Gene Amplification of Ribosomal Protein S6 Kinase-1 and -2 in Gastric Cancer

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Abstract. *Background: The gene amplification of ribosomal protein S6 kinase 1 and 2 (S6K1 and S6K2) and its clinical relevance in gastric cancer remain unclear. Materials and Methods: A comparative genomic hybridization analysis and DNA copy number assay were performed for nine cancer cell lines. The gene amplification of S6K1 and S6K2 were determined using a DNA copy number assay of 213 gastric cancer tissues. Results: S6K1 and S6K2 amplifications were observed in one and three cancer cell lines, respectively. No amplification of S6K1 was detected in the gastric cancer tissues, while S6K2 amplification was observed in 4.7% of the gastric carcinoma tissues. Patients with stage IV gastric cancer whose tumors exhibited amplification had a significantly shorter overall survival. Conclusion: S6K2 amplification was frequently observed in gastric cancer and was related to a poor prognosis. Our findings may provide novel insight into the dysregulation of mammalian target of rapamycin signaling by S6K2 amplification in gastric cancer.*

Many investigations on treatment of gastric cancer have been performed over the past decades; however, the prognosis for patients with advanced gastric cancer remains poor (1, 2). Thus, detailed information on genomic alterations in clinical samples is needed in order for new treatment modalities of molecular-targeted drugs to be developed.

The serine/threonine kinase mammalian target of rapamycin (mTOR) is a downstream effector of the phosphatidylinositol 3-kinase (PI3K)/ v-akt murine thymoma viral oncogene homolog 1 (AKT) pathway and regulates transcription, mRNA translation, cellular growth, proliferation, and survival of cells in response to various stimuli such as growth factors, nutrients, energy, and stress signals (3). mTOR forms two complexes, which are known as mTORC1 and mTORC2, and these multiple-protein complexes are activated by phosphorylated AKT (4). Activated mTORC1 further activates downstream ribosomal protein S6 kinases by phosphorylation (5). Ribosomal protein S6 kinase-1 (*S6K1*, also known as *p70-S6K/RPS6KB1*) and ribosomal protein S6 kinase-2 (*S6K2*, also known as *p70S6Kbeta/ RPS6KB2*) are key target molecules of mTORC1 and belong to the AGC [protein kinase, cAMP-dependent (PKA), protein kinase, cGMP-dependent (PKG) and protein kinase C (PKC)] kinase family (6). S6 kinases possess consensus domains, including a TOR signaling motif in the *N*-terminal domain, a kinase domain and a C-terminal domain (7). S6K1 phosphorylates numerous downstream molecules and regulates many cellular process including mRNA processing, translation initiation, translational elongation, protein folding, cell growth, motility, and survival (6). Therefore, mTORC1-S6K1 signaling is considered to be important for cellular physiology, and its deregulation often leads to disease.

In oncology, both cancerous and non-cancerous cells that contribute to the formation of tumor tissue, such as leukocytes, endothelial cells, and fibroblasts, depend on the mTORC1-S6K1 signaling pathway, and frequent dysregulation of this signaling has been associated with the development of cancer (8). Thus, many mTOR inhibitors are now under clinical development, and some mTOR inhibitors have shown clinical benefits as molecular-targeted drugs (9). However, very limited information on *S6K1* and *S6K2* amplification and its clinical relevance to gastric cancer is available.

In this study, we retrospectively studied the DNA copy numbers of *S6K1* and *S6K2* using formalin-fixed, paraffin-embedded (FFPE) samples from patients with gastric cancer who had undergone surgery, and evaluated the clinical significance of these amplifications.

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Materials and Methods

Cell cultures. Gastric cancer cell lines (44As3, 58As1, HSC43 and OKAJIMA) were maintained in RPMI-1640 medium (Sigma, St. Louis, MO, USA) except for IM95, which was maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; Gibco BRL, Grand Island, NY, USA). The esophageal cancer cell lines KYSE170 and KYSE180 were maintained in a 1:1 mixture of Ham's F12 medium and RPMI-1640 with 2% heat-inactivated FBS. KYSE70 was maintained in DMEM with 2% FBS. KYSE150 was maintained in Ham's F12 with 2% FBS. The cell lines were maintained in a 5% CO_2-humidified atmosphere at 37˚C. The IM95 and KYSE series were obtained from the Japanese Collection of Research Bioresources (Ibaraki, Osaka, Japan), while the others were provided by the National Cancer Center Research Institute (Tsukiji, Tokyo, Japan).

Patients and samples. A total of two-hundred thirteen patients with histologically-confirmed gastric cancer who had undergone surgery at the National Cancer Center Hospital were included in this study, as previously described (10). All the patients in this series had an Eastern Cooperative Oncology Group performance status of 0 to 2. The present study was approved by the Institutional Review Board of the National Cancer Center Hospital.

Genomic DNA samples were extracted from surgical specimens preserved as FFPE tissues using a QIAamp DNA Micro kit (Qiagen, Hilden, Germany), as previously described (10). Macro dissection of the FFPE samples was performed to select for a cancer region, which was marked by a pathologist. The DNA concentration was determined using the NanoDrop2000 (Thermo Scientific, Waltham, MA, USA).

DNA copy number assay for S6K1 and S6K2. The copy numbers for S6K1 and S6K2 were determined using commercially available and pre-designed TaqMan Copy Number Assays, as previously described (10). The primer IDs used were as follows: S6K1, Hs03958357_cn; S6K2, Hs05250093_cn. The telomerase reverse transcriptase (*TERT*) locus was used for the internal reference copy number. Human Genomic DNA (TaKaRa, Otsu, Japan) and DNA from non-cancerous FFPE tissue were used as normal controls. Real-time genomic PCR was performed in a total volume of 20 μl in each well, containing 10 μl of TaqMan genotyping master mix, 20 ng of genomic DNA, and each primer. The PCR conditions were 95˚C for 10 min, 40 cycles of 95˚C for 15 s and 60ºC for 1 min; the resulting products were detected using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Data were analyzed using the SDS 2.2 software and CopyCaller software (Applied Biosystems).

Real-time reverse-transcription PCR. cDNA was prepared from the total RNA of each cultured cell line using a GeneAmp® RNA-PCR kit (Applied Biosystems). Real-time RT-PCR amplification was performed as described previously (11). The primers used for the real-time RT-PCR were as follows: *S6K1*, forward 5'-CAC ATA ACC TGT GGT CTG TTG CTG-3' and reverse 5'-AGA TGC AAA GCG AAC TTG GGA TA-3'; *S6K2*, forward 5'-CTT CCA GAC TGG TGG CAA ACT CTA-3' and reverse 5'-CAG CGT GAT CTC AGC CAG GTA-3'; glyceraldehyde-3-phosphate dehydrogenase (*GAPD*), forward 5'-GCA CCG TCA AGG CTG AGA AC-3' and reverse 5'-ATG GTG GTG AAG ACG CCA GT-3'. *GAPD* was used to normalize the expression levels in the subsequent quantitative analyses.

Immunoblotting. A western blot analysis was performed as described previously (11). The following antibodies were used: anti-S6K1 (Cell Signaling Technology, Beverly, MA, USA), anti-RPS6KB2 (Sigma), anti-β-actin and horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology).

Array-based comparative genomic hybridization (CGH). The array-based CGH analysis was performed as previously described (12). The Genome-wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA, USA) was used for each of the esophageal cancer cell lines. The GeneChip Human Mapping 250K Nsp Array (Affymetrix) was used for the gastric cancer cell lines. A total of 250 ng of genomic DNA was digested with *Nsp*I (250K) or both *Nsp*I and *Sty*I in independent parallel reactions (SNP6.0), with the restriction enzymes ligated to the adaptor, and then amplified using PCR with a universal primer and TITANIUM Taq DNA Polymerase (Clontech, Palo Alt, CA, USA). The PCR products were quantified, fragmented, end-labeled, and hybridized onto a GeneChip Human Mapping 250K Nsp Array or a Genome-wide Human SNP6.0 Array. After washing and staining in Fluidics Station 450 (Affymetrix), the arrays were scanned to generate CEL files using the GeneChip Scanner 3000 and the GeneChip Operating Software Ver.1.4. In the array-CGH analysis, sample-specific copy number changes were analyzed using Partek Genomic Suite 6.4 software (Partek, St. Louis, MO, USA).

Statistical analysis. The statistical analyses of the clinico-pathological features were performed using the Student's *t*-test and the χ^2 test using PAWS Statistics 18 (SPSS Japan Inc., Tokyo, Japan). The overall survival (OS) curves were estimated using the Kaplan-Meier method.

Results

Gene amplification of S6K1 and S6K2 in cancer cell lines. An array-CGH analysis was performed to detect the gene amplifications of *S6K1* and *S6K2* in five gastric cancer and four esophageal cancer cell lines. Among the nine cell lines that were examined, the chromosomal region of 17q23 around *S6K1* was amplified in the 44As3 cell line (Figure 1A). The chromosomal region of 11q13 around *S6K2* was amplified in the Okajima, KYSE170, and KYSE180 cell lines (Figure 1B).

To evaluate the high-throughput method for detecting the gene amplification of *S6K1* and *S6K2*, real-time PCR-based DNA copy number assays were performed using DNA from the cancer cell lines (Figure 1C and 1D). The DNA copy number assay demonstrated that copy numbers greater than four copies were observed for *S6K1* in 44As3 cells (4.7±0.3 copies) and for S6K2 in Okajima, KYSE170, and KYSE180 cells (4.4±0.3, 4.0±0.5, and 4.6±1.0 copies, respectively). Collectively, similar results were observed for the gene amplifications of *S6K1* and *S6K2* between the array-CGH and DNA copy number assay. These results indicated that *S6K1* and *S6K2* were amplified in gastric and esophageal cancer cell lines.

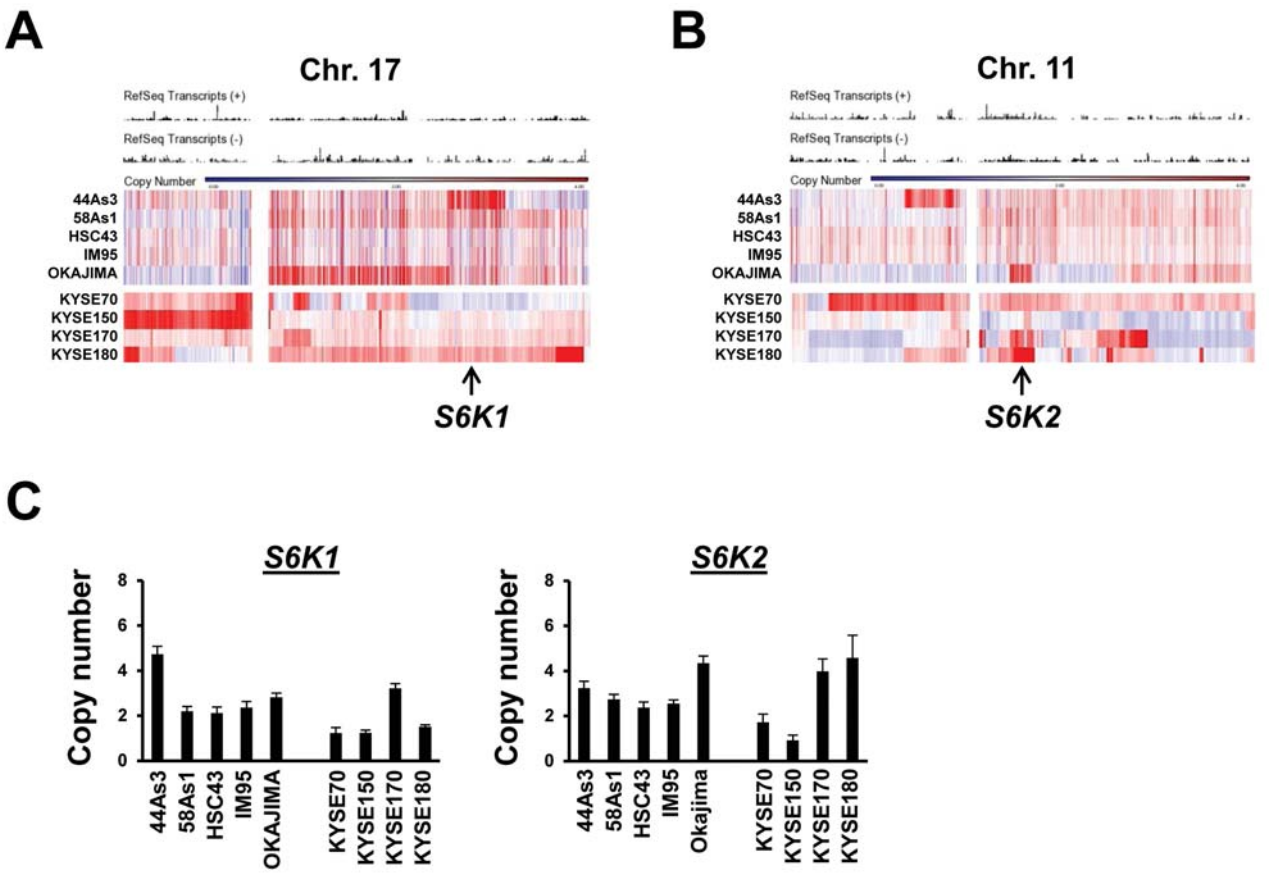

Figure 1. *Gene amplification of ribosomal protein S6 kinase-1 and -2 (S6K1 and S6K2) in gastric cancer and esophageal cancer cell lines. The DNA copy numbers of S6K1 on chromosomal 17 (A) and of S6K2 on chromosomal 11 (B) were evaluated using an array-comparative genomic hybridization analysis. The arrows indicate the genomic loci of the S6K1 and S6K2 genes. (C) Evaluation of DNA copy numbers in cancer cell lines. A DNA copy number assay was performed to determine the copy numbers of the S6K1 and S6K2 genes.*

mRNA and protein expression levels of S6K1 and S6K2 in cancer cell lines. To investigate the correlation between gene amplification and mRNA and protein expression, we examined the mRNA expression levels of *S6K1* and *S6K2* using real-time RT-PCR for gastric and esophageal cancer cell lines. Real-time RT-PCR demonstrated that the *S6K1* mRNA was up-regulated in Okajima cells and that *S6K2* mRNA was up-regulated in Okajima and KYSE180 cells (Figure 2A). A western blot analysis revealed that S6K1 protein expression was observed in all the cell lines, with differences in expression being relatively small, while a high S6K2 protein expression level was observed in Okajima and KYSE180 cells (Figure 2B). In *S6K2*-amplified cell lines, although the mRNA and protein expression levels of S6K2 were not increased in KYSE170 cells, those of Okajima and KYSE180 cells were clearly increased. When compared with the results for the DNA copy numbers, *S6K2* amplification seemed to mediate the mRNA and protein up-regulation, but the effects of *S6K1* were unclear.

Gene amplification of S6K1 and S6K2 in clinical gastric cancer samples. *S6K1* and *S6K2* amplification was evaluated using a DNA copy number assay of 213 FFPE samples of primary gastric cancer. The *S6K1* copy number ranged from 0.4 to 3.8 copies, and no obvious *S6K1* amplification was observed in gastric cancer samples (copies ≥4, Figure 3A). The *S6K2* copy number ranged from 0.5 to 9.5 copies. Overall, 4.7% (10/213) of gastric carcinomas harbored the *S6K2* amplification at a level of more than four copies (copies ≥4, Figure 3B). The copy numbers of S6K2 amplification were 4.1, 4.2, 4.3, 4.4, 4.8, 4.9, 5.5, 7.1, 7.3 and 9.5. The mean copy number in the non-amplified *S6K1* and *S6K2* cases was 1.6±0.5 and 2.5±0.6, respectively. The results indicate that *S6K2* was actually amplified in the clinical gastric cancer samples.

Clinicopathological features of S6K2-amplified gastric cancer. The patient characteristics according to *S6K2* amplification are shown in Table I. The median age was 63 years, and 69% (147/213) of this cohort were male. The

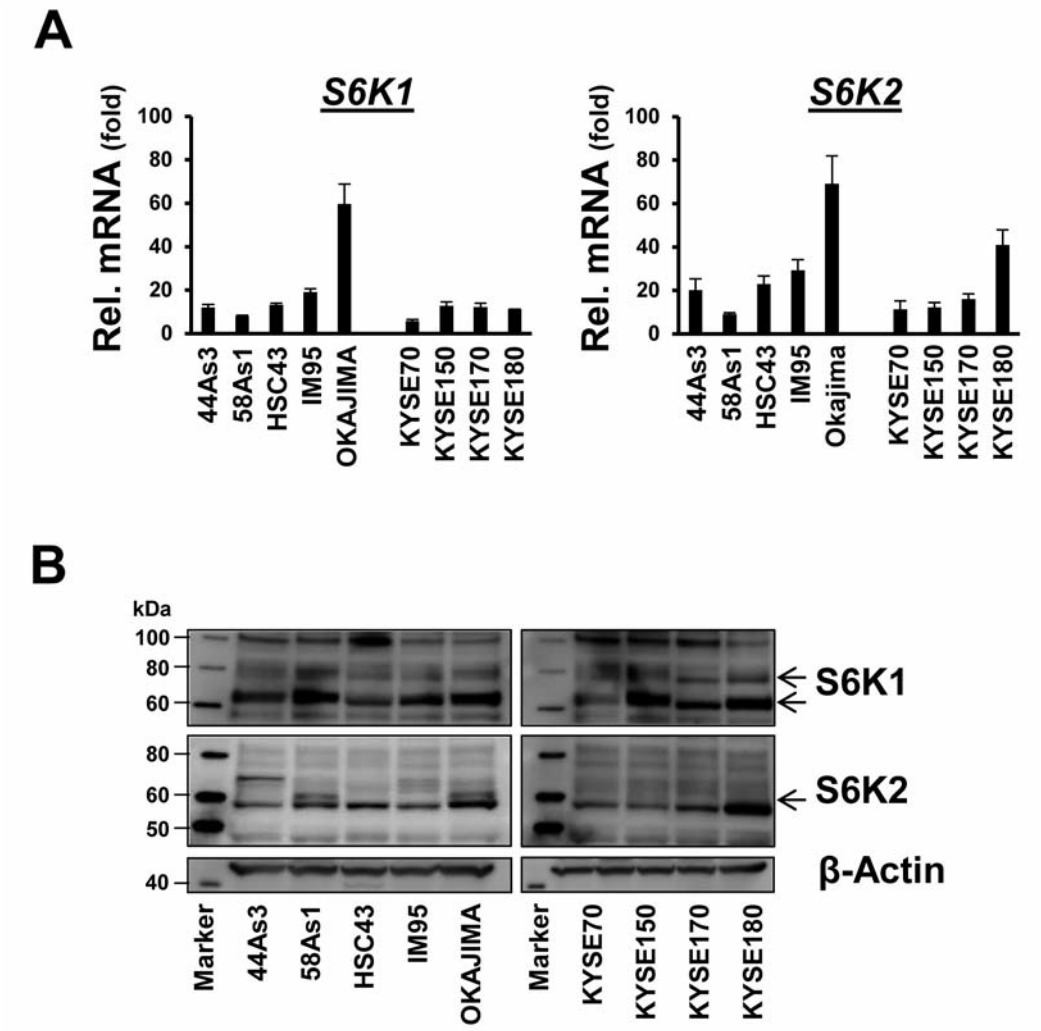

Figure 2. *S6 kinase-1 (S6K1) and S6K2 expression in cancer cell lines. A: The mRNA expression levels of S6K1 and S6K2 were determined using real-time RT-PCR. Rel. mRNA: Relative mRNA expression levels (target genes/GAPD ×10^3). B: Western blot analysis for S6K1 and S6K2 protein. The arrows indicate the target proteins. β-actin was used as an internal control.*

percentages of the pathological stages were as follows: stage I, 8%; stage II, 12%, stage III, 29% and stage IV, 51%. Intestinal-type gastric cancer was observed in 42% (89/213) of the histologically-examined gastric carcinomas. The patient age, sex, pathological stage, and histology were not significantly associated with *S6K2*-amplification (Table I). The characteristics of the S6K2-amplified gastric carcinomas are summarized in Table II.

Finally, we examined the prognostic impact of *S6K2* amplification on OS after surgery. Although the sample size was relatively small (n=108), *S6K2* amplification was associated with a significantly shorter OS, compared with non-amplified cases, among patients with stage IV gastric cancer (log rank test, p=0.02; Figure 3C). Thus, *S6K2* amplification may be a novel prognostic factor for gastric cancer.

Discussion

Accumulating evidence demonstrates that the level of phosphorylation of S6K1 protein as detected using immunohistochemistry, which reflects activated mTORC1-S6K1 signaling, is increased in various types of cancer, including breast, lung, melanoma, hepatocellular carcinoma and glioma (3). Most of these studies showed that an increased phospho-S6K1 level was positively correlated with a poor prognosis, such as nodal metastasis and overall survival (3, 13-14). Therefore, the activation and overexpression of S6K1 enhances the malignant potential of the cancer cells. Regarding the gene amplification of *S6K1*, amplification is mostly observed in breast cancer, with a detection frequency of 6% to 14% using a Southern blot

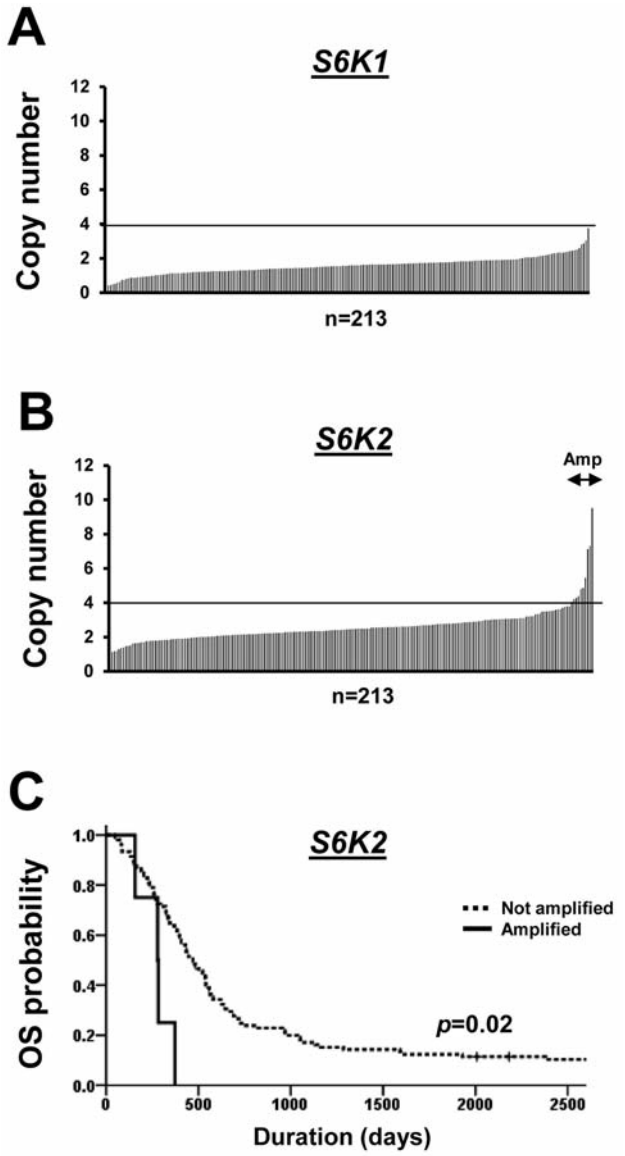

Figure 3. *Gene amplification of S6K1 and S6K2 in clinical gastric cancer samples. S6K1 (A) amplification and S6K2 (B) amplification were determined using the DNA copy number assay for 213 gastric cancer samples. S6K2 amplification over four copies was observed in 10 cases. C: Kaplan-Meier curves for overall survival (OS) in patients with stage IV gastric cancer. Patients were divided into two groups according to the S6K2 amplification. Amp, Gene amplification.*

Table I. *S6 kinase-2 (S6K2) amplification and clinicopathological features in gastric cancer.*

			S6K2 amplification		
		Total n=213	+ n=10	– n=203	*p*-Value
Age (years)	Range	31-91	45-75	31-91	0.94
	Median	63	63	63	
Gender	Male	147	7	140	0.78
	Female	66	3	63	
pStage	I	18	0	18	0.70*
	II	25	3	22	
	III	61	3	58	
	IV	108	4	104	
	Unknown	1	0	1	
Histology	Tub1	32	1	31	0.66**
	Tub2	44	1	43	
	Pap	5	0	5	
	Muc	8	1	7	
	Sig	11	2	9	
	Poor1	25	0	25	
	Poor2	88	5	83	

Tub, Tubular adenocarcinoma; Pap, papillary adenocarcinoma; Muc, mucinous adenocarcinoma; Sig, signet ring-cell carcinoma; Poor, poorly-differentiated adenocarcinoma; pStage, pathological stage. *Comparison between pStage I+II and III+IV. **Comparison between intestinal (Tub1, Tub2, Pap and Muc) and others. *p*-Values were calculated using the *t*-test for age and the χ^2 test for the other variables.

analysis, CGH, and fluorescence *in situ* hybridization (15-19). Thus, breast cancer is widely recognized to harbor a *S6K1* amplification in around 10% of samples. For other types of cancers, *S6K1* amplification was observed in three out of 16 (19%) medulloblastomas, in seven out of 38 (18%) diffuse large B-cell lymphoma combinations, and in two out of 17 (8%) ovarian cancer cell lines (20-22). In this study, we found that the 44As3 gastric cancer cell line harbored *S6K1* amplification, but amplification was not detected in clinical gastric cancer samples, suggesting that *S6K1* amplification is relatively rare in gastric cancer.

A limited number of studies have focused on the molecular and biological function of S6K2, despite the great number of studies that have examined S6K1 to date. Similarly, the dysregulation of S6K2 in cancer remains largely unknown. A recent study demonstrated that *S6K2* amplification was observed in nine out of 207 (4%) breast carcinomas, whereas the *S6K1* amplification was observed in 22 out of 206 (11%) (23). In addition, the *S6K2* amplification was correlated with a high mRNA expression level and was associated with a poor prognosis (23). Of note, another study from the same research group showed that the chromosomal region of 11q13, which includes the *S6K2* gene, was frequently co-amplified with the chromosomal region of 8p12, which includes another key downstream molecule of mTOR signaling, the eukaryotic translation initiation factor 4E binding protein-1 gene (24). Further study on such co-amplification is needed to understand the dysregulation of mTOR signaling. In addition, *S6K2* amplification may alter sensitivity to mTOR inhibitors, and thus further studies are warranted.

Table II. *Summary of patients with S6 kinase-2 (S6K2)-amplified gastric cancer.*

No.	Age	Gender	Macroscopic type*	Histology	pStage	OS (days)	S6K1 (CN)	S6K2 (CN)
1	45	M	2	Poor2	IIIa	394	1.5	4.1
2	64	M	4	Sig	IV	283	2.5	4.2
3	63	F	0-IIc	Sig	IIIb	4732+	1.8	4.3
4	52	M	2	Tub2	II	3935+	1.2	4.4
5	64	M	3	Tub1	II	1907	1.8	4.8
6	66	M	3	Poor2	IV	157	1.9	4.9
7	71	F	3	Poor2	II	835	1.3	5.5
8	75	M	3	Poor2	IV	280	2.0	7.1
9	73	M	4	Poor2	IV	373	1.7	7.3
10	57	F	3	Muc	IIIb	2742	1.6	9.5

No., Sample number; pStage, pathological stage; OS, overall survival; CN, copy number; *classification is based on the definitions of the Japanese Research Society for Gastric Cancer; + for OS, indicates the patient was still alive at the time of writing. Tub, Tubular adenocarcinoma; Muc, mucinous adenocarcinoma; Sig, signet ring-cell carcinoma; Poor, poorly-differentiated adenocarcinoma; pStage, pathological stage.

In conclusion, we found that the *S6K2* amplification was observed in gastric cancer at a frequency of 4.7%, and its amplification was related to a poor outcome. Our results may provide an insight into the dysregulation of mTOR signaling in gastric cancer.

Conflicts of Interest

None.

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