GROWTH FACTORS, CYTOKINES, AND CELL CYCLE MOLECULES

Sperm-Associated Antigen 4, a Novel Hypoxia-Inducible Factor 1 Target, Regulates Cytokinesis, and Its Expression Correlates with the Prognosis of Renal Cell Carcinoma

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Accepted for publication
February 21, 2013.

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Hypoxia plays a crucial role in many pathophysiological conditions, including cancer biology, and hypoxia-inducible factor (HIF) regulates transcriptional responses under hypoxia. To elucidate the cellular responses to hypoxia, we performed chromatin immunoprecipitation with deep sequencing in combination with microarray analysis and identified HIF-1 targets. We focused on one of the novel targets, sperm-associated antigen 4 (SPAG4), whose function was unknown. SPAG4, an HIF-1–specific target, is up-regulated in various cultured cells under hypoxia. Examination of SPAG4 expression using a tissue microarray consisting of 190 human renal cell carcinoma (RCC) samples revealed that SPAG4 is an independent prognostic factor of cancer-specific mortality. Live-cell imaging revealed localization of SPAG4 at the intercellular bridge in telophase. We also studied cells in which SPAG4 was knocked down. Hypoxia enhances tetraploidy, which disturbs cell proliferation, and knockdown of SPAG4 increased tetraploid formation and decreased cell proliferation under both normoxic and hypoxic conditions. Studies using deletion mutants of SPAG4 also suggested the involvement of SPAG4 in cytokinesis. Microarray analysis confirmed dysregulation of cytokinesis-related genes by knockdown of SPAG4. In conclusion, SPAG4 is an independent prognostic factor in RCC and plays a crucial role in cytokinesis to defend against hypoxia-induced tetraploid formation. This defensive mechanism may promote survival of cancer cells under hypoxic conditions, thus leading to poor prognosis. (*Am J Pathol* 2013, 182: 2191–2203; <http://dx.doi.org/10.1016/j.ajpath.2013.02.024>)

Hypoxia plays a crucial role in a number of physiological and pathophysiological phenomena, including cancer biology. Because the abnormal tumor microvasculature cannot provide a sufficient blood supply, hypoxia is a common characteristic of all solid tumors.¹ Although hypoxia is inevitably deleterious to cells, including those in tumors, cancer cells show a range of genetic changes that enable them to adapt to hypoxic conditions and improve their survival.

A key transcription factor that regulates these responses to hypoxia is hypoxia-inducible factor (HIF), which composed of an α subunit (HIF-1 α ; HIF-2 α , alias EPAS-1; or HIF-3 α)

and a β subunit (HIF-1 β ; alias ARNT).² Although HIF-1 β is constitutively present, the HIF- α subunits are highly unstable, except under low oxygen tension. The stability of an HIF- α subunit is determined mainly by its degradation by von Hippel-Lindau protein (VHL) in an oxygen-dependent manner.^{3,4} Under hypoxic conditions, HIF- α escapes degradation and

Supported by Grant-in-Aids for Scientific Research 24390213 from the Japan Society for the Promotion of Science and grants from SENSHIN Medical Research Foundation (M.N.).

T.M. and I.M. contributed equally to this work.

HIF activates the transcription of target genes with hypoxia-responsive elements.

HIF plays a crucial role in tumorigenesis, and HIF-1 α and HIF-2 α are overexpressed in many human cancers, including renal cell carcinoma (RCC), as a result of intratumoral hypoxia and genetic alterations.^{5–7} Furthermore, these elevated levels correlate with cancer-related mortality and treatment failure.⁸

To elucidate the cellular responses to hypoxia, we performed chromatin immunoprecipitation with deep sequencing (ChIP-Seq) in combination with microarray analysis, and identified a number of novel HIF targets. One of the targets, SPAG4, was originally identified as a testis-specific protein localized to microtubule-containing spermatid structures.^{9,10} SPAG4 belongs to the family of SUN domain-containing proteins, and SUN is a conserved domain in eukaryotes.^{11–13} Recent studies demonstrated the ubiquitous expression of human SPAG4 in various normal tissues and neoplastic tissues at the mRNA level.^{14–16} Little, however, is known about its function. We investigated SPAG4 expression in RCC. We also performed functional analyses to reveal a biological function of SPAG4.

Materials and Methods

Cell Culture and Hypoxic Conditions

Human umbilical vascular endothelial cells (HUVECs; ATCC, Manassas, VA) were cultured in EGM-2 MV complete medium (Lonza Japan, Tokyo, Japan). HeLa cells and human kidney-2 (HK-2) cells were cultured in Dulbecco's modified Eagle's medium (Nissui Seiyaku, Tokyo, Japan) containing 10% fetal bovine serum. Cells were grown in a humidified, 5% CO₂-enriched atmosphere at 37°C. Hypoxic conditions were established by exposure to 1.0% O₂ and 5% CO₂ in an multigas incubator (APM-30D; Astec, Fukuoka, Japan). The duration of hypoxic incubation was 24 hours, except as otherwise specified.

ChIP-Seq Analysis

We performed ChIP-Seq analysis using anti-HIF-1 α antibody (NB 100-134; Novus Biologicals, Littleton, CO) in HUVECs, as described previously.¹⁷ A next-generation sequencer (Genome Analyzer II; Illumina, San Diego, CA) was used, and all mapped sequences were analyzed using QuEST software version 2.4 (<http://mendel.stanford.edu/SidowLab/downloads/quest>).¹⁸

DNA Microarray and Data Analyses

Human Genome U133 Plus 2.0 microarrays (Affymetrix, Santa Clara, CA) were used. At 48 hours after transfection of siRNA, the treated cells were incubated for a further 24 hours under normoxia or hypoxia, and their gene expression profiles were examined as described previously.¹⁷ Preparation of cRNA and hybridization of probe arrays were

performed according to the manufacturer's instructions. The raw data in the Affymetrix Human Genome U133 Plus 2.0 array were exported to GeneSpring GX software version 11.5 (Agilent Technologies, Santa Clara, CA) and were normalized by robust multiarray average (RMA) normalization algorithms. For identification of genes significantly altered by hypoxia, total detected entities were filtered by signal intensity value (upper cutoff, 100th percentile; lower cutoff, 20th percentile) and error (SD < 0.1) to remove very low signal entities and to select reproducible signal values of entities among the experiments.

To investigate biological functions associated with the gene lists, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID),^{19,20} an online gene database provided by the NIH (version 6.7; <http://david.abcc.ncifcrf.gov>). Benjamini and Hochberg false discovery rates (5% false positive discovery rate) were used to correct for multiple testing.

siRNA Transfection

siRNAs were obtained from Life Technologies—Invitrogen (Carlsbad, CA). Transfection was performed with Lipofectamine RNAiMax reagent in HUVECs and with Lipofectamine 2000 reagent in HeLa cells, according to the manufacturer's protocols (Life Technologies—Invitrogen). At 48 hours after transfection, siRNA-treated cells were processed for further experiments. The target sequences of siRNA were as follows: human HIF-1 α , forward 5'-AUAU-GAUUGUGUCUCCAGCGGCUGG-3' and reverse 5'-CC-AGCCGUGGAGACACAAUCAUAU-3'; human HIF-2 α , forward 5'-UACAUUUGCGCUCAGUGGCUUGUCC-3' and reverse 5'-GGACAAGCCACUGAGCGCAAUGUA-3'; human SPAG4-1, forward 5'-CCUCCAGGUUAUG-AUGAAACUGA-3' and reverse 5'-UUCAGUUUCAUA-UAAACCUGGAGG-3'; and human SPAG4-2, forward 5'-ACUCCACAAGGAGGUGUCCACUGUU-3' and reverse 5'-AACAGUGGACACCUCUUGUGGAGU-3'. Stealth RNAi Negative Control Med GC Duplex 2 or 3 (Life Technologies—Invitrogen) was used as control.

RNA Isolation and RT-qPCR

RNA extraction, RT-PCR, and real-time quantitative RT-PCR (RT-qPCR) were performed as described previously.²¹ Primer sequences were as follows: human SPAG4, forward 5'-ATGAGGATTTTGTGCGGAAG-3' and reverse 5'-GC-GTAATCGTGGGATGTCTT-3'; human HIF-1 α , forward 5'-CGAGATGCAGCCAGATCTCGGCGAAGTAA-3' and reverse 5'-CTCTCATTTCTCATGGTCACATGGATG-3'; human HIF-2 α , forward 5'-GAAGTCCCGGGATGCTGC-GCG-3' and reverse 5'-ACTATGTCCTGTTAGCTCCAC-CTGTG-3'; and human β -actin (internal control), forward 5'-TCCCCCAACTTGAGATGTATGAAG-3' and reverse 5'-AACTGGTCTCAAGTCAGTGTACAGG-3'.

Luciferase Reporter Assay

To generate the human *SPAG4*-promoter-luc plasmid and *SPAG4*-enhancer-luc plasmid, we isolated fragments from the human *SPAG4* promoter from –1000 to +118 relative to the transcription start site, and from the enhancer from +1633 to +892 relative to the transcription start site by PCR, using human genomic DNA as template. We cloned these fragments into the pGL3-basic vector (Promega, Madison, WI). Mutagenesis was conducted using mutated primer pairs. HUVECs were transiently transfected with plasmid DNA using FuGENE HD reagent (Roche Applied Science, Mannheim, Germany; Indianapolis, IN), and luciferase activity was measured using a dual-luciferase assay kit by cotransfection with pRL-CMV (Promega) to normalize transfection efficiency, as described previously.^{17,22}

Immunoblotting

Immunoblotting was performed as described previously.²³ The primary antibodies used for immunoblotting were anti-SPAG4 (1:1000; sc-85927; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-actin antibody (1:2000; A2066; Sigma-Aldrich, St. Louis, MO). Densitometry analysis was performed using ImageJ software version 1.47b (NIH, Bethesda, MD).

Study Population

The clinical and pathological data of 190 adult patients who had been diagnosed with sporadic clear cell RCC (ccRCC) and had been treated with partial or radical nephrectomy between 1998 and 2003 at the University of Tokyo Department of Urology were reviewed. The diagnosis of ccRCC was based on the evaluation of H&E-stained sections by pathologists at the University of Tokyo in accordance with the World Health Organization classification.²⁴ Tumor staging was revised according to the 2009 TNM staging system.²⁵ Nuclear grading was based on the appearance of the nuclei of tumor cells and was categorized as grade 1 (nuclei of tumor cells were smaller than those of normal renal tubular cells), grade 2 (nuclei were the same size), or grade 3 (nuclei were larger, and sometimes pleomorphic and bizarre).²⁶ The Eastern Co-operative Oncology Group (ECOG) scale was used to measure performance status (PS).²⁷ Incidentaloma was defined as a renal tumor detected during evaluation or surveillance of an unrelated medical condition, and symptomatic tumor was defined as a renal tumor detected on evaluation of hematuria, ipsilateral flank pain, a mass, or systemic symptoms such as loss of appetite.²⁸ Written informed consent was obtained from all participants, and institutional review board approval was obtained from the University of Tokyo.

TMA and Immunohistochemical Analysis

Tissue microarrays (TMAs) consisting of 190 sporadic ccRCC samples were constructed for the immunohistochemical

analysis of SPAG4. All tissue samples were fixed in formalin and embedded in paraffin. Representative tissue areas were punched out of the paraffin block using a 2.0-mm punch, and duplicate cores per sample were arrayed on a recipient paraffin block, to decrease sampling error.

Indirect immunoperoxidase staining was performed as described previously.²⁹ The primary and secondary antibodies used in this analysis were anti-SPAG4 rabbit polyclonal antibody (1:3000)⁹ and biotinylated goat anti-rabbit IgG antibody (1:1000; BA-1000; Vector Laboratories, Burlingame, CA). TMAs were scored in a masked manner by two independent observers (K.S. and M.N.). For the few instances of discrepant scoring, a consensus score was determined.

Plasmids and Stable Transfection

cDNA encoding human SPAG4 was obtained from HUVECs by RT-PCR and was subcloned into pEGFP-N1 (Clontech Laboratories, Mountain View, CA) and pcDNA4/TO/myc-HisA (Life Technologies—Invitrogen). cDNA encoding human calnexin was amplified from human embryonic kidney (HEK 293) cells by RT-PCR, and subcloned into the modified pIRESHyg2 (Clontech) carrying PhiC31 attB sequence that had been excised from pJTI Fast DEST (Life Technologies—Invitrogen). mCherry (the generous gift of Dr. Roger Tsien)³⁰ was fused to the C terminus of calnexin or SPAG4. Deletion mutants of human SPAG4 were constructed by PCR using pEGFP-N1-human SPAG4 as a template.

Transient transfection was performed with Lipofectamine 2000 reagent (Life Technologies—Invitrogen). Stable transfectants of HeLa cells expressing tagged SPAG4 were generated using the Jump-In system (Life Technologies—Invitrogen) according to the manufacturer's protocol. To generate double stable transfectants, HeLa cells stably expressing SPAG4-EGFP were cotransfected with the modified pIRESHyg2 carrying calnexin-mCherry and pJTI PhiC31 (Life Technologies—Invitrogen).

Live-Cell Imaging

Live-cell imaging was performed with a high-speed confocal laser scanning microscope system, as described previously.³¹ HeLa cells were grown on collagen-coated glass-bottom dishes and were observed using a Nipkow-disk confocal laser scanning unit (CSU22; Yokogawa Electric, Tokyo, Japan) equipped with an argon-krypton ion laser (488 and 568 nm excitation). Images were acquired with an electron-multiplying charge-coupled device camera (EMCCD C9100; Hamamatsu Photonics, Shizuoka, Japan) at a rate of 466 ms per image. For multicolor observations, cells were observed using a W-view system (model 8509; Hamamatsu Photonics). For long-term live-cell imaging, cells were observed using a CellVoyager CV1000 confocal scanner system (Yokogawa). Images were acquired every 5 minutes at a rate of 200 ms per image.

To visualize tubulin structures during live-cell imaging, HeLa cells were transduced with CellLight Tubulin-RFP

BacMam 2.0 (Life Technologies—Invitrogen) according to the manufacturer's protocol.

Analysis of Tetraploid Formation by Flow Cytometry

Cells were collected by trypsinization and were fixed with 70% ethanol overnight at -20°C . The cells were then stained with a solution containing 50 $\mu\text{g/mL}$ propidium iodide (Dojindo Molecular Technologies, Kumamoto, Japan) and 5 mg/mL RNase A (Sigma-Aldrich) in 0.1% bovine serum albumin in PBS for 20 minutes in the dark at room temperature. For each sample, 10,000 cells were analyzed using a flow cytometer (BD Biosciences, San Jose, CA), and data were collected using Cell Quest software (Becton Dickinson). The population of tetraploid cells was quantitated based on scatter analysis using ModFIT LT software version 3.1 (Verity Software House, Topsham, ME).

Immunocytochemistry

Cells were fixed with methanol/acetone (1:1) or 2% paraformaldehyde, followed by permeabilization with 0.3% Triton X-100 surfactant in PBS. After a washing, the cells were incubated with anti-c-Myc antibody (1:200; sc-40; Santa Cruz Biotechnology), anti- α -tubulin antibody (1:1000; T6199; Sigma-Aldrich), or anti- β -catenin antibody (1:200; 610154; BD Biosciences). For c-Myc and α -tubulin staining, anti-mouse IgG1-FITC antibody (1:200; 1070-02; SouthernBiotech, Birmingham, AL) was applied. For β -catenin, biotinylated anti-mouse IgG antibody (1:1000; BA-2001; Vector Laboratories) was applied, followed by Alexa Fluor 594-conjugated streptavidin (1:500; S-32356; Life Technologies—Invitrogen). Nuclei were stained with Hoechst 33342 dye (Sigma-Aldrich). An Eclipse E600 microscope (Nikon, Tokyo, Japan) was used for observation.

BrdU Assay

siRNA-treated HeLa cells were plated in 100 μL of medium on 96-well plates. Forty-eight hours after plating, cell incorporation of bromodeoxyuridine (BrdU) was measured by enzyme-linked immunosorbent assay with a BrdU Labeling and Detection Kit III (Roche Applied Science) according to the manufacturer's protocol. Absorbance at 405 nm was measured using a Biochrom plate reader (Asys Hitech, Eugendorf, Austria; Cambridge, UK).

Cell Proliferation Assay

siRNA-treated HeLa cells were plated in 100 μL of medium on 96-well plates. At 1, 24, 48, and 72 hours after plating, we performed CellTiter 96 AQueous one-solution cell proliferation assay (Promega) as described previously.²¹ Absorbance at 490 nm was measured using a Biochrom plate reader (Asys Hitech).

Statistical Analysis

All data are reported as means $\pm$ SD. Data for two groups were analyzed with an unpaired *t*-test, and those for more than two groups were compared by analysis of variance with Tukey's post hoc test. Nonparametric data were analyzed with the *U*-test or Kruskal-Wallis test when appropriate. Cancer-specific survival was estimated using the Kaplan-Meier method. The log-rank test was used to compare survival between different groups. The Cox proportional hazards regression model was used to determine the effect of clinicopathological parameters on survival. The hazard ratio was used as a measure of risk of death in different categories. Differences with a *P* value of <0.05 were considered significant. GraphPad Prism software version 5.04 for Windows (GraphPad Software, San Diego, CA) and JMP software version 9.0.0 (SAS Institute, Cary, NC) were used for data analysis.

Results

SPAG4 Is a Novel HIF-1-Specific Target

To elucidate the cellular responses to hypoxia, we performed ChIP-Seq analysis using HUVECs.¹⁷ We identified 2060 HIF-1 binding sites under hypoxia in HUVECs. We defined the nearest known transcript within 50 kbp from the HIF-1 binding site as the corresponding gene. In this manner, the 2060 HIF-1 binding sites were matched to 1240 genes (Supplemental Table S1).

We also performed microarray analysis in HUVECs treated with control siRNA or siRNA targeting the HIF-1 α gene (*HIF1A*), and examined gene expression profiles under both normoxia and hypoxia.¹⁷ Hypoxia-regulated probes were defined as having a >1.0 log₂-fold up-regulation in the presence of control siRNA under hypoxia, compared with normoxia. To identify genes regulated by HIF-1, we set the following criteria for downstream HIF-1 targets: the base-2 logarithm of fold-change value was >1.6 in normoxia, compared with hypoxia, and the base-2 logarithm of fold-change value was <1.2 in control siRNA, compared with HIF-1 siRNA, under hypoxia. Using these criteria, we identified 276 genes that are regulated by HIF-1 directly or indirectly under hypoxia (Supplemental Table S2).

The overlap of the HIF-1 target genes identified in ChIP-Seq and microarray analysis should correspond to genes that are regulated by HIF-1 directly, and we identified 78 such genes (Figure 1A and Supplemental Table S3). Among these targets, we focused on a novel HIF-1 target, *SPAG4*, because *SPAG4* was the sixth highest gene in the increase rate of mRNA expression after 24 hours of hypoxia and because the function of the *SPAG4* product was unknown.

We confirmed up-regulation of *SPAG4* under hypoxia in HUVECs by RT-qPCR (Figure 1B). Our ChIP-Seq analysis showed HIF-1 binding in the enhancer of human *SPAG4* gene (Figure 1C), and we investigated the HIF-1 dependency

Figure 1 Identification of *SPAG4* as a novel HIF-1 target. **A:** HIF-1 target genes were identified by two different methods in HUVECs: ChIP-Seq analysis and microarray analysis using siRNA targeting HIF-1α. The 78 overlapping genes (ie, genes identified with both methods) are directly regulated by HIF-1. **B:** RT-qPCR demonstrated up-regulation of *SPAG4* mRNA levels under hypoxia in HUVECs. **C:** ChIP-Seq analysis with anti-HIF-1α antibody indicated HIF-1 binding to one site in the enhancer region of *SPAG4* gene under hypoxia. **D:** Luciferase reporter assay demonstrated the up-regulation of *SPAG4* promoter activity by hypoxia via the hypoxia-responsive element at the enhancer of *SPAG4*. The design of luciferase vector pGL3 with the *SPAG4* promoter and/or *SPAG4* enhancer is shown at left, and the results in HUVECs are shown at the right. **E:** RT-qPCR showed that *SPAG4* mRNA levels were regulated by HIF-1α, but not HIF-2α. **F** and **G:** *SPAG4* mRNA expression, estimated by RT-qPCR, was up-regulated under hypoxia in a time-dependent manner in HK-2 cells (**F**) and HeLa cells (**G**). **H:** Immunoblotting analysis confirmed up-regulation of *SPAG4* at the protein level under hypoxia in HK-2 cells and HeLa cells. The duration of hypoxic incubation was 48 hours. Data are expressed as means ± SD. $n = 3$ (**B**, **D**, and **E**) or 5 (**F** and **G**) per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and † $P < 0.0001$.

of *SPAG4* regulation by luciferase reporter assay using this enhancer region. Addition of the *SPAG4* enhancer to the *SPAG4* promoter-luc resulted in the 3.6-fold up-regulation of reporter activity under hypoxia, compared with normoxia (Figure 1D). Furthermore, mutation of the hypoxia-responsive element motif of the *SPAG4* enhancer (ACGTG→AAATG) resulted in a 76.5% reduction in reporter activity. These results demonstrated that a hypoxia-responsive element in the enhancer of *SPAG4* is responsible for hypoxic induction.

To investigate whether *SPAG4* is a specific target of HIF-1, we evaluated *SPAG4* mRNA levels using siRNA targeting HIF-1α or HIF-2α under hypoxia. The knockdown efficiencies of HIF-1α and HIF-2α at the mRNA level were 83% and 90%, respectively. *SPAG4* mRNA levels were decreased only when *HIF-1α* mRNA levels were suppressed (Figure 1E). These results suggest that *SPAG4* expression is regulated by HIF-1, but not by HIF-2. An online database

search using Gene Expression Omnibus profiles (GEO Profiles; <http://www.ncbi.nlm.nih.gov/geo/profiles>) revealed the same results in MCF-7 breast cancer cells (GEO profile records GDS2760 and GDS2761), showing HIF-1-specific regulation of *SPAG4* without cell-type dependency. We also confirmed oxygen-dependent regulation of *SPAG4* in other cell types, such as human proximal tubular epithelial HK-2 cells and human cervical cancer HeLa cell (Figure 1, F–H).

SPAG4 Expression Is an Independent Prognostic Factor in ccRCC

Hypoxia plays a crucial role in cancer, and we decided to investigate *SPAG4* expression in ccRCC. First, we performed an online database search using GEO Profiles and found up-regulation of *SPAG4* in ccRCC, compared with healthy kidney (GDS505 and GDS2880).

To confirm and extend these observations, we performed TMA analysis consisting of 190 ccRCC samples. Demographic and clinical characteristics of the patients are listed in [Table 1](#). The median follow-up time was 98 months (range, 1 to 206 months); during follow-up, 33 (17.4%) and 21 (11.1%) of the patients died of cancer-specific causes and of other causes, respectively. The mean time to cancer-specific death was 22 months (range, 4 to 111 months). Cancer-specific survival for the whole group was 87.2% at 5 years and 79.3% at 10 years. Cancer-specific survival in our study population was dependent on TNM stage, nuclear grading, ECOG PS, and mode of presentation, which is consistent with previous reports^{28,32–34} ([Figure 2, A–D](#)).

In immunohistochemical analysis, only the intensity of SPAG4 staining was scored for each tissue (negative, score 0; low, score 1; high, score 2), because tumor cells stained homogeneously in each sample ([Figure 2E](#)). Of the 190 samples, 32 (16.8%) were categorized as score 0, 97 (51.1%) as score 1, and 61 (32.1%) as score 2. There was no significant difference in SPAG4 score between clinicopathological features except for nuclear grading ([Table 1](#)), which is a well-known prognostic factor of ccRCC.^{32,33,35,36}

Table 1 Characteristics of Patients in Tissue Microarray Analysis

Variable	no. (%)	SPAG4 score (means ± SD)	P value
Total	190 (100)	1.15 ± 0.68	
Sex			
Male	144 (75.8)	1.17 ± 0.67	0.6688
Female	46 (24.2)	1.11 ± 0.74	
ECOG performance status			
PS = 0	159 (83.7)	1.13 ± 0.69	0.2325
PS ≥ 1	31 (16.3)	1.29 ± 0.64	
Pathological TNM staging			
pT			
T1	143 (75.3)	1.10 ± 0.69	0.1944
T2	13 (6.8)	1.15 ± 0.80	
T3	32 (16.8)	1.38 ± 0.61	
T4	2 (1.1)	1.50 ± 0.71	
pN			
N0+NX	186 (97.9)	1.15 ± 0.68	0.7239
N1+N2	4 (2.1)	1.25 ± 0.96	
pM			
M0	172 (90.5)	1.14 ± 0.68	0.3812
M1	18 (9.5)	1.28 ± 0.75	
Overall staging			
Stage I	138 (72.6)	1.09 ± 0.68	0.1724
Stage II	11 (5.8)	1.09 ± 0.83	
Stage III	24 (12.6)	1.38 ± 0.58	
Stage IV	17 (9.0)	1.35 ± 0.70	
Nuclear grade			
Grade 1	35 (18.4)	0.86 ± 0.73	0.0226
Grade 2	125 (65.8)	1.21 ± 0.66	
Grade 3	30 (15.8)	1.27 ± 0.64	
Mode of presentation			
Incidentaloma	126 (66.3)	1.14 ± 0.69	0.6141
Symptomatic tumor	64 (33.7)	1.19 ± 0.69	

Although the difference in cancer-specific mortality between SPAG4 score 1 and score 2 did not reach statistical significance, patients with higher SPAG4 score showed higher mortality ([Figure 2F](#)). Patients with SPAG4 expression (score 1 and score 2) showed significantly higher cancer-specific mortality than those without expression (score 0) ($P = 0.0215$) ([Figure 2G](#)).

Univariate and multivariate analyses of significant clinicopathological factors are listed in [Table 2](#). SPAG4 expression was a significant prognostic factor of cancer-specific deaths in both univariate and multivariate analyses. We further examined the significance of SPAG4 expression in the subgroup of nuclear grades 2 and 3, because there was no cancer-specific death in the grade 1 population ([Figure 2B](#)). Demographic and clinical characteristics of the patients in this subgroup are listed in [Supplemental Table S4](#). Patients with SPAG4 expression showed a trend toward poor cancer-specific mortality, compared with those without expression ([Figure 2H](#)), and even in this subgroup of malignant nuclear grading, SPAG4 was an independent prognostic factor in both univariate and multivariate analyses ([Table 2](#)).

SPAG4 Localizes at the Intercellular Bridge during Telophase

Our analysis of these 190 patients with ccRCC showed that SPAG4 is a significant prognostic factor. We decided to determine the biological role of SPAG4, and therefore studied subcellular localization of SPAG4 using HeLa cells as a representative epithelial cancer cell line. To perform live-cell imaging for this purpose, we generated a stable HeLa cell line expressing fluorescent SPAG4. EGFP was fused at the C-terminus of full-length SPAG4, and expression of the fused protein was confirmed using anti-SPAG4 antibody ([Figure 3A](#)). Live-cell imaging of SPAG4-EGFP HeLa cells with high-speed laser scanning confocal microscopy demonstrated localization of SPAG4 mainly at the endoplasmic reticulum (ER) in interphase ([Figure 3B](#)). It was confirmed that SPAG4 was colocalized with calnexin, an ER marker ([Figure 3C](#)).

The most conspicuous finding was localization of SPAG4 at the intercellular bridge during telophase ([Supplemental Video S1](#)). We confirmed this observation by transducing SPAG4-EGFP HeLa cells with fluorescent tubulin ([Figure 3D](#)). Furthermore, imaging of double stably transfected cells expressing both SPAG4-EGFP and calnexin-mCherry showed that this structure was specific for SPAG4 and was not derived from the ER ([Figure 3E](#)). These data were obtained under normoxic conditions, for technical feasibility, but essentially the same results were confirmed under hypoxic conditions (data not shown).

Next, to examine the influence of the tag, we generated a stable HeLa cell line expressing c-Myc-tagged SPAG4 and then performed immunocytochemistry. Localization of c-Myc-tagged SPAG4 was consistent with that of SPAG4-EGFP during interphase and telophase ([Figure 3F](#)).

Figure 2 Expression of SPAG4 in ccRCC on tissue microarray. **A–D:** Kaplan-Meier analyses based on TNM staging (**A**), nuclear grading (**B**), ECOG PS (**C**), and mode of presentation (**D**) were performed for all 190 patient samples. All groups showed significantly different cancer-specific survival ($P < 0.0001$). **E:** Representative immunohistochemical images showing the expression of SPAG4 in adjacent normal kidney and representative images of negative (score 0), low (score 1) and high (score 2) SPAG4 staining in ccRCC. **F** and **G:** Kaplan-Meier analyses based on SPAG4 expression were performed in all 190 patients. Although the difference in cancer-specific mortality between SPAG4 score 1 and score 2 did not reach statistical significance ($P > 0.05$) (**F**), patients with SPAG4 expression (positive) showed significantly higher cancer-specific mortality than those without expression (negative) ($P = 0.0215$) (**G**). **H:** Kaplan-Meier analysis was performed in 155 patients whose nuclear grade was 2 or 3. Patients with SPAG4 expression (positive) showed a trend toward poor cancer-specific mortality, compared with those without expression (negative) ($P = 0.065$). Original magnification, $\times 200$.

Furthermore, we confirmed that SPAG4-mCherry showed the same localization as SPAG4-EGFP (Figure 3G).

Finally, to confirm the presence of SPAG4 at the intercellular bridge, HeLa cells were transfected with mCherry-tagged SPAG4 plasmid and stained with anti- α -tubulin antibody to identify the telophase cells (Figure 3H). From microscopic observation of more than 2000 cells, we identified 23 telophase cells, of which 22 (96%) exhibited SPAG4 at the intercellular bridge.

SPAG4 Is Involved in Cytokinesis

Given the localization of SPAG4 in the intercellular bridge, we speculated that SPAG4 plays a role in cytokinesis. We therefore studied the functional role of SPAG4 in cytokinesis in HeLa cells with SPAG4 knockdown. The knockdown efficiencies of two independent SPAG4 siRNAs (SPAG4-1 and SPAG4-2) at the mRNA level were 89% and 82% under normoxia and 94% and 76% under hypoxia,

respectively. Knockdown of SPAG4 was also confirmed at the protein level (Figure 4A).

Given that failure in cytokinesis leads to tetraploidization, we evaluated the number of tetraploid cells in nontreated HeLa cells by flow cytometry under both normoxia and hypoxia. Hypoxia increased the number of tetraploid cells significantly, compared with normoxia (Figure 4B). We also confirmed hypoxia-induced tetraploidization in control siRNA-treated HeLa cells (Figure 4C). Knockdown of SPAG4 by either of the two siRNAs independently increased the number of tetraploid cells under both normoxia and hypoxia (Figure 4C). This increase in tetraploidy by knockdown of SPAG4 paralleled the knockdown efficiency of the two siRNAs. Microscopic observation confirmed the increase of binucleated or multinucleated cells by SPAG4 knockdown (Figure 4D).

Given that cells with knockdown of SPAG4 exhibited dysregulated cell division, we investigated the effect of SPAG4 expression on cell proliferation. BrdU assay

Table 2 Univariate and Multivariate Cox Proportional Hazards Model for the Predictive Factors of Cancer-Specific Survival

Variables	Univariate analysis			Multivariate analysis		
	HR	P value	95% CI	HR	P value	95% CI
All patients (n = 190)						
Sex (male vs female)	1.64	0.25	0.72–4.39	1.37	0.53	0.53–4.18
Age (years)	1.02	0.32	0.99–1.05	0.98	0.23	0.95–1.01
SPAG4 (positive vs negative)	7.30	0.0060*	1.57–129.89	7.12	0.0116*	1.43–129.48
Nuclear grade (3 vs 1 + 2)	4.26	0.0002*	2.07–8.48	1.33	0.52	0.55–3.07
TNM stage (reference = stage I)						
II	10.13	0.0023*	2.51–38.28	8.10	0.0080*	1.81–33.56
III	18.28	<0.0001*	6.25–59.99	14.00	<0.0001*	4.62–47.24
IV	48.01	<0.0001*	18.31–149.40	23.04	<0.0001*	7.51–81.14
ECOG PS (≥ 1 vs 0)	6.36	<0.0001*	3.09–12.81	3.82	0.0026*	1.60–9.31
Symptomatic tumor vs incidentaloma	4.43	<0.0001*	2.19–9.49	1.53	0.35	0.63–3.82
Patients of nuclear grade 2 or 3 (n = 155)						
Sex (male vs female)	1.33	0.52	0.59–3.56	1.40	0.52	0.53–4.33
Age (years)	1.01	0.41	0.98–1.05	0.98	0.25	0.95–1.01
SPAG4 (positive vs negative)	5.31	0.0291*	1.14–94.47	6.89	0.0138*	1.38–125.33
Nuclear grade (3 vs 2)	3.24	0.0020*	1.57–6.44	1.24	0.61	0.52–2.84
TNM stage (reference = stage I)						
II	7.49	0.0067*	1.85–28.31	6.44	0.0164*	1.45–26.54
III	13.89	<0.0001*	4.75–45.52	11.44	<0.0001*	3.80–38.38
IV	35.44	<0.0001*	13.52–110.20	17.17	<0.0001*	5.59–60.56
ECOG PS (≥ 1 vs 0)	5.71	<0.0001*	2.77–11.48	3.83	0.0023*	1.62–9.19
Symptomatic tumor vs incidentaloma	4.96	<0.0001*	2.45–10.64	1.71	0.24	0.69–4.33

*Parameters examined in multivariate analysis: demographics (sex and age), SPAG4 score, nuclear grade, TNM stage, ECOG PS, and mode of presentation. HR, hazard ratio.

indicated that the proliferation of SPAG4 siRNA-treated HeLa cells was significantly suppressed, compared with control siRNA-treated cells, under both normoxia and hypoxia (Figure 4E). MTS assay also indicated significantly fewer SPAG4 siRNA-treated HeLa cells than control siRNA-treated cells (Figure 4F). These observations were confirmed by cell number counting under a microscope (data not shown).

To confirm SPAG4 function in cytokinesis, we generated stable HeLa cell lines expressing fluorescent SPAG4 with mutations. Structurally, SPAG4 is composed of the N-terminal domain, transmembrane domain, coiled-coil domain, and SUN domain. We generated five different mutants (Supplemental Figure S1) in which deletion of both N-terminal domain and coiled-coil domain diminished SPAG4 localization at the intercellular bridge (Figure 4G) and induced cytokinesis failure, enhancing formation of binucleated or multinucleated cells (Supplemental Video S2). Microscopic observation confirmed a significant increase of binucleated or multinucleated cells in this deletion mutant (Figure 4H).

Changes of Expression of Cytokinesis-Related Genes by Knockdown of SPAG4

To investigate whether expression profiles of genes involved in cytokinesis are regulated by SPAG4, we performed DNA microarray analysis in HeLa cells treated with control siRNA

or two independent siRNAs targeting SPAG4 (<http://www.ncbi.nlm.nih.gov/geo>; accession number GSE46054). Functional annotation analysis (DAVID analysis) indicated that those genes which were up-regulated by SPAG4 knockdown (>0.5 log₂-fold up-regulated probes in the presence of SPAG4 siRNA, compared with control siRNA, under both normoxia and hypoxia) were associated with cell cycle and with chromosome segregation, and that these were significantly enriched (Tables 3 and 4).^{19,20} The 77 genes in the cell-cycle category are listed in Supplemental Table S5. This category includes genes involved in cytokinesis, such as Aurora A kinase (*AURKA*) and Aurora B kinase (*AURKB*), and the enrichment of cell cycle–related genes by knockdown of SPAG4 was also confirmed even in the DAVID functional annotation of KEGG–Kyoto Encyclopedia of Genes and Genomes pathways (enrichment score ES = 6.04; Benjamini and Hochberg false discovery rate = 1.10×10^{-6}) (Supplemental Table S6).³⁷

Discussion

In the present study, we used CHIP-Seq analysis in combination with microarray analysis and identified *SPAG4* as a novel HIF-1–specific target. RT-qPCR analysis showed up-regulation of *SPAG4* in hypoxic cells. Subcellular localization analysis indicated that SPAG4 localizes at the intercellular bridge in telophase, and functional studies demonstrated a crucial role of SPAG4 in cytokinesis. TMA analysis of

Figure 3 Live-cell imaging of SPAG4 in HeLa cells expressing fluorescent SPAG4. **A:** Immunoblotting analysis detected the EGFP-fused SPAG4 protein in SPAG4-EGFP HeLa clone. **B:** SPAG4 localization in interphase. **C:** Cells expressing SPAG4-EGFP and calnexin-mCherry confirmed localization of SPAG4 in the ER. **D:** SPAG4 localization during telophase. SPAG4-EGFP HeLa cells were transduced with red fluorescent tubulin to illustrate the midbody. SPAG4 localization to the intercellular bridge is indicated by an **arrow**. **E:** Cells expressing SPAG4-EGFP and calnexin-mCherry showed that localization of SPAG4 at the base of the intercellular bridge was specific and independent of the ER. **F:** HeLa cells expressing c-Myc-tagged SPAG4 were fixed and stained with anti-c-Myc antibody. Cells are shown during interphase (**left**) and during telophase (**right**). **G:** HeLa cells were transiently transfected with SPAG4-mCherry plasmid. Cells are shown during interphase (**left**) and during telophase (**right**). **H:** HeLa cells were transiently transfected with SPAG4-mCherry plasmid and stained with anti- α -tubulin antibody. Representative images of telophase cells are shown. Original magnification: $\times 1000$ (**B–E**); $\times 400$ (**F–H**). RFP; red fluorescent protein.

ccRCC demonstrated SPAG4 to be an independent prognostic factor. These findings suggest that SPAG4 may be up-regulated to play a defensive role against hypoxia-induced polyploidy, which promotes survival of cancer cells under hypoxic conditions and thus leads to poor prognosis.

Although SPAG4 was originally identified as a testis-specific protein,^{9,10} recent studies have demonstrated ubiquitous expression of human SPAG4 in various normal tissues, including the kidney, at the mRNA level.^{14,15} Furthermore, *SPAG4* mRNA is also detected in a wide range of neoplastic tissues,^{15,16} suggesting that SPAG4 expression may be a potential clinically relevant cancer marker, although the biological function remained to be elucidated.

Cytokinesis, the physical separation of two daughter cells during cell division, is the final stage of the cell cycle.³⁸ Failure to complete cytokinesis can lead to formation of a persistent connection between the two daughter cells and subsequent tetraploidy, and tetraploidy is a genetically unstable state that promotes chromosomal instability, leading to eventual halt of proliferation and cell death.^{39,40}

Localization of SPAG4 in the intercellular bridge, which was confirmed by SPAG4 with three different tags to exclude an artifact by tagging, suggested the involvement of SPAG4 in cytokinesis. Among 2000 cells transiently transfected with mCherry-tagged SPAG4 plasmid, 22/23 (96%) telophase cells expressed SPAG4 at the intercellular

bridge. For the small proportion of telophase cells that did not express SPAG4 at the intercellular bridge, we supposed that this was due to the overall low expression level of SPAG4-mCherry in these cells. We concluded that SPAG4 localizes at the intercellular bridge during telophase, involved in cytokinesis. Next, our knockdown studies of SPAG4 showed an increase in tetraploid cells, suggesting that SPAG4 may play a role in organized cytokinesis and prevent the formation of tetraploidy. Hypoxia increased the number of tetraploid cells in HeLa cell, compared with normoxia. A previous study had also demonstrated that exposure to acute hypoxia induced tetraploidization of diploid human tumor cells *in vitro*.⁴¹ We speculate that SPAG4 in minute amounts serves a defensive role against tetraploidy under normoxia and is up-regulated by hypoxia to act as a safeguard against hypoxia-induced tetraploidy.

A functional role of SPAG4 in cytokinesis was confirmed by studies using mutated SPAG4. Although the precise molecular mechanism remains to be elucidated, our studies of deletion mutants support involvement of SPAG4 in cytokinesis, showing that both N-terminal domain and coiled-coil domain are essential to induce intact cytokinesis.

We also showed that knockdown of SPAG4 changed the expression level of genes involved in cytokinesis, including *AURKA* and *AURKB*.⁴² Because SPAG4 is localized at the intercellular bridge, its role in cytokinesis likely directly influences the mechanics of cell division. The completion of

Figure 4 SPAG4 plays a defensive role against hypoxia-induced polyploidy. **A:** SPAG4 siRNA decreased SPAG4 protein expression at 48 hours after transfection in HeLa cells. Data are expressed as ratios of SPAG4 and actin band intensity, standardized to the mean of the control siRNA, under normoxia. **B:** Flow cytometric analysis in HeLa cells showed that the proportion of tetraploid cells increased under hypoxia, compared with normoxia. **C:** Flow cytometric analysis in HeLa cells showed that the proportion of tetraploid cells increased in SPAG4 siRNA-treated HeLa cells, compared with control siRNA-treated cells, under normoxia (**left**) and hypoxia (**right**). **D, left:** Microscopic observation revealed a significant increase in binucleated and multinucleated cells in cells treated with SPAG4 siRNA (SPAG4-1) under normoxia. Cells were fixed and stained with anti- β -catenin antibody and Hoechst 33342 dye to visualize cell membranes and nuclei. At least 300 cells were counted in each group, and three independent experiments were performed. **D, right:** A representative image of binucleated cells treated with SPAG4 siRNA (SPAG4-1) under normoxia is shown. **E:** BrdU assay showed that knockdown of SPAG4 significantly decreased intake of BrdU under both normoxia (**left**) and hypoxia (**right**). **F:** MTS assay showed that the number of living cells was significantly less in SPAG4 siRNA-treated cells under normoxia. **G:** Mutated SPAG4-EGFP, in which both N-terminal domain and coiled-coil domain were deleted, failed to localize at the intercellular bridge. **H, left:** Microscopic observation revealed a significant increase in binucleated or multinucleated cells in cells expressing mutated SPAG4-EGFP (in which both N-terminal domain and coiled-coil domain were deleted). At least 300 cells were counted in each group, and three independent experiments were performed. **H, right:** A representative image of binucleated cells in deletion mutant. Data are expressed as means $\pm$ SD. $n = 4$ (**A** and **B**) or 6 (**E** and **F**). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and $^{\dagger}P < 0.0001$ versus control siRNA or normoxia. Original magnification: $\times 400$ (**D** and **H**); $\times 1000$ (**G**).

cytokinesis requires the precise and coordinated regulation of the genes involved, and we speculate that abnormal cytokinesis by SPAG4 knockdown resulted in the dysregulation of the cytokinesis-related genes.

Knockdown of SPAG4 suppressed cell proliferation. It is widely acknowledged that the proliferation of tetraploid cells is limited by a variety of mechanisms.⁴⁰ Tetraploid cells arrest their cell cycle, and this arrest depends on the tumor suppressor protein p53.^{43–45} Even in transformed cell lines with p53-defective pathways (which includes HeLa cells), cleavage failure can also be lethal, via failure of further cleavage and eventual cell death.³⁹ In a study exploring the consequences of binucleation, binucleated

HeLa cells showed delay in cell-cycle progression and more cell death, compared with their mononucleated counterparts.⁴⁶ In the present study, we observed that knockdown of SPAG4 increased tetraploidy of the cultured cells, resulting in subsequent suppression of cell proliferation due to disturbance of cell division.

To clarify clinical relevance of SPAG4, we studied ccRCC, which is the most common malignancy of the kidney in humans.⁴⁷ Half of the patients who undergo resection with a curative intent are expected to have a relapse during the course of the disease, and metastatic RCC is one of the most treatment-resistant malignancies.⁴⁸ HIF-1 α and HIF-2 α are overexpressed in the majority of

Table 3 Functional Annotation Clustering by DAVID Analysis

Functional annotation	Gene number	FDR
Cluster 1 (ES = 23.90)		
Cell-cycle phase	58	9.67×10^{-25}
Cell cycle	77	1.60×10^{-24}
M phase	51	8.26×10^{-24}
Cell cycle process	64	4.32×10^{-23}
M phase of mitotic cell cycle	42	1.52×10^{-22}
Mitotic cell cycle	52	1.30×10^{-22}
Mitosis	41	5.56×10^{-22}
Nuclear division	41	5.56×10^{-22}
Organelle fission	41	2.34×10^{-21}
Cell division	37	8.19×10^{-14}
Cluster 2 (ES = 8.86)		
DNA metabolic process	42	7.26×10^{-10}
Response to DNA damage stimulus	32	1.61×10^{-07}
DNA repair	27	4.31×10^{-07}
Cellular response to stress	36	1.60×10^{-05}
Cluster 3 (ES = 6.32)		
Regulation of cell cycle	30	1.57×10^{-07}
Regulation of mitotic cell cycle	19	2.16×10^{-06}
Regulation of cell cycle process	16	7.66×10^{-06}
Cell cycle checkpoint	13	1.17×10^{-04}
Regulation of mitosis	10	4.08×10^{-04}
Regulation of nuclear division	10	4.08×10^{-04}
Regulation of organelle organization	17	0.0034
Cluster 4 (ES = 4.58)		
Microtubule-based process	23	1.31×10^{-05}
Microtubule cytoskeleton organization	15	6.48×10^{-04}
Spindle organization	8	0.0043
Cluster 5 (ES = 2.95)		
Chromosome segregation	17	8.36×10^{-09}
Mitotic sister chromatid segregation	10	1.09×10^{-05}
Sister chromatid segregation	10	1.33×10^{-05}
Establishment of chromosome localization	5	0.0176
Chromosome localization	5	0.0176

Genes up-regulated by SPAG4 knockdown were clustered by gene ontology (biological process). ES ≥ 1.5 was considered significant.

FDR, Benjamini and Hochberg false discovery rates (5% false positive discovery rate).

ccRCCs, and some studies reported that patients with high HIF-1 α expression had significantly worse survival than patients with low expression.^{7,49–51} HIF is not suitable as a clinical prognostic marker, however, because its very short half-life period (less than 5 minutes) allows degradation of the protein during processing of tissue samples.² Thus, the need remains for biomarkers to improve diagnosis, identify

tumors with a high risk of recurrence, allow easier follow-up, suggest earlier adjuvant treatment, and select the best treatment option for metastatic patients in renal oncology.

We performed TMA analysis with 190 ccRCC samples. We observed relatively few cancer-specific deaths in our study population, because most patients (72.6%) were classified at TNM stage I. It is likely that our university hospital is a flagship hospital of cancer treatments, and that many patients with early-stage cancer who need surgery are referred from regional hospitals. Although cancer-specific survival for the whole group was good, 5-year cancer-specific survival for TNM stages I through IV (96.9%, 72.7%, 63.7%, and 26.5%, respectively) were equivalent to those reported previously.³²

Our TMA analysis of ccRCC demonstrated for the first time that SPAG4 expression is associated with higher cancer-specific mortality. Although SPAG4 expression was associated with higher nuclear grading, which is a well-known prognostic factor in ccRCC,^{32,33,35,36} SPAG4 was an independent prognostic factor in multivariate analysis after correction for TNM stage, nuclear grade, ECOG PS, and mode of presentation. In addition, even in patients with nuclear grade 2 or 3, who have a poor prognosis, SPAG4 expression can predict worse cancer-specific survival. Thus, SPAG4 may be a clinically relevant cancer marker in the prediction of poor survival. Although there was statistical significance in our study, the relatively small number of SPAG4 nonexpressors is a potential limitation of our study. Further study in a larger population is needed to establish the usefulness of SPAG4 as a marker. In ccRCC, carbonic anhydrase 9 (CA9) is one of the most studied and most promising biomarkers.⁵¹ Patients with low CA9 expression were reported to have worse prognosis; in metastatic disease, high CA9 expression was associated with better survival.⁵¹ SPAG4 could be a clinically useful biomarker in combination with other biomarkers, such as CA9.

SPAG4 belongs to the SUN family of proteins, which contain the SUN domain.^{11–13} Most SUN-domain proteins are localized at the inner nuclear membrane, but in previous reports SPAG4 did not localize specifically at the nuclear envelope but instead localized throughout the ER,^{13,52} which is consistent with the present results. A function of SPAG4 in the ER is beyond the scope of the current investigation, however, and remains a subject for future study.

In conclusion, we have identified SPAG4 as a novel HIF-1 target that plays a crucial role in cytokinesis. SPAG4 is up-regulated to play a defensive role against hypoxia-

Table 4 Representative Genes for the Five Cluster Categories

Category	Genes	Enrichment score
Cell cycle	<i>AURKA</i> , <i>AURKB</i> , <i>BIRC5</i> , <i>CCNA2</i> , <i>CCNB1</i> , <i>CDC20</i> , <i>CDK1</i> (previously <i>CDC2</i>), <i>CEP55</i>	23.9
DNA metabolic process	<i>EXO1</i> , <i>NFIC</i> , <i>PARP2</i> , <i>POLK</i> (alias <i>POLQ</i>), <i>RFC3</i> , <i>RFC4</i>	8.86
Regulation of cell cycle	<i>AURKAIP1</i> (alias <i>AKIP</i>), <i>BUB1</i> , <i>CDKN3</i> , <i>CHEK1</i> , <i>PLK4</i>	6.32
Microtubule-based process	<i>CENPA</i> , <i>CKAP5</i> , <i>KIF2C</i> , <i>KIF9</i> , <i>KIF14</i> , <i>KIF18B</i> , <i>KIF20A</i>	4.58
Chromosome segregation	<i>CENPE</i> , <i>CENPO</i> , <i>ESPL1</i>	2.95

induced polyploidy, which promotes survival of cancer cells under hypoxic conditions and leads to poor prognosis.

Acknowledgment

We thank Dr. Teppei Morikawa (University of Tokyo) for constructing the TMA.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2013.02.024>.

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