Mitochondrial Resequencing Arrays Detect Tumor-Specific Mutations in Salivary Rinses of Patients with Head and Neck Cancer

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Abstract

Purpose: Alterations of the mitochondrial genome have been identified in multiple solid tumors and in many head and neck squamous cell carcinomas (HNSCC). Identification of mitochondrial mutations in the salivary rinses of patients with HNSCC has potential application in disease detection. In this study, we used the MitoChip v2.0 mitochondrial genome resequencing array to detect minor populations of mitochondrial DNA in salivary rinses of patients with HNSCC.

Experimental Design: Salivary rinses from 13 patients with HNSCC, whose tumors carried mitochondrial mutations, were collected before surgical resection. DNA isolated from salivary rinses and serial dilutions of DNA derived from HNSCC-derived cell lines with known mitochondrial mutations were sequenced using the MitoChip, and analyzed using a quantitative algorithm which we developed to detect minor populations of mitochondrial DNA from MitoChip probe intensity data.

Results: We detected heteroplasmic populations of mitochondrial DNA up to a 1:200 dilution using MitoChip v2.0 and our analysis algorithm. A logarithmic relationship between the magnitude of assay intensity and concentration of minor mitochondrial populations was shown. This technique was able to identify tumor-specific mitochondrial mutations in salivary rinses from 10 of 13 (76.9%) patients with head and neck cancer.

Conclusions: Minor populations of mitochondrial DNA and disease-specific mitochondrial mutations in salivary rinses of patients with HNSCC can be successfully identified using the MitoChip resequencing array and the algorithm which we have developed. This technique has potential application in the surveillance of patients after resection and may have applicability in the surveillance of body fluids in other tumor types.

Head and neck squamous cell carcinoma (HNSCC) is the sixth leading cause of cancer worldwide and is a consequence of tobacco and viral exposure. Alterations of the mitochondrial genome have been identified in multiple solid tumors, as well as in HNSCC, and are associated with progression (1 – 6). A high-throughput mitochondrial resequencing array approach (MitoChip v2.0) showed mitochondrial mutation in half of the HNSCCs tested (7).

Mutations of the mitochondrial genome have been identified in a variety of human cancers (8). Mutation of a variety of functional genes is present in some form in most cancer types. These mutations are widespread throughout the genome with a diversity of mutations present in tumors of the same type. The high frequency of mitochondrial mutations found in a variety of solid tumors, and the abundance of mitochondrial DNA, lends it potential for use in the detection of mitochondrial mutations in body fluids from patients with cancer.

The advent of sensitive oligonucleotide sequencing arrays has led to novel possibilities for the molecular detection of cancer. Studies of a previous version of the MitoChip have proven its fidelity and reproducibility, and showed its promise in the detection of tumor-specific mitochondrial alterations in body fluids (9). These studies are limited by the ability to reliably and reproducibly detect disease-specific mutations from tumor mitochondrial DNA, a small percentage of the total DNA from a body fluid.

We designed a study to determine the threshold of the MitoChip v2.0 resequencing array to detect mutations in mixed samples of DNA using a simple, quantitative method using the signal probe intensity of the second most dominant peak

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Received 1/29/07; revised 7/16/07; accepted 7/31/07.

Grant support: Clinical Innovator Award from the Flight Attendant Medical Research Institute, and the National Institute of Dental and Craniofacial Research 1R01DE015939-01, and National Cancer Institute Head and Neck Specialized Programs of Research Excellence P50 CA96784 (J.A. Califano); and NIH grant no. T32DC00027 (I.M. Smith).

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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doi:10.1158/1078-0432.CCR-07-0220

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Table 1. Demographic information

	Specimen tested	Total cohort
	Patients (%)	Patients (%)
Median age (SD)	64 (13.4)	58 (13.3)
Sex		
M	6 (46.2)	51 (71.8)
F	7 (53.8)	20 (28.2)
Smoking history		
Current or former	8 (61.5)	54 (76.1)
Nonsmoker	5 (38.5)	17 (23.9)
Median pack years (range)	32.5 (14-110)	30 (5-100)
T stage		
1	2 (15.4)	13 (19.1)
2	3 (23.1)	17 (25.0)
3	5 (38.5)	27 (39.7)
4	2 (15.4)	11 (16.2)
N stage		
0	5 (38.5)	24 (34.3)
1	0 (0)	11 (15.7)
2A	1 (7.7)	6 (8.6)
2B	5 (38.5)	22 (31.4)
2C	0 (0)	6 (8.6)
3	0 (0)	1 (1.4)
Tumor site		
Oral cavity	7 (53.8)	29 (41.4)
Oropharynx	5 (38.5)	21 (30.0)
Larynx	1 (7.7)	20 (28.6)

relative to the primary peak to determine a "secondary base intensity." We applied our secondary base intensity algorithm to the detection of mutations found in head and neck tumors in salivary rinses from these patients prior to tumor resection and successfully detected tumor-specific mutations in 10 of 13 salivary rinses tested.

Materials and Methods

Design of human mitochondrial v2.0 oligonucleotide microarray. MitoChip v2.0 was obtained from Affymetrix (commercially available GeneChip Human Mitochondrial Resequencing Array 2.0). Sequences comprising both strands of the entire human mitochondrial genome were synthesized as overlapping 25-mers on high-density oligonucleotide arrays with 8 × 8 μm features. To query any given site from the human mitochondrial reference sequence, four features were tiled on the MitoChip. The four features differ only by the 13th base, which consists of each of the four possible nucleotides.

Cell lines. HNSCC cell lines, JHU-013 and JHU-019 were established in the Department of Otolaryngology-Head and Neck Surgery, Johns Hopkins University. Cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. All media components were obtained from Life Technologies Invitrogen Corporation.

DNA sample source and preparation. An initial cohort of 84 patients with HNSCC who had undergone enrollment in institutional review board–approved protocols after informed consent was obtained and identified. Mitochondrial mutation status in these tumors were identified by sequencing of tumors and matched leukocyte DNA samples using the MitoChip v2.0 mitochondrial resequencing array (Affymetrix) as previously described (7), to identify true mutations rather than polymorphisms. Forty-one of 84 (49%) of these patients harbored mitochondrial mutations. Specimens from 13 of the patients with mitochondrial mutations of the tumor DNA had been previously collected before surgical resection by rinsing/gargling with normal saline and using an exfoliating brush on oral and oropharyngeal mucosal surfaces (Table 1). Cells were pelleted by centrifugation, and stored at -80°C until DNA extraction according to institutional review board–approved protocols. DNA from salivary rinses and cell lines was obtained by digestion with 1% SDS/proteinase K, extracted by phenol-chloroform, and ethanol precipitated.

MitoChip preparation. The entire mitochondrial DNA sequence was amplified in three overlapping PCR reactions using 50 ng of genomic DNA each (9). Reagents, conditions, and purification were accomplished as described in the previous report (7). Pooling, DNA fragmentation, labeling, and chip hybridization were done using Affymetrix CustomSeq Resequencing protocol instructions. The chips were washed on the Affymetrix fluidics station using CustomSeq Resequencing wash protocols.

Automated batch analysis of microarray data. Analysis of microarray data for the v2.0 MitoChips was done using GeneChip Sequence Analysis Software (GSEQ) v4.0 (Affymetrix). GSEQ uses an objective statistical framework, based on the ABACUS algorithm (10), to assign base calls to each position which meets quality criteria in the mitochondrial genome. GSEQ software was used according to the manufacturer's instructions, with genome model set to "diploid" and quality score threshold set to "3" to maximize base call percentage and fidelity.

Determination of secondary base intensity. For dilution experiments and detection of mutations in saliva, GSEQ software was used to determine the primary presence or absence of previously identified mutations. At all mitochondrial positions where (*a*) mutations had previously been identified, (*b*) the primary base call was not a mutated base, and (*c*) the software algorithm was able to make a primary call of the base, the signal intensity of the second most prominent base was identified by evaluation of the magnitude of the signal of each base from the probe intensity data at this position. The intensity of the secondary base was expressed as the ratio of the difference between the average of the signal intensities of the two least intense bases and the height of the secondary peak to the difference between the average of the signal intensities of the two least intense bases and the height of the primary peak, i.e., SBI = $[H_2 - (H_3 + H_4) / 2] / [H_1 - (H_3 + H_4) / 2]$. This percentage was calculated for both the sense and antisense strands and averaged to yield the secondary base percentage (Fig. 1). Separate evaluation of relative intensities of the sense and antisense strands minimizes error that may be associated with using absolute intensities of the probe data due to differing magnitudes of binding between the two strands.

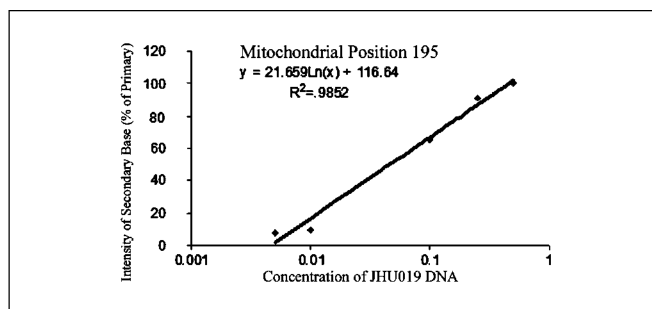

Fig. 1. Calculation of secondary base percentage from MitoChip probe intensity data. Probe intensity data is shown for position 1323 of mitochondrial sequencing of DNA from salivary rinse from patient 1 in the study. This patient's tumor harbors a G-A mutation relative to the germ line (leukocyte DNA) sequence at this position. Guanine remains the base with the highest intensity at this position. Adenine has the second highest base intensity on both the coding and noncoding strands. Secondary base intensity (*SBI*) is calculated for adenine as shown, and secondary base percentage for adenine is derived by averaging this value from both the sense and antisense strand. In this case, the secondary base percentage is 77.38%.

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Table 2. Secondary base intensity generated by 019 base calls at varying dilutions as a percentage of 013-generated primary peak

Position	Cell line base call		Serial dilutions of 019 DNA by 013 DNA				
	JHU-013	JHU-019	1-200	1-100	1-10	1-4	1-2
195	T	C	7.95	9.6	65.23	90.98	100
1811	G	A	—	—	29.11	49.7	57.33
2706	A	G	—	—	26.98	60.35	67.81
3197	T	C	—	—	100	100	100
3915	A	G	—	—	—	20.3	46.11
4093	G	A	—	—	36.24	63.16	82.41
5027	T	C	—	—	87.67	100	100
6216	C	T	5.82	4.66	55	67.61	100
9477	G	A	—	—	19.89	42.82	51.11
11120	T	C	10.66	13.56	100	100	100
13617	T	C	—	6.59	57.15	91.55	100
13802	C	T	—	—	42.99	62.64	93.98
14793	A	G	28.16	32.83	98.11	100	100
15218	A	G	—	—	37.27	60.01	68.78
16320	C	T	—	—	17.9	39.41	56.81

NOTE: The first three columns summarize the mitochondrial positions and base differences between the JHU-013 and JHU-019 cell lines. The last five columns indicate the dilutions at which secondary bases are detected which correspond to the JHU-019 primary base call for the corresponding mitochondrial positions. Secondary base percentage values are given for secondary bases which were detected. Secondary bases detected did not correspond to JHU-019 primary base call at the corresponding mitochondrial position (—).

This algorithm was developed due to the unsuitability of the standard heteroplasmy detection algorithm of the GSEQ sequence analysis software to quantitate minor populations bearing mutations in a body fluid. The specific formula was developed to assess the height of the second highest base probe intensity as a percentage of the difference between the lowest two bases and the primary base and combining the information for the sense and antisense strands. Utilizing this proportion rather than the absolute intensity for both the sense and antisense strands minimizes the effect of binding variability of each strand while combining probe intensity data from each strand. For each base which is evaluated for tumor-specific mutations in the salivary rinse specimen, the primary base call and the calculated secondary base could be compared with tumors in order to determine the detection of a mutation.

Results

Limit of detection of mitochondrial resequencing array. We resequenced two cell lines (JHU-019 and JHU-013) derived from primary head and neck cancers and determined differences in sequence at various positions in the mitochondrial genome (Table 2). We did serial dilutions of JHU-019 DNA by JHU-013 DNA at concentrations of 1:2, 1:4. 1:10, 1:100, and 1:200, and analyzed the mixtures compared with sequence data from the pure cell line population. At all positions and dilutions identified and tested, the analysis software was able to make a primary base call. We found that in all but the least dilute of the samples tested (1:2, 1:4) the majority of bases were called in congruence with the sequence present in larger proportion, JHU-013 (data not shown). The analysis provided by the GSEQ software is designed to detect heteroplasmy in a mixed population of mitochondria, and as such, base and heterozygosity calls are not generated in an optimal manner which detects mutations located in small proportions to a background, such as in applications to screen body fluids for tumors. To take advantage of the sensitivity of the MitoChip, a simple algorithm was developed which examined the raw probe intensities at a mitochondrial position whose base could be called by the GSEQ software and determined the identity of the second highest base probe intensity. The second most intense base at a given mitochondrial position was termed the secondary base. This algorithm identified secondary bases at positions of difference between the two cell lines corresponding to the primary base call for the JHU-019 cell line in dilutions of up to 1:200. Four of 15 (26.7%) base differences tested were detected at a 1:200 dilution. Five of 15 (33%) base differences tested were detected at 1:100 dilution using the secondary base method. Fourteen of 15 (93.3%) base differences were detected at 1:10 dilution (Table 2).

Relationship of secondary base percentage intensity to dilution. Applying the algorithm to our dilution data, we were able to quantitate the intensity of the secondary base previously identified as a percentage of the difference between the median of the two least intense bases at a given mitochondrial position and the most intense base. This calculated value was termed the "secondary base percentage" (Table 2). Secondary base percentages increased in intensity at each mitochondrial position tested as a logarithmic function of dilution (Fig. 2). This relationship was consistent among all 15 mutations tested, with a median R^2 value of 0.9673 and SD of 0.07 (range, 0.73-0.99).

Detection of tumor-specific mitochondrial mutation in salivary rinses from HNSCC patients. To test the applicability of mitochondrial mutation detection as surveillance tools, we extracted DNA from salivary rinses of patients with HNSCC with mitochondrial mutations and analyzed them with the MitoChip. MitoChip results were analyzed in GSEQ and mitochondrial positions in which mutations were identified and confirmed by conventional sequencing in HNSCC were analyzed to determine the base call in saliva. Eighty-four

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Fig. 2. Representative standard curve relating secondary base percentage corresponding to the detected JHU-019 primary base for position 195 to dilution. The relationship is a logarithmic function and the R^2 value is 0.9852. All 15 base differences between JHU-013 and JHU-019 were assessed similarly, with a median R^2 value of 0.9673 with a SD of 0.07 (range, 0.73-0.99).

mutations from 13 tumors were evaluated. The number of mitochondrial mutations in each tumor ranged from 1 to 24. Seventy-two of 84 (85.7%) base calls at positions mutated in tumors evaluated in saliva were found to be congruent with the germ line (leukocyte DNA) base calls by MitoChip v2.0 resequencing array (Supplementary Table S1). The remaining mutations were the primary base call in salivary DNA. Probe intensity raw data from resequencing array of saliva mitochondrial DNA was analyzed by determination of the base with the second highest intensity and determination of secondary base percentage at mitochondrial positions in which corresponding tumors showed mutation. Thirteen positions showed the base call from the tumor as the primary base call (Table 3), with the secondary peak being the patient's baseline. In salivary rinses, secondary bases corresponding to mutations in tumors were identified for 31 of 71 (43.6%) of mutations for which the primary base call was not the tumor-specific mutation. Ten of 13 (76.9%) patients had at least one mitochondrial mutation identified in the salivary rinse as a secondary base. To establish a threshold for identification of mutation-associated secondary bases relative to nonspecific secondary bases, we calculated the median secondary base percentage across all sequenced nucleotides in the mitochondrial genome for all 13 salivary rinse DNA samples to be 8.9% (SD, 5.2%). We analyzed the secondary base percentages for the tumor mutations found in salivary rinses. Using base percentages corresponding to roughly 1, 2, and 3 SDs above the median, we identified 12 of 71 (16.9%), 11 of 71 (15.5%), and 6 of 71 (8.4%) mutations from primary tumors not already identified as primary base calls as secondary bases in the salivary rinse DNA (Table 3). This corresponded to the identification of tumor-specific mitochondrial mutations in 9 of 13 (69.0%) samples tested as a primary base call or secondary base at up to a 20% threshold. Utilizing an equation derived from the median of all the standard curves from the dilution experiments, the secondary base thresholds correspond to concentration thresholds of 0.02, 0.029, and 0.036.

Discussion

In this study, we have shown, using a simple algorithm, that the MitoChip v2.0 mitochondrial resequencing array has an adequate resolution to identify mitochondrial mutations in a

Table 3. Number of total mutants detected as secondary bases using different dilution thresholds

Sample	No. of mutations in tumor	Mutations detected in saliva				
		No. of mutations, primary base call	No. of mutations, secondary base call	Secondary base, >15%	Secondary base, >20%	Secondary base, >25%
1	16	0	10	6	6	2
2	24	0	7	1	1	0
3	3	0	3	1	1	1
4	21	2	6	1	0	0
5	1	0	0	0	0	0
6	3	0	2	2	2	2
7	1	1	0	0	0	0
8	2	1	1	1	1	1
9	2	0	2	0	0	0
10	8	8	0	0	0	0
11	1	1	0	0	0	0
12	1	0	0	0	0	0
13	1	0	0	0	0	0
Total	84	13	31	12	11	6

NOTE: The number of mutations detected in each tumor for which corresponding salivary rinse DNA was assessed is indicated, as are the number of mutations in each specimen which were either the primary base call, or the secondary base. Number of mutations for each sample whose secondary base percentage values exceeded the thresholds are also indicated. The thresholds indicated corresponded to roughly 1, 2, and 3 SDs greater than the median secondary base percentage value of all nonmutated bases in the mitochondrial genome. This was done to distinguish true secondary bases detected from nonspecific background. The thresholds correspond to detection concentration limits of 0.02, 0.029, and 0.036.

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minor population of DNA in a mixture to a limit of 1:200 for some mitochondrial positions. We have also shown that a consistent, mathematical relationship exists between the quantitative value which we have assigned to identify secondary bases and actual concentrations of mutant DNA throughout the range of dilutions. Additionally, we applied our method for secondary base identification and quantitation to DNA extracted from salivary rinses of patients with HNSCC and successfully identified tumor-specific mitochondrial mutations in 10 of 13 samples tested. This is the first reported use of this technology to evaluate oral rinse specimens. These results support further study and possible use of the MitoChip v2.0 resequencing array as a method for surveillance of body fluids to detect tumor-specific mitochondrial mutations.

There are several potential mechanisms by which the salivary rinses of patients with HNSCC are preferentially enriched with mutant mitochondrial DNA, making this fluid a good surveillance medium. A significant proportion of primary HNSCCs have a spectrum of genetic changes occurring in the area surrounding the tumor due to the lateral expansion of clonally related cells. Additionally, mitochondrial content has been shown to increase with tumor progression in this disease (11), increasing the proportion of mutated mitochondrial DNA to normal mucosal mitochondrial DNA and improving the ability to identify tumor-specific mutations.

The functional significance of mitochondrial mutations in cancer lends additional credence to its use in a surveillance strategy. Cancer-associated germ line mutations of mitochondrial DNA with oncogenic potential have been identified in cohorts of patients with breast (12), endometrial (13), and prostate cancers (14). The oncogenic ability of specific mitochondrial mutations in a variety of cancer types has been linked to the dysregulation of oxidative phosphorylation and stimulation of cellular proliferation (15, 16), as well as escape from apoptotic stimuli. In HNSCC, our group has identified mutant mitochondrial respiratory chain proteins based on primary tumor mutants which replicate a tumorigenic phenotype in cells, including increased anchorage-dependent and -independent growth, reactive oxygen species production, and an aerobic glycolytic metabolic phenotype with HIF-1α induction (17).

This method provides several advantages over existing molecular techniques: (*a*) usage of an automated, standard platform and a uniform method for calculating signal thresholds should minimize variability between laboratories, (*b*) the presence of multiple mitochondrial mutations in individual tumors allows for redundancy in the detection of mutations to allow the confirmation of the presence of mutated cells, and (*c*) the presence of mitochondrial mutations in nearly all solid tumor types allows for the applicability of this technique to a variety of solid tumors. The minimal detection threshold in this technique is not as sensitive as other applications, but was adequate to detect mutations in salivary rinses from patients with HNSCC, and may be potentially applicable to body fluids in which some form of enrichment of tumor DNA may occur, including urinary sediment, serum, plasma, surgical margins, and selective bronchial washings.

Dilution studies show an almost uniform ability to detect heteroplasmic populations at up to 1:10 dilution (14 of 15 mutations evaluated), with the occasional ability to detect mutations at up to 1:200 dilution (4 of 15). This variability may be due to the differential binding of mutated mitochondrial DNA fragments to the probe sequences for various mitochondrial positions, affecting the specificity of their binding. In our cell lines, in which dilutional analysis was done, four of eight (50%) T-C transversions tested were detected at 1:100 dilution or higher, whereas only one of eight (12.5%) A-G transversions were detected at dilutions greater than 1:10. This sharply contrasted with our data from salivary rinse detection. T-C transversions make up 36 of 84 (42.9%) mutations in the tested samples and 102 of 228 (44.7%) mutations in all tumors sequenced, whereas A-G transversions make up 37 of 84 (44.0%) mutations in the tested samples and 103 of 228 (45.2%) mutations in all tumors sequenced. Combined, these two types of transversions comprise 90% of the mutations seen. However, of the 44 mutations detected by MitoChip as either primary or secondary base, A-G transversions comprise 25 mutations (67.6% detection rate) whereas T-C transversions comprise only 11 mutations (30.5% detection rate). It is unclear whether the type of transversion has a role in detection sensitivity, but our data is suggestive that although a higher proportion of A-G transversions are identified in the salivary samples, T-C transversions are detected with higher sensitivity in our cell line dilutional analysis. This might potentially be explained by the notion that mitochondrial DNA may be preferentially shed from HNSCC, and that MitoChip identification of mutation may not approach the threshold of detection. Further investigation is required to identify transversion-specific sensitivity.

The ability to detect a subset of mutations from a particular tumor may be dependent on the number of mutations carried by that tumor. We were able to detect tumor mutations in salivary rinses from 6 of 10 (60%) patients with less than 10 mutations, but in all 3 (100%) of the remaining patients whose salivary rinses had more than 10 mutations. It serves to reason that an increased number of mutations may yield a more likely ability for detection, but this sample size remains too small to definitively draw that conclusion.

A study using the MitoChip resequencing array tested previous versions of the array and attempted to detect heteroplasmic mitochondrial alleles (18). This study used a precursor to the GSEQ software to analyze serial dilutions of tumors into wild-type DNA and revealed a 1:50 threshold for the detection of mitochondrial mutation in a qualitative fashion. Initial studies of body fluid detection of tumor-specific mitochondrial mutations in pancreatic juice and urine presented were promising, but our follow-up studies in pancreatic juices did not reflect the findings presented.[4]

A single study has attempted an algorithm-based approach to quantitate the proportion of a heteroplasmic population of mitochondrial DNA which is comprised by a minor allele (19). This technique used a summation of the probe intensities of both the sense and antisense strands at a given mitochondrial position and assessed heteroplasmy (what we term the secondary base percentage) using the difference of the secondary base probe intensity from the lowest base probe intensity and quantitating heteroplasmy of this proportion compared with the primary base probe height. Summation of

[4] Unpublished data.

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the probe intensities of the sense and antisense strands may not be as advantageous because we found binding to, at times, be quite variable between the two strands leading to high degrees of variability in even the primary base probe intensity between the two strands. In the development of the algorithm presented, we observed differential binding, but were able to discern that proportions of peak height remained relatively constant for each strand. The validation of our algorithm by correlation of secondary base percentages and dilution amount support its applicability and fidelity.

Our study, using the updated version of the MitoChip containing the entire mitochondrial genome combined with a validated approach to quantitate heteroplasmy, represents a pilot study for the potential use of resequencing arrays such as the MitoChip array in the surveillance of body fluids. Follow-up studies involving a larger cohort of patients and evaluation of the method in different control groups are indicated to assess the value of this technique for other applications. These include appropriately case-matched and stratified evaluation of patients with no history of cancer or any oral complaints, patients with a history of HNSCC who show no evidence of disease, and patients with a history of benign disease. For the purpose of evaluation of the MitoChip and detection algorithm for surveillance purposes, a prospective study to test salivary rinses of patients who have undergone resection of HNSCC would be of interest to determine the prognostic significance of this approach as a surveillance technique for local recurrence of disease.

It is for the purposes of surveillance after extirpation of cancer that we feel that this technique may have the most immediate applicability. Because there is a broad range and diversity of mitochondrial mutations present in HNSCC, each tumor has its own mutational "fingerprint." This study represents an initial evaluation of a tool which can be used for individualized surveillance after tumor resection. Important questions remain as to the sensitivity of this technique in the detection of residual or recurrent disease that is not grossly detectable. Given the propensity of the tumor cells to slough, and the limits of detection which we have identified for this technique, it is likely that sensitivity for tumor-specific mitochondrial mutations in DNA derived from salivary rinses as we have described would remain quite high for the detection of recurrent disease; however, larger studies are required to define the role of application of this technique to surveillance after extirpation of tumor. We envision that the applicability of the MitoChip for surveillance of salivary rinses is the demonstration of a correlation between tumor-specific mutation after extirpation with recurrent or residual disease.

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