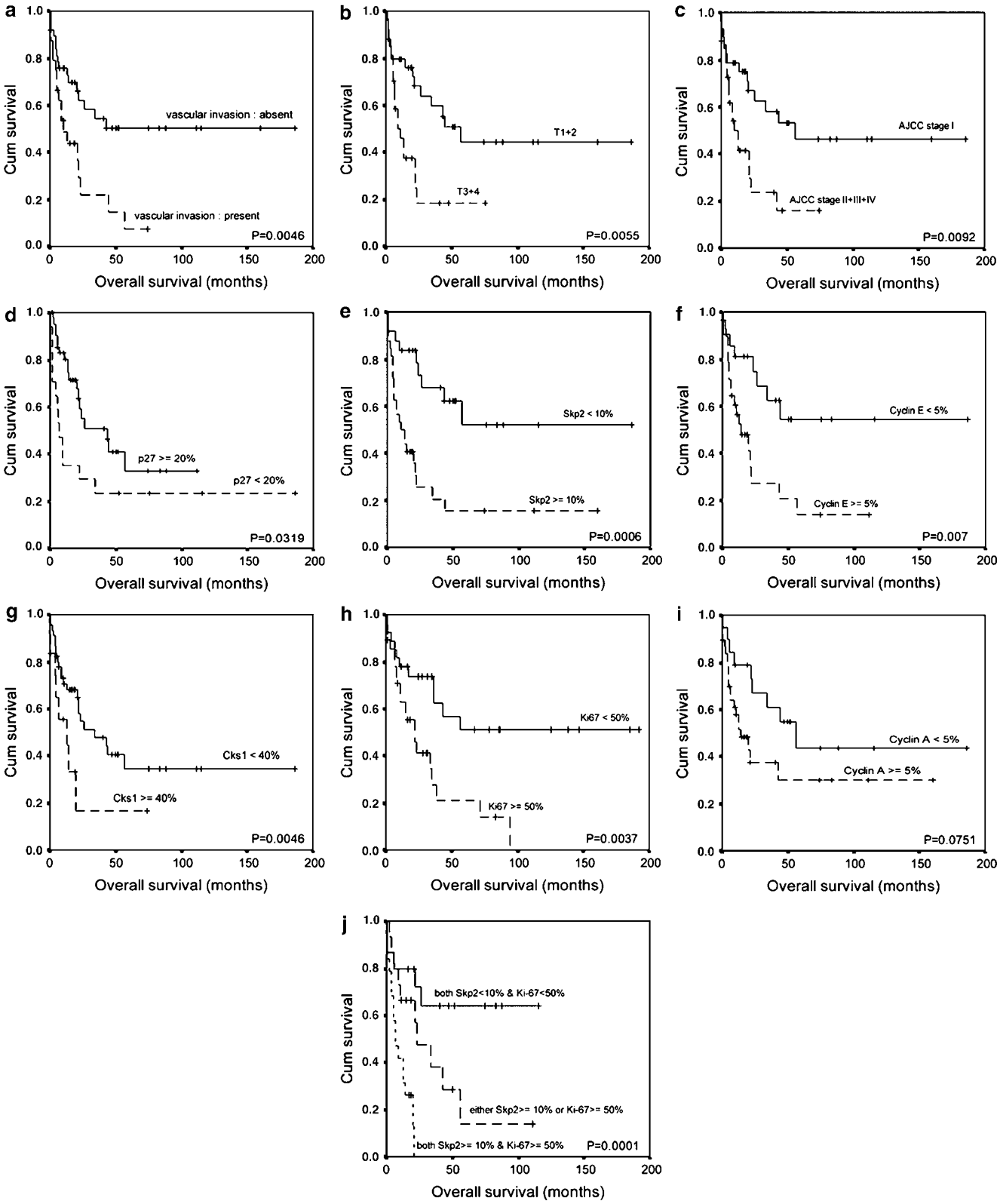


**Figure 2** By log-rank tests, OS of patients with gallbladder carcinoma were significantly associated with the status of vascular invasion (**a**), T stage (**b**), AJCC stage (**c**), and expression levels of $p27^{Kip1}$ (**d**), Skp2 (**e**), cyclin E (**f**), Cks1 (**g**), and Ki-67 (**h**). However, expression level of cyclin A (**i**) only reached a trend to predict prognosis. In addition, the combination of Skp2 and Ki-67 (**j**) effectively classify three prognostically different groups of patients with gallbladder carcinoma.

**Table 3** Results of univariate log-rank analysis of prognostic factors

| *Factors* | *No. of patients* | *Overall survival* | |
|---|---|---|---|
| | | *No. of events* | P |
| *Age* | | | |
| <70 years | 35 | 15 | 0.0111* |
| ≥70 years | 27 | 19 | |
| *Sex* | | | |
| Male | 22 | 12 | 0.2521 |
| Female | 40 | 22 | |
| *Histologic type* | | | |
| Tubular | 47 | 25 | 0.6477 |
| Others | 15 | 9 | |
| *Histologic grade* | | | |
| 1 | 35 | 18 | 0.0957 |
| 2+3+4 | 27 | 16 | |
| *Vascular invasion* | | | |
| Absent | 38 | 16 | 0.0046* |
| Present | 24 | 18 | |
| *Necrosis* | | | |
| Absent | 36 | 22 | 0.6136 |
| Present | 26 | 12 | |
| *Primary tumor* | | | |
| T1+2 | 35 | 15 | 0.0055* |
| T3+4 | 27 | 19 | |
| *AJCC stage* | | | |
| I | 33 | 14 | 0.0092* |
| II+III+IV | 29 | 20 | |
| *$P27^{kip1}$* | | | |
| ≤20% | 17 | 13 | 0.0319* |
| ≥20% | 42 | 19 | |
| *Cyclin E* | | | |
| <5% | 21 | 8 | 0.007* |
| ≤5% | 29 | 19 | |
| *Cyclin A* | | | |
| <5% | 19 | 9 | 0.0751 |
| ≥5% | 37 | 21 | |
| *Cks1* | | | |
| <40% | 45 | 23 | 0.046* |
| ≥40% | 12 | 8 | |
| *Skp2* | | | |
| <10% | 24 | 9 | 0.0006* |
| ≥10% | 32 | 24 | |
| *Ki-67* | | | |
| <50% | 27 | 11 | 0.0037* |
| ≥50% | 28 | 20 | |
| *Skp2/Ki-67* | | | |
| Both Skp2≥10% & Ki-67≥50% | 19 | 16 | 0.0001* |
| Either Skp2≥10% or Ki-67≥50% | 15 | 10 | |
| Both Skp<10% & Ki-67<50% | 15 | 5 | |

AJCC, American Joint Committee on Cancer; Cks1, cyclin-dependent kinase subunit 1.
*Statistically significant.

**Table 4** Results of multivariate Cox regression analysis

| *Factors* | *Overall survival* | |
|---|---|---|
| | *RR (95% CI)* | P |
| Skp2≥10% | 5.538 (1.738–17.649) | 0.004* |
| Ki-67≥50% | 3.254 (1.244–8.512) | 0.016* |
| AJCC stage II+III+IV | 3.163 (1.271–7.874) | 0.013* |
| Age≥70 | — | 0.065 |
| $p27^{kip1}$≥20% | — | 0.088 |
| Cyclin A≥5% | — | 0.169 |
| Vascular invasion | — | 0.446 |
| Cyclin E≥5% | — | 0.491 |
| Cks1≥40% | — | 0.706 |
| T3+4 | — | 0.568 |
| Grade 2–4 | — | 0.708 |

95% CI, 95% confidence interval; Cks1, cyclin-dependent kinase subunit 1; RR, risk ratio.
*Statistically significant.

might confer selective advantage on cells of gallbladder carcinoma.

Although $p27^{Kip1}$ downregulation had a negative prognostic impact at the univariate level, we could not, like previous series, identify significant correlations between this aberration and clinicopathologic variables, such as TNM staging.[19,20] This discrepancy might be ascribed to the following reasons. First, we found 20% reactivity of $p27^{Kip1}$ to be the most prognostically effective cutoff point by using TMA technology, unlike the 50% reactivity for whole sections adopted by previous studies.[19,20] Insufficient formalin fixation in some specimens has been shown to result in artificial reduced labeling of $p27^{Kip1}$ in TMA, which might therefore hamper its scoring wherein a loss or decrease in nuclear staining is interpreted as aberrant.[34] More likely, the root cause might stem from the distribution of presenting stages in different study cohorts. In our institutes, many patients preoperatively with known advanced gallbladder carcinoma gave up the attempt to undergo extended operation, and, therefore, more than a half of gallbladder carcinomas (53%) in this series were stage I disease. Actually, a considerable proportion of these stage I gallbladder carcinomas were incidentally found and treated by simple cholecystectomy for other clinical manifestations. This was different from the series of Hui *et al*[20] and Filpits *et al*,[19] which respectively enrolled as high as 70 and 92% of gallbladder carcinomas with advanced presenting stage.

In this study, Skp2 overexpression significantly correlated with vascular invasion, advanced T stage, and higher histological grades and proliferative rate, implying its importance in disease progression and inherent biologic aggressiveness of gallbladder carcinoma. Although staging was still reaffirmed to be prognostically valid, Skp2 overexpression represented the strongest adverse prognosticator that independently increased the risk of inferior OS by

5.54-fold. Furthermore, we also substantiated that the combined expression pattern of Skp2 and Ki-67 could efficiently define three different prognostic groups of gallbladder carcinomas with a balanced case distribution. Recently, Sanada *et al*[35] have identified Skp2 overexpression as an independent poor prognosticator in a small series of biliary tract cancers, including 12 gallbladder carcinomas. Moreover, they also demonstrated gene amplification to be one principal mechanism leading to Skp2 protein overexpression, as increased DNA copy number of Skp2 gene within chromosome 5p11-13 was observed in 53% (nine out of 17) of cases tested.[35] Given the critical prognostic role of Skp2 overexpression, it appears to be of interest to elucidate specifically the incidence and implication of Skp2 gene amplification in gallbladder carcinoma.

Ectopic overexpression of Skp2 has been shown in transformed cells to promote $Thr^{187}$-phosphorylated $p27^{Kip1}$ degradation, thereby allowing the generation of cyclin A-dependent kinase activity and inducing progression of S phase.[25] However, neither Sanada *et al*[35] nor we could validate an inverse relationship between $p27^{Kip1}$ and Skp2 in biliary tract cancers or gallbladder carcinomas, as described previously in other types of carcinomas.[21,24,29] Recently, several lines of evidence have indicated that the regulatory mechanisms of $p27^{Kip1}$ abundance at the subcellular level turn out to be more complex.[26,36,37] For instance, heightened expression of Jab1 in mammalian cells can specifically interact with $p27^{Kip1}$ and promote its nuclear export to accelerate degradation.[36] Alternatively, Skp2-independent downregulation of $p27^{Kip1}$ can occur at the G0–G1 transition by a novel translocation-coupled cytoplasmic Kip1 ubiquitylation-promoting complex.[37] Additionally, *in vitro* models have identified Cks1 as an essential cofactor for efficient Skp2-dependent ubiquitination of $p27^{Kip1}$[26] and its overexpression was found strongly associated with poor prognosis in carcinomas of the colorectum, stomach, and breast.[28,29,38] Furthermore, gene amplification and overexpression of Cks1 at chromosome band 1q21 is associated with reduced levels of $p27^{Kip1}$ and an aggressive clinical course in multiple myeloma.[39] In our univariate analysis, Cks1 overexpression also correlated with inferior OS in gallbladder carcinomas, although it did not remain prognostically independent in multivariate comparison.

Few, if any, pervious studies had specifically addressed the prognostic utility of cyclin E in gallbladder carcinomas.[40] Eguchi *et al*[40] demonstrated that cyclin E overexpression only correlated with increased proliferative activity but not with any clinicopathological variable or clinical outcome. Conversely, we found that cyclin E overexpression was preferentially detected in females and in gallbladder carcinomas with vascular invasion, tumor necrosis, and higher histological grades and proliferation rate. In addition, cyclin E overexpression, albeit not independent, was also predictive of patient survival by univariate analysis. The reason for the discrepancy between series is unclear. However, it must be added that Eguchi *et al*[40] adopted a simplified semiquantitative scoring method instead of meticulous counting of LI. Cyclin E is a critical cell cycle regulator in mammalian cells that activates and binds to cyclin-dependent kinase 2 (cdk2) to form the cyclin E-cdk2 complex.[8,9] In normal cells, its protein level is periodic, peaking at the G1/S phase transition and declining after the beginning of S-phase program.[8,9] The oncogenic potential of deregulated cyclin E has been exemplified by its ectopic expression in inducing premature onset of DNA synthesis in cultured cells.[8,9] Furthermore, aberrant expression of cyclin E has been shown to induce chromosomal instability, leading to increased aneuploid and polypoid cells as well as accumulation of additional genetic aberrations.[8,41] Currently, it has become apparent that several mechanisms deregulate cyclin E expression in tumors, including gene amplification, enhanced transcription by unleashed E2F protein activity, and disrupted proteolysis caused by inactivation of Fbw7 gene.[8,9,15,41,42] In future investigations, it seems plausible to explore further the underling molecular mechanisms responsible for cyclin E deregulation in gallbladder carcinomas.

In conclusion, it is not uncommon in gallbladder carcinoma to display aberrations of proteins within the Skp2/$p27^{Kip1}$-associated ubiquitin-proteasome proteolytic pathway. Skp2 overexpression is an independent poor prognosticator and associated with intrinsic biological aggressiveness of gallbladder carcinoma. The combined assessment of Skp2 and Ki-67 LIs robustly define three different prognostic groups, which deserve to be prospectively validated in future study. The lack of an inverse association between $p27^{Kip1}$ and Skp2 or Cks1 suggests that additional cellular events may be operating, requiring further elucidation of the underlying mechanisms regulating these interacting proteins in gallbladder carcinoma. As several avenues to anticancer therapy converge at $G_1$/S transition of cell cycle and ubiquitin-proteasome system,[33] our findings provide a useful insight into future targeted drug design for gallbladder carcinomas. The tight correlations of $p27^{Kip1}$ and its interacting cell cycle regulators with aggressiveness in gallbladder carcinoma suggest that these regulatory proteins may well be considered as novel targets of therapeutic intervention.

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