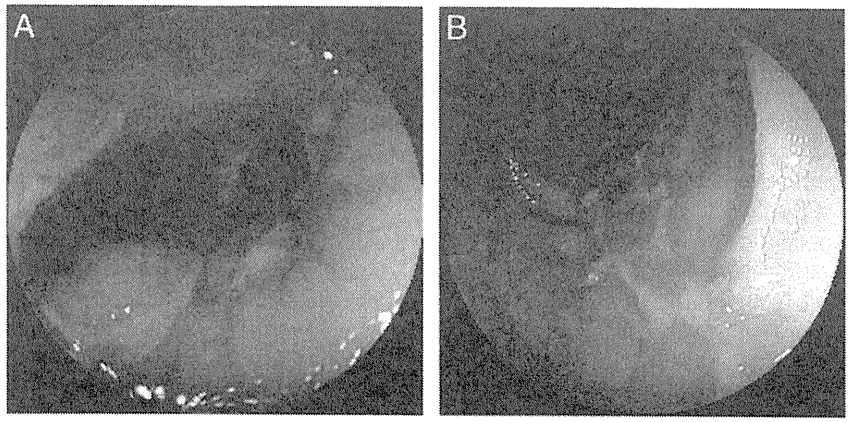

Figure 2 Enteroscopic findings of Case 13. This case is a daughter of a consanguineous marriage of 3 degrees, who has an elderly sister with protein-losing enteropathy. A; DBE reveals a severe concentric stenosis in the middle ileum. The stenostic area is accompanied by circular and sharply demarcated ulcer. B; DBE also shows a shallow, linear mucosal effect with clear margin in the distal ileum.

Small-bowel ulcers are known to occur in various types of chronic enteropathy of obscure etiology. These include Crohn's disease, chronic ulcerative duodenojejunoileitis,[17 19] cryptogenic multifocal ulcerous stenosing enteritis (CMUSE),[9] and diaphragm disease of the small bowel without apparent NSAID use.[8] CNSU shares common clinical manifestations with CMUSE and diaphragms unrelated to NSAID with respect to less severe inflammatory infiltrates and stenosing lesions of the ileum. We thus cannot conclusively distinguish CNSU from those two conditions. There also seems to be an argument that CNSU, together with CMUSE and diaphragms, belongs to a peculiar phenotype of Crohn's disease with less severe inflammation. The occurrence in adolescents with predominant involvement of the ileum in CNSU apparently mimics Crohn's disease, although the ileal phenotype is different between the two diseases.

In 1990s, data on the familial acquisition of Crohn's disease were accumulated. Analyses of those data from all over the world showed that the occurrence of Crohn's disease in the first-degree relatives of a proband ranged from 2.2% to 13.6%.[20 26] A common trend in those analyses was that the siblings of a proband were at the highest risk for the occurrence of the disease while parents have the lowest risk. Although a similar trend was also found in our patients with CNSU, the occurrence of enteropathy in the siblings was much higher, with a value of 23%. In contrast, the consanguinity has rarely been described in Crohn's disease. It thus seems likely that CNSU is genetically different from Crohn's disease.

So far as we reviewed the literature, two types of enteropathy are described in association of consanguinity. The first one is an intractable ulcerating enterocolitis of infancy characterized by diarrhea in the first year of life with large and deep ulcers in the colon.[27] The other enteropathy, referred to as intestinal epithelial dysphasia, has also been characterized by severe diarrhea in infants with disorganization of entrecotes in the epithelium and basement membrane abnormalities of the small-bowel.[28,29] The clinicopathologic features of the infantile enteropathy are obviously different from those of CNSU with respect to the age of onset and the clinical course.

Glocker et al.[16] recently analyzed two unrelated consanguineous families with an early onset of colitis, and they identified homozygous mutations in *IL10RA* and *IL-10RB* genes in the families. Even though the predominant site of involvement and other phenotypes are different between the cases reported by Glocker et al.[16] and those of CNSU, *IL-10R* may be one of the candidate genes associated with CNSU. Adler et al.[10] reported on another peculiar form of enteropathy with a life-long history of occult gastrointestinal

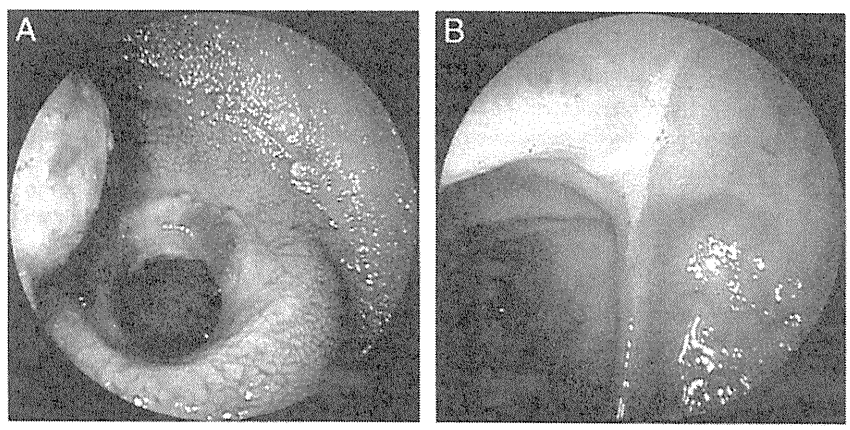

Figure 3 Enteroscopic findings of Case 6. This case is a daughter of a consanguineous marriage of 5 degrees. A; DBE shows a concentric stenosis with a clear ulcer in the ileum. B; in the distal ileum, sharply demarcated and linear mucosal defects are also seen.

Please cite this article as: Matsumoto T, et al, Chronic nonspecific multiple ulcer of the small intestine segregates in offspring from consanguinity, *Journal of Crohn's and Colitis* (2011), doi:10.1016/j.crohns.2011.05.008

Table 4 Consanguinity and family history of patients with CNSU.

Case no.	Consanguinity (degrees)	Family history of enteropathy
1.	Present (3)	None
2.	Absent	None
3.	Unknown	Unknown
4.	Absent	None
5.	Absent	A sibling
6.	Present (5)	None
7.	Present (5)	None
8.	Present (3)	None
9.	Unknown	Unknown
10.	Present (3)	A sibling
11.	Unknown	None
12.	Absent	None
13.	Present (3)	A sibling

blood loss, iron deficiency anemia and relapsing abdominal pain. The male patient had multiple, sharply demarcated ulcers and stenoses in the jejunum and in the ileum during his middle-aged period. Histological examination of the resected small-bowel disclosed nonspecific ulcers with minimal inflammatory infiltrates. Furthermore, Adler et al.[10] confirmed that the patient had inherited compound heterozygosity in *cPLA2α* gene, which resulted in a reduction in eicosanoid biosynthesis in platelets and leukocytes. Based on these observations, it was suggested that homozygous or compound heterozygous mutations of *cPLA2α* gene and a consequent reduction in substrates for arachidonic acids result in an enteropathy with recurrent small-bowel ulcers. It thus seems possible that *cPLA2α* is another candidate gene for CNSU. This hypothesis is under investigation.

The present case series has some limitations due to a retrospective analysis of historically accumulated patients. First, we cannot completely deny undisclosed use of NSAID, because we did not measure its metabolites in blood or urine samples.[8,30] However, we believe the enterosopic findings and the extra-ordinary long-term clinical course of CNSU to be completely different from NSAID enteropathy.[3] Second, we could not serologically deny chronic jejunoileitis complicating celiac disease in 12 of 13 patients. However, we consider celiac disease to be unlikely, because the patients did not have any villous atrophy, and furthermore, the disease is extremely rare among Asians.

In conclusion, a retrospective analysis of patients with CNSU revealed that the disease is possibly an enteropathy segregating in offsprings from consanguineous marriage. This concept may explain the rarity of the disease, and suggests that CNSU is a disease distinct from Crohn's disease. Further accumulation of the patients together with genetic analyses will be needed to conclude that CNSU is an autosomal recessive disorder.

Acknowledgment

TaM contributed to the analysis of the data and the writing of the manuscript. NK collected all the demographic and endoscopic data. ToM, MI and TY contributed to the concept of the manuscript and the management of the study subjects.

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Journal of Crohn's and Colitis (2012) 6, 160–173

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Adalimumab for the induction and maintenance of clinical remission in Japanese patients with Crohn's disease

Mamoru Watanabe [a,*], Toshifumi Hibi [b], Kathleen G. Lomax [c], Susan K. Paulson [c], Jingdong Chao [c], M. Shamsul Alam [d], Anne Camez [e] On behalf of the study investigators [1]

[a] *Tokyo Medical & Dental University, Tokyo, Japan*
[b] *Keio University School of Medicine, Tokyo, Japan*
[c] *Abbott, Abbott Park, IL, United States*
[d] *Abbott Park, IL, United States*
[e] *Abbott GmbH & Co. KG, Ludwigshafen, Germany*

Received 24 February 2011; received in revised form 17 June 2011; accepted 21 July 2011

KEYWORDS
Crohn's disease;
Japan;
Adalimumab;
Double-blind trials;
Induction;
Maintenance

Abstract

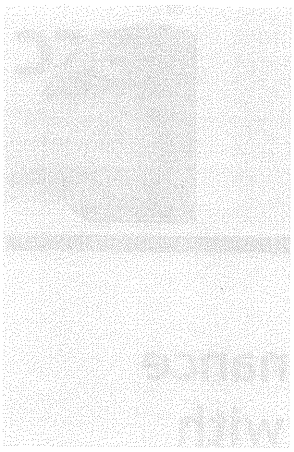
Background and aims: Adalimumab has been shown to be efficacious and well-tolerated in Western patients with Crohn's disease. These 2 randomized, double-blind clinical trials evaluated adalimumab efficacy and safety in Japanese patients with moderate to severe Crohn's disease. *Methods:* 90 patients enrolled in the induction trial and were randomized to receive adalimumab 160/80 mg, adalimumab 80/40 mg or placebo at Weeks 0/2. At Week 4, patients who achieved a decrease in CDAI ≥ 70 points versus Baseline entered the maintenance trial and were randomized to adalimumab 40 mg every other week or placebo for 52 weeks. All other patients received 4 more weeks of blinded adalimumab before entering the open-label portion of

Abbreviations: CD, Crohn's disease; PPD, purified protein derivative; TPN, total parenteral nutrition; CDAI, CD activity index; CR-70, decrease in CDAI score from Baseline ≥ 70; CR-100, decrease in CDAI score from Baseline ≥ 100; eow, every other week; 6-MP, 6-mercaptopurine; BCG, Bacillus Calmette-Guérin; IOIBD, International Organization of Inflammatory Bowel Disease; PCS, physical component summary; MCS, mental component summary; SF-36, Short Form-36 Health Survey; IBDQ, Inflammatory Bowel Disease Questionnaire; AAA, anti-adalimumab antibody; AAA+, patients positive for anti-adalimumab antibody; FAS, full analysis set; mFAS, modified full analysis set; HRQOL, health-related quality of life; IMM, immunomodulator; 5-ASA, 5-aminosalicyclate.

* Corresponding author at: Department of Gastroenterology, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8510, Japan. Tel.: +81 3 3813 6111; fax: +81 3 5803 0262.
E-mail address: mamoru.gast@tmd.ac.jp (M. Watanabe).

[1] Conference Presentation: American College of Gastroenterology Annual Meeting, Orlando, FL, USA, October 2008; American College of Gastroenterology Annual Meeting, San Diego, CA, USA, October 2009; United European Gastroenterology Week, London, United Kingdom, November 2009; European Crohn's and Colitis Organization Annual Meeting, Prague, Czech Republic, February 2010; Digestive Disease Week, New Orleans, LA, USA, May 2010.

1873-9946/$ - see front matter
doi:10.1016/j.crohns.2011.07.013



the maintenance trial. At/after Week 4 of the maintenance trial, blinded patients who flared/failed to respond entered the open-label portion. Open-label maintenance patients received adalimumab 40 mg every other week with the option of 80 mg every other week for flare/non-response.

Results: Clinical remission rates at Week 4 in the induction trial were 33.3%, 17.6% and 13.0% in the adalimumab 160/80 mg, adalimumab 80/40 mg and placebo groups, respectively. Maintenance remission rates were 38.1% for adalimumab and 9.1% for placebo at Week 52. Anti-TNF naïve patients achieved greater efficacy than anti-TNF exposed patients. Patients randomized to adalimumab achieved greater quality of life improvement versus placebo. There were no clinically relevant differences in safety between adalimumab and placebo.

Conclusions: Adalimumab is effective and well-tolerated for inducing and maintaining clinical remission in Japanese patients with moderate to severe Crohn's disease. NCT00445939; NCT00445432.

1. Introduction

The incidence and prevalence of Crohn's disease (CD) in Japan are lower than in other regions, although these have been shown to be increasing in Japan,[1] as they are in Western countries. Characteristics of CD in Japanese patients are the same as in Western patients. Though the positioning of nutritional therapy differs, the same drugs are used in Japanese and Western patients, and data supporting the use of anti-tumor necrosis factor (TNF) agents in the treatment of Japanese patients with CD are available.[2]

Adalimumab (HUMIRA, Abbott Laboratories, Abbott Park, Illinois, USA), a fully human monoclonal antibody that targets TNF, is approved for the treatment of rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, CD, psoriasis, and juvenile idiopathic arthritis in the United States, Europe, and elsewhere.[3] In Japan, adalimumab is approved for the treatment of rheumatoid arthritis, psoriatic arthritis, psoriasis, and ankylosing spondylitis. Adalimumab was also approved for the treatment of CD in Japan on the basis of the present trials. Adalimumab has been shown to be effective for the induction and maintenance of remission in Western patients with moderate to severe CD.[4] Adalimumab is also effective in patients who have had an inadequate response to conventional therapy or who have lost response to or are unable to tolerate infliximab, the chimeric monoclonal antibody to TNF.[5] In addition, adalimumab has been shown to provide rapid and sustained improvements in quality of life, both physical and psychological, in adults with active CD in Western countries.[6–9]

The objectives of these 2 multicenter clinical trials were to determine the efficacy and safety of adalimumab in inducing and maintaining remission, and to determine the effect of adalimumab on quality of life in Japanese patients with moderate to severe CD. In addition, the pharmacokinetics and immunogenicity of adalimumab treatment in Japanese patients were assessed.

2. Methods

2.1. Patients

This report describes results from 2 related clinical trials conducted in Japanese patients: first, a multicenter, randomized, double-blind, placebo-controlled trial (NCT00445939, January 2007 to December 2007) for induction of remission of CD; then a follow-on multicenter, placebo-controlled 52-week trial (NCT00445432, March 2007 to December 2008) for maintenance of remission.

Japanese adults and adolescents ≥15 and ≤75 years of age, with a diagnosis of moderate to severely active CD (CD Activity Index [CDAI] score of 220–450) for >4 months and a diagnosis of ileal, colonic or ileocolonic CD confirmed by endoscopy or radiologic evaluation, were included. Previous exposure to anti-TNF agents other than adalimumab was allowed; primary non-responders to prior anti-TNF therapy were excluded. Women of childbearing potential were not pregnant or breast-feeding and were practicing an acceptable method of birth control throughout the trial and for 150 days after the last study drug administration. Patients provided written informed consent and complied with the requirements of this study protocol. Written informed consent was provided by a parent or legal guardian if the patient was <20 years old.

Patients were excluded for a diagnosis of ulcerative colitis or indeterminate colitis; history of cancer, lymphoma, leukemia or lymphoproliferative disease; active tuberculosis, chest X-ray findings suggestive of previous or current tuberculosis infection, "strongly positive" purified protein derivative (PPD) skin test with induration and erythema ≥ 10 mm with either bulla, necrosis or double redness (concentric surrounding of strong redness by weaker redness)[10]; human immunodeficiency virus infection; persistent chronic infections or recent infections unrelated to CD, requiring hospitalization or treatment with anti-infectives (intravenous within 28 days or oral within 14 days); history of neurologic symptoms suggestive of central nervous system demyelinating disease; presence or suspicion of abscess; surgical bowel resections within the past 6 months; positive *C. difficile* stool assay at Screening; body weight <30 kg; clinically significant abnormalities found during the electrocardiogram evaluation or laboratory assessment at the Screening visit; or a poorly controlled medical condition or any condition which, in the opinion of the investigator, would put the patient at risk by participation in the trial. Patients who received total parenteral nutrition within 14 days before Baseline or enteral nutrition >1200 kcal/day were excluded, as were patients who used infliximab or any biological agent within 8 weeks of Baseline. Previous treatment with adalimumab or participation in an

adalimumab clinical trial, any prior exposure to natalizumab, and receipt of any investigational chemical agent in the past 28 days or 5 half-lives prior to Baseline were not allowed.

2.2. Study design

In the induction trial, patients were randomly assigned (3:3:2) to receive induction therapy with adalimumab 160/80 mg, adalimumab 80/40 mg, or placebo at Baseline and Week 2 (Fig. 1). Patients achieving a clinical response 70 (CR-70, decrease from Baseline in CDAI ≥ 70 points) at Week 4 in the induction trial entered the blinded portion of the 52-week maintenance trial and were randomly assigned (1:1) to receive adalimumab 40 mg every other week (eow) or placebo. CR-70 non-responder patients at Week 4 in the induction trial continued for a further 4 weeks in the induction trial and were given double-blind adalimumab, with the dose of adalimumab depending on the induction regimen to which the patients were initially randomized. Week 4 CR-70 non-responder patients who had been randomized to placebo in the induction trial were given blinded adalimumab 160/80 mg induction therapy at Weeks 4 and 6 in the induction trial, and then entered the open-label portion of the maintenance trial. Week 4 CR-70 non-responder patients who had been randomized to adalimumab at either induction dose in the induction trial were given blinded adalimumab 40 mg at Weeks 4 and 6, and then entered the open-label portion of the maintenance trial. To accommodate the differing lengths of the induction trial for the CR-70 responders (4 weeks) and the CR-70 non-responders (8 weeks), the Baseline week of the maintenance trial is called Week 0x and the numbering of the following weeks includes "x" to describe the follow-on nature of the maintenance trial.

At or after Week 4x, patients in the blinded portion of the maintenance trial who flared could enter the open-label portion. Patients in the open-label portion received adalimumab 40 mg eow with the ability to increase the adalimumab dose to 80 mg eow in case of flare or non-response; the escalation dose of 80 mg eow has been approved in Japan, instead of 40 mg weekly, for other indications including rheumatoid arthritis, psoriasis, and ankylosing spondylitis. Flare was defined as a recurrence of very active disease, specifically an increase of ≥ 70 points in CDAI when compared with Week 0x in the maintenance trial and a CDAI > 220. Lack of response was defined as not attaining a CDAI decrease of ≥ 70 points compared with Week 0 of the induction trial for 2 consecutive visits, at least 2 weeks apart.

Concomitant use of immunomodulators (azathioprine and 6-mercaptopurine), aminosalicylates, and Crohn's-related antibiotics, was permitted in the induction and maintenance trials, provided that the doses remained stable. Stable doses of corticosteroids (≤40 mg/day of prednisolone or equivalent), and stable enteral nutrition ≤1200 kcal/day were permitted. No changes of Crohn's-related concomitant therapies were allowed during the induction trial. At Week 4x of the maintenance trial, patients who had experienced a significant improvement in their Crohn's symptoms (defined as a CDAI decrease of ≥ 70 points compared to Baseline of induction trial) could taper enteral nutritional therapy and corticosteroid doses, with the possibility to increase back to the amount at Week 0x if the disease was aggravated. In the open-label treatment group in the maintenance trial, patients were allowed to reduce or discontinue Crohn's-related concomitant treatments after 12 weeks of exposure to open-label adalimumab, with the possibility to increase back to the initial dose if the patient experienced a loss of clinical response. In both the induction and maintenance trials, patients could receive isoniazide for the prophylaxis of tuberculosis infection in patients with induration ≥ 5 mm on the PPD skin test, irrespective of the Bacillus Calmette–Guérin (BCG) vaccination status.

2.3. Endpoints

2.3.1. Induction trial

The CDAI score and International Organization of Inflammatory Bowel Disease[11] (IOIBD) score were assessed at Baseline and every 2 weeks. The mental component summary (MCS) and physical component summary (PCS) of the Short Form-36 (SF-36) Health Survey and the Inflammatory Bowel Disease Questionnaire (IBDQ) were assessed at Baseline and every 4 weeks.[12–14] The primary endpoint was the proportion of patients in clinical remission (CDAI < 150) at Week 4. Secondary endpoints included the proportion of patients in clinical remission at Week 2 and with clinical response CR-100 or clinical response CR-70 (CDAI decrease of ≥ 100 or ≥ 70 from Baseline, respectively) at Week 2 and Week 4. Additional secondary endpoints included changes from Baseline in CDAI and IOIBD at Week 2 and Week 4 and changes from Baseline in SF-36 MCS and PCS, and IBDQ scores in each treatment group at Week 4. In *post-hoc* analyses, clinical remission, CR-100, and CR-70 at Week 4 were assessed in each treatment group after stratification by patients' previous use of anti-TNF therapies.

2.3.2. Maintenance trial

The CDAI and IOIBD were measured at Baseline (Week 0x) and every 4 weeks to Week 52x in the maintenance trial. The SF-36 MCS and PCS and the IBDQ were assessed at Baseline and Weeks 8x, 24x, and 52x. The primary endpoint was clinical remission (CDAI < 150) at Week 52x in the double-blind portion. Secondary endpoints included the proportion of patients in clinical remission, CR-100, or CR-70 every 4 weeks until Week 52x; changes from Baseline of the induction trial (Week 0) to Week 52x in CDAI, IOIBD, SF-36 MCS and PCS scores and IBDQ in the double-blind portion.

2.3.3. Pharmacokinetics and immunogenicity

Blood samples for serum adalimumab and anti-adalimumab antibody (AAA) assays were collected at Week 1 and prior to dose administration at Weeks 0, 2 and 4 in the induction trial and Weeks 0x, 4x, 8x, 12x, 16x, 20x, 24x, 36x and 52x in the maintenance trial. Adalimumab and AAA samples were analyzed at MDS Pharma Services (Switzerland AG) using validated enzyme-linked immunosorbent assay (ELISA) methods based on a double-antigen technique.[15] The lower limit of quantitation (LLOQ) for adalimumab concentration was established at 3.1 ng/mL in diluted serum or 31.3 ng/mL in undiluted serum. The coefficient of variation (CV) for adalimumab concentration was ≤7.0% and the analytical

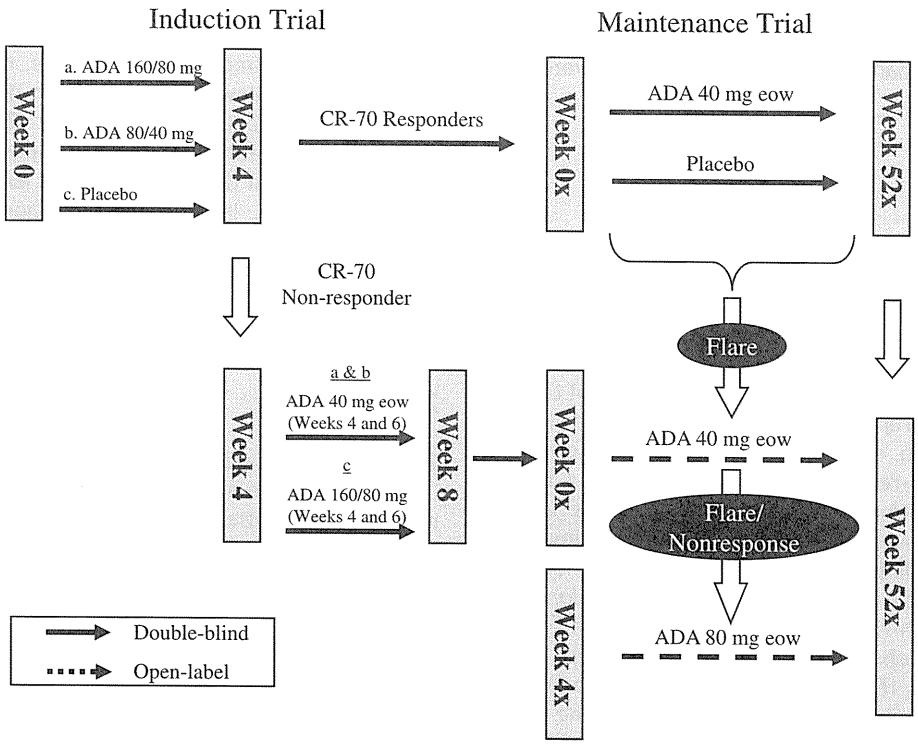


Figure 1 Study design. The primary efficacy analysis for the induction trial is at Week 4 and for the maintenance trial is at Week 52x. The "x" designation beside the week numbers indicates the follow-on nature of maintenance trial. ADA = adalimumab; eow = every other week; CR-70 = decrease in CDAI from Baseline ≥ 70.

recovery ranged from 97.5% to 108.5%. The LLOQ for AAA was established at 1.0 ng/mL in diluted serum or 10.0 ng/mL in undiluted serum. The CV for AAA was ≤ 23.4% and the percent bias ranged from −11.0% to 9.5%.

2.4. Statistics

The purpose of the induction trial was to show that at Week 4 the clinical remission rate in the adalimumab group was numerically higher than in the placebo group. This trial did not have a statistical hypothesis, and sample size was not calculated using statistical techniques. The small sample size of these trials relates to the overall low prevalence of CD in the Japanese population and therefore it was not possible to design the trials with power to reach statistical significance. The purpose of these trials was to show only a numerical difference between adalimumab and placebo. The study protocol stipulated 30 patients per adalimumab group and 20 patients per placebo group. Eighty patients in total were to be enrolled in the induction trial. All patients who completed the induction trial were eligible for the maintenance trial.

All patients enrolled in the induction trial who were randomly assigned to 1 of the 3 treatment groups and received at least 1 dose of study drug constituted the full analysis set (FAS) of the induction trial and were included in the primary and secondary efficacy analyses, and in the safety analyses for the induction trial. In the maintenance trial, patients who entered the double-blind portion and who received at least 1 dose of study drug comprised the FAS of the maintenance trial, for which the safety analysis was performed. Among the FAS of the maintenance trial, patients who received adalimumab in the induction trial constituted the modified FAS (mFAS), for which the primary and secondary efficacy analyses were performed. Patients who received at least 1 dose of adalimumab either during the induction trial or the maintenance trial comprised the all-adalimumab set. The dose escalation set included patients who received adalimumab 80 mg eow in the open-label portion of the maintenance trial.

In both trials, the primary and secondary efficacy analyses were performed using descriptive statistics. *Post-hoc* statistical analyses of rates of clinical remission and response were performed using Chi-square test or Fisher's exact test. Quality of life scores for adalimumab versus placebo and changes in scores from Baseline of the induction trial were compared using Student's *t*-tests. For all analyses of the proportion of patients in clinical remission, clinical response CR-100 and clinical response CR-70, patients who had missing values for the endpoint in question were not considered to have met the endpoint. Patients in the mFAS of the maintenance trial who switched to the open-label portion were also not considered to have met the endpoint in question after the switch. For the analyses of mean change from Baseline in CDAI, IOIBD, SF-36 MCS, SF-36 PCS and IBDQ, missing data were imputed using the last observation carried forward (LOCF) method. However, the measurements at Week 0x were not used to impute the missing values after Week 0x using the LOCF method.

Adalimumab concentrations were summarized by treatment group at each time point using descriptive statistics. Serum AAA concentrations were listed by treatment group at

each collection time. The proportion of patients positive for AAA (AAA+) was calculated and efficacy and safety for AAA+ patients assessed.

2.4.1. Safety

Adverse events (AE), laboratory values, and vital signs were assessed on a routine basis throughout both the induction and maintenance trials. AEs were analyzed using a descriptive analysis. In the induction and maintenance trials, a treatment-emergent AE (TEAE) was defined as any AE with onset from the first dose of study drug, with differing endpoints depending on whether the patient continued in the adalimumab development program or not. Specifically, for patients who continued in the adalimumab development program, the end-date for reporting TEAEs was at the trial end (Week 4 or 8 for the induction trial; Week 52x for the maintenance trial); for patients who discontinued the trial and did not continue in the adalimumab development program, the end-date for reporting TEAEs was up to 70 days following the last study drug administration. For analysis of the any adalimumab set (combines induction and maintenance trial), a TEAE was defined as any AE with onset from the first dose of adalimumab the patient ever received (which could have been in the induction trial for those randomized to adalimumab, or in the maintenance trial for those randomized to placebo in the induction trial) through

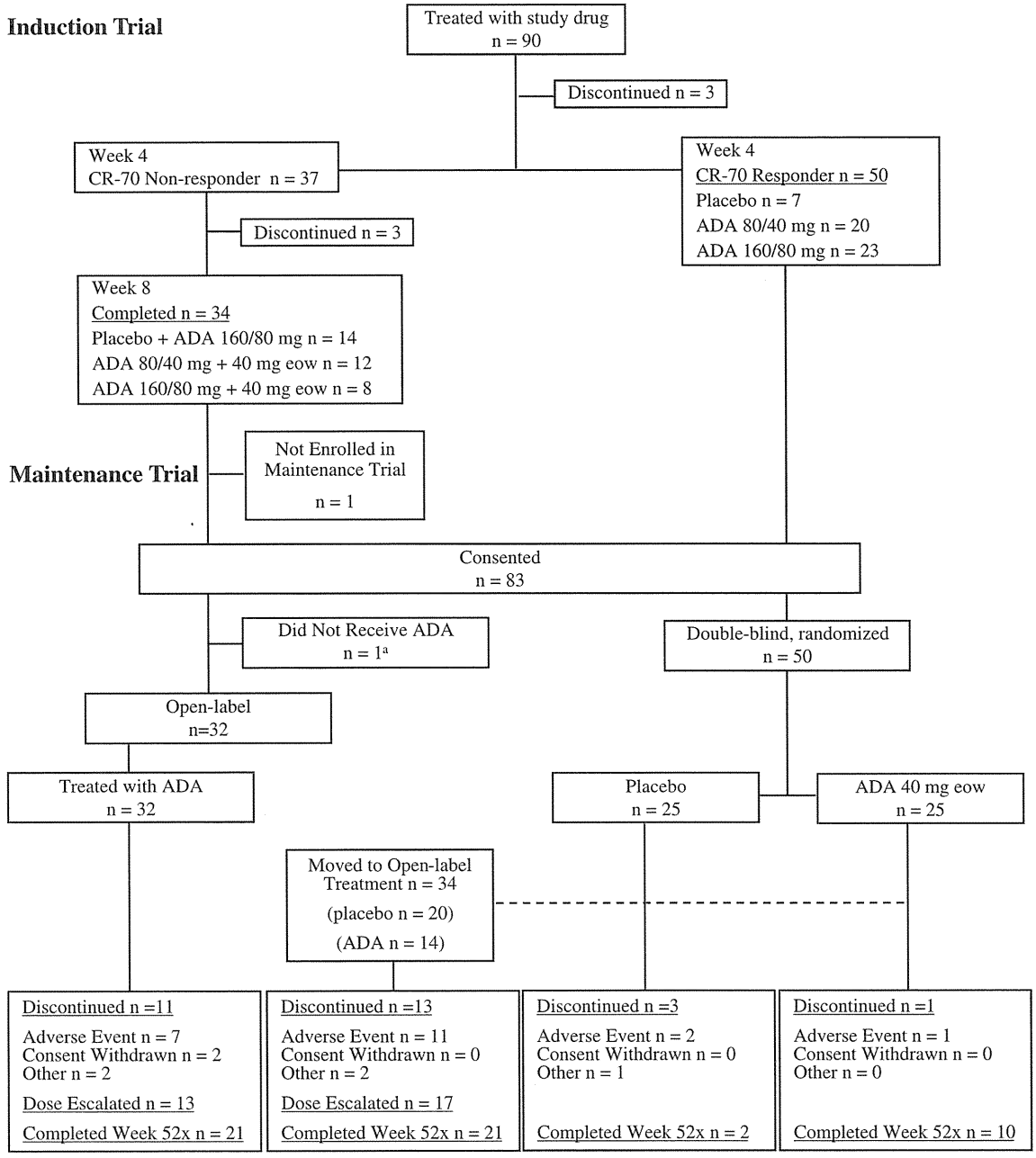


Figure 2 Patient disposition. The "x" designation beside the week numbers indicates the follow-on nature of maintenance trial. [a]One patient in the adalimumab 160/80 mg+40 mg eow group in the induction trial enrolled in the maintenance trial but was not dosed. ADA = adalimumab; CR-70 = decrease in CDAI from Baseline ≥ 70; eow = every other week.

an end-date up to the last dose received in the maintenance trial if the patient continued after Week 52x, or up to 70 days after the last dose of adalimumab if the patient discontinued either trial. Of note, for any adalimumab set, events that occurred during the placebo period prior to receipt of any adalimumab were excluded.

3. Results

3.1. Patient population

3.1.1. Induction trial

Informed consent was obtained from 108 patients, of which 90 were randomly assigned to receive study drug (adalimumab 160/80 mg, n=33; adalimumab 80/40 mg, n=34; placebo, n=23). 18 patients dropped out of the study prior to randomization. The reasons were ineligibility (n=13), serious adverse events (n=3), withdrawal of consent (n=1) and aggravation of CD (n=1).

3.1.2. Maintenance trial

Of 90 patients enrolled in the induction trial, 83 consented to continue in the maintenance trial (for the 7 patients who did not continue, 6 were terminated early from the induction trial due to adverse events and 1 did not continue in the maintenance trial because of the patient's demand); 50 entered the double-blind randomized portion (FAS) and 32 entered the open-label portion (1 patient was not treated). Of the 50 patients who entered the double-blind portion, 43 received adalimumab in the induction trial and were included in the mFAS. Patient disposition for the follow-on maintenance trial is summarized in Fig. 2.

3.2. Baseline characteristics

Baseline demographics, assessments of disease activity, efficacy parameters, health-related quality of life (HRQOL) scales, and concomitant medication use are summarized in Tables 1 and 2. There were no clinically important

Table 1 Baseline characteristics. [a]

	Induction trial				Maintenance trial		
	Full analysis set [b]				Full analysis set [c]		
	Placebo n=23	Adalimumab 80/40 mg n=34	Adalimumab 160/80 mg n=33	Total N=90	Placebo n=25	Adalimumab 40 mg eow n=25	Total N=50
Female, n (%)	7 (30.4)	18 (52.9)	13 (39.4)	38 (42.2)	10 (40.0)	9 (36.0)	19 (38.0)
Age (years)							
Mean±SD	30.4±6.9	30.6±9.3	32.0±9.6	31.1±8.8	30.8±10.9	31.6±7.2	31.2±9.2
<30 years	9 (39.1)	15 (44.1)	16 (48.5)	40 (44.4)	11 (44.0)	12 (48.0)	23 (46.0)
Weight (kg)							
Mean±SD	56.5±8.4	55.3±10.4	54.1±10.5	55.2±9.9	56.5±9.2	58.0±11.0	57.3±10.1
Min–Max	43.9–80.3	37.3–80.0	37.0–81.4	37.0–81.4	38.7–74.8	42.1–81.4	38.7–81.4
Tobacco, never used (n, %)	17 (73.9)	19 (55.9)	22 (66.7)	58 (64.4)	11 (44.0)	16 (64.0)	27 (54.0)
Alcohol, non-drinker (n, %)	14 (60.9)	21 (61.8)	24 (72.7)	59 (65.6)	14 (56.0)	15 (60.0)	29 (58.0)
Duration of CD (years)							
Mean±SD	7.9±4.7	9.2±6.6	11.0±7.1	9.5±6.4	8.2 (7.4)	9.9 (5.3)	9.1 (6.4)
Range	0.7–19.2	0.4–27.4	0.3–24.2	0.3–27.4	0.3–27.4	2.4–21.3	0.3–27.4
CDAI score							
Mean±SD	308.1±63.8	302.7±66.6	300.5±66.5	303.3±65.2	296.7±65.3	325.5±62.3	311.1±64.9
Range	221–444	221–448	221–448	221–448	221–448	223–448	221–448
CDAI score (n, %)							
<300	13 (56.5)	19 (55.9)	18 (54.5)	50 (55.6)	13 (52.0)	11 (44.0)	24 (48.0)
≥300	10 (43.5)	15 (44.1)	15 (45.5)	40 (44.4)	12 (48.0)	14 (56.0)	26 (52.0)
IOIBD score							
Mean±SD	3.7±1.2	3.4±1.6	3.3±1.5	3.4±1.5	3.2±1.8	3.1±1.2	3.2±1.5
Range	2–7	1–6	1–7	1–7	1–7	1–6	1–7
IBDQ score, Mean±SD	139.4±26.8	148.6±27.9	145.9±25.2	145.2±26.6	151.6±26.2	144.2±23.1	147.9±24.7
SF-36 summary score, Mean±SD							
Mental component	39.0±11.7	39.5±10.3	38.7±10.5	39.1±10.6	41.4±10.7	38.2±10.4	39.8±10.6
Physical component	43.8±7.6	42.9±7.8	43.3±6.3	43.3±7.2	45.0±6.5	42.9±7.7	43.9±7.1
C-reactive protein (mg/dL), Mean±SD	2.5±2.0	3.0±2.8	2.2±2.0	2.6±2.3	2.6±2.0	2.2±1.8	2.4±2.0

Eow = every other week; CD = Crohn's disease; CDAI = Crohn's disease activity index; IOIBD = International Organization for the Study of Inflammatory Bowel Disease; IBDQ = Inflammatory Bowel Disease Questionnaire; SF-36 = Short Form-36.

[a] Baseline is Week 0 of induction trial for both the induction and maintenance trials.

[b] Patients enrolled in the induction trial who were randomly assigned to 1 of the 3 treatment groups and received at least 1 dose of study drug.

[c] Patients who entered the double-blind portion of the maintenance trial and received at least 1 dose of study drug.

Table 2 Crohn's disease-related medication prior to or at baseline of induction and maintenance trials. [a]

Baseline medication use	Induction trial				Maintenance trial		
	Full analysis set [b]				Full analysis set [c]		
	Placebo n=23	Adalimumab 80/40 mg n=34	Adalimumab 160/80 mg n=33	Total N=90	Placebo n=25	Adalimumab 40 mg eow n=25	Total N=50
Aminosalicylates	23 (100)	27 (79.4)	32 (97.0)	82 (91.1)	19 (76.0)	25 (100.0)	44 (88.0)
Immunosuppressants	8 (34.8)	11 (32.4)	10 (30.3)	29 (32.2)	7 (28.0)	11 (44.0)	18 (36.0)
Corticosteroids	5 (21.7)	6 (17.6)	8 (24.2)	19 (21.1)	5 (20.0)	3 (12.0)	8 (16.0)
CD-related antibiotics	2 (8.7)	1 (2.9)	2 (6.1)	5 (5.6)	1 (4.0)	1 (4.0)	2 (4.0)
Enteral nutrition	16 (69.6)	22 (64.7)	23 (69.7)	61 (67.8)	12 (48.0)	17 (68.0)	29 (58.0)
Anti-TNF [d]	13 (56.5)	20 (58.8)	19 (57.6)	52 (57.8)	14 (56.0)	13 (52.0)	27 (54.0)

Data are n (%).
Eow = every other week; CD = Crohn's disease.
[a] Baseline is Week 0 of induction trial for both the induction and maintenance trials.
[b] Patients enrolled in the induction trial who were randomly assigned to 1 of the 3 treatment groups and received at least 1 dose of study drug.
[c] Patients who entered the double-blind portion of the maintenance trial and received at least 1 dose of study drug.
[d] Previous use of infliximab or biologic, if any, had to be discontinued at least 8 weeks before Baseline.

differences in Baseline characteristics across treatment groups for either trial.

3.3. Efficacy

3.3.1. Induction trial

Patients who received adalimumab had rapid improvement in disease activity by Week 2, with continued improvement at Week 4 (Fig. 3A). Patients treated with adalimumab 160/80 mg had the highest rate of clinical remission at Week 4, compared with patients treated with adalimumab 80/40 mg or placebo (Fig. 3A). Anti-TNF-naïve patients were more likely to reach remission at Week 4 compared with patients who had prior anti-TNF exposure in all 3 treatment groups (Fig. 3B). Among patients with prior anti-TNF exposure, the clinical remission rate with adalimumab 160/80 mg treatment was more than double than that with adalimumab 80/40 mg treatment (Fig. 3B).

Significantly more patients treated with adalimumab reached CR-100 at Week 4 compared with placebo ($p<0.05$ for adalimumab 160/80 mg versus placebo and adalimumab 80/40 mg versus placebo) (Fig. 4A). Treatment with adalimumab was effective regardless of previous exposure to anti-TNF agents, although patients who had not been previously treated with anti-TNF therapy were more likely to reach CR-100 than those previously exposed to anti-TNF agents (Fig. 4A). The percentage of patients achieving CR-70 at Week 4 was greater in the adalimumab groups compared with placebo. However, results were significant only in the group treated with adalimumab 160/80 mg ($p=0.0062$, Fig. 4B). Adalimumab-treated patients who had not received previous anti-TNF therapy were more likely to reach CR-70 (Fig. 4B).

The mean changes in CDAI from Baseline to Week 2 and Week 4 were, respectively, −75.9 and −101.3 in the adalimumab 160/80 mg group, −74.4 and −81.3 in the adalimumab 80/40 mg group, and −27.2 and −37.5 in the placebo group. The mean changes in IOIBD score from Baseline to Week 2 and Week 4 were, respectively, −1.2 and −1.5 in the adalimumab 160/80 mg group, −0.7 and −0.8 in

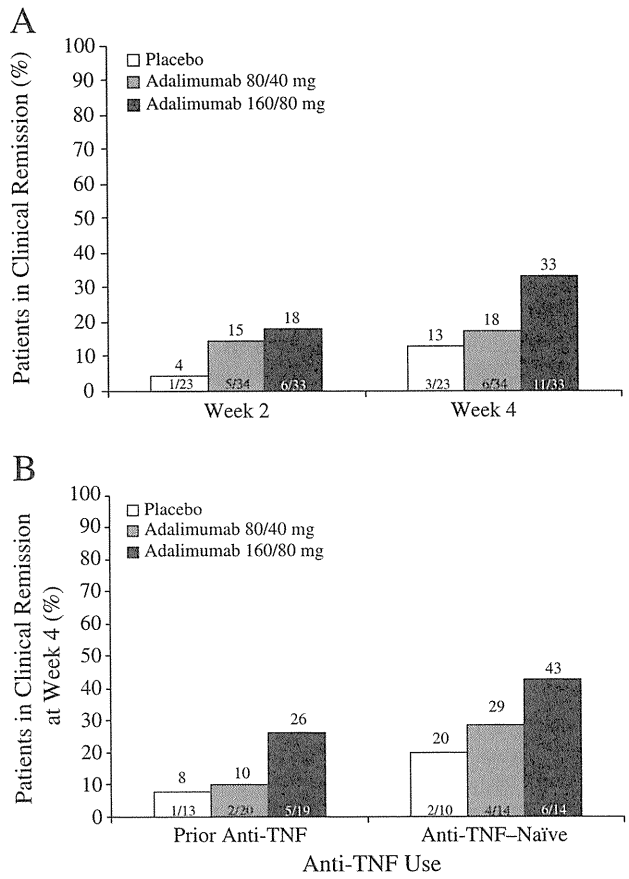


Figure 3 Clinical remission during the induction trial: (A) Remission at Weeks 2 and 4; (B) Remission at Week 4 stratified by prior anti-TNF exposure. Clinical remission is defined as CDAI<150. Analyses were conducted using non-responder imputation in the FAS (patients who enrolled in the induction trial, were randomly assigned to 1 of the 3 treatment groups and received at least 1 dose of study drug). CDAI = Crohn's disease activity index; FAS = full analysis set.

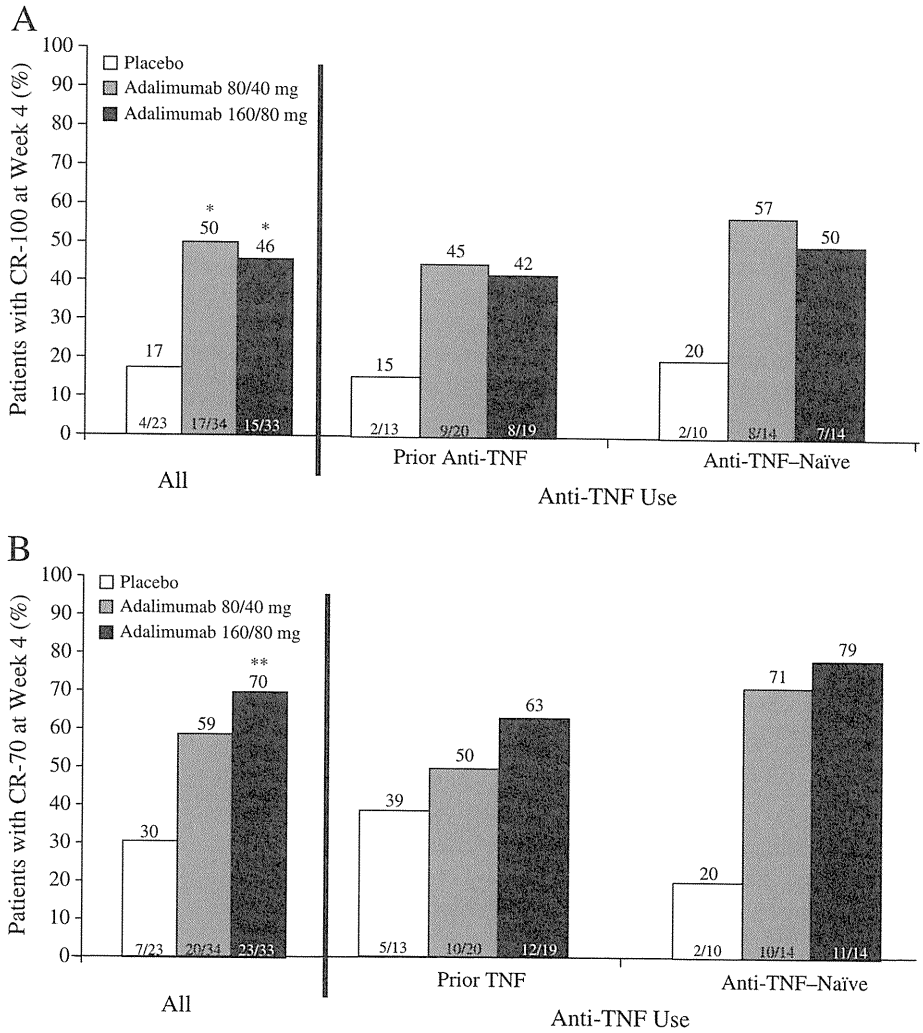


Figure 4 Clinical response during the induction trial: (A) CR-100 at Week 4 [All Patients and Stratified by Prior Anti-TNF Exposure]; (B) CR-70 at Week 4 [All Patients and Stratified by Prior Anti-TNF Exposure]. Analyses were conducted using non-responder imputation in the FAS (patients who enrolled in the induction trial, were randomly assigned to 1 of the 3 treatment groups and received at least 1 dose of study drug). *$p<0.05$ versus placebo; **$p=0.0062$ versus placebo; all other p-values>0.05. CR-100 = decrease in CDAI from Baseline $\geq$ 100; CR-70 = decrease in CDAI from Baseline $\geq$ 70; CDAI = Crohn's disease activity index; FAS = full analysis set.

the adalimumab 80/40 mg group and −0.4 and −0.5 in the placebo group.

3.3.2. Maintenance trial

In the mFAS, a significantly greater percentage of adalimumab-treated patients achieved clinical remission at Week 52x compared with placebo ($p<0.05$, Fig. 5A). Adalimumab therapy was more effective than placebo in maintaining clinical remission, CR-100, and CR-70 over the course of the trial (Fig. 5A–C). Eight (16%) randomized patients (5 placebo-treated and 3 adalimumab-treated) were on corticosteroids when entering the maintenance trial (FAS). Of these, 6 patients (4 placebo-treated and 2 adalimumab-treated) were included in the mFAS population. Among these 6 patients, only 1 (adalimumab-treated) was in steroid-free remission at Week 52x (mFAS). Most of the placebo patients in the double-blind portion (20 of 25) relapsed and switched to open-label adalimumab, whereas 14 out of the 25 adalimumab patients moved from the double-blind portion to the open-label portion (Fig. 2).

In the mFAS, the mean changes (using LOCF) in CDAI from Baseline of the induction trial to Week 0x and 52x were −147.7 and −83.7 in the adalimumab-treated patients and −139.0 and −9.1 in the placebo-treated patients, respectively. The mean changes in IOIBD from Baseline of the induction trial to Week 0x and Week 52x were −2.0 and −0.8 in adalimumab-treated patients and −1.2 and −0.2 in placebo-treated patients, respectively.

The dose escalation set consisted of 30 patients receiving open-label treatment in the maintenance trial for whom the adalimumab dose was increased from 40 mg to 80 mg eow after Week 4x. Within this dose escalation set, 13 patients entered the open-label portion of the maintenance trial at Week 0x and 17 patients moved to the open-label portion after switching from the double-blind portion of the maintenance trial. In the dose escalation set, the percentage of patients who achieved clinical remission, CR-100 or CR-70

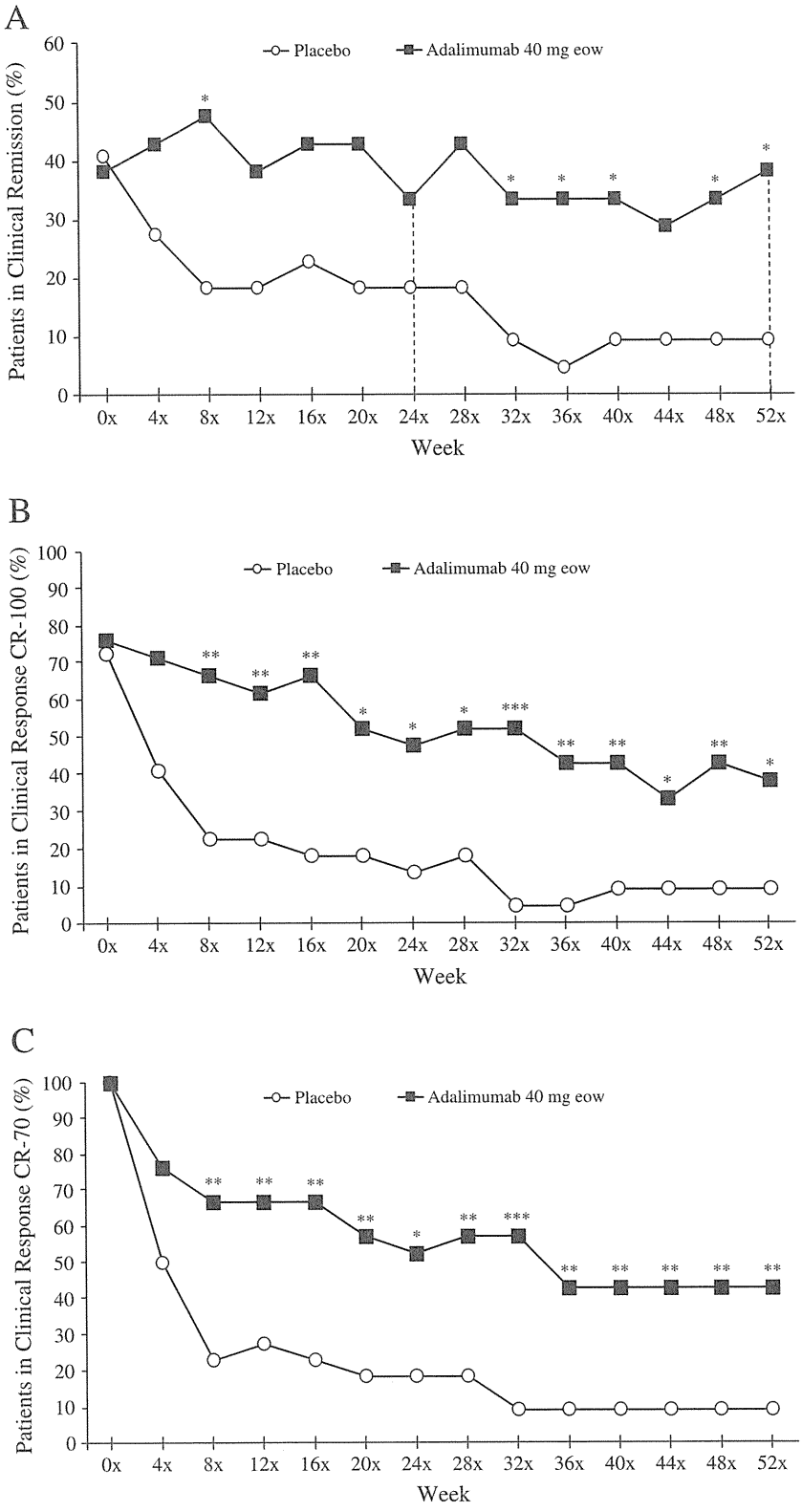


Figure 5 (A) Clinical remission, (B) Clinical response CR-100, and (C) Clinical response CR-70 over time in the maintenance trial. Clinical remission is defined as CDAI<150. Analyses were conducted using non-responder imputation in the mFAS (patients who received adalimumab in the induction trial, entered the double-blind portion of maintenance trial and received at least 1 dose of study drug); n=22 for placebo; n=21 for adalimumab. The "x" beside the week number designates the follow-on nature of the maintenance trial. *$p<0.05$, **$p<0.01$; ***$p<0.001$; all other p-values>0.05. CR-100 = decrease in CDAI from Baseline≥100; CR-70 = decrease in CDAI from Baseline≥70; CDAI = Crohn's disease activity index; mFAS = modified full analysis set; eow = every other week.

at least once after dose escalation was 20.0%, 40.0%, and 53.3%, respectively. The percentage of patients who showed clinical remission, CR-100 or CR-70 at the time of the last dose was 16.7%, 33.3%, and 40.0%, respectively.

3.4. Quality of life

3.4.1. Induction trial

Induction therapy with adalimumab 160/80 mg or 80/40 mg significantly improved SF-36 MCS from Baseline to Week 4 compared with placebo. The mean change from Baseline for adalimumab 160/80 mg was 6.2 versus −1.6 for placebo ($p=0.0005$); for adalimumab 80/40 mg the mean change from Baseline was 5.5 versus −1.6 for placebo ($p=0.0002$). There was a trend toward a greater improvement in SF-36 PCS and IBDQ for patients in the adalimumab 160/80 mg group compared to placebo, although the changes were not significant

3.4.2. Maintenance trial

In the mFAS, adalimumab 40 mg eow treatment led to significantly greater improvement from Week 0 to Week 8x in SF-36 MCS and IBDQ compared with placebo (12.0 versus 2.0, $p=0.03$ and 34.8 versus 8.3, $p=0.05$, respectively); improvement was sustained. Although the difference between the 2 groups was not statistically significant through Week 52x, the improvement in SF-MCS scores from Week 0 to Week 52x was 9.6 with adalimumab 40 mg eow treatment versus 0.3 for placebo. The improvement in IBDQ from Week 0 to Week 52x was 27.9 for adalimumab-treated patients versus 1.8 for placebo-treated patients. The average IBDQ score at Week 52x for adalimumab-treated patients was 170.1, which is above the threshold of remission (170), whereas the average IBDQ at Week 52x for placebo-treated patients is below the remission threshold (Fig. 6).

SF-36 PCS scores did not differ significantly between the 2 treatment groups at Week 52x. However, from Week 0x through Week 52x, patients treated with adalimumab were able to maintain the Week 0x improvement of 5.8 while patients treated with placebo showed a decrease in improvement from 2.4 at Week 0x to 0.2 at Week 52x.

3.5. Pharmacokinetics and immunogenicity

Overall, during the induction phase the mean serum adalimumab concentrations from the adalimumab 160/80 mg dose groups were approximately twice those of the adalimumab 80/40 mg group (Fig. 7A). In the maintenance trial, serum concentrations in patients who remained on adalimumab 40 mg eow through Week 52x (n=15) remained relatively constant from Week 4x to Week 52x (Fig. 7B–C). Mean serum concentrations tended to increase in patients who increased from adalimumab 40 mg eow to 80 mg eow (Fig. 7B–C).

The overall AAA+ rate was 0% in the induction trial, and 6.1% in the maintenance trial (5 of 82 patients who received at least 1 dose of adalimumab in the trial). In the 5 patients who developed AAA, including 1 patient who increased from adalimumab 40 mg to 80 mg, the serum adalimumab concentrations decreased to below or near detectable limits of the assay by the end of the maintenance trial. Three of the 5 patients discontinued early from the trial due to adverse events (abdominal abscess in 1 patient, liver abscess in 1 patient, aggravation of CD in 1 patient) and 2 patients were not in clinical remission at Week 52x. None of the 5 patients were treated with concomitant immunosuppressant therapy.

3.6. Changes in C-reactive protein

Baseline CRP values from the induction and maintenance trials are reported in Table 1. The mean change in CRP values from Baseline to Week 52x was 0.4±0.7 mg/dL for placebo-treated versus −1.3±1.2 mg/dL for adalimumab-treated patients (mFAS).

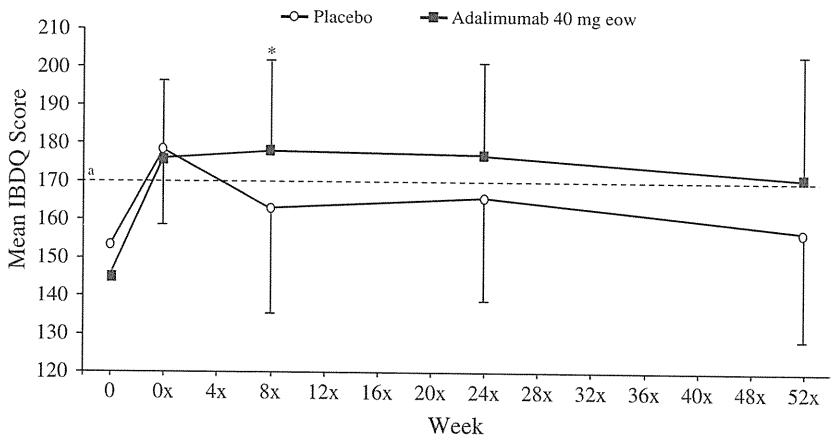


Figure 6 Mean IBDQ scores by week in the maintenance trial. Analyses were conducted using last observation carried forward in the mFAS (patients who received adalimumab in the induction trial, entered the double-blind portion of maintenance trial and received at least 1 dose of study drug); n=22 for placebo; n=21 for adalimumab. Week 0 is Baseline of the induction trial; Week 0x is Baseline of the maintenance trial; "x" beside the week number designates the follow-on nature of the maintenance trial. *$p=0.05$ versus placebo; all other p-values>0.05. [a]Cutoff point for IBDQ remission (IBDQ score≥170). mFAS=modified full analysis set; eow = every other week; IBDQ = Inflammatory Bowel Disease Questionnaire.

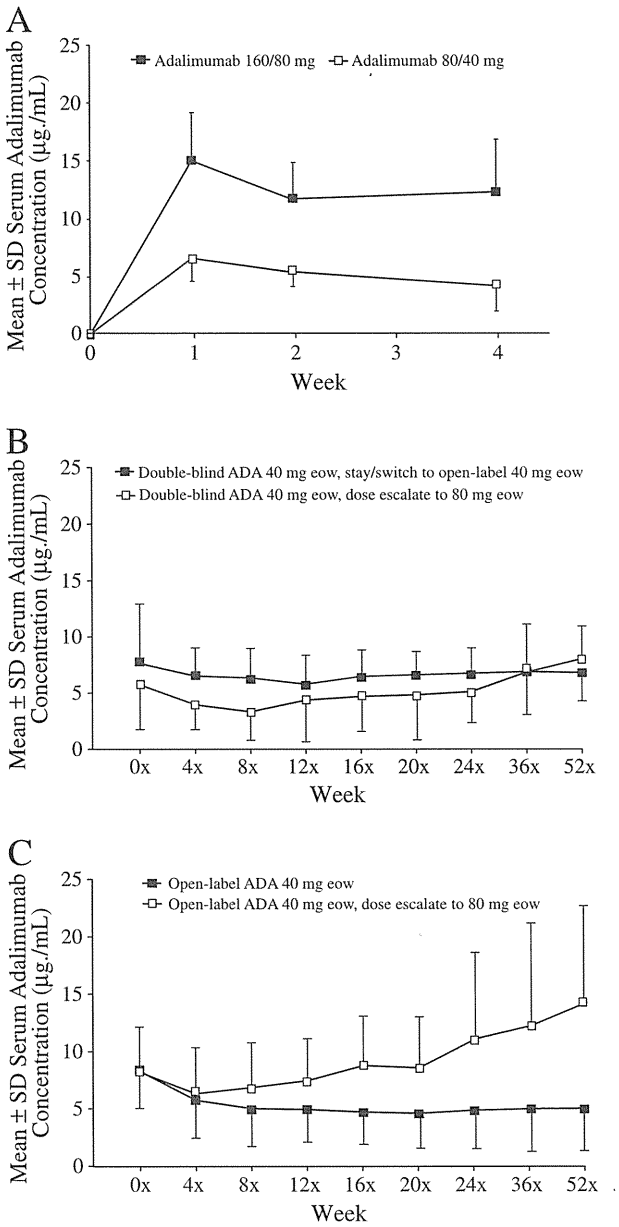


Figure 7 (A) Mean serum adalimumab concentrations in the (A) Induction trial; (B) Double-blind phase of the maintenance trial and (C) Open-label phase of the maintenance trial. Week 0 is Baseline of the induction trial; Week 0x is Baseline of the maintenance trial; "x" beside the week number designates the follow-on nature of the maintenance trial. n = 33 for adalimumab 160/80 mg; n = 34 for adalimumab 80/40 mg; n = 15 for double-blind adalimumab 40 mg eow, stay/switch to open-label adalimumab 40 mg eow; n = 10 for double-blind adalimumab 40 mg eow, dose escalate to adalimumab 80 mg eow; n = 19 for open-label adalimumab 40 mg eow; n = 13 for open-label 40 mg eow, dose escalate to 80 mg eow. ADA = adalimumab; eow = every other week; SD = standard deviation.

3.7. Safety

Serious adverse events (SAEs) were reported in 6.0% of adalimumab-treated patients versus 8.7% in the placebo group of the induction trial (FAS), and 8.0% of adalimumab-treated patients versus 24.0% in the placebo group of the maintenance trial (FAS) (Table 3). Most SAEs were related to the underlying CD. No deaths, lupus-like syndromes, demyelinating diseases, or cases of tuberculosis were reported in either trial. The incidence of injection site pain was low and similar in all groups in both trials.

4. Discussion

The purpose of the present trials was to demonstrate that efficacy of adalimumab was numerically greater than placebo for the treatment of Japanese patients with CD. Due to the low prevalence of CD in Japan, these trials were of small size, however, were expected to demonstrate similar trends with regard to efficacy and safety of adalimumab as previously shown in Western patients.

Induction treatment with adalimumab increased remission and response rates at Week 4 compared with placebo treatment in Japanese patients with moderate to severe CD. The effect was more pronounced at the higher adalimumab dose (160/80 mg). In Japanese patients previously exposed to anti-TNF, only adalimumab 160/80 mg was able to induce clinical remission at Week 4. The results of this trial are consistent with induction results of adalimumab in Western patients from the CLASSIC I and GAIN clinical trials.[5,16]

Maintenance treatment with adalimumab increased the clinical remission and response rates at Week 52x compared with placebo treatment in this trial. These results are similar to maintenance results from the CHARM clinical trial.[9]

Only 1 maintenance regimen of adalimumab, 40 mg eow, was tested in a parallel blinded design in the maintenance trial. However, the efficacy of adalimumab 80 mg eow was explored in patients that had an inadequate response to adalimumab 40 mg eow in the open-label portion of the maintenance trial. From the 90 patients included at Week 0 of induction trial, 30 patients subsequently moved to the open-label arm and increased their dose of adalimumab to 80 mg eow, which corresponds to the reported dose-escalation rate of 27% in Western patients.[17] More than half of the Japanese patients who increased to adalimumab 80 mg eow achieved a clinical response CR-70, which is similar to data from Western studies in which 63% of the patients who increased to adalimumab 40 mg weekly dosing achieved CR-70 at any time after dose escalation.[17]

Adalimumab therapy resulted in a trend toward an improvement in quality of life, as measured by SF-36 MCS, SF-36 PCS, and IBDQ scores, in Japanese patients evaluated in these trials. The improvement of quality of life was most pronounced for SF-36 MCS and IBDQ scores.

Serum adalimumab concentrations reached a steady state following induction, and remained relatively constant from Week 4x to Week 52x in the maintenance trial, as was previously observed in studies of adalimumab in Western patients with CD.[5,16] The rate of immunogenicity through 1 year of treatment with adalimumab in the Japanese patients enrolled in these trials (6.1%) was slightly higher than the 2.6% rate observed in the CLASSIC II clinical trial in Western patients with CD.[18] A trend towards a lower plasma level of adalimumab and a higher rate of AAAs in Japanese patients compared with Western patients has already been noted in rheumatoid arthritis.[19] The difference of AAA

Table 3 Treatment-emergent adverse events. [a]

	Induction trial			Maintenance trial		Any ADA set [d]
	Full analysis set [b]			Full analysis set [c]		Total N=90
	Placebo n=23	Adalimumab 80/40 mg n=34	Adalimumab 160/80 mg n=33	Placebo n=25	Adalimumab 40 mg eow n=25	
All adverse events	12 (52.2)	20 (58.8)	17 (51.5)	21 (84.0)	20 (80.0)	85 (94.4)
Serious adverse events	2 (8.7)	3 (8.8)	1 (3.0)	6 (24.0)	2 (8.0)	35 (38.9) [e]
Adverse events leading to discontinuation of therapy	1 (4.3)	2 (5.9)	1 (3.0)	6 (24.0)	1 (4.0)	21 (23.3)
Any infection	2 (8.7)	5 (14.7)	4 (12.1)	9 (36.0)	15 (60.0)	65 (72.2)
Serious infection	0 (0.0)	1 (2.9) [f]	0 (0.0)	2 (8.0)	1 (4.0)	6 (6.7)
Malignant adverse events	0 (0.0)	1 (2.9)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.1)
Injection-site-related pain	2 (8.7)	5 (14.7)	4 (12.1)	1 (4.0)	2 (8.0)	16 (17.8)
Related opportunistic infections (excluding tuberculosis)	0 (0.0)	0 (0.0)	1 (3.0)	0 (0.0)	0 (0.0)	1 (1.1)
Related congestive heart failure	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Demyelinating disease	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Tuberculosis	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Related allergic reactions	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (2.2)
Lupus-like syndrome	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Death	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

Data are n (%).
ADA = adalimumab; eow = every other week.
[a] In the induction and maintenance trials, a treatment-emergent AE (TEAE) was defined as any AE with onset from 1st dose of study drug, with differing end points depending on whether the patient continued in the adalimumab development program or not. Specifically, for patients who continued in the adalimumab development program, the end-date for reporting TEAEs was at the trial end (Week 4 or 8 for induction trial; Week 52x for maintenance trial); for patients who discontinued the trial and did not continue in adalimumab development program, the end-date for reporting TEAEs was up to 70 days following the last study drug administration. For analysis in the any adalimumab set (combines induction and maintenance trial), a TEAE was defined as any AE with onset from 1st dose of adalimumab the patient ever received (which could have been in the induction trial for those randomized to adalimumab, or in the maintenance trial for those randomized to placebo in the induction trial) through an end-date up to the last dose received in the maintenance trial if the patient continued after Week 52x, or up to 70 days after the last dose of adalimumab if the patient discontinued either trial. Of note, for the any adalimumab set, events that occurred during the placebo period prior to receipt of any adalimumab were excluded.
[b] Patients enrolled in the induction trial who were randomly assigned to 1 of the 3 treatment groups and received at least 1 dose of study drug.
[c] Patients who entered the double-blind portion of the maintenance trial and received at least 1 dose of study drug.
[d] Patients who received at least 1 dose of adalimumab either, in the induction trial or, in the maintenance trial.
[e] SAEs included: 6 SAEs occurred in ≥2 patients, aggravation of Crohn's disease (20 patients), abdominal abscess (5 patients), ileus (2 patients), intestinal obstruction (2 patients), peritonitis (2 patients) malnutrition (2 patients). No SAE of malignancy, demyelinating disorder, tuberculosis was reported.
[f] Abdominal abscess developed 25 days after the last dose of study drug, considered by the investigator to be probably not related to adalimumab.

production between Japanese and Western patients may relate to the different distribution in the IgG1 allotype $GM1^{za}$ across racial groups; 84% of Japanese carry the allotype $GM1^{za}$, compared with only 29% of Western Europeans. Circulating adalimumab, which is an IgG1 $GM1^{za}$, may reach a more optimal integration into the immunoregulatory network in $GM1^{za}$-carrying patients, and cause a higher rate of physiological anti-idiotypic antibodies.[20]

Adalimumab therapy was well-tolerated, and no deaths were reported in either the induction or maintenance trial. Adverse event profiles were similar to those reported in other clinical trials of adalimumab in Western patients with CD.

The similarity of the clinical remission and response rates in these small trials to the results of larger pivotal clinical trials of adalimumab in CD supports the use of adalimumab for induction and maintenance of remission in Japanese patients with moderate to severely active CD. However, because of the limited number of patients enrolled, these trials lacked statistical power to conclusively demonstrate the efficacy of adalimumab in this population. A further limitation of these trials was the exclusion of patients who had previously been exposed to but had failed to respond to infliximab (primary non-responders). The response of such Japanese patients to adalimumab treatment cannot be estimated from the results. Nevertheless, the results of these 2 trials in Japanese patients with CD are consistent with results reported in prior clinical trials of adalimumab. Our findings show that adalimumab is an effective and well-tolerated therapeutic option for inducing and maintaining clinical remission of moderate to severe CD in this population.

Conflict of interest statement

Disclosures of financial conflict of interest are summarized below:

- M Watanabe: Consulting and/or other fees from Abbott Japan, Ajinomoto Pharma, Asahi Kasei Kurary Medical, Eisai, JIMRO, Kyorin Pharmaceutical, Mitsubishi Tanabe Pharma, Otsuka Pharmaceutical, UCB Japan, ZERIA Pharmaceutical
- T Hibi: Consulting and/or other fees from Abbott Japan, Ajinomoto Pharma, Asahi Kasei Kurary Medical, AstraZeneca, Eisai, Janssen, JIMRO, Kyorin Pharmaceutical, Mitsubishi Tanabe Pharma, Mochida Pharmaceutical, Otsuka Pharmaceutical, UCB
- Japan, ZERIA Pharmaceutical
- KG Lomax: Employee of Abbott
- SK Paulson: Employee of Abbott
- J Chao: Employee of Abbott
- MS Alam: Former employee of Abbott
- A Camez: Employee of Abbott.

Acknowledgements

Sources of funding and role of the funding source: This study was sponsored by Abbott Japan Co, Ltd., Tokyo, Japan and Eisai Co, Ltd., Tokyo, Japan. *Contributors*: The authors wish to thank Ashish Kumar, Ph.D., for conducting the statistical analysis and reviewing the manuscript; financial support for this service was provided by Abbott. The authors also wish to thank Eileen M. Burkart-Hartman, Ph.D., and Laurinda A. Cooker, Ph.D., of Abbott, for their assistance in the writing and editing of this manuscript. Support for this manuscript was funded by Abbott. The authors would like to thank the investigators who participated in these trials: S Motoya, Hokkaido P.W.F.A.C Sapporo-Kosei General Hospital; Y Kinouchi, Tohoku University Hospital; Y Suzuki, Toho University Sakura Medical Center; T Katsuno, Chiba University Hospital; K Ohtsuka, Showa University Northern Yokohama Hospital; S Okamura, Toyohashi Municipal Hospital; I Hirata, Fujita Health University Hospital; T Ando, Nagoya University Hospital; H Nakase, Kyoto University Hospital; N Oshitani, K Watanabe, Osaka City University Hospital; H Ito, Kitano Hospital, The Tazuke Kofukai Medical Research Institute; T Matsumoto, The Hospital of Hyogo College of Medicine; S Tanaka, Hiroshima University Hospital; H Inoue, Kagawa University Hospital; Y Sakai, Matsuyama Red Cross Hospital; T Matsumoto, Kyushu University Hospital; K Mitsuyama, Kurume University Hospital; N Tomiyasu, H Kumemura, Omuta City General Hospital; F Kinjo, University of the Ryukyus; T Hisamatsu, Keio University School of Medicine; T Matsui, Fukuoka University Chikushi Hospital; K Shiratori, Tokyo Women's Medical University Hospital; M Miyata, M Mizuno, Aichi Medical University Hospital; Y Fujiyama, A Andoh, Shiga University of Medical Science Hospital; J Kato, Okayama University Hospital; T Ashida, M Fujiya, Asahikawa Medical College Hospital; T Ohkusa, T Osada, Juntendo University Hospital; M Takazoe, Social Insurance Central General Hospital; H Hanai, Hamamatsu South Hospital; T Ichimori, Susaki Kuroshio Hospital; K Okawa, Osaka City General Hospital. *Statements of Authorship*: MW participated in the study design, carried out the studies, analyzed the data, and helped draft the manuscript. TH participated in the study design, carried out the studies, analyzed the data, and helped draft the manuscript. KGL analyzed the data and helped draft the manuscript. SKP analyzed the data and helped draft the manuscript. JC analyzed the data and helped draft the manuscript. MSA participated in the study design, analyzed the data, and helped draft the manuscript. AC participated in the study design, carried out the studies, analyzed and reported the data, and helped draft the manuscript. All authors read and approved the final manuscript.

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RUNX3 copy number predicts the development of UC-associated colorectal cancer

TOSHIAKI WATANABE[1], TAKASHI KOBUNAI[1,2], HIROKI IKEUCHI[3], YOKO YAMAMOTO[1], KEIJI MATSUDA[1], SOICHIRO ISHIHARA[1], KEIJIRO NOZAWA[1], HISAE IINUMA[1], TAKAMITSU KANAZAWA[4], TOSHIAKI TANAKA[4], TADASHI YOKOYAMA[4], TSUYOSHI KONISHI[4], KIYOSHI ESHIMA[2], YOICHI AJIOKA[5], TOSHIFUMI HIBI[6], MAMORU WATANABE[7], TETSUICHIRO MUTO[8] and HIROKAZU NAGAWA[4]

[1]Department of Surgery, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605; [2]Tokushima Research Center, Taiho Pharmaceutical Co., Ltd., 224-2 Ebisuno, Hiraishi, Kawauchi-cho, Tokushima 771-0194; [3]Second Department of Surgery, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501; [4]Department of Surgical Oncology, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655; [5]Department of Pathology, Niigata Univeristy, 754 Ichibancho, Asahimachidori, Niigata 951-8510; [6]Division of Gastroenterology, Department of Internal Medicine, Keio University School of Medicine, Tokyo 160-8582; [7]Department of Gastroenterology, Tokyo Medical and Dental Univeristy, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8501; [8]The Cancer Institute Hospital of Japanese Foundation for Cancer Research, 3-10-6 Ariake, Kotoh-ku, Tokyo 135-8550, Japan

Received July 12, 2010; Accepted September 2, 2010

DOI: 10.3892/ijo_00000839

Abstract. *RUNX3* is a tumour suppressor gene that plays an important role in the development of various cancers. The present study aimed to compare *RUNX3* mRNA expression levels and DNA copy numbers in the non-neoplastic rectal mucosa between ulcerative colitis (UC) patients with and without UC-associated colorectal cancer (UC-Ca). We further aimed to build a predictive model of the development of UC-Ca based on the *RUNX3* DNA copy number. *RUNX3* mRNA expression levels were quantified by RT-PCR. The hypermethylation and DNA copy number of *RUNX3* were also determined. Thirty-five UC patients were examined, 17 of whom had UC-Ca (UC-Ca group) and 18 who did not (UC-NonCa group). The UC-Ca group had significantly lower mRUNX3 expression levels and smaller DNA copy numbers than the UC-NonCa group (p=0.04, p=0.0016, respectively). *RUNX3* expression levels correlated with DNA copy numbers. Classification of the UC-Ca and UC-NonCa group based on DNA copy number gave an accuracy of 82.9%. *RUNX3* expression levels in the non-neoplastic rectal mucosa was significantly decreased in the UC-Ca group and it is suggested that this was attributable to the decrease in *RUNX3* DNA copy number. The present predictive model may be useful in the selection of high risk UC-Ca patients and to improve the efficacy of surveillance colonoscopy. The present study suggests that *RUNX3* might play an important role in the development of UC-Ca.

Correspondence to: Dr Toshiaki Watanabe, Department of Surgery, Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan
E-mail: toshwatanabe@yahoo.co.jp

Key words: ulcerative colitis, colorectal cancer, RUNX3, copy number, prediction

Introduction

Patients with long-standing ulcerative colitis (UC) have an increased risk of developing colorectal cancer. This risk increases with the duration of the disease, and the estimated cumulative risk of UC-associated colorectal cancer (UC-Ca) 30 years after the onset of UC has been reported by meta-analysis to be 18% (1). Therefore, for the early detection of neoplastic lesions, surveillance colonoscopy is recommended for patients whose duration of the disease is 7-8 years or more (2-4). However, to further improve the efficacy of surveillance, new markers to predict the development of UC-Ca are urgently needed.

Previous studies have shown that patients with UC-Ca have widespread genetic alterations in the non-neoplastic colonic mucosa, suggesting that such changes might be effective predictors of UC-Ca development (5-8). Indeed, we previously demonstrated this by DNA microarray gene expression analysis (9). However, to date, specific molecular markers that can be used in clinical settings have not been established.

RUNX3 belongs to the Runt domain family of transcription factors, and is involved in T-cell differentiation, the TGF-ß-induced tumour suppressor pathway (10,11) and is a known tumour suppressor gene in gastric cancer (12). Previous studies

reported that alterations in *RUNX3* gene expression were present in various types of cancer including breast, lung, hepatocellular, prostate, bile duct, pancreatic and colon cancers (10-12). Studies have also shown that *RUNX3* gene expression levels are influenced by the hypermethylation of CpG islands in the *RUNX3* promoter region (13-19). On the other hand, a recent report suggested a role for *RUNX3* in UC susceptibility, the development of colitis and gastric mucosal hyperplasia (20,21). Weersma *et al* showed that *RUNX3* mRNA expression is increased in the colonic mucosa of UC patients compared with controls (20).

In our previous microarray analysis, *RUNX3* was expressed at lower levels in the non-neoplastic mucosa of patients with UC-Ca compared to those without (9). However, microarray analysis is known to lack reproducibility as a means of quantitative analysis of gene expression. In order to accurately validate gene expression levels, it requires verification by an alternative quantitative approach, such as real-time PCR (22,23). Another problem with microarray analysis is the large number of genes used in prediction models. In our previous model, gene expression levels of 72 genes were examined to predict the development of UC-Ca. Because of these problems, the use of microarrays in clinical practice remains limited, and recent studies have shown that RT-PCR analyses of a small number of selected genes are useful in predicting the outcome of patients in various diseases (22,23).

Therefore, in the present study, we aimed to confirm the decreased expression of *RUNX3* by RT-PCR in patients with UC-Ca. We also analysed possible mechanisms for decreased *RUNX3* expression by examining the hypermethylation of CpG islands in the promotor region, and *RUNX3* DNA copy numbers. Lastly, we aimed to use this copy number to establish a predictive model of patients with UC-Ca.

Materials and methods

Patients and samples. Thirty-five UC patients were examined. Informed consent was obtained from all patients for the collection of specimens, and the study protocol was approved by the local Ethics Committee. All UC patients had total colitis and their disease had persisted for more than seven years; they were therefore considered to be at high risk of cancer and/or dysplasia development. Among the 35 UC patients, 17 had UC-associated adenocarcinoma (UC-Ca group) and 18 had no neoplastic lesions (UC-NonCa group). Patient characteristics are shown in Table I.

In all UC-Cases, specimens were obtained from non-neoplastic rectal mucosa for DNA and RNA extraction (Fig. 1). Samples were taken either from surgically resected specimens or during surveillance colonoscopic examination. Samples were snap-frozen in liquid nitrogen and stored at -80°C until use. Paralleled tumour specimens were formalin-fixed and paraffin-embedded for histological examination as described previously (24). Microscopic examination of rectal mucosa in UC patients verified that no neoplastic cells were present in any of the samples.

RNA isolation and RT-PCR. Total RNA was isolated from frozen samples using the RNeasy Mini kit (Qiagen, Valencia, CA). Gene expression levels were determined using TaqMan real-time PCR (Applied Biosystems, Foster City, CA) as described previously (Fig. 1) (25). First-strand cDNA was synthesized from total RNA using the High Capacity cDNA Archive kit (Applied Biosystems) in 50 μl reactions using cDNA samples for TaqMan real-time PCR analysis according to the manufacturer's instructions. cDNA (10 ng/μl) was added to 9.15 μl RNase-free water, 12.5 μl 2 x TaqMan Universal PCR Master Mix (Applied Biosystems) and 1.25 μl 20 x Primer Probe mix. ACTB (actin, ß) was used as the endogenous control gene (Applied Biosystems). Assay IDs were Hs00231709_m1 and Hs99999903_m1 for RUNX3 and ACTB, respectively. PCR amplification was carried out using the Prism 7900HT Sequence Detection System (Applied Biosystems) under the following thermal cycler conditions: 2 min at 50°C and 10 min at 94.5°C for 40 cycles (30 sec at 97°C and 1 min at 59.7°C). Relative *RUNX3* gene expression was calculated by comparing the δ CT values as previously described (26).

Genomic DNA preparation and RUNX3 methylation-specific PCR. Hypermethylation of the promoter region of the *RUNX3* gene was determined by methylation-specific PCR. Genomic DNA was extracted using the QIAAMP DNA mini kit (Qiagen), according to the manufacturer's instructions. Bisulfite conversion of DNA samples was carried out essentially as described and was based on the principle that treatment of DNA with bisulfite would result in the conversion of unmethylated cytosine residues into uracil. Methylated cytosine residues, on the other hand, would remain unchanged (19). Thus, the DNA sequences of methylated and unmethylated genomic regions following bisulfite conversion would be different and distinguishable by sequence-specific PCR primers.

Bisulfite conversion was carried out using an EZ DNA methylation kit (ZYMO research, Orange, CA). DNA (2 μg) was treated with sodium bisulfite following the manufacturer's recommendations and resuspended in a total volume of 10 μl. Hypermethylated DNA treated with Sss I and normal genomic DNA were used as methylation-positive and -negative control samples, respectively, in the following PCR assay: sense and antisense primers for the bisulfite-converted methylated sequence were: mRUNX3-F (5'-TTA CGA GGG GGG GTC GTA CGC GGG-3') and mRUNX3-R (5'-AAA ACG ACC GAC GCG AAC GCC TCC-3') respectively. Sense and antisense primers for the bisulfite-converted unmethylated sequence were: uRUNX3-F (5'-TTA TGA GGG GTG GTT GTA TGT GGG-3') and uRUNX3-R (5'-AAA ACA ACC AAC ACA AAC ACC TCC-3'), respectively (19).

Real-time PCR was performed on a PRISM 7900 sequence detector (Applied Biosystems) using a QuantiTect SYBR Green kit (Qiagen). Conditions for quantitative PCR were as follows: one cycle at 94°C for 15 min, 45 cycles at 94°C for 20 sec, 56°C for 20 sec, and 70°C for 30 sec. The methylation status was judged from the calculated delta Ct value derived from the methylated and unmethylated sequence primer CT values.

Genomic DNA preparation and quantitative real-time PCR for determination of the RUNX3 DNA copy number. The *RUNX3* DNA copy number was determined by real-time PCR.

Table I. Characteristics of patients.

Characteristic	CUC-Ca group (n=17)	UC-NonCa group (n=18)	P-value
Age at biopsy (years)			NS
Mean, SD	54.3 (34-70), 10.5	48.8 (25-70), 11.8	
Age at UC diagnosis (years)			NS
Mean, SD	36.6 (15-52), 13.1	31.4 (15-55), 9.8	
Gender			NS
Male	12	10	
Female	5	8	
Duration of disease (years)			NS
Mean, SD	17.7 (9-29), 6.8	14.8 (9-25), 6.9	
Extent of UC			NS
Total colitis	15	16	
Left-sided colitis	2	2	
Proctosigmoiditis	0	0	
Proctitis	0	0	
Inflammation			NS
None	4	3	
Mild	12	12	
Moderate	1	3	
Severe	0	0	
Medication			NS
Mesalamine	12	11	
Corticosteroids3	5		
6MP/AZA	2	2	
CSA	0	0	
PSC			NS
Present	0	0	
Not present	17	18	

SD, standard deviation; UC, ulcerative colitis; 6MP/AZA, 6-mercaptopurine/azathioprine; CSA, cyclosporin A; PSC, primary sclerosing cholangitis.

Genomic DNA was extracted from samples using the QIAAMP DNA mini kit (Qiagen) as before. Quantitative real-time PCR was performed on a PRISM 7900 sequence detector with a QuantiTect SYBR Green kit as before. We quantified the DNA by comparing the target locus to the reference Line-1, a repetitive element for which copy numbers per haploid genome are similar among all human normal and neoplastic cells. The relative target copy number level was also normalized to normal human genomic DNA as a calibrator. Copy number changes of the target gene relative to Line-1 and the calibrator were determined using the formula $(T_{target}/T_{Line-1})/(C_{target}/C_{Line-1})$, where T_{target} and T_{Line-1} are quantities from tumour DNA using target and Line-1, and C_{target} and C_{Line-1} are quantities from the calibrator using target and Line-1. PCRs for each primer set were performed in triplicate or more, and mean values and standard deviations reported (19,26).

Conditions for quantitative PCR were as above. At the end of the PCR reaction, samples were subjected to melting analysis to confirm amplicon specificity. PrimerExpress software, ver.2.0 was used to design the primers to span a 200 bp non-repetitive region, and primers were then synthesized by Operon Biotechnologies Inc. (Tokyo, Japan). The primer set was subsequently compared with the human genome using a basic local alignment search tool algorithm to determine its uniqueness. Primer sequences for *RUNX3* are: Forward primer (intron 3), 5'-CCAACCACCTGCCTCT ATTCC-3'; Reverse primer (exon 4), 5'-TTGGTGAACACA GTGATGGTCA-3'.

Prediction of UC-associated neoplasm development by RUNX3 mRNA expression and DNA copy number. Areas under the receiver operator characteristic (ROC) curve (AUCs) were