TABLE 1. *DNA hypomethylation on pericentromeric satellite regions and clinicopathological parameters in urothelial carcinomas*

Tissue Specimens	No. Analyzed	No. Hypomethylation (%)	p Value (chi-square test)
	Satellite 2		
Histological grade:			
G1–2	15	2 (13)	
G3–4	12	9 (75)	0.0012
Invasion depth:			
Superficial (pTa, pT1)	11	1 (9)	
Invasive (pT2–4)	16	10 (63)	0.0055
Histological structure:			
Papillary	21	6 (29)	
Nodular	6	5 (83)	0.0161
	Satellite 3		
Histological grade:			
G1–2	15	3 (20)	
G3–4	12	9 (75)	0.0043
Invasion depth:			
Superficial (pTa, pT1)	11	2 (18)	
Invasive (pT2–4)	16	10 (63)	0.0228
Histological structure:			
Papillary	21	7 (33)	
Nodular	6	5 (83)	0.0297

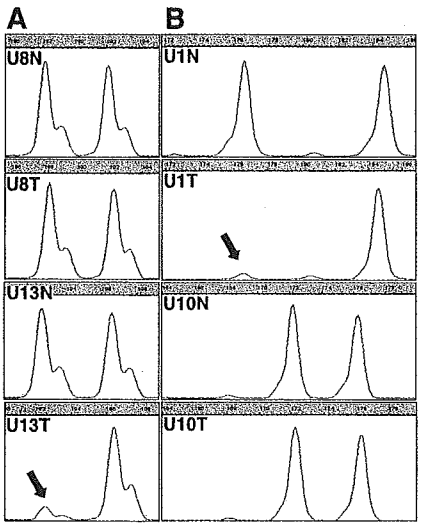

FIG. 2. Examples of results of allelic status analyses in cases of urothelial carcinoma. U8 and U13 DNA samples were amplified for D9S747 (*A*), while U1 and U10 samples were amplified for D9S775 (*B*). Genotypes derived from noncancerous U8N, U13N, U1N and U10N tissues, and corresponding U8T, U13T, U1T and U10T cancerous tissues are shown. Allele size in bp is indicated on top of horizontal axis. In all 4 noncancerous samples PCR products showed polymorphism, indicating that these cases were informative. U8T for D9S747 and U10T for D9S775 were classified as retention of alleles because signal intensity for tumor alleles was not changed significantly relative to matched normal alleles. LOH was identified when signal intensity for tumor allele was decreased by more than 50% relative to matched normal allele, that is in U13T for D9S747 and U1T for D9S775 (arrows).

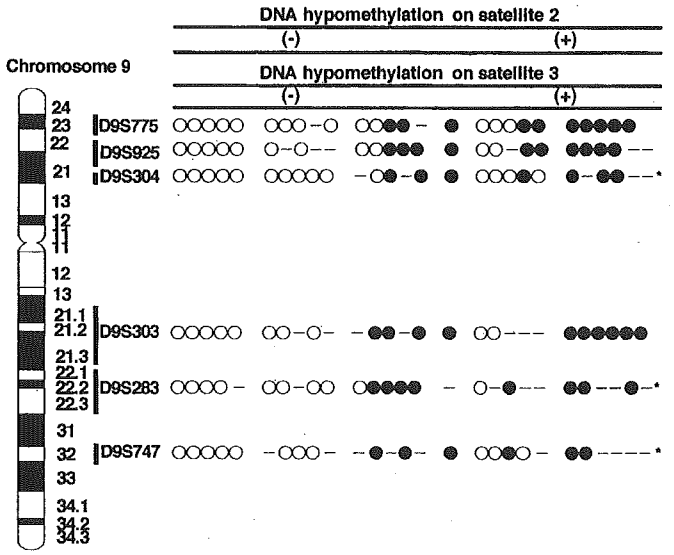

FIG. 3. Allelic status of each locus in urothelial carcinomas. Vertical lines indicate each carcinoma. Open circles indicate retention of 2 alleles. Filled circle indicate LOH. Bar indicates uninformative case. Asterisk indicates replication error. −, negative. +, positive.

TABLE 2. *LOH on chromosome 9 in urothelial carcinomas*

Locus	No. Analyzed	No. Informative	No. LOH (%)
9p:			
D9S775	27	24	10 (42)
D9S925	27	21	10 (48)
D9S304	27	22	7 (32)
Any on 9p	27	26	11 (42)
9q:			
D9S303	27	20	10 (50)
D9S283	27	18	8 (44)
D9S747	27	17	6 (35)
Any on 9q	27	26	12 (46)
Any on chromosome 9	27	27	14 (52)

hypomethylation on pericentromeric satellite regions significantly correlated with the presence of LOH on at least 1 locus on chromosome 9 in urothelial carcinomas (chi-square test p = 0.0098 and 0.0034 for satellites 2 and 3, respectively, table 4).

DISCUSSION

DNA hypomethylation on satellites 2 and 3 was observed frequently in urothelial carcinomas but it was extremely rare in noncancerous tissues, suggesting that DNA hypomethylation on satellites 2 and 3 is associated with urothelial carcinogenesis. We have previously reported that DNA hypomethylation on satellites 2 and 3 is a frequent and early event during hepatocarcinogenesis,[18] whereas it is rare in colorectal and stomach cancers.[19] These and the current findings suggest that DNA hypomethylation on pericentromeric satellite regions is organ specific during human carcinogenesis. In the current study DNA hypomethylation correlated with tumor aggressiveness (eg histological grade and invasion depth), indicating that it may participate in the malignant progression of urothelial carcinomas. In addition, DNA hy-

TABLE 3. *LOH on chromosome 9 and clinicopathological parameters in urothelial carcinomas*

Parameters	No. Analyzed	No. LOH (%)	p Value (chi-square test)
Histological grade:			
G1–2	15	5 (35)	
G3–4	12	9 (75)	0.0318
Invasion depth:			
Superficial (pTa, pT1)	11	4 (36)	
Invasive (pT2–4)	16	10 (63)	0.1817
Histological structure:			
Papillary	21	8 (38)	
Nodular	6	6 (100)	0.0074

TABLE 4. *DNA hypomethylation on pericentromeric satellite regions and LOH on chromosome 9 in urothelial carcinomas*

Chromosome 9 LOH	Hypomethylation		p Value (chi-square test)
	Neg	Pos	
Satellite 2:			
Neg	11	2	
Pos	5	9	0.0098
Satellite 3:			
Neg	11	2	
Pos	4	10	0.0034

pomethylation was associated more frequently with nodular invasive carcinomas showing an aggressive clinical outcome than with papillary carcinomas. Nodular invasive carcinomas arise from their precursor lesions, that is widely spreading flat carcinoma in situ, and rapidly invading suburothelial tissues, whereas papillary carcinomas usually remain noninvasive for a long period, even after recurrence in the bladder following cystoscopic resection.[13]

LOH on chromosome 9 was detected in more than half of the cases and in these cases rather large regions of 9p and/or 9q were lost, consistent with other reports that loss of an entire chromosome arm is frequent (fig. 3).[11] The observed high incidence of LOH on chromosome 9 in urothelial carcinomas may indicate the existence of tumor suppressor genes important for urothelial carcinogenesis on this chromosome.[11] DNA hypomethylation on satellites 2 and 3 significantly correlated with LOH on chromosome 9 in urothelial carcinomas. After the induction of DNA hypomethylation in cultured cells by treatment with 5-azacytidine, a DNA methyltransferase inhibitor, chromosomal recombination occurred between satellite regions.[3] In patients with ICF syndrome DNA hypomethylation on satellites 2 and 3, and multiradiate chromosomes composed of chromosomes 1, 9 and 16 are characteristic.[2] During hepatocarcinogenesis DNA hypomethylation on satellite 2 significantly correlates with chromosome 1 q-arm copy gain with pericentromeric break points.[8] By analogy with these findings DNA hypomethylation on satellites 2 and 3 could be the underlying molecular background for the frequently observed LOH on chromosome 9 in urothelial carcinomas.

DNMT3b has been identified as a DNA methyltransferase specifically targeting satellites 2 and 3 during mouse development.[20] In human hepatocarcinogenesis over expression of DNMT3b4, a splice variant of DNMT3b that lacks methyltransferase activity and competes with the major variant in normal liver tissues, DNMT3b3, for targeting to pericentromeric satellite regions, results in DNA hypomethylation on these regions.[21] Although further studies are needed to understand the molecular mechanism causing DNA hypomethylation on satellites 2 and 3 during urothelial carcinogenesis, this hypomethylation may have a role in the development and progression of urothelial carcinomas by inducing chromosomal instability. These data highlight the practical significance of correction of DNA methylation status for the prevention and/or therapy of urothelial carcinomas.

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The Prostate 63:56 – 64 (2005)

Effectiveness of Adjuvant Intermittent Endocrine Therapy Following Neoadjuvant EndocrineTherapy and External Beam RadiationTherapy in Men With Locally Advanced Prostate Cancer

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PURPOSE. To clarify the optimal duration and methods for adjuvant endocrine therapy after external beam radiation therapy (EBRT) in patients with locally advanced prostate cancer.
MATERIALS AND METHODS. Between 2001 and 2003, 215 patients with locally advanced prostate cancer were enrolled in the study. Patients were registered as primary candidates of the study and were treated with 6 months of LHRH agonist, with short-term of antiandrogen treatment for flare-up prevention. Patients with PSA levels below 10 ng/ml after the 6-month endocrine treatment were randomly divided into two arms. Then, a total dose of 72 Gy was given to the prostate. After 14 months of the protocol treatment, patients were treated with continuous androgen ablation (arm 1) or intermittent androgen ablation (arm 2).
RESULTS. A total of 188 cases (87%) remained in the protocol. The median PSA level at entry was 25.3 ng/ml. The Gleason score was 2–6 in 32 cases (16%), 7 in 94 cases (48%), and 8–10 in 68 cases (35%). The median PSA level showed a remarkable decrease to 1.1, 0.2, and 0.1 ng/ml, after 6, 8, and 14 months of the protocol treatment, respectively. Of the 157 cases treated with EBRT, 153 cases (97.5%) had no biochemical failure in the mean follow-up of 17.3 months.

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CONCLUSIONS. The present study may reveal the possibilities of intermittent endocrine therapy after EBRT. However, the follow-up interval is short and little can be said about the results observed so far, exception of acute tolerance and patient acceptance of the protocol. *Prostate 63: 56–64, 2005.* © 2004 Wiley-Liss, Inc.

KEY WORDS: **prostate cancer; intermittent hormonal therapy; external beam radiation therapy**

INTRODUCTION

Treatment of prostate cancer has been one of the most important issues for elderly males, especially in Western countries. In Japan, prostate cancer is the eighth leading life-threatening cancer in males [1]. However, in the past 10 years, the probability of cause of death from prostate cancer has increased and will increase rapidly in the future [1,2]. In the present study, we have conducted a prospective randomized control trial (RCT) for locally advanced prostate cancer in order to clarify how to treat it with adjuvant endocrine therapy after external beam radiation therapy (EBRT). The previous RCT for locally advanced prostate cancer already revealed that cancer causes of death and also all causes of death may decrease in men treated with both EBRT and endocrine therapy (neoadjuvant and/or adjuvant) in comparison with those treated with EBRT alone [3–5]. Bolla et al. [3] demonstrated that 5-year disease-free survival was higher at 85% in patients with locally advanced prostate cancer treated with EBRT and 3 years of endocrine therapy than in those treated with EBRT alone. However, the optimal timing and duration for endocrine therapy as adjuvant or neoadjuvant treatment with EBRT have not been solved. Furthermore, those issues should be discussed in terms of not only survival advantage, but also improvement of QOL.

Alternatively, the concept of intermittent endocrine therapy was proposed as a possible treatment to prolong the hormone naïve status of prostate cancer. According to basic research on androgen-dependent Shionogi carcinoma in mice, androgen-dependent status recovered after endocrine treatment was stopped in hormone-independent prostate cancer. This phenomenon would result in induction of apoptosis several times during intermittent androgen deprivation [6]. Although the treatment efficacy of intermittent hormonal therapy has not been confirmed in clinical settings, there may be some advantages in the cost for treatment, prevention of osteoporosis development, and recovery of libido.

The present assessment of combination therapy with EBRT and endocrine therapy for locally advanced prostate cancer may be of positive concern. However, it may be difficult to answer how long neoadjuvant and/or adjuvant endocrine therapy should be used. Several RCTs have been carried out or are ongoing in Europe and the USA. However, there have been no RCTs comparing the treatment efficacy and QOL between long-term adjuvant endocrine therapy and intermittent adjuvant endocrine therapy after treatment with EBRT and neoadjuvant endocrine therapy for locally advanced prostate cancer. To answer uncertainties on the above issues, the present multi-center RCT was conducted as a national cancer research project, which has been supported by the Ministry of Health, Labor and Welfare in Japan.

The primary endpoint of this study is biochemical relapse-free survival and the secondary endpoints are overall survival, cancer-specific survival and longitudinal QOL assessment between two groups. It is expected that the survival advantage by means of biochemical relapse-free survival in the continuous adjuvant endocrine treatment group may be better than that in the intermittent endocrine treatment group. Alternatively, adverse effects in patients treated with long-term androgen deprivation may increase in comparison with those treated with intermittent androgen deprivation. After completing this RCT, we expect to be able to distinguish patients who can benefit more from continuous hormonal treatment by means of survival with minimized adverse effect from those who can benefit more from intermittent hormonal treatment by means of maintaining QOL without dying of prostate cancer or suffering cancer-related complications.

MATERIALS AND METHODS

Study Protocol

Patients were eligible to participate in the protocol at any of 15 medical centers if they had biopsy-proven untreated adenocarcinoma of the prostate with clinical stage T3N0M0 or T4N0M0 (bladder neck invasion alone) and were younger than 80-years-old. Clinical stage was confirmed according to UICC 1997 by digital rectal examination (DRE), transrectal ultrasonography (TRUS), chest X-ray, bone scan, abdominal-to-pelvic CT and pelvic MRI. Patients who were treated with antiandrogen or any adrenocortical steroid hormones, or had undergone subcapsular prostatectomy or transurethral resection of the prostate including laser ablation for benign prostatic hyperplasia, were

eliminated from this study. Pelvic MRI was conducted before or 3 months after prostate biopsy.

Patients were registered as primary candidates of the study and were treated with 2 weeks of steroidal antiandrogen (chlormadinone acetate; CMA), then with both luteinizing hormone-releasing hormone (LHRH) agonist (leuprorelin or goserelin) and another 2 weeks of antiandrogen, and thereafter with LHRH agonist alone. After 6 months of endocrine treatment with LHRH agonist, only patients with PSA levels lower than 10 ng/ml, with a PSA level lower than the pretreatment level and without clinically apparent metastatic disease were enrolled in the following protocol as final candidates (2nd-line registration). All Gleason scores were reviewed by one urologic pathologist (M.H.) before the 2nd-line registration. After the 2nd-line registration was done, the patients were randomly divided into two groups according to institutions, age (younger than 70, 70 years, or older), PSA levels after 6 months of endocrine treatment (4.0 ng/ml or lower, 4.1 ng/ml or greater), and Gleason score (7 or less, 8–10) as follows: (1) continuous androgen ablation group (arm 1), (2) intermittent androgen ablation group (hormonal therapy must be stopped 6 months after the day of final EBRT treatment) (arm 2) (Fig. 1). All of these patients were treated with EBRT immediately after completing 2nd-line registration.

Details on the procedures of radiation therapy were specified in the protocol as follows: (1) radiation field should be limited to the prostate in all cases, and the seminal vesicle should be included in radiation fields only in cases with seminal vesicle involvement being highly suspected by imaging. Elective pelvic lymph node irradiation is not performed. (2) Conformal radiation therapy, 4-field oblique or box technique, or pendulum methods are recommended in order to minimize adverse effects in the rectum and bladder. (3) A total dose of 72 Gy should be given in 36 fractions, 5 fractions per week. (4) Verification films should be taken at least two times during the radiation therapy. (5) The gross tumor volume (GTV) and clinical target volume (CTV) are the prostate gland in cases without seminal vesicle involvement. The planning target volume (PTV) margin is 10 mm from the CTV. In cases with seminal vesicle involvement, the GTV and CTV include the seminal vesicles in addition to the prostate gland. In multi-portal treatment, every portal should be irradiated in every treatment. (6) Only photon beam energy of 6 MV or more is accepted.

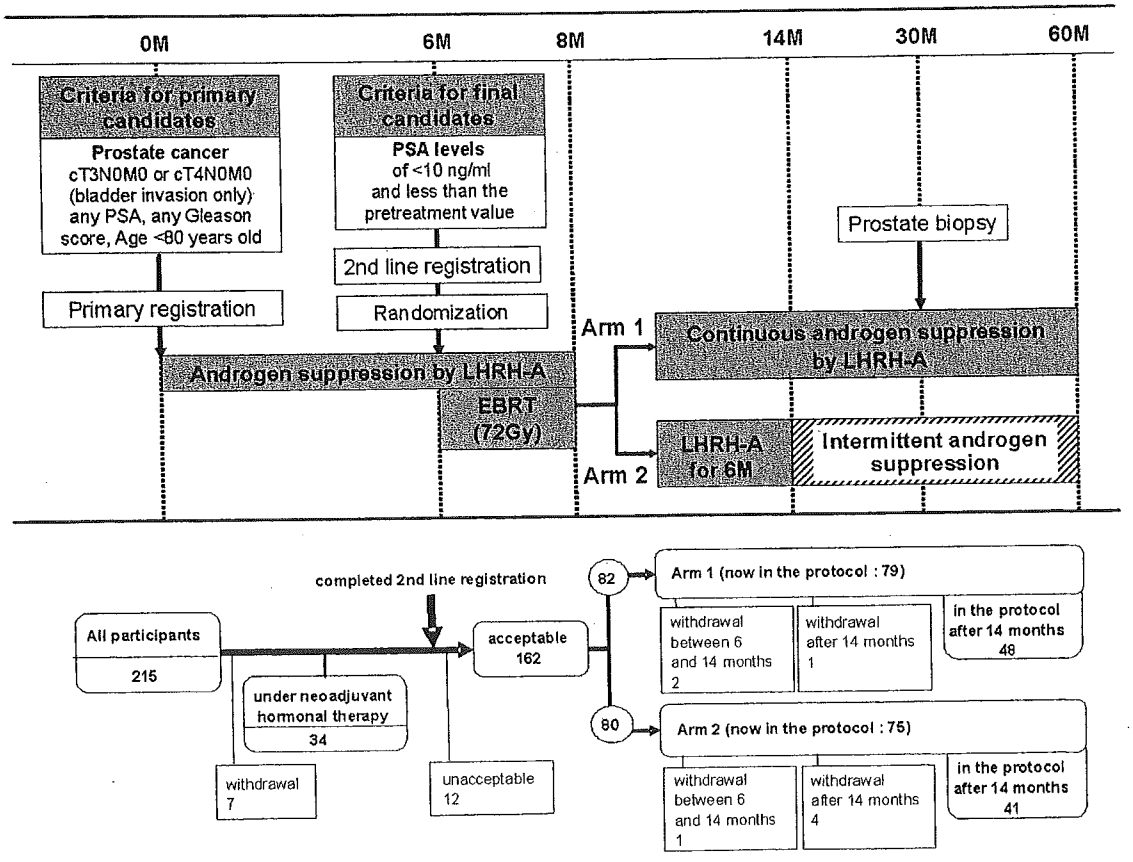

Fig. 1. Scheme of the study protocol, the number of patients registered and the present status of those patients in this study protocol. LHRH-A, LHRH agonist; EBRT, external beam radiation therapy.

Acute radiation morbidity should be evaluated by using common toxicity criteria of NCI within 90 days after radiation therapy, and late radiation morbidity should be evaluated by using the late radiation morbidity criteria of RTOG/EORTC.

Patients assigned to the intermittent androgen ablation group (arm 2) resumed hormonal therapy if they had PSA level of 10 ng/ml or greater or a clinical recurrence of disease. Resumed hormonal therapy would continue until the PSA levels decreased to below 1.0 ng/ml. If the PSA levels did not decrease to below 1.0 ng/ml, the possibility of biochemical recurrence of disease would be evaluated using the criteria in the study.

Biochemical failure was defined according to modified ASTRO criteria as follows: (1) three consecutive PSA increases in every 3-month interval and with a PSA velocity per 3 months of 0.5 ng/ml or greater, or (2) PSA levels increasing to 10 ng/ml or more. If three consecutive monthly-checked PSA levels increased rapidly at a PSA velocity per month of 0.17 ng/ml or greater, the researchers could designate that phenomenon a biochemical recurrence. The day of biochemical recurrence was defined between the day immediately before PSA increase and the day of initial PSA increase.

Clinical relapse was defined as progressive disease at a new site, an increase in the size of a nodule or cancer lesion on any images of the prostate, worse performance status, or body weight loss due to progression of prostate cancer.

Figure 2 shows the clinical assessment schedule of evaluation of treatment efficacy, QOL and adverse effects. PSA levels are measured monthly. Bone scan, abdominal-to-pelvic CT and chest X-ray must be conducted every 6 months for 1 year, and yearly thereafter. Pelvic MRI is conducted yearly. Prostate biopsy is recommended at around 2 years after the first date of EBRT. QOL can be assessed using FACT-P and part of the UCLA prostate cancer index before the initial endocrine therapy (0 months), immediately before EBRT (6 months), immediately after EBRT (8 months), 6 months after EBRT is completed (14 months), and 6 months after dividing the patients into two arms (20 months).

In the present study, treatment efficacy, adverse effects and QOL were compared between the two groups. The primary endpoint was biochemical (PSA) relapse-free survival. The secondary endpoints were overall survival, cause-specific survival, and longitudinal QOL assessment.

Cost effectiveness was also compared between men treated with continuous endocrine therapy and those with intermittent hormonal therapy.

The study protocol of this RCT and the documents of informed consent for the participants were approved by the IRB of all facilities, and a copy of the IRB approval document has been stored in the research bureau.

Statistical Consideration on Primary Endpoint of the Study

There has been no conclusive information on the optimal treatment strategy of adjuvant endocrine therapy after EBRT in patients with locally advanced prostate cancer. Therefore, the present study was conducted on the basis of the following two hypotheses. First, there was the non-recessive hypothesis, that the cumulative biochemical relapse-free survival rate in the intermittent endocrine therapy group (arm 2) would not be remarkably worse than that in the

Variables	Months after enrollment																				
	0	3	6	9	12	15	18	21	24	27	30	33	36	39	42	45	48	51	54	57	60
PSA measurement	◎	←							monthly check-up												◎
Digital rectal examination	◎	○	◎	○	◎	○	◎	○	◎	○	◎	○	◎	○	◎	○	◎	○	◎	○	◎
Transrectal ultrasonography	◎	○	◎	○	◎	○	◎	○	◎	○	◎	○	◎	○	◎	○	◎	○	◎	○	◎
Abdominal and pelvic CT	◎		◎		◎				◎				◎				◎				◎
Pelvic or endorectal MRI	◎				◎				◎				◎				◎				◎
Bone scintigraphy	◎		◎		◎				◎				◎				◎				◎
Chest X-P or Chest CT	◎		◎		◎				◎				◎				◎				◎
Prostate biopsy	◎										◎										◎
QOL assessment	◎		◎	◎(8M)		◎(14M)		◎(20M)													
Uroflowmetry	○				○				○				○				○				○
Residual urine	○				○				○				○				○				○
Blood test	◎	◎	◎	◎	◎		◎		◎		◎		◎		◎		◎		◎		◎
Performance status	◎				◎				◎				◎				◎				◎

◎ Essential assessment
○ Recommended assessment

Fig. 2. Assessment protocol for treatment effects, adverse effects and QOL in the study.

continuous endocrine therapy group (arm 1). If intermittent endocrine therapy after definitive EBRT is acceptable, the present study may be worthwhile from social, economic, and QOL points of view. The study would verify that the cumulative biochemical relapse-free survival rate in the continuous endocrine therapy group (arm 1) can be significantly better than that in the intermittent endocrine therapy group (arm 2). The second hypothesis was that continuous androgen suppression after EBRT may be worthwhile in terms of treatment efficacy, because of the specific characteristics of treatment for prostate cancer, which is famous for being hormone-naïve for a while in most cases. It would be possible to verify both of the above-mentioned hypotheses simultaneously by investigating the interval estimation of the hazard ratio, if the linearity can assume either hypothesis by carrying out the interval estimation of the hazard ratio, if the linearity can assume the recurrence hazard. Then, the 90% confidence interval for the hazard ratio (intermittent group/continuous group) can be calculated at both sides. If the upper limit is within the acceptable threshold, then the non-recessive hypothesis has been verified. On the other hand, the survival rate of the continuous group (arm 1) would be considered significantly excellent if the lower limit surpasses 1.

The main subjects for the analyses are qualified patients from whom the protocol treatments have been properly conducted. The analysis is limited to cases without remarkable contravention and deviation is carried out. The survival curve and recurrence-free survival will be estimated using Kaplan–Meier methods, and the confidence interval of the proportion at 3 and 5 years calculated by the formula of Greenwood. The hazard ratio is estimated by score statistic values from the log rank test results. Supplemental, by the hazard ratio is estimated by the Cox's proportional hazard model using the allocated factors at the 2nd registry, except for that of the facilities. The verification of the proportion hazard is done by double logarithm plotting, and the necessary analysis is carried out for the interpretation of results, such as the appliance of the Cox's proportional hazard model for time-dependent changes of the effects, when there is a remarkable dissociation from the proportion hazard. Prognostic factors which seem to be important are analyzed by means of each allocated factor at the 2nd registry except for that of the facilities, and the uniformity of differences between the two groups is examined. If necessary, the interaction between each facility and its remaining allocated factors at the 2nd registry will be analyzed, and also the differences between one facility and another.

The upper limits for the determination of non-recessiveness are 1.5 and 1.333. These upper limits may be acceptable if the hazard for combination treatment with EBRT and long-term endocrine therapy is outlining these thresholds compared with that for EBRT alone. These consequences have already been clarified by Bolla et al. [3], in which the confidence interval of hazard for disease-free survival was demonstrated between 1/0.15 and 1/0.32. According to the results of the Bolla study [3], an upper limit for the determination of non-recessiveness of 1.5 may be acceptable. On the other hand, the upper limit of 1.333 will also be used for an alternative analysis, because it may be a reference threshold for RCTs comparing treatment efficacy for other cancers.

Intermediate Assessment and the Possibility of Withdrawal of This Protocol

At the time when the number of enrolled cases reaches half of the expected adequate number of cases, an intermediate analysis will be performed to investigate whether the main purpose of the test has already been achieved, and another at the time when the expected adequate number of cases is fully registered. The intermediate analysis will be investigated blind by one statistician (Y.O.) at the registration center of the study in Tokyo University. If the disease-free survival in one group is significantly worse than that in the other group after careful consideration of the intermediate analysis, it will be decided whether the study protocol should continue or not.

Number of Cases Required for the Study, When to Close the Registration, and the Follow-Up Period

Considering that the cumulative PSA recurrence rate within 5 years in treatment with endocrine monotherapy for locally advanced prostate cancer in Japanese was demonstrated at about 40% [7], and that in combination therapy with EBRT and endocrine therapy was demonstrated between 15 and 64% [3,4], the cumulative PSA recurrence rate within 5 years in men treated with 3 years of adjuvant endocrine therapy and EBRT, in the present study, was assumed to be 30% [3]. For non-recessive verification using a hazard ratio of 1.5 as an upper limit, 75 events are necessary in each group in order to have 80% statistical power on the basis of the alternative hypothesis, in which there is no difference in the disease-free survival rate between both groups. Alternatively, on the basis of the alternative hypothesis which uses a hazard ratio of 2, the necessary event number for the dominance verification in both groups is 55, for 80% statistical power. There may be 90–100 events in 300 patients in the protocol during 5 years of observation. Therefore, if the cumulative disease-free survival rate in the continuous endocrine group is better with a hazard ratio of 2 or

more than that in the intermittent endocrine group, it may be possible to verify the dominance with high probability, which would be 93–95% if the number of the events is 90–100. Alternatively, if the cumulative rates for disease-free survival are similar between the two groups, pursuing non-recessive verification can not be avoided. In fact, the power decreases to 61–65% if there are 90–100 events.

It is worthwhile to consider that the significance of the study is the reevaluation by meta analysis with other clinical researchers around the world, who have almost the same hypothesis for verification, when non-recessiveness and dominance can not be verified. On the other hand, it is also possible to continue registration for another few years in some cooperative facilities, because randomization to one of two arms may be permitted even in the ethics target. Furthermore, it would also be possible to conduct a multi-factorial experiment, containing the LHRH administration period as a factor, and then performing a meta analysis.

The number of expected registered cases was set at 300 and the registration period 3 years in the protocol.

Patient Characteristics Registered

Between February 2001 and November 2003, 215 patients were registered in the protocol. Table I shows the clinicopathological features of patients registered in the present study. Age ranged from 54 to 79 years (70.6 ± 5.6, mean ± SD; 72.0, median). The median PSA level at entry was 25.3 ng/ml (45.1 ± 64.3; mean ± SD). The clinical stage was T3N0M0 in 202 (94.0%) and T4N0M0 in 13 (6.0%). The Gleason score diagnosed by the central urologic pathologist was 2–6 in 32 cases (16%), 7 in 94 cases (48%), and 8–10 in 68 cases (35%).

Details in the progression of this protocol in all participants are shown in Figure 1. On November 15, 2003, 188 patients (87.4%) were still in the protocol and 27 patients (12.6%) had withdrawn from the protocol. A total of 19, 3, and 5 cases were excluded from the protocol during 0–6 months, 6–14 months, and after 14 months of the protocol treatment, respectively. Of the 27 cases excluded from the protocol, 3 cases (11%) had adverse effects, 6 cases (22%) withdrew their agreement to this protocol, 1 case (4%) had other life-threatening cancer during the protocol treatment, 4 cases (15%) had recurrence of disease, 12 cases (44%) did not meet the criteria at the 2nd registration, and 1 case (4%) was excluded from the protocol by a contravention issue.

Of the 188 cases in the protocol, 34 patients (18%) received neoadjuvant hormonal therapy between 0 and 6 months of the protocol treatment, 64 patients (34%) were treated with EBRT and adjuvant endocrine therapy between 6 and 14 months, and 90 patients (48%) were treated with continuous or intermittent androgen ablation after 14 months of the protocol treatment.

Of the 95 cases who continued the protocol treatment after 14 months, 49 were treated with continuous endocrine treatment (arm 1) and 46 were treated with intermittent endocrine treatment (arm 2). The mean follow-up duration was 22.2 months (ranged from 14 to 30 months) in arm 1 and 23.0 months (ranged from 14 to 30 months) in arm 2. Of the 49 patients registered in arm 1, 1 case (2.0%) was excluded from the protocol because of recurrence of disease. Of the 46 cases registered in arm 2, 4 cases (8.7%) were excluded from the protocol treatment, because of recurrence of disease in 2 cases, contravention of the protocol in 1 case, and their own decision in 1 case.

TABLE I. Clinicopathological Features at Entry

Age	
Mean ± SD	70.6 ± 5.6
Median	72
Age distribution	
54–59	7 (3.3%)
60–64	28 (13.0%)
65–69	38 (17.7%)
70–74	80 (37.2%)
75–79	62 (28.8%)
PSA level (ng/ml)	
Mean ± SD	45.1 ± 64.3
Median	25.3
PSA distribution	
0.0–4.0	3 (1.4%)
4.1–10.0	38 (17.7%)
10.1–20.0	41 (19.1%)
20.1–50.0	79 (36.7%)
50.1–100.0	33 (15.3%)
100.1–∞	21 (9.8%)
Gleason score by (hospital pathologists)	
2–6	26 (12.1%)
7	106 (49.3%)
8–10	83 (38.6%)
Primary Gleason grade (hospital pathologists)	
–3	92 (42.8%)
4–5	123 (57.2%)
Clinical stage	
T3N0M0	202 (94.0%)
T4N0M0	13 (6.0%)
Gleason score by (central pathologist)	
2–6	32 (16.5%)
7	94 (48.5%)
8–10	68 (35.1%)
Primary Gleason grade (central pathologist)	
–3	99 (51.0%)
4–5	95 (49.0%)

RESULTS

Changes in the PSA levels within 1 month before prostate biopsy (pretreatment), after 6 months of endocrine treatment, 8 months of endocrine treatment (immediately after EBRT), and 14 months of endocrine treatment (6 months after EBRT) are shown in Table II. The PSA levels showed a remarkable decrease to median (mean ± SD) levels of 1.1 ng/ml (2.7 ± 5.0), 0.2 ng/ml (0.6 ± 1.0) and 0.1 ng/ml (0.3 ± 0.5) after 6, 8, and 14 months of the protocol treatment, respectively. The proportion of patients with PSA levels of 1.0 ng/ml or lower was 49% (85/173), 81% (118/145), and 91% (86/95) at 6, 8, and 14 months of the protocol treatment.

Of the 157 cases treated with EBRT, excluding eliminated cases without recurrence of disease, 153 cases (97.5%) had no biochemical failure in the mean follow-up of 17.3 months (range from 6.7 to 34.3 months).

A total of 44 cases were treated by intermittent hormonal therapy. Of the 44 cases, 41 cases have had no endocrine treatment according to the criteria after 14 months of the protocol treatment. Of the 401 months of the post-intermittent phase (i.e., after 14 months in the protocol treatment), in all 44 cases, 394 months (98.3%) were without treatment with endocrine therapy according to the criteria (off-treatment).

Of the 44 cases within the intermittent treatment protocol, 3 cases (6.8%) resumed endocrine therapy, because of clinical progression in 1 case and PSA levels increasing to greater than 10 ng/ml in 2 cases.

DISCUSSION

Although the treatment efficacy of intermittent endocrine therapy has not been clarified, it would be expected to have significance in the QOL, cost and prevention of decreasing bone mineral density. Several investigators have demonstrated the possibility of the clinical utility of intermittent endocrine therapy. The proportion of off-treatment periods were 38–50% during 24–30 months of follow-up periods in men with prostate cancer treated with endocrine monotherapy [8–10]. Most of the non-randomized trials have reported a response to the reintroduction of hormonal therapy in 90% of patients, with an on-treatment/off-treatment ratio of about 40–60% [8,11–17]. However, there had been no RCT to investigate the possibility of intermittent endocrine therapy in combination with EBRT in men with locally advanced prostate cancer. The biochemical recurrence rate may be higher in men treated with intermittent endocrine therapy than in those with continuous endocrine therapy. However, additional EBRT may improve disease-free survival for men with locally advanced prostate cancer. The present study revealed that the on-treatment/off-treatment ratio was extremely low at 1.8%. Therefore, the present RCT can solve uncertainties of treatment efficacy and QOL for intermittent endocrine therapy in combination with EBRT for men with locally advanced prostate cancer.

In the present study, disease-free survival was defined as a primary endpoint, because a previous study demonstrated a high 5-year overall survival rate of 92% and a relatively low 5-year biochemical disease-free survival rate of 61% in patients with locally advanced prostate cancer treated with LHRH agonist alone [7]. To set biochemical disease-free survival as the primary endpoint, it may be possible to have enough statistical power during a 5-year follow-up. The validity of this setting may be acceptable, because there is a limitation to the treatment after developing hormone-insensitive prostate cancer. Furthermore, any endocrine treatments will not be effective after recurrence of disease and the life span may be limited.

TABLE II. Changes in the PSA Levels After 6, 8, and 14 Months of the Protocol Treatment

	0 month	6 months	8 months	14 months
n	215	173	145	95
PSA level (ng/ml)				
Mean ± SD	45.1 ± 64.3	2.7 ± 5.0	0.6 ± 1.0	0.3 ± 0.5
Median	25.3	1.1	0.2	0.1
PSA distribution				
0.0–1.0	0 (0.0%)	85 (49.1%)	118 (81.4%)	86 (90.5%)
1.1–2.0	0 (0.0%)	29 (16.8%)	14 (9.7%)	9 (9.5%)
2.1–4.0	3 (1.4%)	33 (19.1%)	11 (7.6%)	0 (0.0%)
4.1–10.0	38 (17.7%)	15 (8.7%)	2 (1.4%)	0 (0.0%)
10.1–20.0	41 (19.1%)	6 (3.5%)	0 (0.0%)	0 (0.0%)
20.1–50.0	79 (36.7%)	5 (2.9%)	0 (0.0%)	0 (0.0%)
50.1–100.0	33 (15.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
100.1–∞	21 (9.8%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

The rates of biochemical no evidence of disease (bNED) control for patients with stage T3/T4 disease treated with a conventional dose of radiation therapy alone are poor, between 25 and 32% at 5 years [18,19] and 37% at 6 years [20]. The 5-year bNED in patients treated with EBRT alone for stage T1 to T4 disease decreased as pretreatment PSA levels increased, that is a bNED of 82–100%, 44–66%, 27–72%, and 11–14% for patients with pretreatment PSA levels of 4 ng/ml or less, 4–10 ng/ml, 10–20 ng/ml, and greater than 20 ng/ml, respectively [18,20–22]. The bNED control rate is higher in men treated with 3DCRT than in those treated with conventional EBRT even for cases with high levels of PSA. However, the bNED at 5 years is still low at 75 and 32% in patients treated with a high radiation dose of 76 Gy, in the PSA range of 10–20 ng/ml and greater than 20 ng/ml, respectively [23]. These treatment failures might result from the limitation of EBRT for large volume cancer on one side and the existence of clinically undetectable metastasis on the other side.

These poor outcomes of EBRT for locally advanced prostate cancer led to several randomized controlled trials on the effectiveness of neoadjuvant or adjuvant hormonal therapy in comparison with EBRT alone by the Radiation Therapy Oncology Group (RTOG) and The European Organization for Research and Treatment of Cancer (EORTC).

The RTOG 86-10 was conducted to investigate the usefulness of androgen ablation 2 months before and during EBRT compared with EBRT alone for locally advanced prostate cancer [5]. The biochemical disease-free survival and cause-specific mortality were significantly better in men undergoing androgen ablation before and during EBRT than in those treated with EBRT alone, especially in patients with Gleason 2–6 tumors.

Bolla et al. [3] conducted an RCT comparing overall survival and the disease-free interval between men treated with EBRT alone and with EBRT in combination with 3 years of adjuvant endocrine therapy starting from the initial date of EBRT (EORTC 22863) [3]. They demonstrated that the 5-year overall survival rate was significantly higher at 79% in patients treated with combination therapy than that in those treated with EBRT alone, which was 62%. The 5-year disease-free survival rate was also significantly higher at 81% in patients treated with combination therapy than that in those treated with EBRT alone.

The effectiveness of adjuvant endocrine therapy in combination with EBRT for patients with locally advanced prostate cancer can be clarified. Although cancer volume may be a very important factor in the treatment of EBRT, clinical data addressing the potential value of hormonal cytoreduction before radiotherapy have been quite limited. Therefore, it can also be valuable to investigate whether neoadjuvant endocrine therapy before EBRT is useful for locally advanced prostate cancer. In the present study protocol, all patients were initially treated with endocrine therapy for 6 months, and only patients with PSA levels after 6 months of endocrine therapy of 10 ng/ml or lower and also lower than the pretreatment levels were enrolled as final candidates in this study. The eliminated cases without sufficient effects after 6 months of endocrine treatment should be treated with other treatment protocols like chemoendocrine treatment. Therefore, our study protocol, which selects only patients with sufficient effects by neoadjuvant endocrine treatment, may be acceptable by means of ethical issues and also scientific validity.

At present, EBRT in combination with adjuvant endocrine therapy for locally advanced prostate cancer can be recommended in terms of survival benefit. However, it has not been clarified when and how long additional endocrine therapy should be conducted with respect to not only survival but also QOL. The compliance of this RCT may be high, so it is expected that long-term follow-up of the participants in the present study will reveal the possibilities of intermittent endocrine therapy after EBRT in patients with locally advanced prostate cancer.

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Original

Lack of Chemoprevention or Promotion Effects of Docosahexaenoic Acid on Small Intestine, Colon, Liver, Lung, Thyroid, Esophagus, Kidney, and Forestomach Carcinogenesis in a Rat Medium-Term Multi-Organ Carcinogenesis Model

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Abstract: Modifying effects of docosahexaenoic acid (DHA) were examined using a medium-term multi-organ carcinogenesis model (DMBDD model). Groups of twenty F344 male rats were treated sequentially with *N*-diethylnitrosamine (DEN, i.p.), *N*-methyl-*N*-nitrosourea (MNU, i.p.), 1,2-dimethylhydrazine (DMH, s.c.), *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN, in drinking water) and dihydroxy-di-*N*-propylnitrosamine (DHPN, in drinking water) during the first 4 weeks (DMBDD treatment), and then DHA-ethyl ester (DHA-E), DHA-triglyceride (DHA-TG) and/or tocopherol were administered intragastrically 3 times a week for 31 weeks. Significant inhibition of the development of glutathione *S*-transferase placental form (GST-P) positive foci was observed in DMBDD treated 30% DHA-TG 404 mg and 128 mg + tocopherols groups and with tocopherol alone; however, this appeared to be due to the tocopherol. DHA treatment did not influence the development of aberrant crypt foci in the large intestine. Histopathologically, the incidences of preneoplastic and neoplastic lesions in other organs were also not increased or decreased by DHA treatment. Thus, the results indicate a lack of chemopreventive and tumor promotion effects of any type of DHA in male rats under the present experimental conditions. (J Toxicol Pathol 2005; **18**: 53–59)

Key words: docosahexaenoic acid, medium-term multi-organ carcinogenesis model, F344 rat, promotion

Introduction

The n-3 polyunsaturated fatty acid, docosahexaenoic acid (DHA) is a major component of fish oil, which has been frequently reported to have chemopreventive potential for colon, mammary gland and pancreas carcinogenesis in rats[1–6]. For example, DHA was found to suppress aberrant crypt foci (ACF) in the colon induced by azoxymethane (AOM) or 1,2-dimethylhydrazine (DMH)[1,3]. Furthermore, induction of ACF by the heterocyclic amine, 2-amino-1-methyl-6-phenylimidazo[*4,5-b*]pyridine (PhIP), was also inhibited by DHA treatment[4]. Furthermore colon cancer multiplicity was significantly decreased in another study[2,3]. In the mammary gland, development of tumors was also reduced by a low dose of DHA or eicosapentaenoic acid (EPA) treatment after carcinogen (DMBA) injection[6]; however, in a clinical trial with familial adenomatous polyposis (FAP) patients a high risk group for colorectal cancer, it was without major influence[7]. The three FAP patients were administered concentrated DHA in fish oil capsules (2.2 g of DHA-TG and 0.6 g eicosapentaenoic acid (EPA) per day) for one or two years. The patients with FAP developed endometrial cancer after 12 months, colon cancer after 24 months and lung cancer after 12 months, respectively[7].

It is well established that a chemical may act as a tumor inhibitor in one organ and as a promoter in others[8–10]. It is

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Table 1. Fatty Acid Contents for a Rat in Dosing Solvent (mg)

Groups		16:0 Palmitic acid	18:0 Stearic acid	18:1 Oleic acid	18:2 Linoleic acid	20:1 Gadoleic acid	20:4(n-6) AA	20:5 EPA	22:5 DPA	22:6 DHA	Other FA	tocopherol	Total
1,6	128 mg 97% purify DHA-E	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	120.0	4.0	4.0	128.0
2,7	404 mg 30% purify DHA-TG	56.8	13.6	74.0	5.2	10.0	8.0	32.8	12.0	113.2	74.4	4.0	404.0
3	128 mg 30% purify DHA-TG	17.6	4.2	22.9	1.6	3.1	2.5	10.2	3.7	35.1	23.1	4.0	128.0
4	4 mg tocopherol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.0	4.0

AA: arachidonic acid. EPA: eicosapentaenoic acid. DPA: docosapentaenoic acid. FA: Fatty acid.

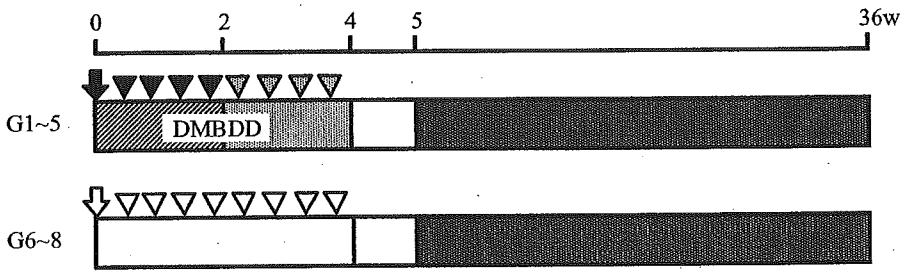

Fig. 1. Experimental protocol for the medium-term multi-organ carcinogenesis model. Animals: male F344/DuCrj rats, 6 weeks old; ▼, DEN, 100 mg/kg body wt. i.p.; ▼, MNU, 20 mg/kg body wt. i.p.; ▽, DMH, 40 mg/kg body wt. s.c.; ▨, BBN, 0.05% in drinking water; ▩, DHPN, 0.1% in drinking water; ⇩▽, saline injection; ▩, G1 and 6, 128 mg 97% purity DHA-E, G2 and 7, 404 mg 30% purity DHA-TG, G3, 128 mg 30% purity DHA-TG, G4, 4 mg tocopherol, G5 and 8, no treatment.

therefore important to examine modification potential not in a single organ, but rather in the whole body. This requires *in vivo* experimental models which can detect effects in a wide spectrum of organs, and for this purpose several multi-organ wide-spectrum initiation models have been established[11–14]. The medium-term approach has clear benefits for the examination of modifying effects of chemicals in multiple organs in a single experiment within a relatively short experimental period[15–17] and is based on the proven good agreement between the multi-organ carcinogenesis model and long-term experimental results[18].

The ethyl ester formed by DHA (DHA-E) has been used in many chemoprevention studies[1–6], and DHA-TG has been used in a clinical trial study[7]. Therefore, we thought it important to investigate the difference in the modifying effects on carcinogenesis of DHA-E and DHA-TG. In the present study, we investigated the post-initiation-phase modifying activity of DHA-E and DHA-TG at the whole organ level using a rat medium-term multi-organ carcinogenesis model developed in our laboratory[8,15,19,20]. Furthermore, a tocopherol group was included as a comparative control.

Materials and Methods

Animals

Male F344 rats, aged 5 weeks, were obtained from Charles River Japan Inc. (Kanagawa Japan), and housed five to a plastic cage with wood chips for bedding in an air-conditioned room at 22 ± 2°C with a 12-h light: 12-h dark cycle. They were maintained on Oriental MF diet (Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum*. The study was started after 1 week of acclimatization.

Chemicals

N-Diethylnitrosamine (DEN), *N*-methyl-*N*-nitrosourea (MNU), 1,2-dimethylhydrazine (DMH) and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) were obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and dihydroxy-di-*N*-propylnitrosamine (DHPN) was obtained from Nacalai Tesque Co. (Osaka, Japan). The DHA dosing solution was supplied by Nippon Suisan Kaisha, Ltd. (Tokyo, Japan). DHA naturally exists in fish oil as a triglyceride (DHA-TG). DHA-E was chemically synthesized from DHA-TG by removing other fatty acids such as oleic acid and EPA. The contents in the dosing solution used in the present study are shown in Table 1. They were stored in sealed ampules under anaerobic conditions at –20°C in the dark.

Experimental methods

Medium-term multi-organ carcinogenesis study

The experimental protocol is shown in Fig. 1. The animals were randomly allocated to 8 groups of 10 – 20 animals. Those in groups 1 to 5 received the combined carcinogen treatments, consisting of a single *i.p.* injection of 100 mg/kg body wt. of DEN, four *i.p.* injections of 20 mg/kg body wt. of MNU, four *s.c.* injections of 40 mg/kg body wt.

Table 2. Final Body and Organ Weights Data

Groups	DMBDD	Treatment	Effective no. of rats	Body wt.[a] (g)	Liver wt.[a] (g)	Liver wt.[a] (%, b.w.)	Kidneys wt.[a] (g)	Kidneys wt.[a] (%, b.w.)
1	+	128 mg 97% DHA-E	19	312.4 ± 14.8[b]	6.60 ± 0.45	2.11 ± 0.08	2.00 ± 0.44	0.64 ± 0.15
2	+	404 mg 30% DHA-TG	17	318.6 ± 15.5	7.01 ± 0.51	2.23 ± 0.11[c][d]	3.12 ± 3.15	1.00 ± 1.00
3	+	128 mg 30% DHA-TG	19	306.8 ± 20.5[c]	6.53 ± 0.59	2.13 ± 0.09	2.03 ± 0.65	0.67 ± 0.25
4	+	4 mg Tocopherol	18	314.8 ± 24.7	6.74 ± 0.54	2.14 ± 0.10	1.97 ± 0.22	0.62 ± 0.06
5	+	no treatment	19	324.6 ± 18.2	6.83 ± 0.47	2.10 ± 0.08	2.03 ± 0.36	0.63 ± 0.11
6	–	128 mg 97% DHA-E	10	356.6 ± 18.2	7.56 ± 0.71	2.13 ± 0.24	1.93 ± 0.16	0.54 ± 0.02
7	–	404 mg 30% DHA-TG	10	373.0 ± 11.7	7.80 ± 0.24	2.09 ± 0.06	2.05 ± 0.11	0.55 ± 0.03
8	–	no treatment	10	369.5 ± 14.9	7.61 ± 0.36	2.06 ± 0.05	2.00 ± 0.11	0.54 ± 0.03

a) Mean ± SD.
b), c) Significantly different from group 5 at $P<0.05$ and 0.01, respectively.
d) Significantly different from group 4 at $P<0.05$.

of DMH, together with 0.05% BBN for 2 weeks and then 0.1% DHPN for 2 weeks (both given in the drinking water), during the initial 4 week period for multiple initiation (DMBDD treatment) as described previously[21–23]. Animals in groups 1 to 5 were then given intragastric injections, 1 ml of 128 mg/ml of 97% purity DHA-E, 404 mg/ml of 30% purity DHA-TG, 128 mg/ml of 30% purity DHA-TG, each with 4 mg/ml of tocopherol, or tocopherol alone or distilled water, 3 times a week from 1 week after completion of the DMBDD treatment to the end of the experiment. Animals in groups 6 to 8 were given 128 mg/ml 97% purity DHA-E, 404 mg/ml 30% purity DHA-TG and distilled water as a solvent control without DMBDD treatment from week 5. The treatment times per week and concentration of DHA dosing solution were decided according to a trial study[7]. Animals were weighed once a week in the initial 14 weeks, then once every 2 weeks until the end of the study period, at week 36, when all surviving animals were sacrificed by exsanguination under ether anesthesia and subjected to complete necropsy.

All experimental procedures were performed in accordance with the in-house guideline for the Care and Use of Laboratory Animals at DIMS Institute of Medical Science.

Aberrant crypt foci assay

Nine or 10 rats for each treatment with DMBDD initiation and 5 rats each without DMBDD were analyzed for colon ACF. The colon was removed, slit open from the anus to the cecum along the longitudinal axis, flattened between sheets of filter paper, and fixed in buffered 10% formalin. Then it was stained with 0.2% methylene blue solution by the procedure of Bird[24] to observed aberrant crypts. The number of aberrant crypt foci per colon, the number of aberrant crypts in each focus, and the location of each focus was determined by microscopy.

Histopathological examination

At necropsy, the brain, liver, kidneys, spleen, heart, lungs, thymus, testes and adrenals were excised and weighed, and the relative percentage organ weights were calculated on the basis of final body weights. These and the other major organs including small and large intestines were fixed in 10% buffered formalin, and routinely processed. Paraffin-embedded sections were stained with hematoxylin and eosin for histopathological examination. Liver slices fixed in 10% buffered formalin were also prepared for quantitative assessment of immunohistochemically demonstrated glutathione *S*-transferase placental form (GST-P) positive foci, as previously described[25]. GST-P positive foci larger than 0.2 mm in diameter and the total areas of the liver sections examined were quantitated using a video image processor (SPICCA-II, Nippon Avionics, Tokyo, Japan) and the data expressed as numbers and areas (mm^2) per unit area of the liver section (cm^2).

Statistical analysis

The significance of intergroup differences in numerical data obtained for body and organ weights was assessed using the two-tailed Student's t-test. Insufficient homogeneity of variance was corrected with respect to the degrees of freedom according to the method of Welch. The significance of differences in the incidences of histopathological findings between treated and control groups was evaluated using Fisher's exact probability test.

Results

No post-initiation treatment-related clinical signs or mortalities were noted in any of the groups in the current experiment. Eight rats were found dead in the course of study, one in group 1, three in group 2, one in group 3, two in group 4 and one in group 5, and the deaths were all considered to have been caused by the DMBDD treatment.

The average body weights of rats in the DMBDD treated groups were significantly less than in the non-DMBDD initiated groups, throughout the study period. After DMBDD initiation, 30% DHA-TG was associated with retardation of body weight increase from week 7. The body weights in the other DMBDD treated groups were not

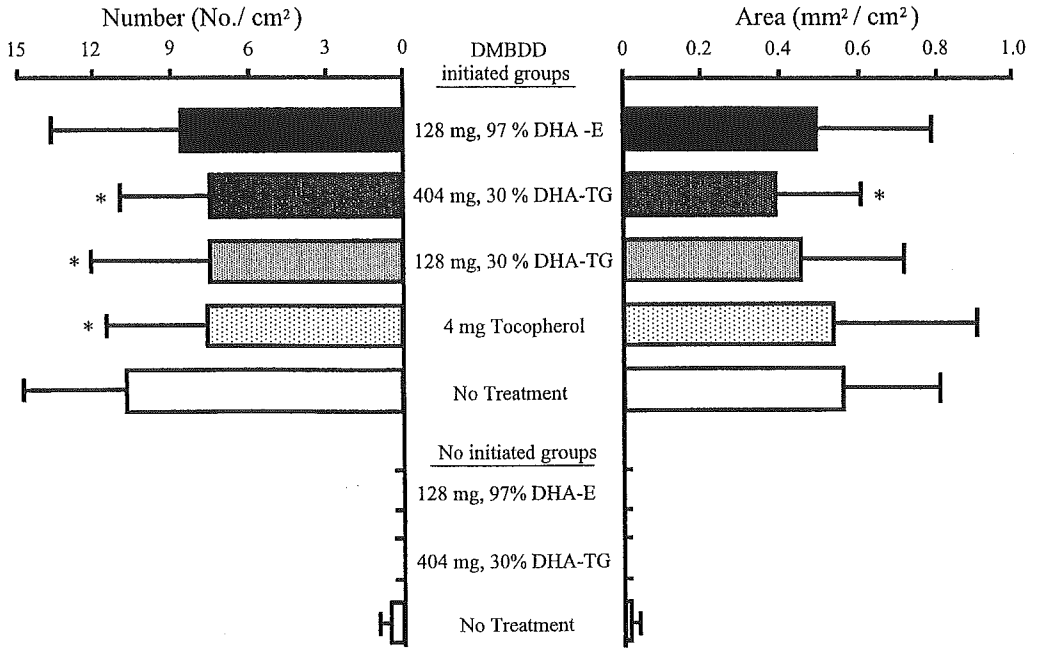

Fig. 2. Areas and numbers of GST-P positive foci in rat livers. * P<0.05 versus DMBDD initiated no treatment group.

Table 3. Number of ACF in Rats Treated with and without DHA during the Post-Initiation Stage

Groups	DMBDD	Treatment	Effective no. of rats	ACF / Colon			AC / Focus
				< 4 crypts	4 crypts≦	Total	
1	+	128 mg 97% DHA-E	9	27.1 ± 6.9	6.2 ± 3.9	33.3 ± 9.7	2.5 ± 0.3
2	+	404 mg 30% DHA-TG	9	27.0 ± 14.3	6.2 ± 6.1	33.2 ± 18.3	2.3 ± 0.4
3	+	128 mg 30% DHA-TG	9	26.6 ± 7.5	7.9 ± 4.6	34.4 ± 11.6	2.5 ± 0.4
4	+	4 mg Tocopherol	9	25.9 ± 16.9	5.2 ± 3.8	31.1 ± 19.3	2.4 ± 0.3
5	+	no treatment	10	26.8 ± 10.1	8.2 ± 5.7	35.0 ± 13.5	2.7 ± 0.4
6	–	128 mg 97% DHA-E	5	0.3 ± 0.5	0	0.2 ± 0.5	0.4 ± 0.9
7	–	404 mg 30% DHA-TG	5	0.3 ± 0.5	0.2 ± 0.5	0.4 ± 0.6	1.4 ± 0.2
8	–	no treatment	5	0.3 ± 0.5	0	0.2 ± 0.5	0.2 ± 0.5

AC: aberrant crypts.

significantly changed.

A significant increase in relative liver weight and a tendency to increase in relative kidney weights were noted in group 2 (Table 2).

Quantitative analysis of GST-P positive foci (Fig. 2) showed the numbers and areas were significantly decreased by the 404 mg/ml 30% DHA-TG treatment. The numbers were also suppressed by 128 mg/ml 30% DHA-TG and tocopherol alone.

No significant difference was observed in ACF between DHA and/or tocopherol treatment groups and the DMBDD alone group (Table 3).

Histopathological examination revealed hyperplastic and neoplastic lesions in various organs/tissues in the rats initiated with the five carcinogens (Tables 4, 5). However, no DHA treatment-related alteration in their incidences was evident. No proliferative lesions were noted in any of the rats given DHA and tocopherol without DMBDD treatment.

Discussion

The present investigation of the modifying potential of DHA in a rat medium-term multi-organ carcinogenesis model found no modifying effects on lesion development in any organ. Decreases of number and/or area of GST-P positive foci in the liver given 404 mg and 128 mg 30% DHA-TG were demonstrated, but similar results were obtained with tocopherol alone, so the latter was considered responsible, in line with its reported inhibitory potential[26,27].

The effect of dietary sardine oil including 28.5% DHA on rat hepatocarcinogenesis was examined with administration in the initiation and post-initiation period[28]. The sardine oil inhibited the number of DEN-induced GST-P positive foci when administered in the initiation period, but enhanced the area of GST-P positive foci when administered in the post-initiation period. However, in another study, fish oil inhibited AOM-induced GST-P positive foci in the post-

Table 4. Incidences of Neoplastic Lesions in the Large and Small Intestines

Groups	DMBDD	Treatment	Effective no. of rats	Small intestine		Large intestine	
				Adenoma	Adenocarcinoma	Adenoma	Adenocarcinoma
1	+	128 mg 97% DHA-E	20	0	1 (5)	1 (5)	1 (5)
2	+	404 mg 30% DHA-TG	20	2 (10)	4 (20)	0	1 (5)
3	+	128 mg 30% DHA-TG	20	1 (5)	1 (5)	2 (10)	1 (5)
4	+	4 mg Tocopherol	20	1 (5)	1 (5)	1 (5)	1 (5)
5	+	no treatment	20	2 (10)	2 (10)	1 (5)	1 (5)
6	–	128 mg 97% DHA-E	10	0	0	0	0
7	–	404 mg 30% DHA-TG	10	0	0	0	0
8	–	no treatment	10	0	0	0	0

Table 5. Incidences of Preneoplastic and Neoplastic Lesions in Other Organs in DMBDD Treated Groups

Organ / Findings		DMBDD treatment				
		128 mg 97% DHA-E	404 mg 30% DHA-TG	128 mg 30% DHA-TG	4 mg Tocopherol	No treatment
No. of rats examined		20	20	20	20	20
Spleen:	Hemangioma	0	0	0	0	1 (5)
Thyroids:	Follicular cell hyperplasia	13 (65)	17 (85)	10 (50)	12 (60)	10 (50)
	Follicular cell adenoma	4 (20)	10 (50)	9 (45)	5 (25)	6 (30)
	Follicular cell carcinoma	5 (25)	7 (35)	6 (30)	5 (25)	5 (25)
Nasal cavity:	Hyperplasia	17 (85)	19 (95)	17 (85)	20 (100)	20 (100)
	Adenoma	2 (10)	0	0	1 (5)	0
Lung:	Alveolar hyperplasia	20 (100)	20 (100)	20 (100)	20 (100)	20 (100)
	Adenoma	8 (40)	6 (30)	8 (40)	10 (50)	8 (40)
	Adenocarcinoma	1 (5)	4 (20)	2 (10)	3 (15)	4 (20)
Tongue:	Squamous cell hyperplasia	0	0	2 (10)	1 (5)	0
	Papilloma	0	0	0	1 (5)	0
Esophagus:	Squamous cell hyperplasia	18 (90)	17 (85)	19 (95)	20 (100)	17 (85)
	Papilloma	0	2 (10)	0	0	1 (5)
Stomach:	Squamous cell hyperplasia	10 (50)	8 (40)	12 (60)	12 (60)	13 (65)
	Squamous cell papilloma	3 (15)	1 (5)	2 (10)	0	3 (15)
	Squamous cell carcinoma	0	1 (5)	0	1 (5)	0
Liver:	Hepatocellular adenoma	3 (15)	1 (5)	2 (10)	1 (5)	5 (25)
	Hepatocellular carcinoma	0	0	0	1 (5)	0
Kidneys:	Atypical tubules	11 (55)	13 (65)	7 (35)	10 (50)	9 (45)
	Renal cell hyperplasia	0	0	1 (5)	0	0
	Transitional cell hyperplasia	6 (30)	8 (40)	4 (20)	4 (20)	8 (40)
	Renal cell adenoma	1 (5)	1 (5)	3 (15)	1 (5)	1 (5)
	Nephroblastoma	5 (25)	7 (35)	6 (30)	4 (20)	10 (50)
	Transitional cell carcinoma	0	3 (15)	0	0	1 (5)
Urinary bladder:	Simple hyperplasia	12 (60)	12 (60)	14 (70)	13 (65)	11 (55)
	PN hyperplasia	4 (20)	2 (10)	4 (20)	3 (15)	3 (15)
	Papilloma	0	0	0	0	1 (5)
	Transitional cell carcinoma	1 (5)	1 (5)	0	1 (5)	0
Other site:	Histiocytic sarcoma	0	0	0	1 (5)	0
	Leiomyosarcoma	0	0	0	1 (5)	1 (5)
	Malignant lymphoma/ leukemia	1 (5)	1 (5)	1 (5)	1 (5)	0

initiation stage[29]. These results suggest that the effects of fish oil appear to be dependent on the types of carcinogens. In the present study, DHA did not enhance hepatocarcinogenesis initiated with five carcinogens.

Some previous studies indicated the chemopreventive effect of DHA on colon[1–4], mammary glands[6] and pancreas carcinogenesis[5] in rats. DHA exerted significant inhibitory effects on implanted tumor growth and metastasis to the lungs in a subcutaneously implanted and highly metastatic colon carcinoma model[30]. However, no chemoprevention was observed for rat colon and other organ carcinogenesis with DHA treatment in the present study. The reason for the discrepancy with the many previous studies which showed chemopreventive effects on colon carcinogenesis and ACF development[1–4,27,31,32], may be due to the different number of treatment times per week. DHA was injected five times a

week or administered in diet continuously in the other chemopreventive studies, but injected three times a week in this study, according to the clinical trial study[7].

In the multi-organ model (DMBDD model)[20,21,23,33] incidences of colon tumor development have been reported to range from 10 to 80%, therefore the figure of 10% achieved in the present study was relatively low. Thus, one reason for the lack of obvious influence of DHA could have been due to weak initiation.

Recently, different results regarding the chemopreventive effect of DHA-E in diet using the same model were published by an other group[33]. They showed an inhibitory effect on carcinogenesis in the small intestine, large intestine and lung by DHA. They used synthetic diet (modified AIN-93) as basal diet for the experiment, but we used a conventional diet (Oriental MF), this may have been the cause of the different results.

DHA has been reported as an useful chemopreventive agent in many rodent studies[1–6]. However, in a long-term trial using concentrated DHA in fish oil capsules containing about 30% DHA in triglyceride form, for patients in a high-risk group for colorectal cancer, three patients with FAP developed cancers, one endometrial, one colon and one lung[7]. During the trial, no marked increase or decrease in the number of polyps was observed.

In the present experiment, no promotion activity of either DHA-E or DHA-TG was found in any organ including the large intestine. The reason for the tumor development seen in the clinical trial study with DHA-TG treatment could not therefore be clarified in the present study.

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NK105, a paclitaxel-incorporating micellar nanoparticle formulation, can extend *in vivo* antitumour activity and reduce the neurotoxicity of paclitaxel

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Paclitaxel (PTX) is one of the most effective anticancer agents. In clinical practice, however, high incidences of adverse reactions of the drug, for example, neurotoxicity, myelosuppression, and allergic reactions, have been reported. NK105, a micellar nanoparticle formulation, was developed to overcome these problems and to enhance the antitumour activity of PTX. Via the self-association process, PTX was incorporated into the inner core of the micelle system by physical entrapment through hydrophobic interactions between the drug and the well-designed block copolymers for PTX. NK105 was compared with free PTX with respect to their *in vitro* cytotoxicity, *in vivo* antitumour activity, pharmacokinetics, pharmacodynamics, and neurotoxicity. Consequently, the plasma area under the curve (AUC) values were approximately 90-fold higher for NK105 than for free PTX because the leakage of PTX from normal blood vessels was minimal and its capture by the reticuloendothelial system minimised. Thus, the tumour AUC value was 25-fold higher for NK105 than for free PTX. NK105 showed significantly potent antitumour activity on a human colorectal cancer cell line HT-29 xenograft as compared with PTX ($P<0.001$) because the enhanced accumulation of the drug in the tumour has occurred, probably followed by its effective and sustained release from micellar nanoparticles. Neurotoxicity was significantly weaker with NK105 than with free PTX. The neurotoxicity of PTX was attenuated by NK105, which was demonstrated by both histopathological ($P<0.001$) and physiological ($P<0.05$) methods for the first time. The present study suggests that NK105 warrants a clinical trial for patients with metastatic solid tumours.
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Keywords: NK105; paclitaxel; polymer micelles; DDS; EPR effect

Paclitaxel (PTX) is one of the most useful anticancer agents known for various cancers including ovarian, breast, and lung cancers (Carney, 1996; Khayat *et al*, 2000). However, PTX has serious adverse effects, for example, neutropenia and peripheral sensory neuropathy. In addition, anaphylaxis and other severe hypersensitive reactions have been reported to develop in 2–4% of patients receiving the drug even after premedication with antiallergic agents; these adverse reactions have been attributed to the mixture of Cremophor EL and ethanol, which was used to solubilise PTX (Weiss *et al*, 1990; Rowinsky and Donehower, 1995). Of the adverse reactions, neutropenia can be prevented or managed effectively by administering a granulocyte colony-stimulating factor. On the other hand, there are no effective therapies to prevent or reduce nerve damage, which is associated with peripheral neuropathy caused by PTX; therefore, neurotoxicity constitutes a significant dose-limiting toxicity of the drug (Rowinsky *et al*, 1993; Wasserheit *et al*, 1996).

The above problems of PTX have been attributed to its low therapeutic indices and limited efficacy due to the nonselective nature of its therapeutic targets and its inability to accumulate selectively in cancer tissue. Therefore, there is an urgent need to develop modalities by which cytotoxic drugs can selectively target tumour tissue and effectively act on cancer cells in the scene. The roles of drug delivery systems (DDSs) have drawn attention in this context. Drug delivery systems are based on two main principles: active and passive targetings. The former refers to the development of monoclonal antibodies directed against tumour-related molecules that allow targeting of the tumour because of specific binding between the antibody and its antigen. However, the application of

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DDSs using monoclonal antibodies is restricted to tumours expressing high levels of related antigens.

Passive targeting is based on the so-called enhanced permeability and retention (EPR) effect (Matsumura and Maeda, 1986; Maeda *et al*, 2000). The EPR effect consists in the pathophysiological characteristics of solid tumour tissue: hypervascularity, incomplete vascular architecture, secretion of vascular permeability factors stimulating extravasation within cancer tissue, and absence of effective lymphatic drainage from tumours that impedes the efficient clearance of macromolecules accumulated in solid tumour tissues.

Several techniques to maximally use the EPR effect have been developed, that is, modification of drug structures and development of drug carriers. The first micelle-forming polymeric drug developed was polyethylene glycol (PEG)-polyaspartate block copolymer conjugated with doxorubicin (DXR) (Yokoyama *et al*, 1990; Yokoyama *et al*, 1991; Kataoka *et al*, 1993). PEG constituted the outer shell of the micelle, which conferred a stealth property on the drug that allowed the micellar drug preparations to be less avidly taken up by the reticuloendothelial system (RES) and to be retained in the circulation for a longer time. Prolonged circulation time and the ability of polymeric micelles to extravasate through the leaky tumour vasculature were expected to result in the accumulation of DXR in tumour tissue due to the EPR effect (Kwon *et al*, 1994; Yokoyama *et al*, 1999). A clinical trail of micellar DXR, NK911, is now underway (Nakanishi *et al*, 2001; Hamaguchi *et al*, 2003). Recently, we succeeded in constructing NK105, a polymeric micelle carrier system for PTX, which conferred on PTX a passive targeting ability based on the EPR effect. In the present paper, we describe the details and characteristics of NK105. We also discuss differences between NK105 and other DDS formulations containing PTX.

MATERIALS AND METHODS

Materials

PTX was purchased from Mercian Corp. (Tokyo, Japan). All other chemicals were of reagent grade. Following cell lines, MKN-45, MKN-28, HT-29, DLD-1, HCT116, TE-1, TE-8, PC-14, PC-14/TXT, H460, MCAS, OVCAR-3, AsPC-1, PAN-9, PAN-3, and MCF-7 cells were purchased from American Type Culture Collection. Colon 26 cells were dispensed from the Japan Foundation for Cancer Research (Tokyo, Japan). Female BALB/c *nu/nu* mice were purchased from SLC (Shizuoka, Japan). Female CDF1 mice and IGS rats were purchased from Charles River Japan Inc. (Kanagawa, Japan).

All animal procedures were performed in compliance with the guidelines for the care and use of experimental animals, which had been drawn up by the Committee for Animal Experimentation of the National Cancer Center; these guidelines meet the ethical standards required by law and also comply with the guidelines for the use of experimental animals in Japan.

NK105, a PTX-incorporating micellar nanoparticle formulation

NK105 is a PTX-incorporating 'core-shell-type' polymeric micellar nanoparticle formulation. Polymeric micellar particles were formed by facilitating the self-association of amphiphilic block copolymers in an aqueous medium. Novel amphiphilic block copolymers, namely NK105 polymers, were designed for PTX entrapment. NK105 polymers were constructed using PEG as the hydrophilic segment and modified polyaspartate as the hydrophobic segment. Carboxylic groups of polyaspartate block were modified with 4-phenyl-1-butanol by esterification reaction, consequently the half of the groups were converted to 4-phenyl-1-butanolate. Via the self-association process, PTX was incorporated into the inner core of the micelle system by physical entrapment through hydrophobic interactions between the drug and specifically well-designed block copolymers for PTX.

Pharmacokinetics and pharmacodynamics of PTX and NK105

Colon 26 tumour-bearing CDF1 mice aged 8 weeks were given intravenously (i.v.) via the tail vein PTX 50 and 100 mg kg^{-1} or NK105 at corresponding PTX-equivalent doses. Mice were killed at 5 and 30 min, as well as 2, 6, 24, and 72 h after injection. Blood was collected, and tumours were removed; plasma and tumours obtained were then stored at −20°C until the analysis. Each time point for collection represented three samples from three different mice. PTX was extracted from plasma obtained by deproteinisation using acetonitrile, followed by liquid–liquid extraction with *t*-butylmethylether. Tumours obtained were homogenised in 0.5% acetic acid, and the resultant homogenate was deproteinised and extracted according to the same method as that used for plasma. The blood and tumour extracts were analysed for PTX by liquid chromatography/tandem mass spectrometry. Reversed-phase column-switching chromatography was conducted using an ODS column and detection was enabled by electrospray ionisation of positive mode. The mean plasma and tumour concentrations of PTX at each sampling point were calculated for both PTX and NK105. Pharmacokinetic modelling was completed using a WinNonlin Standard software version 3.1 (Pharsight Corp., California, USA).

In vitro cytotoxicity

Various human cancer cell lines were evaluated in the present study. The cell lines were maintained in monolayer cultures in Dulbecco's modified Eagle's medium containing 10% (v v^{-1}) foetal calf serum and 600 mg l^{-1} glutamine. WST-8 Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) was used for the cell proliferation assay. In all, 2000 cells of each cell line in 90 μl of culture medium were plated in 96-well plates and were then incubated for 24 h at 37°C. Serial dilutions of PTX or NK105 in a volume of 10 μl were added, and the cells were incubated for 48 or 72 h. All data were expressed as mean ± s.e. of triplicate cultures. The data were then plotted as a percentage of the data from the control cultures, which were treated identically to the experimental cultures, except that no drug was added.

Evaluation of the antitumour activity of PTX and NK105

The antitumour activity of PTX and NK105 was evaluated using nude mice implanted with a human colonic cancer cell line, HT-29. One million tumour cells of HT-29 were inoculated at a subcutaneous (s.c.) site on the back skin of BALB/c female nude mice aged 6 weeks. When tumour size reached approximately 5–8 mm in diameter, mice were randomly allocated to the PTX administration group, NK105 administration group, and control administration group, each of which was made up of five animals. Each treatment was carried out as follows: free PTX group was administered at a dose of 25, 50, or 100 mg kg^{-1}; NK105 group was with same PTX-equivalent doses; and in control group, animals were given saline. Mice were administered a single i.v. injection of PTX or NK105 weekly for 3 weeks. The antitumour activity of PTX and NK105 was evaluated by measuring tumour size ($a \times b$, where a is the major diameter and b is the minor diameter) at various time points after injection. Changes in body weight were also monitored for mice, which were used in the present study.