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Screening for Oral Precancer with Noninvasive Genetic Cytology

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Abstract Oral squamous cell carcinomas develop in precancerous fields consisting of genetically altered mucosal epithelial cells. These precancerous fields may appear as clinically visible lesions, in particular, oral leukoplakia, but the large majority remains clinically undetectable. The aim of this study was to assess the potential value of a noninvasive screening approach to detect precancerous fields. As a first step, we developed a suitable assay and investigated 25 leukoplakia patients and 20 noncancer control subjects. Exfoliated cells were removed by a brush from multiple small areas of the oral mucosa, including the leukoplakia. Brushed samples were investigated for allelic imbalance (AI) at chromosomes 3p, 9p, 11q, and 17p using microsatellite markers known to show frequent alterations in oral precancer. AI was absent in all (137) of the samples of the 20 control subjects, yielding a specificity of 100%. AI was detected in exfoliated cell samples of 40% (10 of 25) of the leukoplakia lesions studied. Genetic changes were also found outside the leukoplakia lesions. Most frequent was AI at 9p (9 of 10). The noninvasive assay was validated against the biopsy results of the leukoplakia lesions yielding an estimate of sensitivity of 78% (7 of 9) and a positive predictive value of 100% (7 of 7). Altogether, these results show the feasibility of a noninvasive genetic screening approach for the detection and monitoring of oral precancer. This assay could therefore contribute to the secondary prevention of oral squamous cell carcinoma. The assay also shows promise for the detection of precancerous changes that are not macroscopically visible.

Early diagnosis of oral squamous cell carcinoma may have a major effect on survival and quality of life. It is well-known that the majority of oral squamous cell carcinomas, if not all, develop in precancerous fields characterized by specific genetic alterations (1–3). Clinically, oral precancerous lesions may appear as a white or red lesion (leukoplakia or erythroplakia, respectively). The malignant potential of these lesions is assessed by histopathology and mainly based on the presence and the degree of dysplasia in biopsy material, graded as mild, moderate, and severe (4). As histology is still the gold standard, microscopic examination of mucosal biopsies might, in theory, be exploited for early diagnosis of precancerous fields even when these are not visible. However, histopathologic grading has limited value to predict the malignant potential in individual cases (5). In addition, histopathologic grading requires taking a biopsy, and to monitor the progression of a lesion, repeated biopsies need to be taken, which is a large burden for the patient. Furthermore, histopathologic grading may largely depend on the precise location of the biopsy, given the heterogeneity of some lesions. Finally, to identify precancerous fields that are not visible, more or less random biopsies need to be taken, which is too invasive as a screening approach. Hence, screening and monitoring oral precancer by histopathologic examination of tissue biopsies does not seem to be feasible, except for the visible lesions.

Notwithstanding, a noninvasive genetic screening assay might be of large value for populations at high risk for developing oral cancer such as treated oral cancer patients, leukoplakia patients, genetically predisposed subjects such as Fanconi anemia patients, and individuals frequently exposed to environmental carcinogens. Oral cancers are frequently surrounded by nonvisible precancerous changes in the oral mucosa that are often not completely resected causing secondary tumors. Detection and monitoring of such nonvisible precancerous fields by histology would require multiple biopsies surrounding the treated area, and a noninvasive screening tool would be a much more attractive alternative. Such an assay would also be of relevance for leukoplakia patients, as it has been shown that these patients can develop oral squamous cell carcinomas outside the visible lesion (6). Therefore, it seems important to screen and monitor leukoplakia patients not only for precancerous changes in the visible lesion(s), but throughout the whole oral cavity.

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Table 1. Patient characteristics and results of histology and Allelic Imbalance (AI) assay in exfoliated cells and in biopsies of leukoplakia patients

Patient	Gender	Age	Tobacco/ alcohol (u/d)	Location (leukoplakia)	Histology (after review)	AI in exfoliated cells (location)	AI in biopsy (location)
1	M	67	25/4	Tongue border (left)	Mild dysplasia	9p	9p
2	F	67	—/—	Tongue border (left)	Mild dysplasia	9p	9p
3	F	86	—/2	Tongue (diffuse), lower lip (left)	No dysplasia	11q	NA
4	F	69	—/—	Multiple lesions	Verrucous carcinoma	0	0
5	F	74	7/—	Soft palate (right)	No dysplasia	0	0
6	F	54	—/2	Cheek mucosa (diffuse), gingiva (lower left jaw)	No dysplasia	0	0
7	F	72	—/1	Palate	No dysplasia	9p	NA
8	F	66	—/3	Gingiva, buccal fold (lower left jaw)	No dysplasia	0	NA
9	F	61	15/2	Gingiva upper jaw, palate	No dysplasia	9p	NA
10	F	60	—/—	Cheek mucosa (left)	Mild dysplasia	0	0
11	F	44	40/—	Floor of mouth (left)	No dysplasia	0	0
12	F	43	—/3	Tongue border (left)	Moderate dysplasia	9p, 17p	9p, 17p
13	F	66	—/—	Gingiva (diffuse)	No dysplasia	0	NA
14	M	69	5/5	Cheek mucosa (right), lower lip, tongue surface	Proliferative verrucous leukoplakia	0	0
15	M	76	12/2	Floor of mouth	Moderate dysplasia	3p, 9p,17p	3p, 9p
16	F	79	—/—	Tongue (diffuse), floor of mouth, gingiva lower jaw	No dysplasia	9p	9p
17	M	72	—/1	Cheek mucosa (left)	No dysplasia	0	0
18	M	70	20/3	Floor of mouth, tongue (floor)	No dysplasia	9p	9p
19	F	56	—/2	Gingiva upper jaw	No dysplasia	0	NA
20	F	64	9/2	Gingiva upper jaw	No dysplasia	0	9p
21	F	34	—/—	Tongue border (right)	Mild dysplasia	0	9p, 11q, 17p
22	M	76	10/—	Tongue border (right)	Verrucous carcinoma	9p	9p
23	F	77	—/—	Cheek mucosa (left)	No dysplasia	0	0
24	F	78	—/—	Multiple lesions	No dysplasia	0	NA
25	M	61	15/2	Floor of mouth	Severe dysplasia	0	NA

Abbreviations: M, male; F, female; NA, not available. 1 Units per day.

The precancerous fields are characterized by genetic changes based on allelic imbalance (AI), and earlier studies have shown that genetic tests are promising in predicting malignant progression of oral precancerous fields, in particular, those that are visible as leukoplakias. Both the presence as well as the accumulation of genetic changes have been shown to be associated with the risk of malignant transformation (7–9). Such genetic alterations have been assessed on exfoliated cell samples (10, 11), and could be further developed to a reliable noninvasive assay. In a previous study, we developed and evaluated various genetic assays that might allow the detection of oral precancerous fields in small brushed samples (12). We found that measurement of AI, commonly known as allelic loss or loss of heterozygosity, seems most suitable. A marker panel of microsatellite markers, located at chromosomes 3p, 9p, 11q, and 17p, was selected based on the following criteria: high percentage of loss of heterozygosity in HNSCC, frequent loss of heterozygosity in precancerous fields indicating that these occur early in carcinogenesis (2, 3, 13), high percentage of informativity of these particular microsatellites, amplimer lengths that can be combined for multiplex sequence runs (12, 14), and known to be associated with malignant transformation of leukoplakia (7–9).

In this study, we report on the potential of a noninvasive genetic assay based on AI in brushed samples. The feasibility of this approach was investigated in noncancer control subjects and leukoplakia patients. Brushed samples of the leukoplakia lesions were tested against biopsies to validate the noninvasive approach.

Materials and Methods

Patients

Biopsy material, blood, and brushed samples of the oral mucosa were obtained from 25 leukoplakia patients who visited (in the period 2004-2007) the outpatient clinic at the Oral and Maxillofacial Surgery Department of the VU University Medical Center. In 17 cases, an incisional biopsy was taken from the leukoplakia lesion or the lesion was excised, whereas in others the lesion was left untreated. Dysplasia was scored according to the standard criteria of the WHO (4). Brushed samples were taken from the leukoplakia lesion as well as from different oral mucosal sites with a relatively high incidence of oral squamous cell carcinoma: border of tongue (left and right), floor of mouth (left and right), and the alveolar ridge/retromolar trigone (left and right; ref. 15). Exfoliated cells were brushed from the oral mucosa using a small disposable brush (Omnident, Dental Union) and collected as described previously (11). Furthermore, information on tobacco and

alcohol use of the patients was collected. The characteristics of the study population are summarized in Table 1.

In addition, we collected seven brushed samples of the oral mucosa from 20 noncancer control subjects at young age (<30 years) without history of smoking and excessive alcohol consumption, assuming that no precancerous changes would be present in this low-risk population. The brushed samples were obtained at the same standardized locations: six at high-risk sites for oral cancer, one at a lower risk site (soft palate). The study was approved by the Institutional Review Board of the VU University Medical Center, and written informed consent was obtained from all patients and control subjects. The results of the genetic analyses were reported to the treating physician, but not used for clinical decision making. In all cases (leukoplakia patients and noncancer controls) the findings in a brushed sample could be directly linked to the specific location. Samples were always processed separately.

Analysis of biopsy material

From the biopsy material, 10-μm paraffin sections were cut and mounted on microscopic glass slides. The first and last tissue sections were stained with H&E for histologic analysis and to guide microdissection. All H&E sections were reviewed by an experienced pathologist. The other tissue sections were stained with 1% toluidine blue and 0.2% methylene blue in PBS and manually dissected under a stereomicroscope to enrich for preneoplastic cells when present. Regions of dysplasia were marked by a pathologist and separately dissected.

Isolation of DNA

The exfoliated cell samples, microdissected tissues, or the nucleated fraction of the blood samples obtained by hypotonic lysis were treated with 1 mg/mL of proteinase K for 24 h at 52°C in 100 to 300 μL buffer containing 100 mmol/L Tris-HCl (pH 9.0), 10 mmol/L NaCl, 1% SDS, and 5 mmol/L EDTA (pH 8.2). The DNA was purified by phenol-chloroform extraction and collected by ethanol precipitation using 2 μg of glycogen as carrier. The DNA was dissolved in 20 μL LoTE buffer (3 mmol/L Tris-HCl, 0.2 mmol/L EDTA; pH 7.5).

AI analysis

AI was assessed using 12 microsatellite markers located at chromosomes 3p, 9p, 11q, and 17p. The following markers were used: D3S1766, D3S1029, D3S1293, D9S171, D9S162, D9S157, D11S1883, D11S1369, D11S2002, CHRNB1, TP53, and D17S1866 (Table 2). Detailed information including primer sequences can be found at the UniSTS page of the National Center for Biotechnology Information web site.[5] These markers were carefully selected on various criteria as discussed above. AI analysis was done by PCR using primers with fluorochromes as described previously (3, 16, 17). PCR products were run on an Applied Biosystems 3130 sequence analyzer (Applied Biosystems BV).

Data processing and quality control

The peak patterns were inspected by eye and peaks for further analysis selected manually. Data were loaded from the sequence analyzer in an Excel spreadsheet and processed automatically. Stutter correction was done when necessary. Data were inspected by two independent researchers and decisions on quality and presence of AI made in consensus. Data were considered reliably when peak values were within the range of 250 to 7,000 fluorescence units. The median of all samples was used as reference value to score AI. We scored AI when one allele was decreased by >50% in the sample when compared with the median ratios of all samples as normal reference, an internationally accepted but arbitrary standard (3, 14, 17). When AI was found in multiple samples, the median of all samples was unreliable as a reference, and the data were reanalyzed with the ratios of normal blood DNA as reference. The SD of the ratios using normal DNA depend on the marker and the individual patient, but are usually in the order of 10% to 20%. We therefore marked ratio changes between 0.5 to 0.625 and 1.6 to 2.00 as "possible AI", limits determined by approximately thrice this SD. More details on data processing and quality assurance are available upon request.

Table 2. Detailed information on the genetic markers used for AI analysis

Marker	Location	Base pairs
D3S1766	3p14.2	58956715-58956927
D3S1029	3p21.33	44110861-44111030
D3S1293	3p24.3	21902207-21902338
D9S171	9p21	24524210-24524384
D9S162	9p22.1	19669807-19669992
D9S157	9p22.2	17618382-17618526
D11S1883	11q13.1	63130309-63130560
D11S1369	11q13.4	72234643-72234819
D11S2002	11q14.1	79643051-79643288
CHRNB1	17p13.1	7290301-7290466
TP53	17p13.1	7558143-7558252
D17S1866	17p13.3	82571-82745

Results

Frequency and pattern of allelic loss in exfoliated cells of leukoplakia patients

The visible lesion in leukoplakia patients was used to assess proof of principle. The visibility of the lesion ensures that noninvasive sampling is carried out at the location that is also biopsied, and it is known that in at least a subgroup AI can be detected. Besides sampling the leukoplakia lesion, we also sampled the other defined regions in the oral cavity. In total, 157 noninvasive samples were collected from 25 leukoplakia patients, and all samples gave results that passed the quality controls. AI was present in exfoliated cells in 40% (10 of 25) of the leukoplakia lesions studied. In Fig. 1, three examples are shown. The most common imbalances were on 9p; in 90% (9 of 10) we found allelic loss at one or more markers on 9p. Allelic loss at multiple chromosomal arms was present in two lesions, one with AI at 9p and 17p, and one with AI at 3p, 9p, and 17p (Fig. 1C). One case showed a single loss on 11q. The leukoplakia lesions of 10 patients were relatively large, and in these cases, multiple brushed samples were taken. In six cases, heterogeneity of the lesions was seen with AI in some samples and with no AI in others (Fig. 2A). In one case, AI on 9p was measured in exfoliated cells outside the leukoplakia lesion, brushed at clinically normal-appearing oral mucosa. The leukoplakia lesion was located at the palate, whereas the samples showing AI at 9p were located at the left and right buccal mucosa (Fig. 2B). This suggests the presence of a large field of precancerous changes in this patient that is not visible (3). Sevenlesions were scored as dysplastic, of which, four showed AI in exfoliated cells. In three cases, a verrucous carcinoma or proliferative verrucous leukoplakia was diagnosed, whereas AI was found in one case. All data are summarized in Table 1.

[5] http://ncbi.nlm.nih.gov

Comparison of the noninvasive AI assay with biopsies in leukoplakia patients

As already indicated above, the leukoplakia lesions could be exploited to estimate the test variables (sensitivity, specificity, and positive predictive value) of the noninvasive AI assay. These macroscopically visible lesions allow noninvasive sampling at exactly the same location as the biopsy. We used genetic analysis of the biopsy as a gold standard to establish the test variables. It should be noted that the number of patients studied only allows an estimation of these test variables, and proof of concept of the noninvasive approach. In 17 cases, biopsy material taken just after brushing was available, and these were investigated for AI in the tissue material. In all eight lesions that did not show AI in the biopsy material, the corresponding exfoliated cells also did not present with AI (data not shown). This suggests a high specificity of the assay (eight of eight, 100%). From the nine lesions with AI in the biopsy material, seven showed a similar pattern of allelic losses in the corresponding exfoliated cells. The results from a subgroup of patients are shown in Fig. 3. This would indicate a sensitivity of ~80% (seven of nine) for the noninvasive test when genetic analysis of the biopsy is considered as the gold standard. The positive predictive value also seems quite high (seven of seven, 100%); when there is AI in the exfoliated cells, it is also present in the tissue biopsy.

A

Patient 4	LP Exfo 1	Exfo 2	Exfo 3	Exfo 4	Exfo 5	Exfo 6	Exfo 7
D3S1766	1.05	0.86	1.01	0.96	0.92	1.00	1.00
D3S1029	1.00	1.32	0.92	0.95	1.14	0.93	1.02
D3S1293	NI	NI	NI	NI	NI	NI	NI
D9S171	0.78	1.12	1.21	0.95	1.00	0.99	1.00
D9S162	0.93	0.95	0.92	1.00	1.15	1.11	1.21
D9S157	0.99	0.98	0.90	1.15	1.00	1.12	1.00
D11S2002	0.87	0.89	1.12	0.97	1.00	1.05	1.06
D11S1369	0.94	1.08	0.95	0.99	1.02	1.12	1.00
D11S1883	1.00	1.15	0.99	0.92	0.99	1.12	1.07
CHRNB1	0.86	0.91	1.00	1.16	1.01	1.00	1.08
TP53	0.90	0.98	1.01	1.05	1.01	0.99	1.00
D17S1866	1.15	0.80	0.91	1.42	1.00	1.31	0.93

B

Patient 22	LP Exfo 1	LP Exfo 2	Exfo 3	Exfo 4	Exfo 5	Exfo 6
D3S1766	0.97	0.96	1.03	1.05	1.00	0.97
D3S1029	1.10	1.05	0.88	0.86	0.94	1.00
D3S1293	0.90	0.94	1.00	1.08	0.74	1.13
D9S171	0.07	0.08	1.06	1.18	1.00	0.83
D9S162	16.01	8.19	0.87	1.00	0.87	1.43
D9S157	13.98	11.64	0.85	0.88	1.00	1.01
D11S2002	1.02	1.07	1.00	1.35	0.97	0.97
D11S1369	1.08	0.97	0.95	1.00	0.93	1.01
D11S1883	NI	NI	NI	NI	NI	NI
CHRNB1	0.83	0.84	1.03	1.00	1.04	0.98
TP53	0.99	1.01	0.96	0.98	1.00	1.12
D17S1866	1.02	0.99	1.00	1.06	0.95	1.05

C

Patient 15	LP Exfo 1	Exfo 2	Exfo 3	Exfo 4	Exfo 5	Exfo 6
D3S1766	0.33	1.03	1.07	1.03	1.07	1.01
D3S1029	NI	NI	NI	NI	NI	NI
D3S1293	3.67	1.09	0.86	1.00	1.01	1.34
D9S171	0.15	0.86	0.75	0.92	0.70	0.83
D9S162	0.24	1.14	1.03	1.09	1.24	0.96
D9S157	3.75	0.91	1.10	0.89	0.89	0.99
D11S2002	0.87	0.97	0.97	1.03	0.94	1.05
D11S1369	0.93	1.09	0.94	0.96	1.00	0.91
D11S1883	0.84	0.79	0.69	0.93	0.86	0.81
CHRNB1	0.43	1.04	0.98	1.14	1.05	1.00
TP53	NI	NI	NI	NI	NI	NI
D17S1866	NI	NI	NI	NI	NI	NI

Fig. 1. Few typical examples of the noninvasive genetic AI assay on exfoliated cells of leukoplakia patients. *Left*, markers; *top*, samples analyzed. The ratios between the median of all samples and the sample analyzed for each markers are depicted. *A*, a patient without AI. *B*, a patient with AI in the leukoplakia lesion at all markers at 9p. *C*, the results from a patient with multiple chromosomal losses in the leukoplakia lesion. Gray, allelic imbalance (AI: ratio <0.5 or >2); LP, leukoplakia lesion; Exfo, exfoliated cells; NI, not informative).

A

Patient 12	LP Exfo 1	LP Exfo 2	LP Exfo 3	Exfo 4	Exfo 5	Exfo 6
D3S1766	1.07	1.17	1.16	1.00	0.97	0.90
D3S1029	0.99	0.67	0.82	0.82	1.00	0.80
D3S1293	1.28	1.23	1.14	1.09	0.96	1.00
D9S171	1.47	2.55	1.01	0.99	1.11	1.00
D9S162	0.72	0.42	0.98	0.97	1.17	1.00
D9S157	1.50	2.46	1.00	0.89	1.00	0.95
D11S2002	1.03	1.18	1.16	0.88	1.24	1.00
D11S1369	NI	NI	NI	NI	NI	NI
D11S1883	1.07	0.89	1.20	0.91	1.00	0.87
CHRNB1	1.41	1.69	1.06	1.00	1.25	1.19
TP53	0.60	0.26	0.98	1.00	0.99	0.90
D17S1866	1.55	1.75	1.04	1.00	1.15	1.10

B

Patient 7	LP Exfo 1	LP Exfo 2	Exfo 3	Exfo 4	Exfo 5	Exfo 6	Exfo 7
D3S1766	0.86	0.98	1.01	1.12	1.00	0.96	1.04
D3S1029	1.53	1.08	1.02	0.78	1.00	1.05	0.97
D3S1293	1.21	1.00	0.98	1.12	1.06	0.91	1.00
D9S171	NI	NI	NI	NI	NI	NI	NI
D9S162	1.44	0.98	6.98	3.81	0.99	1.00	1.31
D9S157	NI	NI	NI	NI	NI	NI	NI
D11S2002	0.74	1.19	0.98	1.09	1.11	0.97	1.00
D11S1369	NI	NI	NI	NI	NI	NI	NI
D11S1883	0.66	0.81	1.04	1.09	1.00	0.97	1.01
CHRNB1	NI	NI	NI	NI	NI	NI	NI
TP53	1.17	1.14	1.12	1.13	0.98	1.00	1.00
D17S1866	1.10	0.76	0.99	1.13	1.09	0.98	1.00

Fig. 2. Examples of genetic heterogeneity in the leukoplakia lesion (*A*) and genetic changes outside the visible lesion (*B*). Abbreviations are identical as indicated in the legend of Fig. 1.

Frequency and pattern of allelic loss in exfoliated cells of control subjects

As mentioned above, the specificity was estimated on the basis of the data collected in eight patients with leukoplakia

who did not show genetic changes in the lesion. However, the specificity can also, or even more reliably, be assessed in noncancer control subjects who are at a low risk for precancerous changes. We therefore analyzed the exfoliated cells brushed at seven defined locations in the oral cavity of 20 noncancer subjects, volunteers at a young age (<30 years) who did not smoke and had no history of excessive alcohol consumption. Based on the assumption that these control subjects were devoid of nonvisible precancerous changes, we decided not to take and analyze biopsy material. The exfoliated cells from the 20 control subjects brushed at seven locations (140 samples) were analyzed and 137 of 140 (98%) samples gave a reliable result for all markers. None of these samples showed AI, confirming the apparent high specificity of the assay (100%).

Discussion

We explored AI analysis on noninvasive brushed samples of the oral cavity as a method for identification of precancerous changes. This would allow identification and monitoring of precancerous changes in the oral mucosa without the burden of taking biopsies. Previously, we showed that the amount of DNA isolated from brushed samples varied between 150 and 600 ng (11). We assessed the minimal and maximal amount of normal template DNA that needs to be added to every PCR reaction till "false" AI occurred (using the 50% change in ratio taken as cutoff). Between 2 and 40 ng input of normal blood DNA, no significant changes in ratios were found. Below 0.5 to 1 ng input of normal DNA, false AIs started to occur, although dependent on the specific marker and the difference in length between the peaks (data not shown). The reliability of the peak ratios over such a large range of DNA input allows to assay samples without preceding DNA quantitation, although samples with very low fluorescence values on the electropherogram should be interpreted with caution.

Usually, normal DNA of blood is used as the reference DNA for AI ratio calculation. We examined whether the median of all seven samples could be used as the reference. This works very reliably except when many samples show allelic loss. In these cases, the "low-risk sample" of the soft palate can be taken as a reference. In general, the median of all values suffices as a reference value and blood sampling can be omitted. This will facilitate the implementation of this noninvasive cytologic assay in clinical practice and as a screening tool.

As mentioned, we scored AI when one allele was decreased by >50% in the sample when compared with the same allele in normal control DNA. However, we found in some cases one or more borderline results, with AI results of $0.5 < \times < 0.625$ and $1.6 < \times < 2.0$. In particular, when multiple borderline results are present on one chromosome, one could envision that there is a precancerous field present, but that by sampling error wild-type cells or free DNA derived from saliva was introduced into the sample, causing a relatively lower change in AI ratio.

In this study, we focused on AI markers. As head and neck cancers, in particular, those arising in the oropharynx, could also be caused by infection with the human papillomavirus, the use of human papillomavirus DNA as markers of precancer might be considered as well. However, in a previous study, it was shown that human papillomavirus infection is rarely found in oral premalignant lesions, and hardly contributes to malignancies in the oral cavity (18). Therefore, we decided not to analyze our samples for the presence of human papillomavirus.

Two lesions with AI in the biopsy were missed in the brushed samples. One leukoplakia patient (patient 21) even showed AI at three chromosomes in the biopsy sample, which were not detected in the noninvasive sample. After scrutinizing the investigation of all paraffin blocks, we could conclude that the lesion was large and genetically homogeneous. Moreover, we could show that the superficial nucleated cells contained the same precancerous changes as the basal cells. These potential caveats of a noninvasive approach with brush samples could not explain this false-negative case (data not shown). Hence, there was no explanation other than that this case was undoubtedly missed by noninvasive sampling. A possible explanation for the failure was the thick hyperkeratotic surface texture of the lesion, making it difficult to obtain a reliably exfoliated cell sample. The signal of the markers on the electropherogram were not particularly low, suggesting that DNA from other sources, possibly saliva, contaminated the samples. These false-negative cases leave room for improvement of the assay. Better designed brushes that allow sampling of more cells of deeper mucosal layers might improve sensitivity. Also, a test/retest approach with duplicate samples taken at the same sampling round might improve the reliability of the data.

Early diagnosis of oral precancerous but clinically nonvisible fields might be of importance for clinical management,

	Patient 12		Patient 17		Patient 20		Patient 21		Patient 22	
	Biopsy	Exfo	Biopsy	Exfo	Biopsy	Exfo	Biopsy	Exfo	Biopsy	Exfo
D3S1766	0.95	1.17	0.62	0.66	NI	NI	1.42	1.06	0.78	0.97
D3S1029	1.26	0.67	0.90	1.20	0.60	0.99	NI	NI	0.81	1.10
D3S1293	0.73	1.23	NI	NI	0.65	0.88	1.13	0.99	0.79	0.90
D9S171	2.98	2.55	6.13	2.10	1.14	1.02	6.88	1.09	0.21	0.07
D9S162	0.34	0.42	0.00	0.46	0.06	0.94	0.34	0.83	4.31	16.01
D9S157	2.44	2.46	39.35	2.96	0.34	1.01	5.95	0.98	3.95	13.98
D11S2002	0.90	1.18	1.21	1.09	NE	1.22	NI	NI	0.94	1.02
D11S1369	NI	NI	0.96	1.31	NE	0.89	2.62	1.36	1.22	1.08
D11S1883	1.32	0.89	1.62	0.99	NI	NI	NI	NI	NI	NI
CHRNB1	1.80	1.69	NI	NI	1.42	1.13	0.13	1.01	0.69	0.83
TP53	0.31	0.26	0.81	0.95	0.68	0.91	0.12	0.92	0.89	0.99
D17S1866	1.80	1.75	NI	NI	NI	NI	NI	NI	1.57	1.02

Fig. 3. Validation of the non invasive AI assay. Biopsies of the leukoplakia lesion were microdissected, analyzed by the same marker panel, and compared to the results of the exfoliated cells. Results from five patients which showed genetic alterations in the lesion. In general, the same genetic changes are seen in the exfoliated cell samples and the biopsies except for patients 20 and 21. Abbreviations are identical as indicated in the legend of Fig. 1.

particularly in high-risk populations such as patients with leukoplakia, patients with treated oral cancer, and even healthy individuals frequently exposed to environmental carcinogens. The noninvasive genetic assay presented here seems to be a valuable screening tool to detect these nonvisible precancerous fields. The large majority of samples work well (>95%), the specificity seems high (100%), and the sensitivity in leukoplakia lesions is ~80%. It should be particularly noted that the sensitivity estimate was based on a few patients. Notwithstanding, we believe that these preliminary results are suggestive to further develop this approach in larger cohorts. Furthermore, the sensitivity is expectedly higher for nonvisible precancerous fields, as these are usually not hyperkeratotic (which seemed to be the most prominent problem), negatively influencing the sensitivity. Following validation of the test variables in larger cohorts, prognostic studies in high-risk groups to predict oral cancer will have to be carried out to prove the actual clinical value of this approach. Assuming that the clinical value can be established, this assay could be helpful in selecting patients for secondary prevention, and monitoring the effect of interventions. Recently, oncolytic adenoviruses such as the ONYX adenovirus have been applied for the treatment of precursor lesions using mouthwash rinses (19). As Expectedly, other interventions will be developed and tested in clinical trials.

In conclusion, the presented noninvasive test can be used for identification as well as genetic grading of visible and possibly nonvisible precancerous fields, monitoring by follow-up sampling, and selection of location for biopsy. For leukoplakia lesions, however, biopsy should remain the gold standard, although genetic analysis of the specimen may have added value to the histologic diagnosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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