

Table 2 IC₅₀ values (μM) of CDDP and NC-6004 in various cancer cell lines

Cancer	Cell line	Exposure time (h)			
		48		72	
		CDDP	NC-6004	CDDP	NC-6004
Bladder cancer	EJ-1	2.46	25.45	1.86	18.44
	J82	2.78	42.89	2.42	20.27
	MBT-2	15.88	> 100	5.67	71.67
Colon cancer	Colo201	34.77	> 100	28.52	> 100
	Colo320	16.32	> 100	9.71	81.15
	HT-29	14.44	> 100	8.83	> 100
Lung cancer	A549	21.43	> 100	20	> 100
	EBC-1	> 100	> 100	9.36	84.78
	PC-14	16.81	> 100	8.73	87.11
Gastric cancer	MKN-28	> 100	> 100	8.23	76.81
	MKN-45	7.12	68.36	6.94	43.81
Breast cancer	MCF-7	12.78	> 100	5.71	54.71

the CDDP 5 mg kg⁻¹ administration group showed a significant decrease ($P < 0.01$) in tumour growth rate as compared with the control group. In the administration of NC-6004, NC-6004 2.5 mg kg⁻¹ administration group ($P < 0.05$) and 5 mg kg⁻¹ administration group ($P < 0.01$) showed significant decreases in tumour growth rate as compared with the control group. However, the NC-6004 administration groups at the same dose levels as CDDP showed no significant difference in tumour growth rate. The same animal model was used to repeat the study using the drugs at different dose levels, and similar tendencies were observed (data not shown). Regarding time-course changes in body weight change rate, the CDDP 5 mg kg⁻¹ administration group showed a significant decrease ($P < 0.001$) in body weight as compared with the control group. On the other hand, none of the NC-6004 administration groups showed a decrease in body weight as compared with the control group (Figure 3B).

Nephrotoxicity and hepatotoxicity of CDDP and NC-6004

In the CDDP 10 mg kg⁻¹ administration group, four of 12 animals died from toxicity within 7 days after drug administration. No deaths occurred in the NC-6004 10 mg kg⁻¹ administration group and the NC-6004 15 mg kg⁻¹ administration group. Regarding renal function, the BUN concentrations on day 7 after the administration of 5% glucose, CDDP 10 mg kg⁻¹, NC-6004 10 mg kg⁻¹, and NC-6004 15 mg kg⁻¹ were 20.8 ± 3.0, 65.3 ± 44.4, 20 ± 4.5, and 24.6 ± 18.2 mg dl⁻¹, respectively. The plasma concentrations of creatinine on day 7 after the administration of 5% glucose, CDDP 10 mg kg⁻¹, NC-6004 10 mg kg⁻¹, and NC-6004 15 mg kg⁻¹ were 0.27 ± 0.03, 0.68 ± 0.23, 0.28 ± 0.04, and 0.45 ± 0.11 mg dl⁻¹, respectively. The CDDP 10 mg kg⁻¹ administration group showed significantly higher plasma concentrations of BUN and creatinine as compared with the control group ($P < 0.05$ and 0.001, respectively), with the NC-6004 10 mg kg⁻¹ administration group ($P < 0.05$ and 0.001, respectively), and also with the NC-6004 15 mg kg⁻¹ administration group ($P < 0.05$ and 0.05, respectively) (Figure 4A and B). Light microscopy indicated tubular dilation with flattening of the lining cells of the tubular epithelium in the kidney from all animals in the CDDP 10 mg kg⁻¹ administration group. On the other hand, no histopathological change was observed in the kidneys from all animals in the NC-6004 10 mg kg⁻¹ administration group (Figure 4C and D). Regarding hepatic function, the plasma concentrations of GOT on day 7 after the administration of 5% glucose, CDDP 10 mg kg⁻¹, NC-6004 10 mg kg⁻¹, and NC-6004 15 mg kg⁻¹ were 68 ± 6.8, 65.1 ± 5.5, 106 ± 13.1, and 97 ± 16.2 IU l⁻¹, respectively. The plasma

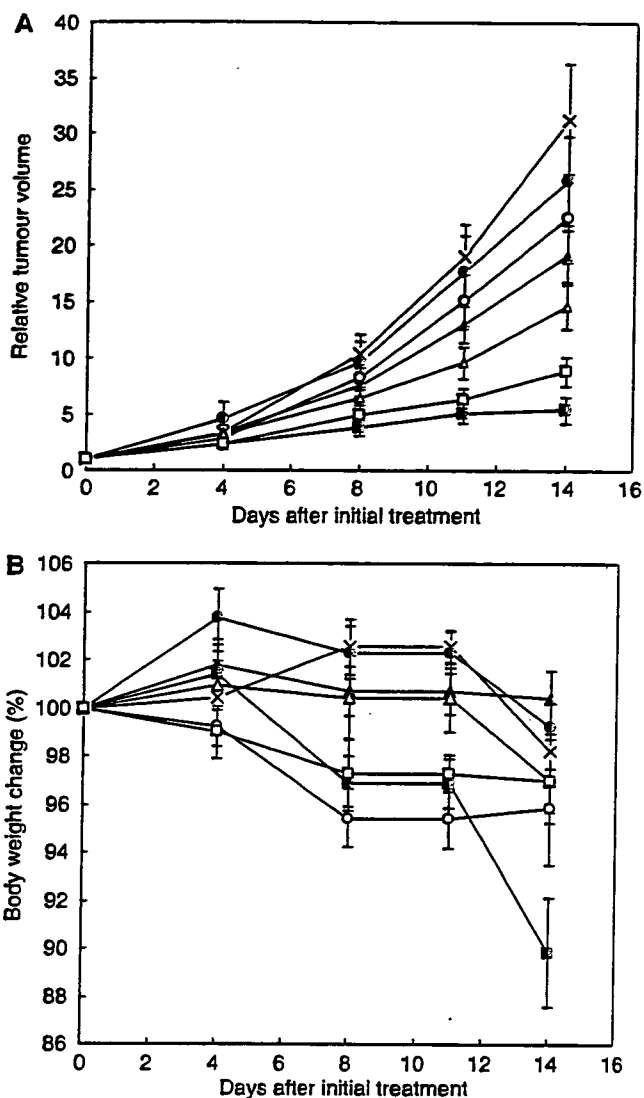


Figure 3 Relative changes in MKN-45 tumour growth rates in nude mice. (A) Cisplatin and NC-6004 were injected i.v. every 3 days, three administrations in total, at CDDP-equivalent doses of 0.5 mg kg⁻¹ (●, ○), 2.5 mg kg⁻¹ (▲, △), and 5 mg kg⁻¹ (■, □), respectively. Glucose (5%) was injected in the control mice (x). (B) Changes in relative body weight. Data were derived from the same mice as those used in the present study. Values are expressed as the mean ± s.e.

concentrations of GPT on day 7 after the administration of 5% glucose, CDDP 10 mg kg⁻¹, NC-6004 10 mg kg⁻¹, and NC-6004 15 mg kg⁻¹ were 39.6 ± 10, 32 ± 6.4, 92 ± 18.9, and 55 ± 11.3 IU l⁻¹, respectively. The CDDP 10 mg kg⁻¹ administration group showed plasma concentrations of GOT and GPT which were comparable to those in the control group. However, the NC-6004 10 mg kg⁻¹ administration group, which presented the same dose level as the CDDP 10 mg kg⁻¹ administration group, showed significantly higher plasma concentrations of GOT and GPT ($P < 0.001$ and 0.01, respectively) as compared with the control group. Furthermore, the NC-6004 15 mg kg⁻¹ administration group also showed significantly higher plasma concentrations of GOT ($P < 0.001$) as compared with the control group. However, the plasma concentrations of GOT and GPT on day 14 after the administration of NC-6004 10 mg kg⁻¹ were comparable to those in the control group (74 ± 2.3 and 42.8 ± 5.1 IU l⁻¹, respectively) (Figure 4E). These results lead to the conjecture that rats which were given NC-6004 10 mg kg⁻¹, i.v., showed transient and reversible hepatotoxicity.

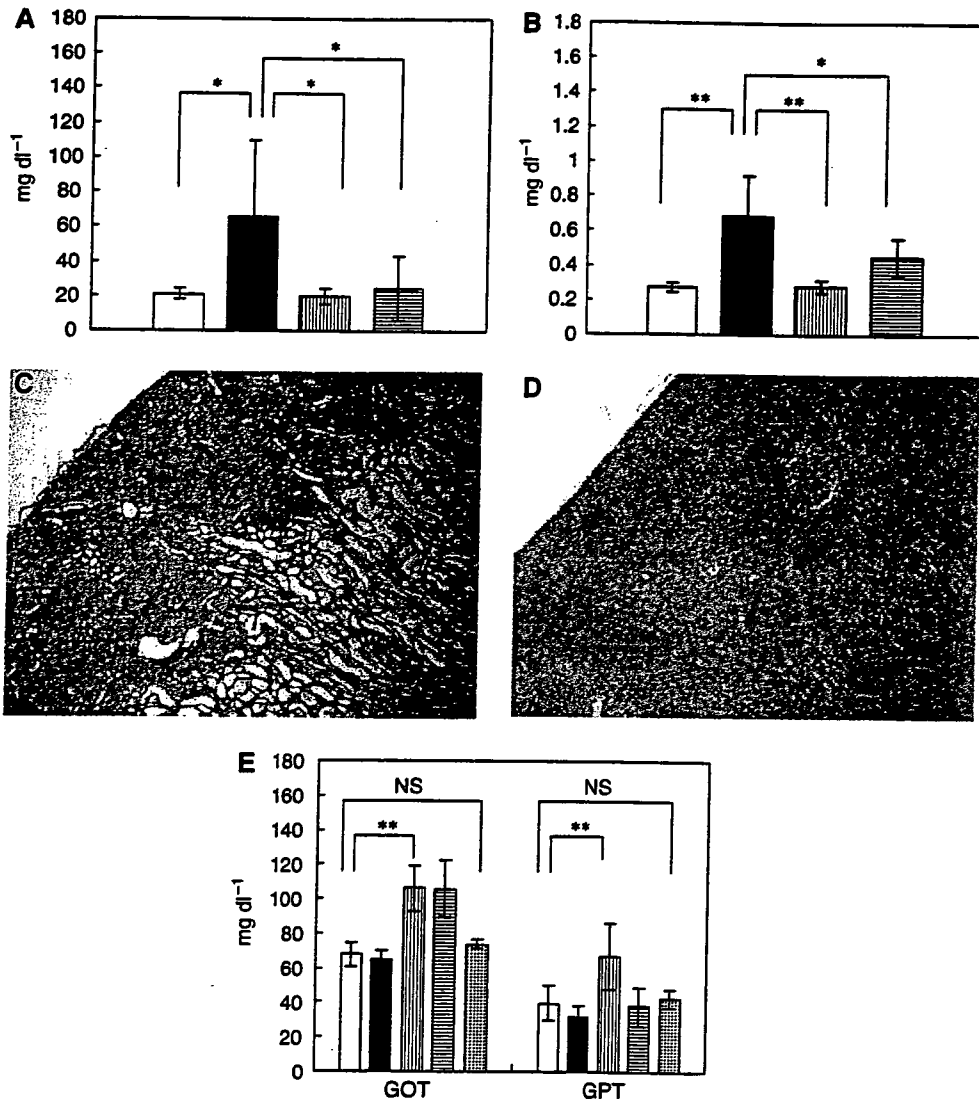


Figure 4 Nephrotoxicity and hepatotoxicity of CDDP and NC-6004. Plasma concentrations of BUN (A) and creatinine (B) were measured after a single i.v. injection of 5% glucose (□) (n = 8), CDDP at a dose of 10 mg kg⁻¹ (■) (n = 12), NC-6004 at a dose of 10 mg kg⁻¹ (n = 13) on a CDDP basis (▨), and at a dose of 15 mg kg⁻¹ on a CDDP basis (▩) (n = 8) to rats. Histopathological changes in the kidney on day 7 after the i.v. injection of CDDP (C, × 4) and NC-6004 (D, × 4) in rats at an equivalent dose of 10 mg kg⁻¹ CDDP. In rats given CDDP, widespread tubular degeneration as indicated by tubular dilation with flattening of the lining cells of tubular epithelium was seen. On the other hand, no histopathological change was observed in the kidney from all animals in the NC-6004 10 mg kg⁻¹ administration group. For hepatotoxicity (E), the plasma concentrations of GOT and GPT were measured on day 7 after administration. When administering NC-6004 at a dose of 10 mg kg⁻¹ on a CDDP basis, five of 13 blood samples were taken on day 14 after administration (▩). The other samples were taken on day 7 administration. In the group given CDDP at a dose of 10 mg kg⁻¹, four of 12 rats died within 7 days. Values are expressed as the mean ± s.d. *P < 0.05, **P < 0.001, NS: not significant.

Neurotoxicity of CDDP and NC-6004

Neurophysiological examination revealed that MNCVs in animals given 5% glucose, CDDP, and NC-6004 were 44.2 ± 3.5, 40.94 ± 5.08, and 40.62 ± 0.63 m s⁻¹, respectively. No significant difference was found among the groups with respect to MNCV. Furthermore, SNCVs in animals given 5% glucose, CDDP, and NC-6004 were 42.86 ± 8.07, 35.48 ± 4.91, and 43.74 ± 5.3 m s⁻¹, respectively. Animals given NC-6004 showed no delay in SNCV as compared with animals given 5% glucose. On the other hand, animals given CDDP showed a significant delay (P < 0.05) in SNCV as compared with animals given NC-6004 (Figure 5A). In addition, histopathological examination with electron microscopy revealed degenerations, as manifested by electron photomicrographs indicating degenerative changes, for example, loss of microtubules,

degeneration in the cytoplasm of Schwann cells, loss of filaments, and an irregular inner loop, in approximately 80% of myelinated segments of the sciatic nerve from animals given CDDP. On the other hand, animals given NC-6004 exhibited nearly normal electron photomicrographs of the sciatic nerve as the control animals did (Figure 5B and C). These results indicate that NC-6004 reduced peripheral neurotoxicity as compared with CDDP. Furthermore, regarding body weight change as an indication of general toxicity, furthermore, the NC-6004 administration groups showed significant inhibition of body weight decrease (P < 0.001) as compared with the CDDP administration group (P < 0.001) (Figure 5D).

The analysis by ICP-MS on sciatic nerve concentrations of Pt could not detect Pt in the sciatic nerve from animals given 5% glucose (data not shown). Sciatic nerve concentrations of Pt in

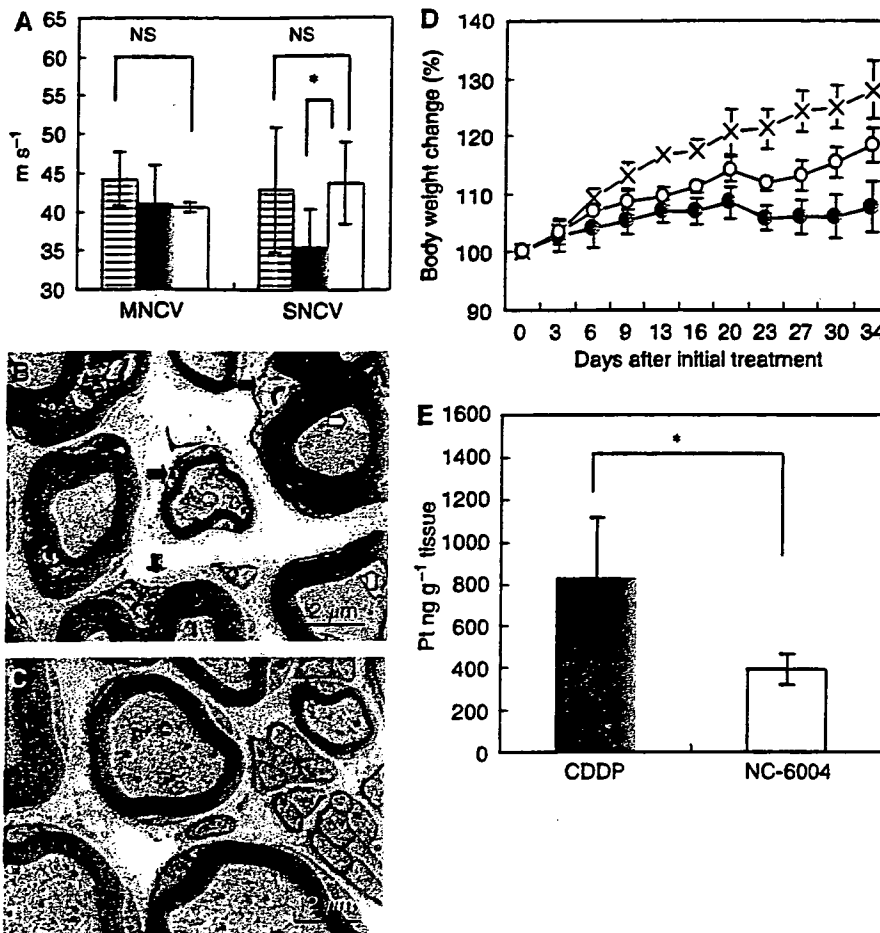


Figure 5 Neurotoxicity of CDDP and NC-6004 in rats. Rats ($n = 5$) were given CDDP (2 mg kg^{-1}), NC-6004 (an equivalent dose of 2 mg kg^{-1} CDDP), or 5% glucose, all i.v. twice a week, 11 administrations in total. (A) Sensory nerve conduction velocity and MNCV of the sciatic nerve at week 6 after the initial administration (control (□), CDDP (●), and NC-6004 (○)). Histopathological changes of the sciatic nerve were examined by electron microscopy after the administration of CDDP (B) and NC-6004 (C). In rats given CDDP, widespread degenerations as indicated by loss of microtubules, loss of filaments, degeneration in the cytoplasm of Schwann cells (■), and an irregular inner loop (⊃) were seen. On the other hand, animals given NC-6004 exhibited nearly normal electron micrographs of the sciatic nerve as the control animals. (D) Changes in relative body weight. Data were derived from the same rats as those used in the present study (control (x), CDDP (●), and NC-6004 (○)). (E) The Pt concentration in the sciatic nerve. Rats were given CDDP (■) (5 mg kg^{-1} , $n = 5$), NC-6004 (□) (an equivalent dose of 5 mg kg^{-1} CDDP, $n = 5$), or 5% glucose ($n = 2$), all i.v. twice a week, four administrations in total. On day 3 after the final administration, a segment of the sciatic nerve was removed and the Pt concentration in the sciatic nerve was measured by ICP-MS. Body weight changes are expressed as the mean \pm s.e. The other data are expressed as the mean \pm s.d. * $P < 0.05$, ** $P < 0.001$, NS: not significant.

animals given CDDP and NC-6004 were 827.2 ± 291.3 and $395.5 \pm 73.1 \text{ ng g}^{-1}$ tissue. Therefore, the concentrations were significantly ($P < 0.05$) lower in animals given NC-6004 (Figure 5E). This finding is believed to be a factor which reduced neurotoxicity following NC-6004 administration as compared with the CDDP administration.

DISCUSSION

The present study indicated that CDDP-incorporating polymeric micelles (NC-6004) are stable nanoparticles with a long blood retention profile as compared with free CDDP. NC-6004 showed 6- to 15-fold less potent *in vitro* cytotoxic activity in several human cancer cell lines as compared with CDDP. These findings are considered attributable to the slow release of free CDDP in the presence of abundant chloride ions because NC-6004 contains coordination bonds between the atoms of Pt(II) of CDDP and the carboxylic group in the side chain of P(Glu). *In vivo*, however, in contrast to the *in vitro* findings, NC-6004 was found to markedly

reduce nephrotoxicity and neurotoxicity – dose-limiting factors of CDDP, while preserving antitumour activity, which was equivalent to or better than that of free CDDP.

Nephrotoxicity of CDDP is considered to depend on the peak urinary CDDP concentration and on the maximum CDDP concentration in the uriniferous tubules (Levi *et al*, 1982). We consider that the reduced nephrotoxicity of NC-6004 may be explained by the following facts: (1) the tendency of micelles to be less prone to filtration by nephrons because of the NC-6004 particle size (approximately 30 nm), and (2) the much lower C_{max} value for CDDP at least in the uriniferous tubules than the value following CDDP administration. NC-6004 possibly facilitates treatment on an outpatient basis because it allows safer administration to patients with decreased renal function and requires no massive fluid replacement to protect renal tissue after the administration of CDDP.

The main neuropathy of CDDP is sensory peripheral neuropathy (van der Hoop *et al*, 1990; Gregg *et al*, 1992). A delay in SNCV due to the injury of dorsal root ganglia and peripheral nerve has previously been reported in rats given CDDP, although MNCV was

preserved in the tail and hind paws of rats (McKeage *et al*, 1994; Tredici *et al*, 1998; Meijer *et al*, 1999; Tredici *et al*, 1999). Furthermore, histopathological examination revealed degenerative changes in the sciatic nerve in similar experimental animals (Cavaletti *et al*, 1992; Tredici *et al*, 1999). In the present study, animals given NC-6004 showed no delay in the SNCV, while animals given CDDP showed a significant delay in the SNCV as compared with animals given NC-6004. Neuropathologically, neuronal degeneration, which was observed following CDDP administration, was not observed with NC-6004 administration. This result is considered attributable principally to the fact that the peripheral nerve concentration of Pt decreased to half or less following NC-6004 administration than with CDDP administration. The nervous tissue concentration of Pt at the time of NC-6004 administration decreased significantly despite the fact that the plasma AUC at the time of NC-6004 administration was high, being 65-fold higher than the plasma AUC concentration with CDDP administration. We consider that this result is attributed to the marked inhibition of Pt distribution into nervous tissue in the NC-6004 administration groups as manifested by V_{ss} of 3.00 ± 0.61 and 0.04 ± 0.0023 l kg⁻¹ in the CDDP and NC-6004 groups, respectively. In any event, we believe that the neurotoxicity of CDDP reduced by NC-6004 allows its long-term administration.

On the other hand, transient hepatic dysfunction was observed in rats. This observation indicates the proneness of Pt to accumulate in the RES of the liver because NC-6004 is, after all, said and done, a macromolecule, although preserving a stealth effect through its outer shell of PEG. We consider that caution should be exercised against hepatic dysfunction in conducting a clinical trial of NC-6004 in the future. However, the accumulation of Pt was lower following NC-6004 administration due to a decrease in V_{ss} in other organs including nerve. As shown by changes in body weight in multiple dose studies in rats, the NC-6004 administration groups have been demonstrated to show a

smaller decrease in body weight as compared with the CDDP administration groups. In single-dose studies, furthermore, one dose of CDDP 10 mg kg⁻¹ was equivalent to the 50% of the lethal dose. In fact, four of 12 animals died within 7 days after administration. However, none of the eight animals in the NC-6004 group died after the administration of NC-6004 at a CDDP equivalent dose of 15 mg kg⁻¹. In terms of haematological toxicity, there was no significant difference between the CDDP and NC-6004 groups in rats (data not shown).

In murine tumour strains, CDDP-incorporating polymeric micelles showed significantly high antitumour activity (Nishiyama *et al*, 2003). In the human gastric cancer strain used in the present study, however, no significant difference was found between the NC-6004 and CDDP administration groups. A significant difference was found in antitumour activity between the NC-6004 low-dose group (2.5 mg kg⁻¹ administration group) and the control group, while no significant difference was found between the CDDP low-dose group (2.5 mg kg⁻¹ administration group) and the control group. Results available to date and the results from the present study lead to the consideration that the incorporation of CDDP into polymeric micelles does not reduce its antitumour activity.

Data from the present study warrant the clinical evaluation of NC-6004. We consider that the protocol for the Phase I clinical trial of NC-6004 should employ a regimen without massive i.v. drip infusion.

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TRANSRECTAL HIGH-INTENSITY FOCUSED ULTRASOUND IN THE TREATMENT OF LOCALIZED PROSTATE CANCER : A MULTICENTER STUDY

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We report a multicenter trial with transrectal high-intensity focused ultrasound (HIFU) in the treatment of localized prostate cancer. A total of 72 consecutive patients with stage T1c-2N0M0 prostate cancer were treated using the Sonablate 500™ HIFU device (Focus Surgery, Indianapolis, USA). Biochemical recurrence was defined according to the criteria recommended by the American Society for Therapeutic Radiology and Oncology Consensus Panel. The median age and prostate specific antigen (PSA) level were 72 years and 8.10 ng/ml, respectively. The median follow-up period for all patients was 14.0 months. Biochemical disease-free survival rates in all patients at 1 and 2 years were 78% and 76%, respectively. Biochemical disease-free survival rates in patients with stage T1c, T2a and T2b groups at 2 years were 89, 67% and 40% ($p=0.0817$). Biochemical disease-free survival rates in patients with Gleason scores of 2-4, 5-7 and 8-10 at 2 years were 88, 72% and 80% ($p=0.6539$). Biochemical disease-free survival rates in patients with serum PSA of less than 10 ng/ml and 10-20 ng/ml were 75% and 78% ($p=0.6152$). No viable tumor cells were noted in 68% of patients by postoperative prostate needle biopsy. Prostatic volume was decreased from 24.2 ml to 14.0 ml at 6 months after HIFU ($p<0.01$). No statistically significant differences were noted in International Prostate Symptom Score, maximum urinary flow rate and quality of life analysis with Functional Assessment of Cancer Therapy. HIFU therapy appears to be minimally invasive, efficacious and safe for patients with localized prostate cancer with pretreatment PSA levels less than 20 ng/ml.

(Hinyokika Kijo 51 : 651-658, 2005)

Key words : Prostate cancer, High-intensity focused ultrasound, Minimally invasive surgery

INTRODUCTION

Prostate cancer is the most common malignancy in men and the second leading cause of death due to cancer in the United States¹⁾. Prostate cancer has been treated in various ways, depending on the severity of the

condition, age of the patient, staging, Gleason score and serum prostate-specific antigen (PSA) level. Radical prostatectomy has long been regarded as appropriate therapy for patients with organ-confined prostate cancer. Despite excellent 5- to 10-year survival rates after radical prostatectomy for organ-confined disease, surgery is

associated with significant morbidity, including blood loss due to transfusion-related complications, erectile dysfunction in 30% to 70% of cases, and stress incontinence in up to 10% of patients^{2,3}). In addition, surgical intervention is not typically considered for patients whose life expectancy is less than 10 years. Recently, a number of alternative less invasive treatments have been developed for patients with localized prostate cancer, either not appropriate for surgery or who do not want to risk the potential side effects of surgery. Three-dimensional conformal radiotherapy (3D-CRT), brachytherapy, intensity-modulated external beam radiotherapy, cryosurgical ablation of the prostate and laparoscopic radical prostatectomy have all been applied for the treatment of this group of patients⁴⁻⁶). However, in the event of treatment failure, these cannot be repeated and salvage radical prostatectomy is associated with a high morbidity rate⁷).

High-intensity focused ultrasound (HIFU) delivers intense ultrasound energy with consequent heat destruction of tissue at a specific focal distance from the probe without damage to tissue in the path of the ultrasound beam⁸). HIFU non-invasively induces complete coagulative necrosis of a tumor without surgical exposure or insertion of instruments into the lesion. This advantage makes it one of the most attractive options for the localized treatment of tumors^{9,10}). We report here a multicenter trial with 72 consecutive patients treated with HIFU for clinical stage T1c-2N0M0 localized prostate cancer.

PATIENTS AND METHODS

Inclusion and Exclusion Criteria

As a rule, the inclusion criteria for treatment were patients with biopsy proven and untreated stage T1c-2N0M0 localized prostate cancer¹¹). Age, serum PSA levels, prostatic volume and WHO performance status should be less than 80 yrs, 20 ng/ml, treatable with a 4.0 focal length probe which means a prostatic volume less than 50 ml and 0-1. Patients with urethral stricture, anal stricture, bleeding tendency, renal dysfunction with serum Cr more than 2.0 mg/dl, hydronephrosis, larger than 5 mm calcifications in the prostate, uncontrolled diabetes mellitus, hypertension, angina, history of cardiac infarction or other malignant diseases were excluded from the study. None of the patients were receiving neoadjuvant hormonal and/or chemotherapy before HIFU. All patients were fully informed of the details of this treatment and gave written consent preoperatively.

HIFU Equipment

For this study, we used the Sonablate 500™ (Focus Surgery, Indianapolis, IN, USA) HIFU machine. This treatment module includes the ultrasound power generator, transrectal probes, the probe positioning system, and a continuous cooling system (Fig. 1). The

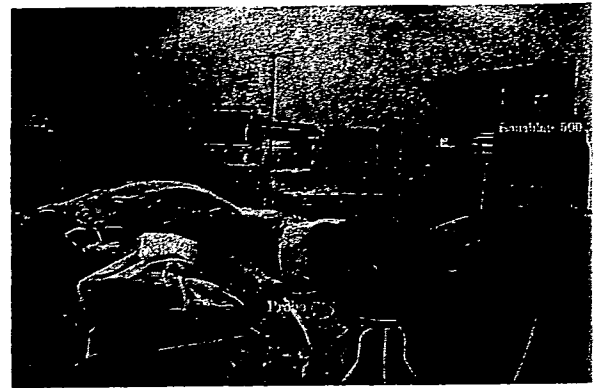


Fig. 1. The Sonablate-500™ type device consists of an operator's console, imaging monitor, transrectal probe and an automatic continuous cooling system.

transrectal HIFU probes use proprietary transducer technology with low-energy ultrasound (4 MHz) for imaging of the prostate and for the delivery of high-energy ablative pulses (site intensity, 1,300–2,200 W/cm²). The single piezoelectric crystal alternates between high-energy power for ablative (3 sec) and low-energy for ultrasound imaging (6 sec)¹⁰).

Prior to beginning the treatment, the operator uses longitudinal and transverse sonograms to obtain an image of the prostate and selects the prostate tissue volume to be ablated by a set of cursors on these images. The probe houses a computer-controlled positioning system that directs each ablative pulse to the targeted region of the prostate. Each discrete high-energy focused ultrasonic pulse ablates a volume of 3 × 3 × 10 mm³ of tissue¹⁰). The total acoustic power is initially set at 24 W and 37 W for 3.0 and 4.0 cm focal length probes, respectively. The individual focal lesion produces almost instantaneous coagulative necrosis of the tissue due to a temperature rise of 80° to 98°C in the focal zone⁸). Under computer control, the ultrasound beam is steered mechanically to produce consecutive lesions in a manner such that all focal lesions overlap

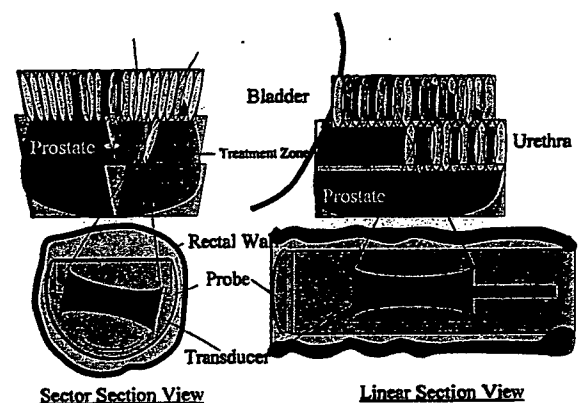


Fig. 2. The computer-controlled transducer ablates the entire prostate tissue. Focal lesions are overlapped in linear rows (left) at each of the lateral sector positions (right) to create a volume lesion.

laterally and longitudinally to ensure necrosis of the entire targeted prostate volume (Fig. 2). An automatic cooling device is used during treatment to maintain a constant baseline temperature of less than 18°C in the transrectal probe that helps to prevent thermal injury of the rectal mucosa.

HIFU Procedure

All patients were anesthetized by general, epidural, spinal or intravenous anesthesia, and were placed in a supine and open leg position. A condom was placed over the probe and degassed water was used to inflate the condom that was covered with ultrasound gel for close coupling of the ultrasound probe to the rectal wall, and the probe was inserted manually into the rectum. The probe was fixed in position by an articulating arm attached to the operating table. After selection of the treatment region of the prostate from the verumontanum to the bladder neck, the treatment was started. Transrectal probes with focal lengths of 3.0 and 4.0 cm were used according to the size of the prostate as determined by transrectal ultrasound (TRUS), with larger glands requiring longer focal lengths. The treatment continued layer by layer (10 mm thickness) from the apex to the base (Fig. 2). Usually, three successive target areas (anterior, mid-part and base) were defined to treat the whole prostate. After treatment was completed, a transurethral balloon catheter was inserted into the bladder¹⁰.

Clinical Follow-up and Definition of Outcome

Patient status and treatment-related complications were followed up by all available means, including periodic patient visits and self-administered questionnaires dealing with urinary continence and erectile function using Functional Assessment of Cancer Therapy (FACT) questionnaire. Urinary symptoms and urinary flow rate analysis were performed using International Prostate Symptom Score (I-PSS) index and uroflowmetry^{12,13}. Serum PSA was assayed every 1 to 6 months during follow-up. A postoperative prostate needle biopsy under TRUS was performed on all patients at 6 months. The American Society for Therapeutic Radiology and Oncology (ASTRO) consensus definition, i.e., three consecutive increases in post treatment PSA after a nadir has been achieved, was used to define biochemical failure¹⁴. The time to biochemical failure was defined as midway between the post treatment PSA nadir and the first of three consecutive PSA increases. None of the patients received androgen deprivation after HIFU or other anticancer therapy before documentation of a biochemical recurrence. HIFU related complications were defined by Japanese version of National Cancer Institute-Common Toxicity Criteria version 2.0¹⁵.

Statistical Analyses

All statistical analyses were performed by the Department Statistics in Indiana University. The chi-square test was used to assess the correlation between

preoperative and postoperative parameters. The distributions of biochemical disease-free survival times were calculated according to the Kaplan-Meier curves and the logrank test was used to compare curves for groups. All *p* values less than 0.05 reflected statistically significant differences.

RESULTS

A total of 75 patients were entered in the trial. The prostate was treated in 1 (75) or 2 (14) HIFU sessions in a total of 89 procedures (1.2 sessions/patient). One patient with stage T1b, 1 patient with a serum PSA of 20.60 ng/ml and 1 patient on whom treatment was stopped during the procedure because of appearance with large microbubbles in the prostate were excluded. The median age, serum PSA level and prostatic volume of the 72 patients analysed were 72 yrs (range 45 to 79), 8.10 ng/ml (range 2.10 to 19.80) and 22.1 ml (range 8.5 to 52.8), respectively. The TNM stage was T1c in 40 patients, T2a in 18 patients and T2b in 14 patients. All patients had a histological diagnosis of prostatic adenocarcinoma according to the Gleason grading system. The Gleason score was 2 to 4 in 9 patients, 5 to 7 in 55 patients, 8 to 10 in 6 patients and unknown in 2 patients (Table 1).

The median time of HIFU treatment and hospitalization was 169 min (range 65 to 485 min) and 5.0 days (range 2 to 55), respectively. The gland size decreased from an initial volume of 24.2 ml to a final median volume of 14.0 ml ($p < 0.01$) in 45 patients. Totally, 49 out of 72 (68%) had negative follow-up biopsies at 6 months after HIFU. Biochemical disease-free survival rates were analyzed in 60 patients. Twelve patients were excluded from the analysis for unsatisfactory followup. The median follow-up period for all patients was 14.0 months (range 2 to 24). Biochemical disease-free survival rates in all patients at 1

Table 1. Characteristics in 72 patients with localized prostate cancer

Median age (range)	72 (45-79)
Median PSA (range)	8.10 ng/ml (2.10-19.80)
Prostate volume (range)	22.1 (8.5-52.8)
Pretreatment PSA (%):	
10 or less	44 (61)
10.1-20	28 (39)
Clinical stage (%):	
T1c	40 (56)
T2a	18 (25)
T2b	14 (19)
Gleason score (%):	
2-4	9 (13)
5-7	55 (76)
8-10	6 (8)
Unknown	2 (3)
Median mos followup (range)	14.0 (2-24)

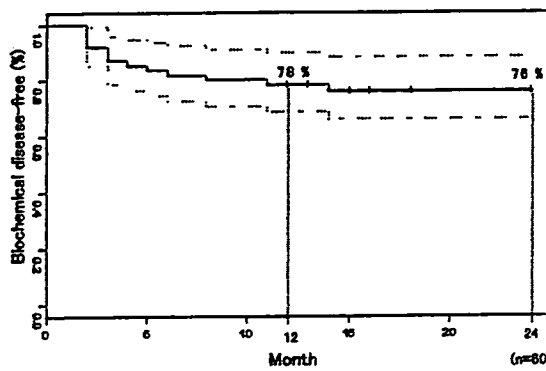


Fig. 3. Kaplan-Meier biochemical disease-free survival curves in all patients.

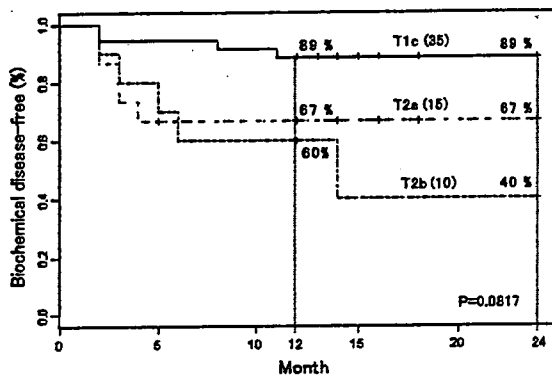


Fig. 4. Kaplan-Meier biochemical disease-free survival curves according to clinical stage.

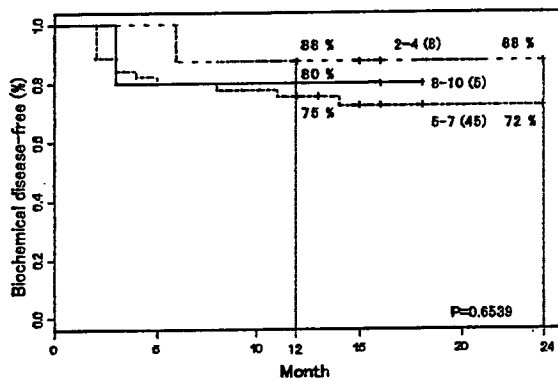


Fig. 5. Kaplan-Meier biochemical disease-free survival curves according to Gleason score.

and 2 years were 78% and 76%, respectively (Fig. 3). Biochemical disease-free survival rates in patients with stage T1c, T2a and T2b groups at 2 years were 89%, 67% and 40% ($p = 0.0817$, Fig. 4). Biochemical disease-free survival rates in patients with Gleason 2-4, 5-7 and 8-10 groups at 2 years were 88%, 72% and 80% ($p = 0.6539$, Fig. 5). The biochemical disease-free survival rate in patients whose serum PSA less than 10 ng/ml and 10-20 ng/ml were 75% and 78% ($p = 0.6152$).

Prostatic volume was decreased from 24.2 ml to 14.0 ml at 6 months after HIFU ($p < 0.01$, Fig. 6). No statistically significant difference was noted in I-PSS, Q-max and FACT quality of life analysis (Fig. 7, 8 and 9).

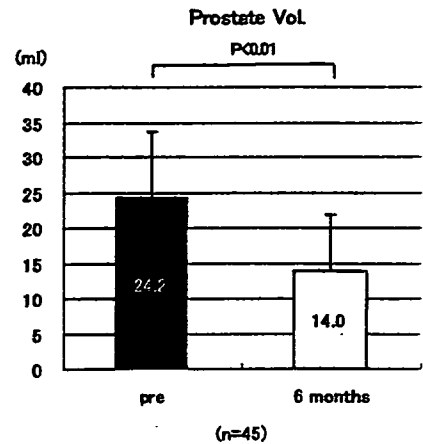


Fig. 6. Changes of prostatic volume.

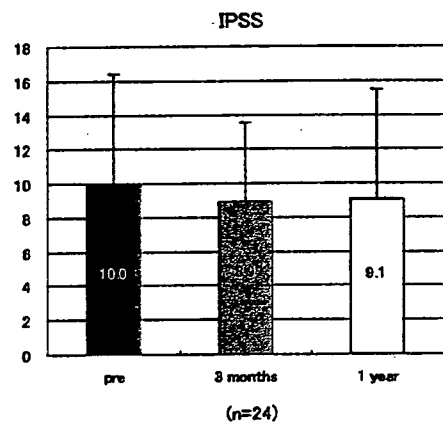


Fig. 7. Changes of International Prostatic Symptom Score.

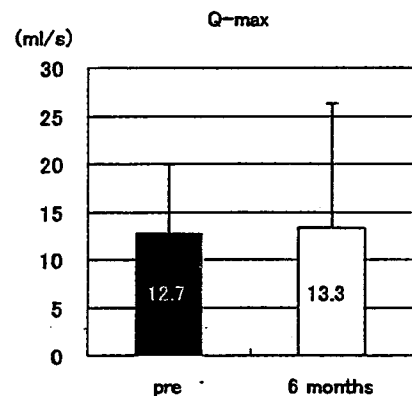


Fig. 8. Changes of maximum flow rate.

Thirteen out of 72 patients developed a urethral stricture, 6 and 4 patients developed epididymitis and prostatitis. Postoperative erectile dysfunction was noted in 12 out of 31 (39%) patients who were potent preoperatively. Nephrotic syndrome, transient urinary incontinence, transit stool incontinence, balanoposthitis or retrograde ejaculation was observed in 1 patient each (Table 2).

For analysis of HIFU treatment using Sonablate 500™, ultrasound imaging for identifying prostate and quality levels were categorized more than good in patients with 92%. A transrectal probe was easily

Table 2. Complications

Complication	Grade 1	Grade 2	Grade 3	Grade 4	Total
Urethral stricture	0	0	13	0	13
Erectile dysfunction (31 potent patients)	0	0	12	0	12
Epididymitis	2	2	2	0	6
Prostatitis	2	0	2	0	4
Nephrotic syndrome	0	0	1	0	1
Balanoposthitis	1	0	0	0	1
Urinary incontinence (grade 1)	1	0	0	0	1
Stooly incontinence	1	0	0	0	1
Retrograde ejaculation	1	0	0	0	1

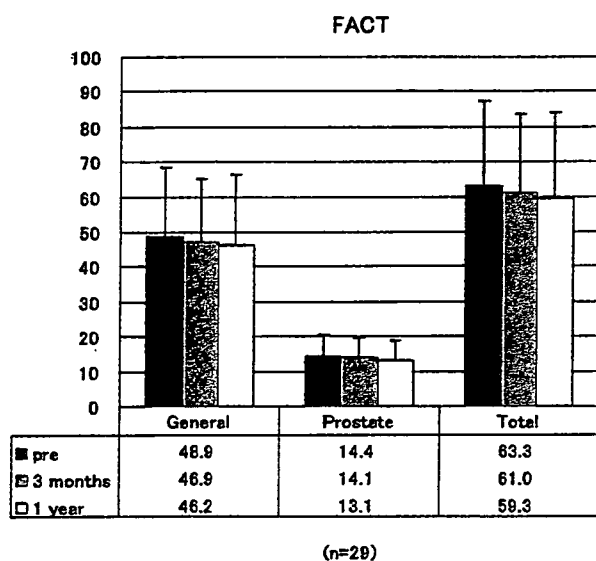


Fig. 9. Quality of life change by FACT general and prostate.

inserted into the rectum in 97% of the patients. Totally, 96% of the HIFU treatment was categorized as an easy procedure.

DISCUSSION

In 1995, Madersbacher et al. reported the effectiveness of HIFU in 10 cases of localized prostate cancer.⁸⁾ Histologically, HIFU-treated lesions of the prostate demonstrated a coagulation necrosis with sharp boundaries. In 1996, Gelet et al. reported preliminary experiences with HIFU using the Ablatherm device (EDAP-Technomed, Lyon, France) for treating localized prostate cancer¹⁶⁾. Beerlage et al. reported the results of HIFU treatments in 111 patients with clinical stage T1-3N0M0 prostate cancer and a PSA level less than 25 ng/ml. The treatment for the first 49 patients was performed selectively (i.e. unilateral or bilateral treatment in one or two sessions depending on findings from TRUS and biopsies) and the whole prostate was treated in the remaining 62 patients. A complete response (defined as a PSA level < 4.0 ng/ml and a negative biopsy) was achieved in 60% of the whole prostate treated patients with and in 25% of selectively treated patients¹⁷⁾.

In 2001, Gelet et al. reported their long-term follow-

up data in which a complete response was obtained in 66% of patients with no residual cancer (regardless of PSA levels) or no increases in PSA levels in three consecutive examinations with a PSA velocity < 0.75 ng/ml/year for patients with negative biopsies¹⁸⁾. More recently, Chaussy and Thuroff summarized clinical outcomes by the ASTRO definition as 84.2% stability rate in the HIFU group and 80% rate in the combination with transurethral resection of the prostate (TURP) and HIFU group in 1 year¹⁹⁾. In summarizing our clinical outcome using the ASTRO definition, the biochemically disease-free survival rate was 76% at 2 years follow-up. Patients with stage T1c, T2a and T2b showed respectively 89, 67% and 40% biochemical disease-free survival rates at 2 years follow-up (p=0.0817). The clinical outcome in our series of patients with preoperative PSA less than 20 ng/ml were comparable to the outcome of patients treated with radical prostatectomy^{2,3)}.

In our series, postoperative urethral strictures at near verumontanum in the prostatic urethra occurred in 21% of the patients. Recently, TURP or bladder neck incision immediately before or after HIFU was found to reduce the treatment-related morbidity such as postoperative prolonged urinary retention, urinary catheterization time and urinary infection^{20,21)}. Neoadjuvant hormonal therapy also might be useful to reduce the volume of the prostate which can reduce the time of treatment and rate of morbidity. However, the upper limit of the gland volume is 50 ml even after reducing the size of the prostate with neoadjuvant androgen deprivation or TURP in our series. Generally, radicalism of prostate cancer and preservation of sexual function are always controversial because postoperative impotence depends on preservation of neuro-vascular bundles that sometimes includes tumor invasion. In our study, 39% of the patients exhibited erectile dysfunction after the HIFU therapy. One out of 12 patients who desired treatment for postoperative erectile dysfunction recovered with sildenafil citrate. We considered this rate to be lower than that compared to radical prostatectomy^{2,3)}. Further experience is required to confirm this important conclusion.

D'Amico et al. compared the outcome of a cohort

treated with 3D-CRT versus a matched cohort treated with brachytherapy plus external radiation therapy. The 5-year estimate of PSA failure-free survival rate after 3D-CRT alone was 45% and 67% when both radiation treatments were combined²²). More recently, Kupelian et al. compared the biochemical disease-free survival rate after permanent seed brachytherapy, external beam radiation therapy (EBRT), combined seeds and EBRT, or radical prostatectomy for clinical stage T1-2 localized prostate cancer²³). The 5-year biochemical disease-free survival rate for radical prostatectomy, EBRT < 72 Gy, EBRT ≥ 72 Gy, permanent seed brachytherapy and combined seeds and EBRT were 81, 51, 81, 83% and 77%, respectively. Although not directly comparable, the results after treatment with HIFU appear to be similar to those after radiotherapy, even when both brachytherapy and EBRT are combined.

For many reasons, transrectal HIFU appears to be highly attractive as a minimally invasive treatment for localized prostate cancer. HIFU treatment requires no incision or puncture, with no bleeding, can be performed on an outpatient basis and is repeatable even when patients with local recurrence have already been treated with radiation therapy²⁴). In addition, radiation therapy including brachytherapy and even surgery can be performed after HIFU.

Transrectal HIFU has considerable potential as a noninvasive treatment modality for patients with localized prostate cancer especially whose PSA less than 20 ng/ml.

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和文抄録

限局性前立腺癌に対する高密度焦点式超音波療法：多施設共同研究

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限局性前立腺癌に対する高密度焦点式超音波療法の多施設共同研究の成績について報告する。対象は、stage T1-2N0M0 の72例の限局性前立腺癌で、治療にはソナプレート500 (Focus Surgery, IN, USA) を用いた。効果判定には、American Society for Therapeutics Radiology and Oncology の効果判定基準を用いた。症例の年齢中央値は72歳、血清 PSA 中央値は 8.10 n/ml であった。また、術後観察期間中央値は14.0カ月間であった。治療効果は、全体では1年78%、2年76%が非再発生存であった。浸潤度別に2年目の生化学的再発生存率を集計したところ、stage T1c が89%、stage T2a 67%、stage T2b は40% (p=0.0817) であった。悪性度別では、Gleason 2~4 群は88%、Gleason

5~7 群は72%、Gleason 8~10 群は80% (p=0.6539) であった。術前の血清 PSA 値別2年非再発生存率は、PSA が10 ng/ml 以下群は75%、10~20 ng/ml 群は78% (p=0.6152) であった。術後6カ月目の前立腺生検では68%において癌細胞は認められなかった。前立腺体積は、術前 24.2 ml から術後6カ月目 14.0 ml と縮小していた (p<0.01)。IPSS, 最大尿流量率, Functional Assessment of Cancer Therapy (FACT) を用いた生活の質項目は術前後に有意な変化は認められなかった。高密度焦点式超音波療法は、術前血清 PSA 値が 20 ng/ml 以下の限局性前立腺癌に対して低侵襲性かつ有用な治療法と思われる。

(泌尿紀要 51: 651-658, 2005)

A New Method for Isolating Colonocytes From Naturally Evacuated Feces and Its Clinical Application to Colorectal Cancer Diagnosis

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Background & Aims: The early detection of colorectal cancer is desired because this cancer can be cured surgically if diagnosed early. The purpose of the present study was to determine the feasibility of a new methodology for isolating colonocytes from naturally evacuated feces, followed by cytology or molecular biology of the colonocytes to detect colorectal cancer originating from any part of the colorectum. **Methods:** Several simulation studies were conducted to establish the optimal methods for retrieving colonocytes from any portion of feces. Colonocytes exfoliated into feces, which had been retrieved from 116 patients with colorectal cancer and 83 healthy volunteers, were analyzed. Part of the exfoliated colonocytes was examined cytologically, whereas the remainder was subjected to DNA analysis. The extracted DNA was examined for mutations of the APC, K-ras, and p53 genes using direct sequence analysis and was also subjected to microsatellite instability (MSI) analysis. **Results:** In the DNA analysis, the overall sensitivity and specificity were 71% (82 of 116) of patients with colorectal cancer and 88% (73 of 83) of healthy volunteers. The sensitivity for Dukes A and B was 72% (44 of 61). Furthermore, the sensitivity for cancers on the right side of the colon was 57% (20 of 35). The detection rate for genetic alterations using our methodology was 86% (80 of 93) when the analysis was limited to cases in which genetic alterations were present in the cancer tissue. **Conclusions:** We have developed a new methodology for isolating colonocytes from feces. The present study describes a promising procedure for future clinical evaluations and the early detection of colorectal cancers, including right-side colon cancer.

cancer in men and women, respectively.¹ However, colorectal cancer is curable by surgical resection if diagnosed at a sufficiently early stage. This incentive has prompted investigators to develop new methods enabling the early diagnosis of colorectal cancer and has led to the introduction of cancer screening programs in many countries. For mass cancer screenings, a simple, economic, and noninvasive method of cancer detection is desired. The Hemoccult test is currently used in many countries for this purpose.²⁻⁶ However, this test is nonspecific and is not sufficiently sensitive to detect early stage colorectal cancer, although a higher sensitivity has been reported for advanced-stage colorectal cancer.⁷ Radioimmunoassays using tumor markers, such as carcinoembryonic antigen, also are not suitable for the detection of early cancer, although such tests can be used to monitor patients for an increasing tumor burden or tumor recurrence. Diagnosis by barium enema study and fiberoptic colonoscopy is accurate but time-consuming, expensive, and invasive. Therefore, an urgent need exists to establish a sensitive, reliable, and noninvasive method for the detection of colorectal cancer at an early stage.

To date, several screening methods for colorectal cancer based on the detection of mutated DNA in feces have been reported.⁸⁻²⁰ These methods, however, are time-consuming and are not sufficiently sensitive. The major reason for this inaccuracy is the fact that

Colorectal cancer is one of the most common malignancies worldwide. In Japan, colorectal cancer is the third and second leading cause of death from

Abbreviations used in this paper: APC, adenomatous polyposis coli; MSI, microsatellite instability; OMIM, Online Mendelian Inheritance in Man.

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nucleic acids in feces are derived from an enormous number and variety of bacteria and normal cells. Accordingly, the proportion of genes derived from cancer cells in feces is as low as 1%, at most.⁹ This makes the application of gene-detecting methods difficult in clinical practice.

We previously reported that the expression of CD44 variants in exfoliated colonocytes isolated from feces according to the Percoll centrifugation method could serve as a noninvasive diagnostic marker for early colorectal cancer.²¹ However, the repetition of the Percoll centrifugation method was found to distort the morphology of the exfoliated colonocytes. Accordingly, the sensitivity of this method also appeared to be unsatisfactory because of the low retrieval rate of the exfoliated colonocytes. Another study described a processing method that involved scraping or washing the stool's surface with a buffer to collect exfoliated colonocytes.²² In the ascending colon, however, the feces remains unformed. Therefore, most cancer cells exfoliated from the walls of the ascending colon would be incorporated into the inner core of the feces during the course of its formation. Thus, recovering cancer cells that originated from the ascending colon might be difficult using methods that involve scraping or washing solid feces.

Under these circumstances, we succeeded in developing a new, very effective methodology that allows the simple isolation of exfoliated colonocytes from not only the surface but also the central portion of feces while maintaining the colonocytes' initial morphology. Currently, we are attempting to apply a molecular biologic tool to purified colonocytes exfoliated into feces to detect cells from early colorectal cancers, including right-side colon cancer.

Materials and Methods

Study Design

This was a prospective study conducted between December 2002 and August 2004. The study protocol was reviewed and approved by the Institutional Review Board of the National Cancer Center, Japan. Written informed consent was obtained from all patients and healthy volunteers. No modifications to the protocol procedures were made during the course of the study.

Study Population

A total of 116 patients with histologically confirmed colorectal cancer and 83 healthy volunteers were enrolled. The healthy volunteers consisted of 37 men and 46 women with no apparent abnormalities, such as adenoma or carcinoma (including hyperplastic polyps), found during a total colonoscopy performed at the National Cancer Center Research Center for

Table 1. Characteristics of Patients and Healthy Volunteers

Characteristic	Patient (N = 116)	Healthy volunteer (N = 83)
Age, y		
Mean	62.0	58.4
Range	32-82	40-70
Sex, no (%)		
Male	69 (59.5)	37 (44.6)
Female	47 (40.5)	46 (55.4)
DNA, ng/gram of stool		
Mean	570.8	175.3
Range	2.0-7462.8	0.2-1907.5
Tumor location, no (%)		
Cecum	6 (5.2)	
Ascending colon	23 (19.8)	
Transverse colon	6 (5.2)	
Descending colon	7 (6.0)	
Sigmoid colon	21 (18.1)	
Rectum	53 (45.7)	
Size, mm		
Mean	40.0	
Range	4.0-120.0	
Histology, no (%)		
W/D	55 (47.4)	
M/D	56 (48.3)	
P/D	2 (1.7)	
Mucinous carcinoma	2 (1.7)	
Carcinoid tumor	1 (0.9)	
Depth, no (%)		
T1	10 (8.6)	
T2	32 (27.6)	
T3	71 (61.2)	
T4	3 (2.6)	
Dukes' stage, no (%)		
A	30 (25.9)	
B	31 (26.7)	
C	53 (45.7)	
D	2 (1.7)	

W/D, Well-differentiated adenocarcinoma; M/D, moderately differentiated adenocarcinoma; P/D, poorly differentiated adenocarcinoma.

Cancer Prevention and Screening. The median age of these volunteers was 58.4 years (range, 40-70 years). The characteristics of the patients and healthy volunteers are summarized in Table 1. All the patients with colorectal cancer had undergone surgical resection of their primary tumor at the National Cancer Center Hospital, Tsukiji, or at Hospital East, Kashiwa, Japan. The median age of the patients was 62.0 years (range, 32-82 years). There were 69 men and 47 women patients. The primary tumors were located in the following sites: rectum in 53 patients, sigmoid colon in 21 patients, descending colon in 7 patients, transverse colon in 6 patients, ascending colon in 23 patients, and cecum in 6 patients. The clinical stage of the patients according to Dukes' classification was as follows: Dukes' stage A in 30 patients, stage B in 31 patients, stage C in 53 patients, and stage D in 2 patients.

Stool Samples

Before surgical resection, stool samples were obtained from 116 patients with colorectal cancer. Stool sam-

plés were also obtained from 83 healthy volunteers a few weeks after they had undergone a total colonoscopy. Naturally evacuated feces from subjects who had not taken laxatives were used as stool samples. Each patient was instructed to evacuate into a polystyrene disposable tray (AS one, Osaka, Japan) measuring 5 × 10 cm in size at home and bring the sample to the reception counter at the outpatient clinic or the Cancer Prevention and Screening Center of the National Cancer Center. The samples were collected and transferred to a laboratory at which they were allowed to stand at room temperature. Preparation of the stool samples for examination was conducted within 1–6 hours after the evacuation.

Magnetic Beads

Dynabeads Epithelial Enrich are uniform, superparamagnetic, polystyrene beads (4.5- μ m diameter) coated with a mouse IgG1 monoclonal antibody (mAb Ber-EP4) specific for the glycopolypeptide membrane antigen Ep-CAM, which is expressed on most normal and neoplastic human epithelial tissues (DynaL, Oslo, Norway). Ep-CAM is widely expressed in the highly proliferative cells of the intestinal epithelium, from the basal cells to cells throughout the crypts at the basolateral membranes, and only the apical membrane facing the lumen is negative. The development of adenomas has been reported to be associated with increased Ep-CAM expression, and Ep-CAM over expression (mAb GA733) has frequently been demonstrated in colorectal carcinomas.^{23–25}

Simulation Studies

A series of simulation studies were conducted to establish the optimal conditions for retrieving HT-29 colorectal cancer cells from feces. Feces from healthy volunteers were divided into several portions, each of which was seeded with 100 μ L HT-29 cells (1×10^6 /approximately 5 g feces). The cells were retrieved under several different conditions as follows: use of a Hank's solution and 25 mmol/L HEPES buffer (pH 7.35); processed feces of 5, 10, or 30 g volume; filter with a pore size of 48, 96, 512, or 1000 μ m; incubation of homogenized solution with magnetic beads at 4°C or room temperature; application of 20, 40, 80, 200, or 400 μ L magnetic beads; incubation of homogenized solution with magnetic beads under gentle rolling at 15 rounds/minute in a mixer for 10, 20, 30, or 40 minutes; and the reaction time between the cell-magnetic bead complexes and a magnet on a shaking platform for 0, 2, 10, 20, 30, 40, 50, or 60 minutes. Finally, the cell retrieval rate calculated for the magnetic beads method under the conditions determined to be the most suitable for this simulation study was compared with that calculated for the Percoll centrifugation method. The retrieval rate was calculated by dividing the number of cells that bound to the retrieved beads by the number of cells initially added to the feces. The cells were counted using a NucleoCounter (ChemoMetec A/S, Allerød, Denmark).

Isolation of Exfoliated Cells From Feces

The procedure was conducted using the most suitable and optimal conditions determined by the simulation study (Figure 1). Approximately 5–10 g of naturally evacuated feces were used to isolate exfoliated cells. Feces were collected into Stomacher Lab Blender bags (Seward, Thetford, United Kingdom). The stool samples were homogenized with a buffer (200 mL) consisting of Hank's solution, 10% fetal bovine serum (FBS), and 25 mmol/L HEPES buffer (pH 7.35) at 200 rpm for 1 minute using a Stomacher (Seward). The homogenates were then filtered through a nylon filter (pore size, 512 μ m), followed by division into 5 portions (40 mL each). Subsequently, 40 μ L of magnetic beads were added to each homogenized solution portion, and the mixtures were incubated for 30 minutes under gentle rolling in a mixer at room temperature. The samples on the magnet were then incubated on a shaking platform for 15 minutes at room temperature. Colonocytes isolated from 5 tubes were smeared onto slides and then stained using the Papanicolaou method. The remainder of the samples was centrifuged, and the sediments were stored at –80°C until DNA extraction.

Extraction of DNA

Fresh tissue samples were obtained from the surgically resected specimens of 116 patients with colorectal cancer. The samples were snap frozen in liquid nitrogen within 20 minutes of their arrival at the pathologic specimen reception area and were stored in liquid nitrogen until analysis.

Genomic DNA was extracted from each tumor tissue specimen using a DNeasy kit (QIAGEN, Valencia, CA). Genomic DNA was also extracted from colonocytes isolated from feces using the SepaGene kit (Sanko-Junyaku, Tokyo, Japan).

Direct Sequence Analysis

Direct sequencing was conducted to identify mutations in the APC codon 1270–1594, in codons 12 and 13 of the *K-ras* gene, and in exons 5, 6, 7, and 8 of the *p53* gene.

The PCR primers used in this study were as follows: APC (5'-AAACACCTCAAGTTCCAACCAC-3', 5'-GGTAATTTGAAGCAGTCTGGGC-3'); *K-ras* (5'-CTGGTGGAGTATTTGATAGTG-3', 5'-CCCAAGGAAAGTAAAGTTC-3'); *p53* exon 5 (5'-GCCGTCTCCAGTTGCTTTAT-3', 5'-CCAAATACTCCACACGCAAAT-3'); *p53* exon 6 (5'-CATGAGCGCTGCTCAGATAG-3', 5'-TGCACATCTCATGGGGTTATAG-3'); *p53* exon 7 (5'-CTTGGCCCTGTGTATCTCTCTA-3', 5'-AAGAAAAGTGGAGCAGT-3'); and *p53* exon 8 (5'-ACCTCTTAACCTGTGGCTTC-3', 5'-TACAACCAGGAGCCATTGTC-3').

The sequence primers used in this study were as follows: APC (5'-CAAAGGCTGCCACTTGCAAAG-3', 5'-AAAATAAAGCACCTACTGCTG-3', 5'-GAATCAGCCAGGCACAAAGC-3'); *K-ras* (5'-CTGGTGGAGTATTTGATAGTG-3'); *p53* exon 5 (5'-CCAAATACTCCACACGCAAAT-3'); *p53* exon 6 (5'-CATGAGCGCTGCTCAGATAG-3'); *p53* exon 7 (5'-AAGAAAAGTGGAGCAGT-3'); and *p53* exon 8 (5'-

(1) Sample



Add feces (5-10g) in Hanks' solution 200mL (25mM HEPES buffer, 10% FBS) in Stomacher Lab Blender bag.

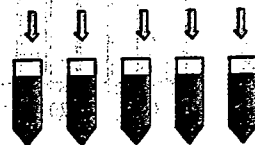
(2) Filtration



Filtrate the homogenates through a nylon filter (pore size, 512 μm).

(3) Incubation

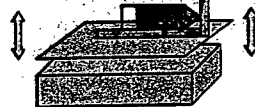
Dynabeads® Epithelial Enrich (40 μL)



50 mL tube

Divide the homogenates into five portions (40 mL each), add 40 μL of magnetic beads into each homogenized solution portion. Incubate for 30 minutes under gentle rolling at 15 rounds/minute in a mixer at room temperature.

(4) Separation



Place the tube in the magnet (Dyna MPC-1®), shake it on the platform for 15min.

(5) Wash



Remove the supernatant, Add 1000 μL of Hanks' solution to the tubes. Transfer the bead suspension to a new microcentrifuge tube. Place the tube in the magnet (Dyna MPC-S®).

(6) Retrieve



Remove the supernatant. Apply Papanicolaou stain, or store at -80° C until DNA extraction.

Figure 1. Schematic of procedure for isolating colonocytes from feces.

ACCTCTAACCTGTGGCTTC-3'). Each fragment was sequenced by direct sequencing using the Big Dye Terminator v 3.1/1.1 cycle kit (Applied Biosystems, Forester City, CA).

All obtained sequences were aligned with previously published sequences (National Center for Biotechnology Information [NCBI] Genbank accession No. M74088 [APC], M54968 [K-ras], and X54156 [p53]) for each of the

target genes and were analyzed using Phred/Phrp/DNASIS pro (Hitachi Software Engineering, Tokyo, Japan). The presence and nature of each mutation were confirmed by repeated PCR and sequencing.

BAT26

The BAT26 gene, an indicator of microsatellite instability (MSI), was amplified by PCR. Each fragment was elec-

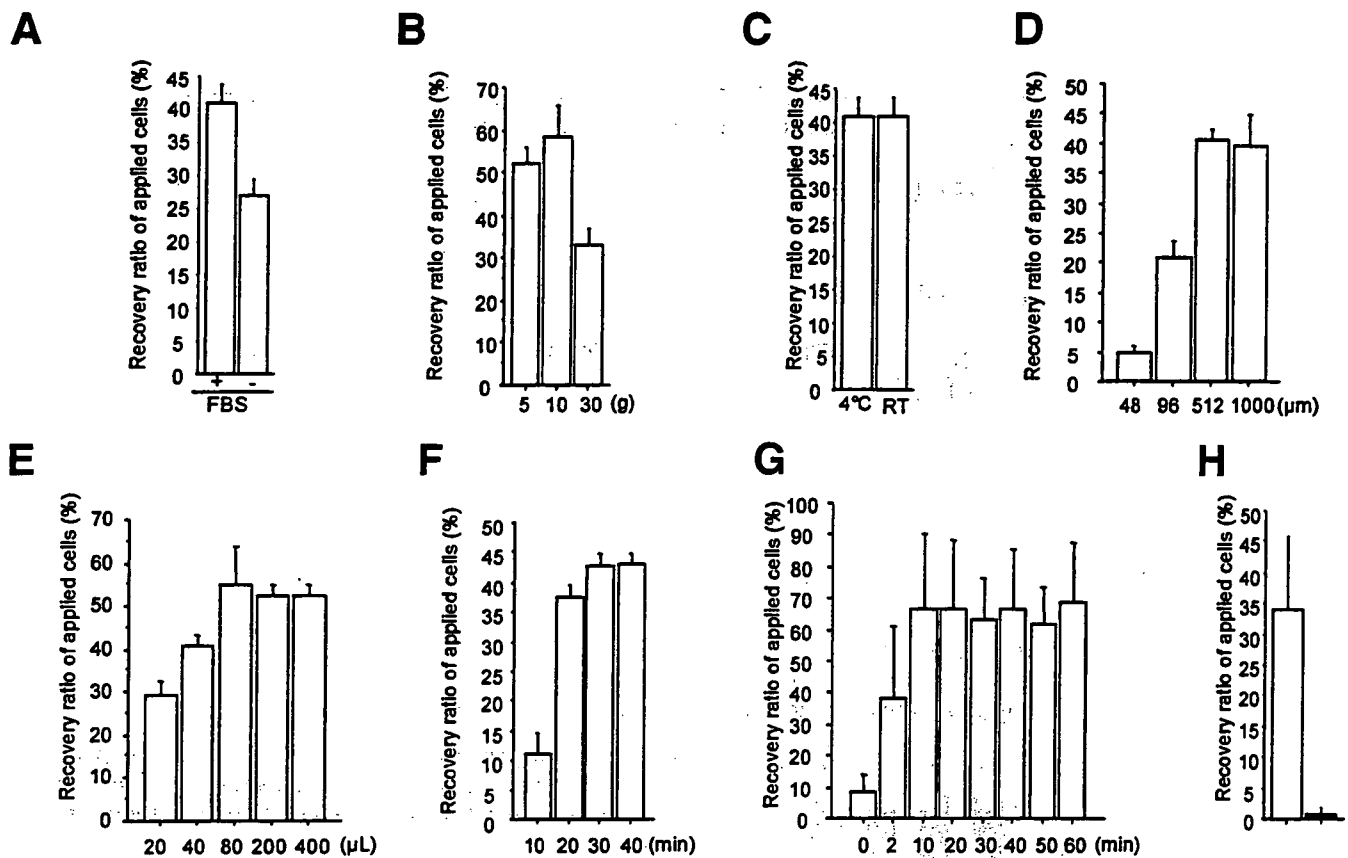


Figure 2. Simulation study to establish the optimal conditions for retrieving HT-29 colorectal cancer cells from feces and to compare the cell retrieval rates for the magnetic beads methods and the Percoll centrifugation method. Feces from healthy volunteers were divided into several portions, each of which was seeded with 100 μ L HT-29 colorectal cancer cells (1×10^6 /approximately 5 grams of feces). The procedure for retrieving the HT-29 cells was conducted under various conditions as follows: (A) homogenizing buffer with or without FBS; (B) stool weight (5, 10, or 30 g); (C) temperature during the cell-yielding procedure (4°C or room temperature); (D) filter pore size (48, 96, 512, or 1000 μ m); (E) volume of applied magnetic beads (20, 40, 80, 200, or 400 μ L); (F) incubation time of the homogenized solution with the magnetic beads under gentle rolling in a mixer (10, 20, 30, or 40 minutes); and (G) reaction time for the cells-magnetic bead complexes and the magnet on the shaking platform (0, 2, 10, 20, 30, 40, 50, or 60 minutes). The cell retrieval ratio (%) was calculated using the following formula: $100 \times$ number of HT-29 cells retrieved/number of applied HT-29 cells. (H) Comparison of cell retrieval rates for the magnetic beads methods (*open column*) and the Percoll centrifugation method (*solid column*).

trophoresed using an ABI PRISM 3100 Genetic Analyser (Applied Biosystems) and then analyzed by GeneScan v 3.7 (Applied Biosystems). The PCR primers used in this study were 5'-TGACTACTTTTIGACTTCAGCC-3' and 5'-AAC-CATTCAACATTTTAAACCC-3'.

Cytology

Colonocytes isolated from feces were examined by 2 experienced cytotechnologists after Papanicolaou staining.

Study Blinding

We followed the guidelines of our medical institution for preparing blinded samples. Technicians processed the stool samples and prepared the slides for cytology and the cell pellets for DNA extraction. The samples were blinded to prevent the identification of individuals and the samples' origins. Two cytologists assessed the blinded samples, and the Life Science Group of Hitachi, Ltd, analyzed the DNA sequences.

Statistical Analysis

A Fisher exact test was used to compare all proportions. All reported *P* values are 2-sided. A value of *P* < .05 was considered statistically significant.

Results

Simulation Studies

The cell retrieval rate was found to decrease when Hank's solution without FBS was used, thus indicating the effectiveness of adding serum to the homogenizing buffer (Figure 2A). The cell retrieval rate was found to decrease when more than 30 g of feces were processed (Figure 2B). The cell retrieval rates were similar when incubation was conducted at room temperature and at 4°C (Figure 2C). Filtering of the stool suspension with the 48- or 96- μ m filter resulted in significant clogging and thus hampered cell retrieval. However, a lot of fecal

residue remained after filtering with the 1000- μm filter, hindering the handling of the stool suspension thereafter. We therefore decided to use the 512- μm filter (Figure 2D). The dose of the magnetic beads applied was also examined. The cell retrieval rate increased in a dose-dependent manner up to 80 μL . In reality, a sufficient amount of genomic DNA derived from exfoliated colonocytes was obtained, even when 40 μL of magnetic beads were used (Figure 2E). Regarding the optimal incubation time of the magnetic beads for the complete binding of HT-29 cells to the beads, 30 minutes of incubation was found to be sufficient for the satisfactory binding of HT-29 cells to the beads (Figure 2F). For the retrieval of the cell-magnetic bead complexes on the magnet, a 10-minute reaction period was sufficient (Figure 2G).

The cell retrieval rates were 0.8% and 33.5% using the Percoll centrifugation method and the magnetic beads method, respectively, thus underscoring the advantage of the magnetic beads method (Figure 2H).

Cytology

Atypical cells were observed in colonocytes isolated from the feces of 32 of 116 patients with colorectal cancer, with a sensitivity rate of 28% (95% CI: 20–37; Table 2, Figure 3A and 3B). No atypical cells were observed in any of the 83 healthy volunteers, with a specificity rate of 100% (95% CI: 96–100). A significant difference ($P < .0001$) was found in the positivity rate between the patient group and the healthy volunteer group. The sensitivity rates for Dukes' A, B, and C or D colorectal cancers were 23% (7 of 30; 95% CI: 10–42), 32% (10 of 31; 95% CI: 17–51), and 27% (15 of 55; 95% CI: 16–41), respectively. No significant differences in the positivity rates were found among any of the stages. Furthermore, the sensitivity rates for cancers on the right side of the colon, including the cecum, ascending colon, and transverse colon, and for those on the left side of the colon, including the descending colon, sigmoid colon, and rectum, were 9% (3 of 35; 95% CI: 2–23) and 36% (29 of 81; 95% CI: 25–47), respectively. Therefore, the positivity rate was significantly higher for cancers on the left side of the colon ($P < .01$).

DNA Analysis

Overall analysis of stool samples. Sequence analysis showed distinct mutations in each of the analyzed genes in the tumor tissue and colonocytes isolated from feces (Figure 3C–F). Genetic alterations were observed in the colonocytes isolated from the feces of 82 of the 116 patients with colorectal cancer, yielding a sensitivity rate of 71% (95% CI: 62–79; Table 2). However, 10 of the

83 healthy volunteers were also positive for genetic alterations, producing a specificity value of 88% (95% CI: 79–94). A significant difference ($P < .0001$) was noted in the positivity rates of the patient group and the healthy volunteer group.

Genetic alterations were observed in 18 of the 30 patients with Dukes' A colorectal cancer, yielding a sensitivity rate of 60% (95% CI: 41–77). Furthermore, genetic alterations were observed among 26 of the 31 patients with Dukes' B colorectal cancer (84%; 95% CI: 66–95) and 38 of the 55 patients with Dukes' C or D colorectal cancer (69%; 95% CI: 55–81). No significant difference in sensitivity was found among any of the stages.

Genetic alterations were observed in colonocytes isolated from feces in 20 out of 35 patients with cancers originating on the right side of the colon (57%; 95% CI: 39–74) and in 62 out of 81 patients with cancers originating on the left side of the colon (77%; 95% CI: 66–85). No significant differences in the sensitivity rates were observed, although the sensitivity rate tended to be higher for cancers on the left side of the colon.

DNA analysis limited to colonocytes isolated from the feces of patients with colorectal cancer tissue involving genetic alterations. We assessed the performance of the present methodology for isolating cancer cells by examining the positivity rate of genetic alterations in colonocytes isolated from the feces of patients who showed alterations in their cancer tissues (Table 3). Among the 116 patients, a total of 93 (80%; 95% CI: 72–87) exhibited genetic alterations in the APC, K-ras, or p53 genes or BAT26 positivity in their cancer tissue: 51 patients exhibited APC mutations (44%, 95% CI: 35–53), 33 patients exhibited K-ras mutations (28%; 95% CI: 20–38), 62 patients exhibited p53 mutations (53%; 95% CI: 44–63), and 6 patients exhibited BAT26 positivity (5%; 95% CI: 2–11). Among the 93 patients with genetic alterations in their cancer tissues, the alterations were also successfully detected in colonocytes isolated from the feces of 80 patients (86%; 95% CI: 77–92). Among the 39 patients with Dukes' C or D advanced cancer who exhibited a genetic alteration in their cancer tissues, 36 patients exhibited genetic alterations in colonocytes isolated from their feces (92%; 95% CI: 79–98). Furthermore, genetic alterations were detected in colonocytes isolated from the feces of 18 of 24 patients with Dukes' A cancer (75%; 95% CI: 53–90) and 26 of 30 patients with Dukes' B cancer (87%; 95% CI: 69–96). No statistically significant difference was found among these 3 groups. In addition, genetic alterations could be detected in colonocytes isolated from the feces of 20 of 27 patients with cancers originating on the

Table 2. Incidences of Genetic Alterations of the APC, K-ras, p53, and MSI (BAT26) Genes as Well as Results From Cytology in all Patients and Healthy Volunteers

Marker	Patient				Healthy volunteer		
	No.	Tumor tissue		Isolated cell		No.	Specificity (%) (95% CI)
		Positivity (%) (95% CI)	Sensitivity (%) (95% CI)	No.	Sensitivity (%) (95% CI)		
Overall	93	80 (72-87)	82	71 (62-79)	10	88 (79-94)	
Patients (n = 116), healthy volunteers (n = 83)	Combined marker	51	44 (35-53)	47	41 (32-50)	1	99 (93-100)
	APC	33	28 (20-38)	33	28 (20-38)	1	99 (93-100)
	K-ras	62	53 (44-63)	45	39 (30-48)	6	93 (85-97)
	p53	6	5 (2-11)	4	3 (1-9)	3	96 (90-99)
	BAT26					0	100 (96-100)
	Cytology			32	28 (20-37)		
Dukes' stage A (n = 30)	Combined marker	24	80 (61-92)	18	60 (41-77)		
	APC	14	47 (28-66)	11	37 (20-56)		
	K-ras	6	20 (7-39)	5	17 (6-35)		
	p53	6	20 (7-39)	9	30 (15-49)		
	BAT26	1	3 (1-17)	1	3 (1-17)		
	Cytology			7	23 (10-42)		
Dukes' stage B (n = 31)	Combined marker	30	97 (83-100)	26	84 (66-95)		
	APC	17	55 (36-73)	17	55 (36-73)		
	K-ras	10	32 (17-51)	9	29 (14-48)		
	p53	18	58 (39-75)	13	42 (25-61)		
	BAT26	2	6 (1-21)	1	3 (1-17)		
	Cytology			10	32 (17-51)		
Dukes' stages C and D (n = 55)	Combined marker	39	71 (57-82)	38	69 (55-81)		
	APC	20	36 (24-50)	19	35 (22-49)		
	K-ras	17	31 (19-45)	19	35 (22-49)		
	p53	27	49 (35-63)	23	42 (29-56)		
	BAT26	3	5 (1-15)	2	4 (0-13)		
	Cytology			15	27 (16-41)		
Right-sided colon cancer (n = 35)	Combined marker	27	77 (60-90)	20	57 (39-74)		
	APC	11	31 (17-49)	8	23 (10-40)		
	K-ras	16	46 (29-63)	12	34 (19-52)		
	p53	17	49 (31-66)	11	31 (17-49)		
	BAT26	2	6 (1-19)	1	3 (1-15)		
	Cytology			3	9 (2-23)		
Left-sided colon cancer (n = 81)	Combined marker	66	81 (71-89)	62	77 (66-85)		
	APC	40	49 (38-61)	39	48 (37-60)		
	K-ras	17	21 (13-31)	21	26 (17-37)		
	p53	45	56 (44-67)	34	42 (31-53)		
	BAT26	4	5 (1-12)	3	4 (1-10)		
	Cytology			29	36 (25-47)		

right side of their colon (74%; 95% CI: 54-89) and 60 of 66 patients with cancers originating on the left side of their colon (91%; 95% CI: 81-97). A statistically significant difference was found between the right- and left-side colon cancer patient groups ($P = .03$).

Discussion

We have devised a simple, highly reliable methodology for isolating colorectal cancer cells from nonlaxative-induced, naturally evacuated feces from most patients with colorectal cancer. To date, several methods of isolating colorectal cancer cells from feces have been reported.^{21,22,26,27}

Our new funnel-shaped filter system extensively improved the filtration efficiency of the stool suspension by

enlarging the filtration area and selecting the optimal pore size; the system was capable of filtering the entire stool suspension without filter clogging. These properties permit the omission of centrifugation and simplify the overall process because all steps can be performed at room temperature. Furthermore, the use of serum successfully increased the cell retrieval rate. We presume that this increase may be attributed to the suppression of protease activity or the inhibition of nonspecific reactions of the antibodies on the bead surface. Consequently, our new methodology also allows the extraction of high-quality DNA or RNA from exfoliated colonocytes. Very recently, Imperiale et al compared a panel of fecal DNA markers and Hemocult II as screening tests for colorectal cancer. It is worth noting that, in their study, colonoscopy as a reference standard was used