Defects in the Fanconi anemia pathway and chromatid cohesion in head and neck cancer


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Failure to repair DNA damage or defective sister chromatid cohesion, a process essential for correct chromosome segregation, can be causative of chromosomal instability (CIN), which is a hallmark of many types of cancers. We investigated how frequent this occurs in head and neck squamous cell carcinoma (HNSCC) and whether specific mechanisms or genes could be linked to these phenotypes. The genomic instability syndrome Fanconi anemia is caused by mutations in any of at least 16 genes regulating DNA interstrand crosslink (ICL) repair. Since patients with Fanconi anemia have a high risk to develop HNSCC, we investigated whether and to which extent Fanconi anemia pathway inactivation underlies CIN in HNSCC of non–Fanconi anemia individuals. We observed ICL-induced chromosomal breakage in 9 of 17 (53%) HNSCC cell lines derived from patients without Fanconi anemia. In addition, defective sister chromatid cohesion was observed in five HNSCC cell lines. Inactivation of FANCM was responsible for chromosomal breakage in one cell line, whereas in two other cell lines, somatic mutations in PDS5A or STAG2 resulted in inadequate sister chromatid cohesion. In addition, FANCF methylation was found in one cell line by screening an additional panel of 39 HNSCC cell lines. Our data demonstrate that CIN in terms of ICL-induced chromosomal breakage and defective chromatid cohesion is frequently observed in HNSCC. Inactivation of known Fanconi anemia and chromatid cohesion genes does explain CIN in the minority of cases. These findings point to phenotypes that may be highly relevant in treatment response of HNSCC.

Introduction

Chromosomal instability (CIN) is a widespread phenomenon in many cancer types and can be caused by replication stress, inappropriate mitosis, aberrant telomere maintenance, or defective DNA double-strand break repair. It can drive tumorigenesis by facilitating the loss of tumor suppressors and gain of oncogenes. The rare disorder Fanconi anemia is one of several genetic syndromes associated with CIN. This disorder is characterized by a broad variety of congenital malformations, bone marrow failure, and high risk of early-onset cancer, in particular acute myeloid leukemia and squamous cell carcinoma of the head and neck region (HNSCC). Currently, biallelic mutations in any of at least 16 Fanconi anemia genes can cause Fanconi anemia.

The observed CIN in Fanconi anemia cells is believed to emerge from impaired DNA interstrand crosslink (ICL) repair, leading to spontaneous and genotoxic-induced chromosomal breaks. As a consequence, Fanconi anemia–deficient cells are hypersensitive to DNA crosslinkers and endogenously produced aldehydes.
Moreover, ICL sensitivity has been reported for a different class of syndromes: the cohesinopathies (e.g., Robert syndrome and the Fanconi anemia–like Warsaw breakage syndrome). These syndromes are caused by mutations in genes involved in sister chromatid cohesion, which is a tightly regulated process and is effectuated by the cohesin complex. This complex consists of several subunits and holds newly replicated chromatids together from S-phase until they separate in mitosis. Thereby, sister chromatid cohesion is essential in regulating the proper distribution of chromosomes over the two dividing daughter cells and preventing CIN.

The high frequency of HNSCC in individuals with Fanconi anemia shows the importance of the Fanconi anemia pathway in maintaining genomic stability and preventing cancer in squamous cells. Inactivation of the Fanconi anemia pathway is not exclusive to inherited cancer, but its significance in sporadic cancer, in particular HNSCC, remains unclear. Epigenetic silencing of FANCF has been reported in 15% of sporadic HNSCC, but this result may be an overestimation as the method used for methylation detection is prone to generate false-positive results. Nevertheless, a comprehensive examination of Fanconi anemia pathway inactivation in sporadic HNSCC is desirable, as more Fanconi anemia genes have been discovered in recent years. Defects in this pathway may be exploited to improve anticancer therapies and yield opportunities to personalize treatment, as Fanconi anemia–deficient cells are hypersensitive to the commonly used chemotherapeutic drug cisplatin. Therefore, we examined CIN in terms of ICL-induced chromosomal breakage in head and neck tumors derived from individuals without Fanconi anemia and whether Fanconi anemia pathway inactivation may underlie this phenotype. Because of the diagnostic overlap in terms of ICL sensitivity between Fanconi anemia and cohesinopathies, we investigated the role of inadequate sister chromatid cohesion in HNSCC in parallel and analyzed candidate genes to explain the observed phenotypes.

**Results**

**Chromosomal breakage and defective sister chromatid cohesion in the majority of sporadic HNSCC cell lines**

Analysis of chromosomal breakage in prometaphase spreads is considered the gold standard for diagnosing Fanconi anemia. The same assay can, in addition, be used to score sister chromatid cohesion defects. We performed this test to determine both spontaneous and ICL-induced chromosomal abnormalities in terms of chromosomal breakage or cohesion defects in 17 HNSCC cell lines derived from non–Fanconi anemia individuals (Table 1). Of note, cell lines UM-SCC-14ABC were derived from “recurrences” in the floor of mouth with 8 months between A and B and 18 months between B and C. Despite that UM-SCC-14A was indicated as a recurrence, it was nonetheless staged as T1N0, which is somewhat peculiar, as
recurrences are not staged by convention. It is very likely based on stage, follow-
up times, and clinical characteristics that UM-SCC-14ABC are independent multiple 
second primary tumors (“second field tumors”) from a single precancerous field 
in the floor of the mouth. To reduce the risk of false-negative findings, we used 
two different ICL agents, mitomycin C and the commonly used chemotherapeutic 
agent cisplatin. Mitomycin C needs to be metabolically activated, of which the rate 
might differ between cell lines, whereas cisplatin response can be influenced by 
metabolic inactivation as well as by influx and efflux transporters. Three HNSCC 
cell lines derived from tumors of patients with Fanconi anemia (FA-HNSCC) and 
their counterparts, in which the Fanconi anemia defect was genetically corrected, 
were used as controls. After treatment with either mitomycin C or cisplatin, almost 
all metaphase spreads (>90%) of FA-HNSCC cell lines exhibited ten or more break 
events per metaphase, whereas a large proportion of the corresponding genetically 
corrected cells had no breaks at all (Fig. 1). Of note, none of these cell lines displayed 
cohesion defects. Sporadic HNSCC cell lines were classified as ICL-sensitive when 
more than 50% of the cells had three or more break events per metaphase. On the 
basis of this phenotype, we found 9 sporadic HNSCC cell lines (VU-SCC-147, UM-
SCC-14A and B, FaDu, UM-SCC-35, VU-SCC-OE, VU-SCC-41, VU-SCC-80, and VU-
SCC-96a) to be sensitive to mitomycin C and/or cisplatin, whereas the remaining 8 
cell lines showed a weak or no response at all. The number of chromosomal break 
events per metaphase was also scored in untreated cells (data not shown). Only 
metaphases of the most sensitive sporadic HNSCC cell lines VU-SCC-147, FaDu, and 
UM-SCC-14B exhibited a small increase of spontaneous breaks compared with other 
HNSCC cell lines (not shown).

While scoring chromosomal breakage in untreated sporadic HNSCC cell lines, 
we also screened for sister chromatid cohesion defects: loss of cohesion at the 
centromere, leading to a railroad phenotype (parallel/railroad chromosomes) or 
total loss of cohesion between sister chromatids, leading to premature chromatid 
separation (PCS). Quantification of railroad chromosomes and PCS revealed that 5 of 
17 sporadic HNSCC cell lines (VU-SCC-147, UM-SCC-14B, VU-SCC-OE, VU-SCC-78, 
and VU-SCC-120) displayed a substantial level (70%) of cohesion defects, almost as 
much as in the positive control cell line VU1199-F SV40 (Fig. 1). This cell line was 
derived from an individual suffering from Robert syndrome, a cohesinopathy caused 
by mutations in ESCO2 (ref 31). Together, these data demonstrate the presence of 
ICL-induced chromosomal breakage and/or defective chromatid cohesion in two 
thirds of the sporadic HNSCC cell lines examined.

ICL-induced accumulation of HNSCC cells in the $G_2-M$ phase of the cell cycle

In addition to increased chromosomal breakage, normal cells from individuals 
with Fanconi anemia are characterized by an ICL-induced accumulation of cells
Table 1. Panel of 18 sporadic HNSCC and 3 FA-HNSCC cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Gender</th>
<th>Stage</th>
<th>Primary tumor site</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sporadic HNSCC cell lines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VU-SCC-40</td>
<td>Female</td>
<td>T3N0</td>
<td>Tongue</td>
</tr>
<tr>
<td>VU-SCC-41</td>
<td>Male</td>
<td>T3N2</td>
<td>Floor of mouth</td>
</tr>
<tr>
<td>VU-SCC-78</td>
<td>Female</td>
<td>T3N2b</td>
<td>Tongue</td>
</tr>
<tr>
<td>VU-SCC-80</td>
<td>Male</td>
<td>Recurrence</td>
<td>Base of tongue</td>
</tr>
<tr>
<td>VU-SCC-94</td>
<td>Female</td>
<td>T3N1</td>
<td>Tongue</td>
</tr>
<tr>
<td>VU-SCC-96A</td>
<td>Male</td>
<td>T4N1</td>
<td>Retromolar trigone</td>
</tr>
<tr>
<td>VU-SCC-120</td>
<td>Female</td>
<td>T3N1</td>
<td>Tongue</td>
</tr>
<tr>
<td>VU-SCC-147</td>
<td>Male</td>
<td>T4N2</td>
<td>Floor of mouth</td>
</tr>
<tr>
<td>VU-SCC-9917</td>
<td>Female</td>
<td>T2N2b</td>
<td>Oral cavity</td>
</tr>
<tr>
<td>VU-SCC-OE</td>
<td>Male</td>
<td>Lymph node metastasis</td>
<td>Floor of mouth</td>
</tr>
<tr>
<td>UM-SCC-11B</td>
<td>Male</td>
<td>T2N2a</td>
<td>Supraglottic larynx</td>
</tr>
<tr>
<td>UM-SCC-14A</td>
<td>Female</td>
<td>T1N0</td>
<td>Floor of mouth</td>
</tr>
<tr>
<td>UM-SCC-14B</td>
<td>Female</td>
<td>T1N0</td>
<td>Floor of mouth</td>
</tr>
<tr>
<td>UM-SCC-14C</td>
<td>Female</td>
<td>T1N0</td>
<td>Floor of mouth</td>
</tr>
<tr>
<td>UM-SCC-22A</td>
<td>Female</td>
<td>T2N1</td>
<td>Hypopharynx</td>
</tr>
<tr>
<td>UM-SCC-35</td>
<td>Male</td>
<td>T4N1</td>
<td>Tonsillar fossa</td>
</tr>
<tr>
<td>FaDu</td>
<td>Male</td>
<td>NA</td>
<td>Hypopharynx</td>
</tr>
<tr>
<td>VU-SCC-1131 (FA-C)</td>
<td>Female</td>
<td>T4N2b</td>
<td>Floor of mouth</td>
</tr>
<tr>
<td>VU-SCC-1365 (FA-A)</td>
<td>Male</td>
<td>NA</td>
<td>Mouth mucosa</td>
</tr>
<tr>
<td>VU-SCC-1604 (FA-L)</td>
<td>Female</td>
<td>NA</td>
<td>Tongue</td>
</tr>
</tbody>
</table>

Abbreviation: NA; not annotated. *Human papillomavirus (HPV)-positive, †local recurrences of a T1N0 carcinoma in floor of mouth.

in the \( G_2 - M \) phase of the cell cycle. We performed cell-cycle analysis to determine whether FA HNSCC and sporadic HNSCC cell lines treated with either mitomycin C or cisplatin also arrested in the \( G_2 - M \) phase of the cell cycle (Fig. 1 and Supplementary Fig. 1). Indeed, despite the presence of tumor-promoting genetic changes, FA-HNSCC cells still accumulated in the \( G_2 - M \) phase of the cell cycle after treatment with cross-linking agents, whereas this accumulation was not observed in the corrected cell lines. Of the 9 sporadic HNSCC cell lines that showed chromosomal breakage in response to mitomycin C and/or cisplatin, 5 cell lines arrested in the \( G_2 - M \) phase of the cell cycle after treatment with mitomycin C and/or cisplatin (Fig. 1 and Supplementary Fig. 1). One plausible explanation for the absence of \( G_2 - M \) arrest in the other 4 sensitive cell lines is a defective \( G_2 - M \) cell cycle checkpoint, enabling the cells to continue growing in the presence of DNA damage. Conversely, of the 8 cell lines that were classified as ICL-resistant in terms of chromosomal breakage, 4 still showed \( G_2 - M \) arrest in response to mitomycin C and/or cisplatin.
Figure 1. ICL-induced chromosomal breakage, G2–M arrest, and spontaneous sister chromatid cohesion defects in HNSCC cell lines.

Metaphase spreads of mitomycin-C (MMC)- and cisplatin (CDDP)-treated HNSCC cell lines were examined and scored for chromosomal breakage events. On the basis of this assay, two groups of sporadic HNSCC cell lines could be distinguished, mitomycin C- and/or cisplatin-sensitive and resistant cell lines.
FA-HNSCC cell lines and the corresponding corrected cells were used as controls. Metaphase spreads of untreated (UT) cells were scored for sister chromatid cohesion defects, railroad chromosomes (RR) and PCS. LN9Sv [wild-type (WT)] and VU1199-F SV40 fibroblasts were used as negative and positive controls, respectively. Percentage of cells with the indicated number of chromosomal break events, railroad chromosomes, and PCS are shown. Mitomycin C- and cisplatin-induced accumulation in the G$_2$–M phase was also analyzed in Fanconi anemia as well as sporadic HNSCC cell lines (see also Supplementary Fig. S1). G$_2$–M arrest was classified positive (filled boxes) as the percentage of cells in G$_2$–M G1. Asterisks indicate that untreated VU-SCC-78 already had a high 4n peak.

(Fig. 1 and Supplementary Fig. 1). Untreated VU-SCC-78 cells already showed a high 4n peak, which will be discussed below.

Somewhat remarkably, ICL-induced G$_2$–M arrest did not correlate with ICL-induced chromosomal breakage. This might be due to the variable genetic alterations that accumulated in these tumor cell lines. Because the chromosomal breakage assay is considered the gold standard in diagnosing Fanconi anemia, cell lines showing chromosomal breakage were classified as ICL-sensitive.

**FANCM mutations in the sporadic head and neck tumor cell line FaDu**

An ICL-induced increase in chromosomal breakage could be indicative for Fanconi anemia pathway inactivation. The Fanconi anemia pathway can be divided into two components: the upstream part, which is required for FANCD2 monoubiquitination, and a downstream part that is not essential for this posttranslational modification. To test for a functional defect in the upstream part of the Fanconi anemia pathway, FANCD2 monoubiquitination and focus formation were analyzed. Mutations in any of the 8 upstream Fanconi anemia core complex genes (FANCA, -B, -C, -E, -F, -G, -L, and -M) abolish or reduce (in case of FANCM mutations) monoubiquitination and nuclear focus formation of FANCD2. Five of the 9 ICL-sensitive sporadic HNSCC cell lines (UM-SCC-14B, FaDu, UM-SCC-35, VU-SCC- 41, and VU-SCC96a) and 1 ICL-resistant cell line (UM-SCC-14C) appeared only moderately able to monoubiquitinate FANCD2 (Fig. 2A). Notwithstanding, FANCD2 nucleus focus formation was observed in all cell lines tested, although slight differences in ICL-induced induction of FANCD2 foci cannot be excluded (Fig. 2B). A previous report already showed that FaDu cells have abnormal FANCD2 monoubiquitination, which is consistent with our data. However, the underlying cause remained unknown. By using the Cancer Cell Line Encyclopedia, we found that FaDu harbors a homozygous nonsense mutation in FANCM (p.Ser1618*), which we confirmed by Sanger sequencing. As a result of this mutation, FaDu cells lack FANCM protein expression (Supplementary Fig. 2), which might explain the observed chromosomal breakage in this cell line.
Figure 2. FANCD2 monoubiquitination and focus formation. (A) Western blotting for FANCD2 monoubiquitination. Sporadic HNSCC cell lines were treated with or without 200 nmol/L mitomycin C (MMC) for 16 hours and whole-cell lysates were immunoblotted. The upper band represents the monoubiquitinated form of FANCD2. (B) Immunofluorescent analysis of FANCD2 focus formation (green). HNSCC cell lines were treated with 200 nmol/L mitomycin C for 16 hours. TO-PRO-3 (red) was used for nuclear counterstaining. Representative confocal images are shown. CDDP, cisplatin.

Whole-exome sequencing revealed several putative pathogenic mutations in Fanconi anemia genes

Nine cell lines in our panel of 17 sporadic HNSCC cell lines were sensitive to mitomycin C and/or cisplatin in terms of chromosomal breakage. To assess whether besides FaDu, other ICL-sensitive HNSCC cell lines contained mutations in any of the 16 known Fanconi anemia or 5 Fanconi anemia–associated genes, we sequenced the whole exomes of 6 ICL-sensitive cell lines (VU-SCC-80, VU-SCC-96a, VU-SCC-147, VU-SCC-OE, and UM-SCC-14B). This revealed possible pathogenic mutations in TP53, HRAS, NOTCH1, PIK3CA, SMAD4, and FAT1, which are frequently altered
in HNSCC (Supplementary Fig. 3A)\textsuperscript{33–35}. Moreover, copy number alterations that often occur in HNSCC were found (e.g., deletions encompassing TP53 and CDKN2A and amplifications of CCND1, MDM2, and EGFR; Supplementary Fig. 3B). These results show that the cell lines are representative HNSCC cell lines.

Besides these oncogenic driver mutations, one hemizygous nonsense mutation and 8 missense variants in various Fanconi anemia genes were found (Table 2). The hemizygous nonsense mutation in FANCD1/BRCA2 (p.Lys3326*), identified in cell line VU-SCC-147, is known as a polymorphic stop\textsuperscript{36} and did not confer mitomycin C or cisplatin sensitivity in a mouse embryonic stem cell–based assay\textsuperscript{37}. To further exclude this variant as possibly pathogenic, we investigated PARP inhibitor response and nuclear RAD51 focus formation in VU-SCC-147 cells, as BRCA2-deficient cells are PARP inhibitor–sensitive and lack nuclear RAD51 focus formation\textsuperscript{38–40}. VU-SCC-147 cells were resistant to PARP inhibitor (Supplementary Fig. 4A) and were able to form RAD51 foci (Supplementary Fig. 4B), suggesting that the resulting truncating BRCA2 mutation did most likely not cause the observed ICL sensitivity in this cell line.

To assess the pathogenic potential of missense variants, we used three tools: (i) the \textit{in silico} algorithms SIFT, MutationTaster, and PolyPhen for the prediction of deleterious mutations, (ii) minor allele frequencies (MAF; Exome Variant Server, http://evs.gs.washington.edu/EVS/), and (iii) the occurrence of these variants in patients with Fanconi anemia (Fanconi anemia gene variant database, www.rockefeller.edu/fanconi/). Variants with low MAFs (≤0.02) and predicted to be pathogenic by at least 2 of 3 \textit{in silico} algorithms were classified as possibly damaging. Four of 8 identified variants with MAFs below 0.02 were predicted to be pathogenic by at least two prediction algorithms (Table 2). Although these variants in FANCA, FANCI, FANCP, and FANCQ occurred heterozygously in the respective cell lines and Fanconi anemia is a recessive disease, haploinsufficiency or a dominant negative effect cannot be excluded, particularly not in cancer cells with their damaged genomes. Interestingly, the heterozygous variant in FANCA (p.Cys625Ser) in cell line UM-SCC-14B had been reported to occur in a patient with Fanconi anemia (Table 2)\textsuperscript{41}. Therefore, this variant might be disease-causing, which is strengthened by the observation that the cell line containing this variant had poor FANCD2 monoubiquitination (Fig. 2A). The other variants were not identified in patients with Fanconi anemia.

Taken together, several sequence variants in Fanconi anemia genes were found, but their pathogenic nature remains elusive. In particular, the variants in FANCA, FANCI, FANCP, and FANCQ predicted to be pathogenic might be disease causing, but their heterozygous nature indicates that they cannot explain the ICL induced chromosomal breakage phenotype.
Table 2. Sequence variants with MAF ≤ 0.02 in Fanconi anemia genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence variant</th>
<th>SIFT</th>
<th>MT</th>
<th>PP</th>
<th>MAF EA/AA</th>
<th>% reads</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell line VU-SCC-80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAAp16</td>
<td>c.370A&gt;G; p.Asn124Asp</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>-</td>
<td>60/86</td>
<td>(70%)          Predicted tolerated</td>
</tr>
<tr>
<td>FANCD2</td>
<td>c.577A&gt;G; p.thr193Ala</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>0.0007/0.0009</td>
<td>19/20</td>
<td>(95%)          Predicted tolerated</td>
</tr>
<tr>
<td>FANCQ/XPF</td>
<td>c.241G&gt;T; p.Val81Phe</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>-/0.0009</td>
<td>24/53</td>
<td>(45%)          Predicted pathogenic</td>
</tr>
<tr>
<td>Cell line VU-SCC-147</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FANCD1/BRCA2</td>
<td>c.9976A&gt;T; p.Lys3326*</td>
<td>0.0084/0.0027</td>
<td>39/41</td>
<td>(95%)</td>
<td>Hemizygous nonsense mutation, polymorphic stop</td>
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<td></td>
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<tr>
<td>FANCM</td>
<td>c.527C&gt;T; p.Thr176Ile</td>
<td>T</td>
<td>T</td>
<td>T</td>
<td>0.0051/0.0005</td>
<td>27/71</td>
<td>(38%)          Predicted tolerated</td>
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<tr>
<td>Cell line VU-SCC-OE</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>FANCI</td>
<td>c.480G&gt;C; p.Leu160Phe</td>
<td>T</td>
<td>P</td>
<td>T</td>
<td>-</td>
<td>66/66</td>
<td>(100%)          Predicted tolerated</td>
</tr>
<tr>
<td>Cell line UM-SCC-14B</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FANCA</td>
<td>c.1874G&gt;C; p.Cys625Ser</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>0.0031/0.0007</td>
<td>12/31</td>
<td>(39%)          Reported in Fanconi anemia patient</td>
</tr>
<tr>
<td>FANCP/SLX4</td>
<td>c.2854_2855delinsAT; p.Ala952Met</td>
<td>-</td>
<td>8/23%</td>
<td>(35%)</td>
<td>Predicted pathogenic</td>
<td></td>
<td></td>
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<tr>
<td>Cell line UM-SCC-35</td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>FANCI/BRIP1</td>
<td>c.517C&gt;T; p.Arg173Cys</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>0.0049/0.0011</td>
<td>39/125</td>
<td>(31%)          Predicted pathogenic</td>
</tr>
</tbody>
</table>

NOTE: Transcript refseq ID: FAAp16 NM_199294.2; FANCA NM_000135.2; FANCD1/BRCA2 NM_000059; FANCD2 NM_033084.3; FANCI NM_00113378.1; FANCI/BRIP1 NM_032043; FANCM NM_020937.2; FANCP/SLX4 NM_032444.4 and FANCO/XPF NM_005236.2. MAF EA/AA represents minor allele frequency European/American and African/American populations, respectively. Abbreviations: MT, MutationTaster; PP, polyphen; P, pathogenic; T, tolerated.

Absence of FANCD2 monoubiquitination in sporadic HNSCC cell line UPCI-SCC-154 due to FANCF methylation

On the basis of hydroxyurea (HU)-induced FANCD2 monoubiquitination analysis of a separate panel of 39 HNSCC cell lines (Supplementary Table 1), one cell line (UPCI-SCC-154) was selected for more detailed investigation. Upon mitomycin C treatment, FANCD2 monoubiquitination and nuclear FANCD2 focus formation were
absent in UPCI-SCC-154 cells (Fig. 3A and B). As with the 17 other sporadic HNSCC cell lines, chromosomal breakage and cell-cycle analysis were performed to examine ICL sensitivity. Upon treatment with either mitomycin C or cisplatin, UPCI-SCC-154 cells accumulated in the G2-M phase of the cell cycle and exhibited in approximately 70% of scored metaphases more than three break events per metaphase (Fig. 3C). This was almost as sensitive as the FANCC-deficient control cell line VUSCC-1131. Furthermore, UPCI-SCC-154 cells were sensitive to cisplatin in a growth inhibition assay (Supplementary Fig. S5). We also found in 60% of metaphases of untreated UPCI-SCC-154 cells sister chromatid cohesion defects, mainly railroad chromosomes (Fig. 3C). Massive parallel sequencing of all known upstream Fanconi anemia genes in UPCI-SCC-154 cells did not reveal mutations that could explain the absence of FANCD2 monoubiquitination. Because promoter methylation of some Fanconi anemia genes has been observed in various tumors, we analyzed the methylation of Fanconi anemia genes using methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA). Promoter methylation of the FANCF gene was detected in UPCI-SCC-154 cells and transfection of this cell line with FANCF cDNA restored FANCD2 monoubiquitination (Fig. 3D) and reduced mitomycin C-induced chromosomal breakage (Fig. 3C). These results demonstrate that FANCF methylation is responsible for the observed Fanconi anemia phenotype in cell line UPCI-SCC-154.

Sister chromatid cohesion defects due to mutations in PDS5A and STAG2

As shown in Fig. 1, we also found sister chromatid cohesion defects in several sporadic HNSCC cell lines. To assess the molecular basis of cohesion defects, we followed a candidate gene approach by first investigating the levels of several known cohesion proteins (SMC1, SMC3, ESCO2, PDS5A, PDS5B, DDX11, STAG1, STAG2, and RAD21) by Western blotting, followed by DNA Sanger sequencing. Remarkably, PDS5A protein expression was absent in one cell line (VU-SCC-41) with only moderate cohesion defects (Fig. 1 and 4A). In contrast, no cohesion defects (data not shown) and normal PDS5A protein expression were observed in primary fibroblasts (VU-41-F) from the same patient (Fig. 4A), suggesting that a somatic mutation in PDS5A had occurred in the tumor. By using cDNA primers spanning exons 3 to 7, we detected a shorter PCR product in VU-SCC-41 cells compared with the wild-type fibroblasts from the same patient (Supplementary Fig. 6A). The shorter cDNA appeared to lack exon 6 sequence, resulting in a frameshift and premature stop (Supplementary Fig. 6B). Further examination of the PDS5A gene revealed an inversion combined with a duplication of part of the inverted sequence at the intron–exon boundary of exon 6, which removes the splice donor site (Supplementary Fig. 6C). This mutation was also found in paraffin-embedded tumor material (data not shown), indicating that the mutation was not induced by cell culture. In the tumor cell line,
Chapter 5

Figure 3. ICL sensitivity, FANCD2 monoubiquitination, and focus formation of sporadic HNSCC cell line, UPCI-SCC-154.

(A) and (B), untreated, or mitomycin C (MMC)-treated (200 nmol/L for 16 hours) UPCI-SCC-154 cells were immunoblotted for FANCD2 (A) or analyzed for FANCD2 focus formation (green; B). VU-SCC-1131 and VU-SCC-1131+FANCC were used as controls. Nuclei were counterstained by TO-PRO-3 (red). (C) ICL-induced chromosomal breakage events and spontaneous sister chromatid cohesion defects were observed in metaphase spreads of UPCI-SCC-154 and FANCF cDNA corrected UPCI-SCC-154 cells. CDDP, cisplatin. (D) Ectopic expression of FANCF in UPCI-SCC-154 cells restored FANCD2 monoubiquitination.

Wild-type PDS5A sequence was absent, suggesting that the other PDS5A allele was deleted. Subsequent comparative genome hybridization (array CGH) indeed showed a heterozygous loss of the entire p-arm of chromosome 4, which is the region where the PDS5A gene is located (Supplementary Fig. 6D).

Absence of STAG2 protein expression was observed in sporadic HNSCC cell line VU-SCC-78 that showed severe cohesion defects (Fig. 1 and 4B). In this cell line, a heterozygous 1 base pair (bp) insertion in STAG2 leading to a frameshift was identified by sequence analysis of gDNA (Supplementary Fig. 7A). Because
STAG2 maps to the X chromosome, a single mutational event could be enough for its complete inactivation. Sequencing of STAG2 cDNA indeed revealed that STAG2 mRNA expression was derived entirely of the mutant allele, indicating that the wild-type allele was not functional by X chromosome inactivation (Supplementary Fig. 7B). To exclude that the mutation was induced during cell culture, STAG2 protein expression was examined and indeed missing in paraffin-embedded tumor material derived from the same tumor of which cell line VU-SCC-78 was derived (Fig. 4C). In addition, it was previously shown that knockdown of STAG2 resulted in the generation of an octaploid population. We detected a similar phenotype in VU-SCC-78 (Supplementary Fig. 1B).

Together, these results strongly suggest that the cohesion defects observed in VU-SCC-41 were due to an acquired mutation in the PDS5A gene, accompanied by a loss of the wild-type allele, whereas the cohesion defects in VU-SCC-78 were caused by a somatic mutation in STAG2 in combination with X chromosome inactivation of the other allele.

Discussion

In the present study, we investigated the occurrence of Fanconi anemia pathway inactivation in sporadic HNSCC by analyzing a panel of Fanconi anemia and sporadic HNSCC cell lines. We found in 9 of 17 (53%) of the sporadic HNSCC cell lines ICL-induced chromosomal breakage, which can be indicative of a defective Fanconi anemia pathway, and in 29% (5 of 17) severe sister chromatid cohesion defects. However, this may be an overestimation as some of these cell lines are related. The UMSCC-14ABC cell lines are most likely derived from multiple primary tumors from a large preneoplastic field based on the clinical history and tumor characteristics. Hence, these could be considered as separate tumors but nonetheless UM-SCC-14A and B share a similar phenotype, whereas UM-SCC-14C clearly behaves differently. Mutational inactivation of FANCM and methylation of FANCF were observed in two sporadic HNSCC cell lines from screened panels of 17 and 39 HNSCC cell lines, respectively. Although a few possible disease-causing variants in Fanconi anemia genes were found in the 6 ICL-sensitive HNSCC cell lines that were analyzed by whole-exome sequencing, their heterozygous nature suggests that they cannot explain the ICL-induced chromosomal breakage phenotype. This indicates that despite a frequent Fanconi anemia–like phenotype (ICL-induced chromosomal breakage), the Fanconi anemia pathway itself seems rarely involved. Silencing of FANCF by promoter hypermethylation has been demonstrated in a wide range of malignancies, including HNSCC. However, the importance of FANCF promoter methylation in HNSCC is contradictory, as it was demonstrated that the methylation-specific PCR method that is routinely used to investigate FANCF methylation is liable to produce false-positive results. By using the more specific method MS-MLPA, we showed that
FANCF methylation does occur in sporadic HNSCC, albeit infrequently. Screening of large sample series by more robust methods, such as quantitative methylation-specific PCR and sequencing, might substantiate our findings.

Interestingly, we found besides ICL-induced chromosomal breakage also spontaneous sister chromatid cohesion defects in a subset of sporadic HNSCC. Because of the role of sister chromatid cohesion in chromatid separation, DNA repair, and gene regulation, this pathway is important for chromosomal stability. Mutations in cohesion genes could therefore lead to CIN by different mechanisms: mis-segregation of chromosomes followed by aneuploidy due to partial or total loss of cohesion or as a consequence of impaired DNA repair as well as altered gene expression. Recently, in several studies, alterations in sister chromatid cohesion genes were identified in a variety of cancers. In particular, inactivating alterations in STAG2 have been frequently found. Likewise, we found mutational inactivation of STAG2 in one HNSCC cell line, whereas in another cell line, PDS5A was mutated.

The cohesion defects in VU-SCC-147 and UPCI-SCC-154 could be explained by the presence of the human papillomavirus (HPV) and subsequent inactivation of the retinoblastoma tumor suppressor proteins (pRb) in these cell lines. Loss of pRb has been demonstrated to alter H4K20 methylation and lead to sister chromatid cohesion defects. The presence of HPV in these cell lines might well explain the inadequate sister chromatid cohesion.

Fanconi anemia and cohesinopathies have some overlapping features. ICL-induced chromosomal breakage is not an exclusive hallmark of Fanconi anemia cells and has been observed in T lymphocytes from individuals with Robert syndrome or Warsaw breakage syndrome as well. Moreover, mitomycin C-induced, but not spontaneous cohesion, defects have been reported to occur in Fanconi anemia cells. Whether these overlapping phenotypes also occur in cancer cells remains to be determined in functional studies. This is of relevance, as the screening for sequence variants in Fanconi anemia genes and cohesion genes might be exploited as biomarkers for cisplatin response. This is supported by previous work that demonstrated that FANCM-deficient cell line FaDu was approximately 6-fold more sensitive to cisplatin than the most resistant HNSCC cell line tested, although not as sensitive as FA-HNSCC cell lines. In addition, in the present study, we showed sensitivity to cisplatin for the FANCF-deficient cell line UPCI-SCC-154 as well.

In summary, CIN in terms of ICL-induced chromosomal breakage or defective sister chromatid cohesion occurs frequently in HNSCC. In few cases, this is caused by defects in the Fanconi anemia pathway or mutational inactivation of chromatid cohesion genes. Further unraveling of the relevant mechanisms and/or genes causing these phenotypes may open new avenues for treatment and provide the biomarkers to predict treatment response.
Figure 4. Mutational inactivation of PDS5A and STAG2 in two HNSCC cell lines. 
(A) Western blot analysis showing a truncated PDS5A protein in VU-SCC-41 and normal expression of PDS5A in fibroblasts (VU-41-F) of the same patient. (B) Western blot analysis showing absence of STAG2 protein expression in VU-SCC-78. Tubulin was used as a loading control. (C) Immunohistochemical analysis of STAG2 expression in paraffin-embedded tumor material of the same tumor of which cell line VU-SCC-78 was derived. STAG2 protein expression was absent in tumor (T) material but present in normal tissue surrounding the tumor.
Materials and Methods

Cell culture
All HNSCC cell lines (Table 1) and control fibroblast cell lines (wild-type controls LN9SV and VU-41-F, ESCO2-deficient VU1199-F SV40, and PALB2-deficient EUFA1341FHV) were cultured in DMEM supplemented with 10% FBS and 1 mmol/L sodium pyruvate (Gibco). Sporadic and Fanconi anemia HNSCC cell lines were established as described previously. All three FA-HNSCC cell lines were genetically corrected. Cell lines UM-SCC-11B, UM-SCC-14A, UM-SCC-14B, UM-SCC-14C, UMSCC22A, and UM-SCC-35 were obtained from Dr. T. Carrey (University of Michigan, Ann Arbor, MI) and FaDu cells from the ATCC. HNSCC cell lines were authenticated by microsatellite profiling and TP53 mutation analysis. For information on cell line UPCI-SCC-154, see previously published data.

Chromosomal breakage assay
HNSCC cell lines were cultured for 48 hours in the absence or presence of 50 nmol/L mitomycin C (Kyowa Hakko Co.) or 1 mmol/L cisplatin (Pharmachemie BV Haarlem). After treatment with 200 ng/mL demecolcin (Sigma) for 30 minutes, cells were harvested, treated with 0.075 mol/L KCl for 20 minutes at room temperature, and fixed with 75% methanol, 25% acetic acid. Subsequently, cells were dropped onto glass slides and stained with 5% Giemsa (Merck). For each cell culture, 50 metaphases were analyzed for chromosomal breakage events and sister chromatid cohesion defects. All scoring was performed on coded slides to prevent counting bias.

Western blot and immunoprecipitation analysis
HNSCC cell lines were treated with or without 200 nmol/L mitomycin C overnight and harvested to examine FANCD2 monoubiquitination. Protein expression of FANCM, PDS5A, STAG1, and STAG2 was only examined in untreated cells. Whole-cell extracts were prepared in lysis buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, and 1% Triton X-100 supplemented with protease (compleTABLE EDTA free tablets, Roche) and phosphatase (PhosSTOP, Roche) inhibitors. For immunoprecipitation reactions, cells were lysed and incubated with antiserum against FANCM (gift from Dr. W. Wang) for 2 hours at 4°C, followed by incubation with Protein A/G PLUS-Agarose (sc-2003, Santa Cruz Biotechnologies) for 30 minutes. Prior to immunoblot analysis, lysates were washed three times with lysis buffer. Proteins were separated on a 3% to 8% Tris-Acetate NUPAGE gradient gel (Invitrogen) and transferred to Immobilon-P membrane overnight. After blocking with 5% dry milk in TBST [10 mmol/L TRIS-HCl (pH 7.5), 150 mmol/L NaCl, 0.05% Tween-20], the membrane was incubated with the indicated primary antibodies overnight [1:500 mouse anti-FANCD2 (Fl17, sc-20022, Santa Cruz Biotechnologies), 1:1,000 rabbit anti-PDS5A (ab17960, Abcam), 1:5,000 goat anti-STAG1 (A300-156A, Bethyl), 1:1,000 goat antiSTAG2(A300-159A, Bethyl)], followed by washing with TBST and incubation with horseradish peroxidase–conjugated secondary antibodies to visualize with ECL (GE Healthcare). Mouse monoclonal anti-a-tubulin (1:5,000, B-5-1-2, SC23948, Santa Cruz Biotechnologies) was used as a control to ensure loading of equal amounts of protein in each Western blot lane.

FANCD2 and RAD51 immunofluorescence
Cells were cultured on sterile chamber slides (Nunc) and treated with 200 nmol/L mitomycin C for 16 hours. After prepermeabilization with 0.25% Triton X-100 in PBS for 1 minute on ice, cells were fixed with 4% formaldehyde [16% formaldehyde solution (w/v), methanol-free (Thermo Scientific Pierce, diluted in PBS)] for 15 minutes at room temperature prior to permeabilization with 0.5% Triton X-100 in PBS (20 minutes at room temperature). Unspecific binding sites were blocked by incubating with 10% FBS (1 hour at room temperature) followed by incubation with rabbit anti-FANCD2 (1:200 NB100-182 Novus Biologicals) or rabbit anti-RAD51 (1:1,000, gift from Dr. R. Kanaar) for 2 hours at room temperature. Slides were washed (0.2% Triton X-100 in PBS) and incubated with goat anti-rabbit ALEXA488 (1:1,000, A-11008, Invitrogen) for 1 hour, washed with 0.2% Triton X-100 in PBS, and counterstained with TO-PRO-3 iodide (1:1,000, T3605, Invitrogen) for 30 minutes. Slides were washed with PBS, mounted by Vectashield (Vector Laboratories), and analyzed by a confocal microscope (Carl Zeiss).

Methylation-specific multiplex ligase–mediated probe amplification
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Whole-exome sequencing
DNA samples were prepared for whole-exome sequencing with NimbleGen SeqCap EZ V3 enrichment kit as described previously\(^{26}\) and sequenced on Illumina’s HiSeq 2000. Three sample libraries were pooled per lane. Sequencing reads were mapped to UCSC genome version hg19 with BWA in paired-end mode. For variant calling, GATK was used to recalibrate base call scores, to realign reads around INDELs, and to call variants using the haplotype caller. Variants with low coverage (depth < 5 reads), low GATK variant quality (GATK variant QUAL < 50), and/or strand bias (FisherStrandBias > 60) were discarded and remaining variants were annotated with ANNOVAR. Annotated variants were further filtered by discarding synonymous variants, mappability > 0.9, mutant allele frequency in exome sequencing project (ESP) < 2%, and the number of samples carrying the variant < 3. For selection of sequence variants in Fanconi anemia and cohesion (-associated) genes, a cutoff of mutant allele frequency in ESP < 2% was used. For copy number analysis, log2 ratios between tumor samples and in-house generated sex-matched nontumor baselines were calculated for each target bait and were segmented using CGH call and CHG regions bioconductor packages into 5 copy number levels. LOH was determined by calculating mutant allele frequencies of polymorphic sites (snp138Common).

Immunohistochemistry (IHC)
IHC was performed on paraffin-embedded sections of an HNSCC tumor biopsy, of which cell line VU-SCC-78 was also derived. Sections were deparaffinized and subjected to Tris-EDTA (pH 9.0) antigen retrieval. A standard protocol was performed using STAG2 antibody (1:25, clone J-12, sc-81852, Santa Cruz Biotechnologies) and the BrightVision Poly-HRP-Anti Ms/Rb/Rt IgG kit (Immunologic BV). The staining was developed with diaminobenzidine as chromogen and counterstained with hematoxylin. The specificity of this antibody was previously verified by others\(^{27}\).

Sanger sequencing
The presence of FANCM, PDS5A, or STAG2 mutations was analyzed by Sanger sequencing. PCR products were purified using a SAP/EXO treatment (Amersham Biosciences) according to the manufacturer’s instructions. Sequencing reactions were prepared using specific primers (available on request) and Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems).

Acknowledgements
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Author Contributions

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Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.
Supplementary materials and methods

Cell cycle analysis
Cell were untreated or exposed for 72 hours to either MMC (15 and 30 nM) or CDDP (250 and 750 nM) and permeabilized in buffer containing 100 mM TRIS-HCl (pH 7.5), 150 mM NaCl, 0.5 mM MgCl2, 1 mM CaCl2, 0.2% BSA and 0.1% IGEPAL (CA-630, Sigma). DNA was stained with PI/RNase staining buffer (BD Pharmingen) for 15 minutes and analyzed by flow cytometry.

siRNA knockdown of BRCA2 and PALB2 in VU-SCC-147
VU-SCC-147 cells plated in 96-well plates were reverse transfected with siRNAs (final concentration 25 nM) targeted against PALB2 and BRCA2 using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s protocol. Non-targeting siCONTROL#2 (Dharmacon) and siRNA targeted against PLK1 were used as a negative and positive control, respectively. Twenty-four hours following transfection, increasing concentrations of PARP inhibitor (KU58948) were added. After 5 days, cell viability was determined by the CellTiter-Blue assay (Promega).

Array CGH
Labeling and hybridization was done as previously described\(^5\). In brief, genomic DNA (500 ng) of tumor and reference were labeled with Cyanine 3-UTP (Cy3) or Cyanine 5-UTP (Cy5) nucleotide mixture (CGH labeling Kit for Oligo Arrays, Enzo Life Sciences), respectively. Labeled DNA of tumor and reference were purified (QIAquick PCR Purification Kit (Qiagen)) and mixed prior to hybridization onto Agilent 180 K oligonucleotide arrays (Agilent Technologies). After hybridization, slides were immediately scanned using microarray scanner G2505B (Agilent technologies) and image analysis was performed using feature extraction software (version 9.1, Agilent Technologies). The Agilent CGH-v4_91 protocol was applied using default settings. Oligonucleotides were mapped according to the human genome build NCBI 6 (May 2006). Of both Cy3 and Cy5 channels, local background was subtracted from the median intensities. The log2 tumor to normal intensity ratio was calculated for each spot and normalized against the median of the ratios of all autosomes.
**Supplementary data**

### A

<table>
<thead>
<tr>
<th>Controls</th>
<th>MMC/CDDP induced chromosomal breaks</th>
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<tbody>
<tr>
<td>untransfected</td>
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<tr>
<td>+ FANCC</td>
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**No ICL induced chromosomal breaks**

- VU-SCC-78
- UM-SCC-22A
- VU-SCC-94
- UM-SCC-11B
- UM-SCC-14C
- VU-SCC-9917
- VU-SCC-120
- VU-SCC-40

### B

**Untreated VU-SCC-78 cells showing a high 4n peak and additional 8n peak upon treatment with 50 or 100 nM MMC.**

**Supplementary Figure 1. Cell cycle analysis in FA and sporadic HNSCC cell lines upon ICL treatment.**

(A) Cells were untreated or continuously exposed to 15 nM MMC, 30 nM MMC, 250 nM CDDP or 750 nM CDDP for 72 hours. G2/M arrest was analyzed by flow cytometry. (B) Untreated VU-SCC-78 cells showing a high 4n peak and additional 8n peak upon treatment with 50 or 100 nM MMC.
Supplementary Figure 2. FANCM protein expression is absent in FaDu cells.
Immunoprecipitation and immunoblot analysis showing absence of FANCM expression in FaDu cells and the control cell line EUFA867-L (FA-M). LE and SE indicate short and long exposures of the blot.

Supplementary Figure 3. Overview of possible pathogenic variants in genes that are frequently altered in HNSCC.
(A) Six cell lines, which were sensitive to ICL agents manifesting as increased chromosomal breakage, were analyzed by whole exome sequencing. Potential pathogenic mutations in genes that are frequently altered in HNSCC are indicated.
(B) Frequency plot of copy number alterations (gains in red, losses in blue) in the six HNSCC cell lines that were analyzed by whole exome sequencing.
Supplementary Figure 4. PARP inhibitor resistance and normal RAD51 focus formation in VU-SCC-147.

(A) PARP inhibitor sensitivity after knockdown of BRCA2 or PALB2 in cell line VU-SCC-147. VU-SCC-147 cells, containing a hemizygous polymorphic nonsense variant in BRCA2 (p.Lys3326*) and a heterozygous missense variant in PALB2 (p.Gly998Glu), were transfected with siRNAs against BRCA2 (siBRCA2) or PALB2 (siPALB2) and treated with increasing concentrations PARP inhibitor (PARPi: Olaparib/KU0058948). Untransfected and VU-SCC-147 cells transfected with non-targeting siRNA (siCON) were used as controls.

(B) RAD51 focus formation upon MMC treatment in VU-SCC-147 cells. Representative images of MMC-induced RAD51 foci (green) in FA (VU-SCC-1131, VU-SCC-1365 and VU-SCC-1604) and sporadic HNSCC (VU-SCC-147) cell lines. Cells were treated with 200 nM MMC for 16 hours. PALB2-deficient EUFA1341FSV cells and FA-HNSCC tumor cell lines were used as controls. Nuclei were counterstained with TO-PRO-3 (red).
Supplementary Figure 5. Cell line UPCI-SCC-154 is sensitive to cisplatin.
FANCF-deficient cell line UPCI-SCC-154 and the control cell lines VU-SCC-1131 and VU-SCC-1131+FANCC were continuously exposed to increasing concentrations cisplatin (CDDP). After three population doublings of untreated cells, cell number for each CDDP concentration was determined using a Coulter counter. The data represent the percentage growth compared to untreated cells.

Right: Supplementary Figure 6. Cell line VU-SCC-41 is mutated in PSC5A.
(A) Analysis of PCR products from cDNA with primers spanning from exon 3 to 7 of the PDS5A gene, showing a shortened PCR product in VU-SCC-41, but not in normal fibroblasts of the same patient. (B) Analysis of the shorter cDNA with sanger sequencing revealed a deletion of exon 6 in VU-SCC-41 cells. (C) Sequencing of genomic DNA showed an inversion of a reverse compliment sequence including a duplication at the start of exon 6 in cell line VU-SCC-41. (D) Whole genome array comparative genomic hybridization (CGH) profiles of VU-SCC-41 compared to VU-41-F and VU-41-F compared to a pool of healthy controls. The X-axis represents the chromosomes and probes on the arrays ordered according to genomic locations, and the Y-axis the log2 ratios of the probes.
FA and cohesion defects in head and neck cancer

A

B

Sequencing cDNA

LN9SV (WT)  

exon 5  
exon 6  
exon 7

VU-SCC-41
VU-41-F

C

Sequencing genomic DNA

LN9SV (WT)  

intron 5  
exon 6

rev. complement

VU-SCC-41

VU-41-F

D

VU-SCC-41 vs. VU-41-F  

VU-41-F vs. healthy controls

Marker VU-41-F VU-SCC-41 LN9SV (WT) H2O
Supplementary Figure 7. Mutational inactivation of STAG2 in cell line VU-SCC-78.

(A) Sequencing of genomic DNA of VU-SCC-78 cells revealed a heterozygous insertion of 1 base pair in STAG2. (B) Sequencing of cDNA demonstrated a homozygous 1 bp insertion, indicating that STAG2 mRNA expression was derived entirely from the mutant allele, whereas the wild type allele is probably inactivated by X chromosome inactivation.

Supplementary Table 1. Separate panel of 39 sporadic HNSCC cell lines

| UT-SCC-10 | UT-SCC-74A | UPCI-SCC-154 | HN |
| UT-SCC-14 | UT-SCC-76A | BICR16 | BHY |
| UT-SCC-16A | UT-SCC-77 | BICR56 | HSC-3 |
| UT-SCC-21 | UT-SCC-87 | SCC-4 | HSC-4 |
| UT-SCC-30 | UPCI-SCC-040 | SCC-15 | OSC-20 |
| UT-SCC-37 | UPCI-SCC-056 | SCC-25 | SAS |
| UT-SCC-40 | UPCI-SCC-070 | RPMI2650 | SIHN-005A |
| UT-SCC-67 | UPCI-SCC-103 | CAL27 | SIHN-006 |
| UT-SCC-73 | UPCI-SCC-122 | CAL33 | |

For further information on these cell lines, see reference (52).
References


