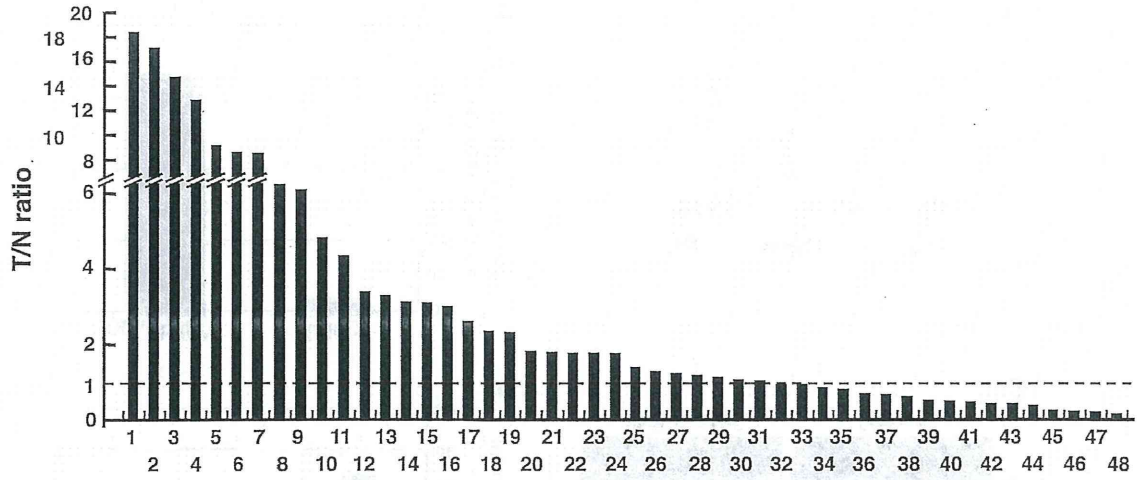

Fig. 2 Upregulation of PLK4 mRNA expression level in human primary gastric cancer. The mRNA expression level of PLK4 variant 1 was compared in cancerous tissues from 48 primary gastric cancers and corresponding non-cancerous gastric tissues using a real-time qRT-PCR analysis. After normalizing the amounts of PLK4 transcripts to those of the GAPDH transcripts, the T/N values were calculated by dividing the amount of normalized transcripts in the cancerous tissue by the amount in the corresponding non-cancerous tissue. The differences between the normalized PLK4 mRNA level in the cancerous tissues and the corresponding non-cancerous tissues were then statistically analyzed using the Wilcoxon matched pairs test (P value = 0.0139)

Table 1 Clinicopathological factors of gastric cancer patients according to the T/N ratio of PLK4 mRNA expression in the stomach

Factor	Patient	PLK4 expression level[a]		P value
		Low ($n = 24$)	High ($n = 24$)	
Gender				
Male	38	20 (83.3 %)	18 (75.0 %)	0.4772[b]
Female	10	4 (16.7 %)	6 (25.0 %)	
Age				
≤60	13	6 (25.0 %)	7 (29.2 %)	0.7453[b]
>60	35	18 (75.0 %)	17 (70.8 %)	
Mean ± SD	65.7 ± 11.9	67.2 ± 11.9	64.3 ± 11.9	0.4010[c]
Histopathology				
Differentiated	21	13 (54.2 %)	8 (33.3 %)	0.1457[b]
Undifferentiated	27	11 (45.8 %)	16 (66.7 %)	
pT stage				
pT1	10	4 (16.7 %)	6 (25.0 %)	0.4772[b]
pT2-pT4	38	20 (83.3 %)	18 (75.0 %)	

SD standard deviation

[a] Low, T/N value <1.5; High, T/N value ≥1.5

[b] χ^2 test

[c] t test

associations were found between the clinicopathological factors of onset age, sex, tumor histopathology, or tumor stage and the PLK4 mRNA expression level (Table 1). These results suggested that the expression of PLK4 is upregulated in a subset of primary gastric cancers.

Induction of centrosome amplification and CIN by PLK4 overexpression in gastric cells

To investigate the effect of PLK4 overexpression on gastric cancer cells, we used the piggyBac transposon vector system [28] to establish human cells capable of inducibly expressing PLK4 variant 1. First, AGS gastric cancer cells were transfected with a piggyBac cumate switch inducible vector for the expression of Myc-PLK4 together with the piggyBac transposase vector; positively transposed cells were then selected using puromycin. We also prepared cells transfected with an empty (parental) piggyBac cumate switch inducible vector and transposase vector. The expression of PLK4 protein was confirmed in cells transposed with Myc-PLK4 vector after cumate induction using a Western blot analysis with anti-PLK4 antibody (Fig. 3a). Then, we examined the centrosome number in Myc-PLK4-transposed cells after cumate induction using an immunofluorescence analysis of γ-tubulin, a major centrosomal protein [29, 30]. The frequency of cells containing ≥3

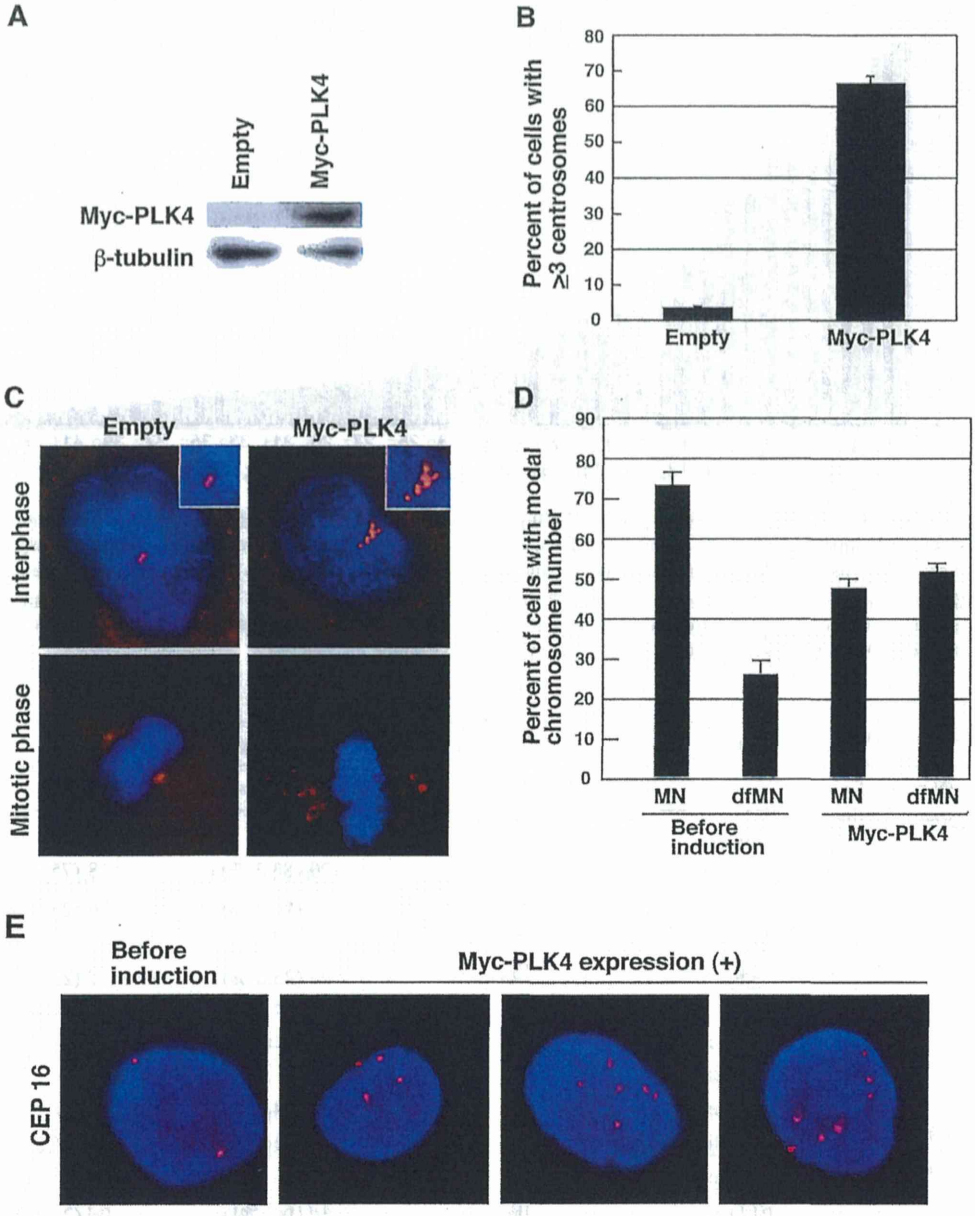

Fig. 3 Induction of centrosome amplification and CIN by PLK4 overexpression in gastric cells. **a** Detection of Myc-PLK4 proteins in cumate-inducible stable cell lines designed to express Myc-PLK4 in the presence of cumate; the Myc-PLK4 proteins were detected using a Western blot analysis with an anti-Myc antibody. Lysates from empty vector-transposed cells and cells inducibly expressing Myc-PLK4 were analyzed. β-tubulin protein was also analyzed as an internal control. **b**, **c** Induction of centrosome amplification in AGS cells as a result of Myc-PLK4 expression. At 72 h after cumate addition, the cells were immunostained with mouse anti-γ-tubulin monoclonal antibody (*red*). The nuclei were stained with DAPI (*blue*). The number of centrosomes per cell was counted, and the counts are shown in **b**. A *t*-test was performed for the statistical analysis (Empty vs. Myc-PLK4, P value <0.00001). Representative immunostaining images are shown in **c**. *Upper right inset* higher magnification of a portion of each panel. **d**, **e** Induction of CIN by PLK4 overexpression in gastric cells. At 72 h after cumate addition, the cells were replated in fresh medium without cumate, cultured for an additional 72 h, and then subjected to a FISH analysis using a Spectrum Orange-labeled probe for the centromere locus on chromosome 16, CEP 16. Cells before cumate induction was also subjected to the FISH analysis. The nuclei were stained with DAPI (*blue*). The percentages of cells with the MN and chromosome numbers dfMN were determined, and the percentages are shown in **d**. A *t* test was used to perform the statistical analysis (before induction vs. Myc-PLK4 overexpression, P value <0.01). Representative FISH images are shown in **e**

centrosomes was significantly higher among the Myc-PLK4-overexpressing cells than among the empty vector-transposed cells (Fig. 3b, c). This result suggests that the upregulation of PLK4 expression causes centrosome amplification in gastric cells. Next, we attempted to determine whether the increased frequency of centrosome

amplification in Myc-PLK4-overexpressing gastric cells resulted in chromosome destabilization using a FISH analysis. When the number of chromosomes per cell was divided into the modal chromosome number (MN) and chromosome numbers different from the modal number (dfMN), dfMN of chromosome 16 were found more frequently in cells after cumate induction than in cells before induction (Fig. 3d, e). Based on the above findings, PLK4 overexpression likely induces centrosome amplification and, in turn, CIN in gastric cells.

Suppression of primary cilia formation using PLK4 overexpression

Since part of the proteins localized in the centrosome modulate ciliogenesis [31], we next examined the effect of the overexpression of the centrosomal protein PLK4 on primary cilia formation. Human 293T cells were transiently transfected with plasmids expressing GFP-PLK4 or GFP only, brought to quiescence by serum starvation, and stained for the presence of acetylated α-tubulin, a ciliary marker [31]. Cells transfected with a control GFP plasmid expressed primary cilia; in striking contrast, however, cells overexpressing GFP-PLK4 exhibited no cilia (Fig. 4a), suggesting that PLK4 overexpression can suppress cilia assembly. Next, to confirm this result, we prepared 293T cells that were capable of inducibly expressing Myc-PLK4 and control cells using the piggyBac transposon vector system. Empty vector-transposed cells expressed primary cilia, whereas cells overexpressing Myc-PLK4 exhibited no cilia (Fig. 4b). In addition, the expression of acetylated α-tubulin was reduced in 293T cells overexpressing Myc-PLK4, compared with empty vector-transposed cells, after serum starvation using a Western blot analysis with anti-acetylated α-tubulin antibody (Fig. 4c). The above results suggest that PLK4 overexpression suppresses primary cilia formation.

Discussion

In the present study, we found that PLK4 variant 1 is predominant among the three PLK4 transcript variants in humans, and the expression of the PLK4 variant 1 mRNA is upregulated in a subset of human gastric cancers. In the analysis, we identified a novel PLK4 variant containing exon 4, but not exon 5; the variant contains 30 amino acids at its C-terminal that are not homologous to those in variant 1. Next, we prepared an AGS gastric cancer cell line capable of inducibly expressing Myc-PLK4 as well as control cells using the piggyBac transposon vector system; as a result, we clearly observed that PLK4 overexpression induced centrosome amplification and CIN in the gastric cells. Finally, we demonstrated that PLK4 overexpression also suppressed primary cilia formation. Our current findings suggested that PLK4 overexpression might be involved in the pathogenesis of gastric cancers by inducing centrosome amplification and CIN. These findings might partly explain the earlier observation of CIN in gastric cancers. As far as we know, this study is the first report to describe aberrant PLK4 expression in human gastric cancer.

In this study, the overexpression of the PLK4 mRNA transcript was observed in a subset of both gastric cancer cell lines and primary gastric cancers. Although the mechanism underlying the upregulation of PLK4 mRNA expression could not be experimentally revealed in this study, some possibilities, such as the abnormal expression of a transcription factor regulating PLK4 expression or miRNAs controlling the level of PLK4 mRNA transcripts in gastric cancer and *PLK4* gene amplification in gastric cancer, can be suggested in view of previous reports regarding gene overexpression in cancer [32–34]. With regards to *PLK4* gene amplification, in our preliminary experiment, amplification was detected in only a small percentage of gastric cancers (data not shown), indicating that this mechanism is not a major cause of PLK4 overexpression. Since some transcription factors involved in PLK4 transcriptional regulation have been previously identified [35, 36], abnormalities in such transcription factors might be involved in PLK4 overexpression in gastric cancer. Future studies examining this point could help to clarify the cause of PLK4 upregulation.

Among human malignancies, the PLK4 expression status has been previously examined in colorectal cancer and hepatocellular carcinoma [17, 18]. Using a semiquantitative RT-PCR analysis, Macmillan et al. [17] detected a higher PLK4 mRNA expression level in cancerous lesions than in non-cancerous tissues in 66 % of their colorectal cancer cases. On the other hand, Liu et al. [18] reported the downregulated expression of PLK4 mRNA in 65 % of hepatocellular carcinomas based on the results of a qRT-PCR analysis. Our present results and the above-mentioned reports suggest that PLK4 is abnormally expressed in some types of human cancers and that its expression status differs among such cancers.

Centrosome amplification and CIN were induced by PLK4 overexpression in the gastric cells of our system. Since PLK4 is active in the control of centrosome duplication, the fact that its dysregulation in gastric cells led to centrosome amplification is not surprising. Our findings regarding centrosome abnormalities in gastric cells are consistent with previous reports describing centrosome amplification induced by PLK4 overexpression in cells other than gastric cells [10, 11]. As possible mechanisms linking centrosome amplification to CIN, abnormal mitotic

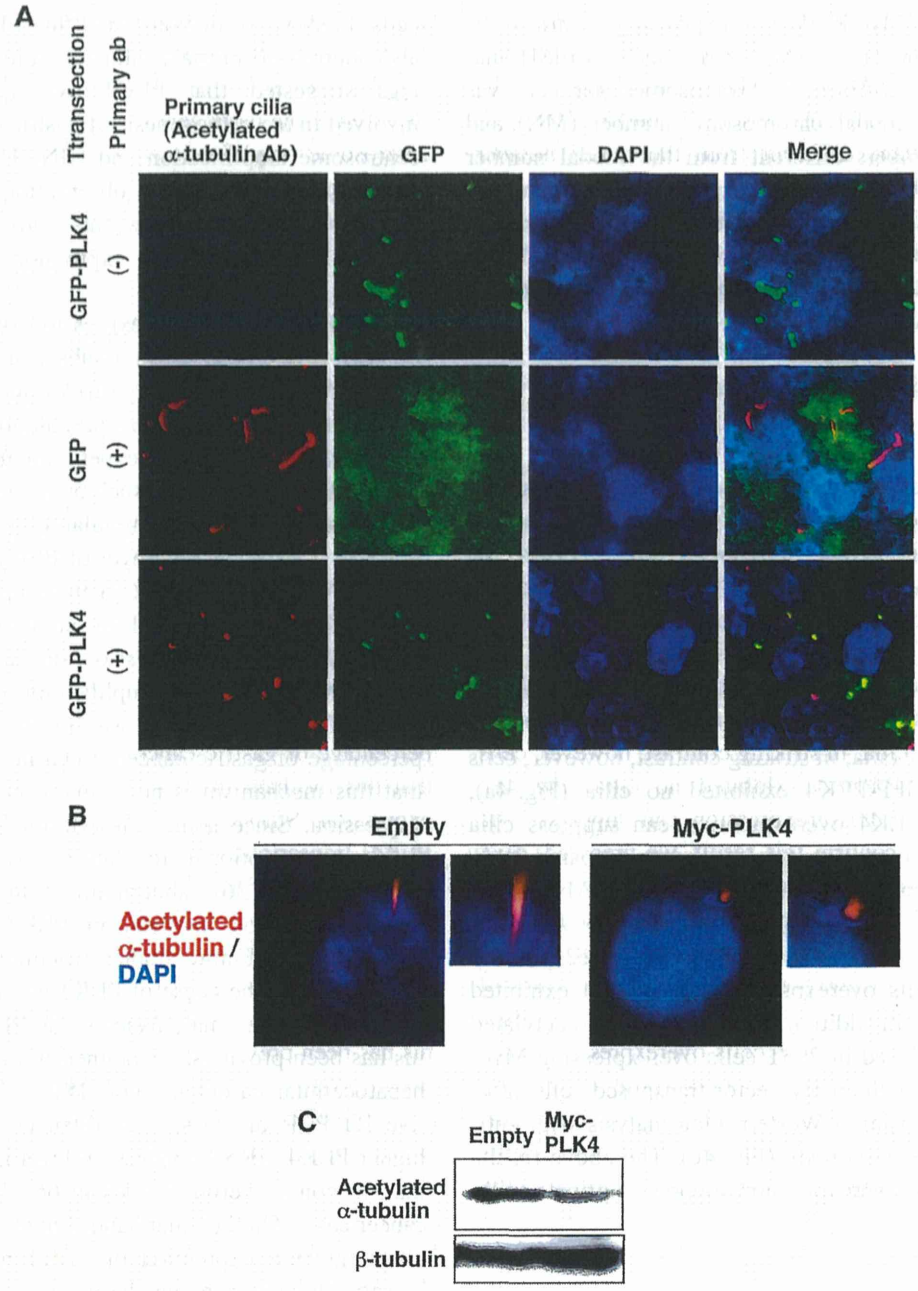

Fig. 4 Suppression of primary cilia formation by PLK4 overexpression in quiescent human cells. **a** Effect of PLK4 overexpression on primary cilia formation in serum-starved 293T cells. The cells were transfected with a GFP-PLK4 expression vector or its parental vector (GFP expression vector), serum-starved for 24 h, and then subjected to an immunofluorescence analysis using a primary antibody against acetylated α-tubulin, a ciliary marker. The primary cilia are shown in *red*, and GFP or GFP fusion proteins are shown in green. The nuclei were stained with DAPI (*blue*). **b** Effect of Myc-PLK4 inducible expression on primary cilia formation in serum-starved 293T cells. 293T cells inducibly expressing Myc-PLK4 and control cells (empty vector-transposed cells) were prepared using the piggyBac transposon vector system. After cumate induction, the cells were serum-starved for 24 h and then immunostained with anti-acetylated α-tubulin antibody (*red*). The nuclei were stained with DAPI (*blue*). Representative immunostaining images are shown. *Right smaller panel* higher magnification of a portion of the *left panel*. **c** Detection of acetylated α-tubulin proteins in serum-starved 293T cells inducibly expressing Myc-PLK4 and control cells (empty vector-transposed cells) using a Western blot analysis with an anti-acetylated α-tubulin antibody. Lysates from empty vector-transposed cells and cells inducibly expressing Myc-PLK4 after serum starvation for 24 h were analyzed. β-tubulin protein was also analyzed as an internal control

spindle formation, lagging chromosome formation, merotelic kinetochore-microtubule attachment errors, and chromosome segregation errors have been proposed [2, 4, 8, 9, 37]. Thus, centrosome amplification as a result of PLK4 overexpression may have resulted in CIN in the gastric cells in the present study. In other words, our results

suggest that PLK4 overexpression contributes to CIN via centrosome amplification in gastric cancer.

A novel PLK4 transcript variant was identified in the present study. Although the mRNA expression level of the novel variant was much lower than that of PLK4 variant 1 and the variant encodes an extremely short protein (105 amino acids long) because of the generation of a premature stop codon, the variant protein contains 30 amino acids at its C-terminal that are not homologous with those of variant 1 (Fig. 1c). Since a homology search using a Basic Local Alignment Search Tool (BLAST) program (**http://blast.ncbi.nlm.nih.gov/Blast.cgi**) showed that part of this 30-amino acid sequence was homologous with various proteins (data not shown), the variant protein might have an important role.

The suppression of primary cilia formation by PLK4 overexpression was shown in human kidney 293T cells in this study. In our preliminary experiment, primary cilia formation was not easily observed in some gastric cancer cell lines including AGS; however, the 293T cell line has been previously used to study ciliogenesis [38]. Therefore, we used the 293T cell line, and an effect of PLK4 overexpression on primary cilia formation was clearly observed. Thus, PLK4 shares some activity with CP110, a protein that is localized at the centrosome and plays an essential role in the control of centrosome duplication and primary cilia formation [31]. Recently, Kim et al. [38] performed a functional genomic screening for modulators of ciliogenesis and cilium length and found 13 negative modulators not including PLK4. The screening process did not detect PLK4 probably because the screening used RNA interference. The results reported by Kim et al. [38] and our results suggest that role of PLK4 on primary cilia formation is shown by a comparison of cells with and without PLK4 overexpression, but not by a comparison of cells with and without PLK4 knockdown. Primary cilia are a feature of epithelial cells, including those of the pancreas, breast, kidney, prostate, and liver, and some epithelial cells in the stomach also exhibit primary cilia [39–41]. Interestingly, pancreatic cancers, but not normal pancreatic cells, are devoid of primary cilia [39]. Thus, the suppression of primary cilia formation by PLK4 overexpression might be related to the pathogenesis of gastric tumors.

Cell cycle progression has an inverse relationship with primary cilia formation [42]. With the exception of some kinds of cells with primary cilia during cell proliferation, primary cilia are disassembled in most cells at cell cycle reentry. Moreover, the forced induction or suppression of primary cilia has been recently shown to affect cell cycle progression; specifically, the exogenous expression of trichoplein, which is concentrated at the centrioles in proliferating cells, suppressed primary cilia formation in G1 phase with the cooperation of Aurora A [43, 44]. Since the suppression of primary cilia formation by PLK4 overexpression was shown in this study, the proliferation of cancer cells showing PLK4 overexpression might be suppressed by the forced reduction of PLK4 expression.

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Image of the Month

Radiation-induced Breast Angiosarcoma with a Confirmative Feature of c-MYC Amplification

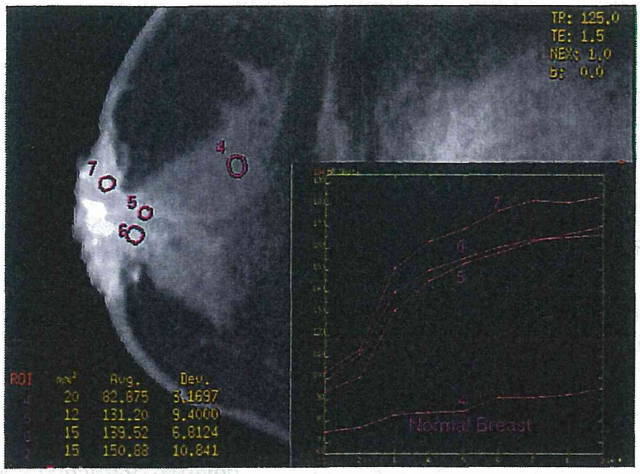

Figure 1.

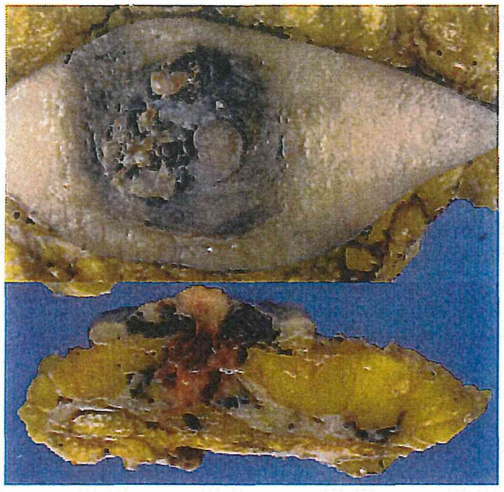

Figure 2.

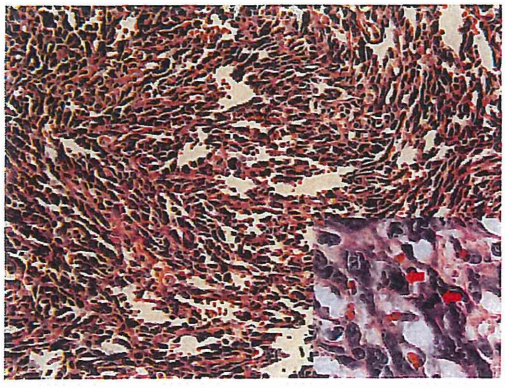

Figure 3.

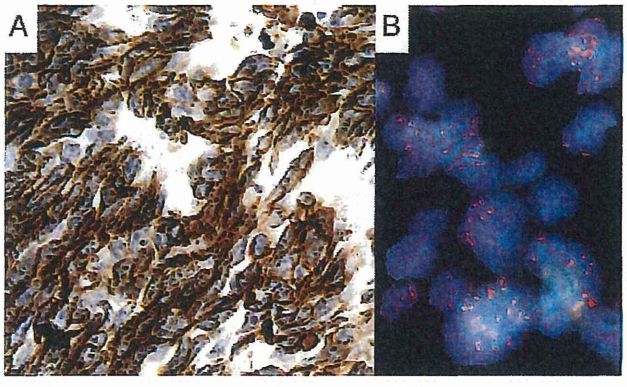

Figure 4.

A 73-year-old female presented with a left axillary lymph node swelling. Histopathological analysis of lymph node biopsy revealed carcinoma that morphologically and immunohistochemically resembled breast carcinoma. The primary site was unidentifiable on mammography and ultrasonography, suggesting an occult carcinoma of the left breast. She received prophylactic chemotherapy and radiotherapy (50 Gy) to the left breast.

Five years afterward, a hypoechoic mass was detected on ultrasonography in the central portion of the left breast. She was kept under observation for 2 years, during when the brownish discoloration near the left areola had increased accompanied by crust formation. Magnetic resonance imaging showed a mass spanning both the breast parenchyma and the superficial skin. Dynamic study displayed gradual enhancement (Fig. 1), in contrast to the characteristic early enhancement of breast carcinoma.

Total mastectomy was performed. Macroscopically, the resected specimen contained a hemorrhagic mass located in both the breast parenchyma and the superficial skin with crust formation (Fig. 2). Histopathological examination revealed atypical spindle cells arranged in the form of fascicles with scattered formation of vascular channels (Fig. 3). Mitotic figures were prominent (Fig. 3, inset, arrows). The tumor was positive for CD31 (Fig. 4A), CD34 and factor VIII on immunohistochemistry, confirming the diagnosis of angiosarcoma. Fluorescence *in situ* hybridization revealed a remarkable amplification of *c-MYC* (orange), clearly distinguishable from the centromere of the chromosome 8 (green) (Fig. 4B). These findings, especially the *c-MYC* amplification, strongly supported the diagnosis of radiation-induced angiosarcoma, and not primary angiosarcoma of the breast, because the existence of *c-MYC* amplification only in the former was reported previously by Ginter et al. (1).

Postoperatively, she underwent adjuvant chemotherapy with paclitaxel for ~1 year and has been recurrence free for 4.5 years.

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Shogo Tajima[1,*], *Ryozo Mochizuki*[2], *Haruhiko Sugimura*[3] *and Shoji Hoshi*[1]
[1]*Department of Pathology, Shizuoka Saiseikai General Hospital, Shizuoka*
[2]*Department of Radiology, Shizuoka Saiseikai General Hospital, Shizuoka*
[3]*Department of Tumor Pathology, Hamamatsu University School of Medicine, Shizuoka, Japan.*
[*]*E-mail: stajima-tky@umin.ac.jp*
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RSPO fusion transcripts in colorectal cancer in Japanese population

Kazuya Shinmura · Tomoaki Kahyo · Hisami Kato · Hisaki Igarashi · Shun Matsuura · Satoki Nakamura · Kiyotaka Kurachi · Toshio Nakamura · Hiroshi Ogawa · Kazuhito Funai · Masayuki Tanahashi · Hiroshi Niwa · Haruhiko Sugimura

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Abstract *R-spondin* (*RSPO*) gene fusions have recently been discovered in a subset of human colorectal cancer (CRC) in the U.S. population; however, whether the fusion is recurrent in CRC arising in patients from the other demographic areas and whether it is specific for CRC remain uncertain. In this study, we examined 75 primary CRCs and 121 primary lung cancers in the Japanese population for EIF3E-RSPO2 and PTPRK-RSPO3 fusion transcripts using RT-PCR and subsequent sequencing analyses. Although the expression of EIF3E-RSPO2 and PTPRK-RSPO3 was not detected in any of the lung carcinomas, RSPO fusions were detected in three (4 %) of the 75 CRCs. Two CRCs contained EIF3E-RSPO2 fusion transcripts, and another CRC contained PTPRK-RSPO3 fusion transcripts. Interestingly, in one of the two EIF3E-RSPO2 fusion-positive CRCs, a novel fusion variant form of EIF3E-RSPO2 was identified: exon 1 of EIF3E was connected to exon 2 of RSPO2 by a 351-bp insertion. A quantitative RT-PCR analysis revealed that RSPO mRNA expression was upregulated in the three CRCs containing RSPO fusion transcripts, while it was downregulated in nearly all of the other CRCs. An immunohistochemical analysis and a mutational analysis revealed that the RSPO fusion-containing CRC had a CDX2 cell lineage, was positive for mismatch repair protein expression, and had the wild-type *APC* allele. Finally, the forced expression of RSPO fusion proteins were shown to endow colorectal cells with an increased growth ability. These results suggest that the expression of RSPO fusion transcripts is related to a subset of CRCs arising in the Japanese population.

Keywords Colorectal cancer · Fusion · RSPO2 · RSPO3 · APC · Lung cancer

K. Shinmura (✉) · T. Kahyo · H. Kato · H. Igarashi · S. Matsuura · S. Nakamura · H. Sugimura
Department of Tumor Pathology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi Ward, Hamamatsu 431-3192, Japan
e-mail: kzshinmu@hama-med.ac.jp

K. Kurachi · T. Nakamura
Department of Surgery 2, Hamamatsu University School of Medicine, Hamamatsu, Japan

H. Ogawa
Division of Pathology, Seirei Mikatahara General Hospital, Hamamatsu, Japan

K. Funai
Department of Surgery 1, Hamamatsu University School of Medicine, Hamamatsu, Japan

M. Tanahashi · H. Niwa
Division of Thoracic Surgery, Respiratory Disease Center, Seirei Mikatahara General Hospital, Hamamatsu, Japan

Introduction

ALK fusion genes, such as *EML4-ALK*, are key oncogenic drivers in a subset of non-small cell lung carcinomas (NSCLCs), and a small molecule inhibitor of ALK has been shown to inhibit the growth of ALK-positive NSCLC in a selective manner [–]. Therefore, molecular data regarding oncogenic fusion can have a significant clinical impact. Numerous reports on oncogenic fusions in NSCLC have been published to date, but only a few papers have discussed recurrent gene fusions in colorectal cancer (CRC). Very recently, Seshagiri et al. [ ] identified *R-spondin* (*RSPO*) gene fusions in a subset of CRCs in the U.S. population. Four members of the *RSPO* gene family exist in humans (*RSPO1*, *RSPO2*, *RSPO3*, and *RSPO4*).

These family member proteins have cysteine-rich furin-like (CR) domains and a thrombospondin type 1 repeat (TSR) domain and are involved in the activation of the Wnt signaling pathway [ ]. Among the RSPO family members, RSPO2 and RSPO3 have been shown to be connected to EIF3E and PTPRK, respectively, in CRCs; specifically, a fusion between exon 1 of EIF3E and exon 2 of RSPO2, a fusion between exon 1 of PTPRK and exon 2 of RSPO3, and a fusion between exon 7 of PTPRK and exon 2 of RSPO3 were identified by Seshagiri et al. [ ]. This group also showed that the RSPO fusions were mutually exclusive with somatic *APC* mutations and that the RSPO fusion proteins were capable of potentiating Wnt signaling, suggesting an important role of RSPO fusion in the development of CRC [ ]. However, since no papers other than that by Seshagiri et al. [ ] have been published regarding RSPO fusions, whether RSPO fusion is a recurrent phenomenon in CRCs arising in patients from other demographic areas and whether it occurs in carcinomas other than CRC are unclear. In this study, we examined the presence of EIF3E-RSPO2 and PTPRK-RSPO3 in a total of 75 CRCs and 121 NSCLCs and further characterized any fusions that were detected. This report is the second published study to describe the RSPO fusion in cancer.

Materials and methods

Primary carcinomas

Samples of surgical specimens from 75 CRC patients and 121 NSCLC patients who had undergone surgery at the Hamamatsu University Hospital or the Mikatahara Seirei General Hospital were obtained. All the patients were Japanese. The clinicopathological profiles of the CRC cases are shown in Table . Regarding the NSCLC cases, the mean age of the 121 patients was 68.3 years (standard deviation, 7.5 years), and they consisted of 94 men and 27 women. The NSCLCs were histologically classified as adenocarcinoma in 70 cases, squamous cell carcinoma in 43 cases, adenosquamous carcinoma in 6 cases, and pleomorphic carcinoma in 2 cases. This study was approved by the Institutional Review Boards (IRBs) of Hamamatsu University School of Medicine and Mikatahara Seirei General Hospital.

Reverse transcription (RT)-polymerase chain reaction (PCR)

Total RNA was extracted from the tissue samples using an RNeasy kit (Qiagen, Valencia, CA, USA) or ISOGEN kit (Nippon Gene, Tokyo, Japan) and was converted to first-strand cDNA using a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the supplier's protocol. PCR was performed in 20-μL reaction mixtures containing HotStarTaq DNA polymerase (Qiagen). For the amplification of the EIF3E-RSPO2 fusion transcript, the following PCR primers were used: 5′-TAC GAC TTG ACT ACT CGC ATC G-3′ for the sequence at exon 1 of EIF3E and 5′-GGG AGG ACT CAG AGG GAG AC-3′ for the sequence at exon 2 of RSPO2. For the amplification of the PTPRK-RSPO3 fusion transcript, two different PCR primer pairs were used. The forward PCR primers were: 5′-AAA CTC GGC ATG GAT ACG AC-3′ for the sequence at exon 1 of PTPRK and 5′-TGC AGT CAA TGC TCC AAC TT-3′ for the sequence at exon 7 of PTPRK. The same reverse PCR primer was used, i.e., 5′-GTT GAA ACA GGT ATC ACA GTC AGC-3′ for the sequence at exon 3 of RSPO3. The PCR products were fractionated using electrophoresis on an agarose gel and were stained with ethidium bromide. PCR-amplified products were purified with Exo-SAP-IT (GE Healthcare Bio-Science, Piscataway, NJ, USA) and were sequenced directly using a BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems, Tokyo, Japan) and the ABI 3130 Genetic Analyzer (Applied Biosystems).

Table 1 Summary of the clinicopathological profiles of the CRC patients

Characteristic	Number
No. of patients	75
Age (years) (mean ± SD)	(63.6 ± 14.5)
Sex	
Male	45 (60.0 %)
Female	30 (40.0 %)
Histology	
Well to moderately differentiated adenocarcinoma	71 (94.7 %)
Poorly differentiated adenocarcinoma	2 (2.7 %)
Mucinous adenocarcinoma	2 (2.7 %)
pT factor	
pTis/pT1	5 (6.7 %)
pT2-pT4	70 (93.3 %)
Lymph node metastasis	
Negative	34 (45.3 %)
Positive	41 (54.7 %)

SD standard deviation

Quantitative RT (QRT)-PCR

The expressions of the RSPO2 and RSPO3 mRNA transcripts were measured using real-time QRT-PCR with a LightCycler instrument (Roche, Palo Alto, CA, USA). PCR amplification of the RSPO transcript and the transcript of the control housekeeping gene *GAPDH* was performed