pathological grade and number of adenomas at the baseline colonoscopy have been reported to be associated with the risk of adenoma recurrence.[24,28–30] However, there are few easily and objectively quantifiable predictors of the likelihood of adenoma recurrence. In this study, the association between the number of ACF in the rectum at the baseline polypectomy and the likelihood of detecting recurrent adenomas at a surveillance colonoscopy performed 3 years later were examined; the recurrence rate at 3 years after the baseline polypectomy was 48.8%. At the baseline colonoscopy, a relationship between the risk of adenoma recurrence and the size, pathological grade and number of adenomas was observed. Also, the association between the risk of adenoma recurrence and the number of ACF at the baseline in the rectum was investigated. Some human studies have revealed the presence of a higher number of ACF in subjects with synchronous advanced adenoma.[11–13,35] The median number of ACF in this study was comparable to that in previous studies in which ACF were counted in the same region of the rectum, the lower rectal region.[11] However, another study counted ACF in a larger region (i.e. the distal 20 cm of the colon) and reported a higher number of ACF.[36] Therefore, the measurement protocol for ACF needs to be unified in the future.

A greater number of ACF was observed in patients who eventually developed colorectal adenoma recurrence than in those who did not. This novel finding suggests that the number of ACF could serve as a useful predictor of the likelihood of colorectal adenoma recurrence. These results also indicate that patients with a larger number of ACF in the lower rectum may be at a higher risk of developing colorectal adenoma recurrence, and therefore, close surveillance colonoscopy is needed for these patients. ACF can be easily and safely measured, within a few minutes, after colorectal polypectomy, and their number can be easily and objectively quantified. Therefore, we suggest that the number of ACF in the rectum could serve as a novel predictor of the likelihood of adenoma recurrence.

Our study had some limitations. Firstly, although all the ACF were recorded on video and evaluated by two independent endoscopists who were unaware of the subjects' clinical histories, the operators were not blinded to their clinical histories. Secondly, no pathological confirmation of ACF was undertaken in this study. In addition, we did not perform surveillance colonoscopy for patients without any adenomas at the baseline colonoscopy.

In conclusion, our study showed that the number of ACF in the rectum was correlated with the likelihood of colorectal adenoma recurrence. These results suggest that the number of ACF is a useful factor for predicting the likelihood of adenoma recurrence, and patients with a large number of ACF detected postpolypectomy need close surveillance colonoscopy. Furthermore, our results indicate that ACF may be advantageous as surrogate lesions of colorectal carcinogenesis.

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STUDY PROTOCOL Open Access

Metformin efficacy and safety for colorectal polyps: a double-blind randomized controlled trial

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Abstract

Background: Colorectal cancer is one of the major neoplasms and a leading cause of cancer death worldwide, and new preventive strategies are needed to lower the burden of this disease. Metformin, a biguanide, which is widely used for treating diabetes mellitus, has recently been suggestive to have a suppressive effect on tumorigenesis and cancer cell growth. In a previous study conducted in non-diabetic subjects, we showed that oral short-term low-dose metformin suppressed the development of colorectal aberrant crypt foci (ACF). ACF have been considered as a useful surrogate biomarker of CRC, although the biological significance of these lesions remains controversial. We devised a prospective randomized controlled trial to evaluate the chemopreventive effect of metformin against metachronous colorectal polyps and the safety of this drug in non-diabetic post-polypectomy patients.

Methods/Design: This study is a multi-center, double-blind, placebo-controlled, randomized controlled trial to be conducted in non-diabetic patients with a recent history of undergoing colorectal polypectomy. All adult patients visiting the Yokohama City University hospital or affiliated hospitals for polypectomy shall be recruited for the study. Eligible patients will then be allocated randomly into either one of two groups: the metformin group and the placebo group. Patients in the metformin group shall receive oral metformin at 250 mg per day, and those in the placebo group shall receive an oral placebo tablet. At the end of 1 year of administration of metformin/placebo, colonoscopy will be performed to evaluate the polyp formation.

Discussion: This is the first study proposed to explore the effect of metformin against colorectal polyp formation. Metformin activates AMPK, which inhibits the mammalian target of rapamycin (mTOR) pathway. The mTOR pathway plays an important role in the cellular protein translational machinery and cell proliferation. Patients with type 2 diabetes taking under treatment with metformin have been reported to be at a lower risk of cancer development than those not taking under treatment with metformin. We showed in a previous study that metformin suppressed the formation of human colorectal ACF. We therefore decided to conduct a study to determine whether metformin might suppress the formation of human colorectal polyps.

Trial registration: This trial has been registered in the University hospital Medical Information Network (UMIN) Clinical Trials Registry as UMIN000006254

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Background

Colorectal cancer (CRC) is a major neoplasm worldwide [1], and both its prevalence and mortality have been increasing [2]. Removal of colorectal polyps has been to shown to reduce the risk of future development of colorectal cancer and advanced adenoma [3,4]. On the other hand, patients with polyps (adenomas and/or hyperplastic polyps) constitute a high-risk group for the development of metachronous colorectal polyps and/or CRC [5]. Therefore, a paradigm shift from surveillance for early detection of cancer or adenomas (polypectomy) to new strategies for prevention, including chemoprevention, is needed to lower the burden of this disease. Several large epidemiologic and/or clinical studies have evaluated the possible preventive effects of more than 200 agents, including fiber, calcium, and non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin and selective cyclooxygenase-2 (COX-2) inhibitors, in protecting against CRC development [6]. NSAIDs, especially COX-2 inhibitors, administered either alone or in combination with other agents, have shown the most promise, until date, for CRC risk reduction [4], although reports have revealed an increased risk of serious cardiovascular events associated with the use of COX-2 inhibitors [7,8]. In light of the adverse cardiovascular effects of COX-2 inhibitors and lack of demonstrable efficacy of the other agents that had initially shown promise in this setting, novel drugs that would be both safe and effective for CRC prevention need to be developed. CRC is associated with lifestyle-related diseases, such as diabetes mellitus and obesity [9-12], therefore, we considered that these conditions might represent potential new targets for CRC chemoprevention.

Metformin (1,1-dimethylbiguanide hydrochloride) is a biguanide derivative that has long been used widely for treating diabetes mellitus [13]. It decreases basal glucose output by suppressing gluconeogenesis and glycogenolysis in the liver and increasing glucose uptake by the muscle. Because metformin does not directly stimulate insulin secretion, it is associated with a lower risk of hypoglycemia than other oral antidiabetic drugs [14]. The molecular mechanism involved in the action of metformin is liver kinase B-1dependent activation of AMP-activated protein kinase (AMPK) [15]. Patients with type 2 diabetes under treatment with metformin have been reported to be at a lower risk of cancer development (including CRC) than those not under treatment with metformin [16,17]. This evidence suggests that metformin might be a candidate agent for CRC chemoprevention in diabetic patients. However, since diabetes mellitus itself is a risk factor for cancer, treatment of diabetes mellitus may reduce the risk; therefore, it is still unclear whether the suppressive effect of metformin against CRC may be exerted by the direct chemopreventive effect of the drug or be mediated by its antidiabetic effect. Therefore, we considered that in order to validate the chemopreventive effect of metformin, a clinical trial in nondiabetic patients needs to be conducted.

In previous studies, we demonstrated the chemopreventive effect of metformin in two rodent models (a genetic model and a chemically-induced cancer model) and one human study of colorectal carcinogenesis. We showed that metformin suppressed the development of intestinal polyps in adenomatous polyposis coli (APC$^{Min/+}$) mise, a murine model of familial adenomatous polyposis [18]; furthermore, we showed that metformin suppressed azoxymethane-induced formation of colorectal aberrant crypt foci (ACF) by activating AMPK [19]. Both studies were performed in nondiabetic mice, which suggested the direct chemopreventive potential of metformin per se. In the study conducted on nondiabetic human subjects, we showed that oral low-dose administration of metformin (250 mg per day) suppressed the formation of colorectal ACF and that the drug was safe [20]. ACF are considered as a reliable surrogate biomarker of CRC [21], although their biological significance still remains controversial. Therefore, in CRC chemoprevention trials, in general, the incidence of polyps or of the cancer itself is set as the study endpoint. Although the incidence rate of CRC would be the most reliable endpoint, use of this endpoint would be unsuitable for chemoprevention trials, because of the relatively low occurrence rate of CRC in the general population [22] and the long-term observation period that it would necessitate. Moreover, observation of polyps detected in annual colonoscopies until they grow into cancer would be fraught with ethical problems. Thus, we set the appearance of colorectal polyps as a suitable endpoint for our chemopreventive trial.

Thus, we devised a prospective randomized controlled trial to evaluate the chemopreventive effect of metformin against the development of metachronous colorectal polyps and the safety of this drug in nondiabetic postpolypectomy patients.

This is the first clinical trial of metformin as a chemopreventive agent against metachronous colorectal polyps in humans.

Methods/Design

Study design and setting

This study is designed as a multi-center, double-blind, placebo-control, randomized controlled trial to be performed in nondiabetic patients with a recent history of undergoing colorectal polypectomy. The study will take place at the Division of Gastroenterology, Yokohama City University Hospital, and its 5 affiliate hospitals. The coordinating office shall be at Yokohama City University

Hospital, with the registration, randomized allocation and data collection to be conducted at this site.

Ethical considerations and registration

The study protocol is in compliance with the Declaration of Helsinki [23] and the Ethics Guidelines for Clinical Research published by the Ministry of Health, Labour, and Welfare, Japan [24]. We obtained approval for this study from the Ethics committee of Yokohama City University Hospital on July 8th 2011. The protocol and informed consent forms were approved by the institutional ethics committee at each of the participating institutions. This trial has been registered in the University hospital Medical Information Network (UMIN) Clinical Trials Registry as UMIN000006254. Written informed consent for participation in the study will be obtained from all the participating patients. The trial results will be reported in conformity with the Consolidated Standards of Reporting Trials (CONSORT) 2010 guidelines [25].

Eligibility criteria

All adult patients visiting the hospital for polypectomy will be recruited for the study.

The inclusion criteria are as follows:

1) No colorectal polyps present after the polypectomy

2) Age 40 to 80 years as on the date of informed consent

3) Willingness to provide written informed consent

The likelihood of development of colorectal polyps in the young is low and the diagnosis history of polyps in the young is usually related to familial adenomatous polyposis or hereditary non-polyposis colorectal cancer; on the other hand, the elderly have various complications. This was the rationale for our setting the age criterion for inclusion in this study as 40 to 80 years.

The exclusion criteria are as follows:

1) History of diabetes mellitus (use of medication and/or HbA1c over 6.5%)

2) History of regular use (defined as at least once per week) of NSAIDs and/or aspirin

3) History of bowel surgery

4) History of malignant disease (excluding carcinoma in adenoma, carcinoma in situ that has already been resected)

5) History of heart failure, renal failure, liver cirrhosis or chronic hepatic failure

6) History of familial adenomatous polyposis

7) History of hereditary non-polyposis colorectal cancer

8) History of inflammatory bowel disease

9) Pregnancy or possibility of pregnancy

10) Patients judged as inappropriate candidates for the trial by the investigators

Intervention

All eligible patients will be allocated randomly to one of two groups, the metformin group and the placebo group. Endoscopists, doctors at the follow-up outpatient clinics and patients will be blinded to the allocation. Patients in the metformin group shall receive oral metformin at 250 mg per day, and those in the placebo group shall receive oral placebo tablet. At the end of 1 year of administration of metformin/placebo, colonoscopy will be performed to evaluate the polyp formation.

Outcome measurements

The primary endpoint shall be the prevalence of colorectal polyps and number of polyps after 1-year's intervention. The endoscopic examinations and polypectomies will be performed using Olympus colonoscopes (model H260AZI). The day before the endoscopy, each patient will be instructed to consume a low-residue diet and shall receive 5 mg of oral sodium picosulfate. On the day of the endoscopy, the patients shall receive 2000 ml of polyethylene glycol (PEG). If the feces are not sufficiently clear, an additional 1000 - 2000 ml of PEG may be given to ensure sufficient bowel cleaning. At the time of the polypectomies, the endoscope shall be inserted into the cecum, and the entire colorectum will be carefully observed as the endoscope is pulled back. If any polyps are detected, polypectomy will be performed. At the end of 1 year of administration of metformin/placebo, the same endoscopists will perform the repeat endoscopic examinations. If a polyp(s) is detected at the repeat colonoscopy (after treatment for one year), a biopsy will be performed. A total of 6 endoscopists from Yokohama City University Hospital and the 5 affiliate hospitals will perform the polypectomies and endoscopic examinations. All procedures will be recorded on DVD, and all the polyps will be photographed. The number of polyps in each patient will first be counted by the operators during the performance of the colonoscopy. To further ensure validity, the number of polyps will be counted again through observation of the recorded DVD by 3 blinded expert endoscopists (H.T, H.E, and E.S). If these expert endoscopists judge the colonoscopic examination as having been inadequate in any case, that case will be excluded. The biopsied polyps will be evaluated by expert pathologists (Y.N and S.Y).

The secondary outcomes are (1) the drug safety; adverse events will be monitored by the doctor at every follow-up visit to the outpatient clinic. Adverse events will be graded according to the National Cancer Institute Common Toxicity Criteria for Adverse Events (NCI-CTCAE), version 4.0. Diarrhea, the most frequent adverse event related to use of metformin, will be watched for spontaneous resolution within a few days and/or managed by antiflatulent drugs. If Grade 3 or more severe adverse events appear,

the follow-up doctor shall report it (them) to the coordinating office and the case will be withdrawn from the study at that point; (2) laboratory data (fasting blood glucose, fasting blood insulin, HbA1c, total cholesterol, LDL-cholesterol, blood urea nitrogen (BUN), creatinine); (3) the number of ACF. At the time of the 1-year colonoscopy and polypectomy, the lower rectal region from the middle Houston valve to the dentate line will be washed thoroughly with water, sprayed with 0.25% methylene blue, which would be left to stand for 2 min, then again washed thoroughly with water. The number of rectal ACF will be counted with a magnifying endoscope [21]; (4) physical examination findings (body weight, body mass index (BMI)). Metformin is widely used as an antidiabetic drug that improves insulin resistance. The effect of metformin on insulin resistance and the plasma lipid profile will be evaluated by comparing these parameters measured at the baseline and at 1 year in the metformin group and placebo group. All participants will receive physical examination and laboratory tests at the time of the 1-year endoscopic examination and polypectomy; (5) effects of metformin on the cell-proliferative and apoptotic activities in the rectal epithelium and polyps (if any). Colonic epithelial samples will be obtained from the same trial patients by biopsy at the time of the 1-year colonoscopy and polypectomy. The cell-proliferative activity will be evaluated by staining for the proliferative cell nuclear antigen (PCNA), and the cell-apoptotic activity by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) method; (6) expression levels of protein (AMPK, mTOR/S6K) in the rectal epithelium and polyps (if any) that are thought to be pharmacological targets of metformin. The expression levels of these proteins shall be determined by western blot analysis.

Randomization

The investigator shall convey the patient's details to the central registration centre via fax. After an eligibility check, the patients will be randomly assigned to receive metformin or placebo at the central registration centre by a computer program, using a minimization method, with stratification by institute, age, gender and BMI. In this way, the patient assignment will be concealed from the investigator. The randomization center will allocate a numbered treatment pack to each patient, which will contain all the drugs or placebos needed to complete a course of the trial treatment for one patient.

Drug supply

Metformin will be purchased from Dainippon Sumitomo Parma Co., Ltd. The placebo (250 mg lactose) will be purchased from Kokando Co., Ltd, Toyama, Japan. All trial drugs will be packaged identically and identified only by number. Subjects will be instructed to take one tablet of the trial drug after breakfast every day, and to visit the hospital every 4 weeks for evaluation of the subjective symptoms and for receiving a new supply of medication. Compliance will be monitored by counting the empty drug packages returned by the patients at every visit to the outpatient clinic.

Sample size estimation

We previously showed that metformin administered at 250 mg/d for 1 month directly suppressed the formation of ACF. In that study, mean number of ACF per patient decreased significantly from 8.78 ± 6.45 (baseline) to 5.11 ± 4.99 (at 1 month, $p = 0.007$) [20]. Based on a similar study, Takayama et al. reported that sulindac administration at 300 mg/d for 2 months to post-polypectomy patients suppressed ACF formation, decreasing the number of ACF from 7.70 ± 4.04 (baseline) to 4.00 ± 2.95 (at 2 months, $p < 0.001$) [26]. From these reports, we estimated that metformin and sulindac may have equivalent effect on suppression of ACF formation. Moreover, from the same study, Takayama et al. reported that in the post-polypectmoy patients who received 2-months' intervention, the number of polyps (hyperplastic polyp and adenoma) at 1 year after the treatment was significantly lower in the sulindac group in comparison with that in the placebo group (26/48 (54.2%) vs. 15/48 (31.3%), $p = 0.025$) [26]. Therefore, we speculated that 1-year's treatment with metformin might yield equivalent suppression of metachronous polyp formation to that with sulindac. To detect the reduction in the number of metachronous polyps in the metformin group using the chi-square test with a two-sided significance level of 5% and a power of 80%, it was found that a sample size of 68 patients per group would be necessary. Assuming a 10% dropout rate, we propose to recruit 75 patients per group, that is, a total of 150 patients.

Statistical analysis

The prevalence of polyps in each group, the primary endpoint, will be compared between the metformin group and placebo group by the chi-square test. The safety, one of the secondary endpoints, will be similarly compared by the chi-square test. The remaining results in the two groups will be compared by the Mann-Whitney U test or Student's t test. A P value of < 0.05 shall be regarded as indicative of significance. The analysis will be performed using SPSS, version 17.0 (SPSS Inc., Chicago, Il.).

Trial steering committee and data monitoring committee

The Trial Steering Committee and Data Monitoring Committee shall be located at the Department of Clinical Research, Yokohama City University School of Medicine. The committee shall consist of three people: Yutaka

Natsumeda, M.D., Satoshi Inoue, M.D., and Yukiharu Yamaguchi, Ph.D. The Management Team will monitor the trial progress status and data by face-to-face and/or telephonic contact with each of the six sites every month.

Study flow

A flow chart of the study is shown in Figure 1.

Discussion

This is the first study proposed to explore the effect of metformin against colorectal polyp formation. Metformin activates AMPK, which inhibits the mammalian target of rapamycin (mTOR) pathway [15]. The mTOR pathway plays an important role in the cellular protein translational machinery and cell proliferation [27]. The best-characterized downstream effector of mTOR is S6 kinase, which regulates the initiation and elongation phases of translation [28]. Activation of the mTOR pathway has been shown to accelerate cell cycle progression from G1 to S in CRC DLD-1 cells [29]. Therefore, AMPK activation may inhibit cell growth and proliferation by suppressing protein synthesis, thereby having a potent antiproliferative effect. Recent evidence indicates that metformin has a suppressive effect on tumorigenesis and cancer cell growth [30-32]. In one study, metformin was demonstrated to activate AMPK and consequently decrease cellular proliferative activity, to produce a general decrease in protein synthesis in vitro in human breast carcinoma cells [30]. Metformin was also shown to inhibit the proliferation of human prostate cancer cells [32].

This trial may have the following limitations. First, we do not propose to conduct a dose-response study of the effect of metformin on colorectal polyp formation. Until now, trails of metformin for cancer prevention and adjuvant treatment have been conducted using high-dose metformin (500 - 2000 mg per day). However, high-dose metformin use is associated with the risk of development of lactic acidosis and gastrointestinal adverse effects (including diarrhea). Gontier et al. reported from a PET/CT study, that subjects treated with antidiabetic agents, including metformin, showed high and diffuse bowel uptake of 18 F-FDG [33]. This suggests that AMPK is present in abundance in the bowel epithelium and that activation of AMPK by metformin up-regulates the expression of glucose transporters. We showed in a previous study that oral low-dose metformin (250 mg per day) suppressed

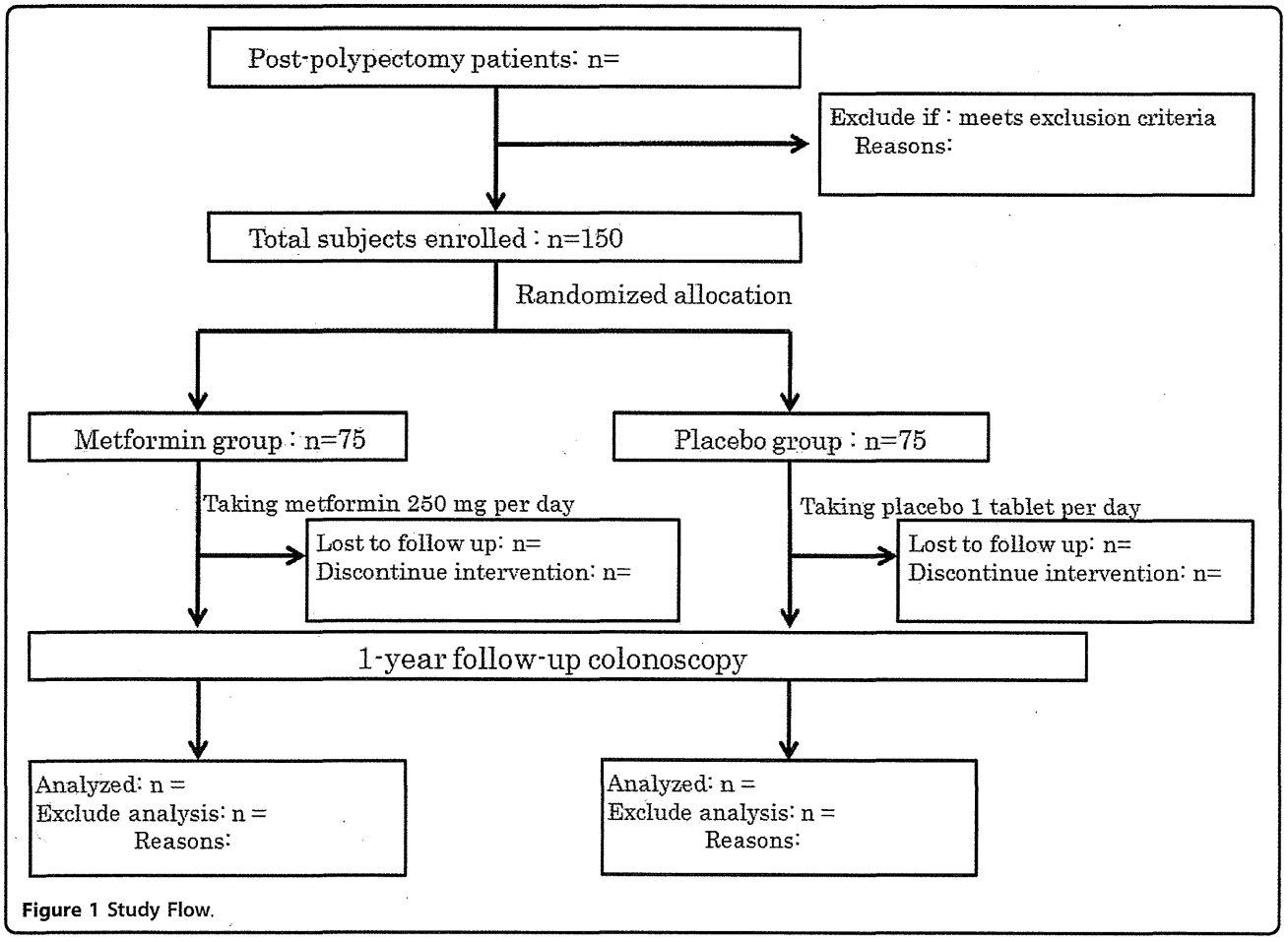

Figure 1 Study Flow.

the formation of human colorectal ACF and was also safe, leading us to surmise that oral low-dose metformin may also be effective for CRC chemoprevention. Therefore, we planned to conduct this trial with low-dose metformin. Second, repeat colonoscopy at 1 year may be too short to allow reliable detection of differences between the groups. However, this is first chemoprevention study of metformin for CRC, long-term administration of placebo to post-polypectomy patients may entail ethical problems. Therefore, this time we planned repeat colonoscopy at 1 year. In order to detect the effect of metformin with less effort, we elected to select participants who had undergone polypectomy for this trial, because these patients constitute a high-risk group for the development of metachronous colorectal polyps and/or CRC [5]. And after safety of chronic metformin administration for non-diabetic patients is confirmed, we would like to follow up the participants of this trial, and conduct long-term chemoprevention trial for CRC.

If metformin were found to be effective for the prevention of CRC, the impact would be extremely large. We consider it of interest, therefore, to determine whether metformin might suppress the formation of human colorectal polyps.

Abbreviations

CRC: Colorectal cancer; NSAIDs: Nonsteroidal anti-inflammatory drugs; COX-2: Cyclooxygenase-2; ACF: Aberrant crypt foci; AMPK: AMP-activated protein kinase; PCNA: Proliferative cell nuclear antigen; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling.

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Current study status

This trial began recruiting patients in September 2011 and shall close to recruitment in December 2012. Data collection is due to be completed in December 2013 and results will be published in March 2014.

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Authors' contributions

TH and AN conceived the study. KH conducted feasibility phase work. HT, TU, NH, US, HK and NM shall perform polypectomy and follow-up colonoscopy. HE, HO and ES will make another count of polyps on DVD record to ensure validity. EY, NH, YH, AE, HN and NM shall recruit participants and follow-up at outpatient clinic. Analysis and interpretation of data is being conducted by SM. SY and YI shall carry out pathological analysis. All authors have read and approve of the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Eicosapentaenoic acid (EPA) efficacy for colorectal aberrant crypt foci (ACF): a double-blind randomized controlled trial

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Abstract

Background: Colorectal cancer (CRC) is one of the most commonly occurring neoplasms and a leading cause of cancer death worldwide, and new preventive strategies are needed to lower the burden of this disease. Eicosapentaenoic acid (EPA), the omega-3 polyunsaturated fatty acid that is widely used in the treatment of hyperlipidemia and prevention of cardiovascular disease, has recently been suggested to have a suppressive effect on tumorigenesis and cancer cell growth. In CRC chemoprevention trials, in general, the incidence of polyps or of the cancer itself is set as the study endpoint. Although the incidence rate of CRC would be the most reliable endpoint, use of this endpoint would be unsuitable for chemoprevention trials, because of the relatively low occurrence rate of CRC in the general population and the long-term observation period that it would necessitate. Moreover, there is an ethical problem in conducting long-term trials to determine whether a test drug might be effective or harmful. Aberrant crypt foci (ACF), defined as lesions containing crypts that are larger in diameter and stain more darkly with methylene blue than normal crypts, are considered as a reliable surrogate biomarker of CRC. Thus, we devised a prospective randomized controlled trial as a preliminary study prior to a CRC chemoprevention trial to evaluate the chemopreventive effect of EPA against colorectal ACF formation and the safety of this drug, in patients scheduled for polypectomy.

Methods: This study is a multicenter, double-blind, placebo-controlled, randomized controlled trial to be conducted in patients with both colorectal ACF and colorectal polyps scheduled for polypectomy. Eligible patients shall be recruited for the study and the number of ACF in the rectum counted at the baseline colonoscopy. Then, the participants shall be allocated randomly to either one of two groups, the EPA group and the placebo group. Patients in the EPA group shall receive oral 900-mg EPA capsules thrice daily (total daily dose, 2.7 g per day), and those in the placebo group shall receive oral placebo capsules thrice daily. After one month's treatment with EPA/placebo, colonoscopic examination and polypectomy will be performed to evaluate the formation of ACF, and the cell-proliferative activity and cell-apoptotic activity in normal colorectal mucosa and colorectal polyps.

Discussion: This is the first study proposed to explore the effect of EPA against colorectal ACF formation in humans.

This trial has been registered in the University hospital Medical Information Network (UMIN) Clinical Trials Registry as UMIN000008172.

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Background

Colorectal cancer (CRC) is amongst the most commonly encountered neoplasms worldwide [1], and both its prevalence and mortality have been increasing [2]. Removal of colorectal polyps has been to shown to reduce the risk of future development of colorectal cancer and advanced adenoma [3,4] and to thereby prevent colorectal cancer death [5]. On the other hand, patients with polyps (adenomas and/or hyperplastic polyps) also constitute a high-risk group for the development of metachronous colorectal polyps and/or CRC [6]. Therefore, a paradigm shift from surveillance for early detection of cancer or adenomas and polypectomy to new strategies for prevention, including chemoprevention, is needed to lower the burden of this disease. Several large epidemiologic and/or clinical studies have evaluated the possible effects of more than 200 agents, including fiber, calcium, and non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin and selective cyclooxygenase-2 (COX-2) inhibitors, in protecting against CRC development [7]. Our group previously reported that sulindac, a NSAID, had the effect of suppressing the development of sporadic colorectal adenoma [8]. Until date, NSAIDs, especially COX-2 inhibitors, administered either alone or in combination with other agents, have shown the most promise for CRC risk reduction [4], although reports have revealed an increased risk of serious cardiovascular events associated with the use of COX-2 inhibitors [9,10]. In light of the adverse cardiovascular effects of COX-2 inhibitors and the lack of demonstrable efficacy of the other agents that had initially shown promise in this setting, novel drugs that would be both safe and effective for CRC prevention need to be developed. CRC is known to be associated with lifestyle-related diseases, such as hyperlipidemia, diabetes mellitus and obesity [11-14], therefore, we considered that these conditions might represent potential new targets for CRC chemoprevention.

Eicosapentaenoic acid (EPA) is an omega-3 polyunsaturated fatty acid (PUFA) that has long been used widely for primary and also secondary prevention of cardiovascular diseases [15]. EPA impacts the biological functions of adipocytes via two distinct mechanisms; the first, via transcriptional activation of lipogenic and adipogenic genes by binding to nuclear receptors such as Peroxisome Proliferator Activator Receptors (PPARs),[16] and the second, via direct competition with arachidonic acid (AA) incorporation into membrane phospholipids and subsequent conversion to eicosanoids, including prostaglandins (PGs). [17] Recent reports have indicated a lower incidence of colon, breast and prostate cancers in many human populations, associated with a high dietary consumption of omega-3 PUFAs. Multiple reports using a variety of rodent models of early-stage colorectal carcinogenesis, including azoxymethane- and dimethylhydrazine-induced colorectal tumorigenesis (using aberrant crypt foci (ACF) or colonic tumors as the primary endpoint), as well as the $Apc^{Min/+}$ mouse model of familial adenomatous polyposis (FAP), have demonstrated the efficacy of the free fatty acid (FFA) form of a combination of EPA plus docosahexaenoic acid (DHA) (as fish oil substituted for the base fat source in chow).[18] In humans, a phase-III randomized placebo-controlled trial of EPA-FFA 2 g daily for 6 months was performed in 55 patients with FAP undergoing sigmoidoscopic surveillance of a rectal stump after total colrectomy. [19] Patients in the EPA-FFA arm had a significantly lower (by 22.4%) number of lower rectal polyps and a 29.8% decrease in the sum of the polyp diameters in the tattooed area of the rectum as compared with the placebo group. Importantly, daily administration of EPA-FFA 2 g was safe and well-tolerated. [19] NSAIDs chemoprevention trial set in past, first conducted to FAP patients, then applied to sporadic colorectal adenoma/cancer. Thus, much evidence suggests that EPA might be a candidate agent for CRC chemoprevention. In CRC chemoprevention trials, in general, the incidence of polyps or of the cancer itself is set as the study endpoint. Although the incidence rate of CRC would be the most reliable endpoint, use of this endpoint would be unsuitable for chemoprevention trials, because of the relatively low occurrence rate of CRC in the general population [20] and the long-term observation period that it would necessitate. Moreover, there is an ethical problem in conducting a long-term trial to determine whether a test drug may be effective or harmful.

Aberrant crypt foci (ACF), defined as lesions containing crypts that are larger in diameter and stain more darkly with methylene blue than normal crypts,[21-24] are considered as a reliable surrogate biomarker of CRC. [25] We previously reported the usefulness of ACF as a biological marker of CRC, [26,27] and carried out a chemoprevention trial for colorectal ACF. [28,29] Chemoprevention trials with colorectal ACF set as the primary endpoint may have some advantages. First, a long-term observation period is not needed to evaluate the drug effect. Our group reported the n.[29] Long-term trials need much effort and may expose the study participants to an increased risk of development of carcinoma. Second, ACF can be estimated quantitatively. Thus, we devised a prospective randomized controlled trial to evaluate the chemopreventive effect of EPA against the formation of colorectal ACF as a preliminary study prior to CRC chemoprevention trials.

This is the first clinical trial of EPA as a chemopreventive agent against colorectal ACF in humans.

Methods/design

Study design and setting

This study is designed as a multicenter, double-blind, placebo-controlled, randomized controlled trial to be

performed in patients with colorectal ACF. It will be conducted at the Division of Gastroenterology, Yokohama City University Hospital, and its affiliate hospital, Chigasaki Municipal Hospital and Yokohama Rosai Hospital. The coordinating office shall be at the Yokohama City University Hospital, and the registration, randomized allocation and data collection shall be conducted at this site.

Ethical considerations and registration

The study protocol is in compliance with the Declaration of Helsinki [30] and the Ethics Guidelines for Clinical Research published by the Ministry of Health, Labour, and Welfare, Japan [31]. We obtained approval for this study from the Ethics committee of Yokohama City University Hospital on May 10, 2012. The protocol and informed consent forms were approved by the institutional ethics committee at each of the participating institutions. This trial has been registered in the University hospital Medical Information Network (UMIN) Clinical Trials Registry as UMIN000008172. Written informed consent for participation in the study will be obtained from all the participating patients. The trial results will be reported in conformity with the Consolidated Standards of Reporting Trials (CONSORT) 2010 guidelines [32].

Eligibility criteria

Patients with both colorectal ACF and resectable polyps will be recruited for the study.

The proposed inclusion criteria are as follows:

1) Age 40 to 80 years as on the date of informed consent.
2) Willingness to provide written informed consent.

The proposed exclusion criteria are as follows:

1) History of regular use of omega-3 PUFA supplements.
2) History of regular use (defined as at least once per week) of NSAIDs and/or aspirin.
3) History of heart failure, renal failure, liver cirrhosis or chronic hepatic failure.
4) History of familial adenomatous polyposis.
5) History of hereditary non-polyposis colorectal cancer.
6) History of inflammatory bowel disease.
7) Pregnancy or possibility of pregnancy.
8) Patients judged as being inappropriate candidates for the trial by the investigators.

Intervention

All eligible patients will be allocated randomly to one of two groups, the EPA group and the placebo group. The endoscopists, doctors at the follow-up outpatient clinics, and patients will be blinded to the allocation. Patients in the EPA group shall receive oral 900-mg EPA capsules thrice daily (total daily dose, 2.7 g), and those in the placebo group shall receive oral placebo capsules thrice a day. At the end of 1 month of administration of EPA/placebo, polypectomy will be performed, and the changes in the number of ACF and in the mucosa will be evaluated.

Outcome measurements

The primary endpoint shall be the change in the number of colorectal ACF after 1-months's intervention. The endoscopic examinations and polypectomies will be performed using Olympus colonoscopes (model H260AZI). Bowel preparation prior to the colonoscopic procedures will be as described [33,34]. At the time of the first colonoscopy, the endoscope shall be inserted into the cecum, and the entire colorectum will be carefully observed as the endoscope is pulled back. If any polyps are detected, biopsy will be performed. Furthermore, colonic epithelial samples will be obtained. The number of rectal ACF will be counted with a magnifying endoscope, as described [25,33]. At the end of 1 month of administration of EPA/placebo, the same endoscopists will perform the polypectomy and counting of the ACF. All procedures will be recorded on DVD, and all the ACF will be photographed. The number of ACF in each patient will first be counted by the operators during the performance of the colonoscopy. To further ensure validity, the number of ACF will be counted again through observation of the recorded DVD by 3 blinded expert endoscopists (H.T, H.E, and E.S). If these expert endoscopists judge the colonoscopic examination as having been inadequate in any case, that case will be excluded.

The secondary outcomes shall be (1) the drug safety; adverse events (AEs) will be graded according to the National Cancer Institute Common Toxicity Criteria for Adverse Events (NCI-CTCAE), version 4.0. All study participants shall be provided with a study diary in order to record the daily dosage of the study treatment and the AEs. Patients developing grade 3 or more severe adverse events will be withdrawn from the study at that point; (2) Mucosal fatty acid analysis: Homogenization, extraction and derivatization of the rectal mucosa and polyp fatty acids (EPA, DHA, docosapentaenoic acid (DPA), AA, linolenic acid, linoleic acid, palmitic acid, stearic acid, etc.) shall be performed as described [35]. Fatty acid content shall be determined by gas chromatography–mass spectrometry and expressed as the percentage of the total fatty acid content [36,37]. (3) Effects of EPA on the cell-proliferative and apoptotic activities in the rectal epithelium and polyps: Colonic epithelial samples will be obtained from the same trial patients by biopsy at the time of the first colonoscopy and

polypectomy. The cell-proliferative activity will be evaluated by staining for the proliferative cell nuclear antigen (PCNA) and estimation of the Ki-67 labeling indices, and the cell-apoptotic activity by the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) method. (4) Laboratory data (HDL cholesterol, LDL cholesterol, triglycerides, fatty acid fractions, fasting blood glucose, fasting blood insulin, HbA1c, blood urea nitrogen (BUN), creatinine); (5) physical examination findings (body weight, body mass index (BMI)). EPA is widely used as an antihyperlipidemic drug that improves the plasma lipid profile. The effect of EPA on the plasma lipid profile will be evaluated by comparing these parameters measured at the baseline with those measured after 1 month of treatment in the EPA group and placebo group. All participants will undergo a physical examination and laboratory tests at the time of the baseline endoscopic examination and polypectomy.

Randomization

The investigator shall convey the patient's details to the central registration center via fax. After an eligibility check, the patients will be randomly assigned to receive EPA or placebo at the central registration center by a computer program using a minimization method, with stratification by age, gender, BMI, and institution. Thus, the patient assignment will be concealed from the investigator. The randomization center will allocate a numbered treatment pack to each patient, which will contain all the drugs or placebos needed to complete a course of the trial treatment for that patient.

Drug supply

Enteric-coated EPA capsules (Ethyl icosapentate granular capsule®) and the placebo capsules (capric, caprylic and lauric acid medium-chain triglycerides) will be purchased from Nipro Pharma Corporation Co., Ltd, Osaka, Japan. All trial drugs will be packaged identically and identified only by number. Subjects will be instructed to take one package of the trial drug after every meal each day. Compliance will be monitored by counting the empty drug packages returned by the patients at polypectomy.

Sample size estimation

In the chemoprevention trial conducted in FAP patients, the NSAID sulindac and selective cyclooxygenase-2 (COX-2) inhibitor celecoxib reduced polyposis of the retained rectum after colectomy with ileorectal anastamosis (IRA). As previously noted, EPA has a suppressive effect for polyp formation and proliferation of FAP [19]. From these reports, we estimated that NSAIDs and EPA may have equivalent effect on suppression of polyp formation and proliferation.

Based on the target in the NSAIDs chemoprevention study for ACF, Takayama et al. reported that sulindac administration at 300 mg/d for 2 months to post-polypectomy patients suppressed ACF formation, decreasing the number of ACF from 7.70 ± 4.04 (baseline) to 4.00 ± 2.95 (at 2 months, $p < 0.001$) [33]. Presuming EPA and sulindac may have equivalent effect in suppressing ACF formation, to detect the reduction in the number of ACFs in the EPA group using the Mann–Whitney U test with a two-sided significance level of 5% and a power of 80%, it was estimated that a sample size of 12 patients per group would be necessary. Assuming a 10% dropout rate, we propose to recruit 15 patients per group, that is, a total of 30 patients.

Statistical analysis

The number of ACFs in each group, the primary endpoint, will be compared between the EPA group and the placebo group by the Mann–Whitney U test. The safety, one of the secondary endpoints, will be compared by the chi-square test. The remaining results in the two groups will be compared by the Mann–Whitney U test or Student's t test. A P values of < 0.05 will be regarded as indicative of significance. The analysis will be performed using SPSS, version 17.0 (SPSS Inc., Chicago, Il.).

Trial Steering Committee and Data Monitoring Committee

The Trial Steering Committee and Data Monitoring Committee shall be located at the Department of Gastroenterology, Kanto Medical Center, NTT East. The committee shall consist of three people: Nobuyuki Matsuhashi, M.D., Toshio Fujisawa, M.D., and Jun Hamanaka, M.D. The Management Team will monitor the trial progress status and data by face-to-face and/or telephonic contact with each of the sites every month.

Study flow

A flow chart of the study is shown in Figure 1.

Discussion

This is the first study proposed to explore the effect of EPA against colorectal ACF formation. Knowledge of the mechanisms underlying the anti-neoplastic activity of EPA remains nebulous. Current understanding of the mechanistic aspects of the anticancer activity of EPA has been reviewed in detail in published reviews [38-41]. In general, the major mechanisms proposed to underlie the anti-neoplastic activities of EPA; (1) modulation of COX activity, (2) alteration of the membrane dynamics and cell surface receptor function, and (3) increased cellular oxidative stress. However, the in vivo relevance of each of the above putative mechanisms and their relative

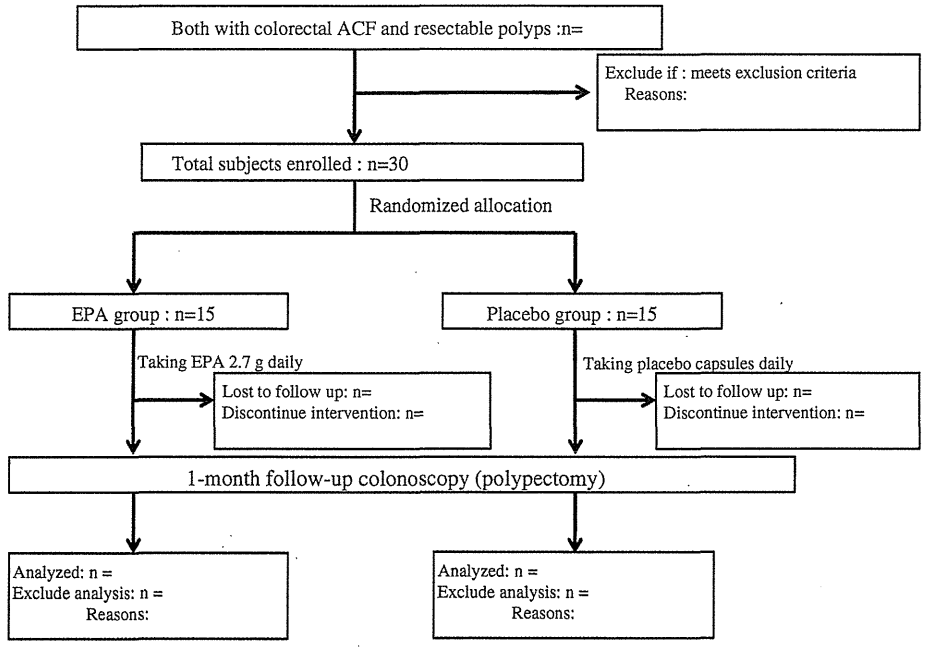

Figure 1 Study Flow.

contributions to the anticancer activity of EPA remain unclear. Several in-vitro studies have explored the antineoplastic activity of omega-3 PUFAs against human CRC cells, and EPA treatment has been shown to reduce cellular proliferative activity and increase cellular apoptotic activity [42-45]. EPA can act as an alternative substrate for COX-2, instead of AA, leading to a reduction in the formation of the pro-tumorigenic '2-series' PGs (e.g., PGE_2) in favor of the 'three-series' PGs (e.g., PGE_3) in several cell types, including CRC cells [46-48]. Furthermore, incorporation of EPA into the cell phospholipid membrane alters the fluidity, structure, and/or function of the lipid rafts or calveolae [49], which are sphingolipid- and cholesterol-rich microdomains that float freely in the cell membrane. The localization of cell surface receptors, such as epidermal growth factor receptor (EGFR) [50], in lipid rafts is believed to be crucial for downstream receptor signaling, controlling proliferation and apoptosis [51,52]. Furthermore, EPA may exert an antineoplastic effect through alteration of the cellular redox state and of the oxidative stress exposure of the cells. PUFAs are highly peroxidizable, which generates reactive oxygen species (ROS), such as superoxide radicals. Many tumor cells display altered cellular pathways for the handling of ROS, including depletion of the major intracellular antioxidant, glutathione. Subsequent elevation of the intracellular ROS levels by EPA has been hypothesized to induce cancer cell apoptosis [53].

This trial may have the following limitations. First, ACF are considered as a reliable surrogate biomarker of CRC, [21] although their biological significance still remains controversial. In CRC chemoprevention trials, in general, the incidence of polyps or of the cancer itself is set as the study endpoint. Although the incidence rate of CRC would be the most reliable endpoint, use of this endpoint would be unsuitable for chemoprevention trials, because of the relatively low occurrence rate of CRC in the general population [18] and the long-term observation period that it would necessitate. We previously reported the usefulness of ACF as a biological marker of CRC [26,27] and carried out a chemoprevention trial for colorectal ACF [28,29]. Thus, we devised a trial using this endpoint (ACF) to evaluate the chemopreventive effect of EPA. Second, we do not propose to conduct a dose–response study in respect of the effect of EPA on ACF formation. Until now, trials of EPA for cancer prevention and adjuvant treatment have been conducted using EPA at doses of 1000 – 4000 mg per day. In Japan, the EPA drug product specification is 0.9 g, and 2.7 g of EPA has been commonly used and very well tolerated. Therefore, we planned to conduct this trial using 2.7 g of EPA per day. Third, an intervention period of 1 month may be too short to allow reliable detection of differences between the groups. However, we showed in a previous study that oral administration of metformin for 1 month suppressed the formation of colorectal ACF in humans [29]. If the intervention agents had a chemopreventive effect, an intervention period of 1 month would be sufficient to evaluate the changes in the number of ACF.

We previously conducted a short-term chemoprevention trial of metformin for colorectal ACF, and showed

the suppressive effect of the drug on the formation of ACF. Thereafter, we are conducting a long-term metformin chemoprevention trial for colorectal polyps, the trial registered in the UMIN Clinical Trials Registry as UMIN000006254 [34]. We propose to repeat the same step for the chemoprevention trial using EPA.

If EPA were found to be effective for the prevention of CRC, the impact would be extremely large. We consider it of interest, therefore, to determine whether EPA might suppress the formation of human colorectal ACFs.

Abbreviations

CRC: Colorectal cancer; NSAIDs: Nonsteroidal anti-inflammatory drugs; COX-2: Cyclooxygenase-2; ACF: Aberrant crypt foci; EPA: Eicosapentaenoic acid; PUFA: Polyunsaturated fatty acid; AA: Arachidonic acid; PG: Prostaglandin; FAP: Familial adenomatous polyposis; FFA: Free fatty acid (FFA); DHA: Docosahexaenoic acid; PCNA: Proliferative cell nuclear antigen; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling.

Competing interests

None of the authors has any financial interests relevant to this trial to disclose.

Authors' contributions

TH and AN conceived the study. HT, TU and AE shall perform the baseline colonoscopy and polypectomy. HE, HO and ES will conduct another count of ACF on a DVD recording to ensure its validity. HI, SU, SM and HN shall recruit participants and follow-up at outpatient clinic. EY and KH shall carry out the pathological analyses. Analysis and interpretation of data will be conducted by YN and SM. All the authors have read the final manuscript and approve of its submission for publication.

Current study status

This trial began recruiting patients in June 2012 and shall complete recruitment in December 2012. Data collection is due to be completed in March 2013, and the results are scheduled to be published in June 2013.

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Independent genetic control of early and late stages of chemically induced skin tumors in a cross of a Japanese wild-derived inbred mouse strain, MSM/Ms

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MSM/Ms is an inbred mouse strain derived from a Japanese wild mouse, *Mus musculus molossinus*. In this study, we showed that MSM/Ms mice exhibit dominant resistance when crossed with susceptible FVB/N mice and subjected to the two-stage skin carcinogenesis protocol using 7,12-dimethylbenz(a)anthracene (DMBA)/ 12-*O*-tetradecanoylphorbol-13-acetate (TPA). A series of F1 backcross mice were generated by crossing $p53^{+/+}$ or $p53^{+/-}$ F1 (FVB/N × MSM/Ms) males with FVB/N female mice. These generated 228 backcross animals, approximately half of which were $p53^{+/-}$, enabling us to search for *p53*-dependent skin tumor modifier genes. Highly significant linkage for papilloma multiplicity was found on chromosomes 6 and 7 and suggestive linkage was found on chromosomes 3, 5 and 12. Furthermore, in order to identify stage-dependent linkage loci we classified tumors into three categories (<2 mm, 2–6 mm and >6 mm), and did linkage analysis. The same locus on chromosome 7 showed strong linkage in groups with <2 mm or 2–6 mm papillomas. No linkage was detected on chromosome 7 to papillomas >6 mm, but a different locus on chromosome 4 showed strong linkage both to papillomas >6 mm and to carcinomas. This locus, which maps near the *Cdkn2a/p19*^{Arf} gene, was entirely *p53*-dependent, and was not seen in $p53^{+/-}$ backcross animals. Suggestive linkage conferring susceptibility to carcinoma was also found on chromosome 5. These results clearly suggest distinct loci regulate each stage of tumorigenesis, some of which are *p53*-dependent.

Introduction

The identification of specific genetic variants responsible for increased susceptibility to familial or sporadic cancers has major implications for prediction of individual cancer risk, as well as for development of strategies for prevention or targeted therapy (1,2). Mouse models of cancer have been extensively used for the analysis of the genetic basis of cancer susceptibility, and have led to the identification of multiple loci that confer, either alone or in specific combinations, an increased cancer risk (3–8).

Inbred strains that were derived recently from mice obtained from the wild are often more resistant to carcinogens and to several types of pathogens compared with the commonly used inbred strains. More specifically, *Mus spretus* shows dominant resistance to several cancers (e.g. skin cancer (9), lung cancer (10) and thymic lymphomas (11)). Exploiting the resistance of *M.spretus* to the two-stage skin carcinogenesis model involving 7,12-dimethylbenz(a)anthracene (DMBA) initiation and subsequent promotion with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), 15 skin tumor susceptibility loci, *Skts1-15*, were identified in an interspecific F1 backcross [(NIH/Ola × *M.spretus*) × NIH/Ola] (NSP) using quantitative trait locus (QTL) analysis (9,12). The *Skts1* locus was also identified in Car-R and Car-S crosses (13), which were generated by the balanced intercross of several inbred strains, including A/J, BALB/C, SJL/J, SWR/J, C57BL/6J, CBA/J, DBA/2 and P/J. In addition to the *Skts* series, several other skin tumor modifier loci were identified using commonly used inbred strains or wild-derived strains. The *Ptch* gene was identified as a *K5Hras* modifier in a C57BL/6J and FVB/N cross (14). *Psl1-4* were identified in a C57BL/6J and DBA cross (15,16). *Skts-fp 1-3* were identified in a cross between the wild-derived inbred PWK strain and FVB/N mice (17). *Skts-fp 1* was also identified in a study involving a cross between a wild-derived outbred stock of *Mus musculus castaneus* and FVB/N (18). The MSM/Ms strain was established from Japanese wild mice, *Mus musculus molossinus* that were collected in 1978 in Mishima, Japan (19). Inbreeding of these mice has reached generation N80 at the end of 2011, and they can now be used as a pure inbred strain for genetic studies. MSM/Ms mice show high breeding performance (100–125 N_2 progeny/pair/year) (19), and have a very low incidence of tumor development (20–24). In addition, large numbers of SSLPs (*S*imple *S*equence *L*ength *P*olymorphisms) and SNPs (*S*ingle *N*ucleotide *P*olymorphisms) are polymorphic between MSM/Ms and laboratory strains of mice, offering a large repertoire of genetic markers for mapping studies (25,26).

In this study, we found that MSM/Ms mice show dominant resistance to chemically induced skin tumor development when crossed with the highly susceptible FVB/N strain (27). To identify genetic determinants of skin cancer susceptibility, we generated a large backcross population FVB/N × (FVB/N x MSM/Ms) F1 mice, and subjected the animals to DMBA/TPA-induced skin carcinogenesis. Previous studies largely focused on papilloma number as the major phenotype because of the overall low incidence of carcinomas. Because *p53* heterozygosity has been shown to be associated with a higher frequency of progression to carcinomas without any effect on papilloma development (28), we used $p53^{+/-}$ mice to facilitate the identification of loci responsible for tumor progression.

Furthermore, we identified stage-specific papilloma modifiers by classifying papillomas into three categories based on diameter. In other words, papilloma multiplicity in each size category was used as a quantitative trait. In this study, we report the identification of early, mid, late stage papilloma, and carcinoma genetic modifiers, as well as the existence of a novel *p53*-dependent modifier locus on mouse chromosome 4.

Materials and methods

Mice

All animal experiments were performed under protocols approved by the Niigata University, Experimental Animal Research Facility. FVB/N mice were purchased from Japan Clea. MSM/Ms had been maintained in the Experimental Animal Facility at Niigata University for >20 years. $p53^{+/-}$ MSM/Ms mice (23) had been maintained on MSM/Ms background for >10 years as well. Female FVB/N mice were crossed with male MSM/Ms and $p53^{+/-}$ MSM/Ms mice, respectively. Male F1 (FVB/N × MSM/Ms) and $p53^{+/-}$ F1 (FVB/N × MSM/Ms) were backcrossed to female FVB/N to generate 121 $p53^{+/+}$ and 107 $p53^{+/-}$ F1 backcross mice, respectively. Mice were genotyped using PCR assays as described previously (23).

Skin carcinogenesis

7,12-Dimethylbenz(a)anthracene (DMBA) was purchased from Sigma Japan, and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was purchased from

Abbreviations: DMBA,7,12-dimethylbenz(a)anthracene; QTL, quantitative trait locus; SNPs, single nucleotide polymorphisms; SSLPs, simple sequence length polymorphisms; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; LOD, logarithm of odds.

Calbiochem. DMBA is used as a carcinogen and TPA as a promoter. 8 MSM/Ms, 10 FVB/N, 12 F1 (FVB/N × MSM/Ms) hybrids, and 228 F1 backcross mice were treated according to the two-stage carcinogenesis protocol. At 8–10 weeks of age, the backs of mice were carefully shaved with an electric clipper. Two days after shaving, a single dose of DMBA (25 μg per mouse in 200 μl of acetone) was applied to shaved dorsal back skin. One week after initiation, tumors were promoted with TPA (10 μg per mouse in 200 μl of acetone) twice weekly for 20 weeks. Papilloma number and size (mm in diameter) of each papilloma was recorded from 10 weeks up to 20 weeks, and carcinoma development was monitored up to 40 weeks post-TPA treatment.

Classification between papillomas and carcinomas

The majority of papillomas was determined by visual inspection. Papillomas appeared as outgrowths on the dorsal skin of mice. Some of papillomas began to convert to carcinomas by getting more flat on the skin and penetrating deeper into the dermis. They were normally easily distinguishable from one another. However, in some intermediate type tumors, we prepared the paraffin section and confirmed histology.

Histological analysis

Tissues were fixed in 10% buffered formalin, progressively dehydrated through gradients of alcohol, and embedded in paraffin. Samples were sectioned on a microtome at 4 μm thickness, deparaffinized in xylene, rehydrated and then stained with hematoxylin and eosin for histological analysis.

DNA isolation and SNP genotyping

Genomic DNA was isolated using a QIAGEN DNeasy kit from ~5 mm of tail tissue from each mouse. A genome-wide scan was performed in the ABI 7900HT system as described previously (29). Single nucleotide polymorphisms (SNP) markers spaced every 10–20 Mb were chosen from the Mouse SNP database (http://www.broad.mit.edu/snp/mouse/) and used for genome-wide scanning (Supplementary Table 1, available at *Carcinogenesis* Online); I.Miura *et al.*, in preparation). We used 10 additional microsatellite markers for a detailed linkage analysis. The physical location of the markers is based on the data from Ensembl database (http://www.ensembl.org) and the Mouse Genome Database (http://www.informatics.jax.org). PCR was performed as described elsewhere (17).

Statistical analysis

Phenotype for papilloma and carcinoma susceptibility was scored by the number of papillomas and carcinomas at 20 weeks and at 40 weeks after initiation, respectively. J/qtl, a Java GUI (graphical user interface) for the popular QTL data analysis software R/qtl (30), was used to analyze phenotype and genotype data from F1 backcross to map QTLs related to susceptibility for chemically induced skin tumors. A permutation test was performed with 10 000 permutations to estimate the empirical threshold value for the mapping.

Results

MSM/Ms is resistant to chemically induced skin tumors

As shown previously, FVB/N mice are highly susceptible to the two-stage skin chemical carcinogenesis, developing on average 30.7±0.9 papillomas at 20 weeks after the initiation (Figure 1A and 1B). In contrast, none of the 8 MSM/Ms mice developed a single papilloma, indicating that MSM/Ms mice are highly resistant to carcinogen-induced skin carcinogenesis. (Figure 1A and 1B). Similarly, F1 (FVB/N × MSM/Ms) mice were resistant to papilloma development, with 7 of 12 mice having one or two papillomas (0.9±1.2) (Figure 1B). Figure 1C shows the size distribution pattern of papillomas of FVB/N mice at the indicated times. In addition, FVB/N mice had a higher incidence and earlier onset of carcinoma compared with MSM/Ms and F1 mice. By 30 weeks after initiation, all FVB/N mice succumbed to their disease while all MSM/Ms and F1 mice were still alive and well (Figure 1D). These results demonstrate that MSM/Ms are highly

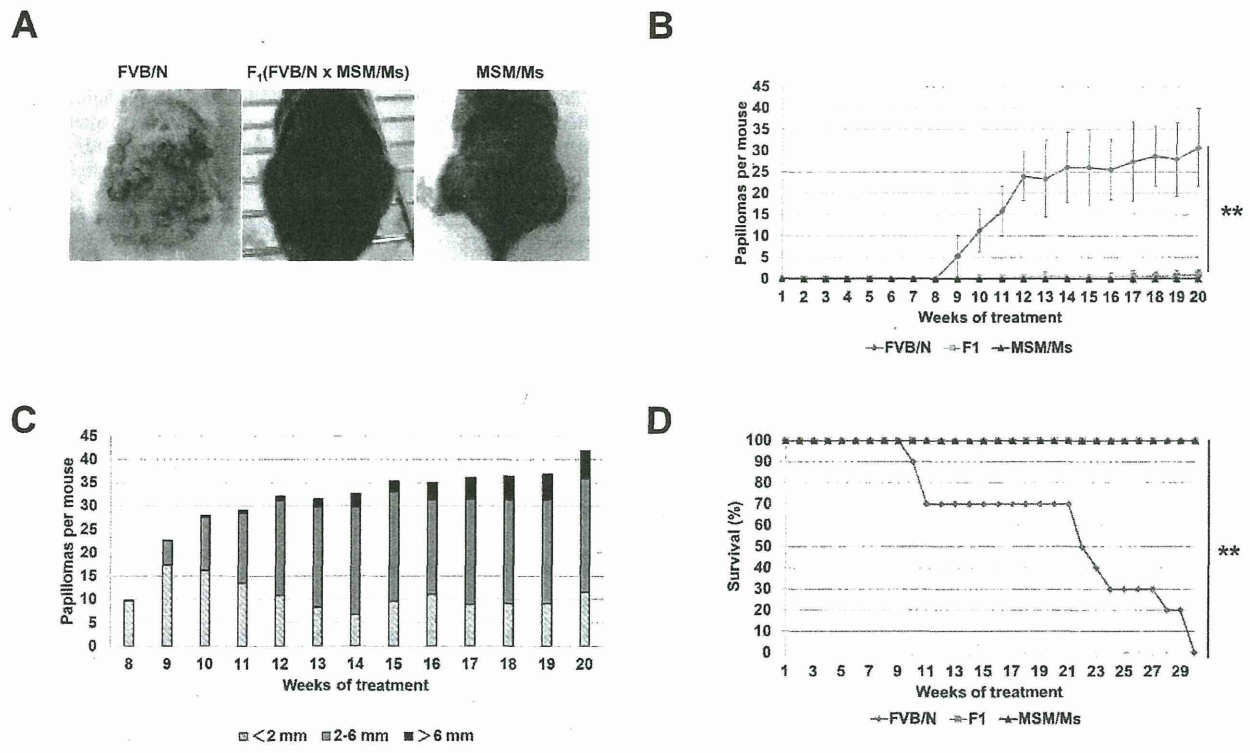

Fig. 1. MSM/Ms shows dominant resistance to DMBA/TPA-induced papilloma. (**A**) A back skin of FVB/N, F1(FVB/N × MSM/Ms) and MSM/Ms at 12 weeks after initiation. (**B**) Comparison of DMBA/TPA-induced papilloma number per mouse among MSM/Ms ($n = 8$), FVB/N ($n = 10$) and F1 ($n = 12$). Error bar is standard deviation (SD). The P value was calculated for papilloma number at 20 weeks by t-test (**$P < 0.01$). (**C**) Size distribution pattern of papillomas on FVB/N mice ($n = 10$). Light gray bars denote <2 mm papillomas, dark gray bars denote 2–6 mm papillomas and black bars denote >6 mm papillomas. (**D**) Survival curve of the DMBA/TPA-treated MSM/Ms ($n = 8$), FVB/N ($n = 10$) and F1 ($n = 12$) (**$P < 0.001$, Kaplan–Meier method). Closed diamonds denote FVB/N, closed squares denote F1 and closed triangles denote MSM/Ms.

resistant to DMBA/TPA-induced skin carcinogenesis, and that this phenotype is dominant.

DMBA/TPA treatment of $p53^{+/+}$ and $p53^{+/-}$ F1 backcross mice

To identify genetic loci that control susceptibility to skin carcinogenesis, we subjected 228 FVB/N × (FVB/N × MSM/Ms) F1 backcross mice, 121 on a $p53^{+/+}$ background and 107 on a $p53^{+/-}$ background, to the DMBA/TPA chemical carcinogenesis protocol and monitored their tumor development for a period of 40 weeks. All mice were genotyped using 107 SNP markers distributed evenly over the genome. For each mouse, we documented the number of papillomas at 20 weeks after initiation as well as the presence of carcinomas at 40 weeks. In addition, papillomas were further categorized into three groups on the basis of size (<2 mm, 2–6 mm and >6 mm in diameter).

Figure 2A–E shows the effect of *p53* on papilloma and carcinoma development in the F1 backcross mice. Only small difference in

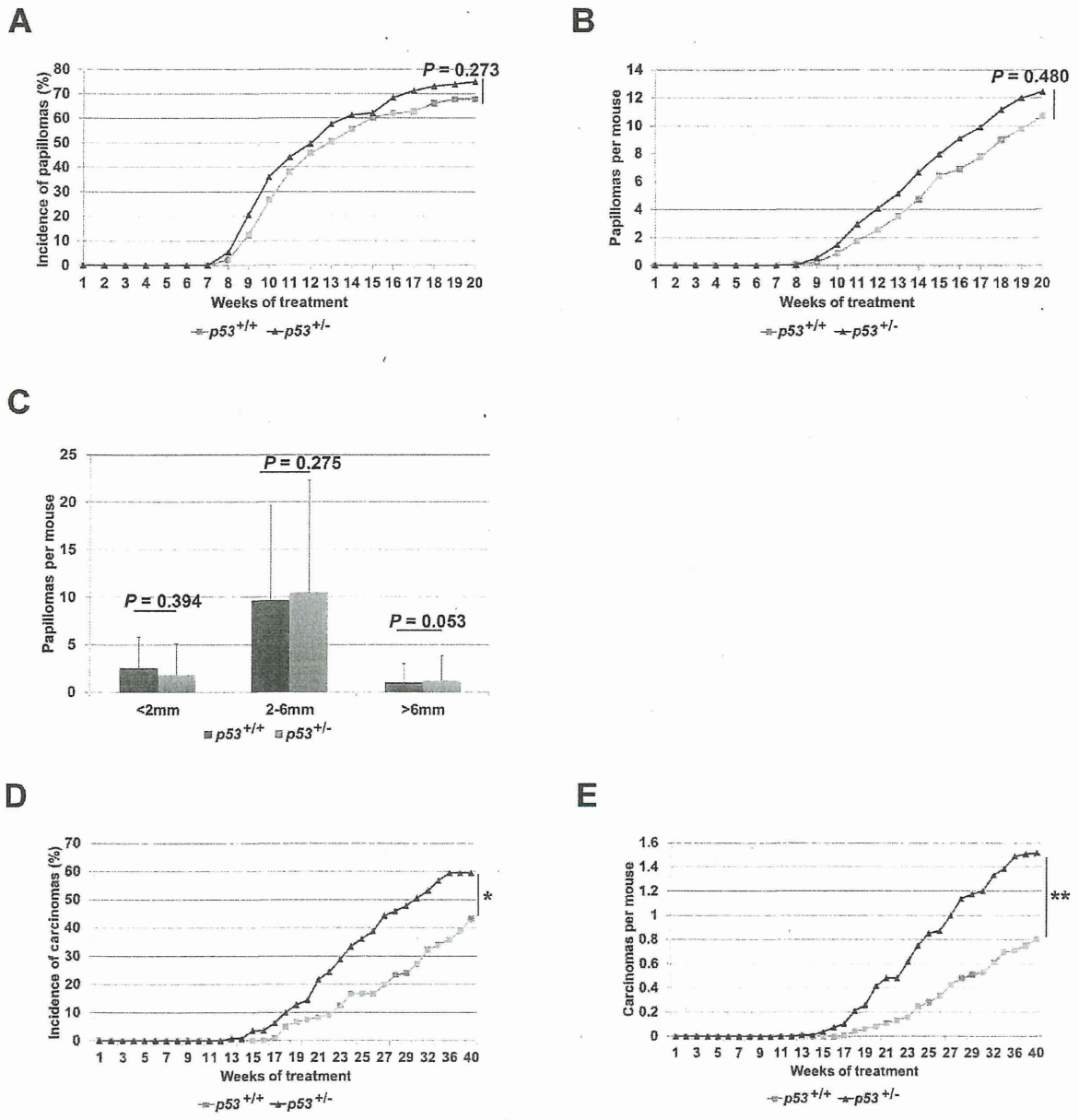

Fig. 2. Papilloma and carcinoma development in $p53^{+/-}$ FVB/N × (FVB/N × MSM/Ms) F1 backcross mice (n = 107) and $p53^{+/+}$ FVB/N × (FVB/N × MSM/Ms) F1 backcross mice (n = 121). (**A**) Percentage of mice bearing at least one papilloma is plotted. Papilloma incidence was not enhanced in the presence of *p53* knockout allele (P = 0.273, Fisher test). (**B**) Average number of papillomas per mouse is plotted. Papilloma number was not greatly enhanced in the presence of *p53* knockout allele (P = 0.480, t-test). (**C**) Comparison of average number of papillomas per mouse in three different size categories (<2 mm, 2–6 mm and >6 mm). *p53* effect was not observed in any category, (P = 0.394 for <2 mm, P = 0.275 for 2–6 mm, P = 0.053 for >6 mm, t-test). Error bar is standard deviation (SD). Black bars denote $p53^{+/+}$ mice and gray bars denote $p53^{+/-}$ mice. (**D**) Percentage of mice bearing at least one carcinoma is plotted. Conversely, carcinoma incidence was enhanced in the presence of *p53* knockout allele during the period of the experiment (**P < 0.01, Fisher test at 30 weeks after initiation). Curves get closer at the end point, but P value is still significant (*P < 0.05). (**E**) Average number of carcinomas per mouse is plotted. Carcinoma number was greatly enhanced in the presence of *p53* knockout allele (**P < 0.01, t-test). Closed squares denote $p53^{+/+}$ mice and closed triangles denote $p53^{+/-}$ mice.

papilloma development is seen between $p53^{+/+}$ and $p53^{+/-}$ F1 backcross mice (Figure 2A–C). On the other hand, a strong effect on carcinoma development is observed (Figure 2D and 2E), as described previously (28).

Highly significant linkage for total papilloma number was found on chromosome 7

When $p53^{+/+}$ and $p53^{+/-}$ mice were combined for analysis, significant linkage was found at 53.6 cM on chromosome 7 (LOD (logarithm of odds) = 6.974) and at 36.0 cM chromosome 6 (LOD = 4.355). Suggestive linkage was found on chromosomes 3, 5 and 12 (Figure 3A). We also found significant linkage based on total papilloma number at 53.6 cM on chromosome 7 (LOD = 5.109) in $p53^{+/+}$ F1 backcross mice, in addition to suggestive linkage on chromosomes 3, 6, 11 and 13 (Supplementary Figure 1A, available at *Carcinogenesis* Online). In $p53^{+/-}$ F1 backcross mice, significant linkage was detected at 86.4 cM on distal chromosome 11 (LOD = 3.555), and suggestive linkage was found on chromosomes 5, 6, 7 and 12 (Supplementary Figure 1B, available at *Carcinogenesis* Online).

Linkage analysis on the basis of papilloma size

Linkage analysis was also conducted on the basis of papilloma size. Significant linkage for number of papillomas <2 mm occurred at 65.4 cM on chromosome 7 (LOD = 5.063), and suggestive linkage was found on chromosomes 4 and 11, but only in $p53^{+/+}$ mice (Supplementary Figure 1C, available at *Carcinogenesis* Online). No linkage was found in $p53^{+/-}$ mice based on this category of papilloma size (data not shown). When $p53^{+/+}$ and $p53^{+/-}$ mice were combined for analysis, significant linkage was found at 53.6 cM on chromosome 7 (LOD = 4.869), and suggestive linkage was found on chromosomes 1 and 4 (Figure 3B).

Next, linkage analysis was performed using papilloma number of the intermediate size (2–6 mm). In $p53^{+/+}$ mice, linkage at 42.0 cM on chromosome 3 (LOD = 3.728) and at 53.6 cM on chromosome 7 (LOD = 3.921) reached statistical significance. Suggestive linkage was detected on chromosomes 6, 11 and 13 (Supplementary Figure 1D, available at *Carcinogenesis* Online). We did not detect any significant linkage in $p53^{+/-}$ mice, but suggestive linkage was found on chromosomes 6, 7, 11, 12 and 17 (Supplementary Figure 1E, available at *Carcinogenesis* Online). When both genotypes were combined, significant linkage was

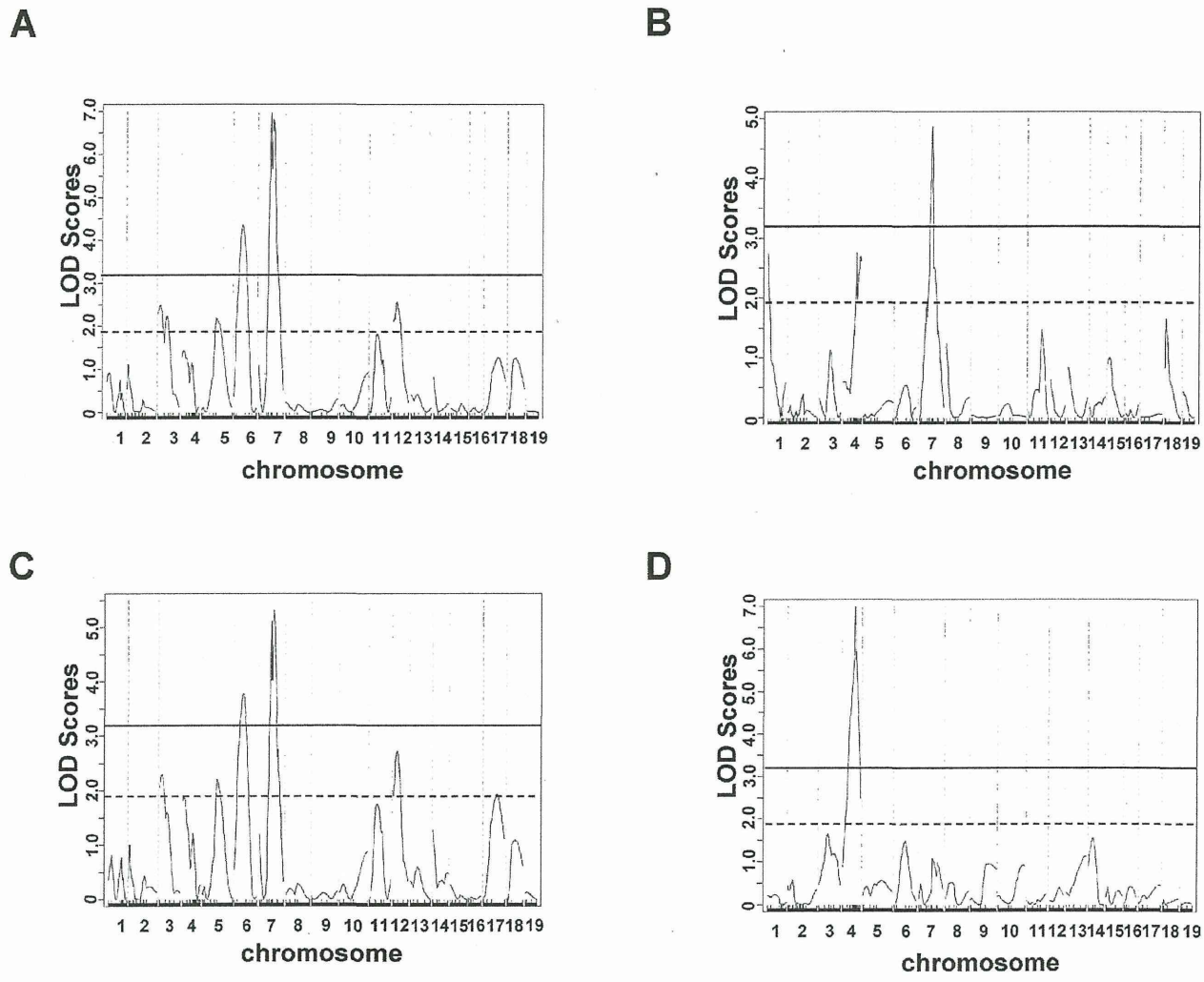

Fig. 3. Whole-genome scanning of 228 ($p53^{+/+}$ and $p53^{+/-}$) FVB/N × (FVB/N × MSM/Ms) F1 backcross mice for papilloma multiplicity at 20 weeks after initiation. Linkage analysis revealed (**A**) significant linkage on chromosomes 6 and 7, and suggestive linkage on chromosomes 3, 5 and 12 for total number of papillomas. (**B**) Significant linkage on chromosome 7 and suggestive linkage on chromosomes 1 and 4 for number of papillomas <2 mm. (**C**) Significant linkage on chromosomes 6 and 7, and suggestive linkage on chromosomes 3, 5, 12 and 17 for papilloma number of the intermediate size (2–6 mm). (**D**) A single highly significant linkage on chromosome 4 for papilloma number >6 mm. Dashed line indicates empirical suggestive and significant linkages of LOD score at 1.9 and 3.3.