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Innovative Rapid Gene Methylation Analysis of Surgical Margin Tissues in Head and Neck Cancer

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Abstract

Background—Securing the negative surgical margin is the first step in surgical cancer treatment. However, tumor recurrence sometimes occurs even with histologically negative surgical margins. To detect minimal residual cancer cells in the deep margin intraoperatively, a time-efficient molecular approach is required.

Methods—We established an innovative rapid quantitative methylation PCR (QMSP) assay, which consists of substantially time-minimized DNA extraction, bisulfite treatment, and QMSP assays. To demonstrate the feasibility of this procedure, 10 serial surgical specimens of primary head and neck squamous cell carcinoma (HNSCC) were evaluated by both rapid and conventional QMSP. Two frequently methylated genes in head and neck cancer, homeobox A9 (*HOXA9*) and endothelin receptor type B (*EDNRB*) were analyzed in 10 HNSCCs and surgical margin tissues, as well as normal muscle and oral mucosa samples.

Results—The product quality of DNA extraction and bisulfite treatment using the time-saving procedure was comparable to the conventional procedure. In the QMSP assay, target gene methylation and reference gene methylation were equally detected by both the rapid and conventional method. Finally, relative results of rapid and conventional QMSP were quite similar to each other in tumors, margins, and normal tissues. The average total time required for the rapid QMSP procedure was less than 3 h and could be accomplished by a single person.

Conclusion—From the viewpoint of accuracy, cost, and time consumption, the innovative rapid QMSP maintains highly sensitive methylation detection accomplished within the time frame of a major ablative and reconstructive procedure.

Head and neck cancers are usually diagnosed in the advanced stage. More than one-third of tumors are classified as Stage IV, and over 50 % recur during the 2 years after treatment.¹ Securing negative surgical margins is a fundamental and critical goal. Jones et al.² reported a high local recurrence rate of 59 % at 5 years in 49 head and neck cancer cases with histologically positive margins. However, 303 cases with negative margins also had a high

recurrence rate of 42 % in the 5 years after surgery. This may be attributed to residual cancer cells not be detected by histological examination.

Tumor budding, which is defined as a single cancer cell or a cluster of <5 cancer cells in the stroma of the invasive front, is reported in various epithelial cancers, including squamous cell carcinoma (SCC).^{3–6} Studies of this phenomenon show that the presence of tumor budding could be a prognostic marker. A tiny cluster of tumor budding in the surgical margin might not be detected by histological examination, especially in the intraoperative frozen sections. Molecular analysis has the advantage of detecting a small number of cancer cells. Brennan et al.⁷ checked the p53 mutation status of histologically negative surgical margins of head and neck squamous cell carcinoma (HNSCC) using a time-consuming plaque assay. The probability of local recurrence was significantly correlated with molecular evidence of positive margins. No local recurrences occurred in the p53 mutation negative group within 2 years after the surgery. Later, Goldenberg et al.⁸ examined epigenetic alterations in margin samples using the quantitative methylation-specific polymerase chain reaction (QMSP). In some histologically cancer-negative margins, tumor-specific methylation was detected.

Although QMSP provides a highly sensitive detection of tumor-related alterations, it is a time-consuming procedure requiring DNA extraction, bisulfite treatment, and QMSP steps. At least more than 5 h of work by two individuals has been required for this procedure until now.⁸ If surgical margin-positivity is reported after the design, inset, and closure of a flap for the surgical defect, the process of clearing the margin surgically may result in severe compromise to the reconstructive procedure, perhaps even necessitating a second flap. In this paper, we describe an innovative and more efficient QMSP pipeline, which can be accomplished within 3 h by one person without compromising the quality of results or additional cost. We demonstrate how this process may be finished during the reconstruction stage of the operation, therefore, paving the way for its clinical use.

METHODS

Patients and Specimens

Surgical samples were collected from 10 consecutive cases of HNSCC in 2013. Institutional review board approval was obtained before collection of the samples and written informed consent was obtained from each patient. Standard surgical margins were taken intraoperatively, frozen, and read as histologically negative prior to sampling for molecular analysis. Molecular margin sampling was performed on the back table after removing the tumor specimen. The sample was blotted to remove liquid (blood) from the surface to reduce dilution of residual tumor cells. A small tissue sample was then collected from the surgical margin from an area closest to the tumor. A sample of tumor tissue was also collected. As negative controls, samples of normal mucosa and muscle were obtained from the surgical defect far from the tumor. All of the tissues were immediately transferred to the laboratory and processed for DNA extraction, which is described as follows.

DNA Extraction

A fragment of tissue (approximately $5 \times 5 \times 1$ mm) was applied to dry bead tubes of UltraClean Tissue & Cells DNA isolation Kit (MO BIO Laboratories, Carlsbad, CA). Preparations with and without Proteinase K (PK) incubation were performed by following the manufacturer's instructions. The shortest procedure without PK incubation took only 20 min for 50 μ L genomic DNA products. As a basis for comparison, we also conducted conventional DNA extraction as follows: microdissected frozen tissue samples were digested in 1 % SDS (Sigma-Aldrich, St. Louis, MO) with 50 μ g/mL proteinase K (Invitrogen, Carlsbad, CA) at 48 °C for 48 h. DNA was then purified by phenol-chloroform extraction and ethanol precipitation, as previously described.⁹ DNA was resuspended in elution buffer (EDTA 2.5 mM and Tris-HCl 10 mM, pH 7.5). DNA concentration was quantified using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA).

Bisulfite Treatment

BisulFlash DNA Modification Kit (Epigentek, Farmingdale, NY) was used for bisulfite treatment of genomic DNA. Two procedures, one with and one without an additional thermal cycler program were performed by following the manufacturer's instructions. The shortest procedure without an additional thermal cycler program took only 30 min in total yielding 20 μ L of bisulfite-treated DNA. As a basis for comparison, we also used two bisulfite treatment kits of general use in our laboratory: Epitect Bisulfite Kit (Qiagen, Valencia, CA) and EZ DNA Methylation-Lightning Kit (Zymo Research, Irvine, CA), following manufacturer's instructions.

Sanger Sequencing of Bisulfite-Treated Genomic DNA Samples

Bisulfite-treated DNA was amplified using primers that were designed to contain no CG dinucleotides in the promoter region of homeobox A9 (*HOXA9*). Forward primer was 5'-TTTGTGTTGGAGTGTTTGG-3' and reverse primer was 5'-CTCCCTATAAATAATCCCTA-3'.

Polymerase chain reaction (PCR) was performed as follows: 96 °C for 5 min, followed by 35 cycles consisting of a 1-min denaturation step at 96 °C, a 1-min annealing step at 50 °C, and a 2-min initial extension step at 72 °C, with a final extension step at 72 °C for 7 min. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). Purified PCR products were then subjected to direct sequencing (Genewiz, South Plainfield, NJ).

Quantitative Methylation-Specific PCR Analysis (QMSP)

Bisulfite-modified DNA was used as template for fluorescence-based real-time PCR, as previously described.¹⁰ The conventional QMSP measurement was performed as follows: fluorogenic PCR reactions were carried out in a reaction volume of 10 μ L consisting of 600 nmol/L of each primer; 200 mmol/L probe; 0.75 units platinum *Taq* polymerase (Invitrogen); 200 mmol/L each of dATP, dCTP, dGTP, and dTTP; 200 nmol/L ROX dye reference (Invitrogen); 16.6 mmol/L ammonium sulfate; 67 mmol/L Trizma (Sigma-Aldrich); 6.7 mmol/L magnesium chloride; 10 mmol/L mercaptoethanol; and 0.1 % dimethylsulfoxide. Triplicates of 1.5 μ L of bisulfite-modified DNA solution were used in

each real-time methylation-specific PCR amplification reaction. Primer and probe sequences were as follows: actin type B (*ACTB*): 5'-TGGTGATGGAGGAGGTTTAGTAAGT-3', 5'-FAM-ACCACCACCCAACACACAATAACAAACACA-3'TAMRA, 5'-AACCAATAAAACCTACTCCTCCCTTAA-3', endothelin receptor type B (*EDNRB*): 5'-GGGAGTTGTAGTTTAGTTAGTTAGGGAGTAG-3', 5'-FAM-TTTTTATTTCGTCGGGAGGAG-3'TAMRA, 5'-CCCGCGATTAAACTCGAAAA-3', *HOXA9*: 5'-AATAAATTTTATCGTAGAGCGGTAC-3', 5'-FAM-GCGCCCCCATTAACCGTACGCGT-3'TAMRA, 5'-CATATAACAACCTTAATAACACCGAA-3'. Amplification reactions were carried out in 384-well plates in a 7900 Sequence Detector (Applied Biosystems, Foster City, CA) and were analyzed by Sequence Detector System (SDS) 2.4 (Applied Biosystems). Thermal cycling was initiated with a first denaturation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Each plate included patient DNA samples, positive standards (Bisulfite Converted Universal Methylated Human DNA standard (Zymo Research), and multiple water blanks, as no template controls. Serial dilutions (2.4, 0.48, 0.096, 0.0192, 0.00384 ng) of positive controls were used to construct a standard curve for each of the plate. The relative level of methylated DNA (relative QMSP) for each gene was determined as a ratio of its QMSP value to *ACTB* gene's QMSP value $\times 100$ for tabulation. For the rapid QMSP assay, the procedure was optimized to minimize thermal cycling time by 54 %. Thermal cycling was changed to 95 °C for 3 min, followed by 40 cycles at 95 °C for 5 s and 60 °C for 15 s, as recommended in the iTaq Universal Probes Supermix protocol (Bio-Rad Laboratories, Hercules, California).

Statistical Analysis

Relative QMSP data were reported as mean $\pm$ standard error. Continuous variables were compared using the Mann-Whitney *U* test. All statistical analyses were performed using JMP 9 software (SAS Institute, Cary, NC). The level of statistical significance was set at $P < 0.05$.

RESULTS

The QMSP pipeline consists of three steps. DNA extraction is followed by a bisulfite treatment step and the QMSP assay step. The conventional QMSP research pipeline takes approximately 4–5 days to complete in our research laboratory. To adapt our QMSP research pipeline for clinical use, we minimized the total sample-to-results time while maintaining convenience and accuracy.

Hepatocellular carcinoma (HCC) specimens were used for process optimization. In the DNA extraction step, we used the shortest method without PK digestion using UltraClean Tissue & Cells DNA Isolation Kit (MO BIO Laboratories). Table 1 shows the results of various kinds of DNA extraction methods. Although the conventional method (number 4) produced the highest amount of DNA, the shortest method (number 1) also yielded enough high-quality DNA in considerably less time (20 min vs. 3 days).

Next, the bisulfite treatment step was evaluated. Table 2 shows four protocols using kits from various manufacturers (numbers 5–8). Genomic DNA (0.5 μ g) was collected with the

fastest protocol (number 1) in step 1, which was used for all four procedures. As a sample of a promoter methylated gene, we selected the homeobox A9 gene (*HOXA9*), which is reported as one of the frequently methylated genes in HCC.¹¹ Direct bisulfite sequencing results of the *HOXA9* promoter region are shown in Fig. 1. The shortest protocol (number 5) showed quality almost equal to the other samples.

We then compared *ACTB* values obtained with the rapid QMSP protocol to those obtained with the conventional QMSP protocol using five serial dilutions of standard DNA. The results of amplification curves and standard curve plots are drawn in Fig. 2. Slope and Y-intercept values for the two groups were very similar to each other. QMSP values of *HOXA9* and *EDNRB* using five serial dilutions of positive standard DNA also showed similar results (data not shown). Thus, minimizing QMSP time setting maintains the accuracy of QMSP results.

Finally, we examined 10 sequential head and neck surgical samples for evaluating the feasibility of this innovative rapid QMSP. All of them were squamous cell carcinoma samples. Patient data is listed in Table 3. Besides *HOXA9*¹² we added another frequently methylated biomarker, *EDNRB*.¹³ DNA extraction (number 1) and bisulfite treatment (number 5) were performed by the fastest methods (Table 1). Relative QMSP values of *HOXA9* and *EDNRB* are displayed in Fig. 3. Seven of 10 tumors showed *EDNRB* methylation, and 9 of 10 tumors showed *HOXA9* methylation (Fig. 3a-1). A few normal mucosa tissues showed low grade methylation of both genes (Fig. 3a-2). Only *EDNRB* methylation of Case 5 showed an inverse pattern that was positive in mucosa and negative in the matched tumor. We also examined methylation status of normal muscle tissue because all margin samples were collected from the deep margin tissue, which mainly consists of muscle (Fig. 3b-2). Only one normal muscle (Case 3) had methylation of either gene. However, margin tissue of Case 3 showed no methylation of both genes. This might be because this margin sample did not contain muscle tissue, but fat or other connective tissue instead. Margin positive cases in this methylation study were Cases 1 and 7 (Fig. 3b-1). In almost all samples, there was no significant difference between relative QMSP values of rapid QMSP (*blue bar*) and conventional QMSP (*red bar*); even in the margin samples, all samples were either both positive or both negative.

The average time for the whole procedure was 162.4 min (150.0–172.0 min) performed by one person. This is almost half the time that was previously reported.⁸ Total cost was the same or lower than using the conventional method.

Average follow-up period was 153 days (43–276 days). During this brief time, one recurrent tumor was detected (Case 1), in which the surgical margin tissue was positive by rapid QMSP analysis, despite histologically negative margins. The recurrent site was localized at the pharyngeal wall.

DISCUSSION

Head and neck cancer has been associated with several hypermethylated genes.¹⁴ One possible mechanism is the exposure to tobacco carcinogens.¹⁵ Cigarette smoke is considered

one of the most powerful environmental modifiers of DNA methylation.^{16,17} Smoking-related cancers, including lung¹⁸ and bladder cancer,¹⁹ are also associated with a higher frequency of methylated gene promoters. As for esophageal SCC, smoking duration has a significant correlation with promoter methylation status of some genes, including *HOXA9*.²⁰ Our results suggest that detection of promoter methylation in HNSCC-specific genes can be one of the most effective approaches to identify head and neck cancer cells in surgical margins.

HOXA9 gene encodes a DNA-binding transcription factor that regulates gene expression, morphogenesis, and differentiation. Frequent promoter methylation of *HOXA9* has been detected in lung cancer, bladder cancer, and head and neck cancer.^{12,21–23} A study from our laboratory showed *HOXA9* promoter methylation had 85 % sensitivity and 97 % specificity for histological diagnosis of HNSCC.¹² The *EDNRB* gene encodes a protein called endothelin receptor type B, which is located on the surface of cells and functions as a regulator of several critical biological processes, including the development of blood vessels and the stimulation of cell growth and division. Frequent promoter methylation of *EDNRB* has been detected in gastric cancer, prostate cancer, and head and neck cancer.^{13,24–26} A study from our laboratory indicated this gene was methylated in 97 % of primary HNSCC tissues and 6.6 % of normal control salivary rinses.¹³

It is necessary for surgical margin analysis that candidate gene methylation is expressed in the tumor. The target genes selected for this study are exquisitely sensitive biomarkers of HNSCC. Even then their methylation levels show some interpatient variability. Thus, a personalized molecular oncology strategy is likely to increase the benefit of the proposed approach to identify head and neck cancer cells in surgical margins. A tumor and a contralateral normal oral mucosa sample can be collected in advance of surgical extirpation (at preoperative biopsy), and genome-wide DNA methylation profiles can be obtained for this individual patient and compared for their normal mucosa, as well as to our database of genome-wide DNA methylation results for normal mucosa samples. Tumor-specific genes can be preselected for analysis of matched margin samples for that patient. Selected genes also need high specificity because, if gene methylation occurs in normal tissues, it becomes necessary to set a clear threshold to indicate the presence of tumor in margin samples. In our cases, a few normal tissues showed candidate gene methylation. Identification of patient-specific and tumor-specific methylated gene promoters will enhance future margin analysis, ushering the era of personalized molecular oncology solutions into the operating room.

The clinical value of molecular margin analysis depends on its positive association of minimal residual disease with recurrence. A previous report from our laboratory summarized the correlation of four methylated genes, including *EDNRB*, with survival outcomes in a cohort of head and neck cancer patients.²⁴ The results showed that promoter gene methylation in tumor tissue is not a significant prognostic factor for HNSCC. However, the prognostic significance of promoter methylation in margin tissues is under examination in our ongoing study. When the predictive value of margin analysis using methylation markers is confirmed, the novel rapid QMSP pipeline we have developed promises to yield useful information portending tumor recurrence intraoperatively. It is provocative and encouraging that during our short clinical follow-up period, a recurrent tumor was found in a patient

whose margin tissue was positive for *HOXA9* methylation while being histologically tumor-free.

In conclusion, the innovative rapid QMSP pipeline is feasible for intraoperative diagnosis with comparable duration, cost, and accuracy when compared with conventional QMSP.

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FIG. 1.

Sequencing results of four types of bisulfite treatment are displayed. There were seven CpGs in the promoter region of *HOXA9* (−680 ~ −632 bp). All four types (#5–8) showed almost the same extent of methylation in every CpG site, which were not completely methylated

(a) Rapid QMSP for ACTB

(b) Conventional QMSP for ACTB

FIG. 2.

Standard curves of both rapid (a) and conventional (b) QMSP of *ACTB* gene are shown. Five serial dilutions of positive controls were examined. *Amplification curves* are illustrated on the *left*, and *dot-plot standard curves* on the *right* by Sequence Detector System (SDS) 2.4 software (Applied Biosystems). Values of *slope* and *Y-intercept* were very similar

FIG. 3. Relative QMSP values of tumors (**a-1**), normal mucosa tissues (**a-2**), margin tissues (**b-1**), and normal muscle tissues (**b-2**) are shown (average $\pm$ standard error). Values from rapid QMSP (blue bar) and conventional QMSP (yellow bar) are compared. Ten sequential samples are displayed on the *x axis*, and relative QMSP values are on the *y axis* in a logarithmic manner

TABLE 1

Four procedures of DNA extraction

Number (#)	Sample	Method	PK digestion	Digestion time	Total time	Final volume (uL)	Concentration (ng/uL)	A260	260/280	260/230
1	HCC	MO BIO	-	-	20 min	50	80.13	1.603	1.83	1.96
2	HCC	MO BIO	+	15 min	35 min	50	147.05	2.941	1.90	2.24
3	HCC	MO BIO	+	30 min	50 min	50	155.43	3.109	1.88	2.14
4	HCC	Conventional	+	2 days	3 days	100	294.57	5.891	2.09	1.80

HCC hepatocellular carcinoma, *MO BIO* MO BIO Laboratories, Carlsbad, CA

TABLE 2

Four procedures of bisulfite treatment

Number (#)	Sample	Method	Incubation time (min)	Total time (min)	Final volume (μL)
5	HCC	BisulFlash DNA Modification Kit (short version)	20	30	20
6	HCC	BisulFlash DNA Modification Kit (long version)	132	142	20
7	HCC	EpiTect Bisulfite Kit	330	360	40
8	HCC	EZ DNA Methylation-Lightning Kit	68	105	10

HCC hepatocellular carcinoma

TABLE 3

Clinical data of 10 sequential HNSCC cases

Case number	Age	Gender	Tumor site	Operation	Size (cm)	Pathology	TNM stage	Pathological margin status	Venous lymphatic invasion	Perineural invasion	Close margin (<1 mm)	
1	60	Male	Hypopharynx	Pyrimform sinus	Pharyngectomy, laryngectomy	3.0	SCC	Poor	pT4aN0M0	Uninvolved	Present	No
2	68	Male	Oral cavity	Mandible	Mandibulectomy	0.5	SCC	Mod	pT1N0M0	Uninvolved	n.a.	No
3	70	Female	Larynx	Supraglottis	Laryngectomy	2.0	SCC	Mod	pT1N0M0	Uninvolved	n.a.	No
4	82	Male	Oral cavity	Tongue	Glossectomy	3.5	SCC	Mod	pT2N2bM0	Uninvolved	n.a.	Yes
5	52	Male	Oral cavity	Floor of mouth	Mandiblectomy, glossectomy	5.2	SCC	Poor	pT4aN2cM0	Uninvolved	Present	Yes
6	65	Male	Hypopharynx		Pharyngectomy, thyroidectomy	5.5	SCC	Mod	pT4aN1M0	Uninvolved	Present	Yes
7	78	Female	Oral cavity	mandible	Mandibulectomy	3.1	SCC	Well	pT4aN2bM0	Uninvolved	Absent	No
8	54	Male	Hypopharynx	pyrimform sinus	Pharyngectomy, laryngectomy	2.0	SCC	n.a.	pT3N2bM0	Uninvolved	Present	No
9	61	Male	Oral cavity	tongue	Glossectomy, laryngectomy	5.0	SCC	Mod	pT4N1M0	Uninvolved	Present	Yes
10	59	Male	Larynx	Glottis	Laryngectomy	3.3	SCC	Mod	pT4aN0M0	Uninvolved	n.a.	No

HNSCC head and neck squamous cell carcinoma, *n.a.* not applicable, *SCC* squamous cell carcinoma