Quantitative detection of circulating tumor-derived mitochondrial NADH subunit variants as a potential prognostic biomarker for oral cancer

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Abstract. Circulating tumor cells (CTCs) and/or their relating molecules are promising determinants during the course of cancer treatment, especially for post-therapeutic monitoring. We recently reported the clinical relevance of detecting circulating tumor-associated mutant mitochondrial DNAs (mut-mtDNAs) at three different regions including the displacement loop, 12S-rRNA and 16S-rRNA in oral squamous cell carcinomas (OSCCs). In the present study, to further investigate if the other mut-mtDNAs have novel efficiency for detecting potential tumoral micrometastasis, mut-mtDNAs on the ND2 and ND3 regions of the genome in 240 clinical samples from patients with OSCC were assessed *in vitro* and *in vivo* by quantitative real-time PCR combined with high-resolution melting curve analysis. Furthermore, the clinical relevance was evaluated by the area under the receiver operating characteristic curve (AUC) analysis. Three discrete sequence variations were identified in OSCC derived cell lines at the regions of ND2 (T:A to C:G at position 5108) and ND3 (A:T to G:C at position 10397 and C:G to T:A at position 10400), whereas no mutation was observed in normal control human normal oral keratinocytes. In OSCC patients examined, the presence of mut-mtDNAs in serum during the postoperative period accurately predicted poor prognoses (ND2 AUC, 0.761; ND3 AUC, 0.704). The data presented here provide a novel approach for detecting the circulating mut-mtDNAs that are promising molecular markers for evaluating tumoral micrometastasis in OSCCs.

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Abbreviations: CTCs, circulating tumor cells; mut-mtDNA, mutant mitochondorial DNAs; OSCC, oral squamous cell carcinoma; qRT-PCR-HRMA, quantitative real-time polymerase chain reaction combined with high-resolution melting curve analysis; hNOKs, human normal oral keratinocytes; ROC, receiver operating characteristic; CI, confidence interval; SNP, single-nucleotide polymorphism

Key words: circulating tumor-derived DNA, mitochondrial DNA, high-resolution melting curve analysis, oral squamous cell carcinoma, prognosis

Introduction

Since conventional approaches cannot detect micrometastasis with high sensitivity, development of novel and effective methods is needed. Circulating tumor cells (CTCs) and/or their specific molecules are important determinants for predicting poor prognosis in patients with cancer (1-4). CTCs also are measured to assess the therapeutic effects of chemotherapy, radiotherapy and chemoradiotherapy (5-8). CTCs or tumor-associated DNAs also have been detected frequently in serum samples from poorly diagnosed patients with oral squamous cell carcinoma (OSCC) (9,10), indicating that this type of blood test is useful during cancer treatment and follow-up to monitor patients for recurrent or metastatic lesions.

Due to low cellular copy numbers of genomic DNAs in serum samples, isolating sufficient DNA for molecular analyses can be difficult. Considering this, we recently reported the clinical relevance of detecting tumor-derived mutant mitochondrial DNAs (mut-mtDNAs) at the regions including the D-loop, 12S-rRNA and 16S-rRNA, the copy numbers of which are much higher than those of genomic DNAs (11). To examine whether discrete mutation(s) may exist in the mitochondorial genome, and moreover, detection of CTCs with the mutant mitochondrial DNA could be useful to predict micrometastasis of patients with OSCC with no histologic evidence of cancer cells in their surgical margins, we used a comprehensive approach for detecting tumor-derived mut-mtDNAs in the ND2 and ND3 regions by quantitative real-time polymerase chain reaction combined with high-resolution melting curve analysis (qRT-PCR-HRMA). This investigation showed

Table I. The primer sequences used for PCR amplification.

	Primer sequence (5'-3')	Nucleotide positions	Product size (bp)
ND2-1F	CCTATCACACCCCATCCTAAA	4382-4402	157
ND2-1R	GCTTAGCGCTGTGATGAGTG	4519-4538	
ND2-2F	TACCATCTTTGCAGGCACAC	4502-4521	175
ND2-2R	GATTATGGATGCGGTTGCTT	4657-4676	
ND2-3F	GTTCCACAGAAGCTGCCATC	4621-4640	191
ND2-3R	TCAGAAGTGAAAGGGGGCTA	4792-4811	
ND2-4F	GGAATAGCCCCCTTTCACTT	4788-4807	215
ND2-4R	ATTTTGCGTAGCTGGGTTTG	4983-5002	
ND2-5F	CCATCATAGCAGGCAGTTGA	4951-4970	228
ND2-5R	GCTTGTTTCAGGTGCGAGAT	5169-5188	
ND2-6F	TCGCACCTGAAACAAGCTAA	5162-5181	208
ND2-6R	GGTGGAGTAGATTAGGCGTAGG	5348-5369	
ND2-7F	CACCATCACCCTCCTTAACC	5318-5337	248
ND2-7R	TGCAACTTACTGAGGGCTTTG	5545-5565	
ND3-1F	CCGTTAACTTCCAATTAACTAGTTTTG	10012-10038	164
ND3-1R	GCACTCGTAAGGGGTGGAT	10157-10175	
ND3-2F	ACCACAACTCAACGGCTACA	10130-10149	169
ND3-2R	TTGTAGGGCTCATGGTAGGG	10279-10298	
ND3-3F	CCCTCCTTTTACCCCTACCA	10267-10286	219
ND3-3R	TGTAAATGAGGGGCATTTGG	10466-10485	

compelling evidence that evaluation of circulating tumor-derived mitochondrial DNAs with ND2 and/or ND3 mutation may be an additional clinical tool to monitor the post-operative patients with OSCC.

Materials and methods

Ethical statement. The study protocol was approved by the Ethics Committee of the Graduate School of Medicine, Chiba University (approval number, 236) and was performed in accordance with the ethical standards laid down in the Declaration of Helsinki. Written informed consent was received from all patients or their families.

All experimental animals were treated and cared for in accordance with the guidelines of Chiba University. Experimental animals were sacrificed by cervical dislocation. We made every effort to relieve the pain of experimental animals. The protocol was approved by the Committee on the Ethics of Animal Experiments of Chiba University (approval number, 25-221).

Mutation detection of mtDNA for OSCC cell lines in vitro and in vivo. The human OSCC-derived cell lines Sa3 and HSC-4 were purchased, respectively, from the RIKEN BioResource Center through the National Bioresource Project of the Ministry of Education, Culture, Sports, Science and Technology (Tsukuba, Japan) and the Human Science Research Resources Bank (Osaka, Japan). A DNA profiling procedure validated the cell lines (11). The cells were cultured in the same manner as previously reported (12).

Ten sets of specific PCR primers were prepared for amplification of regions ND2 and ND3 of the human mitochondrial genome. The primer sequences are shown in Table I. The PCR products were subcloned into a pCR8/GW/TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA), and then sequenced using ABI 3730xl DNA sequencers (Applied Biosystems, Foster City, CA, USA) to validate the identity of the amplified products by comparing them with the MITOMAP database (www.mitomap.org/MITOMAP/Human MitoSeq).

We optimized the conditions and examined the feasibility of using qRT-PCR-HRMA for detecting three discrete sequence variations (ND2-T5108C, ND3-A10397G and ND3-C10400T) in Sa3 and HSC-4 cell lines. Using specific PCR primer sets (Table I), qPCR-HRMA was performed using a LightCycler 480 system (Roche Diagnostics GmbH, Mannheim, Germany) in a final volume of 20 μl of a reaction mixture comprised of 10 μl of LightCycler 480 High Resolution Melting Master Mix (Roche), 3 mM of $MgCl_2$, and 4 μM of the primers, according to the manufacturer's instructions.

The *in vivo* experiments, i.e., detecting Sa3-derived mut-mtDNAs of the ND2 and ND3 regions in serum samples from BALB/cAnNCrj-nu/nu mice (n=2, Charles River Laboratories, Yokohama, Japan), were performed according to our previous methods (11). In brief, to validate whether mut-mtDNA of the ND2 and/or ND3 regions in human oral cancer cells were detectable quantitatively from peripheral blood samples,

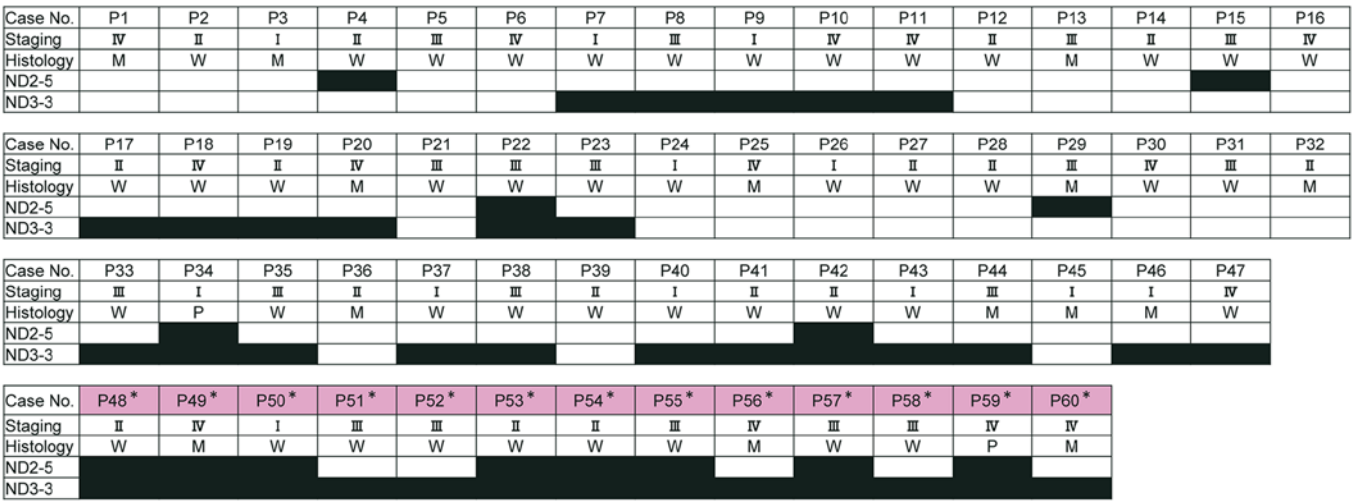

Figure 1. Status of mut-mtDNA from 60 patients with OSCC with surgical malignancy-free margins. Note that the sera at post-operative period were defined as positive (black boxes) for mut-mtDNA in at least one region examined in all poor prognosis patients (light red boxes). W, well-differentiated SCC; M, moderately differentiated SCC; P, poorly differentiated SCC.

we transplanted Sa3 cells ($2x10^6$) by subcutaneous injection into BALB/cAnNCrj-nu/nu mice (n=2, Charles River Laboratories). The mice were sacrificed after 6 weeks as previously described (11). The non-Sa3 transplanted mice (n=2) were used as controls and their serum samples were collected. MtDNA was extracted from them for qRT-PCR-HRMA. All mice were maintained under specific pathogen-free conditions. Environmental conditions were a temperature of 24 ±2˚C, humidity of 50±10%, lighting of 300 lux and a 12:12 light:dark cycle with lights on at 07:00 and off at 19:00. The mice were housed individually in 210x300x225 mm cages.

The committee of the Chiba University Laboratory Animal Center reviewed and approved the protocol.

Determination of mut-mtDNAs in patients with OSCC. Sixty patients with newly diagnosed OSCC with surgical malignancy-free margins were included. The patients were divided into two groups: 47 patients with a good prognosis with no recurrence and/or metastasis and 13 patients with a poor prognosis with a recurrence or metastasis 17 months post-operatively. Additional patient information is shown in Fig. 1.

Overall, we analyzed 240 mtDNAs comprised of normal tissue, tumoral tissue, pre-operative serum samples, and serum samples obtained 4 weeks post-operatively from each patient. To quantify the mut-mtDNAs in each sample, the qRT-PCR-HRMA procedure was performed as previously described (11) with specific primer sets for the ND2 region and the ND3 region (Table I). The amount of each mut-mtDNA in the samples was determined based on the standard curves that were created by diluting mut-mtDNA from Sa3 or HSC-4 with wild-type mtDNAs to prepare 100, 75, 50, 35, 20 and 0% mutated samples for detecting the ND2 or ND3 region in the mtDNA genome as previously described (11).

All results, expressed as the mean ± standard error of the mean, were similar among experiments repeated three times. P-values were analyzed using the Mann-Whitney U-test. $P<0.05$ was considered significant. Statistical analyses were performed using Microsoft Office Excel 2010 (Microsoft, Seattle, WA, USA). For receiver operating characteristic (ROC) curve analysis, EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan) (13) was used. We also utilized the area under the ROC curve (AUC) values with estimated odds ratios and 95% confidence intervals (CIs) to evaluate the diagnostic relevance for predicting the serum mut-mtDNAs in patients with a poor prognosis.

Table II. Comparison of Sa3 specific mut-mtDNA levels between xenografted and control mice.

Mouse	Analyzed regions	Serum
No. 1	ND2	23%
	ND3	50%
No. 2	ND2	17%
	ND3	45%
No. 3	ND2	0%
	ND3	0%
No. 4	ND2	0%
	ND3	0%

Results

Three homoplasmic nucleotide substitutions defined as single-nucleotide polymorphisms (SNPs) were identified in the ND2 region (T:A to C:G at position 5108) in HSC-4 cells and the ND3 region (A:T to G:C at position 10397 and C:G to T:A at position 10400) in Sa3 cells; no mutation was observed in normal control human normal oral keratinocytes (hNOKs) (Fig. 2A and B). In blood samples from Sa3-xenografted mice, we detected mut-mtDNAs identical to Sa3-associated mut-mtDNAs, but control mice did not have mut-mtDNAs (Table II). The results indicated that this blood test is clinically useful for detecting tumor-related mut-mtDNAs. Based on the melting curves separated by the HRMA chromatogram, we created a standard curve by serial dilution of the DNA

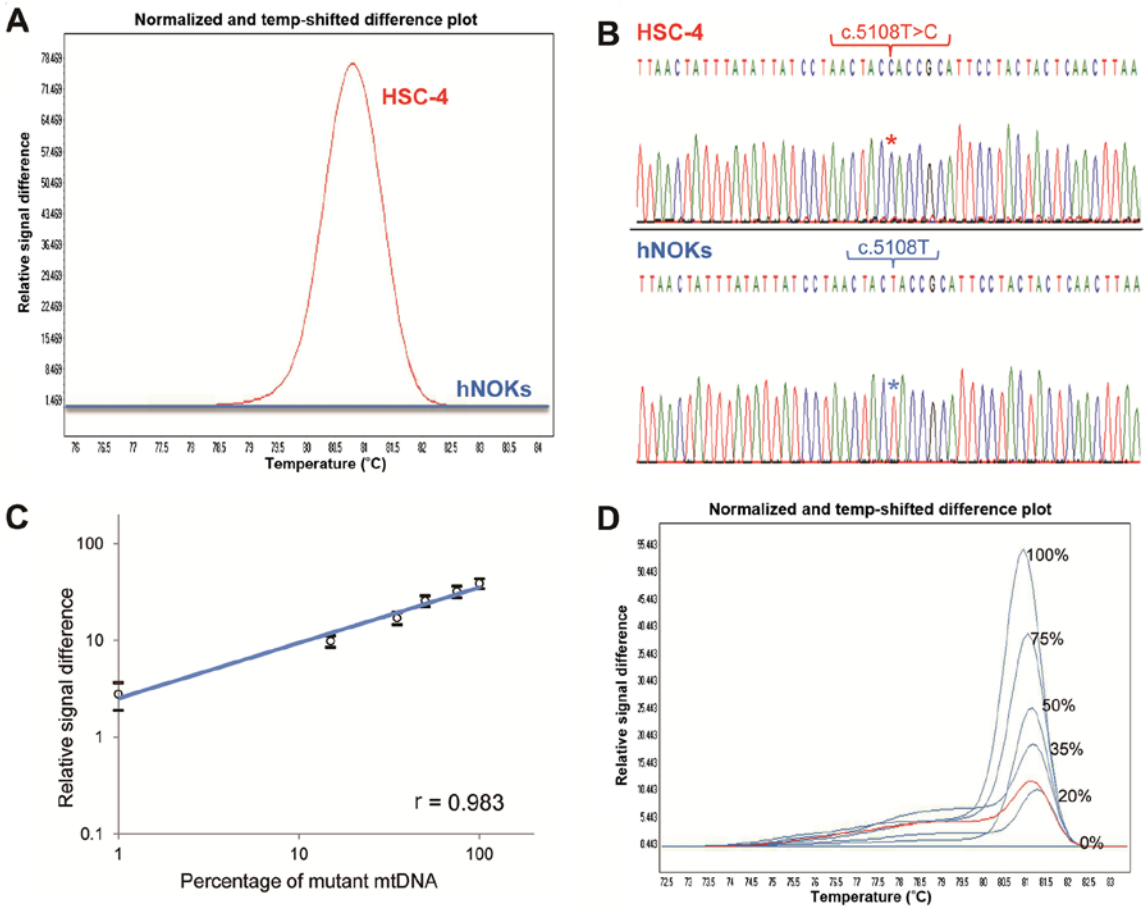

Figure 2. Determination of a novel variation of the human mitochondrial genome in oral squamous cell carcinoma cells. A representative result of quantitative real-time polymerase chain reaction combined with high-resolution melting curve analysis (qRT-PCR-HRMA) followed by DNA sequence analysis of the HSC-4 cells clearly shows a distinguishable peak (red line) compared with human normal oral keratinocytes (hNOKs) (blue baseline) (A) as a result of a variant sequence (c. 5108T>C at the ND2 region), whereas the hNOKs show wild-type (normal) sequences (B). (C) A plotted standard curve for the ND2 region. The levels of the relative signal differences obtained by qRT-PCR-HRMA (y-axis) are reported as percentages of the mutant mitochondrial DNAs (mut-mtDNAs) by Microsoft Office Excel 2010. The coefficient of correlation is high (r=0.98304). (D) Determination of the mut-mtDNA level in the serum from a Sa3-xenografted mouse (mouse 1). The standard curves were created by diluting mut-mtDNAs (ND2-T5108C) with wild-type mtDNAs to prepare 100, 75, 50, 35, 20 and 0% mutated samples for detecting the ND2 in the mtDNA genome. The fluorescence of the serum sample (red line) normalizes as a differential signal against each standard curve in light blue, enabling detection of 23% of mutant mtDNA in the ND2 region.

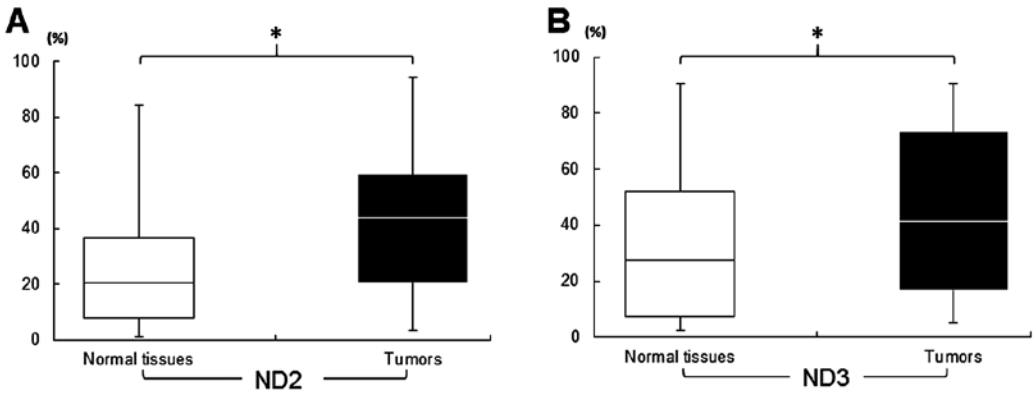

Figure 3. Comparison of mut-mtDNA levels between tumors and corresponding normal tissues. The statistical significance of the data was determined using the Mann-Whitney U test. P<0.05 was considered significant. The data are expressed as the mean ± standard error of the mean. The horizontal indentations in the boxes (white, normal tissues; black, tumor tissues) indicate the medians. *P<0.05 compared with normal tissues. All experiments were performed in triplicate.

from hNOKs (Fig. 2C and D) and detected the mut-mtDNA amounts in samples from the mice and humans examined. Typical results are shown in Fig. 2D.

As previously described (11,14), we isolated sufficient mtDNAs for analysis from clinical samples (n=240) from 60 patients with OSCC (746.8±476 ng/μl, tissue samples;

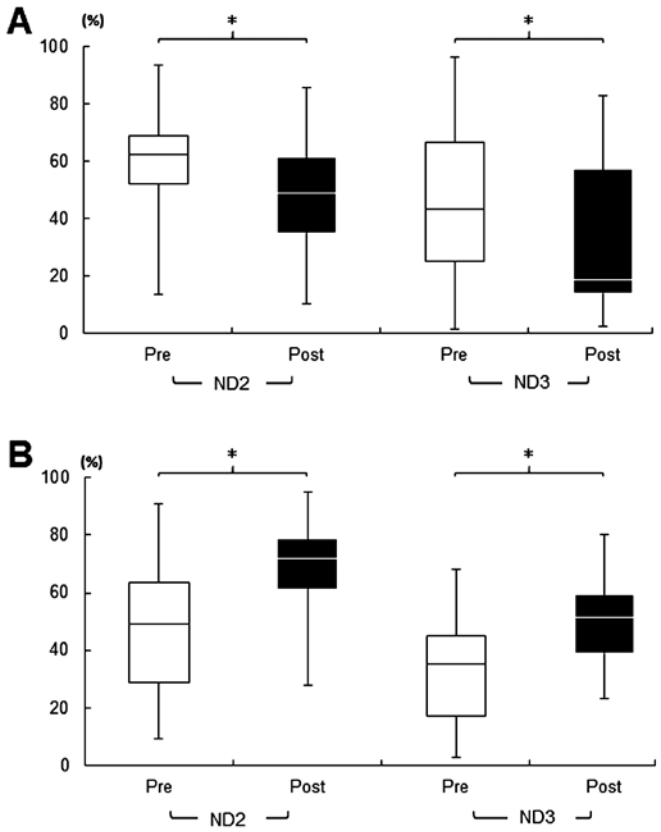

Figure 4. The status of mutant mitochondrial DNA (mut-mtDNAs) levels in patients with oral squamous cell carcinoma preoperatively and postoperatively. The cases without recurrence/metastasis have significantly decreased mut-mtDNAs postoperatively compared to preoperatively (A), whereas significantly increasing mut-mtDNA levels are detected at all regions in serum samples obtained postoperatively from patients with a poor prognosis (B). The statistical significance of the data was determined using the Mann-Whitney U test. P<0.05 is considered significant. *P<0.05. The data are expressed as the mean ± standard error of the mean. The horizontal lines indicate the medians. All experiments were performed in triplicate.

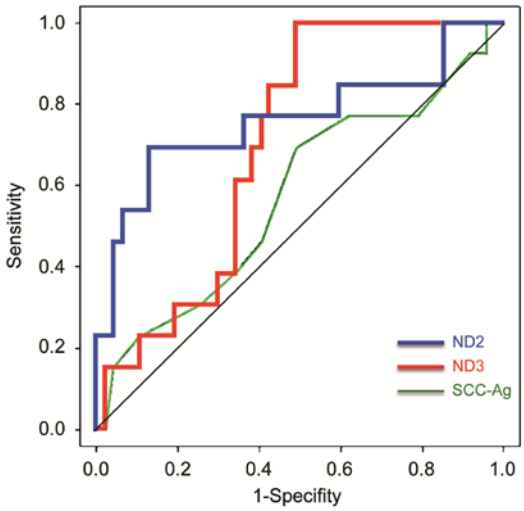

Figure 5. Comparison of the area under the receiver operating characteristic (ROC) curve (AUC) of three diagnostic models based on ND2, ND3 or SCC-antigen (SCC-Ag) in sera. To evaluate the diagnostic relevance for predicting the recurrence/metastasis of serum mut-mtDNAs, we used the ROC curve by plotting the sensitivity vs. the specificity. The AUCs for mut-mtDNAs are 0.761 for ND2 (blue line), 0.704 for ND3 (red line), and 0.574 for SCC-Ag (green line). The AUC is a quantitative measure of the success of predicting mut-mtDNAs through comparisons of poor prognosis. The statistical significance of the study data was determined using the Mann-Whitney U-test. P<0.05 was considered significant.

761.7±340 ng/μl, blood samples). In resected tissues, a significantly (P<0.05) higher concentration of mut-mtDNA was detected in tumoral tissues from patients with a good prognosis and a poor prognosis, compared to each normal counterpart (Fig. 3). The blood test analyzed by qRT-PCR-HRMA indicated that surgery significantly (P<0.05) decreased the circulating mut-mtDNAs in patients with OSCC without recurrence and/or metastasis (Fig. 4A). Compared to the group with a good prognosis, a significant (P<0.05) increase in the circulating tumor-associated mut-mtDNAs was confirmed in the blood samples obtained postoperatively from patients with a poor prognosis (Fig. 4B), all of whom had substantial mut-mtDNA in their serum, without exception, in at least one region examined (Fig. 1).

The area under the ROC curve (AUC) values were more sensitive across a range of mut-mtDNA levels in the sera for the risk of recurrence/metastasis than serum SCC antigen (SCC-Ag) levels (Fig. 5). Using the optimal threshold values of 68% (sensitivity, 61.5%; specificity, 87.2%) for ND2, 22.9% (sensitivity, 92.3%; specificity, 51.1%) for ND3, and 1.0 ng/ml (sensitivity, 69.2%; specificity, 51.1%) for SCC-Ag, each AUC was 0.761 [95% confidence interval (CI), 0.580-0.9421, P<0.05], 0.704 (95% CI, 0.5696-0.838, P<0.05) and 0.574 (95% CI, 0.386-0.761, P=0.793), respectively.

Discussion

CTCs are promising clinical tools in many human cancers (15-17). Evidence indicates that the epithelial cell adhesion molecule is one of the most useful molecular markers for detecting CTCs, including human SCCs (18,19). In contrast, Wirtschafter *et al* (20) reported that CTCs were validated only in a small portion of patients with head and neck SCC, suggesting limited clinical application.

The present study, in which a unique set of human OSCC specimens was used, found that circulating mut-mtDNAs at the ND2 and/or ND3 regions are significant predictive biomarkers for postoperative recurrence/metastasis in OSCC. Nawroz *et al* (21) first reported their potential clinical use by detecting tumor-derived microsatellite alterations in serum genomic DNAs in patients with head and neck cancer. Recently, accumulating data on circulating mtDNAs have been published on malignant tumors (22-24) and other human diseases (25-28). From a clinical standpoint, there are several benefits to adopting mtDNA for clinical blood tests: DNA, including genomic DNA and mtDNA, is more stable than RNA, including mRNA and microRNA, and extracted protein; as He *et al* (14) and we (29) reported, the copy number of the mtDNA is hundreds to thousands of times higher than that of genomic DNA; and a high rate of somatic sequence variations resulting in a pathogenic state are present in patients with OSCC.

We identified cancer-specific somatic variants in the ND2 and ND3 regions (Fig. 1B). These genes encoding ND2 and ND3 are subunits of NADH, which may act as

the rate-limiting enzyme of oxidative phosphorylation (30). Alterations in these genes are correlated with human cancers (31-33). It has been proposed that once these genes function abnormally in cancer cells, enhanced reactive oxygen species induces HIF1α stabilization (34,35). Thus, we speculated that genetic mutations identified in the present study, even in SNPs, may be linked partly to the above-mentioned mechanisms for oral tumorigenesis. In this context, several studies have reported an association between SNPs on mtDNA, especially in the ND3 region, and the risk of developing breast cancer (36-38).

We previously described the usefulness of qRT-PCR-HRMA for searching mtDNA mutations with high sensitivity/specificity. As indicated in the present study, our method, even in different regions on the mitochondrial genome, is sufficient for clinical use as well. However, as Kandel (39) pointed out, several issues need attention such as minimization of cellular contamination, determination of mut-mtDNA characteristics specific for OSCC, and elimination of the effect of other diseases.

The study limitations were the small number of OSCC cases and the absence of other human malignancies. However, our data were highly significant for early detection of high-risk individuals with OSCCs, since subjects expected to have a good prognosis who had recurrence/metastasis postoperatively can be distinguished by the level of tumor-derived mtDNA in their serum 4 weeks postoperatively. When a more precise approach for mut-mtDNA detection of CTCs in cancer patients is established, we will identify earlier the patients with undetectable lesions.

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