表 4-6　センチネルリンパ節生検の feasibility study

| 報告者 | 年 | 方法 | 症例数 | 同定率 | 正診率 | 敏感度 | センチネルのみリンパ節転移 |
|---|---|---|---|---|---|---|---|
| Giuliano[4] | 1997 | IB | 107 | 93% | 100% | 100% | 67%　(28/42) |
| Galimberti[5] | 1998 | CA | 241 | 99% | 98% | 95% | 36%　(39/109) |
| Borgstein[6] | 1998 | CA | 130 | 94% | 99% | 98% | 59%　(26/44) |
| Cox[7] | 1998 | S+IB | 466 | 94% | 100% | 99% | — |
| Krag[8] | 1998 | S | 443 | 93% | 97% | 89% | 53%　(60/114) |
| Veronesi[9] | 1999 | CA | 376 | 99% | 96% | 93% | 44%　(73/168) |
| Hill[10] | 1999 | S+IB | 492 | 93% | 95% | 89% | 61%　(69/114) |
| Imoto | 1999 | RI+IC | 56 | 96% | 98% | 96% | 48%　(13/27) |

CA: technetium-99m ($^{99m}$Tc)-colloidal albumin
S: $^{99m}$Tc-sulfur colloid
RI: $^{99m}$Tc-human serum albumin and $^{99m}$Tc-tin colloid
IB: isosulfan blue
IC: indigocarmine

に伴う有害事象は極めて稀であると考える．まずセンチネルリンパ節生検＋腋窩リンパ節生検すなわち feasibility study を施行することは，標準治療の範囲内であり患者の不利益にはならない．推奨される feasibility study の成績は，少なくとも 30〜50 例程度の症例に実施し，同定率 95% 以上，偽陰性率 5% 以下を目標に腋窩郭清を省略する observation study に進むのが妥当とされている．

センチネルリンパ節生検を行う場合には，この方法について特別なインフォームドコンセントが必要である．内容としては，①試験的な段階にあるリンパ節転移診断法であること，②実施方法，③発生しうる有害事象とその頻度，④患者の利益と不利益，⑤実施に関する費用は研究者負担となること，⑥observation study ならば施設あるいは個人での成績，など説明して被験者の同意を得る必要がある．現時点では，ベネフィットばかり強調して標準治療のごとく説明して同意を得ることは望ましくないと考える．





## 2 適応基準

①臨床的リンパ節転移陰性乳癌である．
②インフォームドコンセントが得られている．
③色素にアレルギー反応の既往がない．
④腫瘍径に関しての基準はないが，早期乳癌（腫瘍径 2cm 以下）から開始して適応を拡げていくのが安全であると考える．
⑤放射線照射に既往のある乳房やステージⅢB 乳癌，炎症性乳癌は適応にすべきではない．

## 3 方法

### 1）準備

センチネルリンパ節生検を始めるにあたって，各診療部門の充分な理解と連携が求められる．センチネルリンパ節生検は，施設内倫理審査委員会あるいはこれに準ずる委員会で承認を経てから実施されるべきである．現時点ではセンチネルリンパ節生検は健康保険の適応ではないので，実施に関する費用は研究者負担であり診療請求することはできない．

### 2）試薬（色素）

①indigocarmine
②indocyanine green
③sulfan blue（patent blue violet）
④isosulfan blue

### 3）試薬（放射性製剤）

①technetium-99m tin colloid
②technetium-99m colloidal rhenium sulphide
③technetium-99m human serum albumin

④technetium-99m phytate

### 4）投与部位

①peritumoral injection
②subdermal injection
③intradermal injection
④subareolar injection

投与部位は推奨されるものはないので，症例によって適宜選択するのが望ましい．

### 5）リンフォシンチグラフィ

センチネルリンパ節を術前に視覚的に捉える方法として，リンフォシンチグラフィは有用である．著者の手技は，手術前日に核医学検査室内にて technetium-99m phytate を腫瘍直上の皮内と乳輪下に 50〜80 MBq を 1，2 カ所に分けて投与する．皮内注は組織圧が高まるため，また乳輪下はリンパ流が豊富なためマッサージは不要である．投与後 30 分間の dynamic early image ならびに 6 時間後の static delayed image を撮影する．著者は斜位像のみ撮影しているが，通常，投与部位からリンパ管流を受けた 1 個から数個のセンチネルリンパ節が腋窩に観察される（図 4-31）．10%弱の症例に穿通枝を介する胸骨傍にもセンチネルリンパ節が観察されることもある（図 4-32）．著者はこのような症例に関しては，現在の胸骨傍リンパ節転移の位置付けから判断して積極的には胸骨傍リンパ節生検は実施していない．図 4-31，32 に紹介した症例は，局所進行例であったため同日のリンフォシンチグラフィの施行前に骨シンチを実施しているためセンチネルリンパ節の解剖学的局在を把握するのが容易である．通常の症例（骨シンチを施行していない）では，テクネシウムをわずかに吸った注射器を用い被験者のボディラインをなぞる．注射器から発するγ線の軌跡をシンチカメラがとらえてセンチネルリンパ節と重ね合わされることにより，センチネルリンパ節の解剖学的局在を容易にイメージできる（図 4-33）．

また，この用意した注射器をシンチカメラのモニター画面下で hot spot であるセンチネルリンパ節に重ね合わせて，皮膚にその局在を示す





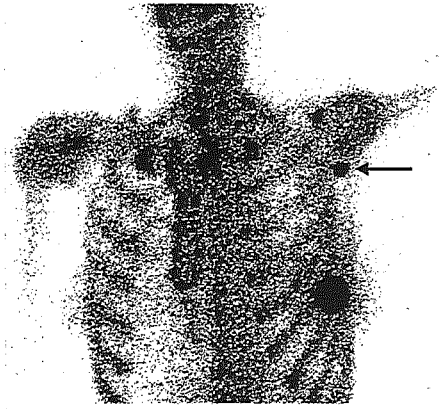

図 4-31　リンフォシンチグラフィ（1）
同日に骨シンチを施行．腋窩にセンチネルリンパ節（矢印）が観察される．

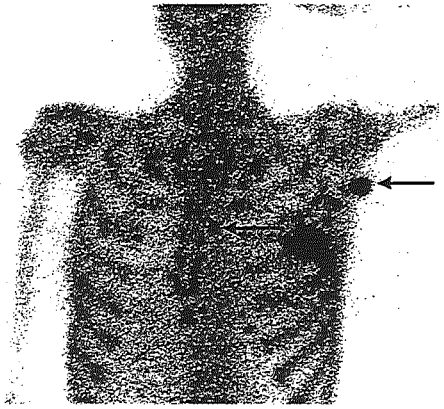

図 4-32　リンフォシンチグラフィ（2）
同日に骨シンチを施行．腋窩と胸骨傍領域にセンチネルリンパ節が観察される．

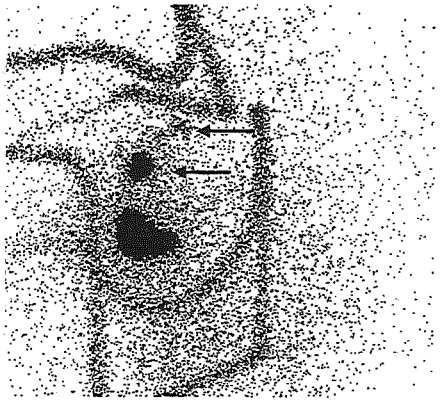

図 4-33　リンフォシンチグラフィ（3）
体輪郭のイメージを重ね合わせて再現している．腋窩にセンチネルリンパ節（矢印）が観察される．

マーキングを実施することも可能である．このようにリンフォシンチグラフィを施行することによりセンチネルリンパ節の解剖学的位置が推定できる．

### 6）色素法

全身麻酔の導入後，青い色素であるパテントブルー 2〜5ml を乳輪下あるいは腫瘍周囲に注射して同部位を数十秒間よくマッサージする（図 4-34，35）．15〜20 分後腋窩のやや尾側に小切開を加える（図 4-36）．小血管からの出血は視野を不良にして，センチネルリンパ節の同定を困難にするので，充分に止血操作をしながら剥離をすすめていく．青く染まったリンパ管を発見し（図 4-37），これを追って流入する青く染まったリンパ節すなわちセンチネルリンパ節に到達し摘出する（図 4-38，39）．色素法は，30〜50 例の手技の経験と学習効果を必要とするが，最終的に 90%近い同定率での実施が可能となる．不成功の理由としては，肥満，腋窩の脂肪組織が厚くリンパ管やリンパ節がみつけにくい場合や剥離の際にリンパ管をすでに切断してしまった場合などが考えられる．

### 7）ガンマプローブ法

ガンマプローブ法は，放射性製剤が移行したセンチネルリンパ節からのγ線を高感度γ線検出装置（図 4-40）を用いて同定する方法である．ガンマプローブの先端部を用いて，最もγ線が検出される部位を同定してマーキングを施行する（図 4-41）．同部位を目指して皮膚を切開し，radioactivity を確認しながら腋窩脂肪組織の剥離を進め，目的とするセンチネルリンパ節を同定し摘出する（図 4-42）．radioactivity の高いリンパ節であれば，色素法よりも容易で初心者でも容易にセンチネルリンパ節を検出できる．また，胸骨傍リンパ節やレベル I 以外のリンパ節がセンチネルリンパ節である場合は，色素法よりガンマプローブ法が同定しやすい．同定されたリンパ節の radioactivity を *in vivo* と *ex vivo* で測定する．測





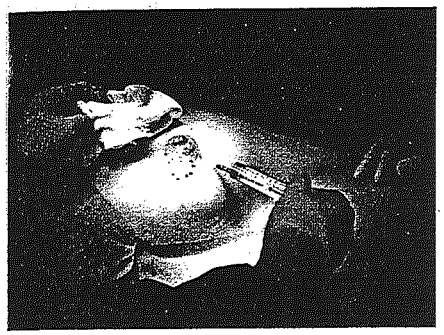

図 4-34　色素法（1）
加刀前に乳輪下に色素 3ml を注射する．

図 4-35　色素法（2）
腋窩方向に向かってよくマッサージする．

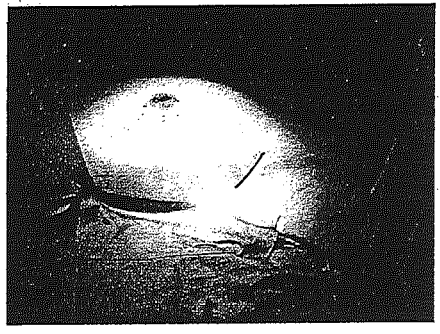

図 4-36　色素法（3）
腋窩のやや尾側に皮切を加える．

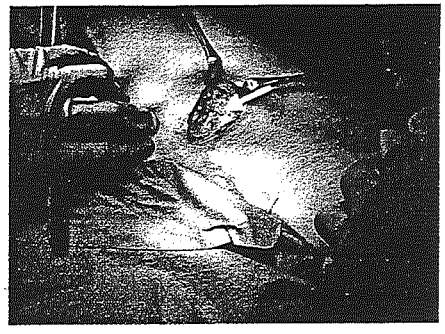

図 4-37　色素法（4）
青く染まったリンパ管をみつけ出す．

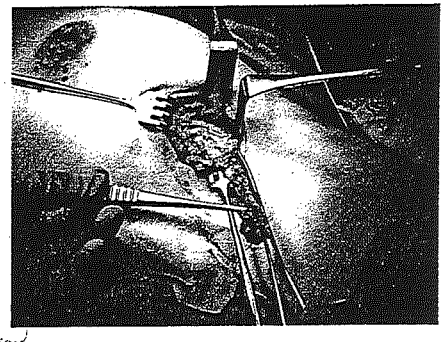

図 4-38　色素法（5）
青く染まったリンパ管を追いかけて青く染まったセンチネルリンパ節を同定し摘出する．

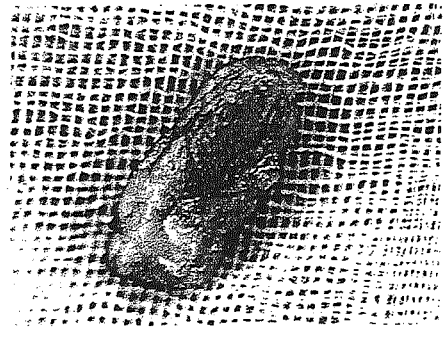

図 4-39　センチネルリンパ節
青く染まったリンパ節．センチネルリンパ節として迅速病理診断にて検査する．

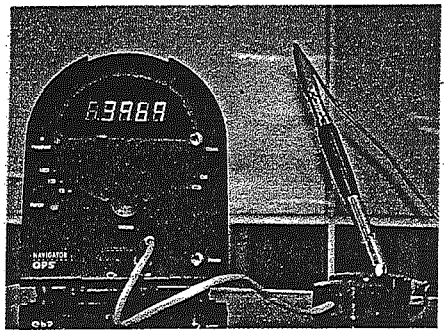

図 4-40　ガンマプローブ法（1）
当院にて使用している高感度γ線検出装置.

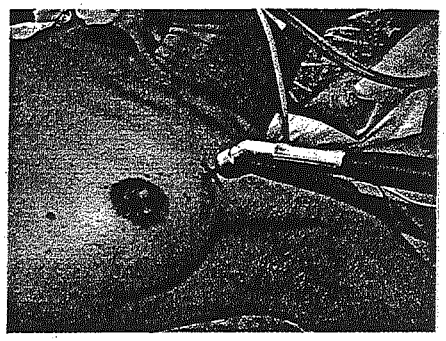

図 4-41　ガンマプローブ法（2）
ガンマプローブを用いて加刀前にマーキングしておく.

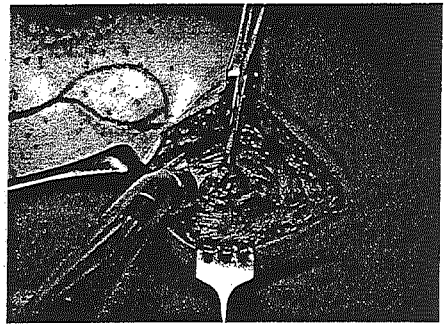

図 4-42　併用法
ガンマプローブと色素を用いて，目的とするセンチネルリンパ節を検出する.

定値は，background（周囲組織）の数十倍～数百倍である.

著者は，術中に先に腫瘍の生検を施行する症例に対しては，色素法が困難となるためガンマプローブ法単独でセンチネルリンパ節生検を施行している.

8）併用法（図 4-42）

上記の色素法とガンマプローブ法を組み合わせて実施する方法である．著者はセンチネルリンパ節生検の精度向上のため色素とガンマプローブの併用法を通常行っている．視覚でも確認ができること，時間の





制約が比較的ないこと，radioactivity で確認しながら容易に目的とするリンパ節を同定できること，レベルⅠ以外のリンパ節にセンチネルリンパ節が存在する場合でも同定可能であることなどが併用法で実施している理由である．この方法によりセンチネルリンパ節をより確実に同定することが可能となる.

## 4 センチネルリンパ節の病理検査

センチネルリンパ節生検によって，摘出された 1 個から数個のセンチネルリンパ節を術中に迅速病理診断をするいくつかの試みが行われている．基本は凍結切片作成による組織診断で，多数切片の作成や抗サイトケラチン抗体などによる免疫組織染色による転移診断や捺印細胞診断などによるセンチネルリンパ節転移診断の向上に関する工夫が報告されている[12-15]．ただし，パラフィン固定後の HE（hematoxylin-eosin）染色による詳細な永久組織診断が基本であり，迅速にて転移陰性とされたセンチネルリンパ節内に転移巣が発見される場合が 10%程度ある．施設毎の病理部門においてセンチネルリンパ節に関する精度の高い検査法を確立することが重要である．また，最近注目されている微小リンパ節転移例（0.2～2mm の転移巣）の取り扱いに関しては，腋窩郭清や放射線治療を追加すべきか結論は得られていない．いずれにしても，永久組織診断で転移ありと判断された場合は，腋窩郭清を追加する方向でインフォームドコンセントをとるべきであると考える.

乳癌診療におけるセンチネルリンパ節生検は，外科手術の個別化・低侵襲化という流れの中で，確固たる地位を築きつつある．腫瘍外科医にとっては，色素法にせよ，ガンマプローブ法にせよ，併用法にせよ，手技の習得は必須である．ただし，一歩間違えるとただのいい加減な治療にもなり得る手法なので，各自が充分な経験と倫理性をもって，患者の充分な理解と同意の後に実施してほしい.

■文献

1) Goldhirsch A, et.al. Meeting highlights: international consensus panel on treatment of primary breast cancer. J Clin Oncol. 2001; 19: 3817-27.
2) Krag DN, et al. Surgical resection and radiolocalization of sentinel lymph node in breast cancer using a gamma probe. Surg Oncol. 1993; 2: 335-9.
3) Giuliano AE, et al. Lymphatic mapping and sentinel lymphadenectomy for breast cancer. Ann Surg. 1994; 220: 391-401.
4) Giuliano AE, et al. Sentinel lymphadenectomy in breast cancer. J Clin Oncol. 1997; 15: 2345-50.
5) Galimberti V, et al. Can sentinel node biopsy avoid axillary dissection in clinically node-negative breast cancer patients? Breast. 1998; 7: 8-10.
6) Borgstein PJ, et al. Sentinel lymph node biopsy in breast cancer; guidelines and pitfall of lymphoscintigraphy and gamma probe detection. J Am Coll Surg. 1998; 186: 275-83.
7) Cox CE, et al. Guidelines for sentinel node biopsy and lymphatic mapping of patients with breast cancer. Ann Surg. 1998; 227: 645-53.
8) Krag D, et al. The sentinel node in breast cancer; a multicenter validation study. N Engl J Med. 1998; 339: 941-6.
9) Veronesi U, et al. Sentinel lymph node biopsy and axillary dissection in breast cancer; results in a large series. J Natl Cancer Inst. 1999; 91: 368-73.
10) Hill ADK, et al. Lessons learned from 500 cases of lymphatic mapping for breast cancer. Ann Surg. 1999; 229: 528-35.
11) Cimmino VM, et al. Allergic reactions to isosulfan blue during sentinel node biopsy? a common event. Surgery. 2001; 130: 439-42.
12) Veronesi U, et al. Sentinel node biopsy to avoid axillary dissection in breast cancer with clinically negative lymph-nodes. Lancet. 1997; 349: 1864-7.
13) van Diest PJ, et al. Reliability of intraoperative frozen section and imprint cytological investigation of sentinel lymph nodes in breast cancer. Histopathol. 1999; 35: 14-8.
14) Gulec SA, et al. Clinical utility of frozen section in sentinel node biopsy in breast cancer. Am Surgeon. 2001; 67: 529-32.
15) Motomura K, et al. Intraoperative sentinel lymph node examination by imprint cytology and frozen sectioning during breast surgery. Br J Surg. 2000; 87: 597-601.

【木下貴之】



# 5 薬物療法

## A. 術前化学内分泌療法

ごく初期の段階をのぞいて，乳癌は全身病であるとの認識に基づき，局所進行癌だけでなく，より早期の乳癌に対しても術前化学（内分泌）療法が行われつつある．特に術前化学療法については多くの研究成果が報告されており，いまや術前化学療法は腫瘍径の大きな乳癌や，リンパ節転移陽性乳癌に対して標準的な治療と考えられるにいたっている．しかし，日常臨床の場において，どこまでが標準であり，何が研究なのかが混同されがちである．どのような対象に何を目的で行うかを明確にしておくことが大切である．術前内分泌療法もまた近年増加している．術前内分泌療法はまだ研究段階の治療と考えられるが，いくつかの有望な成績が示されつつある．

### 1 術前化学療法

局所進行乳癌（Stage IIIB）や炎症性乳癌に対する集学的治療の一環と

*Reprinted from*
*Jpn J Clin Oncol 2005;35(11)667–671*
*doi:10.1093/jjco/hyi177*

# A Flexible Endoscopic Surgical System: First Report on a Conceptual Design of the System Validated by Experiments

**Toshiaki Kobayashi[1], Sean Lemoine[1,2], Akihiko Sugawara[1,2], Takaaki Tsuchida[1], Takuji Gotoda[1], Ichiro Oda[1], Hirohisa Ueda[3] and Tadao Kakizoe[1]**

[1]National Cancer Center, [2]Japan Association for the Advancement of Medical Equipment and [3]Pentax Corporation, Tokyo, Japan

*Jpn J Clin Oncol 2005;35(11)667–671*
*doi:10.1093/jjco/hyi177*

# A Flexible Endoscopic Surgical System: First Report on a Conceptual Design of the System Validated by Experiments

**Toshiaki Kobayashi[1], Sean Lemoine[1,2], Akihiko Sugawara[1,2], Takaaki Tsuchida[1], Takuji Gotoda[1], Ichiro Oda[1], Hirohisa Ueda[3] and Tadao Kakizoe[1]**

[1]National Cancer Center, [2]Japan Association for the Advancement of Medical Equipment and [3]Pentax Corporation, Tokyo, Japan

Received May 17, 2005; accepted August 25, 2005; published online November 8, 2005

**Background:** Surgery is a standard diagnostic and therapeutic procedure. However, its technical difficulty and invasiveness pose problems that are yet to be solved even by current surgical robots. Flexible endoscopes can access regions deep inside the body with less invasiveness than surgical approaches. Conceptually, this ability can be a solution to some of the surgical problems.
**Methods:** A flexible (surgical) endoscopic surgical system was developed consisting of an outer and two inner endoscopes introduced through two larger working channels of the outer endoscope. The concept of the system as a surgical instrument was assessed by animal experiments.
**Results:** Gastric mucosa of the swine could be successfully resected using the flexible endoscopic surgical system, thereby showing us the prospect and directions for further development of the system.
**Conclusion:** The concept of a flexible endoscopic surgical system is considered to offer some solutions for problems in surgery.

*Key words: surgical robot – endoscopic surgery – surgery – robotics – endoscope*

## INTRODUCTION

We recently reported a new concept for endoscopic mucosal resection of gastric cancer with the use of a magnetic anchor. The anchor consisted of microforceps and a magnetic weight in order to grasp, stabilize and pull up the gastric mucosa (1). During the experiments, we thought that the procedure would be easier if one more endoscope was present to hold and stabilize the mucosa instead of the magnetic anchor.

Concerning flexible endoscopes, there are some ultrathin endoscopes that can be inserted into the working channels of standard endoscopes, such as gastrointestinal endoscopes. If the outer endoscope is able to contain larger and multiple working channels, several thin endoscopes could be inserted through the outer endoscope. This would allow for the resecting procedures. Such a system could also be applied to the fields where current surgical robots are targeting.

One of the problems with current surgical robots is inaccessibility to regions located deep inside the body, particularly regions reached through narrow and winding routes, such as the digestive tracts and blood vessels. However, some early gastric cancers can be resected endoscopically with much less invasiveness than surgery. These surgeries cannot be performed by current surgical robot systems because those regions were not originally considered places for the systems to operate.

An experimental flexible endoscopic surgical system was developed to cope with these problems of accessibility, consisting of a flexible outer endoscope with two working channels through which two inner flexible endoscopes could be inserted. These inner endoscopes were designed to have similar functions as flexible gastrointestinal endoscopes allowing for performance of standard endoscopic procedures even when introduced through the outer endoscope.

The uses of the flexible endoscopic system as a surgical instrument, as well as its functionality, were confirmed during gastric mucosal resection of the swine. This is in contrast to the current limitations for surgical robotics in terms of lesion access.

## MATERIALS AND METHODS

### FLEXIBLE SURGICAL ENDOSCOPE

As shown in Fig. 1, the flexible surgical endoscope consists of an outer flexible endoscope and two inner flexible endoscopes inserted into the working channel of the outer endoscope. The specifications of these endoscopes are listed in Table 1.

For reprints and all 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

The outer endoscope also has a 2.8 mm working channel and a charge coupled device (CCD) enabling the endoscope to operate in a similar fashion as standard gastrointestinal endoscopes. The endoscopic images are observed on cathode ray tube (CRT) monitors in the same manner as video-endoscopes.

Each of the inner endoscopes has a 2.0 mm working channel allowing accessories such as forceps and an electrocautery tip to be introduced and used. Unlike the outer endoscope, the inner endoscopes have optic fiber bundles for image visualization, instead of a CCD. These endoscopic images are also observed on CRT monitors. However, a video-adaptor, i.e. a small CCD video camera, must be connected onto each eye piece of the inner endoscopes in order to view the image on the monitors.

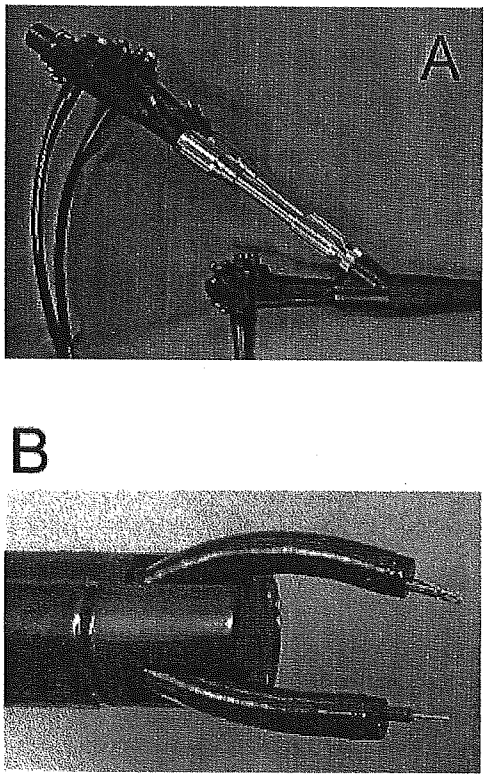


**Figure 1.** The flexible endoscopic surgical system. (A) The inner endoscope is inserted through a telescopic connecting device, which connects to the opening for the working channel of the outer endoscope near its control section. (B) At the tip of the outer endoscope two inner endoscopes protrude laterally, obtaining a certain distance between the two endoscopes.

**Table 1.** Specifications of the flexible endoscopic surgical system

| | Outer endoscope | Inner endoscope |
|---|---|---|
| Total length (mm) | 975 | 1395 |
| Working length (mm) | 665 | 1050 |
| Insertion portion diameter (mm) | 20 | 4.9 |
| Tip bending (degree) (up/down, right/left) | 210/120, 120/120 | 210/120, 120/120 |
| Field of view (degree) | 140 | 120 |
| Depth of field (mm) | 4–100 | 3–50 |
| Channel diameter (mm) | 7, 7, 2.8 | 2 |

These combined endoscopes are manipulated manually by three physicians together with the help of several assistants. The system, as a whole, operates similar to surgical robotic systems.

## Physicians

Two series of experiments were conducted. The first series was performed by a senior endoscopist and three resident physicians in order to assess the system with consideration to its endoscopic nature. The senior endoscopist was trained within the specialty of internal medicine, whereas one of the resident physicians was in training for internal medicine and the other two were for surgery.

The purpose of the subsequent series was to assess the concept of the flexible surgical endoscope from the viewpoint of surgeons. Consequently, the procedure was performed by two senior endoscopists, one having more than 15 year experience as a surgeon and the other having some surgical training, in addition to two residents who were in training for surgery.

These two series were performed on separate occasions, with none of the physicians performing in both series.

## Test Subject

Three female swine, under intravenous anesthesia, were laid on an examination table in the left lateral position. Within the first experiment, a 35.6 kg and a 34.1 kg swine were used. In the following experiment, a 41.8 kg swine was used. During these experiments, the law for the humane treatment and management of animals was observed.

## Procedure

The procedure was similar to standard endoscopic mucosal resection with the exception of one more endoscope for stabilization of the gastric mucosa.

First, an incision was made in the mucosa surrounding the region of stomach intended for resection (2,3). The outer endoscope was inserted through the esophagus into the gastric cavity. Subsequently, using the telescopic connecting devices (Fig. 1), the inner endoscopes were inserted into the working channels of the outer endoscope and introduced into the gastric cavity.

The outer endoscope was placed near the region in which the first incision was made. Thereafter, the resecting procedure was performed using an electrosurgical knife through one of the working channels of the inner endoscopes, whereas the other contained forceps. Within the procedure, the operator decided which side of the working channels would use the electrosurgical knife.

These procedures were observed on three CRT monitors, each of which was connected to its endoscopic counter part.

The resecting procedures were performed on the anterior wall of the gastric angle, the anterior wall of the middle gastric body and the greater curvature of the middle gastric body in the

first series for the assessment of endoscopic features. Within the following series, the resecting procedures were performed on two regions adjacent to the greater curvature of the lower gastric body.

## RESULTS

Concerning insertion of the outer endoscope through the esophagus into the gastric cavity, some difficulties were encountered owing to the large diameter of the outer endoscope and the relatively small size of the swine in both experimental series. However, the outer endoscope was introduced into the gastric cavity.

As for insertion of the inner endoscopes through the working channels of the outer endoscope, there were no difficulties experienced, even when the outer endoscope was bent due to insertion through the esophagus. Access to regions of the gastric wall was limited to the greater curvature due to the rigidity of the outer endoscope.

Maneuverability of the flexible endoscopic surgery system was satisfactory regarding the experiments were the first experiences for the physicians involved, despite some problems to solve.

The images from the outer endoscope were similar to those of standard gastrointestinal video-endoscopes due to the CCD system used in the outer endoscope. However, the images from the inner endoscopes were inferior to those of the outer endoscope. This inferiority was attributed to the limited number of optical fibers within the inner endoscope and deterioration of the image caused by conversion from optical images to electrical images through the use of a video-adapter. Consequently, during most of the procedure, endoscopic images were mainly observed using the monitor for the outer endoscope.

Some differences in use of the inner endoscopes for the resecting procedures between the first series and the second series were noticed. In the first series, the physicians appeared to have difficulties in some of the procedures such as accessing the mucosa, stabilization of the mucosal flap and resection procedures. These procedures were considered standard techniques for actual surgery, which means surgical experiences are required even to maneuver the flexible endoscopic surgical system.

Within the second series conducted by endoscopists with surgical experience, the resecting procedures were satisfactory, despite the fact this was their first experience using the system (Fig. 2). Through cooperation between the operator and assistants using verbal commands, manipulation of the inner endoscopes and the outer endoscope could be achieved. The functions of the inner endoscopes could be modified by changing the instruments inserted into the working channels. The flexible nature of the inner endoscopes allowed additional functions such as stabilization of the gastric wall by the longitudinal flank of the endoscope, as shown in Fig. 2C.

Within all the experiments, resecting procedures were completed without any complications such as perforation of the gastric wall. Consequently, five mucosal pieces, with sizes of

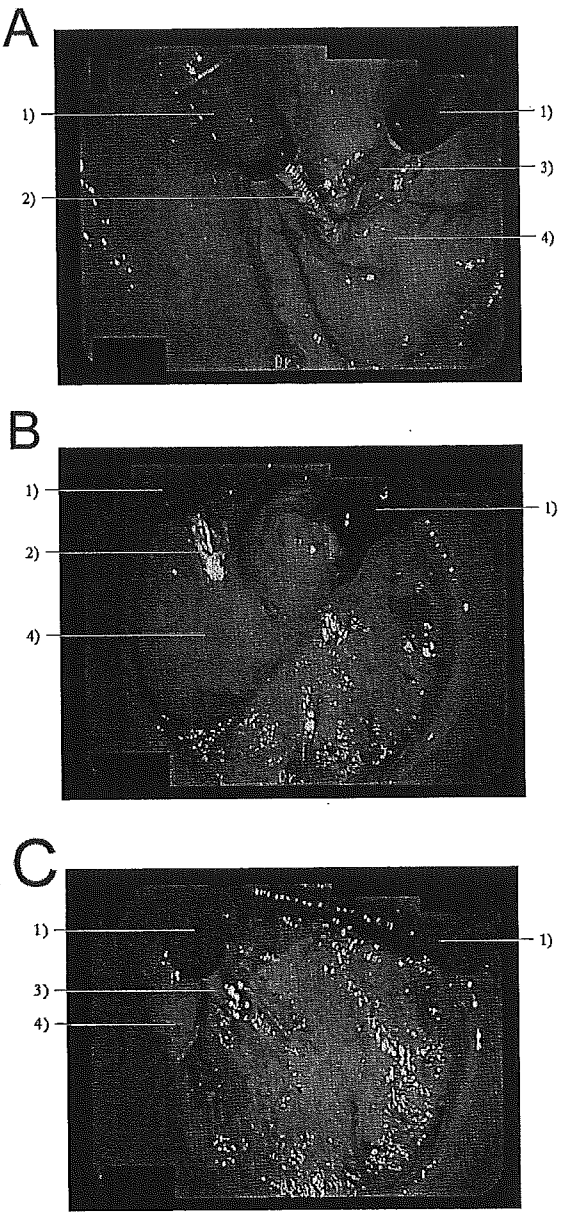


**Figure 2.** Images of the resecting procedures. (1) Inner endoscope, (2) forceps, (3) electrosurgical knife and (4) mucosal flap. (A) The right inner endoscope, with an electrosurgical knife introduced through its working channel, was maneuvered by the operator. The left inner endoscope, with forceps, was maneuvered by an assistant. (B) The tip of the right inner endoscope is holding up the mucosal flap in order to assist the forceps of the left inner endoscope to grasp the mucosal flap. (C) The right inner endoscope is pulling up the mucosal flap using forceps concealed in this image. In addition, using the flexibility of the endoscope, the gastric wall is stabilized by the longitudinal flank of the inner endoscope.

$2.8 \times 1.6$ cm$^2$, $2.8 \times 2.7$ cm$^2$ and $2.6 \times 2.0$ cm$^2$ in the first series, and $3.2 \times 2.7$ cm$^2$ and $4.0 \times 3.4$ cm$^2$ in the second series were each resected in a single piece.

## DISCUSSION

Surgical procedures are good options for diagnosis and treatment providing several advantages over non-surgical

approaches, especially in cases of malignant diseases. Although surgery is well accepted as a standard procedure in medicine there are still some problems left unsettled.

The technical difficulty of surgery is a common problem particularly for trainees, but even for experienced surgeons who have some technical limitations. Surgical procedures are difficult for regions deep in the body because the visual field for surgeons is limited, the number of surgical instruments which can be introduced is limited and the movements of these instruments are limited. One of the exemplary regions of this problem is the pelvic cavity, which includes surgery of rectal and prostate cancers.

Invasiveness is an inherent drawback to surgery, discouraging patients to undergo surgical treatment even when it is appropriate. It is true that surgery should be avoided when there are other less invasive alternatives.

Surgical robots such as the da Vinci system and the Zeus system are highly advanced medical instruments allowing for fine movements when appropriately manipulated by surgical experts. These systems are expected to solve some surgical problems such as invasiveness and the difficulty (4–8). Thus far, the systems have been able to solve some of the problems associated with surgery.

As for the invasiveness of surgery, endoscopic surgeries such as laparoscopy can be performed with robotic systems, utilizing smaller incisions than those of other standard open surgical approaches. The precise movements of surgeons are facilitated by robotic systems. However, laparoscopic procedures can be performed even without the robotic systems with the same amount of invasiveness.

Current robotic systems may also pose problems (4–8), such as the limited number of surgeons who can manipulate the system, which is usually one. Additional training for the specific manipulating methods of the systems is another problem, as well as introduction costs. Consequently, it is currently not clear what the benefits of these robotic systems are, especially when assessed from the patient side. Moreover, problems which even surgical experts suffer from have not been solved.

Flexible endoscopes have been developed to cope with the problems of accessing regions through narrow tracts such as the esophagus and the tracheobronchial tree. Even in these regions flexible endoscopes can perform surgical procedures similar to standard surgery. Therefore, endoscopes are naturally considered functional even in other cavities such as the abdomen and pelvic cavities.

It would be easier and more functional to perform an operation using several endoscopic instruments introduced through the end of one endoscope, rather than conducting resection using only one endoscopic instrument introduced into one endoscope, as done in standard endoscopic procedures. The simplest model for this concept is the flexible endoscopic surgical system we developed and examined within these trials.

We assumed that there would be several problems with the flexible endoscopic surgical system when used clinically as it is merely a conceptual model to confirm its feasibility of use. However, despite those problems, the system was able to perform surgical resection. In addition, the problems encountered within the first experiment were inherent in all technical procedures.

Of interest, these problems showed us that, when indicated for resecting procedures, the flexible endoscopic surgical system is easier to manipulate by surgeons and not by endoscopists despite its endoscopic appearances.

The images of the inner endoscopes were not satisfactory because a CCD was not used in these endoscopes. Consequently, resecting procedures were monitored by images from the outer endoscope which contained the CCD. In this situation, the operator had to control the inner endoscope via observations on the monitor of the outer endoscope. This is different from standard endoscopic procedures in which images are observed on the monitor of the endoscope which the operator is controlling.

In general, it is not easy for trainees to understand appropriate surgical procedures, i.e. where to cut and where to stabilize. Verbal communication during operation is important to facilitate appropriate assistance, which was not adequately utilized in the first series. These issues are to be learned through years of experience and cannot be achieved instantly.

As mentioned above, the difference between the two experiments may reveal that for these flexible endoscopes, surgical experience is an important factor, when the system is indicated for surgical procedures. The limitation of the inner endoscopes, not having CCD may have emphasized this issue. Consequently, the next system is to consist of two inner endoscopes with a CCD for each. This would allow the operators to control the inner endoscopes in such a manner as used for standard gastrointestinal endoscopic procedures.

Furthermore, we think that there should be two styles of design for future flexible endoscopic surgical systems; one with increased surgical maneuverability designed particularly for the techniques of surgeons, the other preserving flexible endoscopic maneuverability for endoscopists. Although it has not been decided yet which design is more appropriate for a future surgical system, endoscopists may be able to become accustomed to the flexible endoscopic surgical system with surgical maneuverability when the system is popularized.

In addition to the merits mentioned above, flexible endoscopic materials can theoretically be made compatible with X-ray systems such as fluoroscopes and computed tomography (CT) systems, exemplified by such procedures as X-ray guided bronchoscopy. In the future, the materials used for flexible endoscopic constructions are expected to acquire compatibility with the magnetic fields of magnetic resonance imaging (MRI) systems.

As mentioned before, limitations in visualization pose surgical problems even for experienced surgeons. This may only partially be solved by the subjective ability of surgeons to presume the identity of invisible objects using their tactile sense and their intuition. Actually, the compatibility with imaging systems was one of the important requirements for the design of the flexible endoscopic surgical system,

allowing visibility of anatomical information invisible to the surgeon's eyes.

In order to make one more step towards the future for less invasive and more effective medical treatments, we believe that future surgical systems should acquire the accessibility to narrow regions located deep inside the body together with the compatibility of imaging systems such as CT and MRI. Thus, from the flexible nature and structural characteristics of a non-jointed, smooth outer sheath, we selected the flexible endoscope as the conceptual basis of development for our system. It is the combination of these and the aforementioned aspects that allows for minimization in invasiveness, through the use of pre-existing natural structures and tracts for lesion access to deep regions, and with the presence of multiple interchangeable inner-scopes, an increase in distal tip functionality at the surgical site. Although there are several factors still to discuss and develop, the concept of the flexible endoscopic surgical system is considered an appropriate development for a future surgical robotic system with this current system being a successful step towards that furture.

## Acknowledgments

This study was supported by a Grant-in-Aid for Research on Medical Devices for Analyzing, Supporting and Substituting the Function of Human Body from the Ministry of Health, Labour and Welfare.

## References

1. Kobayashi T, Gotohda T, Tamakawa K, Ueda H, Kakizoe T. Magnetic anchor for more effective endoscopic mucosal resection. *Jpn J clin Oncol* 2004;34:118–23.
2. Gotoda T, Yanagisawa A, Sasako M, Ono H, Nakanishi Y, Shimoda T, et al. Incidence of lymph node metastasis from early gastric cancer: estimation with a large number of cases at two large centers. *Gastric Cancer* 2000;3:219–25.
3. Gotoda T, Kondo H, Ono H, Saito Y, Yamaguchi H, Saito D, et al. A new endoscopic mucosal resection procedure using an insulation-tipped electrosurgical knife for rectal flat lesions: report of two cases. *Gastrointest Endosc* 1999;50:560–3.
4. D'Anniballe A, Fiscon V, Trevisan P, Pozzobon M, Gianfreda V, Sovernigo G, et al. The da Vinci robot in right adrenalectomy: considerations on technique. *Surg Laparosc Endosc Percutan Tech* 2004;14:38–41.
5. Schiff J, Li PS, Goldstein M. Robotic microsurgical vasovasostomy and vasoepididymostomy: a prospective randomized study in a rat model. *J Urol* 2004;171:1720–5.
6. Ruurda JP, Broeders IA, Simmermacher RP, Borel Rinkes IH, Van Vroonhoven TJ, Theo JM. Feasibility of robot-assisted laparoscopic surgery: an evaluation of 35 robot-assisted laparoscopic cholesystectomies. *Surg Laparosc Endosc Percutan Tech* 2002;12:41–5.
7. Falk V, Diegler A, Walther T, Autschbach R, Mohr FW. Developments in robotic cardiac surgery. *Curr Opin Cardiol* 2000;15:378–87.
8. Hollands CM, Dixey LN. Applications of robotic surgery in pediatric patients. *Surg Laparosc Endosc Percutan Tech* 2002;12:71–6.

0022-5347/05/1731-0243/0
THE JOURNAL OF UROLOGY®

Vol. 173, 243–246, January 2005
*Printed in U.S.A.*
**DOI: 10.1097/01.ju.0000141577.98902.49**

# 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).

Submitted for publication May 20, 2004.
Supported by a Grant-in-Aid for the Second Term Comprehensive 10-Year Strategy for Cancer Control and a Grant-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan, and a Research Resident Fellowship from the Foundation for Promotion of Cancer Research in Japan (TN).
* 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 $\mu$g) was digested for 24 hours with 10 U *Msp* I or *Hpa* II/$\mu$g 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.

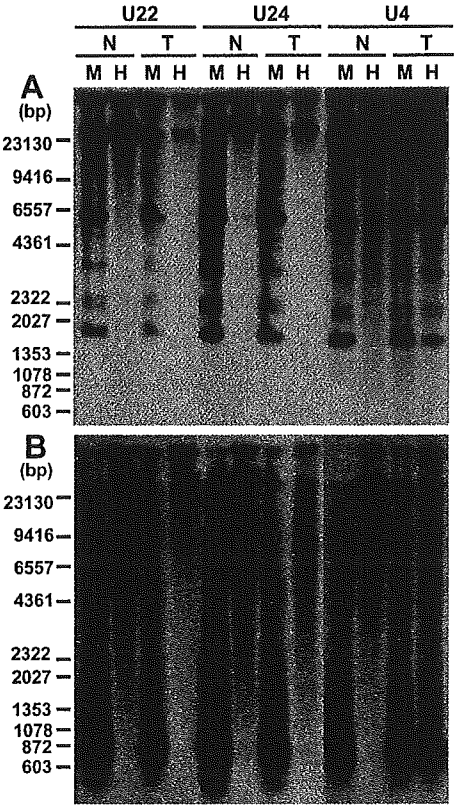


FIG. 1. Examples of Southern blotting for satellites 2 (*A*) and 3 (*B*) in cases of urothelial carcinoma. Methylation status was assessed by digesting DNA with *Msp* I (*Lane M*) and *Hpa* II (*Lane H*). DNA fragments were separated by electrophoresis, transferred to nitrocellulose membranes and hybridized 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 |

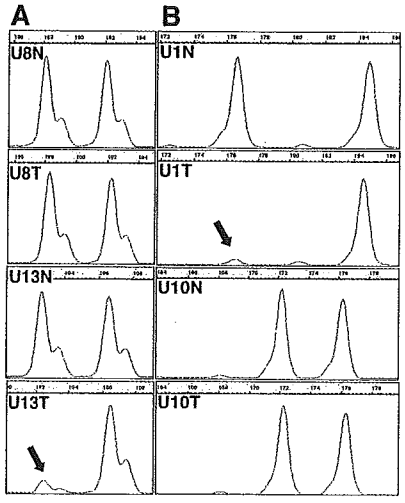


FIG. 2. Examples of results of allelic status analyses in cases of urothelial carcinoma. U8 and U13 DNA samples were amplified for D9S747 (*A*), while U1 and U10 samples were amplified for D9S775 (*B*). Genotypes derived from noncancerous U8N, U13N, U1N and U10N tissues, and corresponding U8T, U13T,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

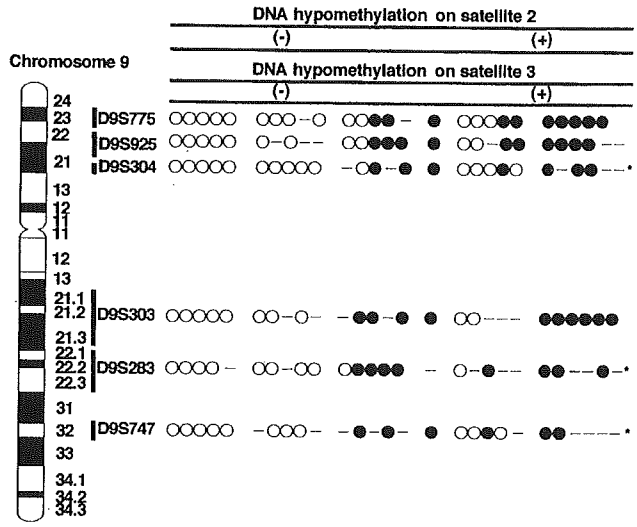


FIG. 3. Allelic status of each locus in urothelial carcinomas. Vertical lines indicate each carcinoma. Open circles indicate retention of 2 alleles. Filled circle indicate LOH. Bar indicates uninformative case. Asterisk indicates replication error. −, negative. +, positive.

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 (60) |
| 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.

REFERENCES


1. Jones, P. A. and Baylin, S. B.: The fundamental role of epigenetic events in cancer. Nat Rev Genet, **3:** 415, 2002
2. Xu, G. L., Bestor, T. H., Bourc'his, D., Hsieh, C. L., Tommerup, N., Bugge, M. et al: Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature, **402:** 187, 1999
3. Kokalj-Vokac, N., Almeida, A., Viegas-Pequignot, E., Jeanpierre, M., Malfoy, B. and Dutrillaux, B.: Specific induction of uncoiling and recombination by azacytidine in classical satellite-containing constitutive heterochromatin. Cytogenet Cell Genet, **63:** 11, 1993
4. Suzuki, T., Fujii, M. and Ayusawa, D.: Demethylation of classical satellite 2 and 3 DNA with chromosomal instability in senescent human fibroblasts. Exp Gerontol, **37:** 1005, 2002
5. Chen, R. Z., Pettersson, U., Beard, C., Jackson-Grusby, L. and Jaenisch, R.: DNA hypomethylation leads to elevated mutation rates. Nature, **395:** 89, 1998
6. Gaudet, F., Hodgson, J. G., Eden, A., Jackson-Grusby, L., Dausman, J., Gray, J. W. et al: Induction of tumors in mice by genomic hypomethylation. Science, **300:** 489, 2003
7. Eden, A., Gaudet, F., Waghmare, A. and Jaenisch, R.: Chromosomal instability and tumors promoted by DNA hypomethylation. Science, **300:** 455, 2003
8. Wong, N., Lam, W. C., Lai, P. B., Pang, E., Lau, W. Y. and Johnson, P. J.: Hypomethylation of chromosome 1 heterochromatin DNA correlates with q-arm copy gain in human hepatocellular carcinoma. Am J Pathol, **159:** 465, 2001
9. Maruyama, R., Toyooka, S., Toyooka, K. O., Harada, K., Virmani, A. K., Zochbauer-Muller, S. et al: Aberrant promoter methylation profile of bladder cancer and its relationship to clinicopathological features. Cancer Res, **61:** 8659, 2001
10. Bornman, D. M., Mathew, S., Alsruhe, J., Herman, J. G. and Gabrielson, E.: Methylation of the E-cadherin gene in bladder neoplasia and in normal urothelial epithelium from elderly individuals. Am J Pathol, **159:** 831, 2001
11. Knowles, M. A.: The genetics of transitional cell carcinoma: progress and potential clinical application. BJU Int, **84:** 412, 1999
12. Sobin, L. H. and Wittekind, Ch.: TNM Classification of Malignant Tumors, 5th ed. New York: John Wiley & Sons, Inc., 1997
13. Friedell, G. H., Parija, G. C., Nagy, G. K. and Soto, E. A.: The pathology of human bladder cancer. Cancer, **45:** 1823, 1980
14. Tagarro, I., Fernandez-Peralta, A. M. and Gonzalez-Aguilera, J. J.: Chromosomal localization of human satellites 2 and 3 by a FISH method using oligonucleotides as probes. Hum Genet, **93:** 383, 1994
15. Hartmann, A., Rosner, U., Schlake, G., Dietmaier, W., Zaak, D., Hofstaedter, F. et al: Clonality and genetic divergence in multifocal low-grade superficial urothelial carcinoma as determined by chromosome 9 and p53 deletion analysis. Lab Invest, **80:** 709, 2000
16. Hartmann, A., Schlake, G., Zaak, D., Hungerhuber, E., Hofstetter, A., Hofstaedter, F. et al: Occurrence of chromosome 9 and p53 alterations in multifocal dysplasia and carcinoma in situ of human urinary bladder. Cancer Res, **62:** 809, 2002
17. Obermann, E. C., Junker, K., Stoehr, R., Dietmaier, W., Zaak, D., Schubert, J. et al: Frequent genetic alterations in flat urothelial hyperplasias and concomitant papillary bladder cancer as detected by CGH, LOH, and FISH analyses. J Pathol, **199:** 50, 2003
18. Saito, Y., Kanai, Y., Sakamoto, M., Saito, H., Ishii, H. and Hirohashi, S.: Expression of mRNA for DNA methyltransferases and methyl-CpG-binding proteins and DNA methylation status on CpG islands and pericentromeric satellite regions during human hepatocarcinogenesis. Hepatology, **33:** 561, 2001
19. Kanai, Y., Ushijima, S., Kondo, Y., Nakanishi, Y. and Hirohashi, S.: DNA methyltransferase expression and DNA methylation of CPG islands and peri-centromeric satellite regions in human colorectal and stomach cancers. Int J Cancer, **91:** 205, 2001
20. Okano, M., Bell, D. W., Haber, D. A. and Li, E.: DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell, **99:** 247, 1999
21. Saito, Y., Kanai, Y., Sakamoto, M., Saito, H., Ishii, H. and Hirohashi, S.: Overexpression of a splice variant of DNA methyltransferase 3b, DNMT3b4, associated with DNA hypomethylation on pericentromeric satellite regions during human hepatocarcinogenesis. Proc Natl Acad Sci USA, **99:** 10060, 2002

The Prostate 63:56 – 64 (2005)

# Effectiveness of Adjuvant Intermittent Endocrine Therapy Following Neoadjuvant Endocrine Therapy and External Beam Radiation Therapy 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.

All authors are members of The National Research Project on Endocrine-Radiation Combination Therapy for Locally Advanced Prostate Cancer.

Grant sponsor: The Ministry of Health, Labor and Welfare in Japan (to Hidetoshi Yamanaka); Grant number: 12–14.

*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
Received 6 June 2004; Accepted 9 August 2004
DOI 10.1002/pros.20171
Published online 5 October 2004 in Wiley InterScience (www.interscience.wiley.com).

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.

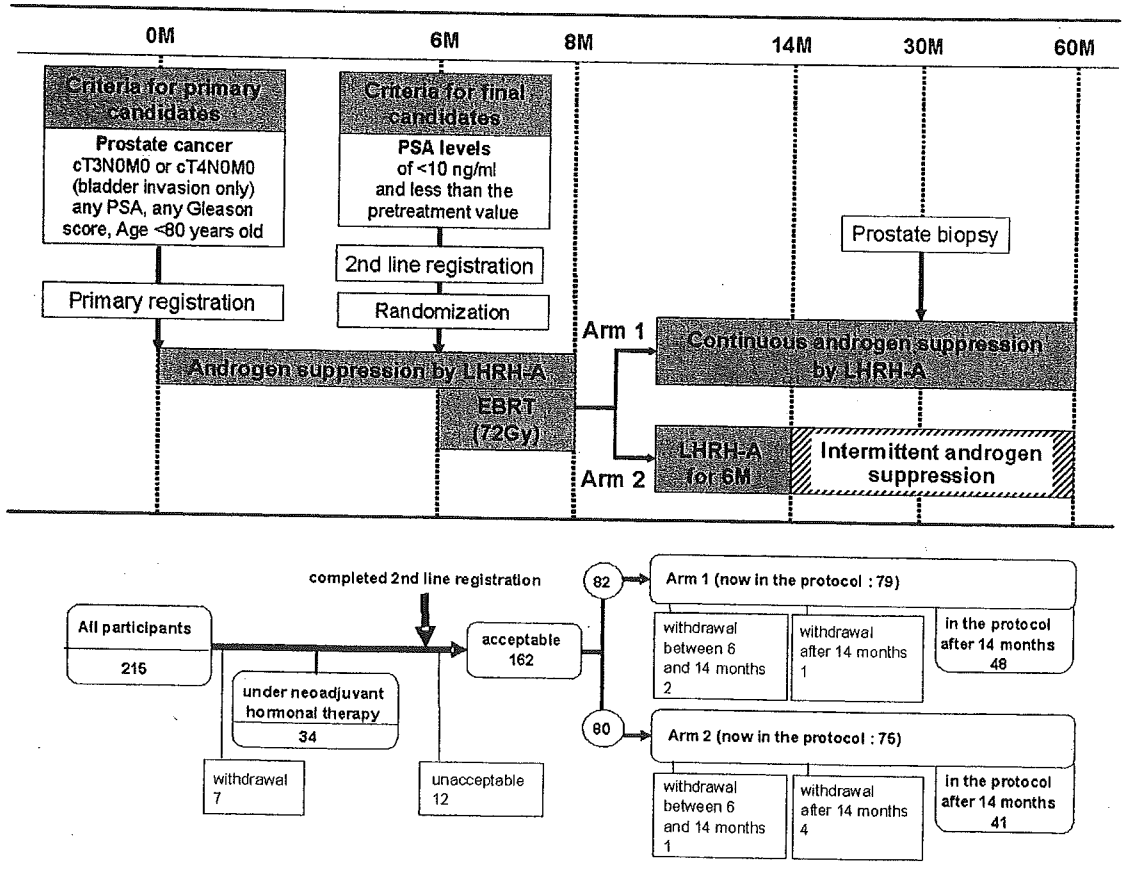


**Fig. 1.** Scheme of the study protocol, the number of patients registered and the present status of those patients in this study protocol. LHRH-A, LHRH agonist; EBRT, external beam radiation therapy.

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