Loss of heterozygosity in *FANCG*, *FANCF* and *BRIP1* from head and neck squamous cell carcinoma of the oral cavity

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Abstract. Recent advances have been made in the understanding of Fanconi anemia (FA), a hereditary disease that increases the risk for head and neck squamous cell carcinomas (HNSCC) by 500- to 700-fold. FA patients harbour germline mutations in genes of cellular DNA repair pathways that are assumed to facilitate the accumulation of mutations during HNSCC development. Mutations in these FA genes may also contribute to HNSCC in general. In the present study, we analysed three FA genes; *FANCF*, *FANCG* and *BRIP1*, that are involved in the repair of DNA inter strand cross-links, in HNSCC and their potential role for patient survival. We measured loss of heterozygosity (LOH) mutations at eight microsatellite loci flanking three FA genes in 54 HNSCC of the oral cavity and corresponding blood samples. Survival analyses were carried out using mutational data and clinical variables. LOH was present in 17% (*FANCF* region), 41% (*FANCG* region) and 11% (*BRIP1* region) of the patients. Kaplan-Meier survival curves and log-rank tests indicated strong clinical predictors (lymph node stages with decreased survival: P=2.69e-12; surgery with improved survival: P=0.0005). LOH in the *FANCF* region showed a weaker association with decreased overall survival (P=0.006), which however, did not hold in multivariate analyses. LOH may predominantly indicate copy number gains in *FANCF* and losses in *FANCG* and *BRIP1*. Integration of copy number data and gene expression proved difficult as the available sample sets did not overlap. In conclusion, LOH in FA genes appears to be a common feature of HNSCC development seen here in 57% of patients and other mutation types may increase this mutation frequency. We suggest larger patient cohorts would be needed to test the observed association of LOH in *FANCF* and patient survival comprehensively.

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Abbreviations: SCC, squamous cell carcinoma; LOH, loss of heterozygosity; FA, Fanconi anemia; HPV, human papilloma virus; HNSCC, head and neck squamous cell carcinomas; PARP, polyADP-ribose polymerase; PCR, polymerase chain reaction; CNV, copy number variants; ESCC, esophageal squamous cell carcinomas; PH, proportional hazards; MSI, microsatellite instability

Key words: squamous cell carcinoma, Fanconi anemia, survival, loss of heterozygosity, microsatellite

Introduction

Squamous cell carcinoma (SCC) is the most frequent tumour entity within head and neck cancers occurring in 90% of patients (1,2) and the 6th most frequent cancer worldwide (3,4). Despite advances in therapy, the prognosis for HNSCC patients remains poor, with a 5-year survival of 46-50% (5,6). As exogenous factors, alcohol consumption and smoking are assumed to cause lesions and HNSCC in a dose-dependent manner (7-9). An infection of human papilloma virus (HPV) is present in ~25% of HNSCC and is associated with an improved prognosis (10,11). Also, endogenous risk factors, such as genetic predisposition may promote hereditary HNSCC (12,13).

The acquisition of somatic mutations during HNSCC development is well understood for the tumour suppressor gene TP53, which is mutated frequently in 30-78% of HNSCCs (14-19). TP53 mutations are associated with a reduced radiosensitivity of HNSCC tumours and with poor prognosis (20). Other potentially important genes in HNSCC are the genes known to be mutated in Fanconi anemia (FA). Patients with this rare autosomal-recessive disorder have a 500- to 700-fold increased risk to develop HNSCC (21-23) and suffer from chromosomal instability, predisposition to congenital anomalies, bone marrow failure and cancer.

FA genes are candidate genes for HNSCC. Sixteen FA genes are currently known, the gene products are involved in the surveillance and repair of DNA crosslinks (24). A recent study found germline mutations of FA genes in 27 analysed FA

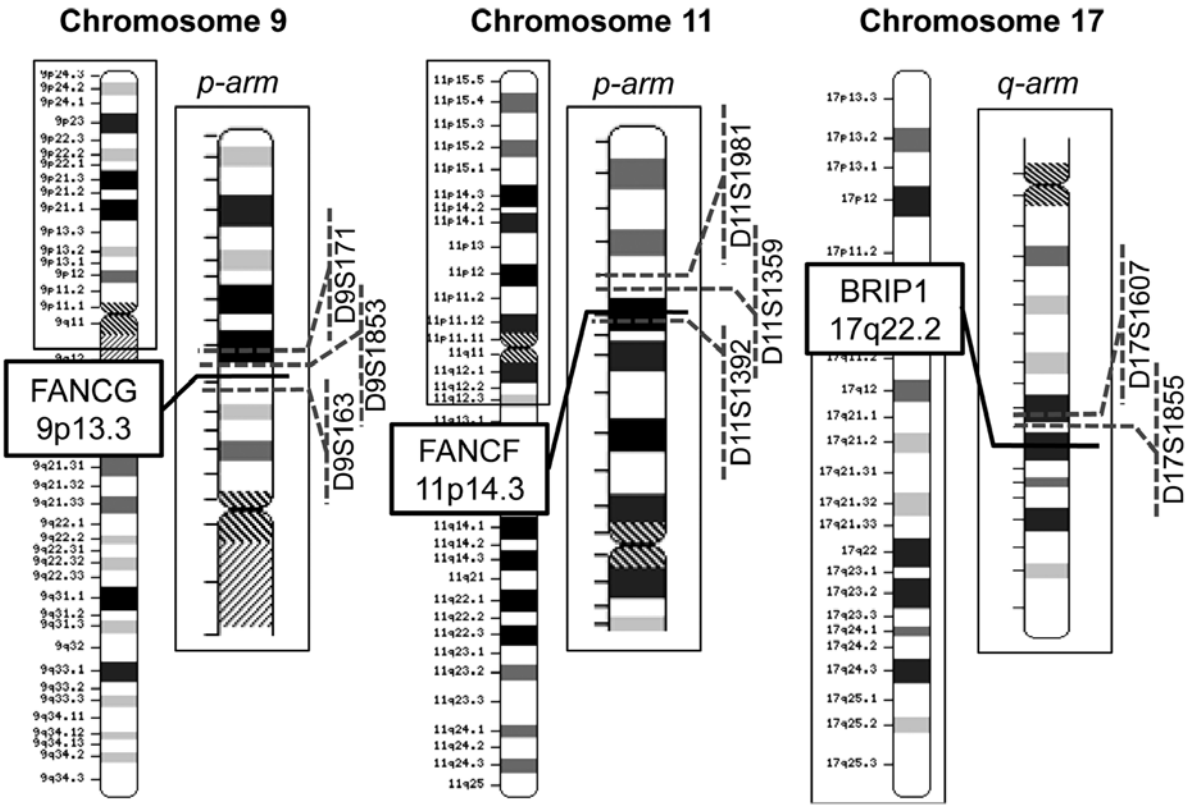

Figure 1. Location of three Fanconi anemia gene regions on chromosome 9, 11 and 17. Three microsatellite markers span the regions of *FANCG* and *FANCF* and two markers are located adjacent to *BRIP1*.

families (25). All FA genes are active in the S-phase of the cell cycle, where DNA damage recognized during DNA replication triggers the accumulation of FA proteins and their interaction with other repair mechanisms. In the present study, we focused on three FA genes that have not been studied extensively, the *FANCF*, *FANCG* and *BRIP1*.

Expression analyses using quantitative PCR showed reduced expression of all three genes, *FANCF*, *FANCG* and *BRIP1* in HNSCC, with a reduction of gene expression by up to 40-fold (26). A possible cause for the reduced expression of FA genes is an allelic gene loss that can be detected as a loss of heterozygosity (LOH). Measurements of LOH have previously indicated FA genes frequently altered in oral HNSCC (27-31). A frequent LOH on chromosome 9 for instance, is potentially overlapping with many genes such as tumour suppressors p16 and *FANCG* (32).

Clinical relevance. LOH in FA genes may impact the prognosis for HNSCC patients under different treatment regimens. It was shown that poly(ADP-ribose) polymerase (PARP) inhibitors can be used to kill specifically *FANCD1* (*BRCA2*)-deficient tumour cells (33). Recently, the assessment of FA genes, such as *FANCD2* was proposed to aid patient stratification for treatment with DNA inter-strand crosslinking agents and PARP inhibitors (34). Thus, FA genes may be used as predictive biomarkers in cancer therapy.

The three FA genes studied here. Fanconi anemia, complementation group F - *FANCF* is localized on chromosome 11p14.3 and involved in stabilization of multimeric FA protein complexes (35,36). The protein, Fancf interacts with Fanca, Fancc and Fancg to stabilize the dimers of Fanca and Fancc as well as Fanca and Fancg which are core components of the FA DNA repair pathway (24,37). Methylation of *FANCF* is associated with a decreased expression of the gene and with ovary, breast, lung, cervix and testis cancer (38-42).

Fanconi anemia, complementation group G - *FANCG* (XRCC9) is localized on chromosome 9p13.3 (43). Fancg interacts with Fanca and Fancf and supports the formation of the DNA repair core complex (44). Mutations in *FANCG* lead to chromosomal instability in a number of different cancers (45).

The *BRCA1* interacting protein C-terminal helicase 1 - *BRIP1 (FANC*J) is localized on chromosome 17q22.2 (46-48). *BRIP1* associates with *BRCA1* and thus, contributes to genomic stability through its role in cellular DNA repair (48,49). Mutations in *BRIP1* are associated with hereditary breast cancer and Fanconi anemia (50).

In this study, we established microsatellite markers for the assessment of LOH in three genetic loci comprising FA genes. To identify a potential effect on patient survival in oral HNSCC, we evaluated LOH in FA genes and clinical parameters in survival analyses. We then discuss an observed weak association of LOH in one of the three FA genes and considerable frequencies of LOH in all three genes in light of potential therapeutic relevance.

Materials and methods

Samples. All patients were enrolled for observation and treatment of HNSCC between 1993 and 2007 at the Department

Table I. Clinical data for patients evaluated for loss of heterozygosity (LOH) in FA gene regions.

Variables	No LOH (n=23)	LOH *FANCF* region (n=9)	LOH *FANCG* region (n=22)	LOH *BRIP1* region (n=6)	Any LOH (n=30)	All (n=54)
Age (median, 1st and 3rd quartile)	59 (46;66.50)	55 (53;68)	59.5 (52.25;66)	57 (51.25;62.75)	60 (53.5;66)	60 (52;66.75)
Gender						
0-Female	6 (26.09%)	2 (22.22%)	4 (18.18%)	2 (33.33%)	7 (23.33%)	14 (25.93%)
1-Male	17 (73.91%)	7 (77.78%)	18 (81.81%)	4 (66,67%)	23 (76.67%)	40 (74.07%)
Tumour stage						
2	2 (8.7%)	1 (11.11%)	2 (9.09%)	0 (0%)	2 (10%)	5 (9.25%)
3	2 (8.7%)	2 (22.22%)	6 (27.27%)	1 (16.67%)	8 (26.67%)	10 (18.52%)
4	19 (82.61%)	6 (66.67%)	14 (63.63%)	5 (83.33%)	19 (63.33%)	39 (72.22%)
Node stage						
0	12 (52.17%)	1 (11.11%)	5 (22.72%)	2 (33.33%)	6 (20%)	19 (35.19%)
1	1 (4.35%)	2 (22.22%)	5 (22.72%)	2 (33.33%)	9 (30%)	10 (18.52%)
2	8 (34.78%)	6 (66.67%)	11 (50%)	2 (33.33%)	14 (46.67%)	22 (40.74%)
3	0 (0%)	0 (0%)	1 (4.55%)	0 (0%)	1 (3.33%)	1 (1.85%)
Metastasis stage						
0	19 (82.61%)	8 (88.89%)	22 (100%)	6 (100%)	29 (96.67%)	49 (90.74%)
1	1 (4.35%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (1.85%)
Tumour grading						
1	1 (4.35%)	0 (0%)	2 (9.09%)	0 (0%)	2 (6.67%)	3 (5.56%)
2	13 (56.52%)	4 (44.44%)	14 (63.63%)	5 (83.33%)	20 (66.67%)	34 (62.96%)
3	3 (13.04%)	3 (33.33%)	4 (18.18%)	0 (0%)	5 (16.67%)	8 (14.81%)
Recurrence						
0	15 (65.22%)	5 (55.56%)	14 (63.64%)	4 (66.67%)	20 (66.67%)	35 (64.81%)
1	8 (34.78%)	4 (44.44%)	8 (36.36%)	2 (33.33%)	10 (33.33%)	19 (35.19%)
Surgery	21 (91.30%)	7 (77.78%)	18 (81.82%)	6 (100%)	26 (86.67%)	48 (88.89%)
Radiotherapy	21 (91.30%)	9 (100%)	20 (90.90%)	6 (100%)	28 (93.33%)	49 (90.74%)
Chemotherapy	8 (34.78%)	4 (44.44%)	5 (22.73%)	1 (16.67%)	7 (23.33%)	15 (27.78%)
Nicotin/Alcohol	18 (78.26%)	7 (77.78%)	19 (86.36%)	3 (50%)	25 (83.33%)	43 (79.63%)
Survival (months, median, 1st and 3rd Qu.)	21.33 (13.61; 73.77)	10.30 (7.53; 22.13)	20.40 (8.30; 98.21)	40.73 (16.10; 73.72)	19.48 (7.80; 78.31)	20.60 (11.30; 78.80)

of Oral and Maxillofacial Surgery, University Hospital Carl Gustav Carus, Technische Universität Dresden (Dresden, Germany). Fifty-four patients, 40 male and 14 female, with a median age of 60 years were included in the study (Table I). We collected clinical information about the tumour-, node- and metastatic stage as well the radiotherapy and chemotherapy used. To study the effects of lifestyle factors we obtained data on smoking and alcohol consumption for each patient.

Tumour specimens were initially chosen to represent primary HNSCC tumours of the locally advanced stages T3 and T4 (51) and were collected in surgeries and biopsies. Five tumour samples were later re-assigned to stage T2. Tumour and corresponding blood samples were collected during the time of surgery or biopsy, snap-frozen in liquid nitrogen and stored at -80˚C. Written informed consent was obtained from all the patients.

Ethics statement. The study was approved by the ethics committee of the Faculty for Medicine at the Technische Universität Dresden, Germany (study ID EK37022001).

DNA extraction, microsatellite amplification and detection of LOH. DNA extraction from tumour samples and corresponding blood was performed with the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following standard procedures. Eight microsatellite markers were used to determine heterozygosity and microsatellite instability (MSI) using their polymorphic repeat length. We used eight pairs of oligonucleotide primers (Applied Biosystems, Darmstadt, Germany) which were previously published (Table II) in polymerase chain reaction (PCR) to amplify loci in proximity to three FA genes (Fig. 1) (43,44,52-59). Six loci were repeats of dinucleotides (D9S1853, D9S171, D9S163, D11S1359, D17S1855 and D17S1607) and two

Table II. Primers used for amplification of microsatellite markers.

Primer	Sequence	Label	Product size (bp)	Refs.
D9S171 s	AGCTAAGTGAACCTCATCTCTGTCT	VTC	158-177	(52-54)
D9S171 as	ACCCTAGCACTGATGGTATAGTCT			
D9S1853 s	GATCCAGCCTCACTGAA	6-FAM	247-265	(44,52)
D9S1853 as	TTGGGCATAGAATTTTTACTTT			
D9S163 s	TGCTGCACATCTTAGGGAGT	NED	270-271	(52,55)
D9S163 as	ACAGCGCTCAGAAATCATATAA			
D11S1359 s	TTGGAAGACACATGCACAAA	NED	148	(43)
D11S1359 as	ATTTTCCAGCCTCCATAATC			
D11S1981 s	AATTCCTTTACTCCAGAAAGG	VTC	134-178	(56)
D11S1981 as	CAGATTTCTGCTTTCCCAGA			
D11S1392 s	TTGCATCCATACGGAAAGTC	6-FAM	200-220	(57)
D11S1392 as	ACATCTGAGACTTGTAGTAGAAGGC			
D17S1607 s	CAGATAAAAAACACAAGTTTCTGAC	NED	103-123	(58)
D17S1607 as	GCTCCACCCCAGACCTA			
D17S1855 s	GGGGACCNCTAGAAACC	PET	219-225	(52,58)
D17S1855 as	GAGAATACATTGTAACAACTCCAGT			

Label, fluorescent labeling of the forward primers; S, sense; As, antisense.

Table III. Combinations of primers and concentrations for multiplex PCR.

Combination	Primer name	Primer concentration	$MgCl_2$ concentration
1	D9S171	0.20 μM	1.8 mM
	D9S163	0.15 μM	
	D17S1607	0.30 μM	
2	D11S1392	0.20 μM	1.5 mM
	D11S1359	0.20 μM	
3	D17S1855	0.20 μM	1.5 mM
	D11S1981	0.20 μM	
4	D9S1853	0.20 μM	1.5 mM

were repeats of tetranucleotides (D11S1392 and D11S1981). We established three multiplex PCR reactions and one singleplex PCR. The reactions contained primers, $MgCl_2$ (concentrations given in Table III), and 100 ng of template DNA in the Qiagen Multiplex PCR kit (Qiagen) and were run in a total reaction volume of 25 μl on a PCR cycler (Bio-Rad Laboratories, München, Germany). After an initial heating step at 95˚C for 15 min, PCR cycling was carried out at 95˚C for 35 sec, 56˚C for 90 sec and 72˚C for 90 sec for 27 cycles. Fluorescent labelling of one primer each per pair was utilized to detect the amplification products in capillary gel electrophoresis using a 3130xl Genetic Analyzer, the GeneScan 500 LIZ size standard and software GeneMapper version 4.0 (all Applied Biosystems).

Larger microsatellite markers may show LOH more frequently than shorter markers, because they amplify less well in PCR. To avoid this problem we used a high amount of input DNA (100 ng). Consequently, the larger microsatellite markers showed a lower frequency of LOH for *FANCF* and *FANCG* and a higher frequency of LOH for *BRIP1* when compared to the smaller markers in the same locus (Table IV). When comparing the two alleles of each microsatellite marker, the LOH affected the larger allele in 33% of the markers (standard deviation 16.6). Thus, a preferential amplification of the smaller microsatellite markers is not apparent in our samples.

To detect LOH all informative microsatellite markers were analysed for their peak area (60-62). The peak area of the higher peak was divided by the area of the lower peak, and a quotient of the values for tumour and blood was calculated. When above 1.5, we called LOH in the tumour tissue as previously described (62). Non-informative and unstable (MSI) markers were excluded.

Statistical analysis of clinical data. Clinical data were obtained from the regional clinic cancer registry Dresden (Table I). To investigate if LOH was more frequent in subgroups of the patients we tested for an association of LOH with age (above vs. below 60 years), gender, tumour stages (T2, T3 and T4), smoking, alcohol consumption and recurrent disease (Table I). For this we used Chi-square tests and Fisher's exact tests if expected frequencies were <5 using IBM SPSS Statistics version 19 (IBM, Ehningen, Germany).

We further investigated the potential impact of LOH on patient survival R v. 3.0. (63). Survival time was obtained and right censored for alive subjects and also if death occurred not due to HNSCC. First, we tested for an association between

Table IV. Loss of heterozygosity (LOH) in FA gene regions.

	FANCG			*FANCF*			*BRIP1*	
Tumour no.	D9S171 158-177 bp	D9S1853 247-265 bp	D9S163 270-271 bp	D11S1981 134-178 bp	D11S1359 148 bp	D11S1392 200-220 bp	D17S1607 103-123 bp	D17S1855 219-225 bp
2	LOH	LOH	n.i.	i.	i.	i.	i.	i.
3	n.i.	i.	i.	i.	i.	i.	i.	i.
7	LOH	n.i.	LOH	i.	i.	n.i.	i.	i.
14	i.	i.	i.	LOH	LOH	i.	LOH	n.i.
20	i.	n.i.	i.	i.	i.	i.	i.	i.
23	i.	LOH	i.	i.	i.	i.	n.i.	i.
26	LOH	n.i.	n.i.	LOH	LOH	LOH	i.	i.
27	i.	i.	i.	n.i.	i.	n.i.	n.i.	i.
30	i.	n.i.	i.	i.	i.	i.	i.	i.
37	i.	i.	n.i.	n.i.	n.i.	n.i.	i.	i.
40	i.	i.	i.	i.	n.i.	i.	n.i.	i.
48	n.i.	LOH	i.	i.	i.	i.	i.	i.
53	LOH	LOH	LOH	i.	i.	i.	n.i.	i.
60	i.	i.	n.i.	i.	i.	i.	n.i.	LOH
101	i.	i.	i.	i.	i.	i.	i.	i.
107	i.	i.	i.	i.	LOH	LOH	i.	n.i.
114	i.	n.i.	n.i.	i.	i.	i.	i.	n.i.
116	LOH	n.i.	LOH	n.i.	LOH	n.i.	LOH	i.
117	i.	i.	n.i.	i.	n.i.	i.	n.i.	i.
118	LOH	n.i.	n.i.	i.	n.i.	n.i.	n.i.	i.
120	i.	i.	n.i.	i.	i.	i.	i.	i.
121	n.i.	i.	i.	n.i.	n.i	i.	i.	i.
123	LOH	LOH	i.	n.i.	i.	i.	i.	i.
144	LOH	LOH	i.	i.	i.	i.	i.	i.
145	i.	n.i.	LOH	i.	n.i.	i.	i.	n.i.
150	LOH	LOH	LOH	i.	i.	MSI	i.	n.i.
152	i.	i.	i.	i.	i.	i.	i.	n.i.
154	LOH	LOH	LOH	i.	i.	i.	i.	i.
155	n.i.	i.	n.i.	i.	i.	n.i.	n.i.	LOH
157	n.i.	i.	i.	i.	i.	n.i.	i.	i.
171	i.	n.i.	i.	LOH	LOH	LOH	i.	i.
179	i.	n.i.	i.	i.	n.i.	i.	i.	i.
180	n.i.	i.	n.i.	i.	i.	i.	n.i.	LOH
181	i.	n.i.	n.i.	i.	i.	n.i.	i.	i.
185	i.	i.	n.i.	i.	i.	i.	i.	i.
193	n.i.	i.	i	n.i.	n.i.	LOH	i.	i.
196	i.	i.	n.i.	i.	i.	i.	i.	i.
206	i.	i.	i.	n.i.	i.	i.	n.i.	i.
213	i.	i.	n.i.	i.	i.	i.	i.	n.i.
325	LOH	LOH	LOH	i.	n.i.	i.	i.	i.
326	LOH	n.i.	n.i.	i.	i.	i.	i.	i.
328	LOH	n.i.	LOH	i.	n.i.	n.i.	i.	i.
336	i.	i.	i.	i.	n.i.	i.	i.	i.
385	LOH	i.	LOH	i.	i.	i.	i.	i.
386	n.i.	LOH	LOH	i.	n.i.	i.	i.	i.
393	n.i.	n.i.	i.	i.	i.	LOH	i.	i.
401	n.i.	i.	i.	i.	i.	i.	i.	i.
409	i.	i.	i.	i.	i.	i.	i.	i.
457	i.	n.i.	i.	i.	i.	i.	i.	i.

Table IV. Continued.

	FANCG			*FANCF*			*BRIP1*	
Tumour no.	D9S171 158-177 bp	D9S1853 247-265 bp	D9S163 270-271 bp	D11S1981 134-178 bp	D11S1359 148 bp	D11S1392 200-220 bp	D17S1607 103-123 bp	D17S1855 219-225 bp
458	n.i.	LOH	n.i.	n.i.	n.i.	LOH	n.i.	i.
474	LOH	LOH	n.i.	i.	i.	i.	i.	i.
477	n.i.	n.i.	i.	i.	i.	i.	n.i.	i.
478	LOH	n.i.	LOH	i.	i.	i.	LOH	n.i.
479	LOH	LOH	LOH	LOH	LOH	n.i.	i.	MSI
∑ i.	42/54	37/54	37/54	46/54%	41/54	44/54	42/54	46/54
	77,78%	68,52%	68,52%	85,19	75,93%	81,48%	77,78%	85,19%
∑ LOH	17/42	13/37	12/37	4/46	6/41	6/44	3/42	3/46
	40,48%	35,14%	32,43%	8,69%	14,63%	13,64%	7,14%	6,52%

Length of amplicons is given for each microsatellite marker. N.i., not informative; I, informative; MSI, microsatellite instability.

LOH in regions around the FA genes, the mentioned clinical parameters and overall patient survival using Kaplan-Meier curves and log-rank tests (Table V). We reported raw P-values using a significance level alpha of 0.05 as well as corrected significance levels (alpha') depending on the number of tests carried out according to the Bonferroni method (64).

To investigate the combined effect of several variables on patient survival we employed Cox proportional hazards (PH) regression models in R v. 3.0. (63). We estimated the proportional hazards for different sets of variables on survival, firstly using all variables in one Cox PH model. Secondly, we tested all variables which were significant in the first model. A third test used backwards elimination, starting with all and deleting the least significant variable at each step until reaching a stage where all remaining variables were significant. Since smoking and alcohol consumption coincided in many patients and both are seen as mutagenic substances we merged them into one binary variable (absence/presence) for survival analyses. For analyses of LOH and survival in Cox PH we evaluated each FA gene region independently. However, we did not evaluate each microsatellite marker independently since those showed similar results as the corresponding FA gene regions in log-rank tests.

Analysis of copy number data. From a published dataset of 106 HNSCC genotyped on microarrays (18) we extracted called copy number variants (CNV) that spanned the genes *FANCF*, *FANCG* and *BRIP1*.

If the reported ploidy deviated from 2 and was <1.8 or >2.2, we noted a loss or a gain, respectively.

Expression data. We accessed publicly available expression profiles of three studies on HNSCC tumours and corresponding normal tissues in NCBI Gene Expression Omnibus. For accessions GDS2520 and GDS3838 we queried the genes of interest directly retrieving lists of expression values. Fold-change in expression was calculated by comparing the cancerous and normal tissue per each tested patient. For GSE55550 we queried for differentially expressed genes by grouping (pooled)

Table V. P-values from log-rank tests comparing survival in HNSCC patients related to clinical variables and LOH at FA gene regions.

		All patients		Patients with tumour stage T4	
Test no.	Variables	P-value	n	P-value	n
1	**Gender**	0.495	53	0.819	39
2	**Tumour stage**	0.636	53		
3	**Node stage**	*2.69e-12*	51	*2.58e-09*	37
4	**Metastasis stage**	0.662	49	0.868	36
5	**Tumour grading**	**0.0418**	44	0.108	32
6	**Surgery**	*0.000466*	53	**0.00368**	39
7	**Radiotherapy**	0.246	53	0.197	39
8	**Chemotherapy**	0.0742	53	0.133	39
9	**Nicotin/Alcohol**	0.24	53	0.434	39
10	D11S1981	0.19	45	0.286	34
11	D11S1359	**0.0248**	40	**0.0617**	32
12	D11S1392	**0.0326**	42	**0.00254**	31
13	*FANCF region*	**0.00617**	52	**0.00595**	38
14	D9S171	0.344	41	0.902	31
15	D9S1853	0.901	36	0.309	27
16	D9S163	0.438	35	0.573	26
17	*FANCG region*	0.682	53	0.452	39
18	D17S1607	0.983	41	0.876	31
19	D17S1855	0.768	44	0.586	30
20	*BRIP1 region*	0.762	53	0.537	39
21	**>1 FA gene region**	0.6	53	0.483	39

Bold indicates significance for raw P-values. Italics indicates P-values significant after correcting for multiple tests (alpha'=0.0024 for 21 tests and alpha'=0.0038 for 13 tests omitting the individual microsatellite markers).

Table VI. P-values from Cox proportional hazards models.

No.	Variables	One Cox PH model with all variables			One Cox PH model with significance in log-rank tests of Kaplan-Meier analysis			Cox PH model with best likelihood ratio, Wald and Score tests		
		P-value	LCI	UCI	P-value	LCI	UCI	P-value	LCI	UCI
1	Gender	0.88885	0.206970	39.176						
2	Tumour stage	0.57460	0.509899	33.673						
3	Node stage	0.34330	0.732887	24.415	0.1584	0.87206	23.155	**0.02539**	107.130	28.550
4	Metastasis stage	0.21420	0.013535	26.236				0.12550	0.01837	16.319
5	Tumour grading	0.10808	0.084520	12.771	**0.0469**	0.09622	0.9838	**0.01890**	0.06185	0.7785
6	Surgery	*0.00298*	0.006957	0.3615	*0.0139*	0.09975	0.7709	*0.00265*	0.04769	0.5267
7	Radiotherapy	0.97083	0.155178	69.173						
8	Chemotherapy	0.53866	0.080760	37.230						
9	Nicotin/Alcohol	0.07382	0.040165	11.592						
10	FANCF region	0.10518	0.631867	1262.340	0.1723	0.74051	53.587			
11	*FANCG* region	0.37524	0.393851	118.390						
12	*BRIP1* region	0.86562	0.187164	73.337						
13	>1 FA gene region	0.09701	0.001591	17.071						
14	Age	0.54531	0.919269	10.455						
Test statistics:										
	n	40			42			41		
	Events	27			28			27		
	Rsquare	0.509			0.335			0.432		
	Likelihood ratio test	28.42 on 14 df, P=0.01249			17.15 on 4 df, P=0.001806			23.19 on 4 df, P=0.0001162		
	Wald test	24.09 on 14 df, P=0.04469			17.48 on 4 df, P=0.001561			22.93 on 4 df, P=0.0001308		
	Score (log-rank) test	35.05 on 14 df, P=0.001444			20.63 on 4 df, P=0.0003744			30.97 on 4 df, P=3.1e-06		

Bold indicates significance for raw P-values. Italics indicates P-values significant after correcting for multiple tests (testing three models, alpha'=0.0167). LCI, lower confidence interval (95%). UCI, upper confidence interval (95%).

normal tissues and tumours. Then we extracted array probes corresponding to our genes of interest from the resulting gene lists and retrieved the fold change in expression when the raw P-value was <0.05.

Differential gene expression analysis using TCGA data. We queried the Cancer Genome Atlas data for head and neck squamous cell carcinoma comprising 521 samples with RNAseq data (queried in November 2016) using the TCGA Browser v0.9 at http://tcgabrowser.ethz.ch:3839/TEST/ (65). The top 200 differentially expressed genes for *FANCG*, *FANCF* and *BRIP1* were subjected to the gene set enrichment analysis (66). We computed overlaps with hallmarks gene sets and with gene sets of known molecular function.

In order to identify protein partners that interact physically with *FANCG*, *FANCF* and *BRIP1*, a network analysis was performed using GeneMANIA (67).

Results

Detection of LOH. The information content of the microsatellite markers was high with 68-85% of informative patients per marker, in 53 of 54 patients at least one microsatellite marker was informative (Table IV). Patient 37 had to be excluded from the analysis of *FANCF* since none of the markers was informative here. We detected LOH in 30 of 53 (57%) patients (Table IV), 23 patients had at least one informative marker but did not show LOH.

LOH was detected most frequently in the gene region containing *FANCG* in 40.74% (22/54) of HNSCCs. The gene region of *FANCF* showed LOH in 16.98% (9/53) and the *BRIP1* region was affected in 11.11% (6/54) of HNSCC. Patient 116 showed LOH in all three FA gene regions. Patients 26, 458 and 479 showed LOH in *FANCG* and *FANCF* regions. Patient 478 showed LOH in *FANCG* and *BRIP1* and patient 14 in *FANCF* and *BRIP1* regions.

Association analysis. We tested if the frequency of LOH was associated with age (either below or above 60 years), gender, tumour stages (T2, T3 and T4), node stage, metastasis stage, histological tumour grading, smoking or alcohol consumption and recurrent disease. None of these variables was significantly associated with an increased or decreased frequency of LOH at the FA gene regions, when combining

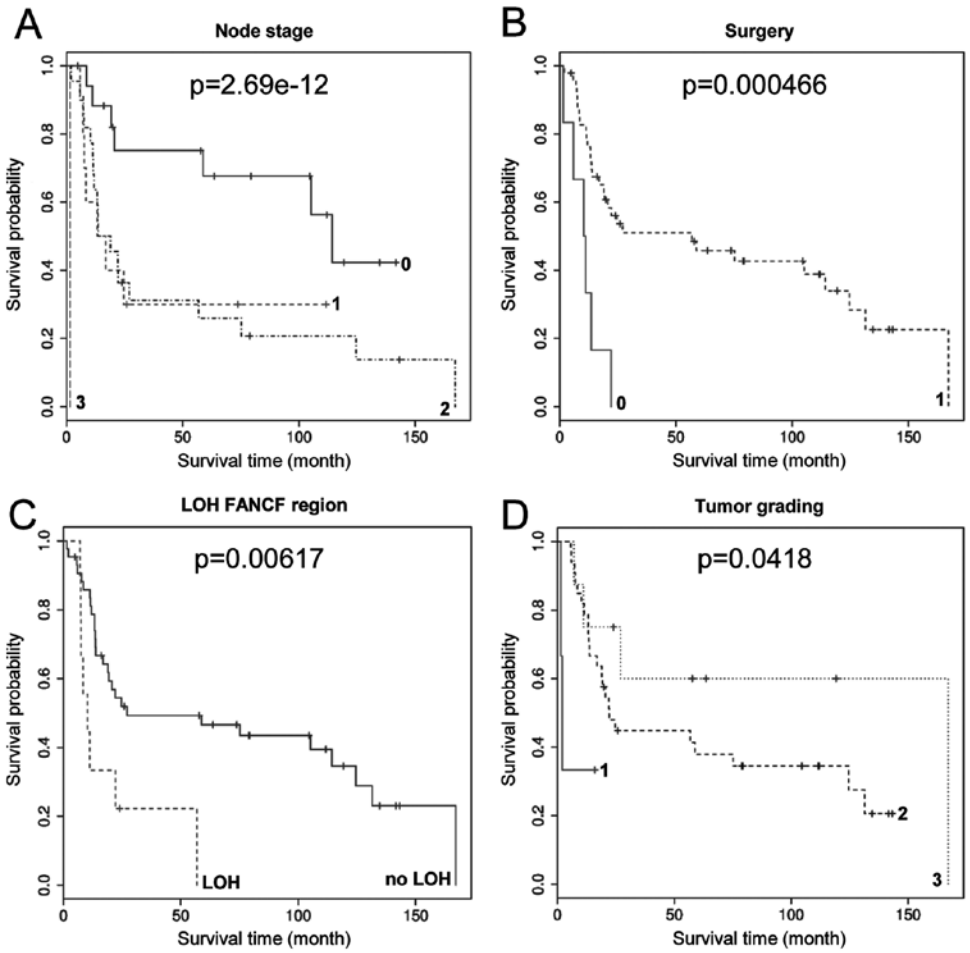

Figure 2. Kaplan-Meier survival curves. Curves shown for (A) node stages 0 to 3, (B) surgery; 0, no and 1, yes, (C) LOH in the *FANCF* region and (D) tumour grading of 1 to 3 (see Table I).

Table VII. LOH, CNV and expression of *FANCF*, *FANCG* and *BRIP1* in HNSCC.

Gene	LOH	CNV (18)			Expression		
	This study	Sum	Gain	Loss	22 HNSCC, 22 normal tissues, dataset GDS2520 (65)	139 HNSCC, 16 normal tissues, dataset GSE55550	17 ESCC, 17 normal tissues, dataset GDS3838 (66)
FANCF	16.98%	20%	16.2%	3.8%	NA	Underexpressed	Underexpressed in14/17
FANCG	40.74%	35.2%	6.7%	28.6%	Overexpressed in 17/22	Overexpressed	Overexpressed in 14/17
BRIP1	11.11%	35.7%	4.1%	31.6%	NA	Overexpressed	Overexpressed in 15/17

LOH in HNSCC measured here and data on CNV previously published match roughly for *FANCF* and *FANCG*. *BRIP1* shows LOH less often. LOH may predominantly indicate copy number gains in *FANCF* and losses in *FANCG* and *BRIP1*. Expression data indicate underexpression of *FANCF* and overexpression of *FANCG* and *BRIP1* in HNSCC. NA, not available.

several microsatellite markers per region (Fisher's exact tests and Chi-square tests, data not shown). Individual microsatellite markers were, moreover, not associated with any factor except for D17S1607 (in the *BRIP1* region) which was associated with smoking or alcohol consumption (P=0.029). However, this association was not significant when corrected for multiple testing (alpha' of 0.006) and LOH overall was more frequent in patients without smoking or alcohol consumption.

Survival analysis. We tested for an association of the LOH in regions of FA genes and overall patient survival using Kaplan-Meier curves and log-rank tests (Table V). Higher lymph node stages were most significantly associated with

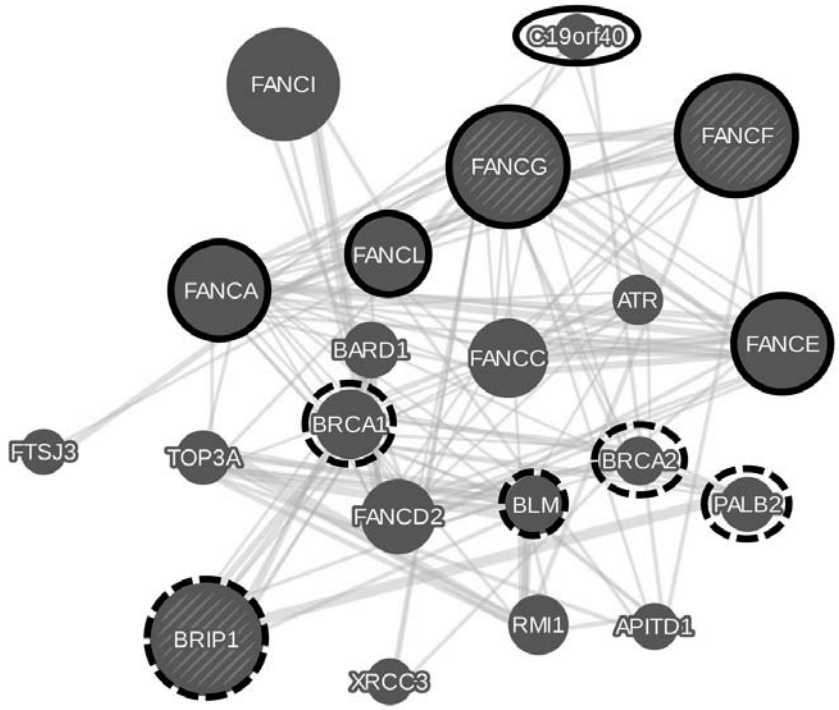

Figure 3. Physical interaction network. FANCF, *FANCG* and 5 other genes (*FANCE*, *C19Orf40*, *FANCC*, *FANCL* and *FANCA*) are members of the Fanconi anemia nuclear complex (solid circles). *BRIP1*, *BRCA1*, *BRCA2*, *BLM* and *PALB2* are involved in double-strand break repair (dotted circles). Physical interactions are indicated by lines.

decreased survival (P=2.69e-12) and surgery was most significantly associated with improved survival (P=0.0004; Fig. 2). Node stage and surgery were significant also when corrected for multiple testing (21 variables: alpha'=0.0024 or 13 variables: alpha'=0.0038, see Table V). LOH in the *FANCF* region was associated with decreased survival (P=0.006; Fig. 2 and Table I) and higher tumour grading was associated with increased survival (P=0.0418; Fig. 2). However, these associations were not significant when corrected for multiple testing. LOH in *FANCG* and *BRIP1* regions were not significantly associated with survival, accordingly LOH in more than one FA gene region was also not significantly associated with survival. Stratification for patients with tumour stage T4 showed similar associations. Only node stage remained significantly associated after correcting for multiple testing (Table V). We did not stratify for T3 and T2 patients since they were too few (≤10).

To increase our understanding of LOH in FA gene regions and survival in the context of clinical covariates we employed Cox PH regression models (Table VI). We evaluated three different Cox PH models: firstly, a model with all variables, secondly one with only those variables which were significant in the previous log-rank tests. We also used backwards elimination of variables to obtain the third Cox PH model with the most significant likelihood ratio test, Wald test and score (log-rank) test. Applying a raw significance level of alpha=0.05, surgery was always significantly associated with survival (P-values between 0.003 and 0.014) and tumour grading was significantly associated with survival when fewer variables were in the model (P-values between 0.047 and 0.019). Node stage was significantly associated only in the Cox PH model with best likelihood ratio, Wald and score tests (P=0.025). LOH in FA gene regions were not significantly associated with survival in the Cox PH models. Moreover, age, gender, tumour stage, metastatic stage, radiotherapy, chemotherapy and smoking or alcohol consumption were not significantly associated with survival. When correcting for multiple testing, applying an alpha' of 0.0167 (due to the three Cox PH models evaluated), only surgery showed a significant association with survival, most prominently in the Cox PH model with the best test statistics.

We did not explore associations of survival and MSI, because of a very small sample for MSI in only two patients.

Analysis of copy number data. To analyse if LOH in *FANCF*, *FANCG* and *BRIP1* may be associated with amplifications or deletions, we checked if published copy number data of HNSCC (18) showed gains or losses of these genes (Table VII). Twenty prercent of HNSCC samples showed copy number variants (CNV) spanning *FANCF*; 3.8% (4/105) were losses and 16.2% (17/105) were gains. *FANCG* was lost in 28.6% (30/105) and gained in 6.7% (7/105), in sum it was affected in 35.2% of samples. *BRIP1* was lost in 31.6% (31/98) and gained in 4.1% (4/98), in sum affected in 35.7%. For comparison, TP53 showed losses in 47% (49/103) of the samples and did not show gains.

Our frequencies of LOH correspond to the frequencies of copy number variants (CNV) recently published in a genome wide screen of HNSCC for *FANCF* (17% LOH, 20% CNV) and *FANCG* (41% LOH, 35% CNV) (18). The estimates depart for *BRIP1* (11% LOH, 36% CNV) possibly reflecting differences between the sample sets. However, at least for *FANCF* and *FANCG* we think that our measurement of LOH approximate the measurement of CNV in array based methods.

Analysis of expression data. One dataset, GDS2520, comprised 22 pairs of HNSCC corresponding normal tissues (68). Here, *FANCG* was overexpressed in 17 HNSCC samples when compared to the corresponding normal tissue. The highest change in expression was 1.6-fold. Data for *FANCF* and *BRIP1* were not available in this dataset (Table VII).

The second dataset (GSE55550, no publication available) contained gene expression profiles of 139 HNSCC and 16 normal samples. For all of our three genes of interest, there was an array probe that showed significant differential gene expression (raw P-value<0.05). Here, *FANCF* was underexpressed in HNSCC compared to normal tissues and *FANCG* and *BRIP1* were overexpressed.

A third dataset (GDS3838) compared 17 esophageal squamous cell carcinomas (ESCC) to corresponding normal tissues (69). *FANCF* was underexpressed in 14 of 17 samples. *FANCG* and *BRIP1* were overexpressed in 14 of 17 and 15 of 17 samples, respectively. The few samples that showed the opposite pattern (overexpression of *FANCF* and underexpression of *FANCG* or *BRIP1*), did not overlap.

Differential gene expression analysis using TCGA data. In order to identify cellular signaling pathways that are affected by mutations in *FANCG*, *FANCF* and *BRIP1* we performed a differential gene expression analysis of the TCGA head and neck squamous cell carcinoma samples (n=521) with subsequent gene set enrichment to identify affected pathways.

The gene set enrichment analysis for *FANCG* revealed a significant overlap with genes that perform transfers of ubiquitin modifications (FDR q-value 0.00005 genes such as UBE2R2 and UBE2W), a process that has been described before to play a role in the damage response of *FANCG* (70).

Differentially expressed genes associated with *FANCG* are also of ribonucleotide binding activity (FDR q-value 0.00006), such as XRCC3 which is together with *FANCG* involved in homologous recombination to maintain chromosome stability and repair DNA damage and also physically interacts with *FANCG* (see below).

The gene set enrichment analysis for *FANCF* revealed a highly significant overlap with genes that have transcription factor activity (FDR q-value 4.86E-022), such as a number of zinc finger proteins. However, these are somewhat inconclusive as a plethora of transcriptions factors is involved in the damage response.

The gene set enrichment analysis for *BRIP1* revealed highly significant overlaps with genes involved in the G2/M checkpoint, as in progression through the cell division cycle (FDR q-value of 2.07e-41), such as e.g. *BRCA2* and *BARD1* (*BRCA1* associated RING domain 1). Also, genes differentially expressed in association with *BRIP1* are preferably genes encoding cell cycle related targets of E2F transcription factors (FDR q-value of 8.14e-30). Some of these, *BRCA2* and *BRCA1* also physically interact with *BRIP1* (see below).

In order to complement the gene set enrichment, protein partners that interact physically with *FANCG*, *FANCF* and *BRIP1* were identified in a network analysis and are shown in Fig. 3. Highly associated protein sets are members of the Fanconi anemia nuclear complex (false discovery rate FDR of 9.72e-18) and proteins involved in double-strand break repair (FDR of 1.79e-3).

Discussion

Frequent LOH in FA gene regions of HNSCC. The microsatellite markers established here were informative for almost all patients and allowed us to assess the frequency of LOH at three FA gene regions in HNSCC tumours. LOH in at least one of the analysed regions containing the genes *FANCF*, *FANCG* and *BRIP1* appears to be a frequent event in HNSCC development in more than half of the cases analysed here. Such a high frequency of LOH supports an assumed role of these genes in HNSCC development, and might also influence the success of HNSCC therapy.

The frequencies of LOH in the three studied genes is much higher than that of reported point mutations (0.3-1.1%): *BRIP1* was mutated in 2 of 172 skin cancers, 4 of 173 esophageal cancers and 1 of 113 upper aerodigestive tract cancers, and the mutational frequencies of *FANCG* and *FANCF* were even lower (71). This suggests minor role of point mutations, however, non-coding point mutations have not been studied and may add onto these frequencies.

Mutations in HNSCC are thought to be induced by mutagens such as tobacco smoke and alcohol (7-9). In the present study we observed a mild negative association between smoking or alcohol consumption and FA mutation for one microsatellite marker, suggesting that in some cases mutagenesis induced by smoking or alcohol is not responsible for HNSCC progression. The frequencies of LOH at FA gene regions reported here should be seen as a minimum estimate since the distance between FA genes and the used microsatellite markers was quite large and result in false negative assignments. The *FANCG* region presented with LOH in 41% of the tumours and is thus a strong candidate for further study. Similarly, the tumour suppressor CDKN2A on chromosome 9p has been implicated in cancer development, and the loss of this gene is thought to be a frequent event in various cancers and in HNSCC with an estimated 25%. A proposed progression model of HNSCC based on CGH data involved an early loss of 9p suggested ~80% of the samples (29). The LOH in this region may therefore be driven by loss of the tumour suppressor CDKN2A rather than that of *FANCG*. However, simultaneous loss of DNA repair mediated by Fancg may promote the accumulation of mutations and a deregulation of apoptosis at the same time (72,73). Future studies on cancer genomes will provide data to test whether heterozygosity is lost in CDKN2A and *FANCG* independently, or whether the loss of *FANCG* is a result of LOH in CDKN2A.

The markers used previously to study LOH in the *FANCF* region on chromosome 11p showed similar frequencies of LOH, partially using the same microsatellite markers (18-33%) (74) and also when markers were 2-3 Mb distant from our markers (17%) (32). These studies focused on primary HNSCCs. A proposed progression model of HNSCC implies that the same gene region 11p14 is lost in up to 60% of metastatic HNSCC (29). If the loss of *FANCF* predominantly happens during the progression of HNSCC to the metastatic stage, the gene may already be downregulated via other mechanisms in primary HNSCC, for instance methylation (40).

Markers previously used on chromosome 17q were more than 10 Mb distant from the *BRIP1* region and showed a higher

LOH frequency (31%) than our estimate (11%), (32). However, another study evaluated loci around 3 Mb distant from our marker positions and did not detect LOH (74).

Thus, our data on LOH in the regions of *FANCG* and *FANCF* are in concordance with previous estimates for LOH in regions containing the studied FA genes. Reports on *BRIP1* vary and it is currently difficult to conclude on the role of LOH adjacent to this gene in HNSCC.

LOH in FA genes and patient survival. Impaired DNA repair in tumour tissue due to mutated FA genes may increase the sensitivity to DNA damage by radiotherapy and even more by alkylating agents and may thus prolong patient survival (72,73,75,76). However, associations of LOH at FA gene regions and patient survival were not strongly supported from our data. We observed a mild association of LOH at the *FANCF* region and decreased survival in our Kaplan-Meier curves and log-rank tests. The loss of *FANCF* as a tumour suppressor is consistent with decreased survival in the affected patients and also with decreased expression of the gene in ovary, breast, lung, cervix and testis cancer (38-42). However, the weak association for *FANCF* is not as strong as that of known predictors for HNSCC survival, such as higher node stages and surgery (77,78). The Bonferroni correction may be overly conservative for the Cox PH models evaluated, as these can be assumed to be positively correlated and also weaker associations might be true positive results (79). Thus, in multivariate analyses with improved statistical power, the weak associations observed here may be correct.

The observed effect of tumour grading and improved survival was very weak and may not hold true, as we had a small sample size for this trait and higher tumour grading has repeatedly been described as associated with decreased survival (80-82). Our analysis may also lack statistical power since the analysed sample of 53 patients had varying clinical data. A rather uniform collection of tumours from patients with similar clinical data, e.g. all with surgery and radiotherapy, would result in a strongly improved study design. A larger sample for only chemotherapy treated individuals may be necessary to explore a potential link of chemosensitivity with *BRIP1* mutation.

Copy number and expression data. From the queried datasets we found that HNSCC tumours tend to overexpress *FANCG* and *BRIP1* (Table VII), however, often exhibit chromosomal aberrations that predominantly involve the loss of these genes. After the induction of both genes for DNA repair, the copy number loss in a progressing tumour could remove both genes and allow the accumulation of mutations. This scenario supports the assumed role of *FANCG* and *BRIP1* as tumour suppressors. These data differ from a previous study that found reduced expression of *FANCG* and *BRIP1* in HNSCC in tongue carcinoma (26). However, a loss of *FANCG* and *BRIP1* would diminish expression of the genes in later stages of HNSCC or in the more aggressive tongue carcinoma. Also in other cancer types *FANCG* was lost more often than gained (breast: 11.5% gains, 24.1% loss; lung: 13.9% gains, 43.9% losses; and pancreas: 20.3% gains, 39.0% losses) (71). However, *BRIP1* was gained more often than lost indicating differences of the various cancer types (breast: 32.8% gains, 12.3% loss; lung: 36.8% gains, 6.9% losses; and pancreas: 27.1% gains, 24.8% losses).

Intergrating copy number data and expression is somewhat inconclusive as well for *FANCF* as it shows underexpression in two sets of HNSCC and is affected by copy number gains in another set. Copy number gains are not characteristic for tumour suppressor genes, which *FANCF* was supposed to be. A decrease in gene expression, may be explained by point mutations or methylation of the gene in 15% of HNSCC as previously reported (40,83). This could also ameliorate copy number gains that involve *FANCF* by chance. In other cancer types *FANCF* is affected by CNV in 30-40% of samples, involving gains and losses (breast: 13.4% gains, 20.4% loss; lung: 13% gains, 27.1% losses; and pancreas: 29.7% gains, 14.8% losses).

Thus, copy number mutations of FA genes are frequent in HNSCC and other cancer types. However, an improved understanding of the mechanisms leading from gene mutations to gene expression changes and a potential clinical relevance could come from data on LOH, CNV, point mutations and gene expression obtained for the same samples.

Differential gene expression analysis using TCGA data. Our pathway analyses using differential gene expression analysis with subsequent gene set enrichment and network analysis showed that physically interacting partners differ for the studied genes. *FANCG* and *FANCF* show some overlap in interacting with members of the Fanconi anemia nuclear complex while *BRIP1* is rather involved in the double-strand break repair pathway.

Clinical relevance. As HNSCC is routinely treated with ionizing radiation and, less frequently, also with chemotherapy, mutations in DNA repair genes may be relevant for treatment success. Since we found LOH in FA gene regions in 57% of the patients and other mutation types may add to this frequency, a substantial proportion of HNSCC patients may be eligible for poly-adenosine diphosphate ribose polymerase (PARP) inhibition therapy. PARP inhibition impairs DNA repair selectively in cancer cells, however, not in normal somatic cells and may enter the clinics for HNSCC treatment (33,84). In theory, cancer cells with an impaired pathway for homologous recombination (e.g. due to mutated FA genes) cannot perform double strand break repair. PARP inhibition then may be used to block the base excision repair pathway for single strand break repair as well. Unlike normal cells, cancer cells will then be sensitized to DNA damage induced by radiation and alkylating chemotherapy (85). In this regard, cells deficient in *FANCA*, *FANCC* or *FANCD2* were previously found to be hypersensitive to PARP inhibition (86). Also human HNSCC cells showed enhanced cytotoxicity with radiation and PARP inhibition compared to either agent alone (87). PARP inhibitors enhanced the effect of radiotherapy in a xenograft model of human HNSCC leading to reduced tumour volume and enhanced apoptosis (88). *FANCF* knockdown has been shown to induce chemosensitivity in cancer cells (89,90). Thus, further studies are needed to explore the observed association of LOH in *FANCF* and decreased HNSCC survival as well as the potential use of FA gene mutations as an indicator for chemo- and radiosensitivity of head and neck tumours.

In conclusion, analysing three FA gene regions, we found LOH in 57% of HNSCC tumours. LOH in *FANCF* showed a weak association with survival of radiotherapy and chemotherapy treated HNSCC patients. Tumours with LOH in FA genes may exhibit an altered sensitivity to cancer therapy utilizing DNA damaging agents. Thus, it is worthwhile to perform further studies screening for other types of mutations in FA genes and involving larger sample sizes to improve the statistical power of survival analysis.

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