Table 1. Chemotherapeutic options and supporting published data (phase III study) in advanced gastric cancer

Regimen	No. of patients	ORR (%)	MST (months)	Reference
FAMTX	105	41	10.5	Wils et al. 1991 [34]
FAM	103	9	7.3	
EAP	30	20	6.1	Kelsen et al. 1992 [35]
FAMTX	30	33	7.3	
FP	103	51	9.2	Kim et al. 1993 [36]
FAM	98	25	7.3	
5-FU	94	26	7.7	
FAMTX	130	21	5.7	Webb et al. 1997 [37]
ECF	126	45	8.9	
FAMTX	133	12	6.7	Vanhoefer et al. 2000 [38]
ELF	132	9	7.2	
FP	134	20	7.2	
MCF	285	44	8.7	Ross et al. 2002 [39]
ECF	289	42	9.4	
FAMTX	98	23	6.9	Cocconi et al. 2003 [40]
PELF	97	51	7.7	
DCF	221	37	9.2	Van Cutsem et al. 2006 [9], Ajani 2007 [10]
CF	224	25	8.6	
FLO	112	34	10.7	Al-Batran et al. 2008 [41]
FLP	106	25	8.8	
ECF	263	41	9.9	Cunningham et al. 2008 [42]
ECX	250	46	9.0	
EOF	245	42	9.3	
EOX	144	48	11.2	
S-1	234	28	11.4	Boku et al. 2008.[43]
5-FU	234	9	12.3	
IC	236	38	10.8	
CS	148	54	13.0	Koizumi et al. 2008 [11]
S-1	150	31	11.4	
XP	139	41	10.5	Kang et al. 2009 [44]
FP	137	29	9.3	

ORR, overall response rate; MST, median overall survival time; FAMTX, 5-FU, doxorubicin, high-dose methotrexate; FAM, 5-FU, doxorubicin, mitomycin; EAP, etoposide, doxorubicin, CDDP; FP, 5-FU, CDDP; 5-FU, 5-FU monotherapy; ECF, epirubicin, CDDP, 5-FU; ELF, etoposide, leucovorin, 5-FU; FLP, 5-FU, leucovorin, CDDP; FLO, 5-FU, leucovorin, oxaliplatin; FP, infusional 5-FU, CDDP; PELF, CDDP, epirubicin, leucovorin, 5-FU; MCF, mitomycin, CDDP, 5-FU; DCF, docetaxel, CDDP, 5-FU days 1–5 continuous infusion; CF, CDDP, 5-FU days 1–5 continuous infusion; ECX, epirubicin, CDDP, capecitabine; EOF, epirubicin, oxaliplatin, 5-FU; EOX, epirubicin, oxaliplatin, capecitabine; S-1, S-1 monotherapy; IC, CPT-11, CDDP; CS, CDDP, S-1; XP, capecitabine, CDDP

nia/neutropenic infection (63% vs 27%), diarrhea (19% vs 8%) and neuropathy (17% vs 6%).

The two docetaxel-based regimens, DCF and DC, were evaluated and compared with ECF in a randomized phase II trial [46]. In that study, chemotherapy-naive patients with unresectable and/or metastatic gastric cancer received ECF (epirubicin 50 mg/m^2 on day 1, CDDP 60 mg/m^2 on day 1, and 5-FU 200 mg/m^2 per day on days 1–21), DC (docetaxel initially 85 mg/m^2 on day 1; later reduced to 75 mg/m^2 as a result of toxicity, and CDDP 75 mg/m^2 on day 1), or mDCF (DC plus 5-FU 300 mg/m^2 per day on days 1–14). Among 119 evaluable patients, ORR was 25% for ECF, 18.5% for DC, and 36.6% for DCF. mDCF was superior to ECF and/or DC also in terms of median TTP (4.6, 4.9, and 3.6 months, respectively), and OS (10.4, 11.0, and 8.3 months), even though the initial dose of docetaxel of 85 mg/m^2 was reduced to 75 mg/m^2 due to a high rate of febrile neutropenia in the mDCF and DC arms, and grade III/IV neutropenia occurred in more treatment cycles with docetaxel (DC, 49%; mDCF, 57%; ECF, 34%). A trend was observed towards increased myelosuppression and infectious complications with mDCF vs DC or ECF. mDCF was more active than ECF, but more toxic. A considerably higher rate of complicated neutropenia and substantially improved quality of life (QOL) with the DCF regimen suggested proper patient selection and management for DCF.

Although the increased toxicity of DCF was controllable and was suggested not to negatively affect QOL or clinical benefit, the regimen was highly toxic. Optimizing studies of the original regimen, in terms of both efficacy and safety, are now ongoing [4, 12, 47] (Table 2). Indeed, in the above-mentioned phase II trials by Roth et al. [46], the dose reduction of docetaxel from 85 to 75 mg/m^2 decreased febrile neutropenia for both docetaxel-containing regimens (mDCF, 28%–12%; DC, 15%–4%) without significant decrease of the activity. In a phase II trial performed by Lorenzen et al. [48], which treated patients with docetaxel (50 mg/m^2) and

CDDP (50 mg/m^2) on days 1, 15, and 29, and 5-FU (2000 mg/m^2) and leucovorin (400 mg/m^2) weekly, in 8-weekly cycles, secondary dose reduction of both docetaxel and CDDP to 40 mg/m^2 was required to decrease the rates of grade III or IV neutropenia, febrile neutropenia, and thrombocytopenia. The T-PLF (docetaxel/CDDP/leucovorin/FU) phase II study provided an ORR of 47%, TTP of 9.4 months, and OS of 17.9 months. Park et al. [49] developed an mDCF regimen with a lower dose of docetaxel (50 mg/m^2) than the regimen of Lorenzen et al. [48]. In the Park et al. [49] regimen, docetaxel was given on day 1 in combination with CDDP (80 mg/m^2) and 5-FU (1200 mg/m^2, days 1–3), and repeated every 3 weeks. The ORR in 47 chemotherapy-naïve patients with metastatic gastric cancer was 40%, while TTP was 4.6 months and OS was 9.7 months. Rates of grade III or IV neutropenia (68%) and febrile neutropenia/neutropenic infection (26%) were less common than those in previously reported high-dose DCF regimens.

Combinations with "new-generation agents"

DCF was proven to be active, but substantial toxicity was observed, which prompted investigators to explore alternative docetaxel-containing regimens for gastric cancer (Table 2). Much attention has been focused on new-generation agents as putative partners of docetaxel.

Combinations with new oral fluoropyrimidines. A docetaxel/fluoropyrimidine combination is a very potent modality for gastric cancer treatment. In a phase II study in patients with advanced gastric cancer without prior chemotherapy, docetaxel combined with a continuous infusion of 5-FU (DF) showed potent efficacy compared with ECF; among patients treated with DF and ECF, respectively, the ORRs were 37.8% and 35.6%, median TTPs were 5.5 and 5.3 months, and median survivals were 9.5 and 9.7 months [15]. Even so, the response of DF is still limited. Instead of 5-FU, new oral 5-FU analogues and prodrugs such as S-1 and capecitabine are becoming key agents in gastric cancer chemotherapy. These agents are very attractive alternative agents for combination with docetaxel [16, 17].

Recent phase III trials have focused on the use of oral 5-FU, especially with S-1, and have demonstrated pivotal activities: a three arm phase III study (Japan Clinical Oncology Group [JCOG] 9912) showed that S-1 monotherapy seemed to be superior to 5-FU and comparable with a CPT-11/CDDP combination, in that there was a significantly lower incidence of grade 3, 4 toxicity than that seen with CPT-11/CDDP [43]. A S-1 plus cisplatin vs S-1 alone for the first line treatment of advanced gastric cancer (SPIRITS) trial, comparing S-1 monotherapy with an S-1/CDDP combination, further demonstrated that the S-1/CDDP combination significantly improved OS (11 vs 13 months; $P = 0.0366$), and progression-free survival (PFS; 4 vs 6 months; $P < 0.0001$) at a median follow up of 34.6 months [11]. On the basis of these findings, treatment with combined S-1 plus CDDP has become a standard treatment option in Japan. Capecitabine has also been shown to be effective in the treatment of advanced esophagogastric cancer in a phase III study comparing capecitabine with fluorouracil in combination with epirubicin/oxaliplatin or epirubicin/CDDP [42]. The randomized ECF for locally advanced esophago-gastric cancer-2 (REAL-2) trial, comparing ECF, EOF (epirubicin/oxaliplatin/5-FU), ECX (epirubicin/CDDP/capecitabine), and EOX (epirubicin/oxaliplatin/capecitabine) showed that the efficacy of capecitabine was equivalent to that of 5-FU, and that EOX significantly improved survival time compared with ECF. An MST of 11.2 months in the EOX regimen was among the longest achieved in this patient setting. Although both of these trials (i.e., the trial carried out by Cunningham et al. [42] and the REAL-2 trial) were designed to assess whether capecitabine was no worse than 5-FU, the findings generally suggested better outcome in patients who received oral capecitabine.

In addition to the observed clinical benefit of S-1 and capecitabine, in an experimental model, Wada et al. [50] suggested that docetaxel could enhance fluoropyrimidine activity through the modification of the intracellular metabolic enzymes—thymidylate synthase (TS), dihydropyrimidine dehydrogenase (DPD), and orotate phosphoribosyl transferase (OPRT)—which are related to fluoropyrimidine resistance, the actions of these agents thereby being synergistically cytotoxic in human gastrointestinal cancer cells. New oral fluoropyrimidines might be the best partners of docetaxel.

Indeed, a combination of docetaxel and S-1 (docetaxel 40 mg/m^2 day 1 and oral S-1 80 mg/m^2 per day on days 1–14 every 3 weeks) was shown to be highly active in advanced and recurrent gastric cancer, and had an acceptable and manageable toxicity profile in a phase II study [16]. The combination achieved promising results for OR rate (56.2%; 95% CI, 38%–66%), median TTP (7.3 months; 95% CI, 4.3–10.0 months), and median OS (14.3 months; 95% CI, 10.7–20.3 months). Nonhematologic toxicities were generally mild and none was greater than grade 3. Stomatitis, the most common grade 3 nonhematologic toxicity, was observed in just 8.3% of patients. The predominant toxicity was myelosuppression, and grade 3–4 neutropenia occurred in 58.3% of patients. However, both the hematologic and nonhematologic toxicities were generally manageable and, in most cases, treatment could be continued in the outpatient setting. The results reported are consistent with

Table 2. Docetaxel combinations with cytotoxic agents and supporting published data (phase II studies) in advanced gastric cancer

Regimen	Dose and schedule	No. of patients	ORR (%)	MST (months)	Reference
Triplet and quartet regimens					
mDCF	Docetaxel 50 mg/m^2 and cisplatin 80 mg/m^2 on day 1, 5-FU, 1200 mg/m^2 per day on days 1–3 (3-weekly cycle)	47	40	9.7	Park et al. 2005 [49]
mDCF	Docetaxel 85→75 mg/m^2 and cisplatin 75 mg/m^2 on day 1, 5-FU 300 mg/m^2 per day on days 1–14 (3-weekly cycle)	61	37	10.4	Roth et al. 2007 (randomized phase II trial) [46]
DC	Docetaxel 85→75 mg/m^2 and cisplatin 75 mg/m^2 on day 1 (3-weekly cycle)	58	18	8.3	
ECF	Epirubicin 50 mg/m^2 and cisplatin 60 mg/m^2 on day 1, 5-FU 200 mg/m^2 per day on days 1–21 (3-weekly cycle)	59	25	11.0	
mDCF (T-PLF)	Docetaxel 50→40 mg/m^2 and cisplatin 50→40 mg/m^2 on days 1, 15, 29, 5-FU 2000 mg/m^2/leucovorin 400 mg/m^2 (8-weekly cycle)	60	47	17.9	Lorenzen et al. 2007 [48]
FLOT	Oxaliplatin 85 mg/m^2, leucovorin 200 mg/m^2, and fluorouracil 2600 mg/m^2 as a 24-h infusion in combination with docetaxel 50 mg/m^2 on day 1 (2-weekly cycle)	59	51	11.3	Al-Batran et al. 2008 [59]
EDOCOX	Epirubicin 50 mg/m^2, docetaxel 60 mg/m^2 followed by oxaliplatin 100 mg/m^2 on day 1 (3-weekly cycle)	40	48	12.1	Di Lauro et al. 2009 [58]
TPC	Docetaxel 30 mg/m^2, cisplatin 25 mg/m^2, and irinotecan 65→50 mg/m^2 (3-weekly cycle)	56	54	11.9	Enzinger et al. 2009 [63]
Doublet regimens					
DI	Irinotecan 160→120 mg/m^2 followed by docetaxel 65→50 mg/m^2 (3-weekly cycle)	132	20	8.9	Sym et al. 2008 [62]
	Docetaxel 30 mg/m^2 and irinotecan 70 mg/m^2 on days 1 and 8 (3-weekly cycle)	46	46	8.2	Park et al. 2006 [61]
DC	Docetaxel 85 mg/m^2 and cisplatin 75 mg/m^2 on day 1 (3-weekly cycle)	76	26	10.5	Ajani et al. 2005 (randomized phase II trial) [45]
DCF	Docetaxel 75 mg/m^2, cisplatin 75 mg/m^2 on day 1, and fluorouracil 750 mg/m^2 per day as continuous infusion on days 1–5 (3-weekly cycle)	79	43	9.6	
DF	Docetaxel 75 mg/m^2 day 1, and fluorouracil 200 mg/m^2 days 1–21 (3-weekly cycle)	45	38	9.5	Thuss-Patience et al. 2005 (randomized phase II trial) [15]
ECF	Epirubicin 50 mg/m^2 day 1, cisplatin 60 mg/m^2 day 1, and fluorouracil 200 mg/m^2 days 1–21 (3-weekly cycle)	45	36	9.7	
DX	Docetaxel 36 mg/m^2 on days 1 and 8, and capecitabine 1000 mg/m^2 twice a day on days 1–14 (3-weekly cycle)	47	40	12.0	Chun et al. 2005 [17]
DS	Docetaxel 40 mg/m^2 day 1 and oral S-1 80 mg/m^2 per day on days 1–14 (3-weekly cycle)	48	56	14.3	Yoshida et al. 2006 [16]
		46	46	14.0	Yamaguchi et al. 2006 [51]
DOCOX	Docetaxel 60 mg/m^2 followed by oxaliplatin 130 mg/m^2 on day 1 (3-weekly cycle)	71	36	8.5	Richards et al. 2008 [55]
	Docetaxel 65 mg/m^2 and oxaliplatin 120 mg/m^2 on day 1 (3-weekly cycle)	39	45	9.9	Kim et al. 2008 [56]

ORR, overall response rate; MST, median overall survival time

reported analyses from a phase II study of a similar regimen for patients with advanced gastric cancer [51].

Capecitabine plus weekly docetaxel (docetaxel 36 mg/m^2 on days 1 and 8 and capecitabine 1000 mg/m^2 twice a day on days 1–14 every 3 weeks) achieved a 40.4% response rate and a median TTP of 4.5 months in a phase II study in patients with metastatic gastric cancer [17]. The median OS time of 12 months was prolonged compared with the survival times reported for DF [15] and was similar to those reported for the other new-generation combinations [52].

Although the phase III First-Line Advanced Gastric Cancer (FLAGS) trial, designed to compare CF with S-1/CDDP, failed to show an advantage of S-1 in OS

compared with 5-FU in Western populations [53], the observed activity and tolerability of docetaxel with S-1 or capecitabine, together with the convenience of oral S-1 dosing, make these highly promising new regimens with the potential to improve survival in patients with advanced or recurrent gastric cancer. Several phase III studies to evaluate these regimens, such as the Japan-Korea Cooperative Study of Docetaxel/S-1 Versus S-1 in Advanced Gastric Cancer (START) are now ongoing; in 2010 the final results of the START study will be reported [54]. The future role of S-1 in gastric cancer could also be the inclusion of this oral drug in a three-drug regimen, making DCF or ECF better tolerated.

Combinations with oxaliplatin. A variety of clinical studies have suggested that both the activity and the toxicity of classical platinum-based chemotherapy in gastric cancer may be improved by the substitution of CDDP by oxaliplatin; thus, oxaliplatin would be another potent partner with docetaxel [2, 3, 6–8, 55].

Kim et al. [56] conducted a phase II study to evaluate the efficacy and safety of a combination regimen of docetaxel/oxaliplatin (docetaxel 65 mg/m^2 and oxaliplatin 120 mg/m^2 on day 1 every 3 weeks). The combination achieved promising results for ORR (45.2%; 95% CI, 32%–59%), median TTP (5.7 months; 95% CI, 4.3–7.2 months), and median OS (9.9 months; 95% CI, 7.8–12.0 months). Among 47 assessable cases, grade 3/4 neutropenia occurred in 11 patients (23.4%) and febrile neutropenia was observed in 7 patients (14.9%). Another docetaxel/oxaliplatin combination regimen (docetaxel 60 mg/m^2 followed by oxaliplatin 130 mg/m^2 on day 1 every 3 weeks) was also investigated as a second-line treatment after failure of fluoropyrimidine and platinum [57]. The docetaxel/oxaliplatin (DOCOX) regimen in 48 pretreated Chinese patients (46 assessable) demonstrated an ORR of 22.9% (95% CI, 0.9%–34.9%), median TTP of 4.4 months (95% CI, 3.4–5.4 months), and median OS of 7.2 months (95% CI, 6.6–12.1 months). Grade III/IV neutropenia was observed in 26% of the patients, and grade 3 thrombocytopenia and febrile neutropenia occurred in 4.3% and 6.5% of the patients. Docetaxel and oxaliplatin have modest activity with predictable hematologic toxicity when given as salvage therapy.

A triplet regimen, epirubicin/oxaliplatin/docetaxel, in metastatic gastric or gastroesophageal junction (GEJ) adenocarcinoma was also evaluated in a phase II study [58]. The regimen (epirubicin 50 mg/m^2, docetaxel 60 mg/m^2 followed by oxaliplatin 100 mg/m^2 on day 1 every 3 weeks) achieved an ORR of 47.5% (95% CI, 32%-63%), median TTP of 6.3 months (95% CI, 5.4–7.2 months), and median OS of 12.1 months (95% CI, 10.7–13.5 months). Grade 3/4 neutropenia occurred in 50% of the patients, with two episodes of febrile neutropenia (5%). Nonhematologic grade 3 toxicities included sensory neuropathy (5%), vomiting and mucositis (5%), and diarrhea in one patient (2.5%). This combination is also active and well tolerated in patients with metastatic gastric or GEJ adenocarcinoma.

Al-Batran et al. [59] investigated the FLOT regimen, which incorporated docetaxel into a tolerable biweekly oxaliplatin-based chemotherapy regimen (oxaliplatin 85 mg/m^2, leucovorin 200 mg/m^2, and fluorouracil 2600 mg/m^2 as a 24-h infusion in combination with docetaxel 50 mg/m^2 on day 1 every 2 weeks). The phase II study demonstrated that the FLOT regimen had an acceptable toxicity profile, while response rates and median survivals of the patients were in the range of those reported with DCF. The study included 59 chemotherapy-naïve patients with metastatic gastric cancer, and provided an ORR of 50.9%, median PFS of 5.2 months, and an OS of 11.1 months. Frequent (>10%) grade 3 or 4 toxic effects in 54 assessable cases included neutropenia in 26 (48.1%), leukopenia in 15 (27.8%), diarrhea in 8 (14.8%), and fatigue in 6 (11.1%) patients, and complicated neutropenia was observed in 2 (3.8%) patients, only.

In addition to the above-mentioned regimens, a variety of possible oxaliplatin-containing regimens such as D-FOX (docetaxel/5-FU/oxaliplatin) are now being intensively investigated [12, 60].

Combinations with irinotecan (CPT-11). Irinotecan (CPT-11) is also active in gastric cancer, showing an ORR of 14%–25% as a single agent, which suggests the topoisomerase I inhibitor to be a possible partner of docetaxel [12, 60]. A doublet regimen, docetaxel/CPT-11, has been investigated by several study groups, but the observed clinical benefit is unlikely to be promising: the regimen had modest activity and was highly toxic. Park et al. [61] evaluated the efficacy and safety of a docetaxel/CPT-11 regimen (docetaxel 30 mg/m^2 and CPT-11 70 mg/m^2 on days 1 and 8 every 3 weeks) in chemo-naive patients with metastatic gastric cancer (48 enrolled and 46 assessable). This study achieved an ORR of 45.7% (95% CI, 31.3%-60.1%), median TTP of, 4.5 months (95% CI, 3.8–5.2 months), and median OS of 8.2 months (95% CI, 5.8–10.6 months). Grade 3/4 neutropenia developed in 57.4% of the patients, and febrile neutropenia/neutropenic infection in 19.1%. Nonhematologic toxicities were moderate; grade 3/4 diarrhea occurred in 19.1% of the patients; however, the toxicity was manageable with dose reduction. Sym et al. [62] studied a doublet regimen in a salvage treatment setting with different doses and schedules (CPT-11 160 mg/m^2 followed by docetaxel 65 mg/m^2 every 3 weeks). The doses of both CPT-11 and docetaxel were reduced to 120 mg/m^2 and 50 mg/m^2 due to unacceptable toxicity in the first ten patients. ORR, median TTP,

and median OS were 20.4% (95% CI, 9.1%–31.7%), 2.7 months (range, 2.1–69.1 months), and 8.9 months (95% CI, 6.6–11.3 months), respectively. Grade 3/4 toxicities included neutropenia (90%), febrile neutropenia (50%), asthenia (40%), and diarrhea (10%) with the higher dose and neutropenia (71%), febrile neutropenia (11%), diarrhea (24%), and asthenia (24%) with the lower dose. There were two possible treatment-related deaths.

Recently, weekly docetaxel, CDDP, and CPT-11 (TPC) was studied in patients with metastatic esophagogastric cancer by Enzinger et al. [63]. Based on the results in the phase I study, docetaxel 30 mg/m^2, CDDP 25 mg/m^2, and CPT-11 65 mg/m^2 were selected for the phase II trial, but the dose of CPT-11 was reduced to 50 mg/m^2 due to severe diarrhea. The phase II trial enrolled 56 patients with previously untreated, metastatic esophagogastric cancer, and demonstrated an ORR of 54%, median PFS of, 7.1 months, and median survival of 11.9 months. At the final CPT-11 dose of 50 mg/m^2, grade 3 or higher toxicity included diarrhea (26%), neutropenia (21%), nausea (18%), fatigue (16%), anorexia (13%), and thrombosis/embolism (13%).

Combinations with biological agents. The incorporation of biological agents, such as cetuximab, bevacizumab, everolimus, and sunitinib, into combination regimens is another innovative approach [2–8, 64]. The optimum combination of these agents is now being intensively investigated, and high response and/or disease control rates have been reported, especially for combinations with epidermal growth factor receptor (EGFR)-targeted cetuximab, such as FOLCETUX (cetuximab alone following CPT-11/5-FU/leucovorin (FOLFIRI) regimen for a maximum of 24 weeks) [65, 66] and combinations with VEGF-targeted bevacizumab [67]. Various randomized phase III studies incorporating targeted agents, such as ToGA (Trastuzumab with Chemotherapy in HER2-Positive Advanced Gastric Cancer) and AVAGAST (Avastin in Gastric Cancer) in first-line regimens have recently been initiated [6, 64]. Combinations of biological agents with docetaxel or docetaxel-containing combinations have also been intensively studied. Among the biological agents, the monoclonal antibodies cetuximab and bevacizumab are the most investigated agents, despite there being sparse published data at present.

Although a study to evaluate a combination of docetaxel with cetuximab (400 mg/m^2 initial dose followed by 250 mg/m^2 weekly for maintenance) with docetaxel (30 mg/m^2 on days 1 and 8 every 3 weeks) in a salvage treatment setting suggested it had limited clinical benefit in terms of ORR, PFS, and OS [68], the incorporation of cetuximab (400 mg/m^2 initial dose followed by 250 mg/m^2 weekly for maintenance) into a doublet docetaxel regimen (DOCETUX; docetaxel 75 mg/m^2 and CDDP 75 mg/m^2 every 3 weeks) showed hopeful results, with an ORR of 40.5% (one complete response) in the front-line setting [69]. The most common grade III/IV toxicities were neutropenia (45.8%), febrile neutropenia (22.9%), and anemia (6.25%); toxicities included fatigue (22.9%), hyponatremia (20%), hypokalemia (16%), skin reaction (31.3%), vomiting (8.3%), and stomatitis (6.3%). Based on these encouraging results, further phase II trials of first-line therapy with cetuximab in combination with capecitabine and CDDP or docetaxel and oxaliplatin are ongoing [12, 64].

For bevacizumab, Enzinger et al. [70] evaluated a combination regimen with docetaxel (bevacizumab 5 mg/kg days 1 and 15, docetaxel 35 mg/m^2 days 1, 8, and 15), and, further, a more aggressive combination (docetaxel 30 mg/m^2, CDDP 25 mg/m^2, CPT-11 50 mg/m^2 on days 1 and 8, bevacizumab 10 mg/kg on day 1, every 3 weeks) in advanced gastroesophageal cancer [12, 64, 70]. Despite a low ORR of 27% in the former regimen, the latter demonstrated a high ORR of 63% in the chemotherapy-naïve patient population, with well-tolerated toxicity.

A modified DCF regimen with bevacizumab (docetaxel 40 mg/m^2, 5-FU bolus 400 mg/m^2, leucovorin 400 mg/m^2, and infusional 5-FU 1000 mg/m^2 ×2 days, and bevacizumab 10 mg/kg day 1, CDDP 40 mg/m^2 on day 3) was also evaluated [12, 64]. The study achieved a significantly higher ORR of 71%, compared with that for DCF, with markedly less toxicity than DCF. However, with the bevacizumab combination, patients should be carefully monitored for gastrointestinal perforation. El-Rayes et al. [71] investigated a combination of docetaxel/oxaliplatin with bevacizumab (docetaxel 70 mg/m^2, oxaliplatin 75 mg/m^2, and bevacizumab 7.5 mg/kg every 3 weeks), and reported that this docetaxel/oxaliplatin/bevacizumab combination resulted in gastrointestinal perforation in two patients after cycle 2.

Future perspectives

As well as the other new-generation (third-generation) agents, docetaxel is now of key importance in the development of new-era systemic chemotherapy. Actually, the incorporation of docetaxel into gastric cancer chemotherapy has yielded a pivotal regimen, DCF, and a variety of regimens that could potentially become standard treatments in gastric cancer. Even so, the DCF regimen has substantial toxicity: optimization studies and the development of alternative docetaxel-containing regimens are now intensively ongoing. The

best partner for docetaxel in the treatment of gastric cancer remains unknown.

In Japan, S-1 is the most widely used drug for the treatment of gastric cancer, based on the results of the Adjuvant Chemotherapy Trial of TS-1 for Gastric Cancer (ACTS-GC) study and a SPIRITS trial [11, 78]. Although differences between Asian and Western patients, such as the relatively low involvement of GE junction tumors in Asian populations and differences in S1 pharmacokinetics caused by CYP2C6 polymorphic differences are suggested, new-generation chemotherapy including S-1 is undoubtedly one of the most promising candidates as a standard treatment for advanced gastric cancer. As a partner of docetaxel, S-1 appears to be more suitable than other third-generation and biological agents. In fact, the reported activity and tolerability of several docetaxel/S-1 regimens make these combinations highly promising ones, with the potential to improve survival in patients with advanced or recurrent gastric cancer. The evaluation of docetaxel/S-1 regimens in large-scale and well-designed randomized phase III clinical trials in various settings, including the perioperative adjuvant setting, is eagerly awaited.

Along with extensive efforts directed toward developing more active docetaxel combination regimens, much attention has been focused on predictive biomarkers of the regimens [72, 73]. Pharmacogenomics is increasingly being recognized as an effective way to optimize therapy and the treatment dose for individuals, and the FDA has validated possible biomarkers and is using corresponding FDA-approved drug labels with three recommendation levels of testing: "required", "recommended", and "information only" (http://www.fda.gov/cder/genomics/genomic biomarkers_table.htm) [4]. At present, however, there is no FDA-approved biomarker for the agents commonly used in gastric cancer chemotherapy, with the exception of C-KIT expression for imatinib mesylate in gastrointestinal stromal tumors. Even so, advances in pharmacogenomics in gastric cancer have provided a number of putative candidate markers for the prediction of tumor response to chemotherapies, including docetaxel and docetaxel-containing combination regimens [4, 72–77]. We look forward to obtaining more data from ongoing trials, and we believe that future large trials will provide the best chemotherapy and predictive biomarkers for indicating individual toxicity risks and therapeutic benefits in gastric cancer patients.

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Fluctuating liver functions in siblings with MPV17 mutations and possible improvement associated with dietary and pharmaceutical treatments targeting respiratory chain complex II

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ABSTRACT

Background/aims: To describe the clinical and biological findings of two Japanese siblings with novel MPV17 gene mutations (c.451insC/c.509C > T) manifesting hepatic mitochondrial DNA depletion syndrome.
Methods: We observed these brothers and sought to determine the efficacy of treatment targeting respiratory chain complex II for the younger brother.
Results: A 3-month-old boy had presented with profound liver dysfunction, failure to thrive, and watery diarrhea. Although he was then placed on a carbohydrate-rich diet, his liver function thereafter fluctuated greatly in association with viral infections, and rapidly deteriorated to liver failure. He underwent liver transplantation at 17 months of age but died at 22 months of age. The younger brother, aged 47 months at the time of this writing, presented with liver dysfunction from 8 months of age. His transaminase levels also fluctuated considerably fluctuations in association with viral infections. At 31 months of age, treatment with succinate and ubiquinone was initiated together with a lipid-rich diet using ketone milk. Thereafter, his transaminase levels normalized and never fluctuated, and the liver histology improved.
Conclusions: These cases suggested that the clinical courses of patients with MPV17 mutations are greatly influenced by viral infections and that dietary and pharmaceutical treatments targeting the mitochondrial respiratory chain complex II may be beneficial in the clinical management of MPV17 mutant patients.

Introduction

MPV17 is a mitochondrial gene encoded by a nuclear gene [1]. Its mutations cause mitochondrial DNA depletion syndrome (MDS)[2], presenting multiple mitochondrial respiratory chain depletions in the manner of autosomal recessive inheritance [1–5]. Recently, the occurrence of patients with MPV mutations has been increasing; the majority of patients have developed liver disease within a few months after birth, with rapid deterioration to liver failure, while the remaining patients have shown relatively slow progression of liver disease or neurological regression [1–5].

However, the number of patients with MPV17 mutations is still small, and the clinical courses according to the mutations or the genotype- phenotype correlation remain unclear. Further, the appropriate internal therapy has yet to be established, although Parini and colleagues recently reported that glucose administration to avoid hypoglycemia is efficient in slowing the progression of liver disease [5].

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[2] *Abbreviations used:* MDS, mitochondrial DNA depletion syndrome; RC, respiratory chain; Co I, complex I; Co II, complex II; Co III, complex III; Co IV, complex IV; CS, citrate synthase.

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In this report, we present the clinical courses of two siblings with novel mutations, c.451insC and c.509C > T, whose liver functions greatly fluctuated according to their respective viral infections. The beneficial effect of treatment targeting mitochondrial respiratory chain (RC) complex (Co) II, including succinate and coenzyme Q, is also described.

Patients and methods

Cases

Case 1: A Japanese boy born as the second child to unrelated healthy parents. Their first son is healthy. The second son, on the other hand, was born without any complications at 37 weeks of gestation age, and weighed 3060 g. At 3 months of age, he was referred to our hospital to receive precise examinations for failure to thrive, hypotonia, mild jaundice, and creamy stools. He had moderate head lag and incomplete head control. He maintained good eye contact and had a sociable smile; he had no seizures or clinical signs of peripheral neuropathy except for moderate hypotonia. Findings of brain computed tomography were normal. The liver was soft and palpated at 4.5 cm below the costal margin with no splenomegaly. Laboratory tests then showed elevated levels of serum bilirubin, total bile acid (TBA), transaminases, and gamma-glutamyl transpeptidase (GGT) as well as prolonged coagulation time (total bilirubin 4.2 mg/dl; direct bilirubin 2.7 mg/dl; TBA 362 μmol/L; GGT 178 IU/L; AST 173 IU/L; ALT 58 IU/L; hepaplastin time 38%). He had no episode of hypoglycemia (his blood glucose level was 65 mg/dl). Simultaneously, low body weight and height were prominent (body weight 4610 g, −2.7 SD; height, 59 cm, −1.8 SD). His plasma amino acid profile and urinary organic acid profile did not suggest any etiology for liver disease, but viral examinations detected an IgM cytomegalovirus (CMV)-specific antibody in the plasma. His liver functions thereafter are shown in Fig. 1 (upper panel).

A liver biopsy specimen obtained at 4 months of age showed moderate inflammatory cell infiltration with destroyed limiting plates and fibrosis in the portal tracts (Fig. S1A1). Two different types of degenerated hepatocytes were found: swollen hepatocytes containing lipid droplets of various sizes with occasional formation of multinuclear giant cells, and small, concentrated acidophilic hepatocytes. Bile plugs were noticed in the cytoplasm of hepatocytes and dilated canaliculi, consistent with the findings of cholestasis (Fig. S1A2).

Immediately during his first visit to our hospital, he was fed with medium-chain triglyceride (MCT) milk (100–105 kcal/kg/day; lipid 25%, carbohydrate 56.6%, protein 13.2%, eight times per day) and received fat-soluble vitamins. His liver dysfunction improved at 7 months of age when the cytomegalovirus-IgM antibody became undetectable. However, 1 month later, he began to frequently vomit, and tube feeding was initiated. Thereafter, he developed recurrent bouts of jaundice and elevations of transaminases accompanied by flu-like signs such as nasal discharge and cough, and his liver dysfunction deteriorated to liver failure with cirrhosis (Fig. S1B shows his liver histology at 15 months of age). He underwent a liver transplantation at 17 months of age but died of recurrent peritonitis and the resultant sepsis at 22 months of age. Afterward, the liver specimens obtained through the explantation, which histologically showed cirrhosis, were subjected to mitochondrial RC examinations when his younger brother received RC examinations.

Case 2: The younger brother of case 1 was born, 15 months after his elder brother died, without any complications at 40 weeks of gestation, with a weight of 3260 g. At the age of 8 months he was referred to our hospital with failure to thrive (height, 62 cm, −3.5 SD; weight 5.5 kg, −3.1 SD) and mild cholestasis. He could sit up alone, but could not crawl yet. He had no seizure and no clinical sign of peripheral neuropathy except for mild hypotonia. He could appropriately respond to changes in emotional content of social interaction. His liver and spleen were not palpated below the costal margin. Liver function tests then showed mild or moderate elevations of serum AST, ALT, GGT, and TBA levels (AST, 150 IU/L; ALT 40 IU/L; GGT, 82 IU/L; TBA 40 μmol/L). The coagulation tests had normal results. He had no episode of hypoglycemia (his blood glucose level was 64 mg/dl). His plasma lactate and pyruvate levels were slightly elevated (lactate, 2.2–3.3 mmol/L; pyruvate 0.1–0.2 mmol/L), but his cerebrospinal fluid lactate and pyruvate levels were entirely normal. His amino acid profile in blood and organic acid profile in urine showed no finding suggesting an etiology, and serological viral tests were all negative. Brain magnetic resonance imaging (MRI) was normal at 9 months of age. His liver functions thereafter are shown in Fig. 1 (lower panel).

Liver histology at 8 months of age displayed mild infiltration of inflammatory cells, fibrosis in the portal tracts, and various-sized lipid droplets in hepatocytes, comparable to the histological findings of his elder brother. He was placed on a carbohydrate-rich, lipid-poor diet using MCT milk (100 kcal/kg/day; lipid 25%, carbohydrate 56.6%, protein 13.2%, eight times per day by nasogastric tube) as his elder brother had been, and was treated with fat-soluble vitamins and ursodeoxycholic acid.

His liver dysfunction thereafter fluctuated as a result of upper respiratory infections, probably due to some viruses, and diarrhea due to rotavirus infection (Fig. 1, lower panel).

Liver histology at 30 months of age showed a rather progressive fibrosis with bridging formation and inflammatory cell infiltration compared with that of the previous biopsy (Fig. S1C). The activity levels of mitochondrial RC Co I and III were decreased in this liver sample, whereas Co II activity remained normal (Table 1). Quantitative PCR revealed a decrease in the amount of mitochondrial DNA, and the boy was diagnosed with MDS. Gene analysis revealed that he is a compound heterozygote for mutations in the MPV17 gene responsible for MDS.

At 31 months of age, he began medication with carnitine (300 mg/day), succinate (2 g/day), and ubiquinone (coenzyme Q10: 30 mg/day). Simultaneously, the carbohydrate-rich, lipid-poor diet using MCT milk was changed to a lipid-rich, carbohydrate-poor diet using ketone milk (lipid 71.8%, carbohydrate 8.8%, protein 15%) and MCT milk (total: 90–100 kcal/kg/day; lipid 56.3–60.1%, carbohydrate 20.8–24.6%, protein 14.4–14.6%, eight times per day by nasogastric tube). After the initiation of these dietary and pharmaceutical treatments, his transaminases decreased to normal (Fig. 1, lower panel).

At the age of 37 months, a liver needle biopsy was again performed; infiltration by inflammatory cells decreased and fibrosis was suppressed, but fatty degeneration remained unchanged (Fig. S1D).

Recently, his body weight and height have increased gradually but are still low (body weight 10.3 kg, −2.6 SD; height 81.5 cm, −4.5 SD). Brain MRI was normal at 42 months of age. At the time of this writing, he is 47 months old, and shows normal psychomotor development.

Materials and methods

Samples for the examination of respiratory chains

The liver sample from the elder brother was the excised part of the liver transplantation at 17 months of age. Liver samples from the younger brother were obtained by liver biopsies at 30 and 37 months of age.

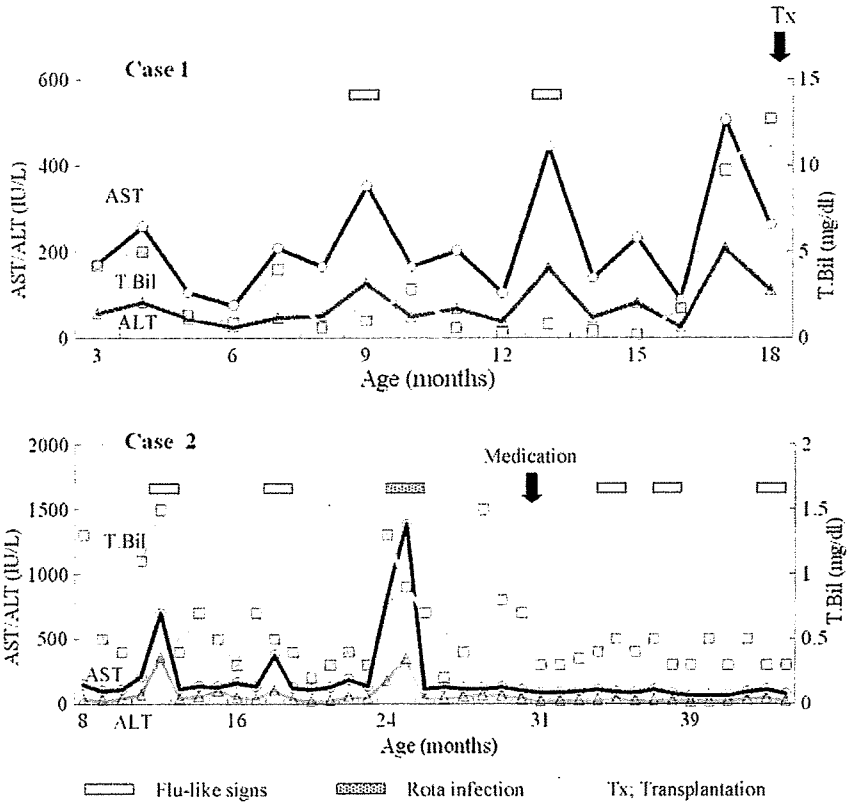

Fig. 1. Clinical courses of cases 1 (upper panel) and 2 (lower panel).

Determination of enzyme activities

Activities of RC Co I, II, III, and IV were assayed for the crude post-600 g supernatant of the liver samples as described previously [6,7]. The activity of each complex was presented as a percentage of the mean value obtained from 35 healthy controls. For each patient, the percentages of Co I, II, III, and IV activities relative to that of citrate synthase (CS) as a mitochondrial enzyme marker or Co II activity were calculated [6].

BN-PAGE Western blotting

Expression levels of the mitochondrial RC Co I, II, III, and IV proteins in the liver were examined by Western blotting using blue native polyacrylamide gel electrophoresis (BN-PAGE) according to the methods described previously [8,9]. Ten micrograms of the protein in the mitochondria-enriched fraction was separated by BN-PAGE. Immunostaining was performed using a monoclonal antibody specific for the 39 kD subunit of Co I, the 70 kD subunit of Co II, the core 1 subunit of Co III, and the subunit 1 of Co IV (Molecular Probes, Eugene, OR).

Quantitative PCR

mtDNA was quantitatively estimated by the real-time amplification of fragments of ND1 in the mtDNA genome, as previously described [10,11]. To determine the overall abundance of mtDNA, we compared the real-time amplification of ND1 with a single-

Table 1
Enzyme assay of respiratory chain and quantitative mtDNA evaluation by qPCR.

%	Co I	Co II	Co III	Co IV	CS	mtDNA/nDNA (%)
Elder Brother (17 months)						7.8
% of normal	0	80	13	41	300	
CS ratio	0	27	4	14	–	
Co II ratio	0	–	16	50	–	
Younger Brother (30 months)						6.6
% of normal	22	80	34	83	397	
CS ratio	6	36	9	21	–	
Co II ratio	15	–	24	57	–	
Younger Brother (37 months)						
% of normal	23	170	28	75	254	
CS ratio	9	67	11	29	–	
Co II ratio	13	–	16	43	–	–

Co I; complex I, Co II; complex II, Co III; complex III, Co IV; complex IV, CS; citrate synthase.
Enzyme activities are expressed as % of mean normal control activity relative to protein, relative to CS, and relative to Co II.

copy nuclear reference gene (exon 24 of the CFTR gene, chosen because it lacks single-nucleotide polymorphisms). For both experiments, DNA from six adult liver samples (from needle biopsies, obtained with informed consent) was used as controls. The results presented were the means of four independent runs, with samples assayed in triplicate in each run.

Mutation detection

Genomic DNA was extracted from liver or peripheral blood leukocyte according to the standard procedures. Detailed sequencing methods appear in the supplemental materials.

Results

Enzyme activities

Both affected siblings showed low activity levels of RC Co I, III, and IV. In particular, their Co I activities were strikingly low. In contrast, their Co II activities were maintained at normal, and those of citrate synthase were greatly elevated (Table 1). Co III and Co IV activity levels were higher in the younger brother than those in the elder brother.

BN-PAGE Western blot analysis

Fig. S2 shows the RC Co amounts by BN-PAGE in each brother. In both brothers, the band corresponding to either assembled Co I or assembled Co IV was invisible, and the band corresponding to the assembled Co III was strikingly weak. On the other hand, the intensity of the Co II band remained normal in both brothers.

Quantitative PCR

Quantitative PCR revealed that liver mtDNA was markedly decreased in both brothers (Table 1). The ratio of ND1 to CFTR in the liver of each brother was lower than those of the six controls (mean ± SD: 7.8 ± 4.6% for the elder brother, 6.6 ± 1.5% for the younger brother).

Mutations in MPV17

Both brothers were confirmed to be compound heterozygotes for c.451insC/c.509C > T (Fig. S3). c.451insC in exon 6 causes a frame-shift predicting an elongated gene product p.Leu151fsX189 (p.Leu151PhefsX39, according to the standard mutation nomenclature guidelines at http://www.genomic.unimelb.edu.au/mdi/mutnomen/). The c. 509C > T in exon 7 causes an amino acid substitution (Ser170Phe). These variations had not registered as genetic polymorphisms in the ensembl_mart_47 database (martdb.ensembl.org) and had not been reported as disease-causing mutations. Moreover, the alignment shows that both amino acid residues (Leu151 and Ser170) mutated in the affected siblings are absolutely conserved in all species (Fig. S4). Therefore, we consider these variations to be novel mutations. A single allele of c.451insC was present in all three siblings and their mother, whereas c. 509C > T was detected in both affected siblings and their father (Fig. S3). The fact that two such mutations were inherited from each parent independently indicated that these mutations were compound heterozygous in both affected siblings. The parents and the unaffected sibling had only one mutation, and had no obvious phenotype (Fig. S3). These observations support an autosomal recessive manner of inheritance for the hepatic dysfunction phenotype segregating within this family.

Discussion

Both brothers had novel compound heterozygous mutations, c.451insC/c.509C > T, but their clinical courses differed greatly. The phenotype of the elder brother was classified as possibly the infantile form, characterized by early onset liver disease that rapidly progresses to liver failure within the first few years of life [4,5].

In contrast, the younger brother exhibited a rather mild course. His liver damage was relatively mild, and he did not show any apparent neurological abnormality.

Such a great difference in the clinical courses between these brothers might be explained, in part, by the differences in their RC activity levels. The degree of reduction in RC activity was generally milder in the younger brother than in the elder. However, several studies have shown that RC activities were not correlated with the clinical course [4,5].

In our MPV17 mutant patients, the fluctuations in liver function were associated with infections that may cause oxidative stress. It was likely that cytomegalovirus infection promoted the onset and progression of liver disease in the elder brother, and that the liver dysfunctions in these siblings were greatly exacerbated by viral infections, in particular rotavirus infection.

Taken together, our experiences with these cases allowed us to assume that the clinical course and prognosis of MDS caused by MPV17 mutations were determined not only by the mutation but also by other factors. We postulated that many complicating factors may arise, including infection.

An effective treatment for mitochondrial RC disorders involving MDS has yet to be established. Liver transplantation is not so promising [6,12–14]. Besides the surgical complication, neurological regression after transplantation has been reported. Collectively, the survival rate is less than 50% [14].

For the younger brother, we tried to administer medications targeting the RC system, including succinate and coenzyme Q. Simultaneously, a lipid-rich carbohydrate-restricted diet using ketone milk was initiated. This combined treatment improved his liver disease biochemically and histologically. However, his liver RC activities did not improve.

Initially, he received a carbohydrate-rich, lipid-restricted diet and fat-soluble vitamins, together with UDCA, as had his elder brother. Recently, Parini et al. reported that glucose administration to avoid hypoglycemia is efficient in slowing the progression of liver disease [5]. However, this dietary treatment did not achieve favorable effects for our patients. Therefore, we resorted to another treatment for the younger brother.

The efficacy of medications with succinate and coenzyme Q, together with a lipid-rich diet, was possibly explained by their biochemical features. The mitochondrial RC system comprises Co I, II, III, and IV. Co I activity was markedly reduced in the younger brother, while his Co III activity remained mildly or moderately decreased. On the other hand, his Co II activity was entirely normal. Co I, an electron and proton acceptor from NADH and H^+, respectively, is the most important reduction-type hydrogen carrier, generating ATP by glucose oxidation [13]. From this context, glucose should hardly have been used as an energy source in the liver of the younger brother. On the other hand, succinate might donate electrons and protons to Co II connected to ubiquinone via $FADH_2$ [15]. In addition, a lipid-rich diet was expected to donate electrons and protons to ETF (electron-transfer flavoprotein): QO (ubiquinone oxidoreductase) connected to ubiquinone by promotion of $FADH_2$ production.

In summary, these cases suggested that the clinical course of MPV17 mutation is not determined solely by the mutation but rather is greatly influenced by viral infection, and that medications targeting Co II, together with a lipid-rich diet, may be beneficial in the clinical management of patients with MDS.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ymgme.2009.04.014.

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miR-210 promotes osteoblastic differentiation through inhibition of *AcvR1b*

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ABSTRACT

Although microRNAs (miRNAs) are involved in many biological processes, the mechanisms whereby miRNAs regulate osteoblastic differentiation are poorly understood. Here, we found that BMP-4-induced osteoblastic differentiation of bone marrow-derived ST2 stromal cells was promoted and repressed after transfection of sense and antisense miR-210, respectively. A reporter assay demonstrated that the activin A receptor type 1B (*AcvR1b*) gene was a target for miR-210. Furthermore, inhibition of transforming growth factor-β (TGF-β)/activin signaling in ST2 cells with SB431542 promoted osteoblastic differentiation. We conclude that miR-210 acts as a positive regulator of osteoblastic differentiation by inhibiting the TGF-β/activin signaling pathway through inhibition of *AcvR1b*.

1. Introduction

MicroRNAs (miRNAs) are classified as regulatory RNAs, and have been reported to play important roles in the development, proliferation and differentiation of various types of cells. Among the various miRNAs, miR-143 regulates adipocyte differentiation, miR-206 regulates muscle differentiation and miR-133 regulates skeletal differentiation [1–3]. miR-223 regulates granulopoiesis by a feedback mechanism and is modulated competitively by the transcription factors nuclear factor I/A (NFI-A) and CCAAT/enhancer binding protein-α (C/EBPα) [4]. miR-223 was also recently reported to regulate osteoclastogenesis in RAW 264.7 cells [5]. Some miRNAs are known to be present in cancer cells and act as oncogenes or tumor suppressor genes [6–8]. miR-21 has been reported to be an oncogene that promotes tumor growth by down-regulating the tropomyosin 1 tumor suppressor (*TPM1*) gene [9,10]. Expression of miR-17 is regulated by the myelocytomatosis (c-Myc) oncogene and acts as an oncogene by regulating cell proliferation through E2F transcription factor 1 (E2F1) [11,12]. On the other hand, the let-7 miRNA suppresses the high mobility group AT-hook 2 (*HMGA2*) oncogene and acts as a tumor suppressor gene [13]. The let-7 miRNA is also known to suppress cell proliferation [14] and induce regression of colon cancer [15].

Although miRNAs are known to play a number of regulatory roles, little is known about their roles in osteoblastic differentiation. As a functional miRNA, we recently reported that miR-125b negatively regulates osteoblastic differentiation by mediating cell proliferation [16]. However, there are no reports of miRNAs that positively regulate osteoblastic differentiation.

In the present study, we demonstrate positive regulation of osteoblastic differentiation by miR-210. The expression profiles of miRNAs during osteoblastic differentiation of mouse ST2 mesenchymal stem cells were obtained by miRNA microarray analyses, and miR-210 was found to be highly expressed in these cells. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) confirmed that miR-210 expression was upregulated during osteoblastic differentiation. We also analyzed the function of miR-210 in osteoblastic differentiation by transfection

Abbreviations: miRNA, microRNA; TGF-β, transforming growth factor-β; AcvR1b, activin A receptor type 1B; Alk, activin-like kinase; ALP, alkaline phosphatase; OC, osteocalcin

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of exogenous miR-210 or its antisense strand into ST2 cells. The miRNA target gene databases MiRanda and TargetScan predicted that the activin A receptor type 1B (*AcvR1b*; also known as Alk4) gene could be a target for miR-210. The regulatory relationship between miR-210 and *AcvR1b* was confirmed by reporter assays. The role of transforming growth factor-β (TGF-β)/activin signaling for osteoblastic differentiation in ST2 cells was also validated.

2. Materials and methods

2.1. Cell culture

ST2 cells and NRG cells were obtained from the RIKEN BioResource Center (BRC, Tsukuba, Japan) and cultured according to the supplier's protocols using RPMI-1640 medium and DMEM, respectively, supplemented with 10% fetal bovine serum (FBS).

2.2. Osteoblastic differentiation

Osteoblastic differentiation was induced by replacing the medium with fresh medium containing 10% FBS and 100 ng/ml of bone morphogenetic protein-4 (BMP-4) (R&D Systems, Minneapolis, MN) every third day. SB431542 (1 μM; Sigma, St. Louis, MO) was added to fresh medium containing the above components for inhibition of TGF-β/activin signaling.

2.3. miRNA microarray analysis

Small RNA fractions of total RNA were purified using a flashPAGE Fractionator System (Ambion, Austin, TX) and labeled with a *mir*Vana miRNA Labeling Kit (Ambion), followed by loading on a custom-made miRNA array on which a *mir*Vana miRNA Probe Set (Ambion) was printed according to the manufacturer's instructions. After 16 h of hybridization, the signals were detected by a microarray scanner (G2565BA; Agilent Technologies, Santa Clara, CA) and quantified by the software Feature Extraction ver. 8.1 (Agilent Technologies).

2.4. Alkaline phosphatase (ALP) staining and its measurement

Cells were fixed with 10% formalin for 20 min, followed by fixation for 1 min in an ice-cold solution of equal volumes of ethanol and acetone, and then washed with phosphate-buffered saline. The ALP staining solution was prepared by dissolving 1 mg of Naphthol AS-MX (Sigma) in one droplet of *N,N*-dimethylformamide (Wako, Osaka, Japan) and suspending the droplet in 10 ml of 0.1 M Tris–HCl buffer (pH 8.5) containing 2 mM $MgCl_2$. Six micrograms of Fast Blue BB salt (Sigma) was added and the ALP staining solution was filtered. The fixed cells were incubated in the ALP staining solution at 37 °C for 20 min. A *p*-Nitrophenyl Phosphate Liquid Substrate System (Sigma) was added to determine the ALP activity and the absorbances at 405 nm were measured using an ARVO MX plate reader (Perkin–Elmer, Norwalk, CT). ALP staining and ALP activity measurements were performed in triplicate wells in two independent experiments.

2.5. Cell proliferation assay

After plating cells and changing to fresh media containing 100 ng/ml BMP-4 and 10% FBS, the total cell count was measured every day for 6 days using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's instructions. The absorbances at 490 nm were measured with an ARVO MX plate reader (Perkin–Elmer). Background absorbances from empty wells were subtracted from those of the sample wells.

2.6. qRT-PCR

Total RNA was isolated from cultured cells using a Nucleospin column (Machery Nagel, Düren, Germany), according to the manufacturer's instructions. A *mir*Vana miRNA Isolation Kit (Applied Biosystems, Forster City, CA) was used for purification of miRNAs. The yield and quality of the RNA samples were determined using a NanoDrop spectrometer (NanoDrop Technology, San Diego, CA). The expression levels were measured by qRT-PCR. Total RNA was reverse-transcribed by Transcriptor (Roche, Mannheim, Germany) with oligo-dT primers or specific primers for miR-210 (Applied Biosystems) according to the manufacturer's instructions. Using the resulting cDNAs as templates, the gene expression levels were measured using an Mx3000P system (Stratagene, La Jolla, CA) and Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. The primer sequences for measurement of mRNA expression were: 5′-TGGAGAAACCTGCCAAGTATG-3′ (glyceraldehyde phosphate dehydrogenase (GAPDH)-forward); 5′-GGAGACAACCTGGTCCTCAG-3′ (GAPDH-reverse); 5′-CTCTGTCTCTCTGACCTCACAG-3′ (osteocalcin (OC)-forward); 5′-GGAGCTGCTGTGACATCCATAC-3′ (OC-reverse); 5′-CGTCCTCTCTG CTTGAGGAA-3′ (Osterix-forward); 5′-TTCCCCAGGGTTGTTGAGT-3′ (Osterix-reverse); 5′-AACGAGATCGAGCTCAGAGG-3′ (Col1a1-forward); 5′-GGGAGGTCTTGGTGGTTTTG-3′ (Col1a1-reverse); 5′-CTG GCTACAAGGTGGTGGAC-3′ (ALP-forward); 5′-GTCTTCTCCACC GTGGGTCT-3′ (ALP-reverse); 5′-GCTTGGCTTATGGACTGAGG-3′ (Osteopontin-forward); 5′-CGCTCTTCATGTGAGAGGTG-3′ (Osteopontin-reverse); 5′-GAGGGCACAAGTTCTATCTG-3′ (Runx2-forward); 5′-GCTCGGATCCCAAAAGAAG-3′ (Runx2-reverse). Specific primers for miR-210 and U6 (Applied Biosystems) were used for measurement of miRNA expression.

2.7. Transfection

The sense strand of miR-210 (miR-210) and the antisense strand of miR-210 (anti-miR-210) (Applied Biosystems) were used to promote and inhibit miR-210 activity, respectively. Negative controls were used for both reactions. For transfection, Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA) was mixed with 20 nM of the above-mentioned RNAs according to the manufacturer's instructions, and these solutions were directly mixed with ST2 cells in 24-well culture plates at a density of 3.2×10^4 cells/well. For osteoblastic differentiation, the medium was replaced with fresh medium containing 10% FBS and 100 ng/ml of BMP-4 at 4 h after transfection.

2.8. Luciferase reporter assay

The miR-210 target region of the *AcvR1b* sequence (5′-ATTCTCCAGACTCAAACGCACAT-3′, part of the NCBI RefSeq ID of NM_007395) or its mutated sequence (5′-ATTCTCCAGAACCAA CTATGTCT-3′) was inserted downstream of the pGL4.13 luciferase plasmid (Promega) and the resulting constructs were named pGL4.13-AcvR1b and pGL4.13-AcvR1-mut, respectively. Cotransfection of 200 ng of pGL4.13-AcvR1b or pGL4.13-AcvR1-mut with 20 nM miR-210 or anti-miR-210 was carried out in 24-well plates. A native pGL4.13 luciferase plasmid was cotransfected with the same concentration of miR-210 or anti-miR-210 in separate wells as a control. Twenty nanograms of a Renilla luciferase vector, pGL4.74 (Promega), was also transfected into all samples for

Table 1
miRNAs whose expression are changed during osteoblastic differentiation (day 14) comparing to Control (without BMP-4) (1 day).

miRNA	log 2 ratio (B14/C1)
Upregulated	
let-7d	2.97
let-7a-1	2.72
miR-210	2.20
let-7f-2	2.16
let-7f-1	1.79
miR-16-1	1.65
let-7b	1.55
Downregulated	
miR-329	−4.31
miR-122a	−2.01
miR-23a	−1.12
miR-147	−1.04
miR-198	−1.01

normalization. Transfections with all plasmid and RNA combinations were repeated three times. After 48 h, the cells were harvested with PLB reagent (Promega) and the firefly luciferase activity was measured in each well using the LARII substrate and a Dual Luciferase Assay Kit (Promega) with the ARVO MX plate reader. The relative firefly luciferase activity was calculated by normalization to the Renilla luciferase activity.

3. Results

3.1. Expression of miR-210 is upregulated during osteoblastic differentiation

To elucidate the differential expression of miRNAs during osteoblastic differentiation, a microarray analysis for miRNAs was performed. We found that the expressions of several miRNAs changed during osteoblastic differentiation (Table 1). Several let-7 family miRNAs, miR-210 and miR-16-1 were listed as upregulated. The let-7 family miRNAs are known to be broadly expressed in many different cells and tissues, and have several known functions. In contrast, the function of miR-210 has been poorly understood to date. Therefore, we focused on the possibility of a regulatory role for miR-210 in osteoblastic differentiation. qRT-PCR with the miRNA measurement protocol was performed to further analyze the expression pattern of miR-210 during osteoblastic differentiation of ST2 cells. As shown in Fig. 1A, miR-210 expression was greatly upregulated at day 12 when ST2 cells were cultured with BMP-4, suggesting its involvement in osteoblastic differentiation of ST2 cells. To test whether the increase in miR-210 was a dose-responsive effect to BMP-4, increasing amounts of BMP-4 were introduced for osteoblastic differentiation. The expression level of miR-210 increased until the BMP-4 concentration reached 50 ng/ml (Fig. 1B), indicating that the induction of

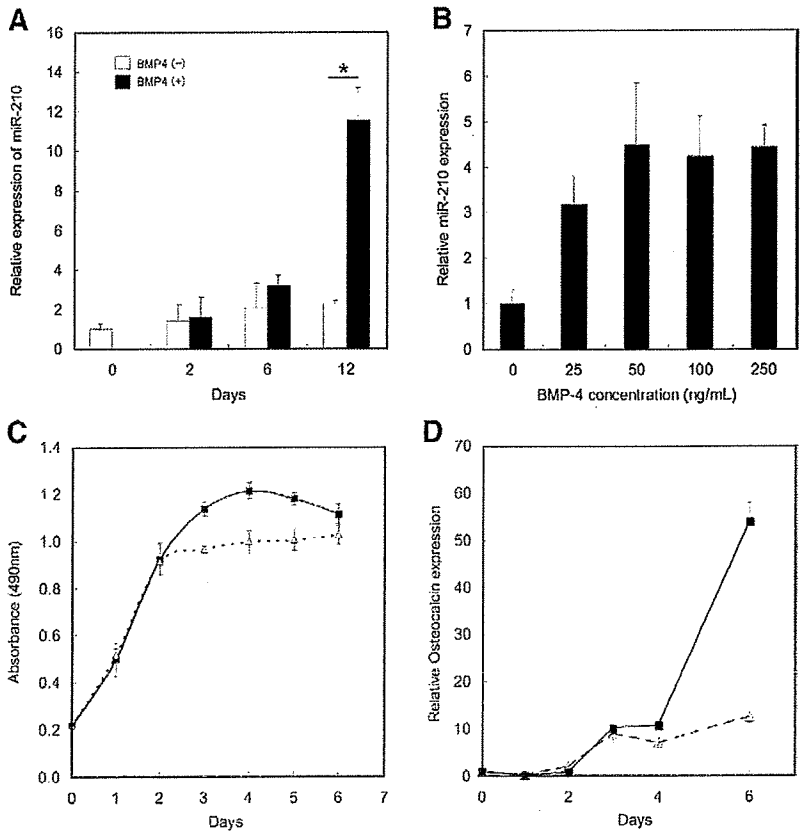

Fig. 1. Relative expression of endogenous miR-210 during osteoblastic differentiation of ST2 cells. (A) After plating ST2 cells, BMP-4 was added at a final concentration of 100 ng/ml. Control cells were cultured without BMP-4. Cells were harvested at 2, 6 and 12 days after the addition of BMP-4, and the relative levels of endogenous miR-210 expression at each time point with and without the addition of BMP-4 compared with day 0 were measured by qRT-PCR. Solid bars and white bars indicate cells incubated with and without BMP-4, respectively. Asterisks indicate significant *t*-test results ($P < 0.01$). (B) BMP-4 was added in increasing amounts (25, 50, 100 and 250 ng/ml) for 6 days, and the expression levels of miR-210 was measured by qRT-PCR. (C and D) Characterization of cell proliferation and osteocalcin (OC) expression during osteoblastic differentiation of ST2 cells. BMP-4 was added at a concentration of 100 ng/ml, then cell proliferation assays (C) and measurement of osteocalcin expression by qRT-PCR (D) were performed. Solid squares and white triangles indicate cultures with and without BMP-4, respectively.

miR-210 is BMP-4 dose-dependent. To clarify the time-dependent profiles of cell proliferation and the expression levels of the osteoblastic marker OC, cell proliferation assays and qRT-PCR were performed, respectively. We found that proliferation stopped at 2–3 days after osteoblastic differentiation (Fig. 1C), and that the expression of OC began to increase at 4 days after osteoblastic differentiation (Fig. 1D).

3.2. Transfection of miR-210 promotes osteoblastic differentiation of ST2 cells

To determine whether miR-210 could influence osteoblastic differentiation, miR-210 and anti-miR-210 were individually transfected into ST2 cells and their effects were assessed by observing the ALP activity and expression level of OC transcripts. ALP staining and activity were increased following transfection of miR-210 compared with transfection of control RNA (Fig. 2A and B). OC expression was also increased following miR-210 transfection (Fig. 2C), suggesting that stimulation of miR-210 activity promoted osteoblastic differentiation of ST2 cells. By contrast, transfection of anti-miR-210 reduced the expression of OC (Fig. 2C). The expression levels of other osteoblastic markers, namely osterix and ALP, were also upregulated following transfection with miR-210 (Fig. 2D). These results indicate that miR-210 positively regulates the osteoblastic differentiation of ST2 cells. To examine whether the regulation of osteoblastic differentiation by miR-210 occurs in other osteoblast-like cells, similar experiments were performed using the mouse NRG cell line. We used this cell line because osteoblastic differentiation can be induced at a lower concentration of BMP-4 and within a shorter period compared with the ST2 cell line. As shown in Fig. 2E, the level of ALP activity was elevated when miR-210 was transfected into NRG cells, as observed in ST2 cells, indicating that the promotion of osteoblastic differentiation by miR-210 is not restricted to the ST2 cell line.

3.3. AcvR1b is a target of miR-210

More than 200 genes were predicted by PicTar or TargetScan to be potential target genes for miR-210 [17,18]. We focused on *AcvR1b* (Fig. 3A), which is a member of the BMP receptor family. In this family, activin-like kinase 2 (Alk2) is known to regulate BMP signal transduction, whereas AcvR1b regulates the TGF-β/activin signaling pathway [19]. We used a luciferase reporter assay system and transfection of sense and antisense strands of miR-210 to determine whether *AcvR1b* was a target for miR-210, and to investigate its effects on osteoblastic differentiation. To test whether the predicted target sequence in the *AcvR1b* gene was regulated by miR-210, we cloned the predicted target sequence downstream of the luciferase reporter gene and cotransfected it with miR-210 or anti-miR-210. The relative luciferase activity was reduced by 50% following cotransfection with miR-210 compared with transfection with control RNA (Fig. 3B). By contrast, transfection with anti-miR-210 slightly increased the luciferase activity, although the difference was not statistically significant. When the targeted sequence of AcvR1b was mutated, the reduction of the luciferase activity by miR-210 was impaired (Fig. 3C). These results suggest that miR-210 targets the predicted site in the *AcvR1b* gene.

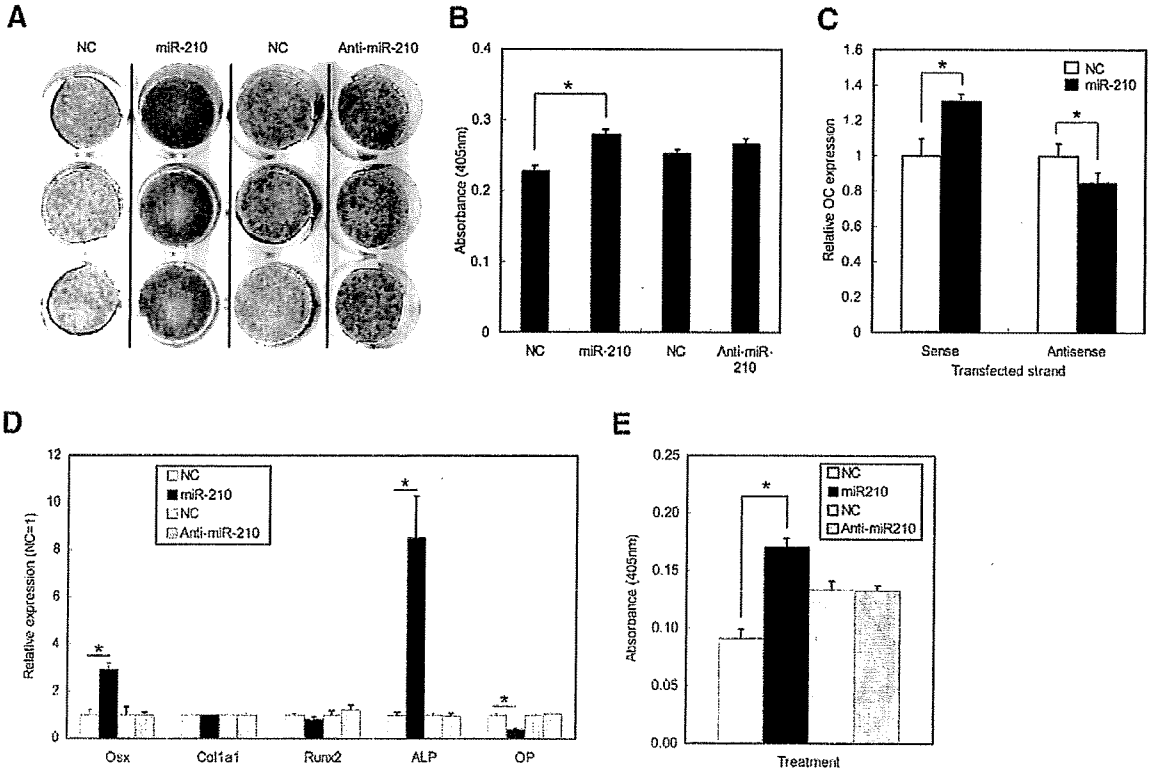

Fig. 2. Influences of transfections with miR-210 and anti-miR-210 on osteoblastic differentiation of ST2 cells. miR-210 (sense), anti-miR-210 (antisense) or a negative control (NC) RNA were transfected into ST2 cells and BMP-4 was added 4 h later. ALP staining was performed (A) and the ALP activity was measured (B), and compared with the NC values. (C) The relative expression of OC transcripts was measured after 5 days. (D) qRT-PCR analysis of other bone markers than OC in miR-210-transfected ST2 cells in which osteoblastic differentiation was induced by BMP-4. Osx: osterix; Col1a1: collagen, type 1, alpha 1; OP: osteopontin. (E) Measurement of ALP activity in miR-210-transfected NRG cells in which osteoblastic differentiation was induced by BMP-4 at 25 ng/ml for 4 days. Each experiment was performed in triplicate wells and on two independent days. Asterisks indicate significant *t*-test results ($P < 0.05$).

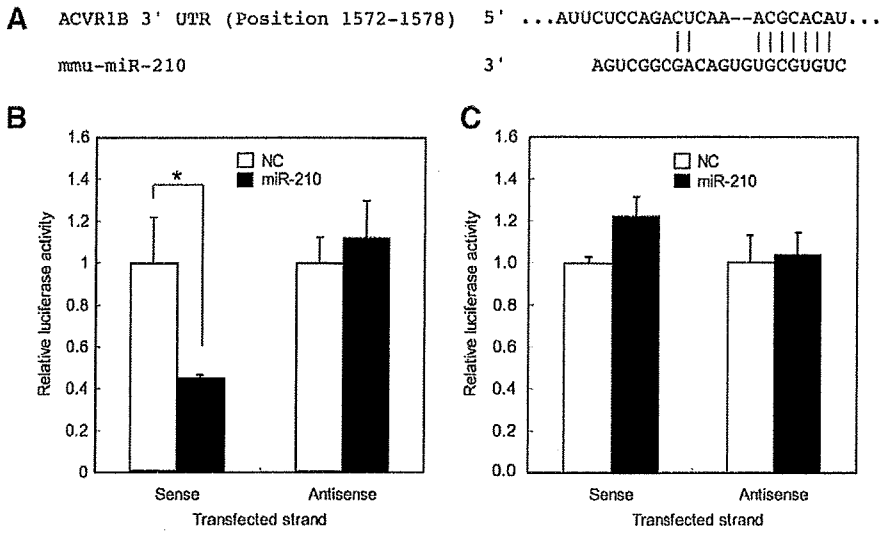

Fig. 3. Evaluation of targeting of *AcvR1b* by miR-210. (A) The region of the mouse *AcvR1b* mRNA 3′ UTR predicted to be targeted by miR-210 (TargetScan 4.1). (B and C) The relative activity of the luciferase reporter of the target sequence of miR-210 in the *AcvR1b* 3′ UTR after cotransfection of sense or antisense miR-210, compared with the transfection of a negative control (NC) RNA. Part of the *AcvR1b* 3′ UTR (the predicted target sequence for miR-210) (B) or its mutated sequence (C) was cloned into a luciferase vector and cotransfected with miR-210, anti-miR-210 or an NC RNA. The luciferase activity was measured at 2 days after transfection. Solid bars indicate transfection of miR-210 or anti-miR-210, and white bars indicate transfection of the NC miRNA. Asterisks indicate significant *t*-test results ($P < 0.05$).

3.4. Inhibition of AcvR1b by SB431542 promotes osteoblastic differentiation of ST2 cells

To further elucidate whether miR-210 promotes osteoblastic differentiation by inhibiting the signaling pathway of AcvR1b, we tried to use the chemical SB431542, which is a specific inhibitor for receptors of TGF-β/activin signaling [19,20]. Following addition of SB431542, ALP activity and OC expression were both elevated (Fig. 4A and B). These results suggest that osteoblastic differentiation of ST2 cells is promoted by downregulation of the TGF-β/activin signaling pathway. Taken together, the inhibition of TGF-β/activin signaling by either miR-210 or SB431542 promotes osteoblastic differentiation of ST2 cells.

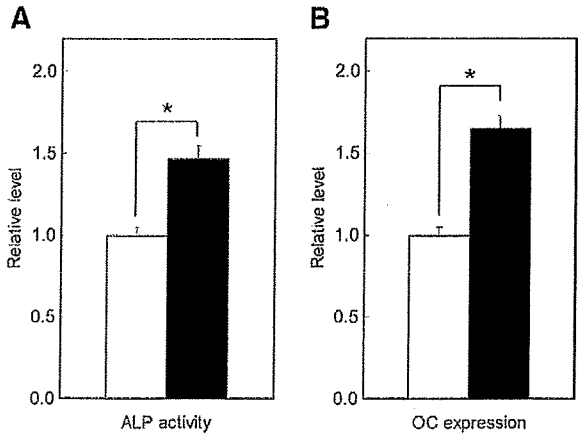

Fig. 4. Inhibition of TGF-β/activin signaling by SB431542 promotes osteoblastic differentiation. The culture medium was changed to fresh medium containing SB431542, an inhibitor of TGF-β/activin signaling, at 4 h and 3 days after plating of ST2 cells. The relative ALP activity (A) and relative expression of OC transcripts (B) compared with mock vector treatment were measured on day 6. Solid and white bars indicate cultures with and without SB431542, respectively. Asterisks indicate significant *t*-test results ($P < 0.05$).

4. Discussion

Osteoblastic differentiation of mesenchymal stem cells can be strongly induced by BMPs [21]. The involvement of miRNAs in various cellular events suggests that they could also have a regulatory function in osteoblast differentiation. However, the regulatory mechanisms of miRNAs in osteoblast proliferation and differentiation are currently poorly understood. We recently reported that miR-125b downregulated osteoblastic differentiation through inhibition of cell proliferation [16]. In that study, in vitro cell proliferation was inhibited when miR-125b was transfected, and osteoblastic differentiation was inhibited owing to the low density of the differentiating cells. That study was the first to demonstrate a function of miRNAs in regulating osteoblastic differentiation. miR-133 and miR-135 have subsequently been reported to inhibit BMP-2-induced osteoblastic differentiation by directly targeting the genes for *Runx2* and *Smad5* [22]. However, the miRNAs studied to date all act as inhibitors of osteoblastic differentiation, and no positive regulation by miRNAs has been reported.

The miRNA array data showed that miR-210 was upregulated during osteoblastic differentiation. Furthermore, the present results showed that miR-210 positively regulated osteoblastic differentiation of mouse mesenchymal ST2 cells. A recent study found that miR-210 was induced by hypoxia and involved in cell cycle regulation through targeting of E2F transcription factor 3 [23,24]. These results suggest that miR-210 could be involved in cell proliferation in cancer and under hypoxic conditions. However, the effects of miR-210 on cell differentiation have not been reported.

The results of the present study indicate that miR-210 was able to positively regulate osteoblastic differentiation via targeting of the TGF-β/activin signaling pathway. AcvR1b is a type I receptor which, together with the type II receptor ActRII, transmits signals from activin. When activated, this receptor is known to transmit signals to the receptor-regulated Smads (R-smad) Smad2 and 3, but not Smad1, 5 or 8, through their phosphorylation, resulting in the transcription of genes that function as inhibitory regulators of proliferation. BMP signals, however, are transmitted via other receptors, such as AcvR1 (Alk2), Bmpr1a (Alk3) and Bmpr1b (Alk6) and their signals are transmitted to Smad1, 5 and 8, thereby

initiating osteoblastic differentiation. It has been reported that Smad2/3 and Smad1/5/8 signaling can interfere with each other by competitive binding to a co-Smad, Smad4 [19]. Inhibition of Smad2/3 signaling is known to activate Smad1/5/8 signaling, resulting in acceleration of osteoblastic differentiation [19,20]. MiRanda and TargetScan both predicted that *Acvr1b* is a target gene for miR-210 in mice and humans. Therefore, we considered the possibility that the promotion of osteoblastic differentiation associated with the stimulation of miR-210 activity arose through its inhibition of *AcvR1b*. Regulation of the predicted 3′ UTR region of *AcvR1b* by miR-210 was demonstrated by luciferase reporter assays, and inhibition of *AcvR1b* was associated with increased osteoblastic differentiation in our culture system using the ST2 bone marrow-derived cell line. Thus the promotion of osteoblast differentiation by miR-210 could be explained by inhibition of the TGF-β/activin signaling pathway, through targeting of *AcvR1b*. Our results could provide an important step toward resolving bone disorders, such as osteoporosis. Elucidation of the roles of miRNAs in osteoblast regulation in vivo could help to clarify the mechanisms of bone metabolism and turnover.

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ORIGINAL ARTICLE

Association of *UGT2B7* and *ABCB1* genotypes with morphine-induced adverse drug reactions in Japanese patients with cancer

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Abstract
Purpose To investigate the effects of genetic polymorphisms on morphine-induced adverse events in cancer patients.
Methods We examined the relation of morphine-related adverse events to polymorphisms in *UDP-glucuronosyltransferase (UGT) 2B7*, *ATP-binding cassette, sub-family B, number 1 (ABCB1)*, and *μ-opioid receptor 1* genes in 32 Japanese cancer patients receiving oral controlled-release morphine sulfate tablets.
Results The T/T genotype at 1236 or TT/TT diplotype at 2677 and 3435 in *ABCB1* was associated with significantly lower frequency of fatigue (grades 1–3) ($P = 0.012$ or 0.011, Fisher's exact test). The *UGT2B7*2* genotype was associated with the frequency of nausea (grades 1–3) ($P = 0.023$). The frequency of nausea was higher in patients without *UGT2B7*2* allele than others. The diplotype at 2677 and 3435 in *ABCB1* was associated with the frequency of vomiting (grades 1–3) ($P = 0.011$). No patient whose diplotype was consisted of no GC allele at 2677 and 3435 suffered from vomiting.
Conclusion Our findings suggest that pharmacogenetics can be used to predict the risk of morphine-induced adverse events.

Keywords Morphine · Cancer patients · Adverse reaction · Pharmacogenetics

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Introduction

Severe pain caused by tumors is therapeutically managed by administration of opioid analgesics. Morphine is one of the most important and widely used opioids for cancer-pain relief, but large interindividual variability in its effectiveness and adverse reactions are a major clinical disadvantage. Clinical pharmacology studies have demonstrated that wide interindividual variability in the response to a drug is associated with considerable pharmacokinetic or pharmacodynamic variability [1], which may be genetically determined [2–4].

Morphine is predominantly glucuronidated by UDP-glucuronosyltransferase (UGT) 2B7 to form morphine 6-glucuronide (M6G) and morphine 3-glucuronide (M3G) [5]. M6G has clinically been shown to be a potent analgesic, and the analgesic properties of morphine are enhanced