Analysis of Beta-Tubulin Gene Exon 4 Mutations in Advanced Stage III or IV Gastric Cancer

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Summary The mechanisms that cause chemoresistance of gastric cancer have yet to be elucidated. Taxanes and promising agents that were recently approved for treatment of advanced or recurrent gastric cancer. Mutations of beta-tubulin, which is a target of taxianes, have been shown to confer chemoresistance against these agents. The aim of the present study is to investigate the presence of mutations of the beta-tubulin in gastric cancer tissues. Sixty-six patients with advanced stage III or IV gastric cancer patients enrolled in this study. Paired samples of gastric cancer tissue and normal mucosa were obtained by endoscopy. The guanosine 5'-triphosphate (GTP)-binding site in exon 4 of the beta-tubulin gene was examined by polymerase chain reaction single-strand conformational polymorphism (PCR-SSCP) analysis, followed by sequencing of the products with abnormally shifted bands. SSCP analysis showed abnormal bands upstream of the GTP-binding site in 7 of the 66 patients, but sequence analysis found no nucleotide substitutions in these patients. Three variant bands were also detected down stream of the the GTP-binding site, but the sequences of the 3 products corresponded to those of two independent pseudogenes. Thus, none of the tumor samples showed mutation of the beta-tubulin exon 4 GTP-binding site. In conclusion, these findings suggest that mutations of the beta-tubulin gene are rare and are unlikely to be an important cause of taxane resistance to taxians.

Key Words: gastric cancer, beta-tubulin, exon 4, mutation, SSCP

Introduction

Gastric cancer is one of the most common and lethal malignancies in Japan. Although the incidence and mortality rate of gastric cancer located outside the cardia have been decreasing over the last few decades, many patients still have advanced disease at diagnosis and not indicated for curative surgery. Chemotherapy with a single agent or a combination of anticancer drugs has generally been used

treat advanced or recurrent gastric cancer, but the response has not been adequate. Taxanes including paclitaxicel and docetaxel have recently been approved for several cancers, including gastric cancer, and have shown promise for the management of advanced or recurrent gastric cancer [*1*, *2*]. Taxanes bind to beta-tubulin, which is one of the major components of microtubules [*3*], and exert a growth-inhibitory effect through the stabilization of microtubules, that causes arrest of tumor cells in the G_2-M phase of the cell cycle [*4*]. The beta-tubulin gene is located in chromosome region 6p21.3 and has four exons [*5*]. The protein is composed sequence of 445 amino acids. Although the mechanism of taxane resistance has not been clearly demonstrated for gastric cancer, Schibler has reported that beta-tubulin gene

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mutations in Chinese hamster ovary (CHO) cells could select for resistance to and/or dependence on paclitaxel [6]. In addition, recently point mutations of the beta-tubulin gene, predominantly in exon 4, have recently been reported to show an association with resistance to paclitaxicel [7–9]. Exon 4 encodes guanosine 5′-triphosphate (GTP)-binding sites, and thus it is speculated that mutations of this region may prevent GTP from promoting microtubule assembly, so that taxane-induced polymerization may not occur [10, 11]. Previous reports about beta-tubulin mutations in gastric cancer have been relatively uncommon. Urano analyzed 50 gastric tumors and found no mutations of the exon 4 GTP-binding site [12], more than half of the tumor samples were obtained from patients with early stage I or II diseases, while chemotherapy is usually given to patients with advanced disease. If beta-tubulin mutations are an acquired change, it is reasonable to expect that such mutations would be more common in advanced tumors. According, the present study examined whether or not exon 4 mutations, of the beta-tubulin gene existed in advanced stage III or IV gastric cancer.

Materials and Methods

Patients and DNA extraction

A total of 66 patients with stage III or IV gastric cancer were enrolled in this study. Each patient underwent endoscopy on admission and samples of tumor tissue and normal gastric mucosa were obtained by endoscopic biopsy. Histologic examination of selected material showed that the tumor samples contained 40% to 90% tumor cells. The specimens were snap frozen in liquid nitrogen and stored at −70°C until use. Genomic DNA was extracted directly from the frozen tissues by incubation in 10 mmol/L Tris (pH 8.1), 50 mmol/L KCL, SDS, EDTA, and 1 mg/mL proteinase K. Then each DNA sample was precipitated with ethanol, dried, in vacuo, and resuspended in 8 mmol/L NaOH. All specimens were collected after written informed consent was obtained from the patients and Ethics Committee of Fujita Health University School of Medicine approved the study protocol.

Polymerase chain reaction

The primer sets were designed to amplify specific regions of exon 4 of the beta-tubulin gene that code for the GTP-binding site (Fig. 1 and Table 1). To confirm that the amplicon was not from a pseudogene, the entire length of exon 4 was amplified first and then divided into two regions, that were amplified by using nested polymerase chain reaction (PCR). The PCR mixture (25 μL) contained 80–100 ng of extracted DNA, 5 pmol of the forward and reverse primers, 200 μmol of dNTP, 0.5 units of Taq polymerase, and 2.5 μL of standard NH₃ buffer with 1.5 μL of 1.5 mmol $MgCl_2$. (Toyobo, Tokyo Japan). PCR was commenced with incubation at 95°C for
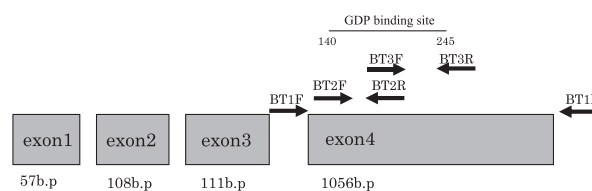
1332 b.p. chromosome 6p-21.3
(Gen Bank accession no. J00314)

Fig. 1. Diagram of the beta-tubulin gene showing its exons, with oligonucleotides used for PCR amplification and sequencing (Table 2) indicated by arrows. The GTP-binding site is shown above the protein.

Table 1. Beta-tubulin primers and annealing temperatures for PCR

Name	Position	Sequence	Annealing Temperature (°C)
BT1F	Forward	tagcttttctcctgactggc	58
BT1R	Reverse	aatcggcagaatgagttgac	58
BT2F	Forward	agagagctgtgactgcctg	52
BT2R	Reverse	aaggtattcatgatgcga	52
BT3F	Forward	cgcatcatgaataccttcag	54
BT3R	Reverse	gtgagagctcgatactgctg	52

BT1: The entire exon 4. BT2: Upstream of the GTP- binding site. BT3: Downstream of the GTP-binding site.

7 min, followed by 35 cycles of 95°C for 30 s, 52 or 57°C depending on the primer for 40 s, and 72°C for 40 s. Final extension was done at 72°C for 7 mins. For amplification of the entire exon 4, the extension time was prolonged for 1.5 min.

SSCP analysis

SSCP analysis was performed by using a Gene Gel Exel 12.5/24 Kit (Amersham Bioscience, NJ). After electrophoresis was done for 80 min at 18°C and 600 V, the bands were visualized by silver staining.

Sequencing analysis

Samples with abnormally shifted bands on SSCP analysis were subjected to direct DNA sequencing by using a Big Dye Terminator version 3 Direct Cycle Sequencing Kit (Applied Biosystems, Tokyo Japan) with electrophoresis of the products in an automatic DNA sequencer (ABI PRISM 310™ Genetic Analyzer, Applied Biosystems, Tokyo, Japan). The sequence data thus obtained were compared with the sequence of class I beta-tubulin in the GenBank data base (J00314).

Results

Patient characteristics

The clinicopathological characteristics of the 66 patients are listed in Table 2. Among the 66 patients, 56 had received no prior treatment, 2 had been treated with TS-1, and 7 had been treated with TS-1 plus cisplatin as first-line chemotherapy. Only one tumor biopsy specimen was a taken after paclitxel treatment.

Mutation analysis of the exon 4 GTP-binding site

SSCP analysis showed abnormal bands upstream of the GTP-binding site in 7 out of 66 patients in the (Fig. 2a, b). However, sequence analysis found no nucleotide substitutions in any of the cases. Three variant bands were detected down stream of the GTP-binding site (Fig. 2c, d). The sequence of

one of these 3 products showed 8 nucleotide substitutions compared with that of class I beta-tubulin in the GenBank data base (J00314), but these corresponded to the sequence of a pseudogene (NT007995; Fig. 3a). The other two products had 4 nucleotide substitutions, but these matched the sequence of another pseudogene (NT086688; Fig. 3b, 3c). Thus, none of the tumors had any mutations at the GTP-binding site of exon 4 in the beta-tubulin gene.

Discussion

The success of anti cancer chemotherapy is limited by the development of tumor resistance. As is frequently observed with other chemotherapy agents, some of the patients who initially respond to taxanes later undergo relapse. Furthermore, some tumors are entirely resistant to taxanes even during initial treatment. Paclitaxel is reported to be effective for gastric cancer, with response rates ranging from 20% to 28% in single-agent phase II studies [1, 2], but more than half of all patients will not show any response to paclitaxel. Thus, understanding the mechanisms of resistance to taxanes is important for developing new approaches.

The rationale for assessing the relationship between beta-tubulin gene mutations and paclitaxel resistance is based on the studies of Giannakakou *et al.* [7] and Gonzarez-Garay *et al.* [8], who found beta-tubulin gene mutations in ovarian cancer cell lines and hamster cells, respectively. Subsequently, Monzo *et al.* [9] reported beta-tublin gene mutations, predominantly at exon 4, in paclitaxel-resistant tumors from patients with advanced non-small cell lung cancer and

Table 2.   Clinicopathologic characteristics of patients

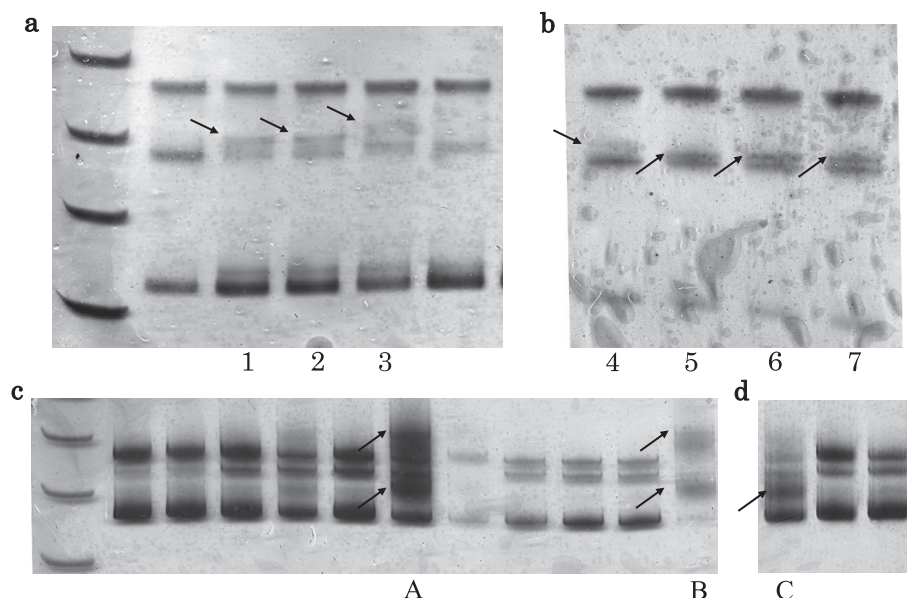| Variable (n)	
Mean age (range/years)	67.4 (29–90)
Sex (Male : Female)	46:20:00
Histologic subtype	
Intestinal	37
Diffuse	29
Stage	
IIIA	23
IIIB	19
IV	24

Fig. 2.   Single-strand conformational polymorphism (SSCP) analysis of the exon 4 GTP-binding site. Arrows indicate the abnormally shifted bands. a, b; Seven of 66 patients had bands upstream of the GTP-binding site (BT2), 1-7. c, d; Three patients had downstream bands (BT3), A, B and C; Fig. 1, Table 2.
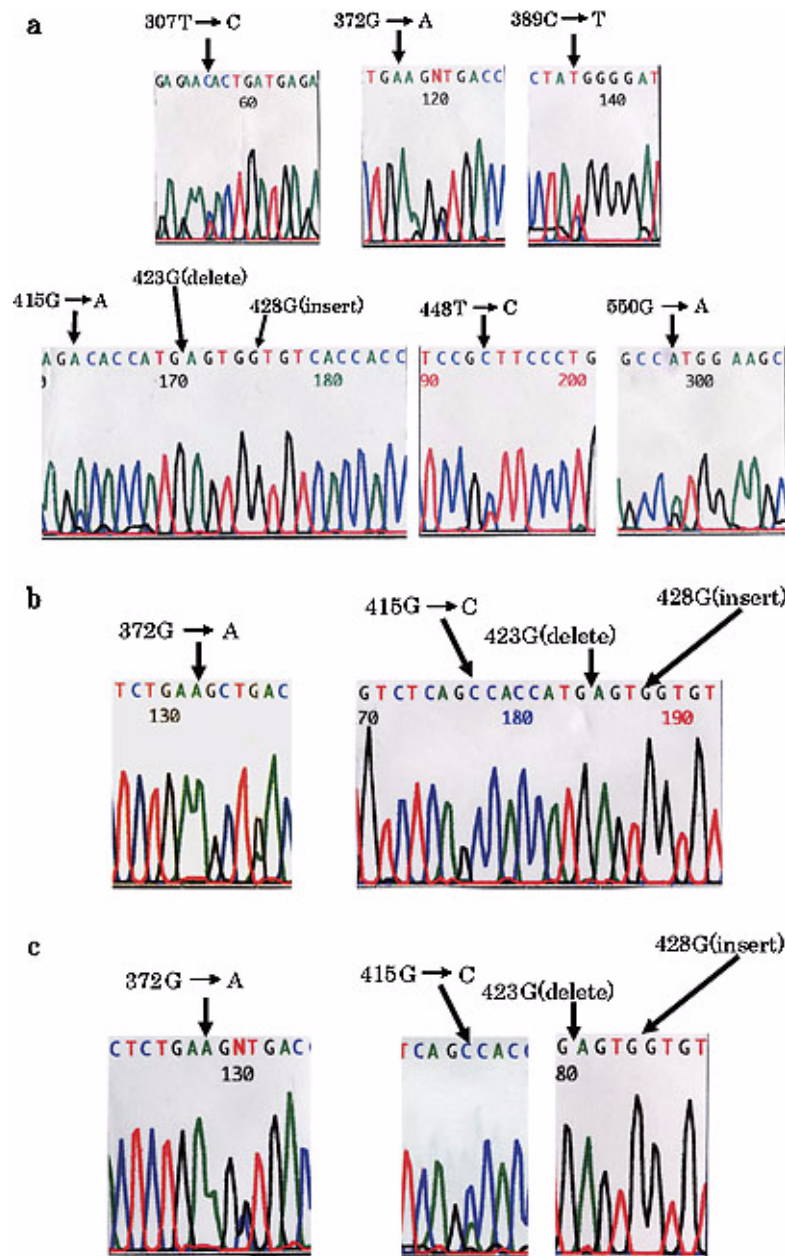
Fig. 3. Sequence analysis of polymerase chain reaction products. a; Case A. b; Case B. c; Case C. (From Fig. 2). The sequence of case A showed 8 nucleotide substitutions compared with the class I beta-tubulin sequence reported in the GenBank data base (J00314), but these all corresponded to the sequence of a pseudogene (NT08688). The sequences of cases B and C had same 4 nucleotide substitutions, but these corresponded to the sequence of another pseudogene (NT007995).

proposed that such mutations could have a possible role in paclitaxel resistance by this cancer. However, in agreement with another recent report about gastric cancer [12], we did not find any mutations of the exon 4 GTP-binding site in advanced stage III and IV gastric cancer. Although we speculated that beta-tubulin gene mutations may be acquired changes that would occur more often in advanced tumors, investigation of 66 all advanced stage III or IV gastric cancers, found no such mutations. The sequences of 7 prod-ucts with abnormally bands upstream of the GTP-binding site did not show any mutations, while the sequences of three downstream products corresponded to those of two previously described independent pseudogenes. In agreement with our results, similar finding have recently been obtained for several other tumors, namely lung cancer [13], breast cancer [14, 15], and ovarian cancer [16, 17]. Taken together, these investigations indicate that beta-tubulin gene mutations are not an important mechanism of paclitaxel resistance. To

explain the findings of Monzo *et al.* [*9*], which have not been reproduced by other investigations including the present study, Kelley *et al.* suggested that the primers used in the early study did not discriminate beta tubulin from its pseudogenes [*13*]. Also, the first reports of beta-tubulin gene mutations were based on investigation of hamster cells [*8*] and ovarian cancer cell lines [*7*] after selection by exposure to paclitaxel, so direct extrapolation of these findings to human tumors is difficult.

In the present study, we analyzed beta-tubulin gene mutations in gastric cancer tissue, obtained by endoscopic biopsiy of the tumor surface. Therefore, it should be noted that the results may not reflect the characteristics of deeper part of the tumor. However, in patients who cannot undergo surgery, we have to collect information about chemoresistance from samples obtained by endoscopic biopsy. Thus, the problem which now confronts us is how to obtain information from such tiny superficial tumor samples. In contexts, beta-tubulin may not be a good marker for predicting chemoresistance to taxanes in patients with gastric cancer. For example, analysis of single nucleotide polymorphism may be a useful marker for expecting chemotherapeutic efficacy or toxity [*18*].

In conclusion, our findings indicate that beta-tubulin exon 4 is very well conserved even in advanced stage III or IV gastric cancer, making it very unlikely that variations in the response to treatment with taxanes are caused by mutations of this gene. Further investigation of alternative mechanisms of taxane resistance is warranted, including P-glycoprotein overexpression [*18*, *19*], differential beta-tubulin isotype expression [*18*, *20*, *21*], and deregulation of apoptosis [*18*, *22–24*]. Such studies are necessary to predict the response of individual patients to this agent.

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