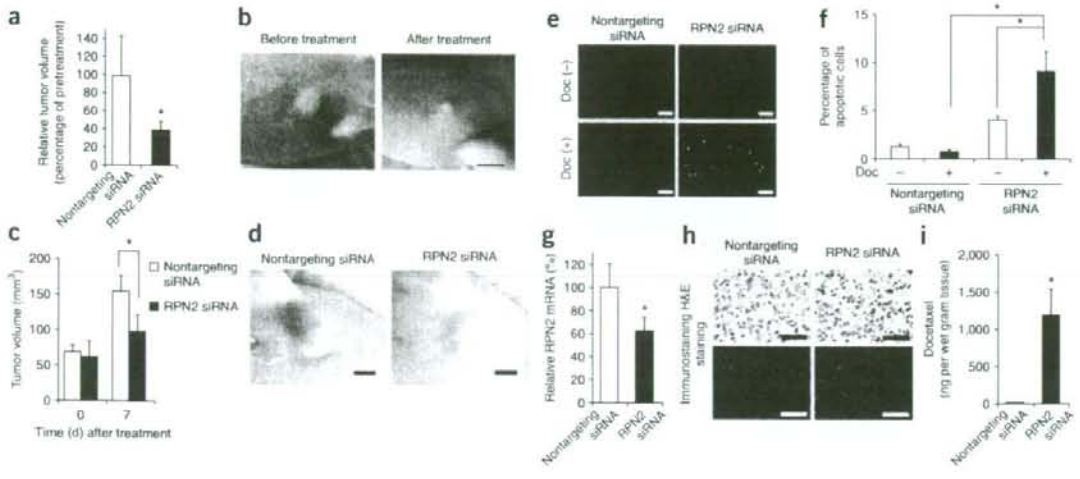

Figure 4 Delivery of RPN2 siRNA to docetaxel-resistant breast tumors. The effect of RPN2 siRNA was examined in orthotopic breast tumor models. (**a**) Reduction of MCF7-ADR breast tumor volume in mice given RPN2 siRNA or control nontargeting siRNA along with docetaxel ($n = 6$ per group, $^*P < 0.01$). (**b**) siRNA-treated MCF7-ADR tumors in mice before and 7 d after docetaxel treatment. Scale bar, 5 mm. (**c**) Growth of MDA-MB-231/MDR1 breast tumor in mice administered RPN2 siRNA or nontargeting siRNA along with docetaxel ($n = 6$ per group, $^*P < 0.002$). (**d**) MDA-MB-231/MDR1 tumors in mice 7 d after treatment with siRNA and docetaxel. Scale bar, 5 mm. (**e**) TUNEL staining of MCF7-ADR tumor tissues treated with RPN2 siRNAs or nontargeting siRNAs in the presence or absence of docetaxel. Scale bar, 50 μm. (**f**) TUNEL-positive cells were counted and are represented in the graph ($n = 3$ per group, $^*P < 0.01$). (**g**) Expression of RPN2 mRNA in MCF7-ADR tumors treated with RPN2 siRNAs or nontargeting siRNAs ($n = 3$ per group, $^*P < 0.01$). (**h**) Expression of RPN2 protein in MCF7-ADR tumors. H&E staining and RPN2 immunofluorescence staining (green, RPN2; blue, nuclei) of tissues treated with RPN2 siRNA or nontargeting siRNA. Scale bar, 50 μm. (**i**) Docetaxel retention in MCF7-ADR tumors in mice treated with RPN2 siRNAs or nontargeting siRNAs ($n = 4$ per group, $^*P < 0.001$). Values are means ± s.d.

in MCF7-ADR cells transduced by RPN2 siRNA (**Supplementary Fig. 4** online). For this reason, and to assess the potential involvement of *RPN2* gene overexpression in MDR1 functions, we tested the glycosylation status of MDR1 protein in MCF7-ADR cells transfected with RPN2 siRNA. We analyzed the glycosylation patterns by western blotting of P-glycoprotein, which appears on blots as mature 170-kDa, immature (partially glycosylated) 150-kDa and unglycosylated 140-kDa bands[28]. The 150-kDa immature and 140-kDa unglycosylated P-glycoproteins were clearly found in MCF7-ADR cells with RPN2 knockdown (90% inhibition of mRNA by real-time RT-PCR analysis; **Fig. 5a**). More than 80% of P-glycoproteins were unglycosylated or partially glycosylated in RPN2-silenced cells (composition of P-glycoproteins, 170 kDa:150 kDa:140 kDa = 18:40:42). In contrast, MCF7-ADR cells transduced with nontargeting control siRNA expressed more than half of their P-glycoproteins as170-kDa mature P-glycoprotein (170 kDa:150 kDa:140 kDa = 52:17:31). This result showed that RPN2 knockdown inhibits glycosylation of P-glycoproteins in MCF7-ADR cells. The western blot of P-glycoprotein, particularly in cells transduced with RPN2 siRNA, showed 'smear' patterns (**Fig. 5a**). We speculated that the smear pattern was caused by the presence of intermediately glycosylated forms in various sizes. We treated the cell lysate samples with peptide:*N*-glycosidase F (PNGase F) to remove *N*-glycan chains, which shifted the P-glycoprotein in the blot from a smear pattern to a 140-kDa unglycosylated protein band in MCF7-ADR cell lysates. After PNGase F treatment, both nontargeting control siRNA– and RPN2 siRNA–transduced cells showed a 140-kDa unglycosylated P-glycoprotein band (**Fig. 5a**). This indicates that the smear pattern resulted from the presence of intermediately glycosylated P-glycoprotein and that there were a number of intermediately glycosylated P-glycoproteins in the RPN2-silenced cells because of inhibition of glycosylation on P-glycoprotein.

We further evaluated the RPN2 siRNA effects on cell surface P-glycoprotein expression in MCF7-ADR cells by immunofluorescence staining. As expected, immunofluorescence staining indicated that P-glycoprotein was predominantly localized to the cell membrane in MCF7-ADR cells transduced with control nontargeting siRNAs, whereas the intensity of membrane P-glycoprotein in RPN2-downregulated cells was considerably reduced (**Fig. 5b**). Moreover, retention of rhodamine-123, which is a substrate of P-glycoprotein, was strongly enhanced in MCF7-ADR cells transfected with RPN2 siRNA compared to those transfected with nontargeting siRNA (**Fig. 5c**). This indicates that downregulation of RPN2 restores drug retention and inhibits P-glycoprotein function by suppressing the glycosylation of P-glycoproteins in MCF7-ADR cells.

To further bolster these findings, we performed immunostaining analysis of RPN2 and P-glycoprotein in MCF7-ADR tumors in mice. The RPN2 shutdown resulted in a marked disappearance of the membrane-bound P-glycoprotein (**Fig. 5d**), an observation that supports our *in vitro* findings that RPN2 downregulation by siRNA in drug-resistant MCF7-ADR cells results in the loss of membrane-bound P-glycoprotein.

Furthermore, we have examined the status of RPN2 and P-glycoprotein in breast cancer tissues from subjects with RPN2 mRNA high expression ($n = 4$) and RPN2 mRNA low expression ($n = 4$) by immunostaining. P-glycoprotein was predominantly localized to the cell membrane in the primary tumor with a strong signal for RPN2, whereas in the primary tumor with low expression of RPN2, P-glycoprotein was found in the cytoplasm (**Supplementary**

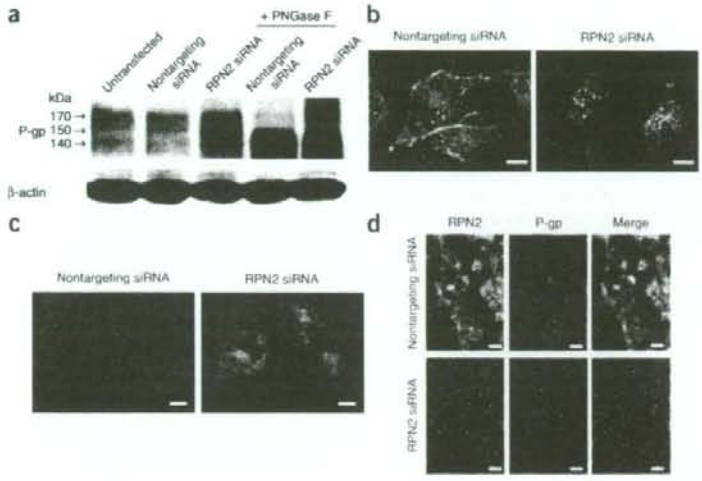

Figure 5 RPN2 siRNA regulates glycosylation of P-glycoprotein (P-gp). (**a**) Western blot analysis shows the glycosylation status of P-gp in MCF7-ADR cells 72 h after transfection of RPN2 siRNA or control nontargeting siRNA. Bands migrating at 170, 150 and 140 kDa represent mature, immature and unglycosylated forms of P-gp, respectively. (**b**) Immunofluorescence staining of P-gp on MCF7-ADR cell membrane surfaces. Cells were treated with RPN2 siRNA or nontargeting siRNA for 72 h. Scale bar, 5 μm. (**c**) Rhodamine-123 retention in MCF7-ADR cells 72 h after transfection with RPN2 siRNA or control nontargeting siRNA. Scale bar, 5 μm. (**d**) Localization of P-gp in tumors of MCF7-ADR in mice. Immunofluorescence staining of RPN2 (green) and P-gp (red) are shown. Nuclei are blue (DAPI). Merged images are also shown. Scale bar, 5 μm.

Fig. 5a,b online). Similar results were observed for other breast cancer tissues (**Supplementary Fig. 5c**).

Thus, these data provide a clear link between the glycosylation status of P-glycoprotein and RPN2 expression in drug-resistant breast cancer cells, and the disappearance of the membrane-bound P-glycoprotein leads to a reversal of the multidrug- resistant phenotype.

DISCUSSION

Cancer researchers today are confronted with how to best identify and select the next generation of molecular targets for oncology. An impressive array of potential new cellular targets, suitable for therapeutic intervention, has been revealed by the recent completion of the human genome sequencing project. Approaches as varied as transcription profiling, proteomics and the use of siRNAs are all being exploited in the race to select the most promising candidate drug targets. We tested the feasibility of using atelocollagen-mediated RNAi delivery *in vitro* and *in vivo* to obtain an unbiased evaluation on the efficacy of a specific siRNA related to drug resistance in human breast cancer. We show here that, among genes whose expression was elevated in nonresponders to docetaxel, the siRNA designed for *RPN2* significantly promoted docetaxel-dependent apoptosis and cell growth inhibition of MCF7-ADR human breast cancer cells that exhibit docetaxel resistance. A clinicopathological study showed that there is a significant association of *RPN2* expression with a pathologic response to docetaxel. Most notably, atelocollagen-mediated *in vivo* delivery of RPN2 siRNA significantly reduced the size of orthotopic MCF7-ADR tumors in mice given docetaxel.

In this study, we demonstrated that the atelocollagen delivery system markedly enhanced the efficiency of siRNA for the inhibition of RPN2 in mouse tumor models of human breast cancer. Because siRNA shows very low efficiency in gene silencing *in vivo*, various delivery methods, such as the use of plasmids and viral vectors encoding siRNA and the use of lipids, have been investigated. We have previously shown that the atelocollagen-mediated systemic delivery of siRNA might be a unique strategy for the inhibition of bone-metastatic prostate tumor growth[22]. The siRNA-atelocollagen complex is a nano-sized particle and is stable *in vitro* and *in vivo*[21,29]. Furthermore, we have previously confirmed that the atelocollagen complex shows low toxicity and low immunogenicity *in vivo*[23,24]. Thus, an atelocollagen-mediated local or systemic delivery system holds great potential for the practical application of gene suppression using siRNAs for cancer therapeutics.

Targeting of P-glycoprotein by small-molecular compounds, antibodies or both is an effective strategy to overcome multiple drug resistance in cancer[30]. Despite promising previous studies showing that the inhibition of P-glycoprotein by pharmacological means can sensitize drug-resistant cells, the ultimate goal of restoring drug sensitivity has met with limited success in clinical trials. Our results indicate that RPN2 is partly responsible for P-glycoprotein–mediated drug resistance in breast cancer and is involved in the regulation of the glycosylation status of P-glycoprotein. In fact, downregulation of *RPN2* restored drug retention, suggesting that P-glycoprotein function is inhibited via suppression of the glycosylation of P-glycoprotein in MCF7-ADR cells. *N*-glycosylation has been shown to contribute to the stability of the P-glycoproteins[31], and it has been reported that reduced glycosylation results in the disappearance of membrane-bound P-glycoprotein, which causes the loss of a multidrug-resistant phenotype[32]. Furthermore, multidrug-resistant cells are hypersensitive to the *N*-linked glycosylation inhibitor tunicamycin, which induces partial inhibition of the glycosylation of GLUT-1, a glucose transporter, and diminishes GLUT-1–mediated transport[33]. Because the amount of MDR1 mRNA was not significantly decreased in MCF7-ADR cells transduced with RPN2 siRNA, it is predicted that RPN2 inhibition may reduce the glycosylation of P-glycoprotein, thereby inducing perturbation of its subcellular localization, inhibition of its protein synthesis and/or acceleration of its degradation, with MCF7-ADR cells inevitably becoming hypersensitive response to docetaxel. In contrast, the RPN2 protein is part of an *N*-oligosaccharyl transferase complex that links to *N*-glycosylation ability; therefore, RPN2 inhibition could affect *N*-oligosaccharyl transferase function, resulting in impaired glycosylation of the P-glycoproteins. We speculate that RPN2 has a key role in drug-resistant tumor cells that overexpress P-glycoprotein and acts as a facilitator, stabilizing factor or both for *N*-glycosylation of P-glycoprotein. The coordinated expression of RPN2 and P-glycoprotein may participate in the mechanism of docetaxel resistance via the glycosylation status of P-glycoprotein.

However, one group has recently reported that the stability of P-glycoprotein is regulated by the ubiquitin-proteasome pathway in multidrug-resistant cancer cells[34]. Furthermore, the P-glycoprotein must be phosphorylated by protein kinase C (PKC) to effectively

function as a drug-efflux pump[35], which suggests that PKC is indirectly involved in the development of the multidrug-resistant phenotype. More recently, it was revealed that wild-type p53, a tumor suppressor, may resensitize soft tissue sarcoma to chemotherapeutic agents by reducing MDR1 phosphorylation via transcriptional repression of PKC expression[36]. There is no direct evidence of RPN2 involvement with the transcriptional repression of PKC. Other transporter proteins mediating drug resistance are the multidrug resistance–associated protein and ABCG2. Whether these different populations of multidrug resistance–associated protein family members and ABCG2 are affected by RPN2 has yet to be determined.

Recently, downregulation of multidrug resistance by the introduction of synthetic siRNAs has been reported[37,38]. However, only partial reversal of the drug-sensitive phenotype of the cells has been obtained. A possible explanation for this low inhibitory effect is that it was the result of a long half-life of P-glycoprotein[39] and the less efficient delivery of synthetic siRNAs into cells. Although the data are not shown, we compared the cell growth inhibition by synthetic RPN2 siRNA versus MDR1 siRNA in the presence of docetaxel *in vitro*. At the mRNA level, the downregulation of RPN2 and MDR1 obtained with the most efficient siRNA was 90% and 80%, respectively. These results indicate that cell growth inhibition was achieved by both siRNAs, although RPN2 siRNA showed a stronger growth inhibitory effect compared to MDR1 siRNA. Thus, though it is impossible at the moment to judge whether MDR1 or RPN2 is a more profitable target for overcoming drug resistance, RPN2 does provide a valuable clue for making multidrug-resistant breast cancer cells sensitive to anticancer drugs.

The continuing interest in apoptosis among cancer biologists has been strengthened by the hope that a molecular understanding of cell death will inform our understanding of cancer drug resistance. In fact, upregulation of antiapoptotic *Bcl2* family genes has been shown to be key in tumor malignancy and drug resistance[40,41]. Overexpression of exogenous Bcl-xL or Bcl-2 suppresses apoptosis[42,43]. In our study, knockdown of RPN2 by siRNA in MCF7-ADR cells selectively downregulated mRNA expression of Bcl-xL and Bcl-w (**Supplementary Fig. 6** online). These results suggest that RPN2 regulates Bcl-xL– and Bcl-w–mediated antiapoptosis and may be partly responsible for the docetaxel resistance of the MCF7-ADR cells. It has already been reported that apoptosis-based therapies[44], such as the downregulation of Bcl-xL expression, Bcl-w expression or both with antisense oligonucleotides, abolish tumorigenicity and enhance chemosensitivity in human malignant glioma cells[45–47]. In addition, Bcl-xL and Bcl-w are upregulated by nuclear factor-κB (NF-κB)[48]. Some chemotherapeutic agents, such as cisplatin and docetaxel, instantly induce the activation of NF-κB in cancer cells, and the cells become drug resistant[49]. In fact, we found that *RPN2* gene expression is also induced by docetaxel treatment of drug-sensitive MCF7 cells. Therefore, it would be useful to know whether RPN2 induces the downregulation of Bcl-xL and Bcl-w in MCF7-ADR cells by direct association with the NF-κB signaling pathway.

It is noteworthy that our findings using docetaxel-resistant human breast cancer cells are commonly found in other multiple cancers. Cisplatin-resistant human non–small cell lung carcinoma cells recover their sensitivity to cisplatin by knockdown of *RPN2* expression and die by apoptosis (Y.Y., K.H. and T.O., unpublished data). In addition, mouse mammary tumor cells resistant to docetaxel express mouse Rpn2, and inhibition of Rpn2 results in apoptotic cell death in the presence of docetaxel (**Supplementary Fig. 2e–g**). Therefore, RPN2 status is responsible for the drug-resistant nature of multiple cancer cell lines both in humans and in mice, and RPN2 expression may confer cross-resistance to a variety of anticancer drugs.

We previously reported that a group of redox genes is useful for the prediction of the clinical response to docetaxel in subjects with breast cancer[16]. Our current results indicate that the RPN2 mRNA level might serve as a predictor of the response to anticancer therapy rather than as a prognostic factor. The determination of the RPN2 mRNA level will be useful in the selection of subjects who are likely to benefit from adjuvant chemotherapy. Furthermore, our animal experiments suggest that treatment of subjects with a pharmacological agent that blocks RPN2 expression or function may induce a complete response to chemotherapeutic drugs. The *RPN2* gene may therefore represent a promising new target for RNAi therapeutics against multidrug-resistant tumors.

METHODS

Cell culture. Human mammary carcinoma cell lines, MCF7 cells and multidrug-resistant MCF7-ADR cells were provided by Shien-Lab, Medical Oncology, National Cancer Center Hospital of Japan. We cultured MCF7, MCF7-ADR and MDA-MB-231 (American Type Culture Collection) cells in RPMI 1640 (Gibco BRL) supplemented with 10% FBS (Gibco BRL) under 5% CO_2 in a humidified incubator at 37 °C. We cultured the mouse mammary tumor cell line EMT6/AR10.0 (European Collection of Cell Cultures), which shows docetaxel resistance, in MEM (EBSS) with 2 mM glutamine, 1% non essential amino acids and 10% FBS. The establishment of bioluminescent MCF7-ADR-Luc cells and docetaxel-resistant MDA-MB-231/MDR1 cells is described in **Supplementary Methods** online.

Design and synthesis of small interfering RNAs. We designed siRNAs and synthesized them with an siRNA duplex for each gene target (Dharmacon) for the preparation of an atelocollagen-based cell transfection array. The siRNA sequences are described in **Supplementary Methods**.

Atelocollagen-based cell transfection array. For RNAi-based functional screening, we prepared an atelocollagen-based cell transfection array, which enables reverse transfection of cells by atelocollagen-mediated gene transfer (**Supplementary Methods**). We performed live-cell luciferase assay for measurement of cell growth, and we performed caspase-7 assays with Apo-ONE Caspase-3/7 Assay Reagent (Promega) and Hoechst staining for apoptosis (**Supplementary Methods**).

Real-time reverse transcription PCR. We purified total RNA from cells and tumor tissues with an RNeasy Mini Kit and RNase-Free DNase Set (QIAGEN) and produced cDNAs with an ExScript RT reagent Kit (Takara). We then subjected cDNA samples to real-time PCR with SYBR Premix Ex Taq (Takara) and specific primers (**Supplementary Methods**). We carried out the reactions in a LightCycler (Roche Diagnostics). We normalized gene expression levels by *HPRT1* or *ACTB*. The cell-direct quantitative RT-PCR method is described in the **Supplementary Methods**.

Atelocollagen-mediated RPN2 small interfering RNA delivery *in vivo*. We performed mouse experiments in compliance with the guidelines of the Institute for Laboratory Animal Research at the National Cancer Center Research Institute of Japan. We used 4-week-old female athymic nude mice (CLEA Japan) to generate an experimental orthotopic breast cancer model. We injected 1.0×10^7 MCF7-ADR cells or MDA-MB-231/MDR1 cells suspended in 100 μl sterile PBS into the fat pad. When the tumor grew to approximately 5 mm in diameter, we injected mice with 200 μl of siRNA-atelocollagen by intratumoral injection. Preparation of the siRNA-atelocollagen complex is described in the **Supplementary Methods**. Simultaneously, we injected docetaxel i.p. into mice. We harvested tumor tissues for analysis of RPN2 mRNA and RPN2 protein at 24 h and 72 h after treatment, respectively.

TUNEL technique. We harvested tumor tissues 72 h after administration of siRNA and prepared frozen sections. We then performed TUNEL staining with an *in situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics), according to the manufacturer's protocol. We stained the nuclei with DAPI. We

determined the number of fluorescein-positive cells in three microscopic fields of each section by fluorescence microscopy.

Docetaxel disposition in tumors. We studied drug disposition of docetaxel in tumors in mice by HPLC with ultraviolet detection at 225 nm after solid-liquid extraction as described elsewhere[30]. Eleven hours after i.p. administration of 20 mg kg^{-1} docetaxel, we harvested the tumors treated with siRNA-atelocollagen complex and then analyzed the docetaxel abundance in the tumor.

Transfection of small interfering RNA. We carried out transfection of MCF7-ADR and EMT6/AR10.0 cells with siRNA using DharmaFECT 1 (Dharmacon) and TransIT-TKO (Mirus), respectively, according to the manufacturers' protocol (**Supplementary Methods**).

Antibodies. We used RPN2-specific antibody (H300, Santa Cruz Biotechnology) and MDR-specific antibody (G-1, Santa Cruz Biotechnology). We visualized staining with Alexa 488 or Alexa 594 (Molecular Probes). We used fluorescence microscopy or confocal fluorescence microscopy (Olympus) for observation of immunofluorescence-stained cells. The procedures of western blotting and immunofluorescence staining are described in the **Supplementary Methods**.

Rhodamine-123 retention assay. We washed cells once with prewarmed Opti-MEM I medium (37 °C, Gibco BRL) and incubated the cells for 30 min at 37 °C in the Opti-MEM I medium containing 10 μM rhodamine-123. We then removed the rhodamine-123 solution from the extracellular medium and washed the cells twice with Opti-MEM I medium. We observed the cells for fluorescence of rhodamine-123 under fluorescence microscopy.

Human samples. The study protocol for clinical samples (results presented in **Table 1**) was approved by the Institutional Review Board of Osaka University Medical School, and written informed consent was obtained from each subject (**Supplementary Note**).

Statistical analyses. We conducted statistical analysis by analysis of variance with the Student's *t*-test. We considered a P value of 0.05 or less as a significant difference.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

K.H. performed the experimental work, data analysis and writing of the first draft of the manuscript. K.K. and T.O. selected the initial set of genes subjected to the screening. K.I.-K., K.K., T.Y. and T.O. participated in the conception, design and coordination of the study. F.T. and Y.Y. performed siRNA delivery *in vivo* and helped with data analysis. K.N. provided drug-resistant cell lines. S.N. provided delivery molecules. The manuscript was finalized by T.O. with the assistance of all authors.

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Research Article

Antitumor Effect of SN-38–Releasing Polymeric Micelles, NK012, on Spontaneous Peritoneal Metastases from Orthotopic Gastric Cancer in Mice Compared with Irinotecan

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Abstract

7-Ethyl-10-hydroxy-camptothecin (SN-38), an active metabolite of irinotecan hydrochloride (CPT-11), has potent antitumor activity. Moreover, we have reported the strong antitumor activity of NK012 (i.e., SN-38–releasing polymeric micelles) against human cancer xenografts compared with CPT-11. Here, we investigated the advantages of NK012 over CPT-11 treatment in mouse models of gastric cancer with peritoneal dissemination. NK012 or CPT-11 was i.v. administered thrice every 4 days at their respective maximum tolerable doses (NK012, 30 mg/kg/day; CPT-11, 67 mg/kg/day) to mice receiving orthotopic transplants of gastric cancer cell lines (44As3Luc and 58As1mLuc) transfected with the luciferase gene (n = 5). Antitumor effect was evaluated using the photon counting technique. SN-38 concentration in gastric tumors and peritoneal nodules was examined by high-performance liquid chromatography (HPLC) 1, 24, and 72 hours after each drug injection. NK012 or CPT-11 distribution in these tumors was evaluated using a fluorescence microscope on the same schedule. In both models, the antitumor activity of NK012 was superior to that of CPT-11. High concentrations of SN-38 released from NK012 were detected in gastric tumors and peritoneal nodules up to 72 hours by HPLC. Only a slight conversion from CPT-11 to SN-38 was observed from 1 to 24 hours. Fluorescence originating from NK012 was detected up to 72 hours, whereas that from CPT-11 disappeared until 24 hours. NK012 also showed antitumor activity against peritoneal nodules. Thus, NK012 showing enhanced distribution with prolonged SN-38 release may be ideal for cancer treatment because the antitumor activity of SN-38 is time dependent. [Cancer Res 2008;68(22):9318–22]

Introduction

Gastric cancer is the second most common cause of death from cancer in the world. The survival rate has remained low in patients with advanced gastric cancer, with a median survival rate of 13 months having been recently reported in a phase III trial, which has been the best outcome thus far (1). Patients with gastric cancer with scirrhous type stroma particularly showed poor prognosis even after curative resection, as well as highly progressed peritoneal dissemination (2). Because peritoneal dissemination causes several refractory symptoms such as massive ascites, intestinal obstruction, hydronephrosis, and obstructive jaundice, the quality of life of patients at the end stage of cancer is severely impaired.

Poor delivery of anticancer drugs to peritoneal metastatic cells may be one of the reasons for the poor prognosis of patients with peritoneal dissemination (3). In peritoneal nodules, the distribution and eventual diffusion of drugs to cancer cells tend to be impeded because of several obstacles such as severe fibrosis and high interstitial pressure (4, 5). On the other hand, angiogenesis was reported to be an essential factor in the development of peritoneal metastasis, and the high expression level of vascular endothelial growth factor (VEGF) in primary gastric tumors or ascitic fluid, which can enhance tumor vascular permeability, was found to be directly associated with the development of ascites and peritoneal dissemination (6–10). In addition, several factors such as kinins and nitric oxide are involved in tumor vascular permeability (11–13). Polymer-conjugated drugs and nanoparticles categorized under drug delivery system agents are favorably extravasated from the vessels into the interstitium of tumors due to the enhanced permeability and retention effect (EPR effect; refs. 14, 15). The EPR effect is based on the following pathophysiologic characteristics of solid tumor tissues: hypervascularity, incomplete vascular architecture, secretion of vascular permeability factors stimulating extravasation within cancer tissue, and absence of effective lymphatic drainage from the tumors that impedes the efficient clearance of macromolecules accumulated in solid tumor tissues. Moreover, macromolecules cannot freely leak out from normal vessels, and thus, the side effects of an anticancer agent can be reduced. Very recently, we have shown that NK012 (i.e., SN-38–releasing polymeric micelles) exerted superior antitumor activity and less toxicity than CPT-11 (15–17). In a series of studies, we showed that NK012 markedly enhanced the antitumor activity of SN-38, particularly against highly VEGF-secreting SBC-3/VEGF tumors compared with SBC-3/Neo tumors. On the other hand, it is conceivable that satisfactory drug delivery cannot be achieved in less-vascularized and highly fibrotic tumors, particularly for macromolecules. However, we observed that NK012 showed a strong antitumor activity even in the xenograft of Capan1 cells, which are pancreatic cancer cells with abundant stromal tissue, compared with CPT-11. This result suggests that NK012 can selectively accumulate in both hypervascular and hypovascular tumors with high interstitial pressure, and then induce sustained

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release of SN-38, followed by SN-38 distribution throughout the entire tumor tissues. In the present study, we evaluated the antitumor activity of NK012 against peritoneal tumor dissemination compared with that of CPT-11 using mouse models orthotopically transplanted with scirrhous gastric cancer cells, as well as against spontaneously progressing peritoneal dissemination (18, 19).

Materials and Methods

Cell cultures. 44As3 and 58As1m were previously reported as human signet-ring cell gastric cancer cell lines that spontaneously metastasize to the peritoneal cavity and produce large volumes of bloody ascites after orthotopic implantion in the gastric wall (18–21). Here, 44As3 and 58As1m cells were transfected with a complex of 4 μg of pEGF-PLuc plasmid DNA (Clontech) and 24 μL of GeneJammer reagent (Stratagene; Cloning Systems) in accordance with the manufacturer's instructions. Stable transfectants were selected in geneticin (400 μg/mL; Invitrogen), and bioluminescence was used to screen transfected clones for luciferase gene expression using the IVIS system (Xenogen). Clones expressing the luciferase gene were named 44As3Luc and 58As1mLuc. 44As3Luc and 58As1mLuc cells were maintained in RPMI 1640 supplemented with 10% FCS (Sigma), 100 IU/mL penicillin G sodium, and 100 mg/mL streptomycin sulfate (Immuno-Biological Laboratories) in a humidified atmosphere containing 5% CO_2 at 37°C.

Orthotopic models *in vivo*. Six-week-old female BALB/c *nu/nu* mice were purchased from CLEA Japan, Inc., and maintained under specific pathogen-free conditions and provided with sterile food, water, and cages. Ambient light was controlled to provide regular cycles of 12 h of light and 12 h of darkness. A total of 1×10^6 cells of 44As3Luc or 58As1Luc were inoculated into the gastric wall of each mouse after laparotomy, as described previously (18–21). *In vivo* photon counting analysis was conducted on a cryogenically cooled IVIS system using Living Image acquisition and analysis software (Xenogen). All animal procedures were performed in compliance with the Guidelines for the Care and Use of Experimental Animals established by the Committee for Animal Experimentation of the National Cancer Center; these guidelines conform to the ethical standards required by law and also comply with the guidelines for the use of experimental animals in Japan.

Drugs. NK012 was prepared by Nippon Kayaku Co., Ltd. (15). CPT-11 was purchased from Yakult Honsha Co., Ltd.

***In vivo* growth inhibition assay.** After inoculation of 44As3Luc or 58As1mLuc cells into the gastric wall (day 0), mice were randomly divided into test groups consisting of 5 mice per group. 44As3Luc mice were i.v. administered the maximum tolerated dose (MTD) of the 2 drugs via the tail vein on days 20, 24, and 28 as previously reported, that is, at 66.7 mg/kg/d for CPT-11 and 30 mg/kg/d for NK012 (15). 58As1mLuc mice were given the drugs in the same manner on days 18, 22, and 26. Photon counting analysis and body weight were measured twice a week. "Visible ascites," which was evident a few days before death in this mouse model, was used as a surrogate for survival time in consideration of animal welfare. Mice were euthanized when ascites became visible, and colonization of gastric wall by cancer cells and metastasis to the peritoneal cavity were confirmed in all the euthanized mice. Differences in relative photon counts between the treatment groups at day 42 in 44As3Luc mice and at day 81 in 58As1mLuc mice were analyzed using the unpaired *t* test.

Assay of free SN-38 in tissues. We next analyzed the biodistributions of NK012 and CPT-11 to orthotopic gastric tumors and peritoneal nodules. Twenty-six days after the inoculation of 44As3Luc cells into the gastric wall of mice, NK012 (30 mg/kg) or CPT-11 (66.7 mg/kg) was administered via the tail vein. Under anesthesia, orthotopic gastric tumor and peritoneal nodule samples were excised 1, 24, and 72 hours after injection.

Measurements of tissue concentration of free SN-38 by high-performance liquid chromatography. Samples were rinsed with physiologic saline, mixed with 0.1 mol/L glycine-HCl buffer (pH 3.0)/methanol at 5 w/w%, and then homogenized. To analyze the concentration of free SN-38, 100 μL of the tumor samples were mixed with 20 μL of 1 mmol/L phosphoric acid/methanol (1:1) and 40 μL of ultrapure water, and camptothecin (CPT) was used as the internal standard (10 ng/mL for free SN-38). The samples were vortexed vigorously for 10 s, and then filtered

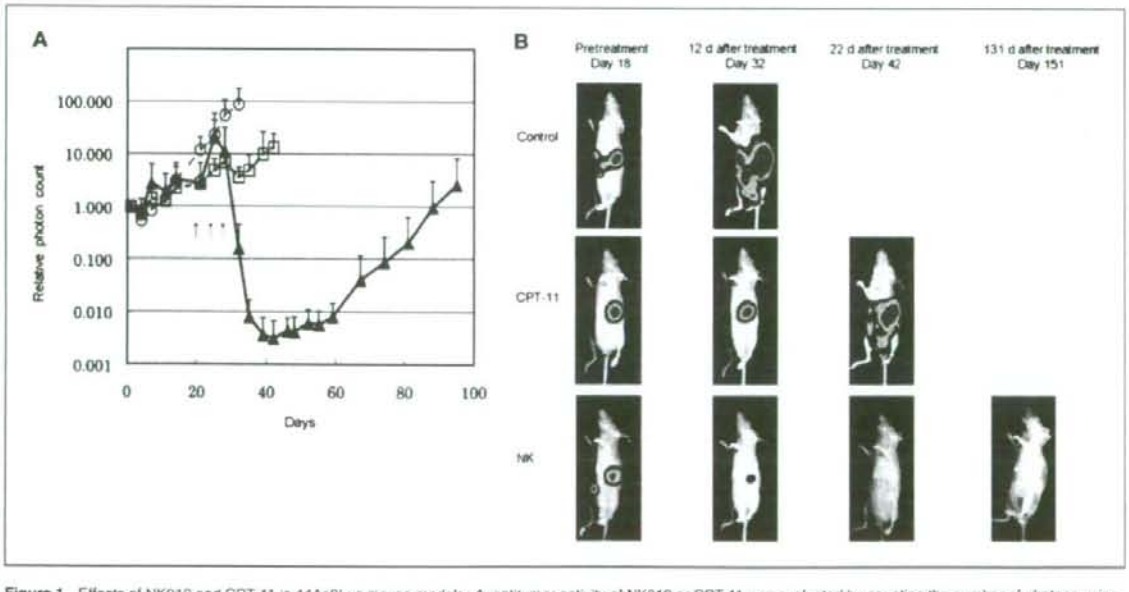

Figure 1. Effects of NK012 and CPT-11 in 44As3Luc mouse models. *A*, antitumor activity of NK012 or CPT-11 was evaluated by counting the number of photons using the IVIS system (*points*, mean; *bars*, SD; *arrows*, drug injections). Antitumor effect of each regimen on days 20, 24, and 28. (○), control; (□), CPT-11 (66.7 mg/kg/d, ×3); and (▲), NK012 (30 mg/kg/d, ×3) in 44As3Luc mouse model. *B*, images of 44As3Luc mouse model administered NK012 taken using the IVIS system on days 18, 32, 42, and 151 after inoculation of 44As3Luc cells. Data were derived from the same mice as those used in the present study.

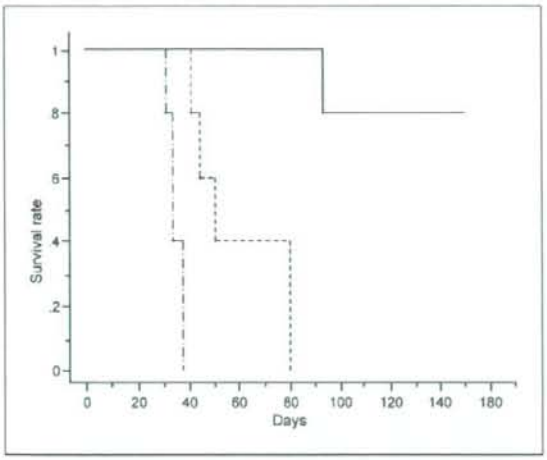

Figure 2. Survival curves of 44As3Luc mouse models. Survival curves of 44As3Luc mouse model in each regimen on days 20, 24, and 28. (– – –), control; (- - -), CPT-11 given at 66.7 mg/kg/d ×3; and (——), NK012 given at 30 mg/kg/d ×3.

through Ultrafree-MC Centrifugal Filter Devices with a cutoff molecular diameter of 0.45 μm (Millipore Co.). Reversed-phase high-performance liquid chromatography (HPLC) was conducted at 35°C on a Mightysil RP-18 GP column (150 × 4.6 mm; Kanto Chemical Co., Inc.). Fifty microliters of a sample were injected into an Alliance Water 2795 HPLC system (Waters) equipped with a Waters 2475 multi λ fluorescence detector. Fluorescence originating from SN-38 was detected at 540 nm with an excitation wavelength of 365 nm. The mobile phase was a mixture of 100 mmol/l ammonium acetate (pH 4.2) and methanol [11:9 (v/v)], and the flow rate was 1.0 mL/min. The content of SN-38 was calculated by measuring the relevant peak area and calibrating against the corresponding peak area derived from the CPT internal standard. Peak data were recorded using a chromatography management system (MassLynx v4.0; Waters).

Visualization of distribution of NK012 and CPT-11 by fluorescence microscopy. Mice were given fluorescein *Lycopersicon esculentum* lectin (100 μL per mouse; Vector Laboratories) to visualize tumor vasculature in the samples 5 min before anesthesia. The samples were then excised and embedded in an optimal cutting temperature compound (Sakura Finetechnochemical Co., Ltd.) and frozen at −80°C. Six-μm-thick tumor sections were then prepared using a cryostatic microtome, Tissue-Tek Cryo3 (Sakura Finetechnochemical Co., Ltd.). Frozen sections were examined under a fluorescence microscope, BIOZERO (KEYENCE), at an excitation wavelength of 377 nm and an emission wavelength 447 nm to evaluate the distribution of NK012 and CPT-11. Both drugs could be detected under the same fluorescence conditions because formulations containing SN-38 bound via ester bonds possess a particular fluorescence.

Statistical analyses. Data were expressed as mean ± SD. Data were analyzed using the Student's *t* test when groups showed equal variances (*F* test), or the Welch's test when they showed unequal variances (*F* test). *P* value of <0.05 was considered as significant. All statistical tests were two sided.

Results

Antitumor activities of NK012 and CPT-11. Comparison of the relative photon counts on day 42 in the 44As3Luc mouse model revealed significant differences in counts between mice given with NK012 and those given with CPT-11 ($P = 0.0282$; Fig. 1*A* and *B*). Similar result was obtained in the experiment with 58As gastric tumor (data not shown). The survival rates on day 150 in the 44As3Luc mouse model were 80% and 0% for the NK012 group and CPT-11 group, respectively (Fig. 2). Similar result was obtained in the experiment with 58As gastric tumor (data not shown). No marked toxic effects in terms of body weight changes were observed in any groups for any mouse models (data not shown). Only 1 mouse in the CPT-11 group of 44As1 mouse models showed diarrhea for 3 d, and any other clinical symptoms were not observed.

Tissue concentrations of free SN-38 after administration of NK012 and CPT-11. We examined the concentration-time profile of free SN-38 in orthotopic gastric tumors and peritoneal nodules in the 44As3Luc mouse model after the administration of NK012 and CPT-11 (Fig. 3*A* and *B*). Either orthotopic gastric tumors or peritoneal nodules exhibited the highest concentration of free SN-38 24 hours after NK012 administration, and 1 hour after CPT-11 administration. The highest concentrations of free SN-38 in the NK012 group were much higher than those in the CPT-11 group in either orthotopic gastric tumors or peritoneal nodules. The concentrations of free SN-38 released from NK012 in orthotopic gastric tumors were higher than those in peritoneal nodules.

Tumor tissue distribution of NK012 and CPT-11 as determined by fluorescence microscopy. Results showed that NK012 accumulation in either orthotopic gastric tumors or peritoneal nodules had been maintained from 1 hour to 72 hours after injection (Fig. 4*A*). On the other hand, CPT-11 showed maximum accumulation in either orthotopic gastric tumors or peritoneal nodules 1 hour after injection and disappeared within 24 hours (Fig. 4*B*).

Discussion

The main purpose of this study was to clarify the advantages of NK012 over CPT-11 as treatment against peritoneal metastasis spontaneously disseminated from orthotopically transplanted scirrhous gastric cancer cells in mouse models. We showed that NK012 exerted more potent antitumor activity in the mouse models used than CPT-11. Therefore, NK012 is considered promising in terms of providing clinical benefit to patients with gastric cancer showing progressing peritoneal dissemination.

CPT-11 is converted to SN-38, a biologically active and water-insoluble metabolite of CPT-11, by carboxylesterases (CE) in the liver and tumors. However, only 2% to 8% of administered CPT-11 is converted by CE in the liver and tumors to the active form SN-38 (22, 23). The conversion of CPT-11 to SN-38 also depends on genetic interindividual variability of the activity of CE (24). Thus, the direct use of SN-38 might be of great advantage and is attractive for cancer treatment. We have recently shown that NK012 (i.e., SN-38–releasing polymeric micelles) exerted superior antitumor activity and less toxicity than CPT-11 (15–17). The mean particle size of NK012 is 20 nm in diameter. NK012 can release SN-38 under neutral conditions even in the absence of CE because SN-38, which is bound to the blockcopolymer by phenolic ester binding, is stable under acidic conditions but relatively labile under neutral and mild alkaline conditions. The release rate of SN-38 from NK012 under physiologic conditions is quite high, that is, >70% of SN-38 is gradually released within 48 hours.

In this study, we used mouse models with orthotopically transplanted human scirrhous gastric cancer cells showing spontaneously progressing peritoneal dissemination, which we

reported previously (18, 19, 21). These models can imitate more realistically the progressing mode of human peritoneal dissemination of gastric cancer than conventional experimental models directly transplanted with cancer cells i.p. Moreover, our models enabled us to quantitatively evaluate drug antitumor effect even against peritoneal dissemination without having to sacrifice the animal and perform autopsy through the use of gastric cancer cells transfected with the luciferase gene and by applying photon counting analysis, having already verified the significant correlation between tumor volume and photon counts in a previous report (19).

For *in vivo* growth inhibition assay, drug administration was started on day 18 or 20 after cell inoculation into the gastric wall, when small peritoneal metastatic nodules and a small degree of ascites had appeared. The present results showed that NK012 had more potent antitumor activity than CPT-11 in the mouse models tested, suggesting its effectiveness against peritoneal dissemination of gastric cancer in the clinical setting.

In the pharmacologic evaluation, we could confirm the more enhanced distribution of NK012 than CPT-11 to not only orthotopic gastric tumors but also peritoneal nodules by quantifying SN-38 concentration in the tumors and visualization of fluorescence originating from NK012 or CPT-11 distributed in the tumors. Because CPT-11 or SN-38 has been reported to possess time-dependent growth-inhibitory activity against tumor cells, this prolonged retention of NK012 in the tumors and the sustained release of free SN-38 from NK012 may be responsible for its more potent antitumor activity observed in the present study (25). On the other hand, CPT-11 disappeared from the tumors before exerting sufficient antitumor activity. For both drugs, however, the concentrations of SN-38 in orthotopic gastric tumors were higher than those

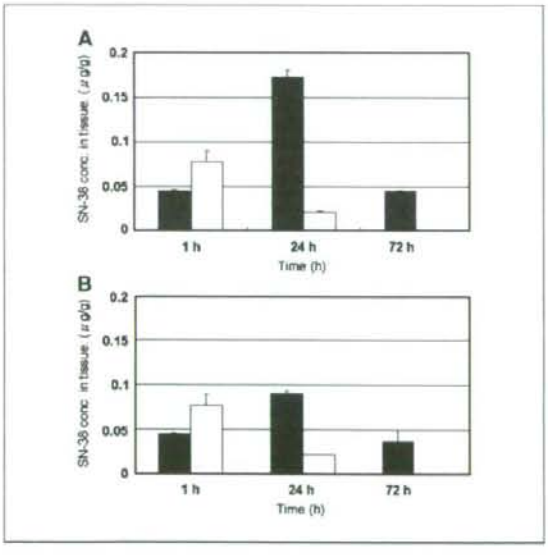

Figure 3. Concentration-time profile of free SN-38. NK012 (30 mg/kg) or CPT-11 (66.7 mg/kg) was injected 26 d after implantation of 44As3Luc gastric cancer cells (*columns*, mean; *bars*, SD). *A*, concentration (*conc.*) of free SN-38 in orthotopic gastric tumor tissue of 44As3Luc mouse model after administration of NK012 (*black column*) and CPT-11 (*white column*). *B*, concentration of free SN-38 in peritoneal nodules of 44As3Luc mouse model after administration of NK012 (*black column*) and CPT-11 (*white column*).

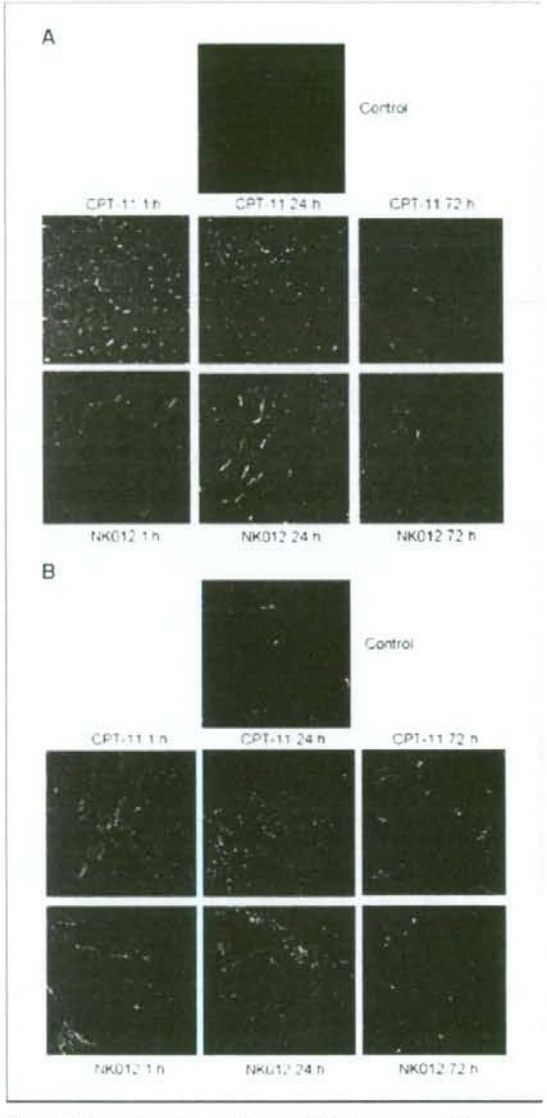

Figure 4. Tissue distribution of NK012 and CPT-11 as determined by fluorescence microscopy. Orthotopic gastric tumors or peritoneal nodules of 44As3Luc mouse model were excised 1, 24, and 72 h after i.v. injection of NK012 (30 mg/kg) or CPT-11 (66.7 mg/kg). Each mouse was i.v. administered with fluorescein-labeled *Lycopersicon esculentum* lectin just before being sacrificed to detect tumor blood vessels. Frozen sections were examined under a fluorescence microscope at an excitation wavelength of 377 nm and an emission wavelength of 447 nm. The same fluorescence condition can be applied for visualizing NK012 and CPT-11 fluorescence. Free SN-38 cannot be detected under this fluorescence condition. *A*, distribution of NK012 or CPT-11 in orthotopic gastric tumors (×100). *B*, distribution of NK012 or CPT-11 in peritoneal nodules (×100).

in peritoneal nodules. This is consistent with previous reports stating the poor delivery of anticancer drugs to peritoneal metastatic cells probably because of some obstacles such as abundant interstitium or high interstitial pressure. To date, we reported that NK012 can

more selectively accumulate and retain longer in various tumor xenografts transplanted s.c. compared with CPT-11 (15–17). In the present study, we succeeded in demonstrating higher accumulation and longer retention of NK012 compared with CPT-11 in orthotopic and peritoneal disseminated gastric cancer model that is closer to human gastric cancer in clinics.

Peritoneal dissemination sometimes causes intestinal obstruction, which enhances the enterohepatic circulation of SN-38 after direct damage to the small intestine, and makes the use of CPT-11 difficult (26, 27). In the present study, no mouse in the NK012 group developed diarrhea. The dose-limiting toxic effects of CPT-11 seem to be neutropenia and diarrhea. In our previous data, however, there was no significant difference in the level of SN-38 in the small intestine between mice treated with NK012 and mice treated with CPT-11 despite the higher plasma area under the concentration of NK012 than CPT-11 (15). Moreover, no serious diarrhea has been reported even at the MTD dose in two phase I clinical trials against advanced solid tumors in Japan and the US (28, 29).

In conclusion, we showed that NK012 exerts significantly more potent antitumor activity against peritoneal dissemination of scirrhous gastric cancer cells than CPT-11, indicating the possibility of the clinical evaluation of this drug in patients with disseminated grastic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Original Article

Long-Term Probucol Treatment Prevents Secondary Cardiovascular Events: a Cohort Study of Patients with Heterozygous Familial Hypercholesterolemia in Japan

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Aim: The POSITIVE study assessed whether long-term treatment with probucol, a potent anti-oxidant and cholesteryl ester transfer protein (CETP) activator, is associated with a lowered risk of cardiovascular events in a very high-risk population: familial hypercholesterolemia (FH).
Methods: The study cohort included 410 patients with heterozygous FH, diagnosed between 1984 and 1999 by cardiovascular and metabolic experts at fifteen centers. Traceable patients were screened using predefined eligibility criteria. The primary outcome measure for comparison between probucol exposure and non-exposure was the time to the first cardiovascular event involving hospitalization.
Results: Analysis revealed significant differences in baseline characteristics and follow-up treatment between exposure and non-exposure. An observed indication bias was the use of probucol in more severe FH at diagnosis, both for primary and secondary prevention. When the multivariate Cox regression procedure was used after adjustment for possible confounding factors, probucol lowered the risk (hazard ratio [HR], 0.13; 95% confidence interval [CI], 0.05–0.34) in secondary prevention ($n=74$) and was statistically significant ($p<0.001$), although not significant (HR, 1.5; 95% CI, 0.48–4.67; $p=0.49$) in primary prevention ($n=233$). Safety assessment found no specific difference between exposure and non-exposure.
Conclusion: Long-term probucol treatment may prevent secondary attack in a higher cardiovascular risk population of heterozygous FH.

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Key words; Atherosclerosis, Antioxidants, CETP activator, Dyslipidemia

Introduction

Cardiovascular (CV) diseases, including coronary heart disease and stroke, are the leading cause of death in Japan. Prevention of fatal CV events is therefore the final goal as well as the rationale of cholesterol-lowering therapy.

Probucol, a conventional cholesterol-lowering drug, originated with the report by Barnhart in 1970[1]. The drug has been used clinically in Japan since 1985. Nearly 60,000 Japanese patients still take probucol; western countries discontinued probucol use after

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the original manufacturer's withdrawal notice to the United States FDA in 1995 after 18 year's use of the drug. Probucol's cholesterol-lowering mechanism has not yet been clearly established, but it is thought to increase catabolic excretion of cholesterol into bile[2]. Later studies[3-5] have described new mechanisms of probucol, including anti-atherogenic and anti-oxidant actions. Another controversial and anti-atherogenic feature of probucol is its paradoxical effect of lowering high-density lipoprotein cholesterol (HDL-C). This action reflects, most likely, its molecular mechanisms: promoting cholesterol efflux, and enhancing reverse cholesterol transport by activation of cholesteryl ester transfer protein (CETP)[6-8] and class B type 1 scavenger receptor[9, 10]. Matsuzawa and his colleagues reported an observed close correlation between the extent of regression in Achilles' tendon xanthoma and probucol-induced decrease in HDL-C levels in patients with familial hypercholesterolemia (FH)[11].

No large-scale, randomized, double blind comparative study has been conducted to justify the use of probucol in the prevention of CV events or diseases. however, clinical studies as well as pre-clinical data have been accumulating evidence of the clinical worth of probucol in arteriosclerotic diseases. Numerous clinical results, including a reduction in Achilles' tendon xanthoma thickness after long-term treatment for FH[12, 13], reduced rates of restenosis after angioplasty[14-16], and a decrease in carotid artery intima-media thickness[17, 18] support the therapeutic and preventative effects of probucol on arteriosclerotic lesions and plaque. To evaluate the risk and benefit of long-term probucol treatment, we conducted a cohort study to determine whether probucol treatment is associated with the risk reduction of CV events in patients with heterozygous FH, a very high-risk population.

Methods

Study Cohort

We registered patients with FH who received treatment between January 1, 1984 and December 31, 1999 at 15 centers specializing in CV and metabolic diseases, including FH, nationwide. Patients were traceable by medical recend and met the diagnostic criteria for heterozygous FH under the Japan Atherosclerosis Society Guidelines (2002) for the Diagnosis and Treatment of Atherosclerotic CV Diseases[19]. Definite heterozygous FH was defined as having at least two of the major features: total cholesterol (TC) of 260 mg/dL and above; tendon xanthoma or xanthoma tuberosum; reduced or abnormal receptor activity noted by LDL receptor analysis. Probable heterozygous FH was defined as having at least one each of the major (as above) and minor features: palpebral xanthoma; arcus juvenilis (<50 years); juvenile (<50 years) ischemic heart disease. For other eligibility criteria, we excluded patients with possible homozygous FH or with severe ventricular arrhythmias (polymorphic premature ventricular contractions). Possible homozygous FH was defined as having any one of the clinical features: defect of homozygous or hetero-polymeric LDL receptors confirmed by gene analysis; no LDLR activity observed by receptor analysis, severe elevation of plasma TC higher than 500 mg/dL; xanthoma or atherosclerotic vascular lesions including symptoms of juvenile ischemic heart disease; hypercholesterolemia confirmed in both parents; history of ischemic heart disease confirmed in both parents; or poor response to any 3-hydroxy-3methyl-glutaryl-coenzyme A reductase inhibitor (statin).

During the study period between June, 2004 and September, 2005, we collected anonymous case report forms with the patients' baseline data, including medical history, findings at clinical examination, medication data, and laboratory data. The investigators transcribed the data on to case report forms (identified by a code) from the stored medical charts of the patients. The observation period was the period for which each patient's clinical course could be traced. The longest observation period exceeded 20 years for patients on stable doses of probucol.

We required a sample size of 200 in both the probucol exposure and non-exposure groups, supposing a difference of 10% in the incidence of CV events for 5 years (15% in exposure and 25% in non-exposure). A least 400 subjects were needed to detect the difference with 80% power and a type I error of 5% at the 5% significance level with two-sided log-rank test based on normal approximation. The study protocol was approved through the process of ethics committee or institutional review board at each center.

Definitions and Endpoints

The primary outcome measure was the time to the first CV event, defined as acute myocardial infarction (MI), angina pectoris (AP), heart failure (HF), stroke, transient ischemic attack (TIA) or arteriosclerotic peripheral artery diseases (PAD) leading to hospitalization or death as well as sudden death within 24 hours of an observed intrinsic event. The obtained baseline data at the first visit of each patient included demographic characteristics: sex, date of diagnosis at the participant medical center, age, height, weight, and habits of smoking and drinking. Body mass index (BMI) was calculated as weight in kilograms divided

by the square of height in meters. The other collected characteristic factors at diagnosis were the presence of xanthoma and its location, prior CV event, onset date if any prior CV event, treatment for the event, and other possible risk factors for CV events, including the presence of hypertension, diabetes, ventricular arrhythmia, and PAD. We collected data on cholesterol-lowering therapy (with or without probucol) and other concomitant therapy with anti-platelet, antihypertensive or diabetic drugs. Dates of drug initiation, discontinuation, re-administration, and termination were entered as elemental information. Treatment period was defined as the length from initiation until medication termination, or until the occurrence of the defined CV event, whichever came first. A lipid profile of TC, triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) and HDL-C, blood pressure, level of fasting blood sugar (FBS), hemoglobinA_{1c} (HbA_{1c}), and thickness of tendon xanthoma in both feet were variables of interest, seen as potential predictors of CV events. We obtained measurements of those variables on a yearly basis after each patient was diagnosed. LDL-C levels were calculated from TC and HDL-C measurements with the Friedewald formula in TG <400 mg/dL. For TG of 400 mg/dL and more than 400 mg/dL, the expression of 0.16 X TG was applied in stead of 0.2 X TG[20]. Most patients had fasted compliantly at periodic checkups of their lipid levels. We set a follow-up period of 10 years for the measurements.

Statistical Analyses

The primary objective of analysis was a comparison between probucol exposure and non-exposure to evaluate whether treatment with probucol (500 mg to 1,000 mg daily) for FH provided CV benefits. The analysis was based on intent-to-treat principles. The secondary objective was to assess whether changes in the lipid profile after probucol treatment predicted CV events in the cohort. Event-free survival, defined as the time from diagnosis to the first CV event, was determined as a response variable. Statistical analysis was performed to evaluate clinical outcomes separately for secondary and primary prevention groups: that is, patients with or without a history of CV events at diagnosis.

Baseline characteristics of each group were explored to detect risk factors for CV events because potential confounders, including indication bias, were anticipated. For baseline comparison, Wilcoxon's rank sum test and Fisher's exact test were used for continuous variables and categorical variables respectively. For detection of risk factors, univariate Cox proportional hazards regression with a baseline variable as covariate was used as a screening step to determine the relationship with CV events. Variables that achieved significance at the level of 20% in univariate analysis were subsequently included in a multivariate Cox proportional hazards regression using backward variable selection. Variables proving significant at the 10% significance level were selected as risk factors to be adjusted. Consequently, probucol treatment effect was evaluated using the multivariate Cox model with adjustment for the selected baseline variables. Finally, the other observed treatment factors: cholesterol-lowering drugs other than probucol, LDL-apheresis, anti-platelet drugs, anti-hypertensive drugs, and diabetic drugs were entered into that model to assess their effects.

For the association between changes in lipid profile after probucol treatment and the risk of CV events, pre-treatment values of TG, LDL-C, HDL-C as well as TC, and each lipid reduction ratio after treatment were used as covariates. Multivariate analyses of time from probucol start to the first CV event used multivariate Cox's proportional hazards models. Statistical analysis was peerformed with SAS version 8.2.

Results

Patient Characteristics

We collected data from the medical records of 541 patients, and excluded the data of 131 patients that did not meet eligibility predefined in the protocol.

The flow diagram (**Fig. 1**) gives reasons for the exclusion. A substantial fraction of probucol-exposed patients, 80.0% and 93.2%, took probucol within two years after diagnosis for in primary and secondary prevention groups, respectively. Baseline characteristics at diagnosis are given for each group (**Table 1**, **2**). The secondary prevention group (**Table 2**) had prior diseases of AP, MI, stroke, HF, and TIA. This group was found to have significant higher proportions of men (60.2%, $p<0.01$), smokers (50.0%, $p<0.01$), hypertension (40.9%, $p<0.001$) diabetes (15.9%, $p=0.02$), and older median age (52 years, $p=0.01$) than the primary prevention group. Moreover, the group tended to have hypo-HDL cholesterolemia of median 42 (20–90) mg/dL, and to receive combined treatments with anti-platelet drugs (56.8%), anti-hypertensive drugs (53.4%), and LDL-apheresis (14.8%).

Comparison between probucol-exposed and non-exposed groups revealed significant differences in some baseline characteristics and treatments, which showed a confounding indication that patients with more severe FH took probucol. For baseline characteristics, the exposed group for primary prevention had more

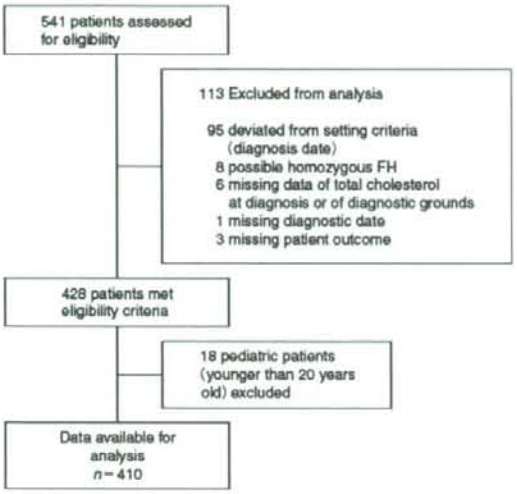


Fig. 1. Patient Flowchart.

We collected data from the medical records of 541 patients, and excluded the data of 131 patients who did not meet the eligibility predefined in the protocol. The flow diagram gives reasons for the exclusion.

palpebral xanthoma (13.4%, $p=0.05$), thicker median measurement of tendon xanthoma (12.5 mm, $p<0.01$), higher median HbA_{1c} (5.8%, $p=0.03$), and more use of antihypertensive drugs (25.3%, $p<0.01$). Their lipid profile was more severe with a higher median baseline TC (325 mg/dL, $p=0.001$), a higher median LDL-C level (253 mg/dL, $p<0.001$), and a lower HDL-C level (47 mg/dL, $p<0.001$) than the unexposed group. The exposed group for secondary prevention had a higher prevalence of post-MI (44.6%, $p<0.01$) than the unexposed group. Observed medications were also significantly different between the exposed and unexposed groups. The exposed group used anti-hypertensive drugs concomitantly at a higher rate (25.3% *vs.* 11.2%, $p<0.01$) for primary prevention.

Descriptive analysis of baseline characteristics and treatments during observation implies that in both primary and secondary prevention, the exposed groups tended to include patients with more severe FH at diagnosis. Arguably, patients considered more severe at diagnosis would receive more intensive treatment, including probucol.

Outcomes

We present the absolute number of CV events requiring hospitalization by prevention group with details of the events (**Table 3**). The incidence of CV events without consideration of confounding factors was 11.6% in the exposed group and 4.5% in the unexposed group for primary prevention. For secondary prevention, the incidence was 27.0% in the exposed group and 64.3% in the unexposed group. The event-free survival curve of the secondary prevention group is given (**Fig. 2**).

To identify risk factors for CV events, we determined the relationship between the incidence and every baseline variable using univariate Cox regression at a significant level of 20%. Variables proving significant at the 10% significance level in multivariate Cox regression were selected as risk factors to be adjusted. We estimated the effect of treatment after adjusting the selected risk factors. We calculated hazard ratios (HRs) with 95% confidence interval (CI) for binary variables, BMI ≥25 vs BMI <25, drinking *vs* no drinking, for example, and the indicated HRs corresponded to a 1 standard deviation increase for continuous variables, including TC. Estimated results are given (**Table 4**).

In the primary prevention group, significant variables were BMI ≥25 (HR 1.86, 95% CI 0.87–3.98; $p=0.11$), drinking (HR 2.17, 95% CI 1.02–4.63; $p=0.05$), tendon xanthoma (HR 2.17, 95% CI 0.76–6.23; $p=0.15$), prior diseases other than CV events (HR 1.87, 95% CI 0.87–3.99; $p=0.11$), PAD (HR 5.23, 95% CI 0.70–39.2; $p=0.11$), diabetes (HR 2.27, 95% CI 0.79–6.50; $p=0.13$), TC (HR 1.37, 95% CI 0.99–1.89; $p=0.06$), HDL-C (HR 0.75, 95% CI 0.50–1.12, $p=0.16$), SBP (HR 1.48, 95% CI 1.00–2.18; $p=0.05$), and the thickness of tendon xanthoma (HR 1.50, 95% CI 1.06–2.14; $p=0.02$). Three of these variables, drinking, TC, and PAD were selected for adjustment at the 10% significance level as a result of a multivariate Cox regression with backward variable selection. After adjustment for these three baseline variables, we found no significant effect by probucol at the 5% significant level. The estimated hazard ratio of probucol use for CV events was 1.50 (95% CI 0.48–4.67; $p=0.49$).

In the secondary prevention group, significance variables were drinking (HR 1.74, 95% CI 0.80–3.79; $p=0.17$), presence of palpebral xanthoma (HR 5.34, 95% CI 2.26–12.61, $p<0.001$), TIA (HR 4.16, 95% CI 0.54–32.21; $p=0.17$), history of coronary artery bypass graft (HR 0.31, 95% CI 0.11–0.90; $p=0.03$), hypertension (HR 0.58, 95% CI 0.26–1.28; $p=0.18$), diabetes (HR 2.89, 95% CI 1.30–6.42; $p<0.01$), and fasting blood sugar (HR 1.31, 95% CI 0.91–1.89; $p=0.15$). Two of these variables, palpebral xanthoma and diabetes, were selected for adjustment at the 10% sig-

Table 1. Baseline characteristics of patients in primary prevention group[†]

Characteristics	Primary prevention No. (%) of patients			*p*
	All *n*=322	Exposed *n*=233 (72.4)	Unexposed *n*=89 (27.6)	
Age, mean (range)	49 (27-74)	50 (20-74)	47 (20-72)	0.18
Men, No. (%)	134 (41.6%)	96 (41.2%)	38 (42.7%)	0.90
BMI ≥ 25	71 (22.5%)	49 (21.4%)	22 (25.6%)	0.45
Smoker	99 (33.2%)	74 (34.1%)	25 (30.9%)	0.68
Drinker	124 (42.2%)	93 (43.7%)	31 (38.3%)	0.43
Xanthoma	259 (80.7%)	190 (81.9%)	69 (77.5%)	0.43
Tendon xanthoma	245 (76.3%)	181 (78.0%)	64 (71.9%)	0.30
Nodular xanthoma	28 (8.7%)	22 (9.5%)	6 (6.7%)	0.51
Palpebral xanthoma	36 (11.2%)	31 (13.4%)	5 (5.6%)	0.05
PAD	4 (1.2%)	1 (0.4%)	3 (3.4%)	0.07
Hypertension	54 (16.8%)	40 (17.2%)	14 (15.7%)	0.87
Diabetes	22 (6.9%)	17 (7.3%)	5 (5.6%)	0.81
Lipid profile, mg/dL				
TC[‡]	320 (188-493)	325 (188-493)	307 (194-464)	0.001
TG[‡]	120 (28-1289)	121 (34-1068)	120 (28-1289)	0.96
HDL-C[‡]	49 (20-108)	47 (20-90)	52 (27-108)	<0.001
LDL-C[‡]	244 (45-425)	253 (98-425)	223 (45-403)	<0.001
Blood Pressure, mmHg				
SBP[‡]	129 (82-190)	128 (82-190)	131 (90-190)	0.57
DBP[‡]	0 (48-120)	80 (48-120)	80 (56-120)	0.91
FBS (mg/dL)[‡]	95 (63-276)	94 (63-140)	95 (81-276)	0.41
HbA_{1c} (%)[‡]	5.7 (4.1-12.4)	5.8 (4.1-9.7)	5.3 (4.3-12.4)	0.03
Tendon xanthoma thickness (mm)[‡]	12.1 (7.5-49.0)	12.5 (7.5-49.0)	10.5 (8.0-20.0)	<0.01
Treatment				
Cholesterol-lowering drugs (non-probucol)	302 (93.8%)	219 (94.0%)	83 (93.3%)	0.80
LDL-apheresis	7 (2.2%)	6 (2.6%)	1 (1.1%)	0.68
Anti-platelet drugs	49 (15.2%)	41 (17.6%)	8 (9.0%)	0.06
Anti-hypertensive drugs	69 (21.4%)	59 (25.3%)	10 (11.2%)	<0.01
Diabetic drugs	15 (4.7%)	12 (5.2%)	3 (3.4%)	0.37

[†]Continuous variables compared by Wilcoxon's rank sum test, distribution of categorical variables by Fisher's exact test. [‡]Data are median (range). All data are number (%) unless otherwise indicated. Each percentage shown is related to the total number with measurement data. BMI, body mass index; PAD, peripheral artery disease; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBS, fasting blood sugar; HbA_{1c}, hemoglobin A_{1c}. LDL-C was calculated with the Friedewald formula.

nificance level as a result of multivariate Cox regression analysis using a backward variable selection. After adjustment for these two baseline variables, the hazard ratio of probucol use for CV events was estimated to be 0.13 (95% CI 0.05-0.34) and significant ($p<0.001$). In sensitivity analyses, we also obtained similar estimation results on probucol for various sets of baseline covariates for adjustment.

The lipid levels of TC, LDL-C and HDL-C were lowered after probucol treatment both in primary and secondary prevention. In the primary prevention group, the median (range) levels of TC, TG, LDL-C and HDL-C closest to before treatment were respectively 305 (165-493), 119 (35-1068), 228 (107-425) and 48 (25-96) mg/dL, and those at 10-year treatment were, respectively, 222 (141-371), 94 (43-335), 157 (91-311) and 39 (17-81) mg/dL. In the secondary prevention, the median levels of TC, TG, LDL-C and HDL-C closest to before atment were, respectively, 320 (191-469), 129 (37-636), 240 (117-381) and 44 (24-90) mg/dL, and those at 10-year treatment were, respectively, 211(135-305), 71 (48-475),

Table 2. Baseline characteristics of patients in secondary prevention group

Characteristics	Secondary prevention No. (%) of patients			*p*
	All *n*=88	Exposed *n*=74 (84.1)	Unexposed *n*=14 (15.9)	
Age, mean (range)	52 (23-71)	51 (29-70)	53 (23-71)	0.62
Men, No. (%)	53 (60.2%)	46 (62.2%)	7 (50.0%)	0.55
BMI ≥ 25	21 (25.3%)	17 (24.3%)	4 (30.8%)	0.73
Smoker	42 (50.0%)	38 (53.5%)	4 (30.8%)	0.23
Drinker	39 (46.4%)	33 (46.5%)	6 (46.2%)	1.00
Xanthoma	75 (85.2%)	63 (85.1%)	12 (85.7%)	1.00
Tendon xanthoma	71 (80.7%)	61 (82.4%)	10 (71.4%)	0.46
Nodular xanthoma	7 (8.0%)	6 (8.1%)	1 (7.1%)	1.00
Palpebral xanthoma	8 (9.1%)	5 (6.8%)	3 (21.4%)	0.11
PAD	2 (2.3%)	2 (2.7%)	0 (0.0%)	1.00
Hypertension	36 (40.9%)	30 (40.5%)	6 (42.9%)	1.00
Diabetes	14 (15.9%)	9 (12.2%)	5 (35.7%)	0.04
Lipid profile, (mg/dL)				
TC†	332 (191-469)	334 (191-469)	322 (229-444)	0.41
TG†	128 (37-636)	128 (37-636)	136 (63-318)	0.85
HDL-C†	42 (20-90)	42 (20-90)	39 (26-73)	0.91
LDL-C†	249 (117-381)	256 (117-381)	245 (138-354)	0.57
Blood Pressure, mmHg				
SBP†	129 (90-180)	128 (96-180)	136 (90-166)	0.97
DBP (mmHg)†	80 (52-114)	80 (52-114)	78 (60-104)	0.33
FBS (mg/dL)†	96 (72-252)	97 (72-197)	94 (79-252)	0.96
HbA1c (%)†	5.8 (4.1-10.6)	5.5 (4.1-8.1)	6.4 (5.3-10.6)	0.06
Tendon xanthoma thickness (mm)†	14.5 (5.8-25.0)	15.0 (5.8-25.0)	10.0 (8.5-18.8)	0.09
Prior CV events				
Angina Pectoris	45 (51.1%)	36 (48.6%)	9 (64.3%)	0.39
Myocardial Infarction	34 (38.6%)	33 (44.6%)	1 (7.1%)	<0.01
Stroke	7 (8.0%)	4 (5.4%)	3 (21.4%)	0.08
Heart failure	2 (2.3%)	2 (2.7%)	0 (0.0)	1.00
TIA	2 (2.3%)	1 (1.4%)	1 (7.1%)	0.29
Treatment				0.08
Cholesterol-lowering drugs (non-probucol)	81 (92.0%)	70 (94.6%)	11 (78.6%)	
LDL-apheresis	13 (14.8%)	11 (14.9%)	2 (14.3%)	1.00
Anti-platelet drugs	50 (56.8%)	44 (59.5%)	6 (42.9%)	0.38
Anti-hypertensive drugs	47 (53.4%)	42 (56.8%)	5 (35.7%)	0.24
Diabetic drugs	6 (6.8%)	3 (4.1%)	3 (21.4%)	0.05

†Data are the median (range). All data are numbers (%) unless otherwise indicated. Each percentage is related to the total number with measurement data. TIA indicates transient ischemic attack.

147 (124-197) and 33 (17-70) mg/dL. Sub-analysis of changes in the lipid profile after probucol treatment detected significant three predictors of CV event risk: higher baseline TC (HR 2.74, 95% CI 1.05-7.16; p=0.04) in the primary prevention group; reduction in TG (HR 0.22, 95% CI 0.06-0.86; p=0.03); and reduction in LDL-C (HR 0.17,95% CI 0.03-0.90; p=0.04) after treatment in the subset of the secondary prevention group on stable doses of probucol. Neither TC nor HDL-C after treatment was associated with CV event risk in the probucol-exposed group, which indicates that reduction of the HDL-C level after probucol treatment is not related to CV event risk for probucol-exposed patients.

We evaluated the safety of probucol for all collected data from 541 patients, and found 56 adverse

Table 3. Incidence of cardiovascular events

		Cardiovascular Event		No event	Total	p
Primary prevention (n=322)	Exposed (n=233)		27 (11.6%)	206	233	
		MI	4			
		AP	18			
		Str.	3			
		TIA	1			
		PAD	1			0.058
	Unexposed (n=89)		4 (4.5%)	85	89	
		AP	1			
		Str.	2			
		TIA	1			
Secondary prevention (n=88)	Exposed (n=74)		20 (27.0%)	54	74	
		MI	6			
		AP	12			
		HF	1			
		Str.	1			0.012
	Unexposed (n=14)		9 (64.3%)	5	14	
		MI	2			
		AP	6			
		Str.	1			

MI, myocardial infarction; AP, angina pectoris; HF, heart failure; Str., stroke; TIA, transient ischemic attack; PAD, peripheral artery disease.
[1]One of the 4 patients died after 12 months of probucol termination.

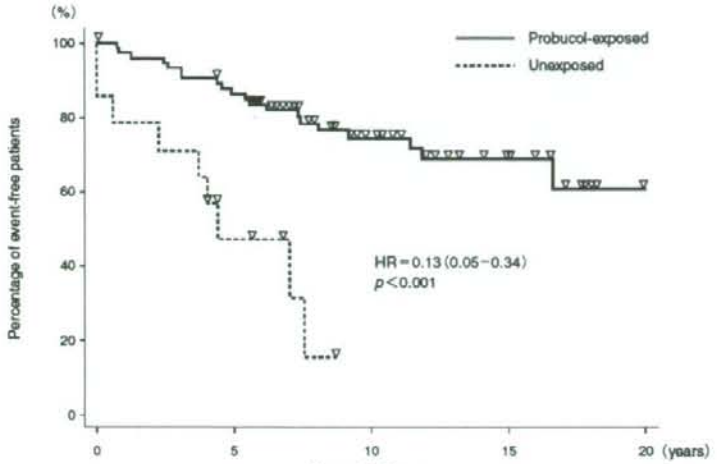
events in 18 patients. Malaise, pruritus, macrocytic anemia and pain in the extremities were recorded as adverse drug reactions associated with probucol. We noted and reported gastric cancer stage III immediately to the Ministry of Health and Welfare as an unexpected serious event, because of an unknown drug relation due to many concomitant drugs, although probucol was found to be non-carcinogenic alone[21]. Six deaths were observed in the population not taking probucol or stopping probucol. There was no other difference in the incidence of adverse events, including serious events, between probucol exposure and non-exposure.

Discussion

Many data from large-scale randomized controlled trials have overwhelmingly demonstrated the clinical benefits of lowering cholesterol with statins[22, 23], yet the rapid and extensive prophylactic use of cholesterol-lowering drugs remains controversial. Few studies have addressed the clinical risks and benefits of long-term treatment of hyperlipidemia among women[24] or elderly patients[25]. The safety of long-term cholesterol-lowering therapy, including the issue of associated cancer risk or benefit, remains inconclusive because of conflicting clinical evidence[26]. More importantly, conclusions from the results of randomized controlled trials are limited by their relatively short follow-up periods (generally less than 5 years) in the analyzed studies.

In long-term treatment for FH, probucol was used with other cholesterol lowering drugs in over 80% of the secondary prevention group–those with a more severe clinical outlook than the primary prevention group: a higher prevalence of hypertension and diabetes, significant thicker tendon xanthoma, more combined therapy with LDL-apheresis, anti-platelet drugs, and anti-hypertensive drugs. The high rate of probucol use in FH was surprising, different from expected. This might partly reflect the prescription behavior of experts with the result that intractable patients responded to the regimen.

In the secondary prevention, the higher-risk group, probucol exposure was associated with a reduction in the risk of cardiovascular events (HR 0.13; 95% CI 0.05–0.34) with high significance ($p<0.001$), while it was not significant in the primary prevention group. This result was also contrary to our expectation that probucol exposure would likely be associated with increased event risk due to a confounding indication–that patients considered more severe at diagnosis would receive more treatment, including probucol. We did not collect the details of non-probucol drugs



Number at risk

Years	0					5					10					15					20
Exposed	74	71	70	68	66	62	54	50	42	38	34	30	25	19	17	13	12	8	3	1	0
Unexposed	14	11	11	10	9	5	4	2	1	0	0	0	0	0	0	0	0	0	0	0	0

Estimates of event-free rates are according to whether patients received probucol. The cumulative probability of remaining without events was higher in patients treated with probucol ($p < 0.001$; log-rank test).

Fig. 2. Kaplan-Meier Estimates of Event-free Rate.

For secondary prevention, the incidence of cardiovascular events was 27.0% in the exposed group and 64.3% in the unexposed group. An event-free survival curve for the secondary prevention group is given.

Table 4. The results of multivariate analysis using Cox regression procedure

Factor	Primary prevention			Secondary prevention		
	HR	95% CI	*p*	HR	95% CI	*p*
Baseline variables						
Total cholesterol	1.58	1.06-2.33	0.02	-	-	-
Drinking	2.43	1.09-5.44	0.03	-	-	-
Peripheral artery disease	5.27	0.51-54.63	0.16	-	-	-
Palpebral xanthoma	-	-	-	2.94	1.02-8.47	0.05
Diabetes	-	-	-	2.58	0.76-8.76	0.13
Treatment in follow-up						
Probucol use	1.50	0.48-4.67	0.49	0.13	0.05-0.34	<0.001
Anti-platelet drug use	-	-	-	2.48	1.00-6.17	0.05

to simplify the study procedure. However, we would likely exclude underused statins because of the reduced use of non-probucol drugs from the possible factors of the higher event rate in the unexposed group, because statins were available when all of the 9 recurrent patients (**Table 3**) started and the patients continued on cholesterol-lowering drugs. We suppose, therefore, that the reasons for this unanticipated great risk reduction include some antioxidant and anti-atherogenic actions[3, 4, 27] of probucol. The finding in second prevention may be suggested by the report[27] that probucol significantly decreased *in vitro* LDL oxidizability measured under typically strong oxidative conditions, and that long-term treatment with probucol had an

anti-atherogenic effect in Watanabe Heritable Hyperlipidemic rabbits. From the observation that the baseline lipid profile was not different between the two groups of exposure and non-exposure in secondary prevention, the drug might exhibit greates effectiveness in post-cardiovascular disease patients, in possibly advanced lipid accumulation and inflammation, which are associated with the circulation of oxidized LDL[28].

In primary prevention, we observed an almost significant increase of events in the exposed group (**Table 3**), and an apparently increased risk (HR 1.5), although not statistically significant after adjustment (**Table 4**). We suppose, however, that the ideal effects of probucol might be concealed by the following factors noted in primary prevention. The exposed group had a worse lipid profile (TC, LDL-C and HDL-C levels), higher HbA_{1c}, and thus definitely a higher risk than the unexposed group. Furthermore, 8 (nearly 30%) of the 27 patients experiencing cardiovascular events in the exposed group discontinued probucol when they had events. This was consistent with the different finding between primary and secondary preventions in the exposed group: less than half of the patients (113 of 233) in primary prevention continued on probucol, while 53 (72%) of 74 patients continued in secondary prevention. This estimation might be conservative.

The controversial and paradoxical action of probucol– lowering HDL-C– level was not associated with the risk of CV events in the cohort, therefore, the association between low levels of HDL-C and an increased risk for CV events or death indicated by the early Framingham Heart Study[29] may not be extrapolated to probucol-treated patients. This proposition is consistent with recent findings that a lowered HDL-C level is not always atherogenic, but that the quality or function of HDL-C is more important than the HDL-C levels[30]. In fact, increased levels of HDL-C with torcetrapib, a CETP inhibitor, were not associated with a significant clinical benefit in patients with coronary disease[31], FH[32] or mixed dyslipidemia[33].

We speculate that enhanced reverse cholesterol transport by CETP activation as a result of probucol treatment also contributed to the detected risk reduction in the cohort. The observed positive outcome of probucol, a CETP activator, might be a mirror image of the negative clinical trial results for the CETP inhibitor[34]. Reports[35, 36] of increased coronary heart disease in CETP deficiency despite increased HDL-C levels, and the molecular approach to review CETP deficiency[37] support our hypothesis, at least in Japanese genealogy. Interestingly, a recent basic research reports that human CETP expression enhances the mouse survival rate in an experimental systemic inflammation model[38], indicating for the first time a role for CETP in the defense against the exacerbated production of proinflammatory mediators.

For the safety evaluation, we found no cardiotoxic adverse drug reaction including QT/QTc prolongation or torsade de pointes, in this study, although probucol can cause them[16, 39, 40].

We obtained these results from an observational study with no control for inaccuracy, unexpected bias or confounding factors. We could not assure the precision of the baseline measurements due to unrecorded data. The participant centers were major hospitals for FH, but not all hospitals in Japan, because the study was conducted as part of a post-marketing study by a pharmaceutical manufacturer within the framework of the Japanese government regulations. Some restrictions on collecting data might have resulted in unexpected small numbers in the unexposed group in secondary prevention, although we think that the study cohort represents nearly a nationwide population of heterozygous FH in Japan. The results derived from patient data in Japan can not necessarily be generalized to patients in western countries.

Despite these limitations of the study, however, we could evaluate the outcome of long-term probucol treatment in the medical practice setting for FH, a high-risk population, for as long as 20 years in Japan. The significant risk reduction of CV events observed in the secondary prevention group holds clinical significance and suggests some beneficial therapeutic actions of this drug in arteriosclerotic diseases. The hypothesis from the findings warrants a randomized controlled trial for verification of the secondary prevention, and needs further research into the molecular mechanisms or roles of CETP in pathogenesis.

Author Contributions

Dr. Yamashita had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Matsuzawa, Kita, Saito, Fukushima, Matsui. Acquisition of data: Yamashita, Bujo, Arai, Harada-Shiba, Saito, Kita, Matsuzawa. Analysis and interpretation of data: Yamashita, Bujo, Arai, Harada-Shiba, Matsui, Saito, Fukushima, Kita, Matsuzawa.

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and Matsuzawa. Statistical analysis: Matsui and Fukushima. Administrative, technical, or material support: Fukushima, Matsui, Kita, Saito, and Matsuzawa. Study supervision: Yamashita, Fukushima, Matsui, Kita, Saito, and Matsuzawa.

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From the formerly Daiichi and Otsuka, Dr. Matsui, Dr. Fukushima, Dr. Matsuzawa, and Dr. Kita received fees and expenses for meetings related to protocol design, statistical and clinical interpretation of the data; Dr. Bujo, Dr. Arai, Dr. Harada-Shiba received honoraria and travel expenses for lectures, Dr.Yamashita, Dr. Bujo, Dr. Arai received fees and travel expenses for a meeting related to clinical interpretation of the data. Dr. Yamashita received consultancy fees from Otsuka. Dr. Matsuzawa is contracted as a short-term adviser to Otsuka in medical science. Dr. Saito received travel expenses only.

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