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Magnetic Anchor for More Effective Endoscopic Mucosal Resection

Toshiaki Kobayashi[1], Takushi Gotohda[1], Katsunori Tamakawa[2], Hirohisa Ueda[3] and Tadao Kakizoe[1]

[1]National Cancer Center, Tokyo, [2]Tamakawa Corporation, Sendai, [3]Pentax Corporation, Tokyo, Japan

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Background: Technical difficulties are involved in endoscopic mucosal resection (EMR) of gastric cancer since it is a 'one handed surgery'. These difficulties prevent this technique from being indicated for larger lesions, even when it can possibly be performed for patients with such lesions. If microforceps could assist EMR, this procedure would become easier and safer. Since magnetic force can control objects without direct contact, it can be applied to control microforceps internally in assistance with EMR.

Methods: We developed a magnetic anchor consisting of three parts: a magnetic weight with dimensions of 1.0 × 1.0 × 1.5 cm, microforceps and a connecting thread. Endoscopic clips used in hemostasis were used as the microforceps of the magnetic anchor in this study. The magnetic control system consisted of a 0.68 kOe/10 cm/100 A electromagnet, 350 mm in diameter and a circumventing positional frame. The microforceps were inserted into a sheath within the endoscope, and the magnetic weight was secured to the tip of the sheath protruding from the endoscope. The magnetic anchor, along with the endoscope, was inserted through an overtube into the gastric cavity of a swine under general anesthesia. The magnetic anchor was used in a manner similar to that in standard surgery, and EMR was thereby performed.

Results: The mucosa to be resected was satisfactorily dragged and stabilized. The magnetic anchor facilitated EMR, regardless of the technical skills of the endoscopist and region of the stomach at which the technique was performed.

Conclusion: The magnetic anchor is considered to have alleviated some technical problems involved in EMR. It has the potential for making EMR a safer and quicker procedure for the treatment of early gastric cancer, when appropriately indicated.

Key words: endoscopic mucosal resection (EMR) – microforceps – magnetic anchor – gastric cancer – endoscopic surgery

INTRODUCTION

Endoscopic mucosal resection (EMR) of gastric cancer is a representative procedure of minimally invasive surgery (1). While this approach seems theoretically appropriate, it has serious problems especially when its indications are extended to lesions larger than those recommended by the Japanese Gastric Cancer Association (2). These problems arise from the fact that all resection procedures are carried out using only one endoscope. Thus, resection is performed without appropriate tissue tension provided by an assistant holding the tip of the mucosa. This 'one handed surgery' is the root cause of several problems, particularly when performing EMR on large lesions.

In EMR, the resection line cannot be fully observed because the ablated mucosa cannot be stabilized and pulled up. Consequently, it is difficult to make an accurate incision into the mucosa. Cutting of unconfirmed blood vessels causes bleeding, and hemostatic procedures are hindered because the bleeding point cannot be confirmed directly by operator's eyes. It is also of consequence that the depth of the mucosa at the site of ablation cannot be confirmed, which may lead to perforation of the gastric wall (1,3). These dilemmas have not caused serious problems so far, because EMR is indicated only for relatively small lesions.

Several new techniques and types of equipment have been developed to overcome these technical difficulties and complications that are problematic even for experienced endoscopists. The insulation-tipped electro-surgical knife (IT knife) is one such device that was designed by covering the tip of the electric knife with a ceramic ball to prevent accidental penetration of the gastric wall (1,4).

The rationale behind this development is that the tip of the electric knife, which is the most penetrative part for surgical incision, cannot be used. Thus, theoretically, resection is limited to a certain extent and the penetration is prevented.

For reprints and áll correspondence: Toshiaki Kobayashi, Cancer Screening Technology Division, Research Center for Cancer Prevention and Screening, National Cancer Center, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan. E-mail: tkobayas@ncc.go.jp

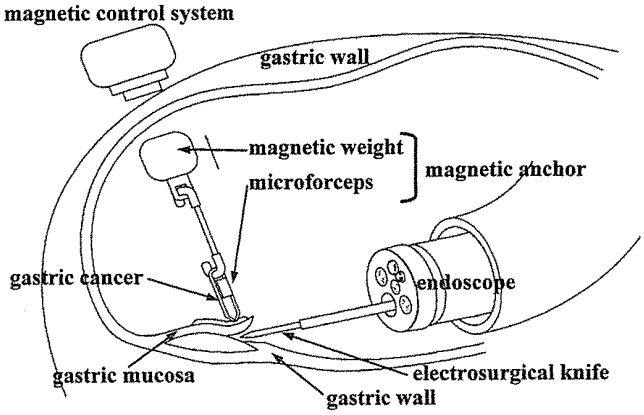


Figure 1. The concept of the magnetic anchor. The concept of the magnetic anchor is shown by microforceps that stabilize and pull up objects by employing a magnetic field. The magnetic anchor consists of three parts: microforceps, a magnetic weight and the connecting thread between them. One application of this concept is the use of the forceps to assist endoscopic resection of gastric cancer. The concept can even be applied to other procedures outside medical practice when stabilization and traction of objects are required and where direct contact is not possible.

However, perforation is still encountered, leading to prolonged resection time. Therefore, EMR for gastric cancer requires an endoscopist with technical skills higher than those required for other endoscopic procedures.

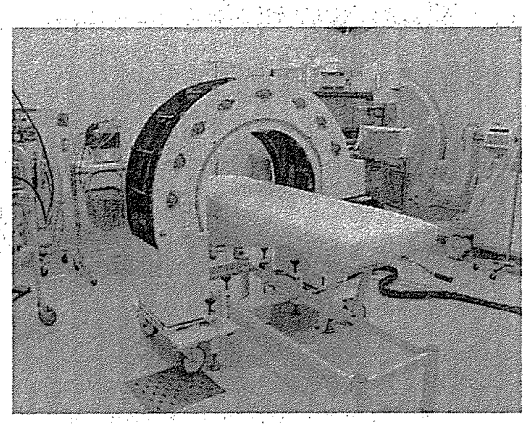
A basic technical principle of surgical resection is the resection of appropriate tissues, which are made to stand out by pulling them up. If this basic technique is integrated into EMR, then the procedure would become easier, less risky and more effective. This, in turn, could make it possible to establish EMR as the standard procedure whenever the nature of the lesions is an indication for resection with EMR, irrespective of their size.

Magnets and magnetic fields have been applied to catheter examinations to control the tips of catheters for years (5). They may also be providing a way to alter tissue contour configurations without any direct contact, such as through electric cables. A direct current magnetic field, as is used in MRI, is regarded as the least invasive, or even the most appropriate, non-invasive procedure that can be applied medically.

If a magnetic field is properly controlled and made to generate enough power to give sufficient force by using microforceps for stabilization of the mucosa during EMR (Fig. 1), then the procedure would be made much easier. If such a device design is developed, then indications for EMR could be expanded to change the current concept of endoscopic surgery for cancer treatment, including gastric cancer.

Thus, this study was initiated to evaluate the potential of magnetically controlled forceps and a magnetic anchor in an animal subject.

a. Positioning frame



b. Electromagnet fixed to the belts

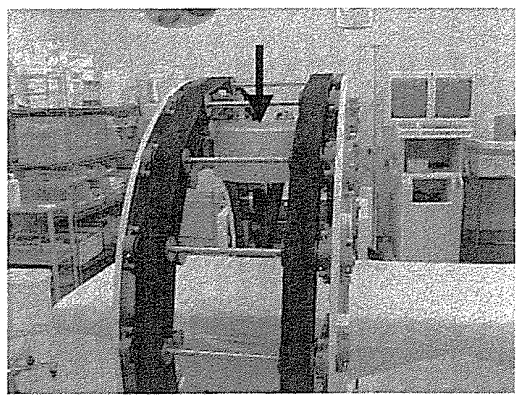


Figure 2. Magnetic control system. (a) Positioning frame: The positioning frame holds the electromagnet and allows its movement around the subject. Its wheels allow lateral movement along the table. (b) Electromagnet fixed to the belts: The electromagnet, shown by the arrow, is fixed to the belts that allow its movement around a subject inside the system. The electromagnet itself is compact; however, the frame is produced on a large scale to keep the distance between the subject and the electromagnet at a minimum.

SUBJECTS AND METHODS

MAGNETIC CONTROL SYSTEM

A magnetic control system was designed for clinical application in a standard endoscopic room, thus limiting the size of the system. The magnetic control system primarily consisted of a 0.68 kOe /100 A electromagnet, 350 mm in diameter, at 10 cm from the center of the magnetic yoke.

The electromagnet was fixed on belts contained in a semicircular positioning frame that revolved around the trails of the frame. In this manner, a pulley system was formed allowing a 180° control of the magnet's position in relation to the patient (Fig. 2).

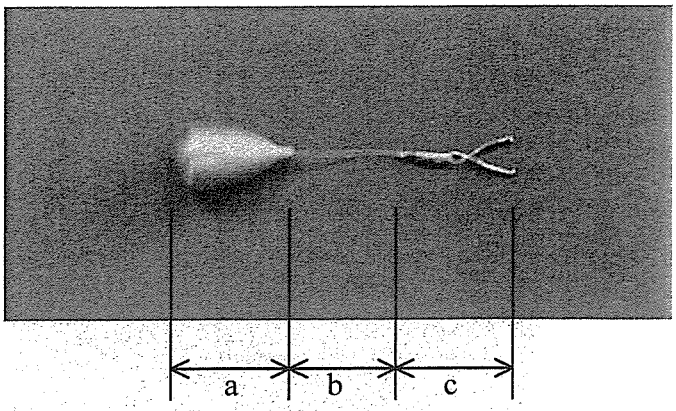


Figure 3. Magnetic anchor. The magnetic anchor consists of three parts: (a) a magnetic weight, (c) microforceps and (b) a thread connecting them.

Magnetic Anchor

The magnetic anchor consists of three parts: a hand-made magnetic weight comprising magnetic stainless steel (SUS420F), microforceps and a connecting thread (Fig. 3). The weight, with dimensions of 1.0 × 1.0 × 1.5 cm, was designed to generate sufficient traction for tissues and to allow insertion into the gastric cavity through the esophagus. The weight of the anchor can be varied by using differently shaped weight components of different weights. The anchor weight used for this procedure is approximately 6 g.

Hemostasis clips (endo-clips) were used as microforceps in order to confirm the feasibility of the magnetic anchor's proposed function of tissue traction. However, it is likely that forceps designed specially for this procedure will be developed in the future.

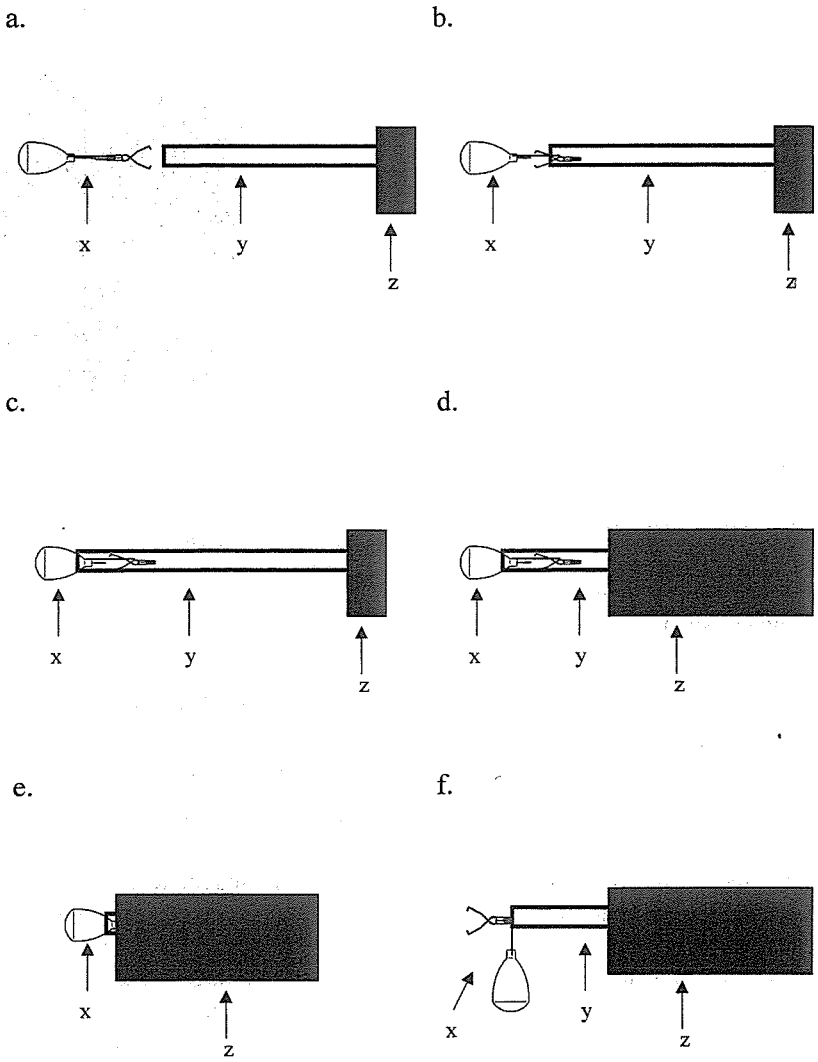


Figure 4. Preparation of the magnetic anchor. (a) The magnetic anchor (x) is prepared by the following procedure: First, the endoscopic hemostasis sheath (y) is inserted into the working channel of an endoscope (z) and pushed out from the tip of the endoscope. (b) The microforceps are connected to the sheath and pulled backwards into the sheath. (c) The thread is pulled further into the sheath together with the magnetic weight, and the magnetic weight is secured at the tip of the sheath. (d) The sheath is pulled into the endoscope. (e) The weight is fixed at the tip of the endoscope. (f) The magnetic anchor is pushed out from the tip of the endoscope at the time of its use. The target is grasped after opening the microforceps. x: magnetic anchor, y: endoscopic hemostasis sheath, z: endoscope.

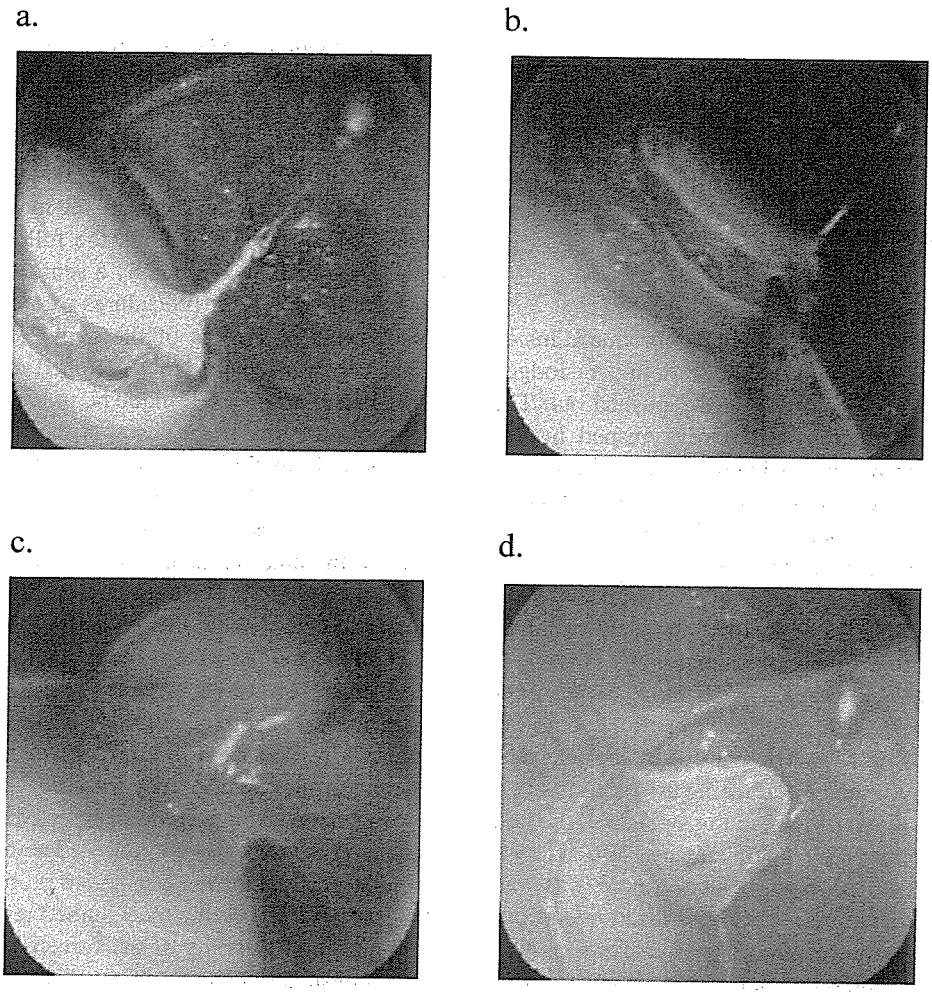


Figure 5. Resection using the magnetic anchor. (a) The mucosa is pulled to create sufficient space to show the line of resection. (b) The resection is performed using an electric knife through an endoscope. (c) The resection line can be clearly shown as a result of traction by the magnetic anchor. (d) The traction of the magnetic anchor is sufficient to allow the mucosal flap to be turned over.

Preparation of the Magnetic Anchor

First, the endoscopic hemostasis sheath is inserted into the working channel of an endoscope as shown in Fig. 4. The microforceps are then connected to the sheath and are pulled backwards into the tip of the sheath protruding from the tip of the endoscope; subsequently, the thread follows and the anchor is secured at the tip of the sheath. The sheath is then withdrawn into the working channel, and the anchor is fixed at the tip of the endoscope.

Test Subject

A 45 kg female swine laid in the left lateral position, was placed on an examination table under intravenous anesthesia.

Procedure

Prior to insertion of the magnetic anchor, an incision was made by the standard EMR technique in the mucosa surrounding the region of the stomach intended for resection (4). An overtube was first inserted into the esophagus to facilitate smooth insertion of the endoscope along with the magnetic anchor to reach the gastric cavity. Inside the gastric lumen, the magnetic weight was pushed out from the sheath, followed by the microforceps. The tip of the mucosa, in which the incision was made in advance, was grasped by the microforceps. In order to lift the tip of the mucosa, a magnetic field was then generated by increasing the electric current through the electromagnet of the magnetic control system.

The EMR procedure was performed by several physicians of the National Cancer Center Hospital at four representative regions of the stomach: the anterior wall of the gastric angle, the lesser curvature of the gastric corpus, the posterior wall of the gastric angle and the greater curvature of the gastric corpus. These areas were selected in order to represent the varying techniques and problems that are incurred with the anatomy of each specific area.

RESULTS

Insertion of the fixed magnetic anchor through the overtube incurred no difficulties. In fact, the magnetic anchor was easily

Table 1. Sizes of resected specimens and the procedure times required

Site in the stomach	Size (cm)	Time (min)
Anterior wall of the gastric angle	5.1 × 2.7	51
Lesser curvature of the gastric corpus	2.4 × 1.7	47
Posterior wall of the gastric angle	2.9 × 1.7	25
Greater curvature of the gastric corpus	9.4 × 5.1	73

introduced even without the overtube. Once introduced inside the gastric lumen, the magnetic weight smoothly dislodged from the sheath and the forceps were easily pushed out. The mucosal target site for traction was easily grasped by the microforceps in the same manner as in endoscopic hemostasis, despite the heaviness of the weight component of the anchor.

As a result of magnetic attraction, the magnetic anchor rose rapidly when the electric current of the electromagnet was sufficient for the operation, pulling up the mucosa in a stable tent-like form (Fig. 5). The direction of the traction for the magnetic anchor could be controlled simply by changing the position of the electromagnet over the animal. Application of magnetic attraction only from above was sufficient to pull the magnetic anchor in the desired direction. The control of the magnetic anchor could also be facilitated by adjusting the position of the swine and/or placing a spacer between the swine and the bed. Thus, the magnetic anchor pulled up the mucosa sufficiently to show the resection line. Moreover, hemorrhage was rare because blood vessels could be clearly visualized endoscopically, and electrocoagulation hemostatic procedures were conducted before cutting the blood vessels. Even on occasions of unexpected hemorrhage, hemostasis proved easier because the site of bleeding was clearly visible by stretching the mucosal folds using the magnetic anchor.

The basic features and functions of the magnetic anchor were similar at all the four tested representative areas of the stomach, and the magnetic anchor could be controlled in the same manner at all sites. Sizes of resected specimens and the procedure time required for each are shown in Table 1.

DISCUSSION

Conventional surgery for cancer is highly stressful and sometimes burdensome for patients. Standard treatment for gastric cancer at present is gastrectomy, which is performed even for early gastric cancer. One alternative to gastrectomy that has recently emerged is EMR, although it has several technical problems related to its one handed surgery approach. This procedure, in turn, demands additional skills that are beyond those required for a standard endoscopic technique.

However, the magnetic anchor is not a technique in itself but just an additional tool which is optionally used by the endoscopist during EMR. In the present experiment, endoscopists used the magnetic anchor throughout the procedure and found that EMR was much easier by using it. This opinion was unanimous even on their first exposure to the new device. The magnetic anchor facilitated resection even with the techniques used for standard EMR, which are different from those used for standard surgery. It is worth noting that it was not necessary for the endoscopists to modify their technique in using the magnetic anchor. In fact, they found that the use of the anchor offered more benefits.

The first EMR procedure using the magnetic anchor was performed by a senior physician, the following two by resident physicians, and the last one by a senior physician with a resident physician. All the procedures were performed with few or no incidents regardless of the skills of the physicians. The senior physician, who has developed skills for using the IT knife, had to modify technique he used for standard surgery in order to maximize the efficacy of the magnetic anchor. However, he did not find it difficult. Even for resident physicians inexperienced with an IT knife, EMR with the magnetic anchor proved easy to perform. According to the endoscopists, one touch to the mucosa made it sufficient for cutting. This was a great contrast to the frustration that they had when performing standard EMR without the magnetic anchor, which demanded much effort and patience.

However, several noteworthy incidents had occurred during the procedure. Prominent among them were separation of the magnetic weight from the thread connecting the forceps and slipping of the microforceps from the mucosa. In these cases, new magnetic anchors could be inserted without any problems, and the procedure was continued. The malfunctioned anchors could be easily removed causing no problem, even when left in the gastric lumen during the resumed resection procedure with a new anchor. Slipping of the forceps occurred twice for the same physician at the same mucosal site. Thus, this may be attributed to his inexperience in surgical techniques, which would be similar to the problems encountered with some assistants in standard surgery. Of greater importance was the unproblematic retrieval of the dislodged magnetic anchor. In fact, one of the reasons for involving various physicians in this experiment was to evaluate the nature of incidents with the device and to consider possible countermeasures for future improvement. Thus, the magnetic anchor will certainly be improved, and refined, and will present with few benign problems that we expect to overcome easily. We emphasize that the magnetic anchors used in this study were merely handmade ones devised to experimentally assess the conceptual feasibility of the technique.

Problems with standard EMR such as perforation and incomplete resection are serious and potentially hinder the EMR procedure from being indicated to lesions proposed for resection by the National Cancer Center Hospital of Japan. Even though indications for EMR procedures may be discussed in later papers, we believe that several current problems with EMR are solved to a great extent by the use of the magnetic anchor.

The results of this study showed that all procedures were satisfactorily performed using an electric current of less than 50 A for the electromagnet, which is comparable to 0.6 kOe/1.0 cm. Consequently, the intensity of the magnetic field required for

the magnetic anchor for EMR was less than what had been expected prior to this procedure. Our next model of the magnetic control system will be smaller and simpler. New concepts of magnetic anchor will expand the indications of endoscopic surgery beyond the treatment of gastric cancer.

Acknowledgments

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DNA HYPOMETHYLATION ON PERICENTROMERIC SATELLITE REGIONS SIGNIFICANTLY CORRELATES WITH LOSS OF HETEROZYGOSITY ON CHROMOSOME 9 IN UROTHELIAL CARCINOMAS

TOHRU NAKAGAWA, YAE KANAI,* SAORI USHIJIMA, TADAICHI KITAMURA, TADAO KAKIZOE AND SETSUO HIROHASHI

From the Pathology Division, National Cancer Center Research Institute (TN, YK, SU, SH), Department of Urology, Faculty of Medicine, Tokyo University (TN, TKi) and National Cancer Center (TKa), Tokyo, Japan

ABSTRACT

Purpose: DNA methylation has important roles in genomic stability. Accordingly DNA hypomethylation on pericentromeric satellite regions may induce chromosomal instability through heterochromatin decondensation and chromosomal recombination enhancement. We elucidated the significance of aberrant DNA methylation on pericentromeric satellite regions during urothelial carcinogenesis.

Materials and Methods: We examined DNA methylation status on satellites 2 and 3 by Southern blotting and determined the allelic status of chromosome 9 using 6 microsatellite markers (D9S775, D9S925, D9S304, D9S303, D9S283 and D9S747) in 27 transitional cell carcinomas of the bladder, ureter or renal pelvis and corresponding noncancerous tissues.

Results: DNA hypomethylation on satellites 2 and 3 was detected in 2 (7%) and no (0%) noncancerous tissues, and in 11 (41%) and 12 (44%) urothelial carcinomas, respectively. DNA hypomethylation in urothelial carcinomas significantly correlated with histological grade ($p = 0.0012$ and 0.0043), invasion depth ($p = 0.0055$ and 0.0228) and morphological structure (papillary vs nodular, $p = 0.0161$ and 0.0297) for satellites 2 and 3, respectively. Loss of heterozygosity on at least 1 locus of chromosome 9 was detected in 14 urothelial carcinomas (52%). DNA hypomethylation on satellites 2 ($p = 0.0098$) and 3 ($p = 0.0034$) significantly correlated with loss of heterozygosity on chromosome 9.

Conclusions: DNA hypomethylation on pericentromeric satellite regions may participate in the development and progression of urothelial carcinomas by inducing loss of heterozygosity on chromosome 9.

KEY WORDS: urothelium; carcinoma, transitional cell; DNA methylation; chromosomal instability; loss of heterozygosity

DNA methylation has important roles in transcriptional regulation, chromatin remodeling and genomic stability.[1] Satellites 2 and 3, which are related families containing a frequent 5 bp repeat, are abundant in pericentromeric heterochromatin regions on chromosomes 1, 9 and 16, and heavily methylated in normal somatic cells.[2] DNA hypomethylation on such pericentromeric satellite regions may induce chromosomal instability through heterochromatin decondensation and chromosomal recombination enhancement.[3,4] DNA hypomethylation on satellites 2 and 3 has been reported to cause chromosomal instability, such as the formation of multiradiate chromosomes composed of chromosomes 1, 9 and 16, in ICF (immunodeficiency-chromosomal instability-facial anomalies) syndrome.[2]

In human cancers overall DNA hypomethylation accompanied by region specific hypermethylation is generally observed.[1] Aberrant DNA methylation may be involved in carcinogenesis by at least three possible mechanisms: induction of genomic instability as a result of decreased methylation level,[5–7] increased gene mutagenicity caused by deamination of 5-methylcytosine to thymine and repression of gene transcription through CpG island methylation in specific gene regulatory regions, including tumor suppressor genes.[1] For example, frequent chromosomal 1q copy gain with a pericentromeric break point has been reported in hepatocellular carcinomas showing DNA hypomethylation on satellite 2.[8]

The role of DNA hypomethylation in urothelial carcinomas is not fully understood, although aberrant hypermethylation on CpG islands around the promoter region and decreased expression of tumor suppressor genes, such as the *p16* and *E-cadherin* genes, have been reported.[9,10] In addition, loss of heterozygosity (LOH) on chromosome 9 is the most common genetic abnormality in urothelial carcinomas.[11] Consequently we focused on the clinicopathological significance of DNA hypomethylation on pericentromeric satellite regions in urothelial carcinomas and examined whether this hypomethylation is the underlying mechanism for LOH on chromosome 9 during human urothelial carcinogenesis.

MATERIALS AND METHODS

Patients and tissue samples. Paired specimens of primary urothelial carcinoma and corresponding noncancerous tissue were obtained from surgically resected specimens from 27 patients (U1 to U27) treated at National Cancer Center Hospital, Tokyo, Japan. The patients were 22 men and 5 women with a mean age ± SD of 67.6 ± 10.5 years (range 50 to 85).

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* Correspondence: Pathology Division, National Cancer Center Research Institute, 5–1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan (FAX: 81–3-3248–2463; e-mail: ykanai@ncc.go.jp).

The primary tumor sites were the bladder, ureter and renal pelvis in 13, 5 and 9 patients, respectively. Based on histological examination 5 (19%), 10 (37%) and 12 (44%) tumors were classified as G1, G2 and G3–4 transitional cell carcinoma, while 11 (41%) and 16 (59%) were superficial (pTa and pT1) and invasive (pT2 to pT4), respectively.[12] Morphologically 21 tumors (78%) were papillary carcinoma and 6 (22%) were nodular carcinoma. Noncancerous specimens were obtained from the urothelium distant from the carcinoma.[13] In cases of widely spreading carcinomas in situ, as diagnosed histopathologically in preoperative biopsy specimens, the muscle layer of the bladder or the renal parenchyma was collected as noncancerous specimens since macroscopic examination cannot necessarily discriminate noncancerous urothelium from carcinoma in situ.

Southern blotting for pericentromeric satellite regions. High molecular weight DNA was isolated from fresh tissue samples by phenol-chloroform extraction and dialysis. DNA methylation status was assessed by digesting DNA with *Msp* I and *Hpa* II, which cut at the sequence CCGG. *Hpa* II does not cut when the internal cytosine is methylated. High molecular weight DNA (5 μg) was digested for 24 hours with 10 U *Msp* I or *Hpa* II/μg DNA. DNA fragments were separated by electrophoresis, transferred to nitrocellulose membranes and hybridized with ^{32}P labeled DNA probes. Previously described oligonucleotides were used as probes for satellites 2 and 3.[14]

Analysis of LOH on chromosome 9. Genomic DNA was amplified by polymerase chain reaction (PCR) using oligonucleotide primers for 6 microsatellite loci on chromosome 9, namely D9S775, D9S925, D9S304, D9S303, D9S283 and D9S747. Primer sequences were D9S775 (9p23) 5′-AAAGTAGCCATCCGTGTGT-3′ and 5′-GCTTTCTTTGATGGTTTACAG-3′, D9S925 (9p21–22) 5′-GTCTGGGTTCTCCAAAGAAA-3′ and 5′-TGTGAGCCAAGGCCTTATAG-3′, D9S304 (9p21) 5′-GTGCACCTCTACACCCAGAC-3′ and 5′-TGTGCCCACACACATCTATC-3′, D9S303 (9q21) 5′-CAACAAAGCAAGATCCCTTC-3′ and 5′-TAGGTACTTGGAAACTCTTGGC-3′, D9S283 (9q22) 5′-TGCTGGATTTCAGGTAGGG-3′ and 5′-ATGGTTATGCGGGTGTATTTCTC-3′, and D9S747 (9q32) 5′-GCCATTATTGACTCTGGAAAAGAC-3′ and 5′-CAGGCTCTCAAAATATGAACAAAAT-3′. The 5′ ends of forward primers were labeled with 6-carboxyfluorescein and PCR amplifications were performed with 20 ng genomic DNA. Subsequently PCR products were fractionated by electrophoresis (ABI 3100 sequencer, Applied Biosystems, Foster City, California) according to the manufacturer protocol. Data were analyzed with the GeneScan, version 3.7 computer program (Applied Biosystems). When 2 amplified bands per locus were detected in the noncancerous tissue specimen, the case was considered informative for LOH analysis. LOH was recorded when signal intensity for a tumor allele was decreased by more than 50% relative to the matched normal allele in informative cases, as described previously.[15–17] Replication error was identified by the presence of band shifts or the presence of novel bands in PCR products.

Statistics. Correlations between any 2 of DNA methylation status, allelic status and clinicopathological parameters were analyzed by the chi-square test with $p < 0.05$ considered significant.

RESULTS

DNA methylation status on pericentromeric satellite regions and its correlation with clinicopathological parameters. Figure 1 shows examples of Southern blotting. In 25 (93%) and all 27 (100%) noncancerous tissue specimens examined significantly larger DNA fragments were detected in *Hpa* II digests compared with *Msp* I digests at satellites 2 and 3, respectively, indicating that these regions were heavily methylated. In 11 (41%) and 12 (44%) urothelial carcinomas smaller fragments were detected in *Hpa* II digest compared with corresponding normal tissues or *Hpa* II digest showed almost the same hybridization pattern as the *Msp* I digest of the same sample and the corresponding normal tissue, indicating that these regions were hypomethylated. In almost all carcinoma samples in which DNA hypomethylation was detected hypomethylation occurred on satellites 2 and 3.

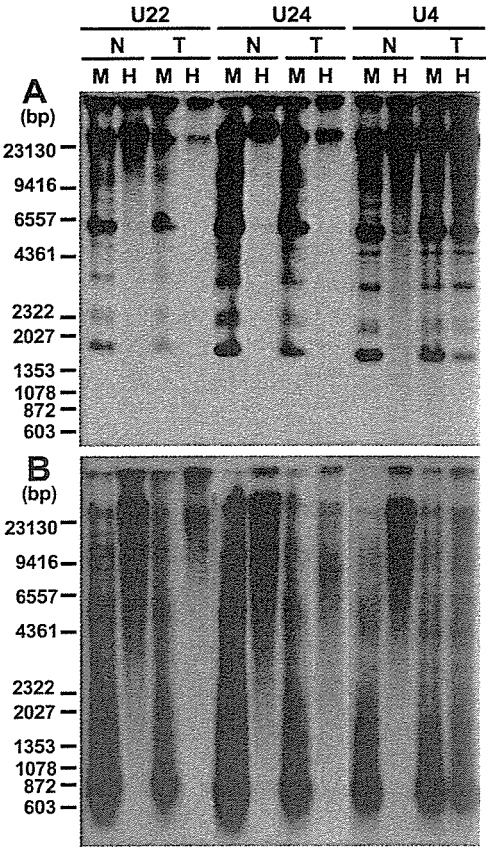

FIG. 1. Examples of Southern blotting for satellites 2 (*A*) and 3 (*B*) in cases of urothelial carcinoma. Methylation status was assessed by digesting DNA with *Msp* I (*Lane M*) and *Hpa* II (*Lane H*). DNA fragments were separated by electrophoresis, transferred to nitrocellulose membranes and hybridized with ^{32}P labeled DNA probes. Larger bands were detected in lane H compared with lane M in all noncancerous tissue (*N*), and in U22T and U24T, indicating that satellite 2 region was heavily methylated (*A*). In U4T lane H showed same hybridization pattern as lane M, indicating that this region was hypomethylated. (*B*) In all noncancerous tissues, and U22T and U24T satellite 3 region was heavily methylated, whereas this region was hypomethylated in U4T. *T*, cancerous tissue.

DNA hypomethylation on pericentromeric satellite regions significantly correlated with histological grade (chi-square test $p = 0.0012$ and 0.0043), invasion depth (chi-square test $p = 0.0055$ and 0.0228) and morphological structure (papillary vs nodular chi-square test $p = 0.0161$ and 0.0297) for satellites 2 and 3, respectively (table 1), but not with age or gender (data not shown).

Allelic status of chromosome 9 and its correlation with clinicopathological parameters. Figure 2 shows examples of electropherograms of PCR products. Figure 3 shows the results of LOH analysis. Table 2 lists the incidence of LOH on each locus. LOH for at least 1 marker was found in 14 of the 27 informative cases (52%) (table 2).

The presence of LOH on at least 1 locus on chromosome 9 significantly correlated with histological grade (chi-square test $p = 0.0313$, table 3). LOH on at least 1 locus was detected in all 6 nodular carcinomas and its incidence (100%) was significantly higher than in papillary carcinomas (chi-square test $p = 0.0074$, table 3).

Correlation between DNA methylation status on pericentromeric satellite regions and allelic status of chromosome 9. DNA

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

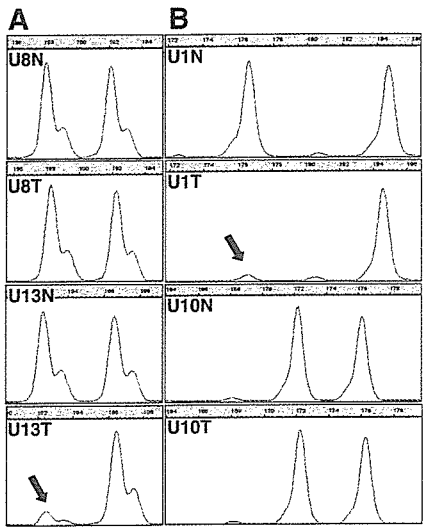

FIG. 2. Examples of results of allelic status analyses in cases of urothelial carcinoma. U8 and U13 DNA samples were amplified for D9S747 (*A*), while U1 and U10 samples were amplified for D9S775 (*B*). Genotypes derived from noncancerous U8N, U13N, U1N and U10N tissues, and corresponding U8T, U13T, 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).

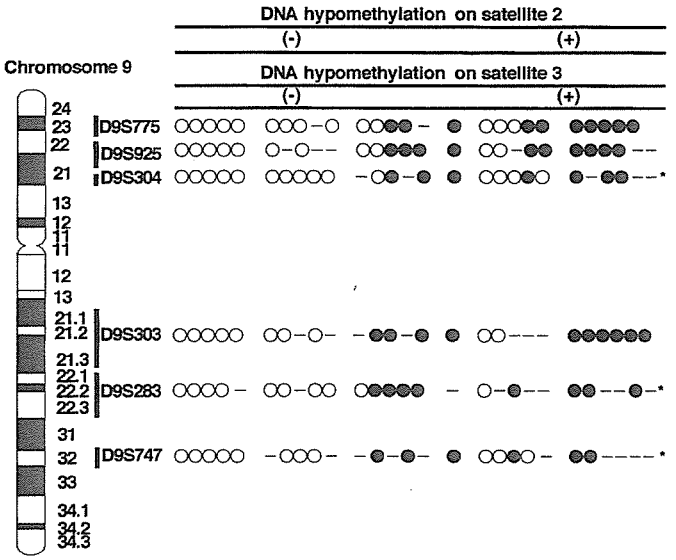

FIG. 3. Allelic status of each locus in urothelial carcinomas. Vertical lines indicate each carcinoma. Open circles indicate retention of 2 alleles. Filled circle indicate LOH. Bar indicates uninformative case. Asterisk indicates replication error. −, negative. +, positive.

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 (33)	
G3–4	12	9 (75)	0.0313
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

Hidetoshi Yamanaka,[1] Kazuto Ito,[2]* Seiji Naito,[3] Taiji Tsukamoto,[4] Michiyuki Usami,[5] Hiroyuki Fujimoto,[6] Naoki Matsuoka,[6] Iwao Fukui,[7] Masaoki Harada,[8] Yasuo Ohashi,[9] Toshihiko Kotake,[10] and Tadao Kakizoe[6]

[1]*Institute for Preventive Medicine, Kurosawa Hospital, Takasaki, Japan*
[2]*Department of Urology, Gunma University Graduate School of Medicine, Maebashi, Japan*
[3]*Department of Urology, Graduate School of Medicine, Kyusyu University, Fukuoka, Japan*
[4]*Department of Urology, Sapporo Medical University School of Medicine, Sapporo, Japan*
[5]*Department of Urology, Osaka Medical Center for Cancer and Cardiovascular Disease, Osaka, Japan*
[6]*Urology Division, National Cancer Center Hospital, Tokyo, Japan*
[7]*Department of Urology, Cancer Institute Hospital, Tokyo, Japan*
[8]*Kanagawa Cancer Center Research Institute, Yokohama, Japan*
[9]*Department of Hygiene and Preventive Medicine, School of Health Sciences and Nursing, University of Tokyo, Tokyo, Japan*
[10]*Kotake Clinic, Osaka, Japan*

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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*Correspondence to: Kazuto Ito, MD, Department of Urology, Gunma University Graduate School of Medicine, 3-39-22, Showa-machi, Maebashi, Gunma, 371-8511, Japan.
E-mail: kzito@med.gunma-u.ac.jp
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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.

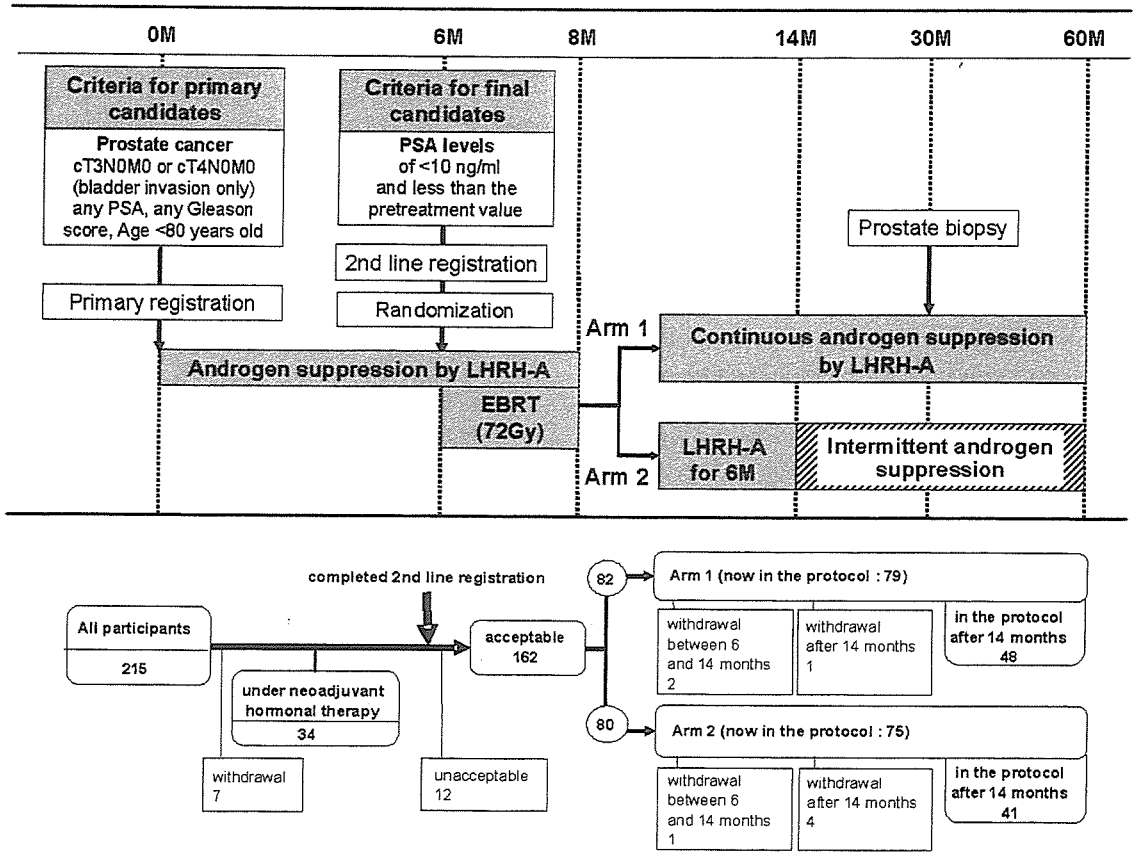

Fig. 1. Scheme of the study protocol, the number of patients registered and the present status of those patients in this study protocol. LHRH-A, LHRH agonist; EBRT, external beam radiation therapy.

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.

Members of the National Research Project on Endocrine-Radiation CombinationTherapy for Locally Advanced Prostate Cancer

Hidetoshi Yamanaka, Hiroyuki Fujimoto, Naoki Matsuoka, Taiji Tsukamoto, Iwao Fukui, Seiji Naito, Michiyuki Usami, Kazuhiro Suzuki, Norio Mitsuhashi, Tetsuo Akimoto, Minako Sumi, Masato Hareyama, Takashi Yamashita, Katsumasa Nakamura, Koichi Tokuuye, Kinji Nishiyama, Yasuo Ohashi, Masaoki Harada, Jun Aoki, Mitsuru Shinohara, Katsuyuki Karasawa, Satoshi Kitahara, Miwako Nozaki, Shin Egawa, Iku Nishiguchi, Masashi Kitano, Takanori Suzuki, Nobuaki Shimizu, Yoshio Tamaki, Mikio Kobayashi, Iku Takahashi, Mikinobu Ohtani, Akio Iwasaki, Tatsuo Tochigi, Shiro Saito, Katsuyoshi Hashine, Tadao Kakizoe, Toshihiko Kotake.

Associate Researchers in the Project

Kazuto Ito, Naoya Masumori, Norio Meguro, Hirofumi Koga, Sadafumi Kawamura, Kotaro Gomi, Yutaka Takezawa, Atsushi Yamauchi, Takumi Yamamoto, Akira Irie, Kazumi Shiojima.

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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

Toshio Ichihara[1,2], Seiko Tamano[1,2], Hiroko Yoshino[1,2], Katsumi Imaida[3], Hideki Ishikawa[4], Tadao Kakizoe[5], and Tomoyuki Shirai[1]

[1]Department of Experimental Pathology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467–0001, Japan
[2]DIMS Institute of Medical Science, 64 Goura, Nishiazai, Azai-cho, Ichinomiya 491–0113, Japan
[3]Onco-Pathology, Faculty of Medicine, Department of Pathology and Host-Defense, Kagawa University, 1750–1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761–0793, Japan
[4]Laboratory of Hereditary Tumor, Institute for Advanced Medical Sciences, Hyogo College of Medicine, 2–3–1–2F Kyomachibori, Nishi-ku, Osaka, 550–0003, Japan
[5]National Cancer Center Hospital, 5–1–1 Tsukiji, Chuo-ku, Tokyo 104–0045, Japan

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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Mailing address: Toshio Ichihara, DIMS Institute of Medical Science, 64 Goura, Nishiazai, Azai-cho, Ichinomiya 491–0113, Japan
TEL: 81-586-51-1201 FAX: 81-586-51-5634
E-mail: ichi@dims.co.jp