CHAPTER 2

Generation and molecular characterization of head and neck squamous carcinoma cell lines of Fanconi anemia patients

Hester J.T. van Zeeburg
Peter J.F. Snijders
Mario A.J.A. Hermsen
Martin A. Rooimans
Grover Bagby
Jean Soulier
Eliane Gluckman
Johan Wennerberg
C. René Leemans
Hans Joenje
Ruud H. Brakenhoff

Cancer Research 2005 65;1271-76
Abstract

Patients with Fanconi anemia (FA) are prone to develop malignancies at an early age. Besides hematological malignancies also squamous cell carcinomas in the anogenital region and head and neck are also frequently found in these patients. The aim of this study was to generate a panel of head and neck squamous carcinoma (HNSCC) cell lines and xenografts of FA HNSCC, and to characterize these cell lines in comparison with a panel of seven cell lines from patients with sporadic HNSCC. Analyses have been done on sensitivity to DNA cross-linking agents, loss of heterozygosity profile, TP53 mutations, TP53 polymorphisms and the presence of human papillomavirus. Four FA HNSCC cell lines were established. Sensitivity to DNA cross-linking agents (cisplatin) in the FA HNSCC cell lines was on average 10 times higher as compared with the sporadic HNSCC cell lines. Human papillomavirus was not detected in any of the FA or sporadic cell lines. No differences were found in loss of heterozygosity pattern, TP53 mutation frequency and TP53 polymorphism between FA and sporadic HNSCC cell lines. This is the first report on the generation of squamous cell lines of FA patients. The FA HNSCC cell lines we have generated may be utilized for future studies and might aid in the development of new preventive therapies for FA patients. The genetic characteristics of these cell lines suggest that FA HNSCC are not very different from sporadic HNSCC, except for the sensitivity to cisplatin which is consistent with the known cellular FA phenotype.
Generation of Fanconi Anemia HNSCC cell lines

Introduction

Head and neck squamous cell carcinomas (HNSCC) comprise about 5% of all newly diagnosed cancer cases in the Netherlands and has a worldwide prevalence of 500,000 new cases each year. HNSCC arises from the squamous epithelial cells in the upper aerodigestive tract, which includes the oral cavity, nasopharynx, hypopharynx and larynx. Risk factors for developing HNSCC are smoking and/or alcohol abuse. A second risk factor is infection with human papillomavirus (HPV), particularly for development of carcinomas in the oropharynx and oral cavity. Finally, a third risk factor for development of HNSCC is genetic predisposition. It has been noted that patients with Fanconi anemia (FA) are genetically predisposed to head and neck cancer\cite{1,2}.

FA is an autosomal recessive disease and has a worldwide prevalence of one to five per million. Cells of FA patients have spontaneous chromosomal instability and a cellular hypersensitivity to DNA cross-linking agents, such as mitomycin C and cisplatin. This hypersensitivity has been exploited for complementation analysis to answer the question whether one or more genes cause FA\cite{3}. Today, 11 complementation groups have been identified (A, B, C, D1, D2, E, F, G, I, J and L) and genes have been identified from 8 of these complementation groups\cite{4,5}. The most common complementation groups are A and C accounting for, respectively, 65% and 15% of all FA cases. Mutations in one of these FA genes prohibit FA protein complex formation, resulting in excessive DNA damage. The disease is characterized by diverse congenital malformations, progressive bone marrow failure and predisposition to develop malignancies. The life expectancy of FA patients is reduced to an average of 20 years, mainly because, at an early age, FA patients develop bone marrow failure and malignancies, particularly acute myeloid leukemia. They also frequently develop squamous cell carcinomas (SCC), particularly in the head and neck, anogenital region and esophagus\cite{3}.

Most FA HNSCC patients seem to develop carcinomas in the oral cavity (63%), in particular on the tongue (32%)\cite{6}. The high incidence of HNSCC and anogenital SCC in FA patients in combination with the specific locations of the tumors, led to the hypothesis that an environmental factor might be associated with HNSCC development in FA patients\cite{6}. Because HPV is involved in the carcinogenesis of some SCC of the anogenital (cervix) as well as head and neck (oropharynx) regions\cite{1}, the environmental factor that might play a role in FA HNSCC development could therefore be this virus\cite{6}.

A recent report showed that HPV is present in most FA HNSCCs (15/18) examined. In addition, none of these 18 FA HNSCCs were found to have a \textit{TP53} mutation\cite{7}, which is in concordance with previous results on HPV-mediated carcinogenesis\cite{1}. HPV expresses two viral oncoproteins, and one of these, E6, inactivates the p53 protein. The exact role of HPV in the pathogenesis of these tumors, however, needs further investigation because in other HNSCC of FA patients no HPV could be detected, while the p53 gene was mutated\cite{8}.
To facilitate these studies, and to evaluate therapeutic strategies for FA HNSCC tumors we aimed at generating cell lines of these tumors. Here, we describe the establishment of four FA HNSCC cell lines. We compared the genetic profiles, HPV status, TP53 mutation frequency and TP53 codon 72 polymorphism of these FA HNSCC cell lines with cell lines derived from sporadic HNSCC.

Materials and Methods

Patient material
Tumor samples were obtained from various sources; OHSU-974, VU-1131-T2.8, and VU1365 cell lines were established according to methods described previously\(^{[9]}\). UHLU-539 could only be obtained as a xenograft. Relevant clinical data of tumors are depicted in Table 1. Cell lines of sporadic tumors were established previously; 92VU040, 92VU041, 92VU059, 92VU078, 92VU080, 93VU094 and 93VU1209; the clinical data of the sporadic HNSCCs are summarized in Table 2. The study was approved by the Institutional Review Board.

Cell culture
Human HNSCC cell lines 93VU078, 93VU094, 93VU120, OHSU-974, VU1131-T2.8 and VU1365 were grown at 37 °C and 5% CO\(_2\) as monolayers in 75 cm\(^2\) flasks. Cell lines OHSU-974, VU1365, 93VU078, 93VU094 and 93VU120 were cultured in minimal essential medium Eagles with Earl’s salts (EMEM) (Cambrex, Verviers, Belgium) supplemented with 10% heat-inactivated FCS, 50 U/ml penicillin, 50µg/ml streptomycin (Cambrex) and 0.4 mM-glutamine. VU1131-T2.8 was cultured in RPMI 1640 medium (Cambrex) supplemented with 5% heat inactivated FCS, 50 U/ml penicillin and 50 µg/ml streptomycin.

Sulforhodamine B assay
Inhibition of cell growth in response to cisplatin and methotrexate was determined with the semi-automated proliferation assay using sulforhodamine B (SRB, Sigma, Zwijndrecht, The Netherlands), as described previously\(^{[10]}\). In short, 1,000 to 3,000 cells in 150 µl medium were plated in 96-wells plates (Greiner, Mannheim, Germany) and incubated for 72 hours at 37°C and 5% CO\(_2\). In total 50 µl medium containing cisplatin or methotrexate (VUmc Pharmacy) was added and cells were incubated for 72 hours at 37°C and 5% CO\(_2\). Cells were exposed to different cisplatin and methotrexate concentrations (1, 10 and 100 nM, and 1 and 10 µM cisplatin; or 0.1, 1, 10 and 100nM and 1 µM methotrexate). Finally cells were fixed with 25% trichloroacetic acid for at least 1 hour at 4 °C, washed and stained for 15 minutes with 0.4% SRB dissolved in 1% acetic acid. Plates were washed with 1% acetic acid and dried. The dye was solubilized with 10 mmol TRIS-HCl (pH 9.0) by shaking for 1 hour; absorbances were measured.
at 540 nm in the microplate reader (Multiskan Bichromatic, Labsystem, Helsinki, Finland).

**RNA and DNA isolation**

RNA was isolated using RNA-bee (Campro Scientific, Veenendaal, The Netherlands); the cell pellet was resuspended into 1 ml RNA-bee and 200 µl chloroform was added, mixed and placed on ice for 5 minutes. This was followed by centrifugation for 15 minutes at 17,000 × g at 4°C. The aqueous layer was transferred to a new vial and the interphase was used for DNA isolation. RNA (450 µl) was precipitated by addition of 450 µL isopropanol, mixed, placed on ice for 5 minutes and centrifuged for 15 minutes at 17,000 x g at 4°C in an Eppendorf (Eppendorf, Hamburg, Germany) tabletop centrifuge. The pellet was washed with 500 µl 70% ethanol, dried and dissolved in 30 µl RNase free water. For DNA extraction 800 µl DNA-STAT60 (Campro Scientific) and 200 µl chloroform was added to the interphase, mixed and centrifuged for 15 minutes at 17,000 x g at 4°C. The aqueous layer was transferred into a new vial and DNA was precipitated as described for RNA. As reference, normal DNA was extracted from stored T lymphocytes of all patients (sporadic SCCs). DNA isolation was performed by treatment with proteinase K as described previously (see also below)[11].

**Isolation of DNA from microdissected tumors**

Snap-frozen tissue of VU-1131-T2.8, VU-1365 and UHLU-539 was serially sectioned (10-20 sections of 5 µm) on a cryostate microtome. Sections were directly used for HPV analysis or the DNA was isolated as follows. Sections were put in 90 µl proteinase K buffer [100 mM TRIS-HCl, pH 9.0, 10 mM NaCl, 1% SDS and 5 mM EDTA] with 10 µl Proteinase K (1 mg/ml) (Boehringer-Ingelheim, Alkmaar, The Netherlands) for 24 hours at 52°C with shaking. Thereafter DNA was isolated by phenol-chloroform-isoamylalcohol (25:24:1) extraction, followed by ethanol precipitation. DNA was dissolved in LoTE-buffer [3 mM TRIS-HCl, 0.2 mM EDTA, pH 7.5]. Paraffin embedded tissue of the sporadic cell lines 92VU040, 92VU041, 92VU059, 92VU078, 92VU080, 93VU094 and 93VU120 and 1 section of 5 µm with 1 cm² tumor tissue was directly used for HPV analysis.

**HPV analysis**

Firstly, a ß-globin PCR generating a 209-bp product was performed to determine the DNA integrity of the samples. All samples positive for ß-globin PCR were subjected to HPV PCR analysis. HPV detection was performed by GP5+/6+-PCR enzyme immunoassay as previously described using an oligoprobe cocktail for 14 high-risk HPV types (i.e. HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68)[12]. This method targets L1 sequences within the HPV genomes. GP5+/6+-PCR enzyme immunoassay-positive cases were subsequently typed by reverse line blot genotyping. Be-
cause failure of HPV detection by GP5+/6+ PCR may be due to integration of the viral genome and disruption of the GP5+/6+ primer region within L1, samples negative in the GP5+/6+ PCR enzyme immunoassay were additionally tested by HPV-16 E7 region type specific PCR, as previously described\[12\].

Microsatellite analysis
Microsatellite analysis to assess loss of heterozygosity (LOH) profiles were done as previously described\[11\]. The following markers were used; D3S1284 (3p12), D3S1766 (3p14), D3S1293 (3p24), D3S1029 (3p21), D3S1217 (3p13), D3S1274 (3p12), D9S171 (9p21), D9S1748 (9p21), D9S157 (9p22), D9S162 (9p22), IFNA (9p21), D9S1751 (9p21), D17S1866 (17p13.3), CHRN81 (17p11-12), TP53 (17p13.1), D13S170 (13q31), D13S168 (13q14.3), D13S158 (13q32), D13S294 (13q14.3), D18S57 (18q12), D18S54 (18q12), D8S1130 (8p23), LPL-GZ (8p22). Statistical analysis of differences in LOH frequencies between FA HNSCC and sporadic HNSCC cell lines of all 23 markers was done using Fisher’s exact test. Two-tailed analyses were done, and \( P \) values <0.05 were considered statistically significant.

TP53 mutation analysis
TP53 mutation analysis was done on both RNA and DNA of all cell lines. Transcript sequencing was performed as previously described\[13\] with a few adaptations. RNA primer III reversed was adapted to (5’ TTA TGG CGG GAG GTA GAC TG 3’) and cycling conditions were 3 minutes 94°C, followed by 35 cycles (94°C for 30 seconds, 58°C or 60 °C for 1 minute and 72°C for 1 minute) and finally an extension for 5 minutes at 72°C and holding at 4°C with the MJ cycler (Biozym, Landgraaf, The Netherlands). PCR products were purified using the Qiagen PCR purification kit (Westburg, Leusden, The Netherlands). Sequencing was performed using the Big Dye primer –21M13 or M13 kit (Amersham Biosciences, Buckinghamshire, UK) using 1 µl of purified PCR product. Sequencing products were mixed (GATC) and precipitated using 100 µl 70% ethanol for 20 minutes on ice followed by 30 minutes centrifugation at 17,000 x g at 4°C. The pellet was vacuum-dried and dissolved in 15 µl TSR (Applied Biosystems, Buckinghamshire, UK) and analyzed on the ABI310 (Amersham Biosciences, Buckinghamshire, UK). TP53 mutation analysis was also done on genomic DNA in exons 5-9 as described before\[11\]. For analysis of codon 72 polymorphism, exon 4 of normal DNA from blood was sequenced.
Results

Cell lines

Four FA HNSCC cell lines could be established from the patients listed in Table 1. Three of them (OHSU-974, VU1131-T2.8 and VU1365) could be cultured in standard medium under standard conditions. Xenograft take rates of these cell lines were 5/6 for OHSU-974, 6/6 for VU1131-T2.8 and 0/6 for VU1365. UHLU-539 could only be propagated as xenograft, and is not available as cultured cell line.

HPV and TP53 mutation

HPV DNA was not detected in any of the FA and sporadic HNSCC cell lines. Mutations in TP53 were detected in all FA-HNSCC cell lines. VU1131-T2.8 and VU1365 were found to have TP53 mutations on well-known hot spots (R273 and R282 respectively). A 1-bp deletion in OHSU-974 at codon 41 (exon 4) results in a very short p53 protein by introduction of a stop codon downstream of this frameshift mutation. UHLU-539 was found to have a deletion of 1.8 kb encompassing exons 8 and 9. Mutations of VU1131-T2.8 and VU1365 were confirmed on the original tumor. The TP53 mutations of OHSU-974 and UHLU539 could not be confirmed as the original tumor material was not available. In the seven sporadic HNSCC cell lines, four were found to have a TP53

Table 1. Clinical information of FA HNSCC patients and tumors

<table>
<thead>
<tr>
<th>FA information</th>
<th>OHSU-974</th>
<th>VU1131</th>
<th>VU1365</th>
<th>UHLU539</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>Oregon</td>
<td>Groningen</td>
<td>Paris</td>
<td>Sweden</td>
</tr>
<tr>
<td>Gender</td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>FA-type</td>
<td>FA-A*</td>
<td>FA-C</td>
<td>FA-A6</td>
<td>FA-A</td>
</tr>
<tr>
<td>BMT</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>Age of BMT</td>
<td>-</td>
<td>13</td>
<td>13</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SCC information</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>Tongue</td>
<td>Floor of mouth</td>
<td>Mouth mucosa</td>
</tr>
<tr>
<td>Age of onset</td>
<td>29</td>
<td>34</td>
<td>22</td>
</tr>
<tr>
<td>Treatment</td>
<td>SURG</td>
<td>SURGRT</td>
<td>SURG</td>
</tr>
<tr>
<td>TNM stage</td>
<td>U</td>
<td>T4N2b</td>
<td>U</td>
</tr>
<tr>
<td>HPV cell line</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
</tr>
<tr>
<td>HPV tumor</td>
<td>NA</td>
<td>neg</td>
<td>neg</td>
</tr>
<tr>
<td>TP 53 codon</td>
<td>del T codon 41</td>
<td>R273L</td>
<td>R282W</td>
</tr>
<tr>
<td>TP 53 tumor</td>
<td>NA</td>
<td>R273L</td>
<td>R282W</td>
</tr>
<tr>
<td>Codon 72 polymorphism</td>
<td>RP</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Pack-years</td>
<td>U</td>
<td>4</td>
<td>U</td>
</tr>
<tr>
<td>Take rates</td>
<td>5/6</td>
<td>6/6</td>
<td>0/6</td>
</tr>
</tbody>
</table>

Pack-years are defined as smoking of one box of cigarettes per day for 1 year.
Abbreviations: R, arginine; RP, arginine/proline; IP, in progress; BMT, bone marrow transplanted; SURG, surgery; SURGRT, surgery and radiotherapy; U, unknown; neg, negative; NA, not available.

*Complementation analysis was not conclusive. Mutation analysis is in progress.
#Complementation group was determined by retroviral transduction.
mutation. Cell line 92VU078 had mutation A161T (exon 5), 92VU080 had a deletion of 300 bp, including a part of exon 6, 93VU094 had a deletion of exon 8 and 93VU120 had mutation A159F in exon 5. The frequencies of p53 mutations in FA and sporadic HNSCC cell lines were not significantly different ($P=0.2$, two-tailed Fisher’s exact test).

**TP53 codon 72 polymorphism**
The TP53 exon 4 codon 72 polymorphism was analyzed for all FA and sporadic HNSCC. The results of the codon 72 polymorphism can be found in Table 1 and 2 for the FA and sporadic HNSCC cell lines, respectively.

**Microsatellite analysis of FA HNSCC and sporadic HNSCC cell lines**
The LOH profiles of the four FA-HNSCC and seven sporadic HNSCC cell lines are depicted in Figure 1. Losses at all chromosome arms studied were detected in most FA HNSCC and sporadic HNSCC cell lines. Exceptions were the FA cell lines VU1365 and OHLU539, and the sporadic cell lines 92VU040 and 93VU120. VU1365 showed no losses at 17p and 18q and OHLU539 had no losses at 13q and 18q. Cell line 92VU040 only shows LOH at 9p and 93VU120 only shows LOH at 3p and 18q. No statistical differences of LOH frequencies between FA HNSCC and sporadic HNSCC cell lines could be found for any of the all 23 markers tested. Recently, results were published on the different LOH patterns of HPV-positive versus HPV-negative tumors\[14\], using E6 expression as an indicator of positivity or negativity. It was shown that tumors that do contain HPV DNA and which are E6-positive show much less LOH than tumors that do not contain HPV DNA. We compared the LOH frequencies of this previous study\[14\] with those of the FA HNSCC and the sporadic HNSCC of the present study. LOH at some regions was significantly more frequent in both FA and sporadic HNSCC than in the HPV DNA/E6-positive tumors reported previously (12/23 and 10/23 markers, respectively; $P<0.05$). No statistical differences were detected between both FA HNSCC and sporadic HNSCC versus the HPV DNA/E6 negative tumors\[14\]. This suggests

<table>
<thead>
<tr>
<th>Location</th>
<th>Tongue</th>
<th>FOM</th>
<th>Ret. Trig.</th>
<th>Tongue</th>
<th>Base of tongue*</th>
<th>Tongue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age of onset</td>
<td>65</td>
<td>60</td>
<td>57</td>
<td>20</td>
<td>40</td>
<td>65</td>
</tr>
<tr>
<td>Treatment</td>
<td>SURGRT</td>
<td>SURGRT</td>
<td>SURG</td>
<td>SURGRT</td>
<td>SURGRT</td>
<td>SURGRT</td>
</tr>
<tr>
<td>TNM stage</td>
<td>T3N0</td>
<td>T3N2</td>
<td>T2N0</td>
<td>T3N2b</td>
<td>NA</td>
<td>T3N1</td>
</tr>
<tr>
<td>HPV cell line</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
</tr>
<tr>
<td>TP53 codon 72 polymorphism</td>
<td>neg</td>
<td>neg</td>
<td>neg</td>
<td>A161T</td>
<td>del 300 bp</td>
<td>del exon 8</td>
</tr>
<tr>
<td>Pack-years</td>
<td>U</td>
<td>84</td>
<td>10</td>
<td>3.5</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>Unit years</td>
<td>U</td>
<td>630</td>
<td>107</td>
<td>U</td>
<td>0</td>
<td>150</td>
</tr>
</tbody>
</table>

Clinical TNM stage is shown. One pack-year is defined as smoking one box of cigarettes per day for 1 year. One unit year is defined as one unit of alcohol per day for 1 year.

Abbreviations; SURG, surgery; SURGRT, surgery and radiotherapy; neg, negative; Ret. Trig., retromolare trigone; R, arginine; P, proline; RP arginine/proline; NA, not applicable/recurrent tumor; NE, not evaluable; U; unknown.

*Recurrent tumor, primary tumor T2N0 at age 38.
Generation of Fanconi Anemia HNSCC cell lines

Figure 1. Results of microsatellite analysis of 23 markers on six chromosome arms of four FA HNSCC and seven sporadic HNSCC cell lines. Black boxes indicate allelic loss, gray boxes indicate no allelic loss. Note that the FA HNSCCs show the LOH patterns as has been described for sporadic HNSCC\(^1\), which are not induced by HPV. XS = chromosome. NI = not informative; MSI microsatellite instability.
that HPV was not involved in the genesis of the studied FA and sporadic HNSCC.

Sulforhodamine B assay
The FA HNSCC cell lines OHSU-974 and VU1131-T2.8 were analyzed for their sensitivity to cisplatin and methotrexate and compared to sporadic HNSCC cell lines 92VU078, 93VU094 and 93VU120 which all had a mutation in the TP53 gene. Figure 2 shows the results of the SRB analysis. The FA cell lines were on average 10 times more sensitive to cisplatin (mean IC$_{50}$ 0.4 versus 4.3 µM; $P=0.019$ with the two-sided Student’s t-test) than the sporadic HNSCC cell lines, although sensitivities to methotrexate in FA and sporadic HNSCC cell lines were not different (mean IC$_{50}$ 53 versus 66 nM respectively; $P=0.26$ with the two-sided Student’s t-test).

Discussion
We have generated four FA HNSCC cell lines, two of which could be propagated in culture as well as in xenografts, although one could only be propagated in culture and one only as xenograft. Comparison of the FA HNSCC cell lines with sporadic HNSCC cell lines that have already been established$^{[9]}$ may provide information about the etiology and characterization of FA HNSCC. Therefore, these cell lines were compared for HPV presence, mutations of the TP53 gene and polymorphism, LOH patterns and sensitivity to DNA cross-linking drugs. Our data show that the FA HNSCC cell lines are very similar to the majority of sporadic

![Figure 2. Sensitivity for cisplatin (A) and methotrexate (B) of two FA HNSCC cell lines (OHSU-974 and VU-1131T2.8) and three sporadic HNSCC cell lines (92VU078, 93VU094 and 93VU120). IC$_{50}$ values are given in nM. Results from two independent experiments (done in duplicate) are shown.](image-url)
Generation of Fanconi Anemia HNSCC cell lines.

We detected no HPV DNA, although previously, a role of HPV in FA HNSCC was proposed. It could be argued that these cell lines were HPV-negative for various reasons including that the virus could be integrated, that the specific sequences for PCR detection are not present, or that the subtype is not recognized by the general primer PCR used. Not all of these reasons seem valid as the majority of HPV types in HNSCC is HPV16 and one copy of HPV can be detected in 5,000 cells by the general primer PCR. Nevertheless, we decided to analyze several surrogate markers including the presence of TP53 mutations, TP53 codon 72 Arg/Pro polymorphism and LOH patterns.

In 50 to 60% of all HNSCC cases, mutations in the TP53 gene are detected. We analyzed both FA and sporadic HNSCC for mutations of TP53 and found mutations of TP53 in all (4/4) FA HNSCC. In two cases, these could be confirmed on the DNA from the primary tumor and in two cases, this was not available. Mutations of TP53 were also found in four (4/7) sporadic HNSCC. It is known that this type of mutation could be related to etiology. Particularly, tobacco use causes particular G to T transitions, which are frequently found in HNSCC. G to A transitions are also frequently found in HNSCC, but this transition might not be associated with tobacco use. In our study, one FA patient (VU-1131T2.8) had used tobacco (6 pack-years) and the TP53 mutation was indeed a G to T transition. The other FA patients had no history of tobacco use and consistent with this, the TP53 mutations were not tobacco-related [C to T transition (VU-1365) and two deletions (OHSU-974 and UHLU-539)]. Of the four sporadic HNSCC that showed a TP53 mutation, three had a history of tobacco and/or alcohol use. 92VU078 (3.5 pack-years) had a G to A transition, which might not be related with tobacco use, cell line 92VU080 (2.5 pack-years) had a deletion of 300 bp, which is not thought to be tobacco-related, whereas cell line 93VU120 (37 pack-years) had two transitions, G to T and C to T, with the G to T transition probably being tobacco-related.

Previously, it was shown that the known codon 72 polymorphism of TP53 (arginine or proline) might play a role in the development of HPV-induced HNSCC. The Arg72 p53 protein is considered to be a risk factor for HPV-induced HNSCC because it is more easily degraded by the E6 protein of HPV16 and HPV18, and therefore we compared the allele frequencies of the two variants. In a control population (no HNSCC), allele frequencies were around 0.75 and 0.25 for arginine and proline, respectively.

<table>
<thead>
<tr>
<th>Allele/Genotype</th>
<th>FA HNSCC</th>
<th>Sporadic HNSCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>Proline</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Arginine/Arginine</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Arginine/Proline</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Proline/Proline</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 3. Allele and genotype frequencies of TP53 codon 72 polymorphism for FA and sporadic HNSCC cell lines.

NOTE: No statistical differences were found between FA and sporadic HNSCC allele frequencies. It should be stressed that the number of samples for these comparisons is very small, and the data should be interpreted with caution.
and we found no major differences in allele frequencies and genotypes (Table 3) in the FA HNSCC cell lines and the sporadic HNSCC cell lines, although it should be noted that the number of samples for these comparisons is small, and these data should be interpreted with caution. The codon 72 polymorphism status of FA patients with and without HNSCC was compared previously, and it was shown that FA patients with Arg72 homozygosity had an increased risk (OR 5.6) to develop SCC\[7\]. Although the Arg72 homozygosity in FA SCC patients is relatively high, it is important to note that this is mainly due to a remarkable low frequency of Arg/Pro heterozygotes (4/24) in the FA SCC patients as compared with the FA patients without SCC\[7\]. We did not observe this low frequency of Arg/Pro heterozygotes (2/4) in the FA HNSCC patients, but it should again be stressed that the number of samples is very small for these comparisons.

As discussed previously, HPV expresses oncoprotein E6, which inactivates p53, and therefore mutations in TP53 are rare in tumors containing transcriptionally active HPV\[1]\]. Recently, it was also shown that these tumors show specific genetic profiles, characterized by a low frequency of allelic loss\[14\]. LOH on 3p, 9p, 17p, 13q, 18q and 8p is a common phenomenon in HNSCC\[11\]. All FA HNSCC and sporadic HNSCC showed losses on all or most of these chromosome arms. Two sporadic HNSCC cell lines are remarkable, 92VU040 and 93VU120, where only losses are found on 9p or 3p and 18q, respectively. The cell line 92VU040 was found to only have LOH at 9p. Previously CGH analysis was done on this cell line and only gains or losses were detected at 3q, 9p, 9q and Xp\[18\]. The other cell line, 93VU120, shows only losses or gains on 3p and 18q with LOH analysis. Previous CGH results showed more genetic aberrations including a gain of chromosome arm 9p\[18\]. In LOH analysis, allelic loss is only scored when the ratio between normal and tumor alleles are <0.5 or >2.0. All 9p markers of 93VU120 ranged from 1.7 to 0.68 and this strongly indicates a single gain of 9p, resulting in borderline LOH ratios. A gain of one allele gives a ratio of 3/2 or 2/3 (1.5 or 0.66).

Based on the observations that none of the FA HNSCCs did contain HPV DNA, all showed a TP53 mutation and all exhibited a pattern of allelic loss associated with sporadic cancers without an HPV etiology, we conclude that HPV is not likely to have a role in the genesis of these FA HNSCC. We do not have an explanation why these data are in contrast to those reported previously\[7\], except for possible differences in clinical history of the patients.

Besides a comparative genetic characterization of the cell lines, we did an SRB assay for testing the sensitivity of the cell lines to DNA cross-linking drugs. FA cells are known to be sensitive to DNA cross-linking drugs, a trait that is used for diagnosis of FA. Two FA HNCC cell lines were tested in the SRB assay. One of the FA cell lines (VU-1365) did not grow when diluted to low cell concentrations and the sensitivity for cytotoxic drugs could therefore not be established for this cell line. The SRB as-
say showed that the FA HNSCC cell lines were on average 10 times more sensitive to cisplatin compared with the sporadic HNSCC cell lines. This observation, although expected, raises hope for future treatment of sporadic HNSCC and other cancers. The Fanconi anemia genes might not only play a role in FA-HNSCC but also in sporadic HNSCC. Recently it was observed that Fanconi anemia gene FANCF can be methylated in sporadic head and neck carcinoma, lung carcinoma and ovary carcinoma[19], and it was shown that this can result in abrogation of the FA pathway. Abrogation of the FA pathway will cause a FA-like phenotype of the tumor characterized by a high sensitivity to DNA cross-linkers as cisplatin. The observation that SCCs arising in FA patients indeed remain cisplatin-sensitive, despite all accumulating genetic changes during carcinogenesis, indicates that sporadic HNSCC with an inactivated FA pathway will also become sensitive to cross-linking drugs such as cisplatin and might thus be more effectively treated by chemoradiotherapy.

In conclusion, we established four FA HNSCC cell lines and these seem to be genetically similar to sporadic HNSCC cell lines, which was also previously reported by cytogenetic characterization on both FA and sporadic HNSCC[20]. Whether the process of carcinogenesis, the progression through precursor stages and the ordered accumulation of genetic changes[21], is comparable with that of sporadic HNSCC is not known at present. If so, diagnosis and monitoring of these preneoplastic fields might be a quantum step forward to early diagnosis and even prevention of HNSCC in FA patients. The FA HNSCC cell lines we have generated can be used further for development of new preventive therapies for FA patients.

Acknowledgments
Grant support: Fanconi Anemia Research Fund.


