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RESEARCH ARTICLE

Nuclear expression of β-catenin predicts the efficacy of meloxicam treatment for patients with sporadic desmoid tumors

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Abstract This study aimed to determine the prevalence of β-catenin nuclear positivity as a prognostic factor in patients with desmoid tumors (DTs) treated with meloxicam, a cyclooxygenase-2 (COX-2) selective inhibitor. Between 2003 and 2012, consecutive 31 patients with extraabdominal, sporadic DTs were prospectively treated with meloxicam as a systemic medical therapy. Immunohistochemistry was performed on formalin-fixed material to quantify the nuclear expression of β-catenin and Ki-67, and cytoplasmic expression of COX-2. All clinicopathological characteristics including the intensity of immunohistochemical staining were analyzed with respect to their prognostic value for meloxicam treatment. Of the 31 patients with meloxicam treatment, there was 1 with complete remission (CR), 7 with partial remission (PR), 12 with stable disease (SD), and 11 with progressive disease (PD). Higher nuclear expression of β-catenin was significantly associated with a poor response (PD/SD) (p= 0.017). The positivity of COX-2 and Ki-67 and none of the other clinical variables were associated with prognosis. The nuclear expression of β-catenin can predict the efficacy of meloxicam treatment for patients with sporadic DTs.

Keywords Desmoid tumor · Meloxicam · Prognostic factor · β-catenin · COX-2

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Introduction

Desmoid tumors (DTs), or aggressive fibromatosis, are mesenchymal tumors characterized by a local severely aggressive nature, but rarely metastasize, and do not cause disease-specific death if not located at anatomically critical sites [1, 2]. Extensive surgical resection has been the standard treatment for decades. However, radical surgical intervention and/or repeated surgery necessitated by a high recurrence rate (range 34–53 % at 5 years) [3, 4] may lead to significant treatment-related morbidity such as amputation or significant functional impairment. Several reports have failed to demonstrate any significance of the surgical margin on recurrence [3, 5, 6], while spontaneous regression has been reported in some patients [5, 6]. Together, the clinical course of DT is unusual and even enigmatic [7]. Several authors have recently outlined various conservative treatment modalities for DT including radiotherapy [8, 9] and drug treatment. Pharmacological treatment includes antihormonal agents, NSAIDs, and targeted and traditional cytotoxic chemotherapies [2, 10–13]. However, considering that tumor-related mortality is rare in patients with extraabdominal DT, cytotoxic agents with severe complications should be avoided as the initial treatment.

Cyclooxygenase-2 (COX-2) has been implicated as a factor in tumor initiation in colonic neoplasia and has also been demonstrated to play a role in the growth of DTs [14]. We have previously reported the clinical results of consecutive patients prospectively treated with meloxicam, a COX-2 selective inhibitor [12, 15]. The results of the studies showed that older age is a significant favorable prognostic factor for meloxicam treatment. However, there is difficulty in predicting the efficacy of meloxicam precisely before treatment. Biological markers are anticipated to be more useful in predicting the efficacy of this treatment.

β-catenin plays an important role in the development of desmoid fibromatoses and has the diagnostic potential to differentiate DT from other lesions [16]. The significance of β-catenin has been reported not only for the pathophysiological process of DT but also as a prognostic factor, particularly after surgical treatment [17, 18]. However, no useful prognostic factors have been identified for conservative treatment. Although we have reported favorable clinical results with meloxicam treatment for patients with extraperitoneal DT [12], the relationship between COX-2 and outcome has not been analyzed. This prompted us to investigate the prognostic value of β-catenin, Ki-67, and COX-2 for meloxicam treatment in patients with extraperitoneal DTs.

Materials and methods

Patients

We reviewed the medical records of 38 consecutive cases with extraabdominal or abdominal wall DTs diagnosed in our institutions since 2003. Seven cases were excluded. Two had been correctly diagnosed with other diseases. Two cases refused meloxicam treatment. Three cases had been followed for less than 6 months. There was no case with familial adenomatous polyposis-associated DT. Finally, this study was composed of 31 consecutive patients with extraperitoneal DT prospectively treated with meloxicam without any other previous treatment. No patients received other medical treatment or radiotherapy during meloxicam treatment. All 31 cases were histologically reevaluated as having DT. Meloxicam was administered orally at 10 mg/day. Baseline imaging of DTs by magnetic resonance imaging (MRI) and/or computed tomography (CT) was obtained before starting treatment. Patients treated with meloxicam have been followed with physical examinations and MRI and/or CT at the outpatient unit of our institution every 3–6 months. The efficacy of meloxicam was evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) [19] measured with MRI or CT at the latest follow-up. When patients were evaluated as showing a complete response (CR), they discontinued meloxicam. Patients with partial response (PR) or stable disease (SD) continued meloxicam.

In cases with progressive disease (PD), patients can choose surgical treatment or low-dose chemotherapy with methotrexate and vinblastine, or continuation of meloxicam treatment after careful discussion of their tumor status. All patients signed an informed consent form, and the protocol was approved by the institutional review board of our institution.

Immunohistochemistry

All tumor samples were obtained at biopsy with no effects of meloxicam or chemotherapy. Biopsy specimens were fixed in 10 % formalin and embedded in paraffin. Paraffin specimens were cut at a thickness of 5 μm. The deparaffinized and rehydrated sections were treated with 0.3 % H_2O_2 in 30 % methanol for 30 min at room temperature to block the internal peroxidase activity, followed by incubation with 1 % bovine serum albumin in PBS as a blocking agent for 1 h at room temperature. The slides were incubated overnight at 4 °C with anti-β-catenin mouse monoclonal antibody (M3539; Dako, Carpinteria, CA; dilution, 1:200 dilution), anti-COX-2 goat monoclonal antibody (sc-1747; Santa Cruz Biotechnology, Santa Cruz, CA; 1:500 dilution), and Ki-67 mouse monoclonal antibody (M7240; Dako, Carpinteria, CA; 1:100 dilution). After rinsing with PBS, the sections were incubated with biotinylated secondary antibodies (Nichirei Biosciences, Tokyo, Japan), and the reaction products were observed using 3,3-diaminobenzidine tetrahydrochloride. Slides were counterstained with hematoxylin, dehydrated, and mounted [20]. Nonimmune mouse and goat serum was substituted for the primary antibody as a negative control.

Assessment for staining positivity

For β-catenin and Ki-67, positivity of nuclear staining was evaluated, whereas positivity of cytoplasmic staining was investigated for COX-2. Staining positivity was evaluated by two independent observers (S.H., N.F.) without any knowledge of the clinicopathological information and divided into 4 groups: 0 % for positive stainable cell number (negative; 0), 1–9 % (weak; 1+), 10–50 % (moderate; 2+), and 51–100 % (strong; 3+) on 10 independent high-power fields. We defined the positivity rate of β-catenin according to a previous report [21]. Ki-67 was divided into two groups; 0–1 % (negative; 0) and more than 1 % (positive; +). Using these criteria, both observers agreed on the degree of positivity or negativity of each case. Patients were divided into two groups as responders (CR, PR) or nonresponders (SD, PD). Age, gender, site (abdominal wall or extraabdominal), tumor size, and positivity of β-catenin, COX-2, and Ki-67 were examined as possible prognostic factors for responsiveness to meloxicam.

Statistical evaluation

Data were analyzed using the Chi-square test with Yates' continuity correction for dichotomous variables to examine correlations between the clinical results for meloxicam and clinicopathological characteristics including β-catenin, COX-2, and Ki-67 expression and clinical response. Continuous variables of age and tumor size were compared between the two groups using unpaired Student's t test. $p<0.05$ was considered significant.

Results

Clinical features

The mean age was 42.1 years (median, 37.0 years; range, 12–75 years). Eleven were male, and 20 were female. The anatomic distribution of the tumors was the abdominal wall in seven patients, four each in the neck and back, three each in the shoulder, thigh, and forearm, two in the chest wall, and one each in the upper arm, calf, foot, groin, and retroperitoneum. The diameter of the tumor ranged from 20 to 220 mm (mean, 83.7 mm; median, 76.0 mm). No patients had received radiotherapy or other treatment for DTs in advance of meloxicam treatment. The mean follow-up was 41.2 months (10–105 months). The median period of medication was 30.3 months (range, 2–105 months). Of the 31 patients evaluated, there was 1 patient with CR, 7 with PR, 12 with SD, and 11 with PD. There were no significant differences in age (p=0.44) or tumor size (p=0.85) between the responders and nonresponders. The number of analyzed patients increased in this study compared with our previous study [12]. It might cause that "age" was no longer a significant prognostic factor. The periods of meloxicam medication were 49 months at the time of CR evaluation and 28.0 months (median, range; 19–36 months) at the time of PR evaluation. These suggest that relatively longer duration of medication might be required to obtain good response.

Immunohistochemical findings

In all 31 cases evaluated, positive nuclear staining for β-catenin was observed; moreover, there was no case with weak positive (1+) (Table 1). Fourteen (45 %) patients showed moderate positive (2+) and 17 (55 %) strong staining (3+) (Fig. 1). Only one patient (13 %) in the responder group showed strong (3+), whereas 16 patients (70 %) in the poor responder group showed strong staining (3+). Of interest, none of the responders showed more than 60 % nuclear positivity of β-catenin.

All 31 cases revealed positive COX-2 staining in cellular cytoplasm. Four (13 %) patients showed weakly positive (1+), 12 (39 %) showed moderate (2+), and 15 (48 %) showed strong staining (Table 2) (Fig. 1). There was a trend in staining positivity between β-catenin and COX-2 (p=0.058). Among 31 cases evaluated for Ki-67 staining, 17 cases showed positivity equal to or less than 1 % (Table 3) (Fig. 1). Positive Ki-67 staining was not correlated with β-catenin (p=0.815) or COX-2 (p=0.301). There was no association between positivity of β-catenin and demographic data including age.

Table 1 Nuclear β-catenin expression and clinical prognosis

Intensity	CR	PR	SD	PD	Total
Negative (0 %)	0	0	0	0	0
Weak (1–10 %)	0	0	0	0	0
Moderate (11–50 %)	1	6	5	2	14
Strong (51–100 %)	0	1	7	9	17

Factors correlated with efficacy of meloxicam

Gender, age (<50 vs ≥50 ys), site (abdominal wall vs extraabdominal), and tumor size (<80 vs ≥80 mm) were not correlated with the efficacy of meloxicam treatment. Positivity of COX-2 staining and Ki-67 was not significantly correlated with responsiveness to meloxicam treatment. However, there was a statistically significant correlation with the intensity of nuclear staining of β-catenin (p=0.017), while strong β-catenin expression independently predicted a poor response to meloxicam with a relative risk of 1.88 (95 % CI, 1.10–3.22) (Table 4).

Discussion

β-catenin has been reported to play an important role in the Wnt signaling pathway involved in tumorigenesis in some tumors [22]. The accumulation of β-catenin caused by CTNNB1 (β-catenin) or APC mutation subsequently activates T cell factor, which in turn causes transcription of target genes in fibroproliferative disease [23]. In DT, this Wnt-β-catenin pathway also plays crucial roles in tumor development, and the nuclear expression of β-catenin has increasingly been used in the differential diagnosis of spindle cell neoplasms due to the high positivity rate of DTs [1, 16–18, 24]. The results of these previous reports are consistent with ours, in which all cases showed moderate (2+) or higher positive nuclear β-catenin expression.

Several previous studies reported the significance of nuclear expression for β-catenin in the prognosis of patients with DTs treated surgically. Gebert et al. analyzed nuclear expression for β-catenin in 37 cases with extremity and trunk DTs and reported that the overexpression of β-catenin is associated with a decreased event-free survival based on 23 cases with available clinical records. However, 6 of the 23 cases received surgery with a wide surgical margin and 17 with a marginal margin. Further, nine cases received radiotherapy primarily, suggesting that therapeutic bias may have influenced the results of β-catenin [17]. In contrast, Lazar et al. reported that the intensity of nuclear β-catenin expression was inversely correlated with the incidence of desmoid recurrence in the analysis of 195 tumor tissues. This study did not describe the details of surgical treatment or adjuvant treatment. Interestingly, the β-catenin 45F mutation exhibited a less intense β-catenin staining as compared with 41A mutation [18]. The association of β-catenin mutation status with

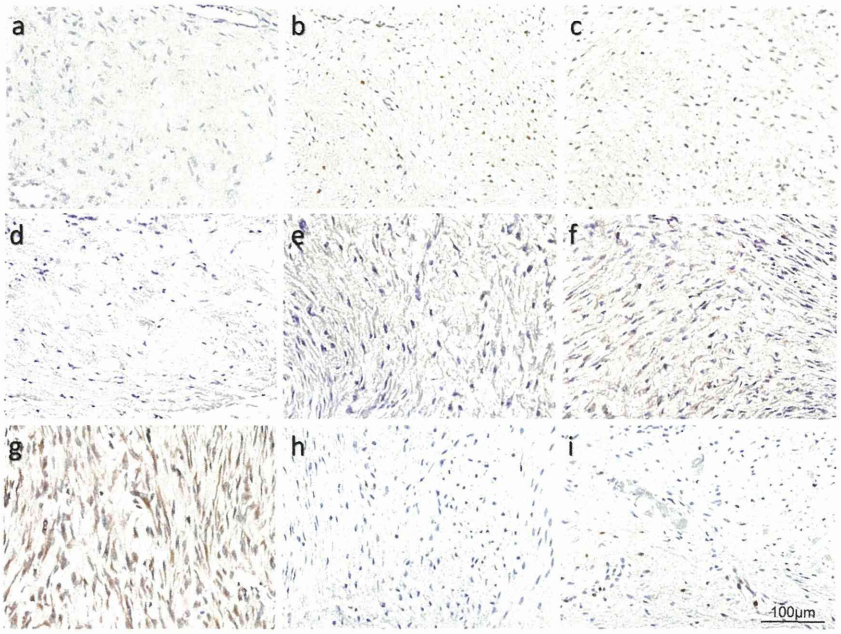

Fig. 1 Immunohistochemical staining of desmoid tumor. **a**, **b**, **c** β-catenin (**a** negative control; **b** moderate; **c** strong). **d**, **e**, **f**, **g** COX-2 (**d** negative control; **e** weak; **f** moderate; **g** strong). **h**, **i** Ki-67 (**h** negative; **i** positive) (counterstain with hematoxylin; original magnification, ×200)

positivity of nuclear β-catenin staining has not been clarified. On the other hand, Huang et al. showed that neither the percentage nor intensity of nuclear β-catenin expression correlated with tumor recurrence in their analysis of 46 cases [25]. In their study, only 6 of 46 cases (13 %) developed recurrence, representing a much better outcome than noted in other studies and suggesting that increasing the number of cases may produce different results. Together, these studies analyzing the significance of nuclear β-catenin staining for recurrence after surgery included heterogeneous and retrospective cohorts of patients. Prospectively designed studies may reveal more precisely the roles of β-catenin in surgically treated patients.

The treatment approach to sporadic DTs has recently changed. Bonvalot et al. proposed an algorithm that commences with more conservative modalities before resorting to treatments with predicted high morbidity [11]. We have prospectively treated sporadic DT patients with meloxicam since 2003. Although the early outcome was favorable [12], we have experienced an increasing number of patients who are resistant to meloxicam treatment. Predictive indicators for meloxicam treatment are required. There have been no informative reports analyzing prognostic factors for conservative treatment with not only COX-2 inhibitors but also hormonal agents. Bocale et al. reported that the response to antiestrogen therapy did not seem to be related to estrogen receptor status [26]. Therefore, we assessed the prognostic value of nuclear β-catenin expression in sporadic desmoid patients with meloxicam treatment. The present study demonstrated that patients with strong nuclear β-catenin staining are significantly resistant to meloxicam treatment, suggesting that this group of patients should be treated promptly with another modality. To the best of our knowledge, our results are the first to suggest that β-catenin might serve as a prognostic index for conservative treatment, particularly of COX-2 inhibitor. Moreover, the cohort of our study is composed of prospective and consecutive patients treated with meloxicam without any antecedent treatment, supporting the credibility of the results in this study.

COX-2 is an enzyme involved in prostaglandin synthesis. A previous study [14] showed that DTs express elevated levels of COX-2, which is consistent with the results in this study that the overexpression (more than weakly positive) of COX-2 was observed in all cases. Poon et al. demonstrated that COX-2 blocking decreased cell proliferation in desmoid cell cultures in vitro and the size of DTs in an in vivo mouse model [14]. Based on the preclinical study, following the IRB approval in our institution, we have applied the COX-2 inhibitor, meloxicam prospectively, to sporadic DTs as

Table 2 Cytoplasmic COX-2 expression and clinical prognosis

Intensity	CR	PR	SD	PD	Total
Negative (0 %)	0	0	0	0	0
Weak (1–10 %)	0	2	0	2	4
Moderate (11–50 %)	0	3	5	4	12
Strong (51–100 %)	1	2	7	5	15

Table 3 Nuclear Ki-67 expression (MIB-1) and clinical prognosis

Intensity	CR	PR	SD	PD	Total
Negative (0–1 %)	1	5	7	4	17
Positive (>1 %)	0	2	5	7	14

Table 4 Clinicopathological characteristics and clinical prognosis with responder (CR/PR) vs nonresponder (SD/PD)

Clinicopathological characteristics	Number	Responder (n=8)	Nonresponder (n=23)	p value
Gender				
Female	20	3 (15 %)	17 (85 %)	0.154
Male	11	5 (45 %)	6 (55 %)	
Age (mean=42.3 ys)				
<40 ys	18	4 (18 %)	14 (82 %)	0.464
≥40 ys	13	4 (36 %)	9 (64 %)	
Location				
Abdominal wall	7	1 (14 %)	6 (86 %)	0.764
Extraabdominal wall	24	7 (29 %)	17 (71 %)	
Size (mean=83.7 mm)				
<80 mm	17	6 (35 %)	11 (65 %)	0.359
≥80 mm	14	2 (14 %)	12 (86 %)	
Nuclear β-catenin staining				
Moderate (2+)	14	7 (50 %)	7 (50 %)	0.017
Strong (3+)	17	1 (6 %)	16 (94 %)	
Cytoplasm COX-2 staining				
Weak (1+)	4	2 (50 %)	2 (50 %)	0.475
Moderate (2+)	12	3 (25 %)	9 (75 %)	
Strong (3+)	15	3 (20 %)	12 (80 %)	
Ki-67 staining				
Negative (0)	17	6 (35 %)	11 (65 %)	0.359
Positive (+)	14	2 (14 %)	12 (86 %)	

conservative treatment since 2003. Nuclear accumulated β-catenin activates the T cell factor (TCF), which in turn causes transcription of target genes, one of which is considered to be COX-2 [1, 14]. However, the intensity of COX-2 expression was not correlated with the responsiveness to meloxicam but rather tended to be associated with nuclear β-catenin expression. There may be two possible explanations for this. One is that COX-2 comprises one part of the downstream of β-catenin/Wnt pathway which partially causes tumorigenesis of DT. This may be attributable partly to differences in the responsiveness to meloxicam treatment of each DT. Another explanation is the sensitivity of COX-2 immunostaining, since we did not confirm the COX-2 expression quantitatively with ELISA or immunoblot, which is a limitation of this study. Precise quantification of COX-2 may provide different information in the future.

In this study, the positivity of Ki-67 staining was very low and did not correlate with the positivity of β-catenin. This finding was consistent with that of a previous study and may reflect the essentially benign nature of DTs [27]. Brueckl et al. reported that low positivity of Ki-67 was of positive prognostic value concerning disease-free survival in a surgically treated series [28]. Considering that the increasing results of the relationship between nuclear β-catenin expression and treatment outcome, and very low positivity of Ki-67 staining in DTs, the prognostic significance of β-catenin might receive more attention.

There are some limitations in this study. First, the sample size was relatively small. However, given that DT is a rare condition and the cohort of this study is composed of prospective patients, the results derived from this study provide meaningful information. Future studies with more accumulated cases will help to clarify the association with the prognosis. Second, we evaluated only the expression of β-catenin and COX-2 immunohistochemically and did not perform quantitative evaluation of mRNA expression. Recently, other gene expressions were reported to correlate with the behavior of DT, including p53, EGFR, sex steroid receptor, and PDGF [18, 29–31]. Furthermore, the correlation of CTNNB1 gene mutation and prognosis has been reported in recent studies [17, 32]. Additional comparisons of such gene expression or mutation analysis with responsiveness to meloxicam may provide additional valuable information.

In conclusion, we suggest for the first time that nuclear β-catenin expression may serve as a prognostic marker in patients with sporadic DT treated with meloxicam. Given that treatment modalities for patients with DTs are now shifting to more conservative treatment, prognostificators for conservative treatment were urgently needed. Larger prospective studies are still necessary to confirm our findings and to further explore the role of β-catenin and other signaling in desmoid responsiveness to medical and surgical treatment.

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Conflicts of interest None

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PLOS ONE

CTNNB1 S45F Mutation Predicts Poor Efficacy of Meloxicam Treatment for Desmoid Tumors: A Pilot Study

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Abstract

We hypothesized that patterns of CTNNB1 (β-catenin) mutations would affect the outcome of conservative therapy in patients with desmoid tumors. This study aimed to determine the significance of CTNNB1 (β-catenin) mutations in predicting the treatment outcome in patients with desmoid tumors treated with meloxicam, a cyclooxygenase-2 (COX-2) selective inhibitor. Between 2003 and 2012, consecutive thirty-three patients with extra-peritoneal sporadic desmoid tumors were prospectively treated with meloxicam as the initial systemic medical therapy. The efficacy of meloxicam was evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST). DNA was isolated from frozen tissue or formalin-fixed materials. CTNNB1 mutation analysis was performed by direct sequencing. Positivity of nuclear β-catenin staining by immunohistochemistry was compared with the status of CTNNB1 mutations. The correlation between the efficacy of meloxicam treatment and status of CTNNB1 mutations was analyzed. Of the 33 patients with meloxicam treatment, one showed complete remission (CR), 7 partial remission (PR), 12 stable disease (SD), and 13 progressive disease (PD). The following 3 point mutations were identified in 21 of the 33 cases (64%): T41A (16 cases), S45F (4 cases) and S45P (one case). The nuclear expression of β-catenin correlated significantly with CTNNB1 mutation status ($p = 0.035$); all four cases with S45F mutation exhibited strong nuclear expression of β-catenin. S45F mutation was significantly associated with a poor response (all cases; PD) ($p = 0.017$), whereas the other mutations had no impact on efficacy. The CTNNB1 mutation status was of significant prognostic value for meloxicam treatment in patients with sporadic desmoid tumors.

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Introduction

Desmoid tumors, also known as aggressive fibromatosis, are mesenchymal tumors that show marked local aggressiveness, but rarely metastasize, and do not cause disease-specific death if they are not located at anatomically critical sites [1], [2]. Extra-peritoneal desmoid tumors, which are usually sporadic in nature, occur across a wide age range, and can arise at virtually any body site. Extensive surgical resection has been the standard treatment for decades. However, radical surgical intervention and/or repeated surgery due to a high recurrence rate (range 34–53% at 5 years) [3], [4] occasionally lead to significant treatment-related morbidity including amputation or significant functional impairment. Several reports have failed to demonstrate the significance of margin status in surgery for local recurrence [3–5], while spontaneous regression has also been reported [5], [6]. Several authors recently demonstrated the effectiveness of conservative treatment for desmoid tumors including radiotherapy and pharmacological treatment [7], [8]. Pharmacological treatment includes anti-hormone, NSAIDs, and targeted and traditional cytotoxic chemotherapies [2], [9–12]. However, the efficacy of these treatments cannot be predicted, and so remains a crucial problem.

β-catenin plays various important roles in the tumorigenesis of desmoid tumors, and has a diagnostic potential to differentiate them from other lesions [13], [14]. The nuclear accumulation of β-catenin causes activation of Wnt signaling, and in turn transcription of target genes in fibroproliferative disease [15]. Most desmoid tumors arise sporadically, with a minority associated with familial adenomatous polyposis (FAP), which is caused by a germline mutation of the adenomatous polyposis (APC) gene [15]. APC protein forms β-catenin destruction complex and is involved in the regulation of Wnt signaling. Several recent studies have reported point mutations of CTNNB1 (β-catenin) exon3. These mutations, occurring at codon 41 and 45, were found in about 64–85% of all sporadic desmoids, with p.T41A (threonine to alanine), p.S45F (serine to phenylalanine), and p.S45P (serine to proline) being the most frequent ones [16–19]. These mutations were considered to lead to stabilization of β-catenin and tumorigenesis in desmoid tumors, suggesting that the status of CTNNB1 mutations might influence the efficacy of various treatments for patients with these tumors.

We previously reported the clinical results of consecutive patients prospectively treated with meloxicam, a cyclooxygenase-2 selective inhibitor [12], [20], [21]. The efficacy of meloxicam treatment varied among patients, indicating that biological

markers are required to predict its efficacy in individual patients. Because consecutive patients were prospectively treated with the same medication, meloxicam, without other treatments, this cohort is particularly suitable to identify a biological marker predictive of the efficacy of this treatment. The aims of this study were to prospectively analyze the status of CTNNB1 mutations in consecutive patients with extra-peritoneal desmoid tumors treated with meloxicam, and to determine the significance of mutational status in predicting the efficacy of meloxicam treatment.

Materials and Methods

Ethics statement

The study protocol was approved by the Institutional Review Board (IRB) of Center for Advanced Medicine and Clinical Research, Nagoya University (approval ID: 1322), and written informed consent was obtained from all participants before the study commenced. The individuals in this manuscript have given written informed consent to publish these case details

Patients and Tumor Tissues

There were 41 consecutive cases with extra-abdominal or abdominal wall desmoid tumors diagnosed in our institutions since 2003. They were all prospectively treated with meloxicam, a selective COX-2 inhibitor. Eight cases were excluded from this study. Three cases had been followed for less than 6 months, and two refused meloxicam treatment. In two cases, biopsy specimens were not available for analysis. One case was subsequently correctly diagnosed with another disease. There was no case with FAP-associated desmoid tumor. Finally, this study was composed of thirty-three consecutive patients with extra-peritoneal desmoid tumor prospectively treated with meloxicam without any other treatment previously. No patients received other medical treatments or radiotherapy during the meloxicam treatment. All thirty-three cases were histologically evaluated as having desmoid tumor by specialized pathologists.

Efficacy of meloxicam treatment

As described in our previous study [12], meloxicam was administered orally at 10 mg/day. Baseline imaging of desmoid tumors by magnetic resonance imaging (MRI) and/or computed tomography (CT) was obtained before starting treatment. Patients treated with meloxicam were followed with physical and radiological examinations with MRI and/or CT at the outpatient unit of our institution every 3 to 6 months. The efficacy of meloxicam was evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) measured with MRI or CT at the latest follow-up or the end of meloxicam treatment. [22]. When patients were evaluated as showing a complete response (CR), they discontinued meloxicam. Patients with partial response (PR) or stable disease (SD) continued meloxicam. Patients with evaluation of PD could stop this treatment and chose other treatment modalities including low-dose chemotherapy or surgery depending on the tumor status, including location and aggressiveness. Considering that meloxicam has minimal side effects, SD status is thought to be preferable. Patients were divided into two groups, namely a favorable group (CR, PR, SD) and unfavorable group (PD). Age, gender, site, tumor size, follow-up period, and mutational status of CTNNB1 were examined as possible prognostic factors for responsiveness to meloxicam.

Mutation analysis of CTNNB1

All patients received needle or incisional biopsy for histological diagnosis. Some of the obtained specimens were snap-frozen, and stored at −80°C for DNA and/or RNA analyses. DNA was extracted from frozen tissue or 5-μm-thick formalin-fixed, paraffin-embedded tissue by the High Pure PCR Template Preparation Kit (Roche Molecular Diagnostics, Mannheim, Germany), according to the manufacturer's instructions. DNA was first amplified by polymerase chain reaction (PCR) of 40 cycles at an annealing temperature of 58°C using LightCycler 480 System (Roche). To analyze the existence of point mutations in codons 41 or 45 of CTNNB1 exon 3, we designed 2 pairs of primers: forward 5′- GATTTGATGGAGTTGGACATGG-3′, reverse 5′-TCTTCCTCAGGATTGCCTT-3′, and forward 5′-TGGAACCAGACAGAAAAGCG-3′, reverse 5′- TCAG-GATTGCCTTTACCACTC -3′. The expected sizes of the amplified products were 149 and 118 bp, respectively. PCR products were isolated by gel electrophoresis in 2% agarose, and amplified bands were extracted and purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA). Purified products were subjected to direct sequencing using the above primers (forward), 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 were compared with those in the databases of NCBI-BLAST to confirm the mutation sites.

Immunohistochemistry

Biopsy specimens fixed in 10% formalin and embedded in paraffin were subjected to immunohistochemical study for β-catenin. As described previously [23], the slides were incubated for overnight at 4°C with anti-human β-catenin mouse monoclonal antibody (M3539; Dako, Carpinteria, CA; dilution, 1:200 dilution), and counterstained with hematoxylin, dehydrated, and mounted [24]. Nuclear positivity of β-catenin was evaluated by two independent observer (S. H., N. F.) without any knowledge of the clinicopathological information, and divided into 4 groups; 0% for positive stainable cell number (negative; 0), 1% to 9% (weak; 1+), 10% to 50% (moderate; 2+) and 51% to 100% (strong; 3+) on 10 independent high-power fields. The relationship between mutation status of β-catenin and nuclear positivity of β-catenin was analyzed.

Statistical evaluation

Data were analyzed using the Fisher's exact test for dichotomous variables to examine correlations between the efficacy of meloxicam and clinical characteristics and between the mutation status of CTNNB1 and clinicopathological characteristics. Continuous variables of age and tumor size were compared between the two groups using the Mann-Whitney U test and between the multinomial groups using one-way analysis of variance. All statistical analyses were performed using SPSS version 20. $P < 0.05$ was considered significant.

Results

Clinical features and efficacy of meloxicam treatment

Of the 33 patients prospectively treated with meloxicam, 11 were male, and 22 were female. The mean age was 41.6 years (median, 37.0 years; range, 10–74 years). The anatomic distribution of the tumors was the abdominal wall in 7 patients, neck 5, forearm and back 4 each, chest wall and thigh 3 each, shoulder 2, and upper arm, calf, foot, groin and retroperitoneum one each. The diameter of the tumor ranged from 20 to 220 mm (mean, 84 mm; median, 72 mm). No patients had received radiotherapy or other treatment for desmoid tumors prior to the meloxicam treatment. The median follow up was 36.6 months (10–120

months). The median period of medication was 15.2 months (range, 3 to 113 months). Of the 33 patients evaluated, there was one patient with CR, 7 with PR, 12 with SD, and 13 with PD. Of the 13 cases with PD, three were subjected to surgical treatment, and 8 to low-dose and/or doxorubicin-based chemotherapy. There were no significant differences in gender ($p = 0.46$), age ($p = 0.34$), tumor size ($p = 0.63$), or site of involvement ($p = 0.22$) between the favorable and unfavorable groups (Table 1).

Mutation status of CTNNB1 and efficacy of meloxicam treatment

We performed genotyping of CTNNB1 exon 3 for all the cases. Point mutations were identified in 21 of the 33 cases (64%), and occurred in only 2 codons (41 and 45). Replacement of threonine by alanine (T41A) in codon 41 was detected in 16 cases (49%). Replacement of serine by phenylalanine (S45F) in codon 45 was detected in 4 (12.1%), and replacement of serine by proline (S45P) in codon 45 was detected in one (3.0%; Figure 1).

In 7 cases with desmoid tumor arising in the abdominal wall, 4 did not have mutations in exon 3 (wild type), 2 had codon 45 mutations (S45F and S45P) and one had a codon 41 mutation (T41A). In other sites, T41A was the most frequent mutation. The mean age of each mutation group was lower than that of the wild-type group, and the mean tumor size of each mutation group was larger than that of wild-type. However, there were no significant differences in gender ($p = 0.67$), age ($p = 0.57$), tumor size ($p = 0.47$), or site ($p = 0.23$) between the groups (Table 2).

In analyses of the correlation of mutation status of CTNNB1 and efficacy of meloxicam treatment, mutation status showed a trend to associate with efficacy ($p = 0.053$, Table 2). Focusing on the S45F mutation, all 4 cases with 45F mutation showed PD, and no cases in the favorable group (0/20 cases) had S45F mutation ($p = 0.017$, Table 3), whereas cases with T41A or wild type did not show a significant association with the efficacy of meloxicam. Representative cases are shown in Figures 2 and 3.

Correlation of nuclear β-catenin staining and mutation type

As reported previously [23], positive nuclear staining for β-catenin was observed in all 33 cases, and there was no case with weak positive staining (1+). Strong positive (3+) indicated a poor prognosis in comparison with moderate (2+) staining ($p = 0.032$).

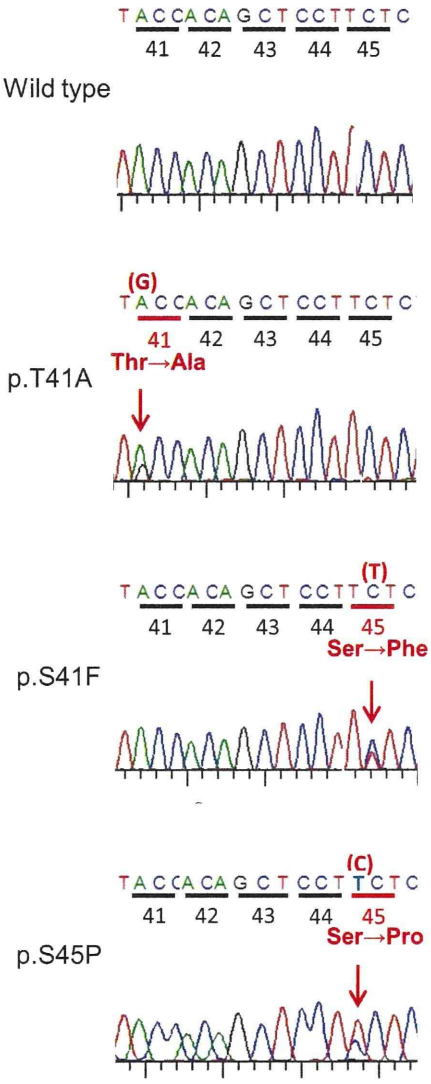

Figure 1. Representative sequencing results of each mutational type. Thr; Threonine, Ala; Alanine, Ser; Serine, Phe; Phenylalanine, Pro; Proline.
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Table 1. Patient characteristics between two prognosis groups.

Variables	Favorable group (n = 20)	Unfavorable group (n = 13)	P value
Gender			0.46
Female	12 (60%)	10 (77%)	
Male	8 (40%)	3 (23%)	
Mean age, years (range)	36.8 (10–74)	44.6 (20–73)	0.34
Mean size, mm (range)	84.5 (23–159)	83.0 (20–220)	0.63
Median follow up, months (range)	35.0 (10–120)	36.9 (13–85)	0.83
Site			0.22
Abdominal wall	3 (15%)	4 (31%)	
Other trunk	5 (25%)	4 (31%)	
Extremities	10 (50%)	2 (23%)	
Neck	2 (10%)	3 (15%)	

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Table 2. Relationship between CTNNB1 mutation status with clinical and pathological characteristics, and efficacy of meloxicam.

	Mutation status				
Variables	**WT (n = 12)**	**T41A (n = 16)**	**S45F (n = 4)**	**S45P (n = 1)**	**P value**
Gender (Female/Male)	7/5	12/4	2/2	1/0	0.67
Mean age, year	45.0	40.7	38.5	26.0	0.57
Mean Size, mm	74.7	82.4	106.5	118.0	0.47
Site					0.23
Abdominal wall	4	1	1	1	
Other trunk	2	5	2	0	
Neck	1	3	1	0	
Extremities	5	7	0	0	
Efficacy of meloxicam					0.053
Favorable group (CR, PR, SD)	8	11	0	1	
Unfavorable group (PD)	4	5	4	0	
β-catenin nuclear positivity[a]					0.035
Moderate (2+)	5	11	0	0	
Strong (3+)	7	5	4	1	

WT; wild type, CR; complete response, PR; partial response, SD; stable disease, PD; progressive disease.
[a]No cases showed negative (0) or weak (1+) positive.
[b]S41F vs other type.
doi:10.1371/journal.pone.0096391.t002

There was a significant correlation between the nuclear positivity of β-catenin and CTNNB1 mutation status ($p = 0.035$), and all four cases with S45F mutation exhibited strong positive (3+) staining (Table 2).

Discussion

Dysregulation of the Wnt/β-catenin signaling pathway is a common molecular event in desmoid tumors [25]. The nuclear accumulation of β-catenin caused by CTNNB1 or APC mutation subsequently activates T-cell factor, which in turn causes transcription of target genes such as c-MYC and cyclin D [25], [26]. In practice, mutations of CTNNB1 exon3 gene are found in a wide variety of human cancers, and occur between codon 32 and 45, the site of phosphorylation by GSK3β or CK1α [15], [27]. Regarding desmoid tumors, β-catenin mutations are restricted to exon 3 of CTNNB1, and several larger studies have described a high frequency of mutations (64%–85%) in codons 41 and 45 [16–19], [28]. In this study, we used not only frozen specimens but also paraffin-embedded tissue extracts, as described in other reports [29], [30]. CTNNB1 point mutations were observed in 21 of the 33 cases (64%). The majority (76%) of them were T41A mutations, whereas the others were codon 45 (S45F and S45P). These results are consistent with the results of previous reports that showed T41A to be the predominant type of mutation [17], [31]. Thus these two specific mutations (T41A and S45F) may play crucial roles in the pathogenesis of desmoid tumors.

Several reports have focused on the correlation between CTNNB1 mutation type and local recurrence after surgery in desmoid tumors [17], [19], [28]. Colombo et al. reported that mutation status was the only poor prognostic factor predictive of local recurrence of desmoid tumors in an analysis of 179 surgical cases [28]. Lazar et al. showed that the five-year recurrence-free survival was significantly worse in 45F-mutated desmoids than either 41A-mutated or nonmutated ones [17]. In contrast, two other reports did not identify any association between mutation type and recurrence risk after surgery [19], [32]. Mullen et al. showed that 2-year recurrence rates in mutated tumors (64%) were slightly worse than those in wild-type (77%), although the difference failed to reach statistical significance [32].

In our study, all four cases with S45F mutation exhibited PD, indicating that S45F status is a significant poor prognosticator for meloxicam treatment. In tumors with other mutation status including T41A mutation and wild-type, the efficacy of meloxicam could not be predicted. Although several previous studies reported the predictive value of CTNNB1 mutation type for the outcome of surgical treatment, the current study analyzed for the first time the significance of CTNNB1 mutations in the prediction of efficacy for conservative treatment. Recently, several reports proposed treatment algorithms for patients with desmoid tumors, mainly based on conservative therapy including a "wait and see" policy [10], [33], although molecular determinants were not described in these studies. As the current study indicated, CTNNB1 mutation type may be a possible determinant for successful conservative treatment including meloxicam treatment and "wait and see" policy.

The nuclear expression of β-catenin has increasingly been used in the differential diagnosis of spindle cell neoplasms because of the

Table 3. S45F mutation status and efficacy of meloxicam.

	S45F (+)	S45F(−)
Favorable group (CR, PR, SD)	0	20
Unfavorable group (PD)	4	9

$P = 0.017$.
doi:10.1371/journal.pone.0096391.t003

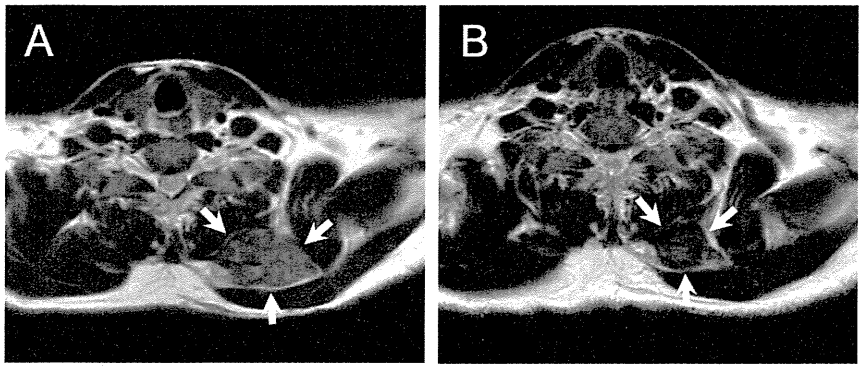

Figure 3. Forty-four year-old man with a desmoid tumor arising in the left posterior neck region. Evaluation for efficacy of meloxicam according to RECIST criteria was PR. This tumor had T41A mutation. (A) A T2-weighed axial MRI of desmoid (arrows) at the first visit. (B)A T2-weighed axial MRI of desmoid (arrows) at last follow-up.
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high positivity rate of desmoid tumors [13], [34], [35]. Results of these previous reports are consistent with those of our study, showing that all cases showed equal or higher than moderate (2+) positive staining of nuclear β-catenin expression. Several previous studies reported the significance of nuclear expression of β-catenin in surgically treated patients with desmoid tumors [17], [36]. However, the results of these studies are controversial, probably because immunohistochemical assessment may depend on the various parameters such as the antibody, technique and/or observation methods selected. The staining intensity may vary at different times, and be heterogeneous in different areas of the same tumors. Our previous study for the first time demonstrated that nuclear expression of β-catenin is a significant prognosticator for conservative treatment with meloxicam [23]. The current study showed a positive correlation between nuclear β-catenin staining and S45F mutation. In contrast, Lazar et al. reported that cases with CTNNB1 S45F mutations exhibited a less intense β-catenin staining compared to those with T41A mutations [17]. Taking these findings together, although immunohistochemical evaluation serves as a useful reference to predict the outcome of surgery or conservative treatment for patients with desmoid tumors, more definitive prognosticators, including mutation type as shown in the current study, are also required.

The limitations of this study include the fact that the sample size was relatively small compared to previous mutation studies with larger numbers of cases. However, the cohort of this study is composed of prospectively treated patients with meloxicam, while no previous studies have reported the significance of CTNNB1 mutation type in conservative treatment. The results derived from this study provide meaningful information. Second, CTNNB1 mutation was analyzed only in exon 3. Other significant mutations may exist in different areas of CTNNB1. Third, due to the small number of cases, we could not analyze the relationship between mutation status and period up to onset of efficacy for meloxicam treatment. Future studies with more accumulated cases with molecular analysis will be needed to clarify the further correlation between the prognosis and mutation status.

In conclusion, we demonstrate for the first time that S45F mutation of CTNNB1 may serve as a prognostic marker in patients with sporadic desmoid tumors treated with conservative treatment with meloxicam. Given the current shift in the treatment modality for patients with desmoid to conservative treatment, the identification of prognosticators for conservative treatment is more important than ever. Mutation status of CTNNB1 may be a promising tool to predict the efficacy of conservative treatment including meloxicam.

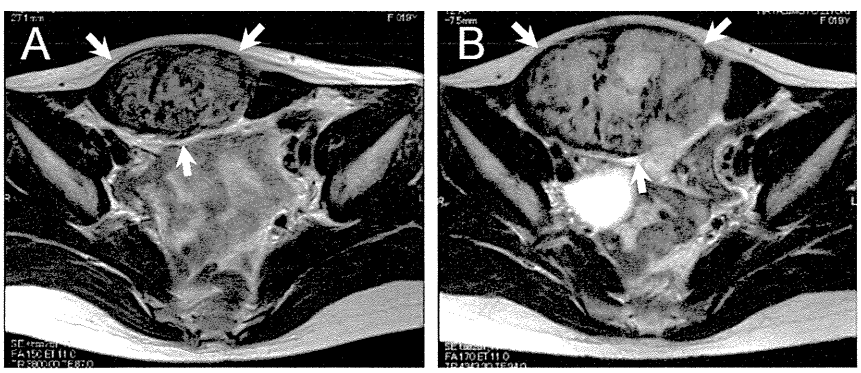

Figure 2. Twenty year-old woman with a desmoid tumor arising in the abdominal wall. Evaluation for efficacy of meloxicam according to RECIST criteria was PD. This tumor had S45F mutation. (A) A T2-weighed axial MRI of desmoid (arrows) at the first visit. (B) A T2-weighed axial MRI of desmoid (arrows) at the end of meloxicam treatment.
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Author Contributions

Conceived and designed the experiments: YN SH HU NI. Performed the experiments: SH NF KI EK. Analyzed the data: SH YN EK NI. Contributed reagents/materials/analysis tools: SH NF KI HU. Wrote the paper: SH YN NI.

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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

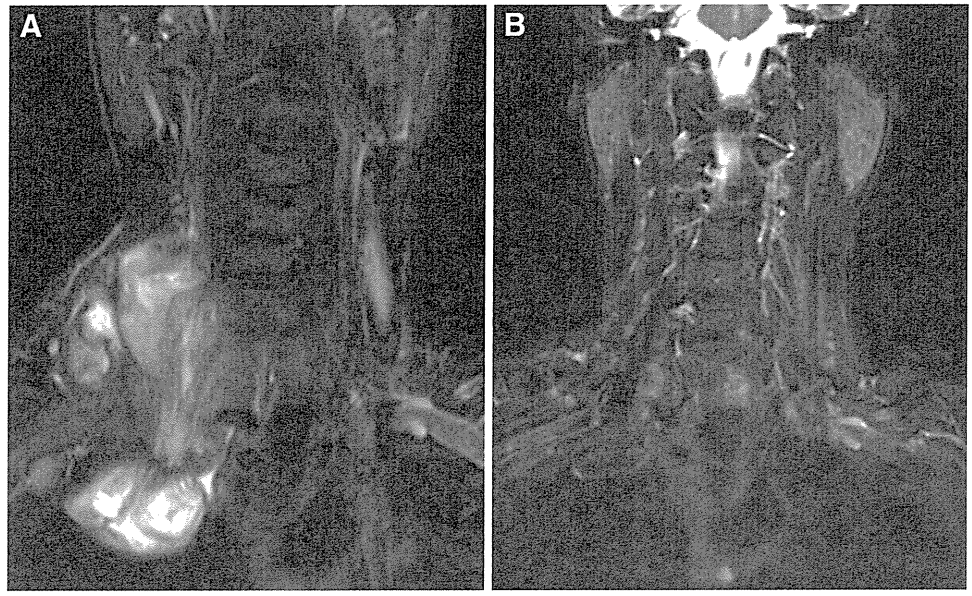

Fig. 1a–b Case 10. A 74 year-old male patient had a tumor in the neck and chest cavity (**a**). Two years' treatment with MTX and VBL reduced the tumor size markedly (**b**)

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

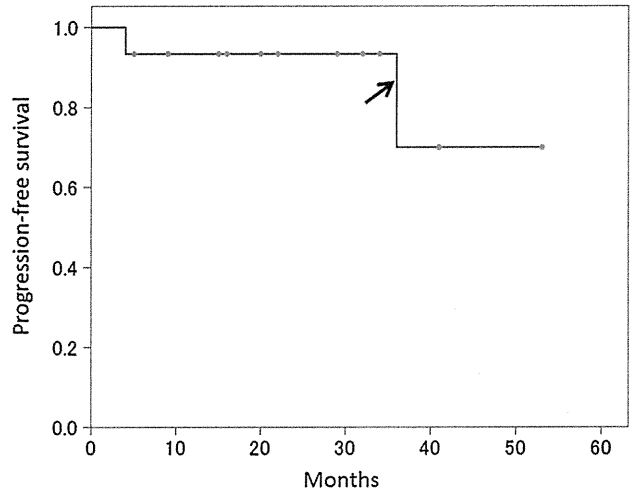

Fig. 2 Progression-free survival after treatment with MTX and VBL. In case 2 with the 45F mutation, the tumor size was reduced by MTX and VBL therapy and treatment was discontinued 28 months after the initiation of therapy. The tumor then regrew 7 months after treatment cessation (*arrow* depicts the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