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ORIGINAL ARTICLE

Low-dose chemotherapy with methotrexate and vinblastine for patients with desmoid tumors: relationship to CTNNB1 mutation status

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Abstract
Background This study was conducted to determine the efficacy and safety of low-dose chemotherapy with methotrexate (MTX) and vinblastine (VBL) for patients with desmoid tumors refractory to meloxicam treatment, focusing in particular on the relationship between the efficacy of this chemotherapy and catenin β-1 (CTNNB1) mutation status.
Patients and methods Since March 2003, patients pathologically diagnosed with extraperitoneal desmoid tumors have been prospectively treated with meloxicam, a COX-2 inhibitor, at our institution. Patients with inoperable tumors who were resistant to meloxicam treatment underwent MTX and VBL therapy every other week. The responses of all patients were evaluated, and factors that were correlated with efficacy were analyzed, including CTNNB1 mutation status.
Results Sixty-eight patients were prospectively treated with meloxicam. MTX + VBL therapy was administered in 15 patients. Six patients showed a partial response. Only one patient presented disease progression. A few patients showed grade 3–4 treatment-related toxicity with the administration of MTX and VBL every other week. Intriguingly, CTNNB1 status did not affect the efficacy of this treatment.
Conclusion MTX and VBL treatment every other week is well tolerated and achieved a favorable response in patients resistant to meloxicam treatment, regardless of CTNNB1 mutation status.

Keywords Desmoid tumor · CTNNB1 · Methotrexate · Vinblastine

Introduction

Desmoid tumors show locally infiltrative growth behavior but do not metastasize to other organs [1]. The pathogenesis of desmoid tumors is considered to be multifactorial. Genetic predisposition, endocrine factors, and trauma may play roles in the development of this disease as well as in its responsiveness to conservative and surgical treatment.

Surgical resection has been the mainstay of desmoid tumor treatment. However, previous studies have reported inconsistent results regarding the association between the microscopic margin status and the recurrence rate [2–9]. No definitive conclusion has been reached regarding the significance of the histological margin status. On the other hand, the mutation status of the catenin β-1 (CTNNB1) gene was recently reported to be a significant influence on the outcome after surgical treatment [10–12].

The potential morbidity associated with surgery and radiotherapy and a high recurrence rate even after radical surgery have led investigators to assess the role of a wait-and-see policy [8, 13, 14] in desmoid tumor treatment, as well as the roles of noncytotoxic [15, 16] and cytotoxic [17, 18] chemotherapy. Because extra-abdominal desmoid tumors do not metastasize and rarely cause disease-specific death, medical treatment with fewer complications is desirable. A previous report on basic research indicating that COX-2 blockade

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slows tumor growth [19] suggested that COX-2 inhibitors may possess antitumor activity against desmoid tumors. Since 2003, we have prospectively treated patients with extraperitoneal desmoid tumors using meloxicam (a selective COX-2 inhibitor) alone, and we have reported its favorable effects [15]. However, as the number of patients being treated with meloxicam has increased, some of them have shown resistance to this treatment [20]. A previous study demonstrated the efficacy of low-dose methotrexate (MTX) and vinblastine (VBL) chemotherapy [21] in patients with inoperable desmoid tumors. Based on the results of that study, outpatient chemotherapy with MTX and VBL was approved and has been used for desmoid patients at our institution. Our treatment algorithm following meloxicam treatment has been either planned simple resection or low-dose MTX and VBL chemotherapy; the option chosen is determined by the characteristics of the desmoid tumor, in particular resectability and predicted functional impairment after surgery. Additionally, in patients undergoing simple resection or MTX and VBL chemotherapy, desmoid tumors were prospectively subjected to mutation analysis of CTNNB1.

To aid with the selection of the appropriate treatment modality or treatment algorithm for patients with desmoid tumors, the prognostic value of the CTNNB1 mutation status needs to be analyzed, not only in relation to surgical treatment but also in relation to systemic therapy, including noncytotoxic agents (hormonal therapy, anti-inflammatory agents, interferon alpha) [15, 16, 22] and cytotoxic agents [17, 18]. One of the aims of this study was to clarify the efficacy of low-dose MTX and VBL chemotherapy in patients resistant to meloxicam therapy, and the complications associated with this therapy in a Japanese cohort. Another was to determine the correlation of CTNNB1 mutation status with the efficacy of MTX + VBL chemotherapy, which has not been reported before.

Patients and methods

Since March 2003, patients with extraperitoneal desmoid tumors have been prospectively and consecutively treated with meloxicam, a COX-2 inhibitor, based on results for genetically modified mice [19]. There were 87 patients who had been pathologically diagnosed with desmoid tumors at extraperitoneal sites. Excluding patients who refused the meloxicam therapy, desired surgical treatment, had stomach disease, had recurrent disease after radiotherapy at the pre-referral hospital, were treated with celecoxib, were followed up for less than 6 months, or who only requested a second opinion from our hospital, 68 patients were prospectively treated with meloxicam. Twenty-four of those 68 patients experienced progressive disease. Among them, excluding resectable cases and patients who did not consent to low-dose chemotherapy, 14 patients received low-dose MTX and VBL therapy after meloxicam treatment, and 1 pediatric patient underwent this therapy too.

Biopsy specimens at our hospital and excised specimens at pre-referral hospitals were all reviewed, and the definitive diagnosis of desmoid tumor was re-confirmed by an experienced pathologist (Y.S.) at our institution. Mutation status of CTNNB1 was determined using DNA extracted from frozen tissue or 5-μm-thick formalin-fixed, paraffin-embedded tissue. The DNA was subjected to PCR amplification using specific primers for CTNNB1 exon 3, as reported previously [23]. Purified products were subjected to direct sequencing using the above primers (forward) along with Applied Biosystems (Foster City, CA, USA) Big Dye Terminator V3.1 and an Applied Biosystems 3730x DNA analyzer at FASMAC Co. Ltd. (Kanagawa, Japan). The studies and treatment regimens were approved by the institutional review board or committee for chemotherapy regimen at our institution. All of the participating patients or their parents signed informed consent forms.

Patients were treated with MTX at a dose of 30 mg/m^2 and VBL at a dose of 6 mg/m^2, both administered by intravenous injection every other week. For the first 2 patients, MTX and VBL were administered every week, which resulted in grade 4 neutropenia and grade 2 anemia or a grade 2 alanine/aspartate aminotransferase increase (ALT/AST increase). For another 13 patients, MTX and VBL were delivered every other week from the beginning. The duration of this treatment was not planned; this chemotherapy was continued until tumor progression (progressive disease, PD) was noted. In cases evaluated as showing complete response (CR), partial response (PR), or stable disease (SD), the interval between the chemotherapy sessions was increased. In the case of PR, treatment was discontinued after much consultation between the physician and patient. Dose reduction due to toxicity was planned when the patient had at least grade 3 myelosuppression or another prolonged grade 2 toxicity.

Patients were followed with magnetic resonance imaging (MRI) at the outpatient unit of our department of orthopedic surgery every 3 months, and occasionally with computed tomography in cases who could not tolerate MRI. The efficacy of MTX and VBL was evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 [24]. Progression-free survival was defined as the time from enrollment onto chemotherapy to disease progression or the date of last follow-up in progression-free cases.

Adverse events were reported according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE v4.0; http://ctep.cancer.gov).

Patients were divided into two groups: responders (CR, PR) and nonresponders (SD, PD). Age, gender, primary or recurrent disease, tumor size, treatment duration, number

Table 1 Clinical data for patients who received low-dose MTX and VBL chemotherapy

Case	Sex	Age	Site	P/rec	Size (cm)	Prior treatment	Treatment duration (months)[a]	No. of cycles	RECIST	Total follow-up (months)	CTNNB1 mutation status
1	F	37	Abd. wall	P	18.0	Meloxicam	6	17	PR	35	WT
2	M	41	Chest wall	P	14.0	Meloxicam	28	50	PR	44	45F
3	M	11	Forearm	P	12.0	Meloxicam	29	26	PR	97	41A
4	F	19	Abd. wall	Rec	6.0	Meloxicam + S	20	28	SD	66	45F
5	F	19	Neck-axillary	P	12.0	Meloxicam	4	12	PD	62	45F
6	M	20	Neck-mediastinum	Rec	5.5	S + meloxicam	38	69	SD	42	45F
7	F	53	Chest wall	P	10.8	Meloxicam	6	5	SD	23	41A
8	F	26	Abdominal wall	P	11.0	Meloxicam	14	20	PR	30	45P
9	F	70	Neck-back	P	8.7	Meloxicam	8	18	SD	30	41A
10	M	74	Neck-chest cavity	P	16.5	Meloxicam	31	32	PR	33	45F
11	F	38	Back	P	23.0	Meloxicam	18	39	SD	23	41A
12	M	39	Chest wall	Rec	4.2	S + meloxicam	8	13	SD	21	41A
13	F	45	Neck-back	P	13.2	Meloxicam	15	29	SD	67	41A
14	M	6	Lower extremity	Rec	14.0	S	9	21	SD	10	41A
15	F	29	Abdominal wall	P	10.8	Meloxicam	5	9	PR	7	WT

Age age at enrollment, *size* greatest dimension

F female, *M* male, *P/rec* primary/recurrence, *S* surgery, *PR* partial response, *SD* stable disease, *PD* progressive disease, *WT* wild type

[a] MTX and VBL treatment

of cycles, and mutation status of CTNNB1 were examined as possible prognostic factors for responsiveness to MTX and VBL. Fisher's exact test was used to assess the significance of the differences between proportions. Treatment duration and number of cycles were compared between the two groups using the unpaired Student's *t* test. *P* values <0.05 were considered significant.

Results

All patients were Japanese. None had familial adenomatous polyposis (FAP) related desmoid tumors (Gardner's syndrome). The mean age of the 15 patients was 35 years, ranging from 6 to 74 years. Six were male and 9 were female. Tumors were located mainly in the trunk, except for 2 cases with tumors in the extremities (Table 1). The median greatest dimension of the tumor was 12.0 cm (4.2–23.0 cm). Fourteen patients received meloxicam treatment to which they showed resistance. Four patients had recurrent tumors after being treated with surgery at the prereferral hospital or at our institution after meloxicam treatment. None of the patients received radiotherapy or any other treatment for desmoid tumors. The median treatment duration and number of cycles of MTX and VBL treatment were 16 months (ranging from 4 to 38 months) and 25 cycles (ranging from 5 to 69 cycles), respectively.

There were 6 patients with PR (Fig. 1), 8 with SD, and 1 with PD according to the RECIST evaluation. The patient with PD status underwent combination therapy with doxorubicin and dacarbazine, which reduced the size of the tumor markedly. None of the analyzed factors, including age, gender, tumor status (primary or recurrent), tumor size, treatment duration, number of cycles, and mutation status of CTNNB1, was significantly associated with the efficacy of MTX and VBL treatment (Table 2). Given that tumors with a mutation status of 45F are reported to have a poorer clinical outcome, the tumors were divided into two groups: those with or without the 45F mutation. No significant difference was observed ($P = 1$) between tumors with or without the 45F mutation.

Five patients who showed disease stabilization or regression with MTX and VBL ceased this chemotherapy; four of these patients remained free of disease progression at a median of 18 months (range 13–27) after cessation of MTX and VBL therapy. Interestingly, among those five patients, one (case 2) experienced regrowth of the tumor after ceasing chemotherapy and had the 45F mutation of CTNNB1 exon 3 (Fig. 2, arrow).

One patient discontinued chemotherapy because of the onset of interstitial pneumonia (case 1). She was cured with medical treatment and the size of the tumor continued to decreace after discontinuing the chemotherapy. No other patients discontinued the treatment due to serious

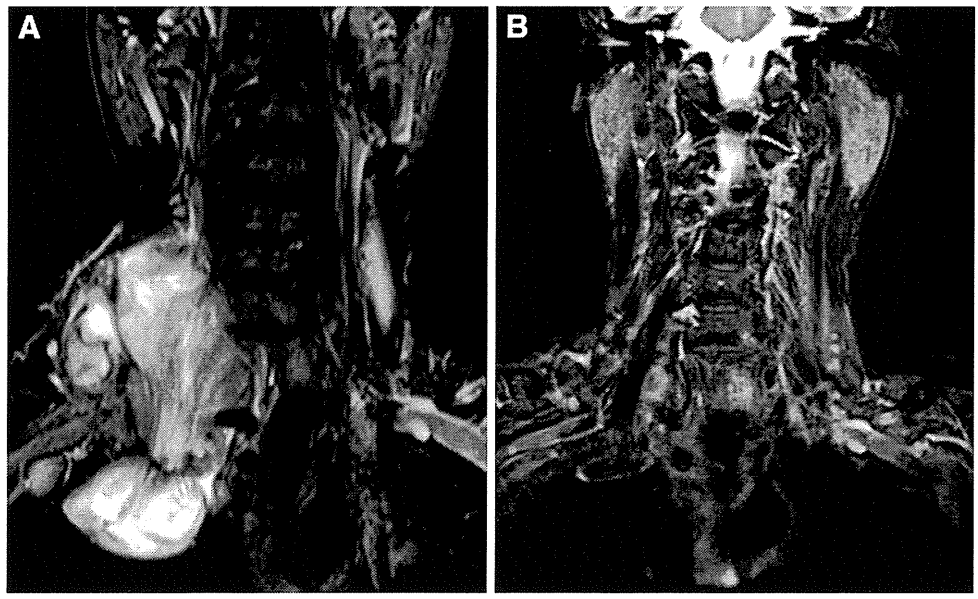

Fig. 1a–b Case 10. A 74 year-old male patient had a tumor in the neck and chest cavity (**a**). Two years' treatment with MTX and VBL reduced the tumor size markedly (**b**)

Table 2 Correlating the efficacy of MTX + VBL with clinical factors

Factor	*P* value
CTNNB1 mutation status	0.52
Sex	0.91
Age	0.75
P/rec	0.19
Tumor size	0.75
Treatment duration	0.45
No. of cycles	0.93

Age: cutoff value is 35 years old

Tumor size: cutoff value is 12 cm

P value: evaluated with the chi-square test; treatment duration and no. of cycles were evaluated with the unpaired Student's *t* test

P/rec primary/recurrence

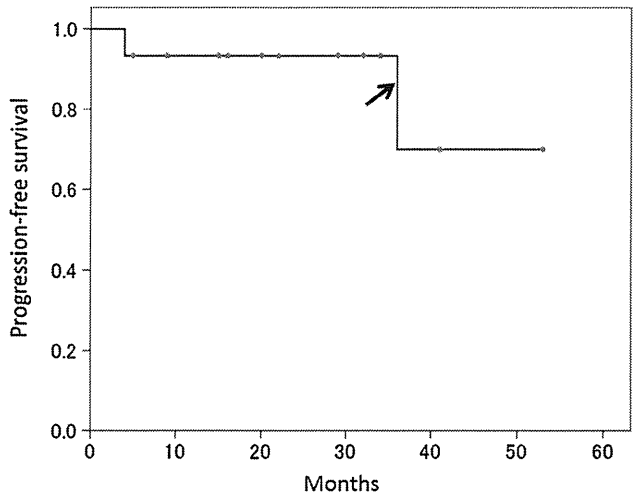

Fig. 2 Progression-free survival after treatment with MTX and VBL. In case 2 with the 45F mutation, the tumor size was reduced by MTX and VBL therapy and treatment was discontinued 28 months after the initiation of therapy. The tumor then regrew 7 months after treatment cessation (*arrow* depicts the regrowth of tumor at 35 months after the initiation of therapy)

adverse events. Although grade 4 neutropenia occurred in one patient with weekly treatment, this adverse event improved with treatment every other week (Table 3). Due to weekly treatment, three patients had a grade 3 asymptomatic increase in alanine aminotransferase or aspartate aminotransferase. Most of the patients suffered grade 1 adverse events (Table 3).

Discussion

This study has demonstrated the feasibility of low-dose MTX and VBL chemotherapy for Asian patients for the first time, based on a relatively homogeneous cohort, namely patients who are resistant to meloxicam treatment without radiotherapy. Treatment every other week reduced the incidence of adverse events and enabled the long-term use of this regimen. In practice, four patients (cases 2, 3, 6, and 10) received this chemotherapy for >2 years without suffering serious side effects.

Several studies have previously reported the efficacy of this treatment. Azzarelli et al. reported the results for 30 patients who received MTX + VBL therapy; the doses (MTX: 30 mg/m^2, VBL: 6 mg/m^2) were the same as those used in the present study and the efficacy was also

Table 3 Degree of toxicity in 15 patients who received low-dose MTX and VBL therapy (values in the table represent numbers of patients)

Toxicity	Grade				
	0	1	2	3	4
Neutrophil count	11	1	1	1	1[a]
Hemoglobin	13	0	2[b]	0	0
ALT, AST	11	0	3[b]	1	0
Abdominal pain	12	3	0	0	0
Diarrhea	14	1	0	0	0
Constipation	12	3	0	0	0
Malaise	0	2	0	0	0
Nausea	5	7	3	0	0
Vomiting	11	4	0	0	0
Anorexia	13	2	0	0	0
Oral mucositis	13	2	0	0	0
Dysgeusia	14	1	0	0	0
Headache	13	2	0	0	0
Vertigo	14	1	0	0	0
Epistaxis	14	1	0	0	0
Pneumonitis	14	0	1	0	0
Dysesthesia	12	3	0	0	0
Myalgia	13	21	0	0	0

[a] At the time of weekly administration

[b] One patient at the time of weekly administration

identical: PR 12 patients (40 %), SD 18 cases (60 %). This treatment regimen was thought to be tolerable because no serious adverse events occurred. Five-year progression-free survival was 67 %, which is comparable to that seen in the present study (70 %) [21]. One of the major concerns regarding this chemotherapy is its efficacy in young patients. Skapek et al. reported the results for 28 pediatric patients (median age 11.5 years) treated with MTX (30 mg/m^2) and VBL (6 mg/m^2). In 26 evaluable patients, the response rates were CR in 1 (4 %), PR in 4 (15 %), MR (minor response) in 3 (12 %), SD in 10 (38 %), and PD in 8 (31 %) [25], indicating a poorer outcome compared with an adult cohort [21] (the present study). They opined that, considering that early responses to therapy are not typical for this tumor and long-term disease control can be achieved in at least some patients, it would be reasonable to continue therapy as long as it is well tolerated in cases with no disease progression. Except in cases showing rapid progression, this treatment regimen could be sustained in Asian patients, as demonstrated in this study. Italian and French groups reported the results of several treatment regimens including MTX and VBL therapy. Meazza et al. reported that 11 (58 %) of 19 patients responded well to MTX + VBL [26], and Garbay et al. demonstrated a significantly superior outcome with anthracycline-containing regimens (response rate: 54 %) compared with other regimens, including MTX + VBL therapy (response rate: 12 %) [27]. However, those previous reports did not investigate the mutation status of CTNNB1 or the correlation of that mutation status with the efficacy of MTX and VBL therapy.

We speculate that chemotherapy with MTX and VBL may stabilize or decrease disease activity, and that once the disease activity regresses, this response may be sustained even after the chemotherapy is stopped. The results of the present study demonstrate that this chemotherapy regimen could be effective in patients with desmoid tumors regardless of the mutation status of CTNNB1. As reported recently, a 45F mutation status of CTNNB1 was associated with a worse outcome after surgical treatment [10–12, 28] and a worse outcome of conservative treatment with meloxicam [23] compared with other mutation statuses (41A, 45P, and WT). However, no studies have clarified the relationship between the mutation status and the efficacy of MTX and VBL chemotherapy. The present study indicates, for the first time, that this chemotherapy may even be effective for tumors with a 45F mutation status of CTNNB1 (4 of 5 such cases showed PR or SD). Given the results of previous studies, we speculate that surgical treatment and/or meloxicam treatment cannot alter tumorigenic potency, particularly of desmoid tumors harboring the 45F mutation, whereas low-dose MTX and VBL can stabilize the tumorigenicity. However, 1 of 5 desmoid tumors with the 45F mutation showed PD. The other tumor with this mutation reduced during chemotherapy (PR) but regrew 7 months after its cessation. This experience suggests that the mutation status of CTNNB1 should be considered when low-dose MTX and VBL chemotherapy is used, particularly if the mutation status is 45F.

Recently, molecular targeted treatment has been administered in clinical trials, with favorable outcomes reported. Progression-free rates at 1 year were reported to be 37–67 % with imatinib treatment [29–31]. Favorable outcomes from using sorafenib and sunitinib have been also reported [32, 33]. However, cost-effectiveness studies will be required for the application of molecular targeted drugs in patients with desmoid tumors in the future.

There are several limitations of the present case series. Only a small number of cases could be collected due to the rarity of this neoplasm, which means that we are unable to draw any definitive conclusions based on our results, and multicenter prospective studies will be needed to better clarify the efficacy of this low-dose MTX and VBL chemotherapy. Because this treatment is not yet covered by insurance in Japan but has been approved for use by individual institutional review boards, there are difficulties concerning its use in some institutions, which can make it problematic to plan multi-institutional studies. Next, there were only

two cases in our series who were younger than 15 years of age and had extremity desmoid tumors. Considering that the clinical outcome of surgery and chemotherapy in young and/or extremity desmoid patients has been reported to be poorer than the corresponding outcome in adult and/or trunk desmoid patients [2, 8, 25, 34], the favorable outcome observed in the present report should be analyzed further, particularly in young patients with extremity tumors.

The correlation between the efficacy of this chemotherapy and the mutation status of CTNNB1 should be further analyzed in a greater number of cases. We are now carrying out a multi-institutional retrospective study to analyze the relationship between clinical outcome (surgery, conservative treatment) and mutation status of CTNNB1. In conclusion, low-dose chemotherapy with MTX and VBL is a well-tolerated and promising treatment for Asian patients with desmoid tumors resistant to other treatment modalities. A favorable response can be expected for desmoid patients with the 45F mutation of CTNNB1 as well.

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Conflict of interest Y.A. received Honoraria from Nippon Kayaku and Pfizer. Y.A. received research funding from Nippon Kayaku. The other authors declare that they have no conflict of interest.

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ORIGINAL RESEARCH

Characteristics of cultured desmoid cells with different CTNNB1 mutation status

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β-Catenin, CTNNB1 mutation, desmoid tumor, meloxicam, Wnt/β-catenin pathway

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Abstract

Desmoid tumors are benign mesenchymal neoplasms with a locally aggressive nature. The mutational status of β-catenin gene (CTNNB1) is presumed to affect the tumorous activity of the cells. In this study, we isolated three kinds of desmoid cell with different CTNNB1 status, and compared their characteristics. Cells were isolated from three patients with abdominal wall desmoid during surgery, all of which were resistant to meloxicam treatment. The mutational status of the CTNNB1 exon 3 was determined for both parental tumor tissues and isolated cultured cells. β-catenin expression was determined with immunohistochemistry. Responsiveness to meloxicam was investigated with MTS assay together with COX-2 immunostaining. mRNA expressions of downstream molecules of Wnt/β-catenin pathway were determined with real-time RT-PCR. Three kinds of cell isolated from desmoid tumors harboring different CTNNB1 mutation status (wild type, T41A, and S45F), all exhibited a spindle shape. These isolated cells could be cultured until the 20th passage with unchanged proliferative activity. Nuclear accumulation of β-catenin was observed in all cultured cells, particularly in those with S45F. Proliferating activity was significantly suppressed by meloxicam (25 μmol/L, $P < 0.007$) in all three cell cultures, of which parental desmoid was resistant to meloxicam clinically. The mRNA expressions of Axin2, c-Myc, and Cyclin D1 differently increased in the three cultured cell types as compared with those in human skin fibroblast cells (HDF). Inhibitors of Wnt/β-catenin pathway downregulated Axin2, c-Myc, and Cyclin D1 significantly. Isolated and cultured desmoid tumor cells harboring any one of the CTNNB1 mutation status had unique characteristics, and could be useful to investigate desmoid tumors with different mutation status of CTNNB1.

Introduction

Desmoid-type fibromatosis is a benign, but locally aggressive fibroblastic tumor. The biological features are enigmatic due to the markedly high recurrence rate after planned surgery (range 34–54%) [1, 2] and occasional spontaneous regression [3]. For this reason, a nonsurgical approach, such as hormone therapy, nonsteroidal anti-inflammatory drugs, or tyrosine kinase inhibitors, has been applied in recent years [4]. Definitive treatment has not yet been established due to the small numbers of desmoid patients and the limited efficacy of previously reported drugs. Moreover, efficacy cannot be predicted in advance of the therapy, which has been the most pressing demand of patients and physicians.

In desmoid tumors, the nuclear accumulation of β-catenin has shown diagnostic potential in differentiating desmoid tumors from other similar fibroblastic lesions [5]. Its nuclear accumulation has been considered to be a trigger of desmoid tumors and its positivity denotes tumor aggressiveness [6, 7]. A correlation between the nuclear positivity of β-catenin and efficacy of conservative treatment has recently been

reported [8]. Thus, aberrant accumulation of β-catenin, which causes activation of the Wnt pathway, is considered to play crucial roles in desmoid tumor biology.

This aberrant accumulation is commonly caused by mutations of the Wnt pathway-related gene, particularly somatic mutations at exon 3 of CTNNB1 (β-catenin gene) in sporadic extraperitoneal desmoid tumors [9–11]. A minority have mutations of the adenomatous polyposis (APC) gene, which is associated with familial adenomatous polyposis (FAP). CTNNB1 mutations of desmoid tumors generally occur at codon 41 or 45, with p.T41A (threonine to alanine), p.S45F (serine to phenylalanine), and p.S45P (serine to proline) being the most frequent [9–11]. Recent studies have suggested that desmoid tumors with different CTNNB1 mutations have diverse tumorigenic potency against various treatment modalities. Desmoid tumors with S45F mutation had higher rates of local recurrence after surgery [10, 12, 13], and greater resistance to meloxicam treatment [14], whereas the efficacy of low-dose chemotherapy was not associated with the mutation status of CTNNB1 [15]. Taking these findings into consideration, the mutation status of CTNNB1 in desmoid tumors would appear to alter not only tumorigenicity, but also the responsiveness to surgical and conservative treatment. However, the mechanism whereby the mutation status affects biological behavior has not been extensively investigated. This prompted us to isolate, culture, and characterize desmoid cells harboring different mutation status of CTNNB1 (wild type, S45F, and T41A). To exclude other possible factors affecting the biological behavior of desmoid cells, we selected these three cell types from tumors resistant to meloxicam treatment located in the abdominal wall of three young female patients.

Material and Methods

Tissue acquisition

Desmoid tissues were collected, and subjected to various experiments including cell culture and CTNNB1 mutation analysis. The experimental protocol was approved by the institutional review board of Nagoya University. Among patients prospectively treated with meloxicam [16–18], three young females with abdominal wall desmoids resistant to meloxicam (progressive disease according to Response Evaluation Criteria in Solid Tumors) were treated surgically. Part of the resected tumors, from which normal tissues were carefully removed, was applied for cell culture. The patients' ages at the time of surgery were 20, 30, and 39 years. All three resected tumors were histologically diagnosed as desmoid tumors by specialized pathologists including immunohistochemical analysis of β-catenin.

Cell cultures

The samples were cut into small pieces with a sterile scalpel, and were dissociated with 0.2 mg/mL proteinase in Dulbecco's modified Eagle's medium (DMEM) at 37°C for 3 h. The resulting cells were seeded into T-75 flasks, and cultured in DMEM containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C in humidified atmosphere air plus 5% CO^2. The medium was changed every 3–4 days. After the cells were grown to near confluency (passage #1), they were trypsinized and divided for continued in vitro culture. Successive experiments were performed with cell cultures of passage 5–15. Human skin fibroblasts (HDF; Detroit 551: ATCC, CCL-110) were cultured in monolayers and used as control cells.

Mutation analysis of CTNNB1

DNA of tumors was extracted from both 5-μm thick formalin-fixed, paraffin-embedded specimens and cell lysate of monolayer cultures, using High Pure Polymerase Chain Reaction (PCR) Template Preparation Kit (Roche, Basel, Switzerland), according to the manufacturer's instructions. Quality of DNA was confirmed with A_{260}/A_{280} ratio; more than 1.8. Extracted DNA was amplified by PCR with 40 cycles at an annealing temperature of 58°C with specific primer pairs for exon 3 of CTNNB1 using the LightCycler 480 System (Roche). We designed specific 2 primer pairs: forward 5′-GATTTGATGGAGTTGGACATGG-3′, reverse 5′-TCTTCCTCAGGATTGCCTT-3′, and forward 5′-TGG AACCAGACAGAAAAGCG-3′, reverse 5′-TCAGGATT GCCTTTACCACTC -3′ (The expected sizes of amplified products were of 149 and 118 bp, respectively). The PCR products were segregated by 2% agarose gel electrophoresis, and gel bands of predicted size were extracted and purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA). The purified products were subjected to direct sequencing using the above described forward primers with Applied Biosystems Big Dye Terminator V3.1 and Applied Biosystems 3730x DNA analyzer (Applied Biosystems, Foster City, CA) at FASMAC Co. Ltd. (Kanagawa, Japan). All sequencing results obtained with two different primer pairs were compared and confirmed as identical. Mutation site was determined with the databases of NCBI-BLAST.

Immunofluorescence staining

Immunofluorescence staining was performed to evaluate the nuclear accumulation of β-catenin. The cells were grown to 40–60% confluency on sterile glass coverslips, fixed with 4% paraformaldehyde, washed once with phosphate buffered saline PBS, and soaked with 3%

bovine serum albumin (BSA) for 30 min for blocking. Then, slides were incubated for 1 h at 37°C with anti-β-catenin rabbit polyclonal antibody (ab47426; Abcam, Cambridge, CA; 1:200 dilution) or anti-COX-2 goat polyclonal antibody (sc-1747; Santa Cruz Biotechnology, Santa Cruz, CA; 1:500 dilution). After rinsing with PBS, fluorescent goat polyclonal anti-rabbit IgG–H&L (Alexa Flour® 488) (ab150077; Abcam; 1:1000 dilution) or donkey polyclonal anti-goat IgG–H&L (Alexa Flour® 488) (ab150129; Abcam; 1:1000 dilution) were used as secondary antibodies. After nuclear staining with DAPI(D1306; Life Technologies, Carlsbad, California), slides were analyzed under a fluorescence microscope. Nonimmune rabbit serum was substituted for the primary antibody as a negative control.

Cell proliferation and apoptosis

Doubling time of the three cultured cell types was determined by cell growth assay. Desmoid tumor cells were seeded in 96-well plates at 5×10^3/well in medium supplemented with 10% FBS and allowed to adhere for 12 h. The subconfluent cells were exposed to 10% FBS medium with and without dimethyl sulfoxide DMSO containing 0–50 μmol/L meloxicam. After treatment for 48 h, cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay using a CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Fitchburg, WI). Absorbance intensity was determined on a microplate reader, Rainbow RC (Tecan Japan, Kawasaki, Japan) at 490 nm. Subconfluent cells were also exposed to 10% FBS medium with and without DMSO containing 25 μmol/L meloxicam or 50 μmol/L actinomycin-D for 24 h, and apoptotic activity was evaluated by Caspase-Glo® 3/7 Assays (Promega) according to the manufacturer's instructions. Luminescent intensity was determined on a microplate reader, PowerScan4 (DS Parma Biomedical, Osaka, Japan).

Real-time RT-PCR

The mRNA expression of target genes, Axin2, Cyclin D1, and c-Myc in the Wnt/β-catenin signaling pathway was determined by real-time RT-PCR. Total cellular RNA was isolated from cultured cells in monolayer using RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. Reverse transcribed cDNA was subjected to real-time RT-PCR for semiquantification of Axin2, Cyclin D1, and c-Myc mRNAs using a LightCycler (Roche Diagnostics, Mannheim, Germany). The relative levels of these mRNA in a sample were expressed after normalization with GAPDH mRNA. The Axin2, Cyclin D1, c-Myc, and GAPDH primer pairs were as follows: Axin2 sense; 5′-TGTCTTAAAGGTCTTG AGGGTTGAC -3′, antisense; 5′-CAACAGATCATCCCATCC AACA -3′ (predicted PCR product of 80 bp); Cyclin D1 sense; 5′-CAGCTCCTGTGCTGCGAAG-3′, antisense; 5′-AC GGCAGGACCTCCTTCTG -3′ (predicted PCR product of 157 bp), c-Myc sense; 5′-TCTGGATCACCTTCTGCTGG -3′, antisense; 5′-AGGATAGTCCTTCCGAGTGG -3′ (predicted PCR product of 126 bp), GAPDH sense 5′-AGGTCGGA GTCAACGGATTTG-3′, antisense, 5′-TGTAAACCATGTAG TTGAGGTCA-3′ (predicted PCR product of 123 bp).

Effects of inhibitors for Wnt/β-catenin pathway on mRNA expression of target molecules

To determine the effects of inhibitors for Wnt/β-catenin pathway on gene expression of Axin2, Cyclin D1, and c-Myc, two inhibitors (IWR-1; Santa Cruz Biotechnology, Santa Cruz, Quercetin; Wako, Osaka, Japan) were used for cell cultures harboring CTNNB1 mutations. IWR-1 (10 μmol/L), which inhibits Wnt-induced β-catenin accumulation through stabilization of the destruction complex member Axin1 [19], and Quercetin (20 μmol/L), which blocks the β-catenin–TCF (T-cell factor)/lef (lymphoid enhancer factor)-1 pathway [20], were added to desmoid cell culture in 60-mm tissue cultured dishes (93060; TPP, Trasadingen, Switzerland) ($2.0 \times 10_4$ cells/cm^2) for 24 h at 37°C. Cultured cells were subjected to total RNA purification and subsequent real-time RT-PCR to determine the effects on mRNA expression of Axin2, Cyclin D1, and c-Myc.

Statistical evaluation

All the in vitro quantitative experiments were performed three times, and analysis of variance followed by Bonferroni–Dunn post-hoc test was used to assess differences between the means. The results are expressed throughout as the mean ± SD. All statistical analyses were performed using SPSS statistics 20 (IBM Corp. Armonk, NY). $P < 0.05$ was considered significant.

Results

Three cultured cell types with different CTNNB1 mutation status

All three cultured cell types of desmoid tumors exhibited spindle-shape, homogeneous fibroblast-like morphology. Doubling time of cultured cells with T41A, S45F, wild type (WT) was 66.6, 62.4, and 53.3 h, respectively (Fig. 1). The three cultured cell types did not become senescent,

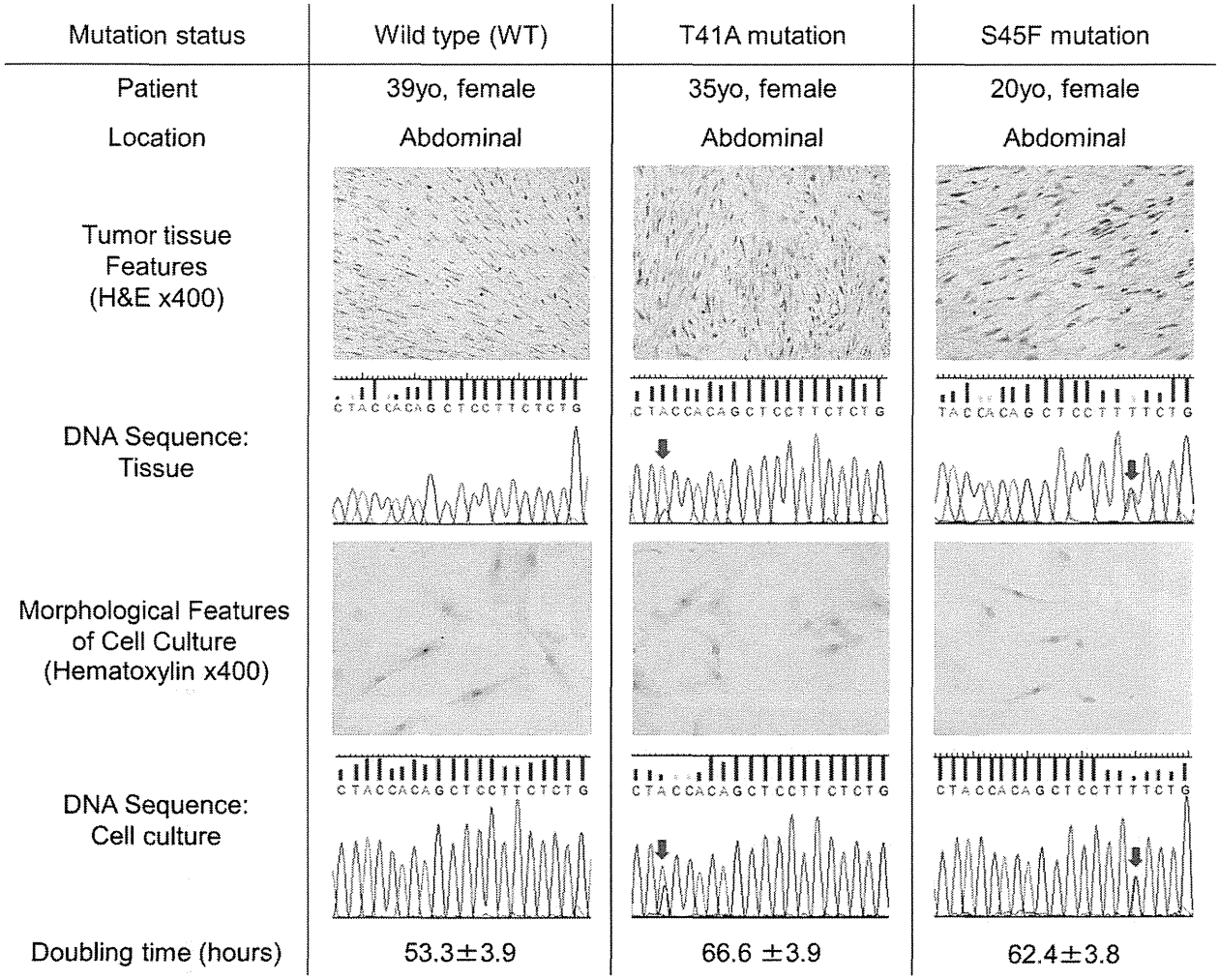

Figure 1. Parental desmoid tissues and isolated cell cultures. Histological and cell morphological features of three different CTNNB1 mutation status. Waveform data of DNA sequence at CTNNB1 exon3 and doubling time of each cell types are shown.

and the growth behavior remained constant until the 20th passage.

Mutation analyses of CTNNB1 revealed that parental desmoid tumors harbored (WT), T41A, and S45F, which were identical with the respective mutation of the isolated cultured cells (Fig. 1).

Expression of β-catenin and COX-2

S45F harboring cells exhibited strong nuclear β-catenin positivity. T41A and WT cultured cells showed intermediate and relatively weak staining (Fig. 2A). IWR-1 treatment could not effectively downregulate nuclear β-catenin accumulation (Fig. 2B). Immunofluorescent study for COX-2 indicated strong positivity in cytoplasm, particularly around the nucleus, of S45F cells as compared with that of WT cells (Fig. 2C).

Effects of meloxicam on cell proliferation and apoptosis

Inhibitory effects of meloxicam were evaluated in three cultured cell types, which were derived from desmoid tumors resistant to meloxicam clinically. Cell proliferation was inhibited in a dose-dependent manner in all of them. It was significantly suppressed with 25 μmol/L meloxicam in all three cell cultures (suppression rate ranging from 16–24%), whereas HDF was not affected (Fig. 3A). Higher dose (50 μmol/L) inhibited HDF proliferation.

Actinomycin-D (50 μmol/L) significantly increased apoptotic activity in HDF, WT, T41A, and S45F cultured cells ($P < 0.01$). However, meloxicam did not induce apoptosis in any of the cultured cells (Fig. 3B).

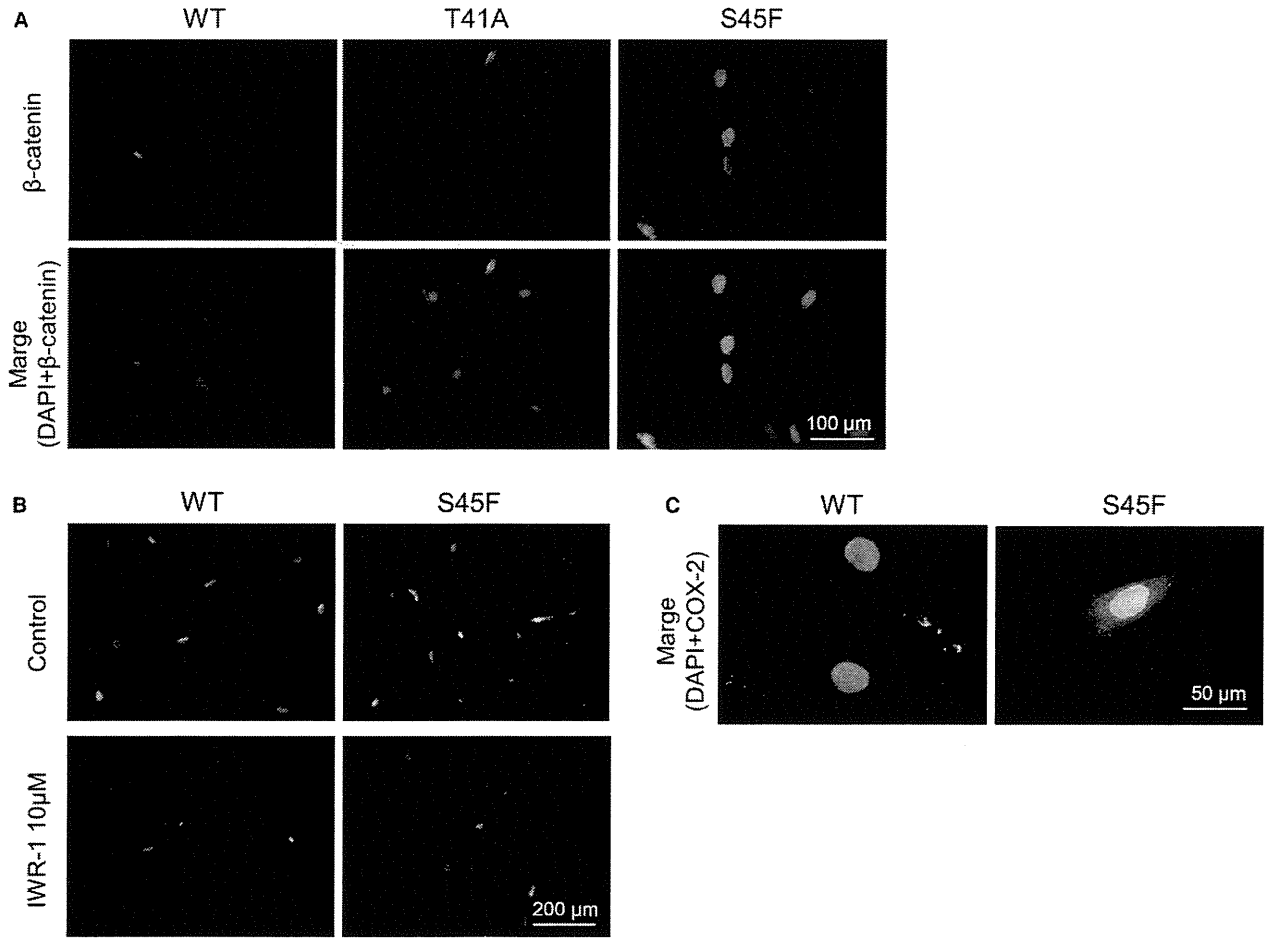

Figure 2. Immunofluorescence of β-catenin and COX-2 in desmoid cell cultures. (A) Immunofluorescence of β-catenin (green) in desmoid cell cultures harboring each CTNNB1 mutation status. The DAPI (blue) staining of cell nuclei were merged with β-catenin staining (original magnification, ×200). (B) Immunofluorescence of β-catenin (green) with 10 μmol/L of IWR-1 (original magnification, ×100). (C) Fluorescent double staining for COX2 (green) and DAPI (Blue) (original magnification, ×400).

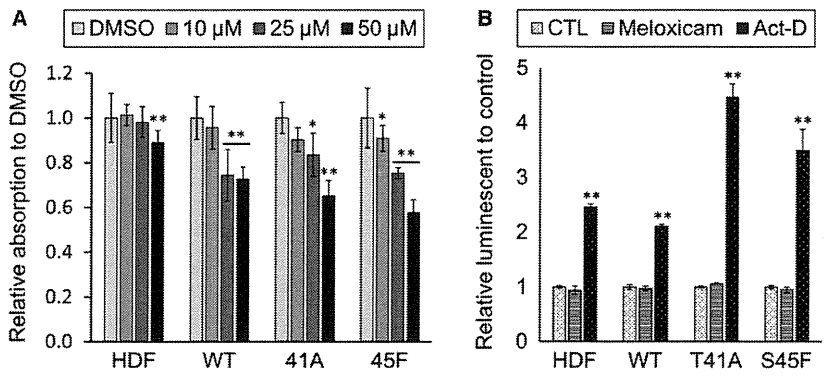

Figure 3. Effects of meloxicam on cell proliferation and apoptotic activity. (A) Cell proliferation of each cell culture was in a dose-dependent manner with meloxicam at 0–50 μmol/L for 48 h with MTS assay kit. (B) Effects of meloxicam on apoptosis in human skin fibroblast cells (HDF) and desmoid cell cultures. Caspase3/7 assay was performed with 25 μmol/L meloxicam or 50 μmol/L actinomycin-D (Act-D), relative luminescent to control (CTL) was exhibited. Bars show one standard deviation (SD) (*$P < 0.05$, **$P < 0.01$).

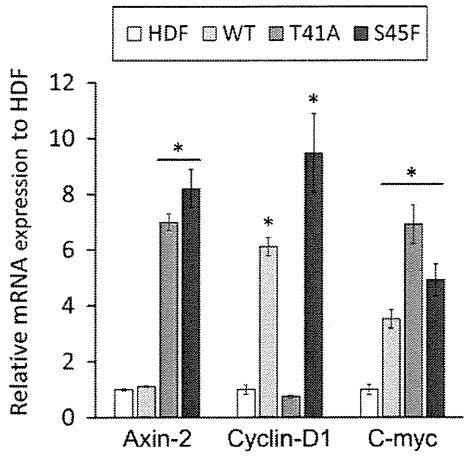

Figure 4. mRNA expression of the target genes of Wnt/β-catenin in cultured cells from desmoid tumors. Expression level is depicted as *n*-fold of the normalized amount of mRNA of human skin fibroblast cells HDF control cells. Bars show one standard deviation (SD) (*$P < 0.01$).

Steady state mRNA expression of Axin2/Cyclin D1/c-Myc in three desmoid cell cultures

The mRNA expression of Axin2, Cyclin D1, and c-Myc was significantly increased in the S45F-mutated cells ($P < 0.001$, 8.2-fold, 9.5-fold, and 4.9-fold, respectively) compared with that of HDF cells. mRNA of Axin2 and c-Myc was increased in cultures with T41A mutation, and that of cyclin D1 and c-Myc was in cultures with WT ($P < 0.001$) (Fig. 4).

Effects of inhibitors for Wnt/β-catenin pathway on mRNA expression of Axin2/cyclin D1/c-Myc

IWR-1, which stabilizes Axin protein and subsequently promotes β-catenin degradation, reduced to some extent the mRNA expression of Axin2, cyclin D1 and c-Myc, whereas quercetin, which blocks the β-catenin–TCF/Lef-1 pathway, more markedly decreased the mRNA expression. The combined use of IWR-1 and quercetin exerted additive inhibitory effects on mRNA expressions (Fig. 5A–C).

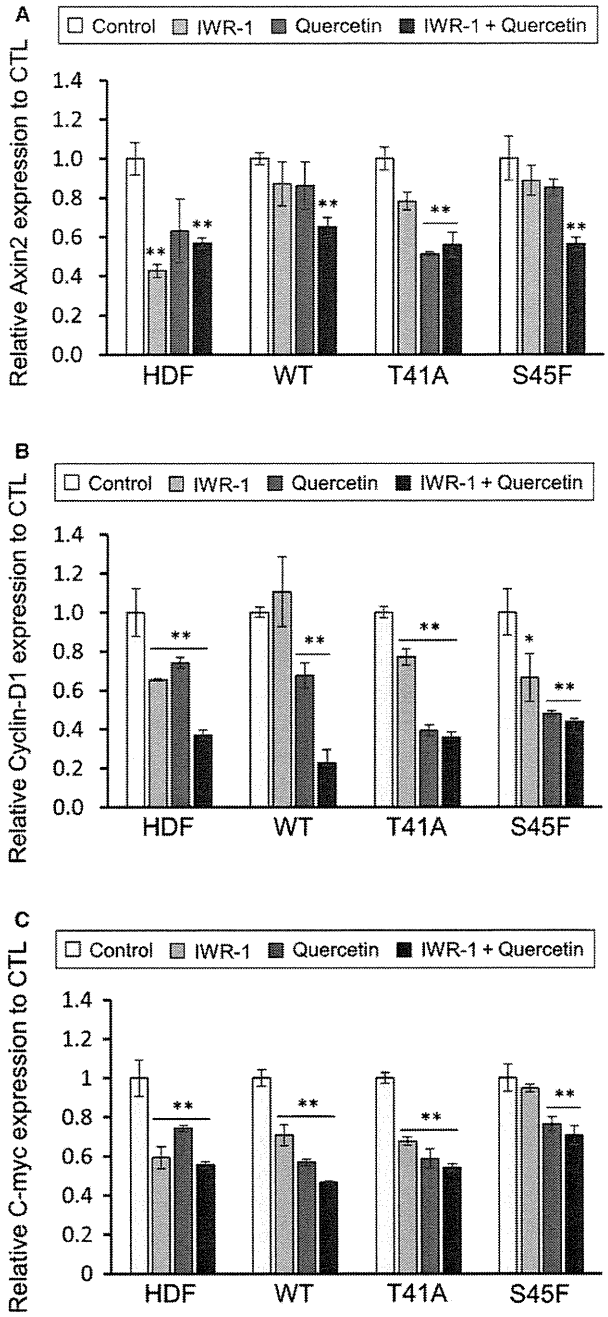

Figure 5. Effects of Wnt/β-catenin inhibitors on mRNA expression of the target genes of Wnt/β-catenin pathway. (A) Axin2. (B) CyclinD1. (C) C-myc. IWR-1 (10 μmol/L) and quercetin (20 μmol/L) were added to each cell culture. Expression level is depicted as *n*-fold of the normalized mRNA of control cells. Bars show one standard deviation (SD) (*$P < 0.05$, **$P < 0.01$).

Discussion

We successfully cultured and characterized desmoid cells harboring three different CTNNB1 mutation status: wild type, T41A, and S45F. In malignant tumors, mutation of CTNNB1 has been reported to locate between codon 32 and 45, the site of phosphorylation by GSK3β or CK1α [21, 22]. It spans a much wider range compared with that in desmoid tumors, possibly explaining the paucity of reports describing correlations between specific mutation status and biological behavior in malignant tumors. On the other hand, several studies have demonstrated the relationship between mutation status of CTNNB1 and

treatment outcome including surgery [10, 12, 13] and conservative therapy [14, 15] in patients with desmoid tumors. To obtain proof of this concept using in vitro study and investigate cell behavior with different molecular features, and to establish and characterize cell cultures with diverse mutation status of CTNNB1 will be important.

Cultured cells had homogenous fibroblast-like features despite the different mutation status of CTNNB1. Although positivity of nuclear β-catenin staining was strong in cells with S45F, doubling time was shortest in WT cells compared with those with T41A and S45F. On the other hand, downstream target gene of Wnt/β-catenin signaling pathway including Axin2, Cyclin D1, and c-Myc were upregulated in S45F cells compared with T41A and WT cells. Considering the results of previous reports that patients with S45F exhibited resistance to surgical and conservative treatment [10, 12–14], cells with S45F may exhibit the most aggressive biological behavior. Results of this study could not completely explain the in vivo behavior of desmoid cells. Part of the biological behavior will be affected by the environmental conditions in vivo, because patients with young age and/or extremity desmoid tumors had significant worse surgical outcome regardless of the mutation status [23], suggesting that host conditions may influence the tumorigenicity.

Based on a previous study in which COX-2 blockade decreased cell proliferation of desmoid tumor in vitro, and inhibited the growth of desmoid tumors in a mouse model [24], a COX-2 inhibitor, meloxicam, has been prospectively used for patients with extraperitoneal desmoid tumors in our institution [16, 18]. Cultured cells in this study were all derived from tumors exhibiting resistance to meloxicam treatment clinically, suggesting that responsiveness to meloxicam may not be different in in vitro experiments among the three cultured cell cultures. Although positivity of COX-2 immunofluorescence staining was stronger in S45F cells compared to other cells, responsiveness to meloxicam treatment was similar among cells. It will be explained by our previous report describing that responsiveness to meloxicam is not correlated with the COX-2 stainability [8]. Despite deriving from tumors resistant to meloxicam, the reason why meloxicam showed inhibitory effects in vitro of three cells may partly be due to the difference between in vitro and in vivo environment. On the other hand, apoptotic activity of actinomycin-D was well characterized in the analyzed cell cultures. A previous study evaluated the proliferation rate of desmoid cell cultures with three COX blocking agents, sulindac, indomethacin, and 5,5-Dimethyl-3-(3 fluorophenyl)-4-(4 methylsulphonal) phenyl-2 (5H)-furanone (DFU), and determined the concentration effective to inhibit the cell viability, although CTNNB1 mutation status was not considered in their study [24].

Nuclear accumulation of β-catenin is considered to activate T-cell factor, which in turn stimulates Tcf/Lef transcriptional gene expression [25, 26]. The expression of these genes including Axin2, c-Myc, Cyclin D1 has been shown to be increased in desmoid tumor [27–29]. In this study, expression patterns of these target genes altered among cells harboring different mutation types. To determine these characteristics may help to understand the biological features of desmoid cells with different mutation type, and moreover, to evaluate the responsiveness of cells to drugs based on mutation type. Effectiveness of drugs had better be analyzed by not only cell viability test, but also gene expression patterns of target genes because downstream of Tcf/Lef transcription pathway should have crucial roles in tumorigenesis of desmoid tumors. Meneghello et al. reported results inconsistent with those of our study, namely that mRNA expression of cyclin D was decreased in all desmoid cells harboring different mutation status compared with control cells, whereas that of Axin2 increased [28]. This discrepancy between our studies might be due to differences in the control cells used. The control cells used in their study showed the greatest proliferation as compared with desmoid cells. Another reason might be the heterogeneous origin of desmoid cells in their study, including male and female patients, age ranging from 31 to 53 years, and location in abdominal wall versus extremity. In contrast, the cells used in this study were all obtained from female patients, located on the abdominal wall, and with age ranging from 20 to 39, and thus seemingly had similar background features except for mutation status. The expression of all target genes in cells with S45F mutation, and two genes in those with WT or T41A mutation were increased compared with that in control cells. Generally, phosphorylation first occurs at S45 by CK1α, and subsequently at T41, S37, and S33 by GSK3β [30]. Phosphorylation of S45 seems to be an essential process for the degradation of β-catenin, suggesting S45F mutation, which may block the entire phosphorylation, and cause the most prominent accumulation of β-catenin.

Different effects were observed of inhibitors (IWR-1 and quecertin) for Wnt/beta-catenin pathway on desmoid cells. Mutation of β-catenin seems to be insulated from degradation by Axin/APC complex. IWR-1, which stabilizes Axin protein and stimulates β-catenin degradation might have less marked effects on TCF/Lef-1, downstream of the Wnt/beta-catenin pathway compared with quercetin, which blocks the TCF/Lef-1 pathway. Present study using inhibitors of Wnt/beta-catenin pathway revealed that desmoid cells harboring different mutation status had partly different response to these inhibitors. Although in vivo conditions should be taken into account in the future, investigating gene expression profile after treatment of various inhibitors may provide meaningful information.

There are several limitations in this study. First, although isolated desmoid cells were all obtained from females and from the abdominal wall with different mutation status, cell characteristics should be determined from different locations including extremity and neck regions where tumors occasionally show resistance to therapies. Another is that the behaviors of desmoid tumors are considerably affected by the host environment including age, gender, and location. Experiments using isolated cells in vitro could not easily mimic in vivo phenomena.

In conclusion, we successfully cultured and characterized three desmoid cell types with different CTNNB1 mutation status. Investigations using these cells will help to clarify the altered responsiveness of cells with different mutations to various therapies.

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Conflict of Interest

None declared.

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Title: Simple resection for truncal desmoid tumors: a case series

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Running title: NISHIDA et al: SIMPLE RESECTION FOR DESMOID

Abstract

The morbidity of aggressive surgery and high recurrence rate have been reported in extra-abdominal and abdominal wall desmoid tumors. Surgery without functional impairment is desired to patients with demoid tumors. Among patients, prospectively and consecutively treated with identical conservative treatment, selected patients were treated with less invasive surgery. Of 60 patients pathologically diagnosed with desmoid tumors, 9 with tumors refractory to, and 4 who refused conservative treatment were treated with planned simple resection. Clinical outcome and CTNNB1 mutational status of tumors were analyzed. Mean age of patients with planned simple resection was 39 years, and locations of tumors were abdominal wall in 6, chest wall in 4, and neck region in 3. All excised specimens were evaluated as microscopically margin positive. However, during the mean follow up of 30 months, 12 of 13 cases did not develop recurrence, which had T41A mutations in 7 and wild type in 5. Only one early case, which had S45F mutation of CTNNB1, developed recurrence. The results of this prospectively treated with simple resection and retrospectively analyzed study suggest that planned simple resection could be a possible therapeutic modality for extra-peritoneal desmoid tumors, particularly of truncal location with wild-type or T41A mutational status.