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Biased Discordance of *KRAS* Mutation Detection in Archived Colorectal Cancer Specimens Between the ARMS–Scorpion Method and Direct Sequencing

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Objective: The concordance of *KRAS* mutation detection between the amplification refractory mutation system–Scorpion assay and direct sequencing was evaluated with clinically available formalin-fixed, paraffin-embedded specimens of metastatic colorectal cancers.
Methods: Genomic DNA from 120 macrodissected specimens was examined by the amplification refractory mutation system–Scorpion assay and direct sequencing. DNA mixtures of wild-type and mutant *KRAS* genes were prepared from the peripheral blood and the SW620 human colon cancer cell line for the model experiments.
Results: *KRAS* mutation was identified in 50 samples (41.7%) by the amplification refractory mutation system–Scorpion assay and 42 samples (35.0%) by direct sequencing. Discordance between the two methods was observed for samples with smaller amounts of amplifiable DNA. The sensitivity of direct sequencing was impaired by the decrease in template DNA and polymerase chain reaction cycles in the experimental models.
Conclusions: Decreased sensitivity of direct sequencing caused by insufficient polymerase chain reaction amplification resulted in biased discordance between direct sequencing and amplification refractory mutation system–Scorpion. Polymerase chain reaction conditions satisfactory for amplifying tens of haploid copies of genomic DNA to the saturation level might be necessary to ensure the robustness of the direct sequencing-based method employed for formalin-fixed, paraffin-embedded specimen-derived DNA samples.

Key words: ARMS–Scorpion – colorectal cancer – direct sequencing – formalin-fixed paraffin-embedded specimen – KRAS

INTRODUCTION

Retrospective subset analyses and prospective randomized Phase III clinical trials strongly suggest that patients with metastatic colorectal cancer (mCRC) containing *KRAS* gene mutations do not benefit from treatment with anti-epidermal growth factor receptor antibodies (1–4). On the basis of this evidence, pre-use *KRAS* mutation testing has been strongly recommended. Various methods have been developed for detecting *KRAS* mutation. A mutation-specific real-time polymerase chain reaction (PCR)-based technique combining amplification refractory mutation system (ARMS) and a unique bi-functional fluorescent primer/probe molecule (Scorpion) is one of the recommended methods for clinical use according to its robustness and convenience (5–11). Mutant alleles are selectively amplified by ARMS, and these amplified PCR products are sensitively and specifically detected by the Scorpion system. A standardized commercial kit based on the ARMS–Scorpion (ARMS/S) system allows the detection of seven major mutations in codons 12 and 13 of the *KRAS* gene present at low allelic concentrations (1%)

in heterogeneous specimens, with detection limits of between 5 and 10 copies. The kit has been used in several Phase III trials and is approved for use in *in vitro* diagnosis in EU countries (3).

Although various methods including ARMS/S have been developed, direct sequencing (DS) of PCR-amplified *KRAS* gene fragments is still one of the most clinically accessible methods. However, the low sensitivity of DS for mutant detection has been argued. DS is regarded as being most suitable for the detection of mutant alleles at an allelic concentration of more than 10–30%. To reduce the rate of false-negative errors in the diagnosis, macroscopic isolation of tissues in which cancer cells occupy more than 70% of the area (macrodissection) is recommended for retrieving genomic DNA samples (7).

We routinely employ both ARMS/S and the conventional DS for *KRAS* testing. Our data obtained from 120 formalin-fixed paraffin-embedded (FFPE) specimens revealed discordance in the detection of *KRAS* mutations between the two methods. Interestingly, the discordance was specifically observed in the samples with lower amounts of amplifiable DNA. Since such biased discordance could not be simply explained by the lower sensitivity of DS, we attempted to clarify the underlying reasons.

PATIENTS AND METHODS

Tissue Samples and DNA Extraction Used for Practice Investigations

Genomic DNA was obtained from primary and mCRC tissues of patients who were scheduled to receive treatment with cetuximab. Tissue samples were collected by surgical resection or biopsy at the National Cancer Center Hospital East (NCCHE) and other hospitals. The collections and investigations were conducted with the approval of the Institutional Review Board. In NCCHE, all specimens were fixed in 10% formalin for 1 day and then embedded in paraffin. From the FFPE tissue blocks, three to five slices of 10 μm-thick unstained sections were cut. Tissue areas in which tumor cells occupied more than 70% were macroscopically dissected and then genomic DNA was isolated using the QIAamp DNA FFPE Tissue Kit (Qiagen).

Control DNA Used for the Model System

Control DNA harboring wild-type *KRAS* gene and mutant *KRAS* gene, in which the 35th G residue of *KRAS* cDNA is mutated to T (c.35G > T), were purified from the whole blood cells of a healthy volunteer and the human colon cancer cell line SW620 which is homozygous for *KRAS* mutation (ATCC: CCL-227), respectively, for the model experiments. Total DNA from these samples was extracted using the DNeasy Blood & Tissue Kit (Qiagen).

ARMS/S Assay

KRAS mutations were detected using the K-RAS Mutation Test Kit (DxS-Qiagen), in accordance with the manufacturer's instructions. Purified genomic DNA (8–900 ng per reaction) was applied for a set of seven known *KRAS* mutation-detecting reactions and a control reaction. Reactions were allowed to proceed on a LightCycler 480 real-time PCR instrument (LC480) (Roche Diagnostics) and analyzed using LightCycler Adapt software, v1.1 (Roche Diagnostics). The presence of mutant alleles was determined by the difference in the cycle threshold (ΔC_t) value between the control and each of the mutant reactions. C_t is a number of the PCR cycles necessary to detect a fluorescent signal above the background signal, as a measure of the target molecules present at the beginning of the reaction. LightCycler Adapt software compares the sample ΔC_t values with the cut-off values for a 1% concentration of mutant alleles to identify the presence/absence of the mutation. To ensure the sensitivity to detect mutants present at 1%, the manufacturer suggests the use of DNA samples with control C_t values of <28.9.

PCR Amplification and DS of the *KRAS* Gene

The *KRAS* exon-2 fragment was amplified according to the method described in a previous report, but with some modification (1,12). Briefly, each 50 μl PCR cocktail contained genomic DNA, 1.5 mM of magnesium chloride, 200 μM of deoxynucleotide triphosphates, 0.2 μM of PCR primers and 2.5 U of HotStarTaq® DNA polymerase (Qiagen). The PCR conditions were as follows: 1 cycle at 95°C for 15 min; 35, 37 or 40 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 1 min; 1 cycle at 72°C for 10 min. Primer sequences were GTGTGACATGTTCTAATATAGTCA and GAATGGTCC TGCACCAGTAA. The PCR products were purified by Microcon centrifugal Filter devices (Millpore), and the amplicon size and amount were confirmed by DNA agarose gel electrophoresis. The purified PCR products were directly sequenced with the same primers as those used for the PCR. The BigDye Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3100 (Applied Biosystems) were used in accordance with the manufacturer's instructions. Analyses of the DNA sequences were performed with the Sequence Scanner Software, ver. 1.0 (Applied Biosystems). The signal intensity of each sequence peak was determined based on both the raw and the analyzed data view of the software.

Statistical Analysis

Fisher's exact test, ANOVA and Tukey's HSD procedure were used to compare the test results. Analyses were conducted with the JMP® 8 package software (SAS Institute, Cary, NC, USA).

RESULTS

Biased Discordance of Mutation Detection Between ARMS/S Assay and DS

A total of 120 colorectal cancer specimens (103 primary and 17 metastatic tumor specimens) collected from 112 patients were analyzed. Tumor DNA was isolated from tumor cell-rich tissue areas obtained by macrodissection. *KRAS* mutation was identified in 50 samples (41.7%) by the ARMS/S assay and 42 samples (35.0%) by DS. The results were stratified according to the C_t value of the control assays of the ARMS/S system. Among the samples with a control C_t value of <28.9, both the ARMS/S assay and DS identified the mutation in 18 of 46 samples (39.1%), whereas among the samples with a control C_t value of >29.0, the mutation was detected in 32 of 74 samples (43.2%) by the ARMS/S assay and in 24 of 74 samples (32.4%) by DS. Thus, the concordance rate was significantly lower among the samples with higher levels of control C_t (Table 1).

Table 1. Correlation between the control C_t value and the frequency of detection of the *KRAS* mutation

Control C_t	Incidence of mutation		Concordance between the two methods (%)
	Direct sequencing	ARMS–Scorpion	
<28.9	18/46 (39.1%)	18/46 (39.1%)	100.0%
>29.0	24/74 (32.4%)*	32/74 (43.2%)	86.5%*
Total	42/120 (35.0%)	50/120 (41.6%)	91.7%

ARMS, amplification refractory mutation system.
*Significantly different from the values in the specimens containing template DNA equivalent to <28.9 of the control C_t at a level of $P = 0.0130$ (two-tailed Fischer's exact test).

Enhancement of Mutant Signal Detection of DS by Increase in the Template DNA Extracted from the FFPE Specimens

The above findings implied that the amount of the template DNA influenced the sensitivity of DS. Therefore, in a subsequent experiment, seven specimens that had been determined as harboring wild-type *KRAS* by DS using a small amount of the template DNA (median: 160.0 ng, range: 54.6–212.9 ng) were selected and retested by DS using an increased amount of the template (median: 408.1 ng, range: 118.5–895.8 ng). Three of the seven samples examined were then found to exhibit apparent traces of the mutation. The three samples had been identified as harboring the *KRAS* mutation by the initial ARMS/S assay (Table 2 and Fig. 1A).

Table 2. Conversion of the nucleotide sequences of the *KRAS* gene by increase in the amount of the template DNA

Sample	Experiment 1		Experiment 2		ARMS–Scorpion
	DNA amount (ng)	Interpretation	DNA amount (ng)	Interpretation	
A	114.5	GGT	895.8	GGT	12Ala
B	179.9	GGT	839.2	GGT	Wild-type
C	160.0	GGT	494.9	G(G/A)T	12Asp
D	126.8	GGT	408.1	G(G/T)T	12Val
E	54.6	GGT	118.5	G(G/T)T	12Val
F	177.5	GGT	329.1	GGT	Wild-type
G	212.9	GGT	385.4	GGT	Wild-type

Decrease in Minor Mutant Signal Detection by DS Caused by Small Amounts of the Template DNA

To confirm the above DNA amount-dependent decreased detection power of DS, the following model experiment was carried out. A DNA mixture containing 10% of colon cancer cell line-derived genomic DNA harboring the homologous c.35G > T mutation and 90% of wild-type DNA was prepared (10% mutation DNA). Since our preliminary result revealed that 3.0 ng of intact genomic DNA yielded a control C_t value of 29 in the ARMS/S system (data not shown), 3.0 ng of 10% mutation DNA was serially diluted and 0.75, 0.19 and 0.05 ng of the DNA mixtures were examined by both the ARMS/S assay and DS. The mutant signals detected by DS using 3.0 ng DNA were specifically diminished when 0.75 and 0.19 ng of DNA were used. Neither wild-type nor mutant signals were detected with 0.05 ng of DNA. In the ARMS/S system, although the control C_t value increased as the amount of DNA decreased, the ΔC_t (difference between the mutation assay C_t and control assay C_t) did not differ among the results obtained using 3.0, 0.75 and 0.19 ng of DNA (Table 3 and Fig. 1B). The control C_t value was over 35 with 0.05 ng of DNA, and no mutant signals were detected even with 40 cycles of amplification when this amount of DNA was used.

Enhancement of the Sensitivity of DS by Increasing the Number of PCR Cycles

We further evaluated whether an increased number of PCR cycles might improve the sensitivity of DS obtained using a small amount of template DNA. The above-mentioned serially diluted 10% mutation DNA was amplified using 35 cycles and also using an increased number of PCR cycles of 37 and 40, then applied to the sequencing. With an increase in the number of PCR cycles, mutant signals became evident even with 0.05 ng of the template DNA (Table 3).

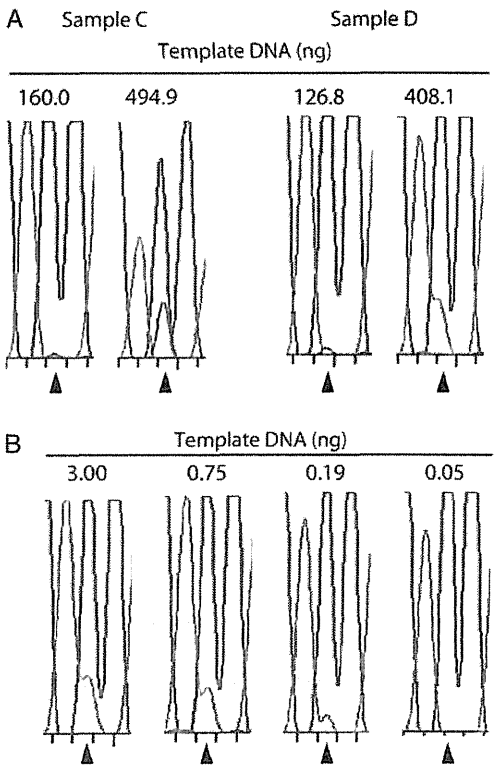

Figure 1. (A) Representative cases of conversion of wild-type to mutant signals. Analyzed data of antisense sequences of codon 12 of the *KRAS* gene for two samples are presented. Arrowheads represent the second letter of anticodon 12. Increase in the amount of the template DNA caused the occult peaks of T (red trace of sample C) and A (green trace of sample D) to become apparent. (B) Serial dilution of 10% mutation DNA and attenuation of the mutant signals. Analyzed data of antisense sequences of codon 12 of the *KRAS* gene for 10% mutation DNA models (37 cycles) are presented. Arrowheads represent the second letter of anticodon 12. As the amount of the template DNA was serially diluted to a quarter of the original and the total amount of DNA decreased, the mutant peak A diminished significantly or altogether disappeared.

DISCUSSION

ARMS/S is known to show a high sensitivity and specificity for the detection of *KRAS* mutations, with a short turnaround time. Meanwhile, DS is still recognized as one of the standard methods for mutation detection and is frequently used for confirming the results obtained with other methods. Comparisons of the sensitivity of the two methods have been reported. ARMS/S allows the detection of mutations in heterogeneous specimens, even at a low allelic concentration (1%), whereas DS allows the detection of heterogeneous mutant alleles present at 10–30%. Such difference in the sensitivity between the two methods has been regarded as the main reason for the discordance in the detection of *KRAS* mutations between the two methods (7,8,11).

In our present study, we showed that the discordance between the ARMS/S and DS for the detection of *KRAS* mutations was biased by the amount of amplifiable template DNA. Although the mutant-positive rates of ARMS/S and DS were not significantly different among samples with large DNA amounts, the mutant-positive rate of DS was significantly lower for samples with lower DNA amounts, suggesting that the discordance cannot be simply explained by the above-mentioned difference in the sensitivity between ARMS/S and DS. Subsequently performed experiments clearly showed that the sensitivity of DS was reduced by insufficient PCR amplification, which was caused not only by a lower template amount, but also by an inadequate number of PCR cycles.

Table 3. Alteration of the mutation/wild-type ratio of the *KRAS* gene in a mixture of 10% SW620 and 90% control genomic DNA (10% mutation DNA)

DNA amount (ng)	ARMS/S		Direct sequencing		
	Control C_t	ΔC_t	Mutant/wild-type ratio		
			35 cycles ($n = 3$)	37 cycles ($n = 5$)	40 cycles ($n = 5$)
3.00	30.73	2.84	0.225 ± 0.012	0.216 ± 0.010	0.242 ± 0.038
0.75	32.80	2.82	$0 \pm 0^{a,*}$	0.209 ± 0.053	0.230 ± 0.082
0.19	34.84	2.91	$0 \pm 0^{a,*}$	0.154 ± 0.108	0.237 ± 0.061
0.05	37.70	ND[b]	ND[b]	$0 \pm 0^{a,**}$	0.243 ± 0.214

[a]Wild-type signals could be detected, whereas mutant signals were not detectable.
[b]Neither wild-type nor mutant signals were detectable.
*Significantly different from the values in obtained with 40 cycles and 37 cycles of amplification of samples with an equivalent amount of DNA at $P < 0.05$ (ANOVA and Tukey's HSD procedure).
**Significantly different from the values obtained with 40 cycles in samples with an equivalent amount of DNA at $P < 0.05$ (ANOVA and Tukey's HSD procedure).

To explain the above mechanism, a schematic model is presented in Fig. 2. Figure 2A represents the traces of the PCR-amplified copy numbers of the template containing three copies of mutant and seven copies of wild-type genes. The absolute copy numbers [(a) and (b) in Fig. 2A] increased exponentially by amplification. On the other hand, the fluorescent signals obtained by cycle sequencing of too small an amount of amplified DNA might not be detected by the capillary DNA sequencers. In this model, a copy number of 1024 is virtually designated as the detection threshold and the difference between the absolute copy numbers and the detection threshold is regarded as the 'detectable copy number' [(c) and (d) in Fig. 2A]. The detectable mutant/wild-type ratio [(c):(d) in Fig. 2A] supposedly represents the actual mutant/wild-type ratio. Throughout the amplification, the ratio of the absolute copy numbers of the mutant to wild-type DNA [(a):(b) in Fig. 2A] was fixed at 3:7; however, the detectable mutant/wild-type ratio was smaller when the absolute copy number was closer to the detection threshold. Figure 2B represents the change of the detectable mutant/wild-type ratio of 10% Mut DNA with a total 10, 100 and 1000 copies. The approximation of the detectable mutant/

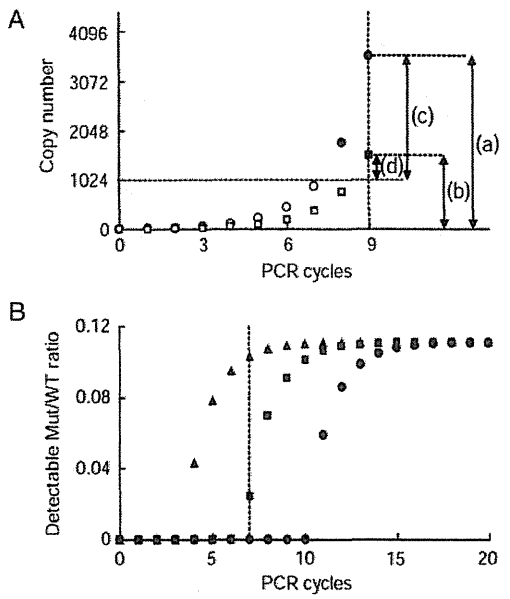

Figure 2. Schematic models of specific attenuation of the mutant signals. (A) Expected copy number of amplicons from a DNA template consisting of seven copies of the wild-type and three copies of the mutant genes. The copy number of 1024 was virtually designated as the detection threshold. Open circles, wild-type amplicons below the detection threshold; closed circles, wild-type amplicons over the detection threshold; open squares, mutant amplicons below the detection threshold; closed squares, mutant amplicons over the detection threshold. (a) and (b): absolute copy number of the wild-type and mutant amplicons, respectively. (c) and (d): detectable copy number of the wild-type and mutant amplicons, respectively. (B) The change of the detectable mutation/wild-type ratio obtained for 10% Mut DNA with a total of 10 (closed circles), 100 (closed squares) and 1000 (closed triangles) copies. WT, wild-type.

wild-type ratio to the absolute mutant/wild-type ratio was delayed when the initial copy number was small. At the seventh PCR cycle, the detectable mutant/wild-type ratio for the 10-, 100- and 1000-copy models were 0, 0.024 and 0.103, respectively. This model also suggests that even with a small initial copy number, the detectable mutant/wild-type ratio approximated the absolute mutant/wild-type ratio when the PCR amplification efficiently amplified the DNA to levels high enough to overshadow the detection threshold. The results obtained with these models are consistent with our finding of the loss of mutant signals in samples containing lower amounts of template DNA or obtained using a smaller number of amplification cycle.

Amplifiable DNA amounts are often limited when FFPE samples are used as the DNA source, since DNA is highly fragmented by formalin treatment. Previous studies have emphasized the harmful effects of fixatives on the efficiency of amplification by PCR. Especially, prolonged fixation periods contribute to further deterioration of the template DNA (13,14). Standardization of the conditions of formalin fixation is, without doubt, important to improve the quality of any DNA testing. However, at present, we have to use archived FFPE samples with various fixation conditions in routine clinical testing. In this study, we tried to obtain detailed data about the fixation conditions; however, the information was not recorded for many of the samples, especially those from other hospitals, and we could not conduct the evaluation in the present study.

To ensure the robustness of DS, it is necessary to set the appropriate conditions for PCR amplification from samples with low-quality DNA. Since various laboratories have established 'home-brewed' DS methods, it is difficult to propose a single standard method that can be demonstrated to be superior to other established methods. Regardless of specific conditions, such as the primer sequences, sizes of the PCR products and brands of DNA polymerases and thermal cyclers, we strongly recommend confirmation of whether the PCR amplification is saturated before the start of cycle sequencing. According to our experimental results, PCR conditions amplifying 0.05 ng of intact genomic DNA, which is equivalent to tens of haploid copies, to saturation level might be required. Since the amplifiable amount of DNA from FFPE specimens is often limited, increase in the number of PCR cycles (more than 40 cycles) may be most effective. The potential risk of detection of artificial mutations with the use of insufficient amounts of the template DNA from FFPE specimens and increased number of PCR cycles has been suggested (15); to avoid such false-positive errors, duplication of the test would be expected to be effective.

Genetic tests for appropriate use of molecular-targeting drugs are becoming increasingly popular and necessary. In many cases, DS will be the primary procedure for the detection of gene mutations in DNA samples from FFPE specimens. As suggested above, validation of the testing conditions, including the PCR settings, would be most required to strengthen the robustness and rigidity of the tests for routine clinical diagnoses.

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Conflict of interest statement

None declared.

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Feasibility and Robustness of Amplification Refractory Mutation System (ARMS)-based *KRAS* Testing Using Clinically Available Formalin-fixed, Paraffin-embedded Samples of Colorectal Cancers

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Background: *KRAS* mutation testing is recommended for the discernment of metastatic colorectal cancer patients who are unlikely to benefit from anti-epidermal growth factor receptor antibodies. A recently developed amplification refractory mutation-Scorpion system is becoming a standard method for *KRAS* mutant detection. The feasibility and robustness of this system using DNA samples from clinically available formalin-fixed, paraffin-embedded specimens were evaluated.
Methods: Genomic DNA from macro-dissected 110 specimens was applied for the *KRAS* mutant detection using a commercial amplification refractory mutation-Scorpion system kit. Success rate and mutant detection rate of the test were evaluated.
Results: Small intra- and inter-lot deviations of the testing kit and a good concordance among different real-time polymerase chain reaction systems suggested the reliability of the amplification refractory mutation-Scorpion system. Though one-third of the 110 samples that were tested did not contain a sufficient amount of DNA to detect a 1% concentration of mutant alleles, the mutant detection rate was not impaired using tumor DNA concentrated by macro-dissection. Using a higher amount of template DNA, which supposedly contained abundant interfering substances, prevented the detection of the exogenous control amplicons, resulting in a reduced success rate. Adjusting the template amount according to the total DNA concentration might reduce the failure rate.
Conclusion: The amplification refractory mutation-Scorpion system with formalin-fixed, paraffin-embedded specimen-derived DNA samples exhibited an acceptable feasibility and robustness suitable for routine clinical practice.

Key words: colorectal cancer – KRAS mutation – ARMS-Scorpion method – FFPE

INTRODUCTION

The clinical benefits of anti-EGFR (epidermal growth factor receptor) antibodies for the treatment of metastatic colorectal cancer (mCRC) have been revealed in randomized clinical trials (1–3). Meanwhile, retrospective subset analyses of these trials have clarified that patients with tumors containing mutant *KRAS* genes, which encode constitutively active forms of KRAS proteins, did not benefit from anti-EGFR antibodies. Based on this evidence, *KRAS* mutation testing is now strongly recommended prior to the use of anti-EGFR antibodies (4,5).

A series of procedures have been developed for detecting *KRAS* mutations in genomic DNA from archived formalin-fixed, paraffin-embedded (FFPE) specimens. Among them, a recently developed mutation-specific real-time polymerase chain reaction (PCR)-based technique, combining an amplification refractory mutation system and a Scorpion fluorescent primer/probe system (ARMS/

S), offers both simple and rapid means of testing (6,7). A single kit detects seven major mutations in codons 12 and 13 of the *KRAS* gene in heterogeneous specimens at a low allelic concentration (1%), with detection limits of between 5 and 10 copies. This assay has been used in several phase III trials examining the use of anti-EGFR antibody treatment in patients with mCRC, and a commercialized, quality-controlled kit has been approved for the CE mark, indicating conformity with European health and safety requirements (4,8).

Though the ARMS/S method is likely to be a valuable tool in clinical practice, the feasibility and robustness of this system using DNA samples from clinically available FFPE specimens have not been well evaluated. In the present study, we explored the feasibility and robustness of this kit and the DNA properties that influence a successful test outcome.

PATIENTS AND METHODS

Tissue Samples and DNA Extraction

Samples from 110 mCRC patients who were planned to receive anti-EGFR antibody treatment between April and November 2009 were available for *KRAS* mutation testing. The test was performed as a part of the Advanced Medical Technology Programs approved by the Ministry of Health, Labour and Welfare of Japan and written informed consent was obtained from all the patients. All the specimens had been obtained from primary or metastatic tumors collected by surgical resection or biopsy. Genomic DNA was isolated from FFPE tissue blocks using the QIAamp DNA FFPE Tissue Kit (QIAGEN) according to the manufacturer's instructions. To enrich the tumor-derived DNA, the tissue areas containing more than 70% tumor cells from each section were macro-dissected. The extracted DNA was spectrophotometrically quantified using Nano Drop 1000 (Thermo Fischer Scientific).

KRAS Mutant Detection

Mutant *KRAS* was determined using the K-RAS Mutation Test Kit (DxS-QIAGEN) according to the manufacturer's instructions. Real-time PCR was performed and analyzed using the LightCycler 480 Real-Time PCR System and LightCycler Adapt software v1.1 (Roche Diagnostics) or the 7500 Fast Real-Time PCR System (Applied Biosystems-Life Technologies).

RESULTS

Negligible Intra- and Inter-lot Differences Obtained with the K-RAS Mutation Kit

To evaluate the deviation in the data among each run, we estimated the Ct (cycle threshold) values for the amplification plots of the kit-attached mixed standard. The mixed standard contains all the mutant fragments. Amplification with a control, which amplifies a region of exon 4 of *KRAS*, and 7 mutant-specific primers provided constant Ct values. Using three different lots of the kit, a total of 27 runs were performed using the Roche LightCycler 480 Real-Time PCR System (LC480). All the plots exhibited the Ct values of the control and mutation assays with a small standard deviation (SD), and these values converged within the range reported by the manufacturer. The differences in the Ct values among the different lots were also negligible (Figure 1 and Table 1).

Good Concordance between Recommended Real-Time PCR Systems

Two real-time PCR systems, the LC480 mentioned above and the Applied Biosystems 7500 Real-Time PCR System (ABI7500), have been approved for *in vitro* diagnostic use in Europe. The deviation in the results obtained using these two systems was assessed. Eleven randomly selected samples that were identified as mutation positive using the LC480 system were re-tested using the ABI7500 system. All the mutation callings were concordant between two systems. Furthermore, once the threshold of the ABI7500 system was adjusted so that it was equal to the control Ct value of the mixed standard identified using the LC480 system, the differences in the control Ct values of the samples became very small (mean and SD, -0.55 ± 0.83). The differences in ΔCt (the difference between the mutation assay Ct and the control assay Ct) between the two systems were also significantly small (mean and SD, 0.20 ± 1.31). Confirmatory direct sequencing did not detect any mutant signals in 4 of the 11 samples, however the other mutation interpretations were concordant with those obtained using the ARMS-Scorpion kit (Table 2).

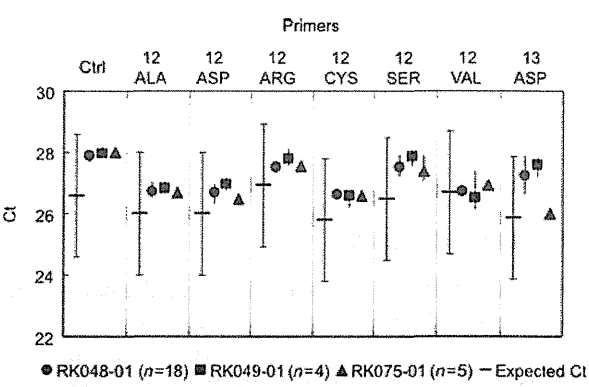

Figure 1. Intra- and inter-lot differences of the tests. The means and ranges of the Ct values for the amplification plots of the mixed standard produced using a total of 27 runs with three different lots were plotted. The expected Ct values and permissible ranges provided by the manufacturer were also plotted.

Table 1. Intra- and inter-lot differences of the test

Primer	Lot	*n*	Ct		Range	
			Mean	SD	Upper	Lower
Control	RK048-01	18	27.90	0.10	28.09	27.70
	RK049-01	4	27.98	0.13	28.21	27.89
	RK075-01	5	28.01	0.04	28.05	27.94
12ALA	RK048-01	18	26.73	0.17	27.04	26.52
	RK049-01	4	26.81	0.15	27.06	26.64
	RK075-01	5	26.68	0.12	26.88	26.52
12ASP	RK048-01	18	26.69	0.16	26.98	26.36
	RK049-01	4	26.97	0.18	27.21	26.71
	RK075-01	5	26.47	0.12	26.64	26.28
12ARG	RK048-01	18	27.52	0.09	27.72	27.34
	RK049-01	4	27.79	0.20	28.09	27.55
	RK075-01	5	27.56	0.06	27.63	27.49
12CYS	RK048-01	18	26.62	0.08	26.81	26.49
	RK049-01	4	26.59	0.24	26.78	26.20
	RK075-01	5	26.60	0.10	26.77	26.50
12SER	RK048-01	18	27.52	0.20	27.94	27.20
	RK049-01	4	27.85	0.21	28.10	27.54
	RK075-01	5	27.38	0.30	27.90	27.07
12VAL	RK048-01	18	26.72	0.10	26.92	26.55
	RK049-01	4	26.53	0.50	27.40	26.15
	RK075-01	5	27.38	0.09	27.08	26.81
13ASP	RK048-01	18	27.24	0.39	27.88	26.53
	RK049-01	4	27.57	0.26	27.85	27.20
	RK075-01	5	25.99	0.12	26.19	25.84

Limits of Sample Assessment Interpretation

LightCycler Adapt Software is recommended for the analysis of data obtained using the LC480 system. The software calculates the sample ΔCt values, which are compared with the cut-off values for a 1% concentration of mutant alleles, to identify a positive or negative amplification plot. A CONF_LEVEL Flag/Warning is displayed in a mutation-negative sample with a control Ct > 28.9, suggesting the absence of an amount of amplifiable DNA sufficient to detect a 1% mutation and indicating that low-level mutations might have been missed. As well, a LIMITED Flag/Warning is displayed in a mutation-negative sample with a control Ct >35 to warn that the system has only detected apparent mutations. Of the 110 samples that were tested, 35 samples (31.8%) were judged as CONF_LEVEL and three samples (2.7%) were judged as LIMITED (Fig. 2). However, the mutant detection rates of the samples with a control Ct below and above 28.9 were 40.9 and 40.0%, respectively (Fig. 2).

Exogenous Control Failure Using a Larger Amount of DNA Templates

The assays contain an exogenous control reaction to assess contamination with PCR inhibitors. If the exogenous control reactions fail and no mutant-specific amplification was detected, an EXO_FAIL Flag/Warning is displayed. EXO_FAIL warnings were displayed in 25 of the 110 samples (22.7%) that were examined. All the samples were mapped onto a two-dimensional plot with the control Ct value and the spectrophotometrically determined gross amount of DNA used in the reaction. The EXO_FAIL samples converged within an area corresponding to a larger amount of DNA and smaller Ct values. The lowest DNA

Table 2. Concordance between runs performed using different real-time PCR systems

Sample	LC480			7500 Fast			dCt (LC480-7500)	ddCt (LC480-7500)	DS interpretation
	Ct	dCt	Interpretation	Ct	dCt	Interpretation			
A	26.69	4.71	12Cys	26.98	2.02	12Cys	−0.29	2.69	12Cys
B	28.64	0.16	12Val	30.93	0.81	12Val	−2.29	−0.65	12Val
C	30.32	2.48	12Cys	31.04	1.80	12Cys	−0.66	0.68	12Cys
D	31.32	3.52	12Ala	32.37	3.69	12Ala	−1.05	−0.17	WT
E	31.50	3.63	12Asp	32.93	2.95	12Asp	−1.43	0.68	12Asp
F	31.72	6.61	13Asp	32.50	3.64	13Asp	−0.78	2.52	13Asp
G	31.83	3.40	12Cys	31.11	4.20	12Cys	0.72	−0.80	WT
H	32.37	0.34	12Ala	33.01	1.04	12Ala	−0.64	−0.70	12Ala
I	32.55	6.36	12Cys	32.76	6.64	12Cys	−0.21	−0.28	WT
J	32.79	2.92	12Val	32.21	4.72	12Val	0.58	−1.80	WT
K	32.96	2.17	12ASP	32.93	2.13	12Asp	0.03	0.04	12Asp

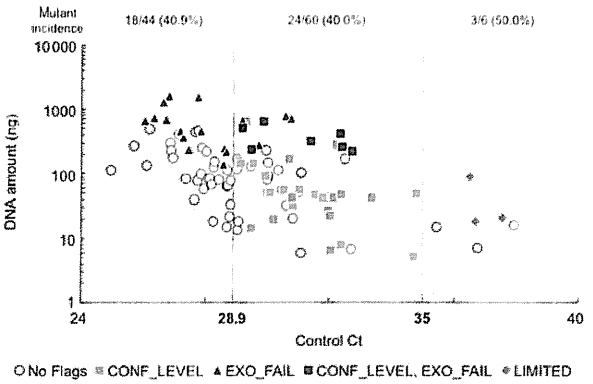

Figure 2. DNA amount and sample assessment interpretation. The spectrophotometrically determined DNA amounts used for the single assay and control Ct values of each sample were plotted. Control Ct values of 28.9 and 35 were used as the cut-off values for CONF_LEVEL and LIMITED warnings, respectively. The Flag/Warning results were overlaid on the plot. The incidence of the mutation was indicated above the plot. The results were stratified according to the control Ct values.

amount and the largest Ct value for the EXO_FAIL samples were 139.2 ng per reaction and 32.71, respectively. The number of mutation-negative samples with more than 139.2 ng of DNA was 27, i.e. the incidence of the EXO_FAIL warning was 92.6% (25/27). Meanwhile, the number of mutation-negative samples with a Ct value of less than 32.71 was 59, i.e. the incidence of the EXO_FAIL warning was 42.4% (25/59, Fig. 2).

DISCUSSION

One of the advantages of the ARMS/S system for *KRAS* testing is its expediency for standardizing operating procedures. The significantly small intra- and inter-lot deviations obtained in the present study suggest the reliability of the meta-chronically obtained data. Furthermore, the good concordance between different real-time PCR systems assures consistency among different facilities.

The limited amount of DNA in archived samples, mainly caused by fragmentation as a result of formalin treatment, often makes *KRAS* testing difficult using FFPE samples (9). Though the standardization of the fixation methods, such as concentration and pH of formalin and fixation time, is highly recommended, controlling these factors of clinically available archived samples is quite difficult. Actually, we could not follow the information of fixation methods of most of the specimens examined in this study, and about one-third of the samples did not satisfy the criteria for assuring sensitivity capable of detecting a 1% mutation rate. However, the mutation incidences were similar between the samples with a control Ct below and above 28.9, suggesting that the limited sensitivity did not lower the detection power, at least using DNA samples that had been enriched by macro-dissection.

In addition to its high sensitivity, robustness is another advantage of ARMS/S system. Discordant mutant interpretation between ARMS/S and direct sequencing was observed in 4 of 11 samples. All the four samples exhibited the control Ct value >28.9, suggesting that the amplifiable DNA amount of these samples was rather limited. Using a larger sample size, we confirmed a similar phenomenon and found that the sensitivity of direct sequencing was impaired by insufficient template DNA amount, resulting false-negative results for mutant detection (Bando, submitted for publication). Taking different sensitivity and robustness into account, which testing method, ARMS/S or direct sequencing, provides better conformity to the clinical benefit of anti-EGFR antibodies should be further investigated.

Unamplifiable DNA and other contaminants can interfere with PCR-based assays. The results showing that EXO_FAIL warnings were observed among the samples with larger amounts of DNA supports this idea. A threshold determined by the amount of total DNA might be useful to reduce the appearance of EXO_FAIL warnings, compared with a threshold determined by the control Ct value. Theoretically, the control Ct stands for the amount of amplifiable DNA, while the spectrophotometrically determined DNA amount reflects the sum of the amplifiable and unamplifiable DNA. Thus, the above finding seems to suggest that the total DNA concentration is correlated with the incidence of EXO_FAIL warnings. The manufacturer's instruction suggests repeating the assays using diluted DNA samples if this warning appears. In such cases, checking the total amount of DNA, rather than the control Ct of the primary assays, might produce better results. However, DNA dilution sometimes causes a trade-off between a decreasing EXO_FAIL incidence and a decreasing sensitivity. Limited DNA yields from clinical specimens can be quite troublesome in such cases. Improving the quality of DNA samples, for example, by eliminating necrotic tissues and the remains of hemorrhage during macro-dissection, the use of alternative DNA extraction methods that do not require spin-columns or filtering out contaminants with lower molecular weights might help to resolve this problem.

In conclusion, ARMS/S testing using FFPE-derived DNA samples exhibited a good feasibility and robustness that might satisfy the requirements for routine clinical practice. To maintain the reliability of this test, authorized quality assurance/control programs for laboratories should be required, and the merits and demerits of this system versus other *KRAS* mutation tests will need to be further evaluated in clinical use.

Funding

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Conflict of interest statement

None declared.

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ORIGINAL ARTICLE

NARROW BAND IMAGING ENDOSCOPY FOR UNKNOWN PRIMARY TUMOR SITES OF THE NECK

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Abstract: *Background.* Examinations used to search for unknown primary tumors of squamous cell carcinomas of the neck include CT, MRI, laryngoscopy, gastrointestinal endoscopy, and positron-emission tomography (PET). Narrow band imaging (NBI) endoscopy in which an optical color-separation filter is used to narrow the bandwidth of spectral transmittance is also used.

Methods. Twenty-eight patients in whom primary squamous cell carcinomas could not be detected with conventional white light laryngoscopy underwent NBI endoscopy and PET.

Results. Primary lesions were detected with NBI endoscopy in 3 patients, but no primary lesions were detected with PET. However, PET was used to detect a lower gingival cancer and a palatine tonsillar cancer.

Conclusion. Both PET and NBI endoscopy is effective for detecting unknown primary tumors of squamous cell carcinomas of the neck. © 2011 Wiley Periodicals, Inc. *Head Neck* **00:** 000–000, 2011

Keywords: narrow band imaging; positron emission tomography; unknown primary tumor; sensitivity for detecting; less invasive examination

Patients with cancers of the head and neck sometimes present with metastases to the cervical lymph nodes. In many cases, a primary tumor cannot be identified with laryngoscopy, CT, or MRI. Two percent to 9% of metastases to lymph nodes in the head and neck are reportedly from an unknown primary tumor.[1–4]

Modalities used to search for unknown primary tumors of squamous cell carcinoma include CT, MRI, laryngoscopy, gastrointestinal endoscopy, and positron-emission tomography (PET). Some authors recommend that unilateral or bilateral tonsillectomy should be performed for patients with adequate lymphoid tonsillar tissue.[5] Some authors report that the combination of ^{18}fluorodeoxyglucose (FDG)-PET and CT is useful for detecting primary tumors.[6–8] However, there is no consensus about which examinations should be included in an optimal diagnostic evaluation.

In the esophagus, Lugol chromoendoscopy facilitates the detection of lesions at an early stage. However, Lugol staining cannot be used in the head and neck region because it causes severe mucosal irritation, which produces pain and discomfort and can result in aspiration into the airway.

In narrow band imaging (NBI) endoscopy, an optical color-separation filter is used to narrow the bandwidth of spectral transmittance. The filter is placed in the optical system of the illumination. The filter decreases illumination in all wavelengths except for 2 narrow wavelengths. The central wavelengths of the bands are 415 and 540 nm. The image is reproduced in the processor with the information from the illumination of the 2 bands (Figure 1). Narrow bandwidth filters increase the likelihood that malignant lesions will be visualized. Lesions with a well-developed microvasculature are particularly well visualized (Figure 2).

The time required for NBI endoscopy is only 15 to 30 minutes when lidocaine spray is used for local anesthesia, and patients stay at the hospital for only 2 hours. Furthermore, NBI endoscopy is less invasive than gastrointestinal endoscopy. Finally, NBI endoscopy for head and neck lesions is less costly than gastrointestinal endoscopy.

In the present study, we used NBI endoscopy to search for unknown primary tumors of squamous cell carcinomas causing metastatic mucosal lesions of the head and neck.

PATIENTS AND METHODS

The study consisted of 28 patients (22 men, 6 women; median age, 60 years; age range, 36–78 years) who visited our clinic from January 2003 through July 2009 and were found by means of needle aspiration cytologic examination to have squamous cell carcinoma with an unknown primary tumor that could not be detected with conventional white-light laryngoscopy. The N classification was N1 in 3 patients, N2a

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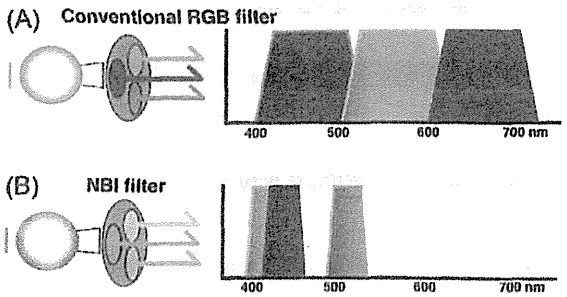

FIGURE 1. Conventional medical video endoscope system with the RGB sequential illumination method (CLV-Q260SL, Olympus Medical Systems, Tokyo, Japan) and the narrow band image (NBI) system. **(A)** The conventional system has a xenon lamp and rotation disk with 3 RGB optical filters. The rotary filter and monochromatic charge-coupled device (CCD) are synchronized, and 3 band images are generated sequentially. Color images can be synthesized by using 3 band images by the video processor. **(B)** The NBI is a novel system using narrow banding filters instead of conventional RGB broadband filters. The center wavelengths of 3 NBI filters using this study were 415, 445, and 500 nm, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in 2 patients, N2b in 17 patients, and N3 in 6 patients. All patients underwent CT, whole-body FDG-PET, NBI endoscopy, and upper gastrointestinal endoscopy. All patients gave written informed consent.

Equipment. The equipment used for NBI endoscopy was a magnifying videoendoscope (Q240Z, Olympus Medical Systems, Tokyo, Japan) and a sequential RGB light source with NBI function (CLV-Q260SL, Olympus Medical Systems). The endoscope had a capability of 80 times optical magnification. The NBI system has been described in detail in previous studies. In this system, the central wavelengths of NBI were 415 and 540 nm, and each had a bandwidth of 30 nm.

RESULTS

Primary tumors were detected in 5 patients (Figure 3). The tumors in 3 patients were detected with NBI endoscopy. In 2 patients, superficial squamous carcinoma were detected in a palatine tonsil, and in the other patient, a thick squamous carcinoma was detected in the pyriform sinus and was identified as a primary lesion that had metastasized to a cervical lymph node. The lesions were thick but were of a color similar to that of normal mucosa, so they could not be visualized with white light (Figure 4). Neither of these primary lesions was detected with PET (Figure 5). However, PET was used to detect a lower gingival cancer and a palatine tonsillar cancer, which were identified as primary lesions metastasizing to the neck. Twenty-three patients were treated for

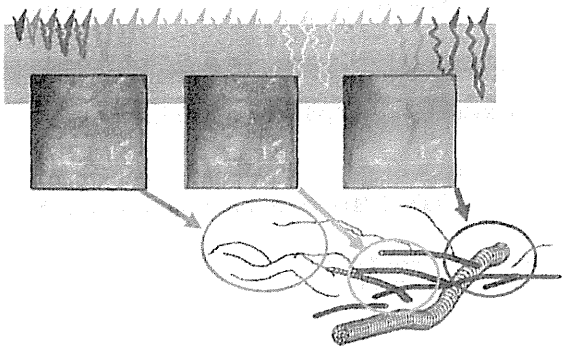

FIGURE 2. Narrow bandwidth filters increase the likelihood that malignant lesions will be visualized. Lesions with a well-developed microvasculature are particularly well visualized. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

unknown primary squamous cell carcinomas. In 1 of these 23 patients, a mesopharyngeal squamous cell carcinoma was detected 2 years after radiation therapy.

DISCUSSION

Classic workups for patients with unknown primary tumors of squamous cell carcinoma include thorough physical examinations of the head and neck and the upper aerodigestive tract, CT, and MRI followed by panendoscopy. Random biopsies of the head and neck region can be useful for detecting primary tumors in patients with squamous cell carcinoma, but the detection rate is only 10%. Ugumori et al[9] have reported that irregular microvascular patterns can be detected more easily with NBI laryngoscopy. Directed biopsy with NBI laryngoscopy may increase the detection rate.[9]

Several studies have evaluated the role of FDG-PET imaging in patients with unknown primary tumors of metastatic squamous cell carcinoma. Based on a literature review by Rusthoven et al,[6] FDG-PET

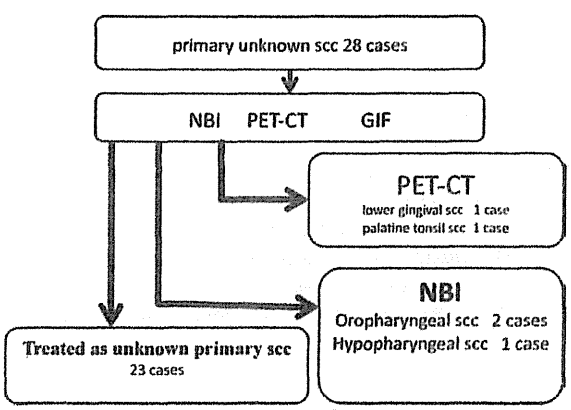

FIGURE 3. Schema of result.

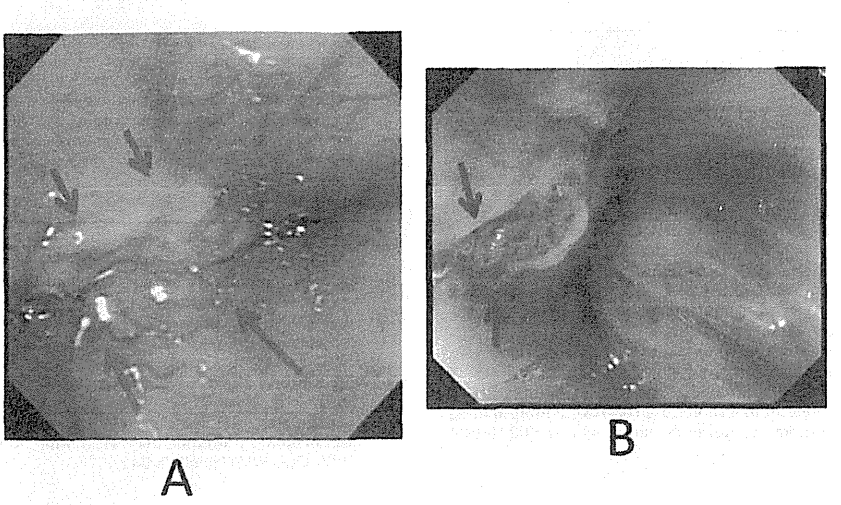

FIGURE 4. Endoscopic findings. **(A)** Conventional white-light image; **(B)** narrow band image (NBI). The cancerous lesion was difficult to visualize with conventional white-light imaging. In contrast, a well-demarcated brownish area in the hypopharynx was detected with NBI. Magnifying observation with NBI revealed an irregular microvascular pattern inside the lesion.

detects approximately 25% of primary tumors not detected with other modalities. In the present series, PET was used to detect tonsillar cancers and gingival cancers.

PET can yield both false-negative and false-positive results. Neither small primary tumors nor low-uptake tumors are detected with PET. Cianchetti et al[8] have suggested that PET is unlikely to improve the probability of detecting unknown primary tumors. On the other hand, when expertise in CT or MRI or both is less developed, PET studies may be useful. We also believe that the discovery of unknown primary tumors will become easier, even without PET, with further technical improvements in CT and MRI.

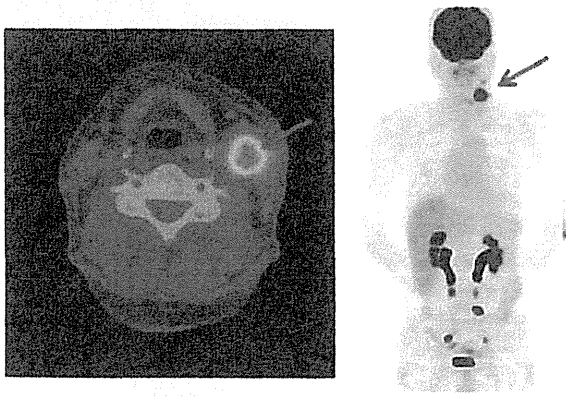

FIGURE 5. With ^{18}fluorodeoxyglucose (FDG)-positron-emission tomography (PET)/CT, only masses in the left upper part of the neck were detected (arrow). The primary lesion of the right hypopharynx was not detected with FDG-PET/CT.

A thorough workup for an unknown primary tumor of squamous cell carcinoma would include examinations of the parenchyma with CT, MRI, PET, and of tubular lumens with endoscopy. A highly skilled endoscopist can often detect cancerous lesions with white-light endoscopy alone, but NBI endoscopy provides greater sensitivity for detecting primary tumors and may decrease the likelihood that lesions will be overlooked.

With NBI endoscopy, cancers of the head and neck can be identified more readily than with conventional white-light endoscopy. Muto et al[10] have reported that NBI can be used to detect superficial cancers of the oropharynx and hypopharynx. Watanabe et al[11] have reported that NBI endoscopy provides high sensitivity and specificity for the diagnosis of superficial laryngeal cancers based on abnormalities of the intraepithelial microvasculature. Hayashi et al[12] have reported NBI endoscopy can be used to detect unknown primary cancers that have metastasized to cervical lymph nodes.

NBI endoscopy is a less invasive examination and is cost-effective. Furthermore, NBI endoscopy provides high sensitivity for detecting abnormal lesions.

Our study supports the usefulness of NBI endoscopy for detecting mucosal lesions. Our findings suggest that the likelihood of detecting unknown primary cancers increases when directed biopsies with NBI laryngoscopy and PET are added to classic workups.

CONCLUSIONS

Both PET and NBI endoscopy are useful for detecting unknown primary tumors of squamous cell carcinoma metastasizing to cervical lymph nodes.

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The Laryngoscope

Salvage Total Pharyngolaryngectomy and Free Jejunum Transfer

Shimpei Miyamoto, MD; Minoru Sakuraba, MD; Shogo Nagamatsu, MD; Ryuichi Hayashi, MD

Objectives/Hypothesis: The objective of this retrospective study was to examine the safety and efficacy of free jejunum transfer after total pharyngolaryngectomy after radiotherapy (RT) or chemoradiotherapy (CRT) to the neck for patients with recurrent or second primary disease.

Study Design: Retrospective study.

Methods: A total of 313 patients were divided into two groups on the basis of a history of RT to the neck: 86 patients had received RT and 227 patients had not. The patients who had received RT were subdivided on the basis of the type of previous treatment: those who had received RT alone (32 patients) and those who had undergone concurrent CRT (54 patients). Postoperative complications were compared between RT and non-RT groups and between the RT-alone and CRT groups.

Results: The rates of complications did not differ significantly between the RT and non-RT groups, but the rates of anastomotic thrombosis and carotid rupture were slightly but not significantly higher in the RT group than in the non-RT group. The overall complication rate did not differ between the RT-alone group and the CRT group.

Conclusions: Pharyngolaryngectomy and free jejunum transfer can be performed safely, even in patients who have received RT, without significant increases in morbidity or mortality. However, a risk of carotid rupture due to pharyngocutaneous fistula remains in patients who have received RT, and prevention and early detection of fistulas are crucial. The risk of postoperative complications is not higher with CRT than with RT alone.

Key Words: Salvage surgery, hypopharyngeal cancer, laryngeal cancer, total pharyngolaryngectomy, free jejunum transfer.

Level of evidence: 3b.

Laryngoscope, 121:947–951, 2011

INTRODUCTION

Salvage surgery after failed organ-preserving therapy for head and neck cancer is becoming increasingly important as radiotherapy (RT) and chemoradiotherapy (CRT) are administered to increasing numbers of patients.[1,2] Reconstruction of the previously irradiated neck is a challenging problem for reconstructive microsurgeons. A history of RT to the neck is associated with an increased risk of wound complications after head and neck surgery.[3–6] To our knowledge, few studies have investigated the effects of previous RT on postoperative complications after total pharyngolaryngectomy (TPL) and free jejunum transfer (FJT).[7–9] In addition, whether the addition of concurrent chemotherapy to RT increases the risk of postoperative complications over that with RT alone is uncertain.[10]

The primary aim of this study was to determine whether previous RT increases the rates of postoperative complications following TPL and FJT. The secondary aim was to determine whether concurrent CRT increases the rates of postoperative complications over those with RT alone.

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MATERIALS AND METHODS

From 1999 through March 2010, a total of 313 patients who underwent FJT after TPL at the National Cancer Center Hospital East, Chiba, Japan, were enrolled in this study. The patients were 264 men and 49 women with a mean age of 64.9 ± 9.0 years (range, 36–88 years).

The 313 patients were divided into two groups on a basis of a history of RT to the neck: 86 patients had received RT and 227 patients had not. The patients who had received RT were subdivided on the basis of the type of previous treatment: those who had received RT alone (32 patients) and those who had undergone concurrent CRT (54 patients).

In the CRT group, the agents used were cisplatin and fluorouracil in 26 patients, cisplatin alone in nine patients, cisplatin and S-1 (a combination of tegafur, 5-chloro-2,4-dihydroxypyridine, and potassium oxonate) in six patients, and other drug combinations in 14 patients. Overall, 48 of 54 patients (88.9%) had undergone platinum-based chemotherapy.

The FJT was performed with our standard method reported previously.[11–13] Barium-swallow examination was performed near the 7th postoperative day in patients who had not received RT. In patients who had received RT, this examination was delayed until about the 14th postoperative day. If the examination showed no leakage, oral feeding was started the same day.

The medical records of 313 patients were analyzed for the following variables: sex, age, medical comorbidity, history of prior neck surgery, and postoperative complications in the acute phase (recipient-site, donor-site, and medical complications). Recipient-site complications analyzed included anastomotic thrombosis, loss of the jejunum graft, infection, hematoma, fistula formation, major fistula formation, neck skin necrosis, and carotid artery rupture. Reoperation for recipient-site complications was also analyzed. Fistula formation was diagnosed on the basis of clinical findings or the findings of barium-swallow

TABLE I.
Comparison of Patient Characteristics Between the Non-RT and RT Groups (N = 313).

	Non-RT	RT	P
No. of cases	227	86	
Sex, no. of patients			
Male	186	78	.08*
Female	41	8	
Mean age, yr (range)	65.6 (36–88)	63.2 (36–79)	.03†
Medical comorbidity (%)	102 (44.9)	34 (39.5)	.44*
Primary site of tumor requiring TPL			
Hypopharynx	176	64	
Cervical esophagus	42	13	
Larynx	7	9	
Thyroid	2	0	
History of neck surgery (%)	10 (4.4)	21 (24.4)	<.01*

Data are numbers of patients unless otherwise indicated.
*Fisher exact test.
†Student *t* test. RT = radiotherapy; TPL = total pharyngolaryngectomy.

examination and therefore included "radiological leakage" that did not progress to pharyngocutaneous fistula. Major fistula was defined as a pharyngocutaneous fistula that required surgical intervention to close or that could not be closed.

Statistical Analysis

Statistical analysis was performed between the RT and non-RT groups and between the RT-alone and CRT groups with a statistical software program (Statcel version 2; OMS Publishing, Saitama, Japan). The Fisher exact test, Student *t* test, and Mann-Whitney *U* test were used. Differences with a *P* value of less than .05 were considered statistically significant.

RESULTS

The data for patients in the RT and non-RT groups are summarized in Table I. Patients in the RT group were significantly younger than those in the non-RT group and were significantly more likely to have a history of neck surgery. The other variables did not differ significantly between the two groups.

Comparisons of postoperative complications between the RT and non-RT groups are shown in Table II. The overall frequency of recipient-site complications did not differ significantly between the groups. However, the rates of anastomotic thrombosis (4.7% vs. 1.3%, $P = .09$) and carotid rupture (2.3% vs. 0.0%, $p = .07$) were slightly but not significantly higher in the RT versus the non-RT group.

The data of patients in the RT-alone and CRT groups are summarized in Table III. The patients in the RT-alone group were significantly older than those in the CRT group and were significantly more likely to have a history of neck surgery. The other variables did not differ significantly between the groups.

A comparison of postoperative complications between the RT-alone and CRT groups is shown in Table IV. The rate of major fistula formation was significantly higher in the RT-alone group than in the CRT group, but the rates of other complications did not differ between the groups.

A comparison of the characteristics of fistulas between the RT and non-RT groups is shown in Table V.

TABLE II.
Comparison of Postoperative Complications Between the Non-RT and RT Groups (N = 313).

Complication	No. of Patients (%) Non-RT (n = 227)	RT (n = 86)	P*
Recipient-site complications	34 (15.0)	18 (20.9)	.23
Anastomotic thrombosis	3 (1.3)	4 (4.7)	.09
Loss of the jejunum	3 (1.3)	3 (3.5)	.35
Infection	20 (8.8)	7 (8.1)	1
Hematoma	6 (2.6)	2 (2.3)	1
Fistula	9 (4.0)	7 (8.1)	.15
Major fistula	3 (1.3)	3 (3.5)	.35
Neck skin necrosis	4 (1.8)	2 (2.3)	.67
Carotid rupture	0 (0.0)	2 (2.3)	.07
Reoperation for recipient-site complications	9 (4.0)	7 (8.1)	.15
Donor-site complications	16 (7.0)	6 (7.0)	1
Medical complications	10 (4.4)	5 (5.8)	.57
Perioperative death	3 (1.3)	2 (2.3)	.62

*Fisher exact test.
RT = radiotherapy.

TABLE III.
Comparison of Patient Characteristics Between the RT-Alone and CRT Groups (n = 86).

	RT Alone	CRT	P*
No. of cases	32	54	
Sex			
Male	30	48	.70*
Female	2	6	
Mean age, yr (range)	67.1 (50–79)	60.9 (36–76)	<.01†
Medical comorbidity (%)	11 (34.4)	23 (42.6)	.50*
Dose of irradiation in Gy, median (range)	69.5 (32–70.4)	70 (38–72)	.15‡
Primary site of tumor requiring irradiation			
Hypopharynx	19	33	
Cervical esophagus	2	11	
Larynx	5	6	
Others	6	4	
Primary site of tumor requiring TPL			
Hypopharynx	25	39	
Cervical esophagus	2	11	
Larynx	5	4	
History of prior neck surgery (%)	12 (37.5)	9 (16.7)	.04*

Data are numbers of patients unless otherwise indicated.
*Fisher exact test.
†Student *t* test.
‡Mann-Whitney *U* test.
RT = radiotherapy; CRT = chemoradiotherapy; TPL = total pharyngolaryngectomy.

TABLE IV.
Comparison of Postoperative Complications Between the RT-Alone and CRT Groups (n = 86).

Complication	No. of Patients (%) RT Alone (n = 32)	CRT (n = 54)	P*
Recipient-site complications	8 (25.0)	10 (18.5)	.58
Anastomotic thrombosis	2 (6.3)	2 (3.7)	.63
Loss of the jejunum	2 (6.3)	1 (1.9)	.55
Infection	3 (9.4)	4 (7.4)	1
Hematoma	0 (0.0)	2 (3.7)	.53
Fistula	4 (12.5)	3 (5.6)	.42
Major fistula	3 (9.4)	0 (0.0)	.048
Neck skin necrosis	0 (0.0)	2 (3.7)	.53
Carotid rupture	1 (3.1)	1 (1.9)	1
Reoperation for recipient-site complications	4 (12.5)	3 (5.6)	.42
Donor-site complications	3 (9.4)	3 (5.6)	.67
Medical complications	3 (9.4)	2 (3.7)	.36
Perioperative death	2 (6.3)	0 (0.0)	.14

*Fisher exact test.
RT = radiotherapy; CRT = chemoradiotherapy.

There were no differences between the groups in the rate of spontaneous closure, the date of fistula onset, or the interval between onset and spontaneous closure. However, fistulas developed in two patients of the RT group more than 3 weeks after surgery. Fistulas in both these patients were resistant to conservative and surgical treatments and remained unclosed even at the time of last follow-up.

There were five perioperative deaths (three in the non-RT group and two in the RT-alone group); therefore, the overall mortality rate was 1.6%. Only one wound-related death occurred in the RT-alone group: a patient died of carotid artery rupture due to pharyngocutaneous fistula. The other causes of perioperative death were acute myocardial infarction in two patients and hepatic failure, cerebral infarction, and ischemic enteritis in one patient each.

DISCUSSION

The present study showed no significant difference in the rates of complications between patients who had received RT and those who had not; however, the rates of anastomotic thrombosis and carotid rupture were slightly but not significantly higher in the RT group than in the non-RT group. There was no significant difference in the rates of complications between the RT-alone and CRT groups, except that major fistula formation was more common in the RT-alone group.

Several studies have found increased rates of major wound complications after head and neck surgery in patients who had received RT.[3,4] However, few studies have focused on salvage TPL, and the practicality of the procedure has not been established.[7,9,14] The results of the present study show that TPL and FJT can be safely performed, even in patients who have received RT, with acceptable rates of morbidity and mortality. On the other hand, life-threatening wound complications, such as carotid rupture, occurred only in patients who had received RT, a result indicating that special care is required for the salvage operation.

The effects of previous RT on anastomotic thrombosis remain controversial.[9,15–18] In the present series, the rate of anastomotic thrombosis tended to be higher in patients who had received RT than in patients who had not. Although the difference did not reach the level of statistical significance, the techniques of dissection and anastomosis for irradiated vessels must obviously be more meticulous. If possible, vessels with less exposure to radiation should be selected by confirming the irradiation field preoperatively.

Fistula formation is the most troublesome complication following TPL and FJT. In the present series, the rate of fistula formation in the RT group was 8.1%, which compares favorably with rates reported previously.[7] However, this rate was still twice that in the non-RT group (4.0%). Fistula formation results in prolonged hospitalization and increased health-care costs. Moreover, fistula formation can easily lead to carotid rupture in patients who have received RT. Both prevention and early detection of fistulas are important after salvage TPL and FJT.

Our patients who did not receive RT routinely started oral feeding 1 week after surgery. However, for patients who have received RT, oral feeding was withheld for at least 2 weeks. The effect of delayed feeding on fistula formation is controversial, and its rationale is unclear.[19,20] We believe that wound healing has progressed sufficiently by 2 weeks, not only at the mucosal suture lines but also around the major vessels, and, therefore, the resulting damage would be localized even if pharyngocutaneous fistula occurred.

Fistulas in patients who have received RT often do not respond to conservative treatment and are less likely to close spontaneously.[4,7] Chang et al. have reported that the rate of spontaneous closure is lower in patients who have received RT (46%) than in patients who have not (90%).[7] In the present series, the rate of spontaneous closure and the time until spontaneous closure did not differ significantly between the RT and non-RT groups.

TABLE V.
Details of Fistulas.

	Non-RT (n = 9)	RT (n = 7)	P
Spontaneous closure (%)	6(66.7)	4 (57.1)	1*
Closure after surgical intervention (%)	1 (11.1)	0 (0.0)	
Persistent (%)	2 (22.2)	3 (42.9)	
Onset of fistula, POD, median (range)	9 (6–15)	14 (4–29)	.11†
Interval between onset and spontaneous closure, d, median (range)	15 (6–34)	18.5 (4–31)	.52†

Data are number of patients except where indicated.
*Fisher exact test.
†Mann-Whitney *U* test.
RT = radiotherapy; POD = postoperative day.

However, fistulas developed more than 3 weeks after surgery in two patients of the RT group. Such late fistulas seem to occur only in patients who have received RT and are often resistant to conservative or surgical treatment. Careful long-term follow-up is necessary for patients who have received RT.

The addition of concurrent chemotherapy to RT increases tissue damage and impairs nutritional status, which are generally believed to lead to poor wound healing and higher rates of postoperative complications.[10,14,21] Multivariate analysis by Ganly et al. has shown that previous CRT is an independent risk factor for both local complications and fistula formation after total laryngectomy.[21] Few studies have reported TPL after failed CRT, and our study involves the largest series reported to date.[8] The results of the present study show that the addition of concurrent chemotherapy to RT does not increase the overall risk of postoperative complications over RT alone in the context of TPL and FJT.

However, the rate of major fistula was significantly higher in the RT-alone group (9.4%) than in the CRT group (0.0%). This paradoxical result can be partly explained by the effect of our learning curve in treating patients who have received RT. The number of patients in whom CRT has failed is increasing in accordance with the recent trend toward organ-preserving therapy.[22,23] In the present series, two thirds of the patients in the CRT group (36 of 54 patients) underwent surgery in the last 6 years of the study period (2005 or later). On the other hand, more than two thirds of the patients in the RT-alone group (22 of 32 patients) underwent surgery in the first 6 years of the study period (2004 or earlier), and all major fistulas in the RT-alone group occurred in this period. This bias should be taken into account when our results are interpreted. In general, tissue fibrosis tends to be more severe with CRT than with RT alone, and, therefore, surgery and postoperative care should be performed with meticulous attention.

Indications for use of an anterolateral thigh flap for TPL reconstruction have recently expanded because of low donor-site morbidity and good speech function with tracheoesophageal puncture.[24] Although fasciocutaneous flaps supposedly have a high fistula rate, Yu et al. have reported that the fistula rate with anterolateral thigh flaps is comparable to that with jejunal flaps.[25,26] They speculated that an extra layer of closure with well-vascularized fascia, which is not available with the radial forearm flap, may contribute to the lower fistula rate of the anterolateral thigh flap.[26] However, few reports of the use of anterolateral thigh flaps for salvage TPL reconstruction, especially after CRT failure, have been published, and the safety of anterolateral thigh flaps in previously irradiated patients has not been established. We believe that FJT should be the procedure of first choice, especially for salvage TPL reconstruction, because of its excellent wound-healing properties and low fistula rate.

We did not consider the difference in the extent of the radiation field due to the primary sites of tumors. In general, RT tends to be more extensive in patients with hypopharyngeal cancers than in patients with laryngeal cancers.[27] For the sake of accuracy, patients with these cancers should not be lumped together because the toxic effects of RT differ significantly. This combining of patients with all cancers into the same treatment-based group is the main limitation of this study. Another limitation is that various CRT regimens were included in this study. Most patients in the CRT group had undergone concurrent platinum-based chemotherapy; however, the intensity of chemotherapy varied among patients. This drawback is a limitation of retrospective studies, and prospective studies are necessary for a more detailed assessment.

CONCLUSION

We retrospectively analyzed the postoperative results of TPL and FJT. This study has shown that TPL and FJT can be performed safely, even in patients who have received RT, without significant increases in morbidity or mortality. However, patients who have received RT are at risk for carotid rupture due to pharyngocutaneous fistula, and prevention and early detection of fistulas are crucial. Furthermore, CRT did not increase the risk of postoperative complications as compared with RT alone.

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