Noninvasive Molecular Screening for Oral Precancer in Fanconi Anemia Patients

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Abstract

LOH at chromosome arms 3p, 9p, 11q, and 17p are well-established oncogenic aberrations in oral precancerous lesions and promising biomarkers to monitor the development of oral cancer. Noninvasive LOH screening of brushed oral cells is a preferable method for precancer detection in patients at increased risk for head and neck squamous cell carcinoma (HNSCC), such as patients with Fanconi anemia. We determined the prevalence of LOH in brushed samples of the oral epithelium of 141 patients with Fanconi anemia and 144 aged subjects, and studied the association between LOH and HNSCC. LOH was present in 14 (9.9%) nontransplanted patients with Fanconi anemia, whereas LOH was not detected in a low-risk group (n = 50, >58 years, nonsmoking/nonalcohol history) and a group with somewhat increased HNSCC risk (n = 94, >58 years, heavy smoking/excessive alcohol use);

Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the most common cancer types worldwide and has high morbidity and mortality. The most important risk factors are tobacco use and alcohol consumption, although a subgroup of HNSCC is caused by human papillomavirus infection. Furthermore, certain inherited disorders, such as Fanconi anemia, predispose to HNSCC (1).

Most HNSCCs develop in large precancerous fields of genetically altered mucosal epithelium, also known as field cancerization. These fields can present as visible white (leukoplakia) or red (erythroplakia) lesions, but the majority are macroscopically not detectable. Field cancerization is not only relevant for cancer development, but also explains in part the high rate of local relapse and second primary tumors in patients with HNSCC (1–7).

LOH at chromosome arms 3p, 9p, 11q, and 17p are well-established oncogenic aberrations in oral precancerous lesions (3, 4, 8). In addition, several groups have shown that LOH, particularly at chromosome 3p and 9p, is a reliable prognostic biomarker associated with progression to oral cancer (9–15). Likewise, presence of LOH in histopathologically tumor-free surgical margins can predict local relapse after surgery (6). The observation that HNSCC develops in large precancerous fields opens a possibility for noninvasive detection of genetic changes using cells brushed from the mucosa. A genetic assay based on the analysis of LOH of 12 selected microsatellite markers located on chromosomes 3p, 9p, 11q, and 17p, mostly flanking areas close to tumor suppressor genes, can be used to detect LOH in exfoliated cells from the oral mucosa (16–18). This noninvasive genetic screening approach might be of value for the detection and monitoring of oral precancer in cohorts at increased risk for HNSCC, such as genetically predisposed patients with Fanconi anemia.

Fanconi anemia is a rare DNA repair disorder that is mainly inherited in an autosomal recessive manner. Fanconi anemia is characterized by congenital malformations, progressive bone marrow failure, endocrine disorders, and a strong predisposition to develop malignancies, particularly acute myeloid...
leukemia and squamous cell carcinoma (SCC; refs. 19, 20). The hematologic abnormalities in patients with Fanconi anemia often require stem cell transplantation (SCT) during the course of the disease. At present 19 Fanconi anemia genes have been identified, and the proteins encoded by these genes act together in a complex to organize repair of DNA interstrand cross-links (21, 22). Fanconi anemia cells are hypersensitive to interstrand cross-links that are induced by chemical agents, such as mitomycin C and cisplatin (22).

Patients with Fanconi anemia have a 500- to 1,000-fold higher incidence of HNSCC than the general population (19, 23). In addition, patients with Fanconi anemia develop HNSCC at a much younger age (median 31 years, range, 15–49 years) with a cumulative incidence of 14% by the age of 40 years (23, 24). HNSCC in Fanconi anemia patients is most often located in the oral cavity (23, 24). An important additional risk factor for HNSCC in patients with Fanconi anemia is SCT and associated graft-versus-host-disease (25, 26). Hence, within the Fanconi anemia patient population, those who received SCT are at highest risk for HNSCC with a relative risk of 4.4 compared with patients with Fanconi anemia who did not receive SCT (25).

Because patients with Fanconi anemia are hypersensitive for cisplatin-based chemoradiation protocols and often show increased toxicity with radiation, treatment possibilities for HNSCC are limited (23, 24). Frequent screening is therefore essential to detect oral (pre)cancer at an early stage when surgery alone is adequate. It is therefore indicated that patients with Fanconi anemia should be screened by oral inspection every 3 months from 10 years of age. As most precancerous changes are not visible as lesions, biopsies should be taken from high-risk areas for histology and genetic analysis, with, for example, 1-year intervals. This screening approach, however, would cause an unacceptable large burden for patients and detecting oral precancer with a noninvasive genetic test would therefore be an attractive alternative for these high-risk patients. LOH analysis of a panel of 21 SCCs from patients with Fanconi anemia, including 16 HNSCCs, showed that the LOH patterns in Fanconi anemia are comparable to those generally found in sporadic non-Fanconi anemia SCC. SCCs of patients with Fanconi anemia seem not to be genetically different from sporadic SCCs, at least at this resolution, and similar genetic changes might be used for noninvasive genetic screening (26).

In this study, we determined the prevalence of LOH in brushed cells of the oral epithelium of aged subjects with a low or somewhat increased risk for HNSCC. In addition, we analyzed brushed samples of genetically predisposed patients with Fanconi anemia who are at highest risk for HNSCC. In this group, we further analyzed the association of LOH with clinical characteristics and HNSCC.

Materials and Methods
Patients
Brushes of oral mucosa were obtained from patients with Fanconi anemia in a cross-sectional study design. Convenience sampling of patients with Fanconi anemia was performed between May 2005 and November 2009. Patients known to pediatricians or family support groups (German Fanconi anemia support group, Dutch Fanconi anemia support group, and Fanconi anemia Research Fund) were actively approached and sampled during hospital visits, at family support meetings, or at home visits. Clinical characteristics and disease history were collected. Smoking and alcohol use was scored as never, former, or current. A number of patients with Fanconi anemia were sampled more than once during the study period. Follow-up was performed from date of first brush until death or last available follow-up.

In addition, two other risk groups were tested, one at low risk for HNSCC and one at somewhat increased risk for HNSCC. These groups were recruited between September 2005 and September 2007 from The Longitudinal Aging Study Amsterdam cohort, a community-based sample of older adults in the Netherlands (27). The low-risk group for HNSCC consisted of men and women >58 years of age with a nonsmoking and nonalcohol use history. The second group at somewhat increased risk for HNSCC consisted of men and women >58 years old with a history of heavy smoking (average number of pack years 47 [range, 10–112.5]) and alcohol use (Garretsen score “excessive” and “very excessive”, refs. 28, 29).

The study was approved by the Institutional Review Board of the VU University Medical Center (protocol number 2005/156) and written informed consent was obtained from each subject or their legal representative. Patient information and collected data were encoded in a dedicated study database.

Transplanted patients with Fanconi anemia
During LOH analysis, we discovered that the presence of donor DNA in brushed oral mucosa of transplanted patients with Fanconi anemia interfered with the microsatellite patterns. To study this in more detail we performed longitudinal sampling in patients undergoing SCT to investigate the appearance and origin of donor DNA in the oral cavity. After informed consent, brushes of the oral mucosa were taken before SCT, weekly after SCT until stable hematopoietic recovery, followed by brushes once every 3 months. Sampling was performed as described below and samples were taken in duplicate: one sample was used for DNA isolation and the other sample was used for cytospin preparation and CD45 immunostaining to quantify the number of leukocytes.

Sampling
Brushed samples of the oral mucosa were taken from six sites with the highest predilection for oral cancer: the retromolar trigone left [A] and right [F], the lateral sides of the mobile tongue left [B] and right [E] and the floor of the mouth left [C] and right [D] (30). Also, macroscopically suspect mucosa, as judged by the inspecting physician, was brushed (indicated as H, I, J, etc.).

Omnident brushes (Dental Union) were used initially. Exfoliated cells were collected in 1.5 mL vials in sterile phosphate buffered saline (PBS). Samples in PBS were centrifuged and cell pellets were stored at −20°C until further DNA analysis. At a later stage in the study, Origenex brushes (Rovers Medical Devices) were used. Exfoliated cells were collected and frozen in Cytolyt (Hologic Benelux). Thawed cells were pelleted by centrifugation before DNA extraction. Changes of brushes or storage had no effect on the robustness of the assay (17). Sample information was stored in an encoded format.

LOH assay
DNA of the exfoliated cell samples was isolated by a column kit (Nucleospin 96 Tissue kit, Macherey-Nagel) on a Hamilton’s Microlab Star platform. A multiplex LOH assay, using 12 selected
microsatellite markers for chromosome 3p, 9p, 11q, and 17p, was performed as previously described (markers are shown in Supplementary Table S1, refs.16, 17). Data were analyzed and processed as previously described (16, 17, 31). LOH was scored if the allele ratio differed more than two times compared with the reference, when necessary after stutter correction. As a reference for LOH calculation, the median ratio of all brushed samples was used. In case of many genetic changes in multiple samples, blood or fibroblast DNA was used as reference in addition.

The percentage of donor DNA in brushed samples of transplanted patients with Fanconi anemia was calculated as follows. First, DNA of the recipient (collected before SCT) and DNA of the donor was analyzed to identify microsatellite markers showing different allele lengths for donor and recipient. Second, the peak intensities of donor- and recipient-specific alleles were measured. Only distinctive heterozygous alleles of donor and recipient or distinctive homozygous alleles of donor and recipient were compared for calculation. The data for all distinctive alleles of the tested markers were averaged as final readout.

**CD45 staining**

CD45 immunostaining was performed to quantify the number of leukocytes in brushed samples of transplanted patients with Fanconi anemia, as CD45 is a routinely used leukocyte biomarker. A cytospin slide centrifuge was used to deposit a thin layer of exfoliated cells onto a glass slide. Anti-CD45 antibody staining of the cytopsin was performed by standard immunocytochemical staining. In short, a three-step staining was applied using monoclonal Mouse Anti-Human CD45 (DAKO M0701, Glostrup), Biotinylated rabbit-anti-mouse (DAKO E0413), and StrepAB-complex/HRP (DAKO K0377). At least 100 cells were counted by microscopic examination and the percentage of CD45-positive cells was calculated.

**Statistical analysis**

Continuous variables are displayed as median and range. Discrete variables are displayed as counts and proportions. A Fisher exact test (two-sided) was used to compare proportions between groups. Donor DNA percentages were compared with CD45-positive cells percentages using least-squares linear regression analysis. Logistic regression was used to study variables potentially associated with LOH. Variables investigated were age, gender, smoking, and alcohol use. Univariate predictors of LOH that were statistically significant ($P < 0.10$) were selected for multivariable analysis. Results were expressed as OR and corresponding 95% confidence intervals. $P$ values of $<0.05$ were considered statistically significant.

**Results**

**Analysis of brushed samples from transplanted patients with Fanconi anemia**

Within the group of patients with Fanconi anemia, those who received an SCT are at highest risk for HNSCC, and we initially enrolled transplanted and nontransplanted patients. However, after LOH analysis of the first patients with Fanconi anemia, we noted aberrant alleles in brushed samples of transplanted patients. These aberrant alleles disturbed LOH analysis and were likely derived from donor cells. We analyzed DNA of the recipient before and after transplantation and compared the DNA profiles with those of the donor as the most likely source of these aberrant alleles (see Fig. 1). As expected, these aberrant peaks were donor derived. Further analysis and quantitation in 15 transplanted patients with Fanconi anemia showed presence of donor DNA in 14 of 15 patients, with donor DNA percentages up to 93% in some samples (see Table 1).

To investigate the appearance and origin of the donor DNA after SCT in more detail, we analyzed longitudinal samples in five additional patients with Fanconi anemia (4 male, 1 female) planned to undergo SCT because of bone marrow failure, myelodysplastic syndrome, or leukemia. Median age at SCT was 10.4 years (range, 3.8–32.7). Median follow-up after SCT was 5.0 months (range, 0–12.2). In total, 31 brushes were performed after transplant (median 8 brushes, range, 0–11). One patient died shortly after SCT and no posttransplant brushes could be performed.

First donor DNA signal was found at a median of 29 days after SCT. Donor DNA was persistent in all consecutive samples, including the samples obtained at last follow-up. Donor DNA percentages varied between individuals and also within individuals and ranged between 0% and 85%. An example of recipient- and donor-specific alleles for one microsatellite marker in consecutive mucosal brushes taken at site D (floor of mouth, right) after SCT is shown in Fig. 2A. The appearance of donor DNA after SCT in the brushes of oral mucosa at all the six sites in this patient is presented in Fig. 2B.

CD45 staining confirmed presence of leukocytes in brushes of the oral mucosa. Comparison of donor DNA percentages and CD45-positive cells percentages by linear regression analysis showed a strong correlation ($r^2 = 0.760, P < 0.001$, see Fig. 2C). To definitively confirm the leukocyte origin of donor DNA a PCR for T-cell receptor and immunoglobulin rearrangements was performed, but the amounts of DNA from the brushed samples were too low for these relatively insensitive methods (data not shown).

**Prevalence of LOH in nontransplanted patients with Fanconi anemia and other risk groups**

As LOH analysis was disturbed by donor DNA, we focused our research on the nontransplanted patients with Fanconi anemia as high-risk group, and, in addition, aged non-Fanconi anemia subjects without and with a smoking/alcohol use history as groups with low and somewhat increased risk for HNSCC, respectively.

A total of 144 aged subjects were brushed and analyzed; 50 in the low-risk group (3 male/47 female, median age 75 years [range, 58–90]) and 94 in the somewhat increased risk group (79 male/15 female, median age 64.6 years [range, 58–82]). In both groups, LOH could not be detected in any of the samples. An example of a subject without LOH is presented in Supplementary Table S2.

Between May 2005 and November 2009, 141 nontransplanted patients with Fanconi anemia were sampled. The characteristics of these patients are summarized in Table 2. Information on pre-study HNSCC screening was available for 116 patients, of whom 64 were older than 10 years of age. Of note, only 16 of these 64 patients (25%) received routine screening by a head and neck surgeon as indicated in the guidelines.

LOH was found in 14 of 141 (9.9%) patients with Fanconi anemia at a median age of 25.5 years (range, 6–52). This prevalence was significantly higher when compared with the low-risk...
group (Fisher exact test, \( P = 0.023 \)) or the somewhat increased risk group (Fisher exact test, \( P = 0.001 \)). LOH at chromosome 9p and/or 3p was found in 13 of the 14 (93%) patients with Fanconi anemia with LOH in the brushed samples. Additional LOH at chromosome 11q and/or 17p was found in three patients.

Next, we analyzed factors associated with the presence of LOH (see Supplementary Table S3). In univariate analysis, age (OR, 1.09, \( P = 0.000 \)) and alcohol use (former alcohol use OR, 16.8, \( P = 0.003 \), current alcohol use OR, 7.00, \( P = 0.016 \)) significantly increased the chance of LOH. In multivariable analysis only age was independently associated with LOH (OR, 1.13, \( P = 0.001 \)), indicating that the association of LOH and alcohol was age dependent, as expected.

**Longitudinal sampling in nontransplanted patients with Fanconi anemia**

Eight of the 14 patients with Fanconi anemia with LOH in the primary sample were sampled more than once. LOH persisted in all consecutive samples and in a few patients progression of LOH or enlargement of the field was seen (see Table 3, LOH at 9p at C, D, and E in the primary sample, addition of LOH at 9p at B in the consecutive sample).

**Follow-up nontransplanted patients with Fanconi anemia**

Follow-up was available for 110 of the 141 nontransplanted patients with Fanconi anemia, including 10 patients with LOH and 100 patients without LOH (see Supplementary Fig. S1).
Median follow-up was 66.1 months (range, 0–93.7). Five patients were diagnosed with HNSCC during the study period, four in the LOH group and one in the non-LOH group. LOH was significantly associated with HNSCC (4 of 10 in the LOH group versus 1 of 100 in the non-LOH group; Fisher exact test, \( P = 0.000 \)). Detailed description of patients with Fanconi anemia with LOH and HNSCC is presented in Table 4.

Two of the five HNSCC patients (F06–11 and F09–31) presented with suspect mucosal lesions at the time of first brush. The lesions of both patients were brushed and showed LOH and appeared to be HNSCC after further investigation. The other three patients (F06–84, F07–06, and F07–13) developed HNSCC during the study period at a median of 26.5 months (range, 17.1–42.3) after the first brush. Patient F06–84 and F07–13 showed LOH in one of the six routinely brushed sites and developed HNSCC within a few centimeters of the initial LOH site. A picture of a SCC of the gingiva in patient F06–84 is shown in Supplementary Fig. S2. LOH analysis of this patient is shown in Supplementary Table S4. Patient F07–06 did not show LOH in first and consecutive brushes. Of note, brushed samples of this patient were always contaminated by blood caused by bleeding of the mucosa during sampling.

Information on visible lesions at the time of first brush was analyzed in 110 patients with Fanconi anemia with follow-up (see Supplementary Fig. S3). Visible lesions were found in 27 of 110 patients (25%). Five patients with visible lesions (4 with LOH, 1 without LOH) developed HNSCC. Twenty-two patients with visible lesions (3 with LOH, 19 without LOH) did not develop HNSCC during the study period. None of the 83 patients without visible lesions (3 with LOH, 80 without LOH) developed HNSCC.

Follow-up time in the three patients with LOH, but without visible lesions, was 65.1, 92.2, and 92.3 months, respectively.

Of the five patients with HNSCC only one is alive at 83.3 months after HNSCC diagnosis. Four patients died at a median age of 44.9 years (range, 26.1–56.4). Cause of death was non-HNSCC cancer (\( n = 4 \)), bone marrow failure (\( n = 1 \)), and SCT-related morbidity (\( n = 1 \)).

Table 1. Donor DNA percentages in 15 transplanted patients with Fanconi anemia.

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NOTE: A, left retromolar trigone; B, left lateral side tongue; C, left floor of mouth; D, right floor of mouth; E, right lateral side tongue; F, right retromolar trigone.

Donor percentage 1%–25%.
Donor percentage 26%–50%.
Donor percentage 51%–75%.
Donor percentage 76%–100%.

Discussion

In this study we used a noninvasive multiplex LOH assay on brushed oral mucosal cells of several HNSCC risk groups: aged subjects with and without a smoking/alcohol use history and genetically predisposed patients with Fanconi anemia, who have a very high risk of developing HNSCC.

In a previous study with 20 young subjects no LOH was found in any of the 140 samples tested (18). As expected, no LOH was found in the low-risk group of aged subjects either. We expected to find LOH in the group at somewhat increased risk, as older age in combination with heavy smoking and excessive alcohol consumption are the well-known risk factors for oral cancer (RR 3.54 compared with never smokers/drinkers; ref. 32). However, the crude, yearly incidence of oral cancer in men and women older than 60 years is less than 55 per 100,000 (33). Notwithstanding that precancerous changes may be undetected for many years, the chance to find subjects with precancer remains very low and requires screening of thousands. This changes when analyzing samples of patients with a very high risk of HNSCC due to their genetic predisposition. We detected LOH in nearly 10% of the sampled patients with Fanconi anemia. The high prevalence of LOH found in these patients is in line with the 500- to 1,000-fold increased HNSCC risk for patients with Fanconi anemia (19, 23).

A technical limitation of the noninvasive LOH assay, which became apparent in this study, is the inability to use it in transplanted Fanconi anemia patients because presence of donor DNA interfered with the LOH analysis. We found donor DNA percentages up to 93% in brushes of the oral mucosa. Recent papers in the field of forensic medicine reported mixed chimerism in DNA of buccal swabs from recipients of hematopoietic SCT, affecting forensic DNA profiling (34, 35). Donor DNA percentages were highly variable between individuals and also within an individual at different sample sites and at different sample dates, and varied between 0% and 100% with a median value of 23%.

Age, time after transplant, type of SCT donor, and type of conditioning regimen were not significantly correlated with donor DNA percentages (35). Also in our study highly variable donor DNA percentages were detected in transplanted patients with Fanconi anemia.

The origin of donor DNA has been the subject of discussion in the last decades and is still a matter of debate. Reported mechanisms that could explain the presence of donor DNA in nonhematopoietic tissues are stem cell plasticity, fusion of bone marrow-derived blood cells with recipient cells, horizontal DNA transfer from donor to recipient cells, and contamination with donor leukocytes (36–42).

Although we cannot exclude other mechanisms, our data strongly indicate a very simple source: leukocyte contamination.

In 2013, Graveland and colleagues reported the results of the noninvasive LOH assay in patients with leukoplakia (17). An analytical sensitivity of 45% was found indicating that with a brushed sample only 45% of the cases with LOH in the biopsy were detected. The analytical specificity and positive predictive value were both 100%. Hence, this assay has limited value for monitoring patients with leukoplakia (17). Whether this low sensitivity was caused by the keratotic characteristics of the
leukoplakia lesion that may hamper sampling, or is also the result of contamination with DNA of other sources, most likely leukocytes, remains uncertain. Consequently, it is possible that the high prevalence of LOH found in brushes of the oral cavity of patients with Fanconi anemia is underestimated due to the suboptimal sensitivity of the LOH assay. In fact, the data suggest that

Figure 2.
Appearance of donor DNA in longitudinal samples of a transplanted Fanconi anemia patient. A, donor- and patient-specific alleles. Patient DNA as well as donor DNA is detected in brushed samples D of patient SCT-1, analyzed with marker D939369, 2 weeks, 4 weeks, 5 weeks, and 7 months after SCT. Patient-specific alleles were analyzed in oral mucosa collected before SCT. Donor-specific alleles were analyzed in DNA of blood of the donor. FU, fluorescence units; bp, base pairs.

(Continued on the following page.)
contamination with DNA of leukocytes might become a limiting factor for all noninvasive approaches.

Zhang and colleagues showed that LOH in oral premalignant lesions is the most significant predictor for malignant progression with a 22.6-fold increase in risk for lesions with LOH at 3p and/or 9p compared with lesions with 3p and 9p retention (15). In our study LOH of chromosome 3p and/or 9p was present in 93% (13/14) of the patients with Fanconi anemia with LOH. In addition, LOH was persistent in all consecutive brushes. We could associate LOH with HNSCC, but we could not indicate LOH as a prognosticator of HNSCC in our cohort, because two of the five HNSCC patients already presented with oral cancer at the time of first brush. However, when excluding these two patients, two of eight patients with LOH at first brush developed HNSCC compared with 1 of 100 in the LOH negative group (Fisher exact test, \( P = 0.014 \)) indicating that LOH might be an important prognostic marker of subsequent HNSCC development in Fanconi anemia, although this is based on the frequency analysis of only a few events. Much larger studies with long follow-up time will be required to prove the predictive value of noninvasive genetic testing in Fanconi anemia.

In our study, the youngest patient with LOH was 6 years of age. Unfortunately, follow-up of this patient was not available and it is unknown whether this patient developed oral cancer. Because LOH was persistent in all the consecutive brushes of the longitudinally brushed LOH patients, we may assume that LOH also persisted in this 6-year-old patient. Several cases of very young Fanconi anemia HNSCC patients are reported in the literature, including one patient of only 10 years of age (43, 44).

As life expectancy of patients with Fanconi anemia has increased markedly due to improved results of SCT, the number of patients developing HNSCC will increase consequently (23, 24, 45). A major problem of HNSCC in Fanconi anemia is that treatment possibilities are limited because patients with Fanconi anemia tolerate radiotherapy and chemotherapy poorly. Morbidity is high and overall survival at 2 years is only 49% compared with 70% in non-Fanconi anemia HNSCC (23, 24, 46). Another problem in the treatment of HNSCC in Fanconi anemia is that relapses are common; 10 of the 19 patients (53%) studied by Kutler and colleagues developed recurrences at a median interval of only 16 months (23). Furthermore, patients with Fanconi anemia often develop multiple primary SCCs; 63% developed one or more second primary tumor of the aerodigestive and anogenital tract (23, 46).

Up to now screening remains the best approach to detect oral (pre)cancer at an early stage and treat these by transoral excision or laser. Patients with Fanconi anemia are therefore advised to undergo inspection of the oral cavity every 3 months from the...
Table 3. Example of a patient with Fanconi anemia with progression of LOH

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Table 4. Patients with LOH and HNSCC

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<th>HNSCC site</th>
<th>LOH at standards sites</th>
<th>LOH at suspect mucosa</th>
<th>LOH at HNSCC site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Code</td>
<td>Age†</td>
<td>M/F</td>
<td>LOH 1st brush</td>
<td>Site</td>
<td>(yes/no/unknown)</td>
</tr>
<tr>
<td>F05-03</td>
<td>26.0</td>
<td>M</td>
<td>3p, 9p</td>
<td>3p, J, 9p, C-J</td>
<td>Unknown</td>
</tr>
<tr>
<td>F05-05</td>
<td>11.0</td>
<td>M</td>
<td>17p</td>
<td>17p</td>
<td>Unknown</td>
</tr>
<tr>
<td>F05-09</td>
<td>10.0</td>
<td>F</td>
<td>3p, 9p, 11q, 17p</td>
<td>H</td>
<td>Unknown</td>
</tr>
<tr>
<td>F05-32</td>
<td>6.4</td>
<td>M</td>
<td>3p</td>
<td>3p</td>
<td>Unknown</td>
</tr>
<tr>
<td>F06-02</td>
<td>16.2</td>
<td>F</td>
<td>9p</td>
<td>C, D, E</td>
<td>No</td>
</tr>
<tr>
<td>F06-14</td>
<td>19.4</td>
<td>M</td>
<td>9p</td>
<td>D</td>
<td>No</td>
</tr>
<tr>
<td>F06-53</td>
<td>40.7</td>
<td>F</td>
<td>9p</td>
<td>A</td>
<td>No</td>
</tr>
<tr>
<td>F06-84</td>
<td>52.3</td>
<td>F</td>
<td>9p</td>
<td>C</td>
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<tr>
<td>F06-101</td>
<td>22.6</td>
<td>F</td>
<td>9p</td>
<td>D</td>
<td>No</td>
</tr>
<tr>
<td>F07-36</td>
<td>36.1</td>
<td>F</td>
<td>no LOH</td>
<td>—</td>
<td>Yes</td>
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<tr>
<td>F07-08</td>
<td>30.1</td>
<td>F</td>
<td>9p</td>
<td>E</td>
<td>F</td>
</tr>
<tr>
<td>F07-11</td>
<td>24.8</td>
<td>F</td>
<td>3p, 9p, 11q, 17p</td>
<td>J, L</td>
<td>Yesc</td>
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<tr>
<td>F07-13</td>
<td>35.2</td>
<td>M</td>
<td>9p</td>
<td>B</td>
<td>No</td>
</tr>
<tr>
<td>F08-61</td>
<td>30.0</td>
<td>M</td>
<td>9p</td>
<td>C</td>
<td>No</td>
</tr>
<tr>
<td>F09-31</td>
<td>48.1</td>
<td>F</td>
<td>3p, 9p, 11q</td>
<td>H</td>
<td>Yes</td>
</tr>
</tbody>
</table>

NOTE: A, left retromolar trigone; B, left lateral side tongue; C, left floor of mouth; D, right floor of mouth; E, right lateral side tongue; F, right retromolar trigone. First sample, performed in June 2006, shows LOH of 9p in C, D, and E. Second sample, performed in March 2007, shows LOH of 9p in B, C, D, and E. Marker not informative.

LOH: allele ratio <0.5 or >2.0.

age of 10 years. Strikingly, in our Fanconi anemia cohort four of the five patients with oral cancer developed SCC of the gingiva. We therefore recommend to carefully inspect the gingiva during the three monthly screening, and consider the gingiva as predilection site for oral cancer in Fanconi anemia.

As a result of our opportunistic screening approach, it was noted that many patients with Fanconi anemia did not receive routine screening for HNSCC at the time of first brush. This study therefore improved the awareness of patients and parents. The presented data in this first observational study do not allow strong recommendations or introduction of genetic screening at routine basis, but the following schedule could be considered. We adhere to the three monthly screening by an experienced head and neck surgeon for all patients with Fanconi anemia older than 10 years, and at least yearly when this is not feasible, including careful inspection of the oral cavity and the gingiva. In addition, we recommend noninvasive genetic analysis of suspect visible lesions and this may be extended to the six sites with the highest HNSCC predilection as well as the gingiva in patients with Fanconi anemia. When visible lesions are present or when areas of premalignant genetic changes are diagnosed by noninvasive genetic analysis, the screening frequency should be increased to at least three monthly intervals and suspicious areas should be documented by photography. When visible lesions are biopsied on clinical indication, the biopsies should be diagnosed by histopathologic examination and by 3p and 9p LOH for risk assessment. A possible further implication of noninvasive genetic screening in the future is identifying patients eligible for chemoprevention trials.

LOH screening in patients with Fanconi anemia is a promising method to identify the patient at high risk for oral cancer. The somewhat unexpected source of leukocyte DNA indicates that we have to develop more sensitive assays, especially for transplanted patients. New genetic assays, using next generation sequencing approaches, might improve the applicability of this noninvasive screening tool and might have a higher sensitivity (47). However, it is important to realize that these approaches are still expensive, usually require larger amounts of input DNA and will also be influenced by the presence of leukocyte DNA.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.E. Smetsers, R. Dietrich, T. Wu, D.J.H. Deeg, B.J.M. Braakhuis, R.H. Brakenhoff
Writing, review, and/or revision of the manuscript: S.E. Smetsers, E. Velleuer, D.J.H. Deeg, C.R. Leemann, B.J.M. Braakhuis, R.H. Brakenhoff

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Study supervision: R.H. Brakenhoff

Grant Support

S.E. Smetsers received a grant from the Dutch Children Cancer Free Foundation (KiKa, project number 34). E. Velleuer received a grant from the German Fanconi Anemia Support Group, and R.H. Brakenhoff received a grant from the Fanconi Anemia Research Fund (FAFE).

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Received June 3, 2015; revised August 3, 2015; accepted August 5, 2015; published OnlineFirst August 14, 2015.

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Publish Online First August 14, 2015; DOI: 10.1158/1940-6207.CAPR-15-0220

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Stephanie E. Smetsers, Eunike Velleuer, Ralf Dietrich, et al.


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