Pyrosequencing-based DNA methylation profiling of Fanconi anemia/*BRCA* pathway genes in laryngeal squamous cell carcinoma

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Abstract. Fanconi anemia (FA) associated genes [*FANCA*, *-B*, *-C*, *FANCD1(BRCA2)*, *-D2*, *-E*, *-F*, *-G*, *-I*, *-L*, *-M*, *FANCN (PALB2)*, *FANCJ(BRIP1)* and FA-linked *BRCA1*] encode proteins of DNA damage response pathways mutated in FA patients. FA is characterized by congenital malformations, chromosomal instability and high cancer susceptibility. FA patients have a 500-700 times higher risk of head and neck squamous cell carcinoma (HNSCC) compared to the non-FA population. As DNA methylation comprises one of the known gene inactivation mechanisms in cancer we have investigated the methylation status of 13 FA and one FA-linked gene in order to assess the role of FA in sporadic laryngeal squamous cell carcinoma (LSCC) tumor samples. Thirteen laryngeal squamous carcinoma cell lines (UT-SCC) and 64 primary laryngeal carcinoma cases were analyzed by bisulfite pyrosequencing. DNA from buccal swabs of 10 healthy volunteers was used as a control group. Promoter regions of *FANCA*, *BRCA1* and *BRCA2* displayed recurrent alterations in the methylation levels in cancer samples as compared to buccal swabs controls. For *FANCA*, hypomethylation was observed in 11/13 cell lines ($p<0.0003$) and all 64 primary larynx samples ($p<0.001$) compared to buccal swabs. For *BRCA1*, 4/13 cell lines ($p=0.04$) and 3/58 primary laryngeal cases ($p=0.22$) showed hypomethylation. In *BRCA2*, all 13 cell lines ($p<0.0001$) 4/63 primary LSCC ($p<0.01$) showed hypermethylation as compared to controls. In conclusion, we show recurrent alterations of DNA methylation levels in three Fanconi anemia genes which might contribute to the pathogenesis of LSCC.

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Introduction

Squamous cell carcinoma of larynx (LSCC) constitutes a serious medical concern, as a low cure efficacy is demonstrated with a 5-year survival rate only 60%. LSCC occurrence is associated with tobacco smoking and alcohol intake (1) as well as viral infections by HPV (2) or EBV (3). A substantial disproportion between smoking and drinking male and female individuals (8-10:1) diagnosed with LSCC is observed. The treatment is complicated by frequent recurrences and occurrence of secondary tumors associated with poor prognosis (4,5).

Fanconi anemia (FA) is a recessively inherited, genetically and phenotypically heterogeneous disorder which manifests with different congenital malformations (6,7). The FA/BRCA pathway comprises 12 known FA genes (*FANCA*, *-B*, *-C*, *-D2*, *-E*, *-F*, *-G*, *-I*, *-J/BRIP1*, *-L*, *-M* and *-N/PALB2*) that cooperate within to DNA damage signaling pathway (8). Moreover, *BRCA1* and *BRCA2/FANCD1* genes are also functionally involved in this DNA repair pathway (9,10). Defects of the FA/BRCA pathway lead to DNA repair deficiencies presenting with increased chromosomal instability and resulting in proneness to hematologic malignancies and solid tumors early in life (11). FA subjects are highly susceptible to develop squamous cell carcinomas (SCC) with particularly high incidence of aggressive forms of head and neck cancer (12-14). The risk ratio of head and neck squamous cell carcinoma (HNSCC) occurrence among FA-individuals is estimated to be over 700 times higher than in the non-FA population (15).

One of the mechanisms leading to aberrant gene expression is DNA methylation. Hypermethylation of tumor suppressor

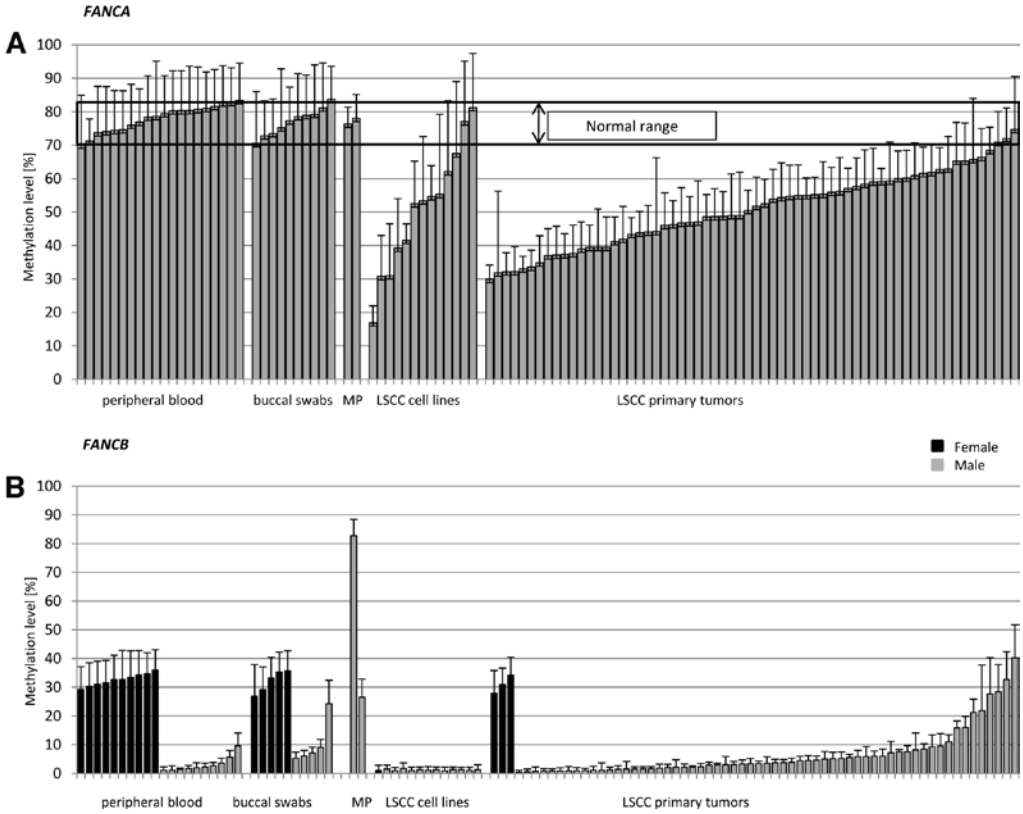

Figure 1. Pyrosequencing analysis results for (A) *FANCA*, hypomethylation is shown in 85% laryngeal cancer cell lines and in 94% LSCC primary tumors compared to buccal swabs. (B) *FANCB*, gender specific pattern; few male-derived samples show elevated methylation level (similarly to control group); X-axis, analyzed samples; Y-axis, methylation level measured (%); M, methylated control; P, pooled blood DNA; LSCC, laryngeal squamous cell carcinoma.

genes (TSG) has been shown to be involved in carcinogenesis (16). The consequence of this epigenetic process is often a loss of function of these genes associated with transcriptional down-regulation in the absence of structural alterations. Many TSG like, cyclin-dependent kinase inhibitor 2A (*CDKN2A*) (17), E-cadherin (*CDH1*), human mismatch repair gene (*MLH1*), retinoblastoma 1 (*RB1*) or TIMP metallopeptidase inhibitor 3 (*TIMP3*) (18) and in HNSCC *p16/CDKN2A*, *p14*, *DAPK*, *RASSF1A*, *RARβ*, *MGMT* (19-23) and many others have been screened and reported to be hypermethylated in tumor cells.

Disruption of FA pathway genes has been previously reported in several cancers including leukemia (24,25), cancer of the breast (26), ovary (27), bladder (28), cervix (29), testis (30) and lung (31). In HNSCC sporadic epigenetic events involving FA/BRCA-related genes have been previously documented in several reports (32-34). *FANCF* has been demonstrated to be often hypermethylated and associated with alcohol and tobacco use and poor prognosis (31). Moreover, altered methylation patterns of *FANCB* and *BRIP1* have been recently demonstrated in HNSCC and *FANCB* inactivation is supposed to contribute to HNSCC pathogenesis (33). However, the defects of FA-genes among sporadic HNSCC still remain poorly explained.

Considering the high prevalence of epigenetic events in HNSCC it is expected that altered DNA methylation in HNSCC plays a significant role in the deregulation of FA/BRCA genes. By impairing DNA repair and inducing high chromosomal instability this might contribute to carcinogenesis. Thus, in this study we assessed the methylation profiles of all Fanconi anemia/BRCA pathway genes in non-Fanconi specimens of laryngeal squamous cell carcinoma (cell lines and patients) using bisulfite pyrosequencing.

Materials and methods

Cell lines. Thirteen LSCC cell lines: UT-SCC-6A, UT-SCC-11, UT-SCC-19B, UT-SCC-22, UT-SCC-29, UT-SCC-34, UT-SCC-35, UT-SCC-38, UT-SCC-42B, UT-SCC-57, UT-SCC-106A, UT-SCC-107 and UT-SCC-116 were derived from patients diagnosed with squamous larynx carcinoma at Turku University Central Hospital in Finland. The cell lines were previously characterized by Jarmuz *et al* (35) or Jarvinen *et al* (36). Cells were grown in 25 cm^2 flasks in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37˚C under 5% CO_2 atmosphere.

Primary laryngeal tumor specimens. Fresh frozen tumor samples were obtained from 64 patients diagnosed with squamous laryngeal carcinoma and undergoing surgical tumor resection followed by radiotherapy in the Department of Otolaryngology, K. Marcinkowski University of Medical Sciences in Poznan, Poland. Patient characteristics are listed in Table III. The Institutional Ethics Review Board of K. Marcinkowski University of Medical Sciences approved the study and informed consent was obtained from all patients. The tumor tissues were assessed histopathologically to confirm the presence of at least 80% cancer cells in the submitted sample.

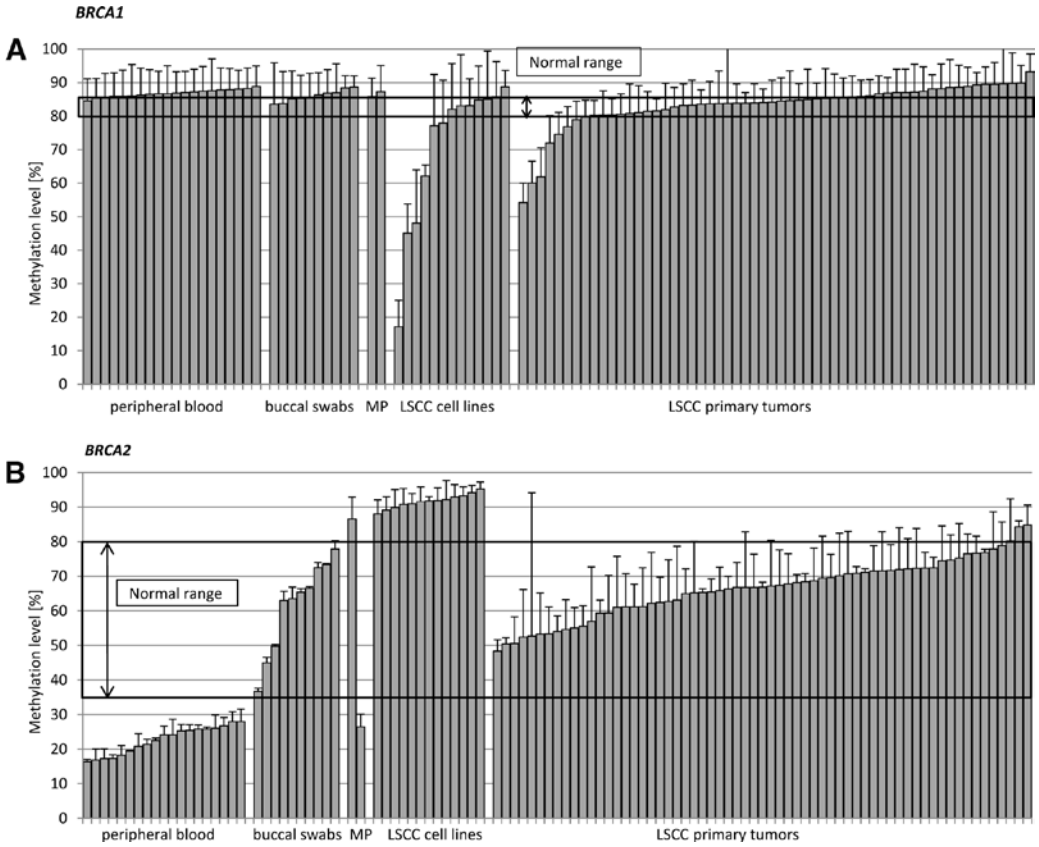

Figure 2. Pyrosequencing analysis results for (A) *BRCA1*, loss of methylation in 46% laryngeal cell lines and 5% primary tumor samples compared to controls. (B) *BRCA2*, hypermethylation of all LSCC cell lines and elevated level of methylation in primary tumor samples compared to control group; X-axis, analyzed samples; Y-axis, methylation level measured (%); M, methylated control; P, pooled blood DNA.

Control group. The epithelial cell samples (buccal swabs) were collected from inner oral cavity surfaces with the use of medical wooden sticks from 10 healthy volunteers (5 men and 5 woman). For every single reaction totally methylated DNA sample (Millipore, Hilden, Germany) and single sample of pooled DNA from peripheral blood as quality control were included. As an extra reference control DNA from peripheral blood of 20 healthy volunteers was analyzed for altered genes (Figs. 1 and 2). Additionally the serial dilutions of totally methylated DNA and whole genome amplificated DNA of pooled peripheral blood DNA (not shown) were applied as sensitivity control (from 0 to 100%, for every 10%) and included into analysis of four genes (*FANCA*, *FANCB*, *BRCA1* and *BRCA2*).

DNA isolation and bisulfite treatment. DNA from cell lines and primary tumors was isolated using standard phenol/chloroform method. DNA isolation from buccal swabs was carried out with High Pure PCR Template Preparation Kit (Roche, Diagnostics GmbH) and eluted with 200 μl sterile water (instead of elution buffer delivered in the kit) and stored in -20˚C until used. The different DNA extraction methods used do not interfere the methylation levels, at least for the majority of the analyzed genes (Figs. 4 and 5). Purified DNA from every sample (1 μg) was converted with bisulfite solution according to EpiTect DNA Modification Kit supplied by Qiagen (Germany).

Primer design. The primers were designed with PyroMark Assay Design Software 1.0 (Biotage, Uppsala, Sweden). All assays were verified for potential SNPs occurrence within primer binding region. Sequencing designs were employed with 5'-biotinylation of the reverse or forward PCR primer, respectively (Table I).

PCR reaction and agarose gel. The conditions of PCR reaction were optimized according to PyroMark PCR kit manual (Qiagen). PCR program was preceded with an initial heat activation step at 95˚C for 15 min (HotStarTaq DNA Polymerase activation). Then followed with 30 sec of denaturation in 94˚C, annealing for 30 sec in variable temperature (each primer pair was primarily tested for optimal temperature, ranging from 55 to 65˚C). Extension step was followed for 30 sec in 72˚C. The PCR reaction consisted of 45 cycles. The reaction was stopped with final extension for 10 min in 72˚C. PCR products were run on 2% agarose gel stained with ethidium bromide and visualized under UV light (Bioanalyzer, Biometra).

Pyrosequencing. Pyrosequencing was performed according to standard protocol. The first step was cleaning up the appropriate PCR products on water pump station in following buffers order: 70% EtOH, 0.2% NaOH and washing buffer (according manufacturer's instructions) (Qiagen). After a final denaturation step at 85˚C for 2 min sequencing primer was hybridized.

Table I. Primer sequences used for pyrosequencing-based methylation analysis of FA genes with gene symbol (NCBI-hg19).

Gene symbol	Primer sequence 5'-3'[a,b]	Primer annealing temperature (˚C)	Amplicon size (bp)[c]	Chromosome locus	Genomic sequence reference (NCBI)
BRCA1/FANCD1	FW: TGGTTTAGGTTAGTTTAGAG[b]	55	164	17q21.31	NC_000017.10
	RV: AAAATTAAAAAATCTATAATTCA				
	SQ: ATCTATAATTCAAAATAC				
BRCA2	FW: TTATTAGGGAGGTTGAGGTAGGAGAATTATTTGATTTTTG[b]	60	220	13q13.1	NC_000013.10
	RV: CAACCCCTTTCCTTACATAAAAAATTATAAAACCCATCTT				
	SQ: AAATTATAAAACCCATC				
BRIP1/FANCJ	FW: GAGTTAGTTGGGTTATTGG[b]	55	137	17q23.2	NC_000017.10
	RV: ACTTCCCCCACCTTATAT				
	SQ: ACCCCTCAACCACAC				
FANCA	FW: TTAGGTTGGTTAAAAGATAGGGT[b]	55	102	16q24.3	NC_000016.9
	RV: TCCCAACCTCAAATAATCC				
	SQ: CCAACCTCAAATAAT				
FANCB	FW: AGAGGTAGATAGAGAAGGGTG	58	134	Xp22.2	NC_000023.10
	RV: AAACCTAAACTAAAAACCTAATC[b]				
	SQ: GGGTGGGAGGGGGAG				
FANCC	FW: AGGAGGAGAGGGGTAAGA	55	103	9q22.32	NC_000009.11
	RV: ACCTTAACCACAACCCTA[b]				
	SQ: GGGTAAGAGGGAAGG				
FANCD2	FW: ATTTGTTTTTGGGTGAGTT[b]	55	124	3p25.3	NC_000003.11
	RV: ATTACTTAACTCCAACATCTACC				
	SQ: TTTTTTGTAGTTTTTTAGTA				
FANCE	FW: GTAGAGGAGAGTTTGTAATAG	60	109	6p21.31	NC_000006.11
	RV: CTAATATAAAACCTCAACTCAAA[b]				
	SQ: AGGAGAGTTTGTAATAGG				
FANCF	FW: GTTAGTTATTATTTGGTGTAGTAAT	55	146	11p14.3	NC_000011.9
	RV: AAATTCTCTCTATAACCATTA[b]				
	SQ: AAACCCAACATATACAC				
FANCG	FW: GGGAATTTAGTAGGATTT	55	166	9p13.3	NC_000009.11
	RV: CCCAAATCCTATCTAACTAAA[b]				
	SQ: TTTTAGAGGTTGTGGTT				
FANCI	FW: TTTAGATTGTGAGTTGGGA	55	197	15q26.1	NC_000015.9
	RV: ATCACCTCCACCTCACCT[b]				
	SQ: TTTAGTTTTTATGGTATA				
FANCL	FW: TTTTAGTTAAGGTTGGGGTTT[b]	55	201	2p16.1	NC_000002.11
	RV: ACCCAAATAAATCCCTCATAC				
	SQ: GACTAAATCCTACACATAC				
FANCM	FW: TAAGGAGTTTTTGTTAGTAGTAG	58	153	14q21.2	NC_000014.8
	RV: CAATTAATAAAATAAATCCACAA[b]				
	SQ: GGAGGTTTAGTTGGAG				
PALB2/FANCN	FW: GTTTTAGGTGGTTTATTGG	55	120	16p12.2	NC_000016.9
	RV: CCCACCAATTAAATCCAC[b]				
	SQ: AGGAGGTTTAGTTTTATTGT				

FW, forward primer; RV, reverse primer (complementary reversed); SQ, sequencing primer (complementary reversed); [a]Optimal primer annealing temperature; [b]Primer marked with biotin; [c]Size of the analyzed amplicon; Chromosomal locus; Annotation gene number (NCBI).

Pyrosequencing was performed using the Pyrosequencer PyroMark ID and the DNA methylation analysis software Pyro Q-CpG 1.0.9 (Biotage), which was also used to evaluate the ratio T:C (mC:C) at the CpG sites analyzed.

Evaluation of pyrosequencing data. All assays were optimized with respect to fully methylated control (in figures marked as M) and a single sample obtained from mix of blood DNA (in figures marked as P), included in each sequencing series.

Methylation levels of particular gene regions were measured in terms of hypo- or hypermethylation status. To date there is no standard approach practiced as best determinant for defining hypo- or hypermethylation. In the current study a non-cancerous (normal range) of DNA methylation level was determined

Table II. *FA/BRCA* genes described by amplicon position in gene and in chromosome, number of CpGs analyzed and their position in the amplicon.

Gene symbol	Amplicon location[a]	Analyzed CpGs no.	Amplicon position (UCSC Genome Browser hg19, Feb. 2009)[b]	CpGs location (bp in amplicon)[c]
BRCA1/FANCD1	Promoter	9	chr17:41278672-41278831	7, 35, 43, 66, 79, 99, 105, 109, 124
BRCA2	Promoter	2	chr13:32888876-32889096	148, 176
BRIP/FANCJ	Promoter	6	chr17:59940795-59940931	21, 31, 39, 46, 54, 59
FANCA	Promoter	6	chr16:89883819-89883920	36, 46, 74, 79, 83, 99
FANCB	Promoter	11	chrX:14891429-14891562	33, 37, 46, 53, 63, 66, 69, 74, 90, 94, 106
FANCC	Intron	10	chr9:98079622-98079724	28, 30, 36, 49, 51, 58, 65, 78, 81, 83
FANCD2	Promoter	5	chr3:10067734-10067857	26, 47, 55, 68, 81
FANCE	5' UTR/exon	8	chr6:35420182-35420290	23, 37, 43, 46, 48, 57, 60, 73
FANCF	CDS/exon 1	10	chr11:22646893-22647038	33, 38, 42, 46, 51, 54, 85, 94, 97, 100
FANCG	5' UTR exon	9	chr9:35079856-35080021	105, 107, 104, 108, 113, 121, 125, 129, 148
FANCI	Promoter	10	chr15:89787084-89787280	67, 71, 82, 86, 89, 100, 108, 123, 130, 141
FANCL	Promoter/5' UTR/CDS	8	chr2:58468358-58468558	99, 106, 116, 112, 118, 129, 140, 170
FANCM	Promoter/5' UTR/CDS	7	chr14:45605348-45605500	42, 46, 64, 66, 69, 73, 83
FANCN/PALB2	Promoter	7	chr16:23652649-23652768	68, 72, 78, 84, 86, 97, 118

[a]Amplicon location in gene sequence (promoter, CDS, 5'UTR, intron or exon); [b]Exact chromosomal position of amplicon (according to UCSC Genome Browser hg19, version 2009); [c]Analyzed CGs location within the amplicon (in bp) counted from 5' side of plus strand.

from control group samples (buccal swabs of 10 healthy donors). The lowest value from the control group has been defined as the lower cut-off and the highest as the upper cut-off for the normal range of methylation (marked in Figs. 1 and 2). Therefore, the studied group samples with higher methylation level compared to the highest of controls were defined as hypermethylated and those with lower than the lowest control were assigned as hypomethylated.

A group of 20 peripheral blood DNA samples served as additional reference controls. Simultaneously methylation values of all samples from each studied group were compared to the control group (buccal swabs) in order to assess statistical significance of differences between measured methylation levels (non-parametric two-tailed t-test with CI=95%).

Pyrosequencing results were obtained as proportional values of each analyzed CpG separately. Different proportions of samples were analyzable by pyrosequencing and depicted on figures. The numerical data were further calculated as average percentage for each gene sequence submitted to pyrosequencing and depicted in bar charts (MS Excel 2007) with standard deviation.

Results

Methylation analysis of promoter sequences in FA-associated genes. The methylation status of selected regions (Table II) in 14 FA/BRCA pathway genes was analyzed in 13 laryngeal squamous cell carcinoma cell lines and 64 primary LSCC samples. Buccal swab samples served as controls. The genes *FANCC*, *FANCD2*, *FANCG*, *FANCI*, *FANCL*, *PALB2* showed no evidence for significantly different methylation level in the cell lines as compared to controls (Fig. 4). Slight changes were identified at the *BRIP1*, *FANCE*, *FANCF* and *FANCM* genes (Fig. 5). However, these differences were not significant between control samples and LSCC cell lines. Moreover, in *BRIP1* a high variation of methylation levels in both cohorts was observed. For that reason we excluded these genes from further analyses.

The genes *FANCA*, *BRCA1* and *BRCA2* demonstrated considerably altered methylation levels in HNSCC cell lines in comparison to buccal swab samples and thus were further investigated in the group of 64 primary LSCC samples. Likewise the gender-specifc *FANCB* was submitted to analysis with primary LSCC samples. Additionally, the methylation levels of these four genes were characterized in 20 DNA samples from peripheral blood, obtained from each 10 male and 10 female healthy volunteers (shown in Figs. 1 and 2) and served as demonstrative reference.

FANCA. The DNA methylation levels at the *FANCA* locus determined in buccal swabs ranged from 69 to 84% (mean 76%). On the basis of these controls hypomethylation of *FANCA* was observed in 11/13 (85%) cell lines (range 17-81%, mean 51%), with 5 cell lines displaying particularly low methylation levels (UT-SCC-34, -22, -116, -57 and -29). In the 64 primary LSCC tumor samples a broad range of DNA methylation levels were observed (range 30-75%, mean 51%). 60 of the 64 primary LSCC (94%) showed hypomethylation at the *FANCA* locus in comparison to controls (Fig. 1A). Overall, both cell lines ($p<0.0003$) and primary LSCC tumors ($p<0.001$) showed significantly lower methylation levels at the *FANCA* locus than the buccal swabs (Fig. 3A).

FANCB. The FANCB gene (located on the X chromosome) showed differences in methylation levels in buccal swabs with respect to gender. In women the range was 27-35% (mean 30%) whereas in men the range was 6-24% (mean 10%). Twelve of 13 LSCC cell lines were male-derived and showed methylation

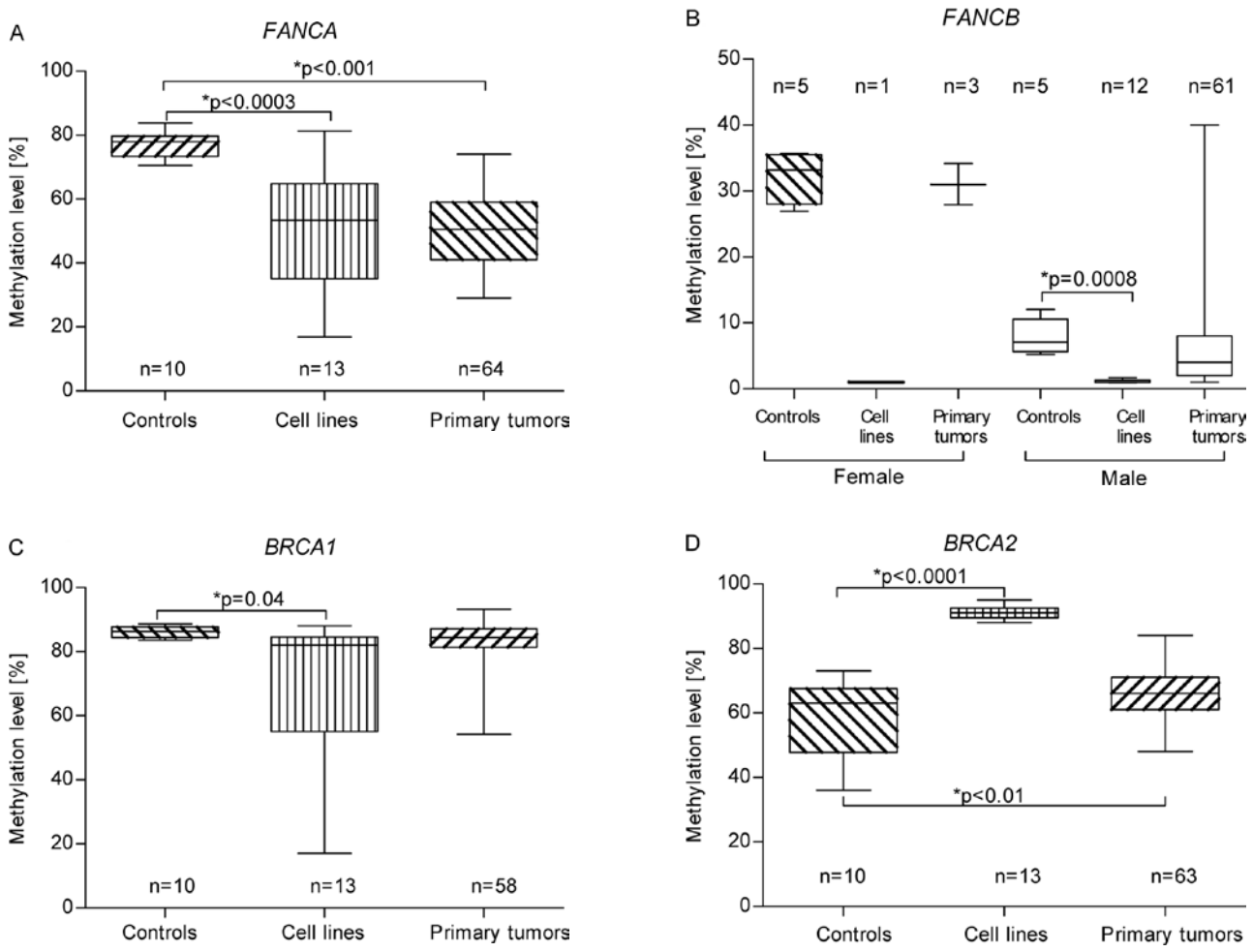

Figure 3. Distribution of methylation values between control group, LSCC cell lines and primary LSCC samples in (A) *FANCA*, (B) *FANCB*, (C) *BRCA1* and (D) *BRCA2*; *Statistically significant (two tailed t-test, CI=95%); N, number of samples in a group; X-axis, samples analyzed; Y-axis, methylation level measured (%). Different proportions of samples shown here were analyzable by pyrosequencing.

ranging from 1 to 2% (mean 1%). Only one cell line was derived from female material and showed a 1% methylation level (Fig. 1B).

The 61 primary LSCC male samples showed methylation levels ranging from 0 to 40% (mean 7%). The 3 female-derived LSCC samples demonstrated levels from 28 to 34% (mean 30%).

In males a significant difference in methylation levels at the *FANCB* locus was seen between controls (buccal swabs) and LSCC cell lines (p=0.0008), but not between controls and primary LSCC (p=0.33). There was also no significant difference between female controls and female primary LSCC tumors (p=0.71) (Figs. 1B and 3B).

BRCA1. All 10 buccal swab samples presented high methylation levels in the region analyzed for *BRCA1* (range 85-89%, mean 86%). The LSCC cell lines showed methylation levels ranging from 17 to 89% (mean 71%). In 6/13 (46%) LSCC cell lines hypomethylation was observed as compared to the control group (buccal swabs). In 58 analyzable LSCC primary tumor samples methylation ranged from 54 to 93% (mean 83%). Only in 3/58 (5.2%) primary laryngeal samples a relevant hypomethylation as compared to buccal swabs was observed.

The comparison of the methylation levels between control group and all 13 LSCC cell lines indicated a statistically lower methylation in the cell lines (p=0.04), but no significant differences were observed between controls (buccal swabs) and primary tumor samples (p=0.22) (Figs. 2A and 3C).

BRCA2. *BRCA2* methylation levels of the buccal swabs controls ranged from 37 to 78% (mean 60%). In all LSCC cell lines the *BRCA2* gene appeared to be hypermethylated (88-95%, mean 92%). Primary LSCC samples methylation levels ranged from 48 to 85% (66%) and thus, were slightly elevated when compared to buccal swabs.

Overall, methylation levels in the control group were significantly lower than in LSCC cell lines (p<0.0001) or in primary LSCC (p<0.01) (Figs. 2B and 3D).

Discussion

Fanconi pathway genes are involved in a specific DNA repair system. The FA activity of genes is induced by DNA damage caused by cross-linking agents (e.g., mitomycin C, cisplatin) or reactive oxygen species (ROS). Not repaired defect in genome integrity leads to DNA damage and causing further cancer-

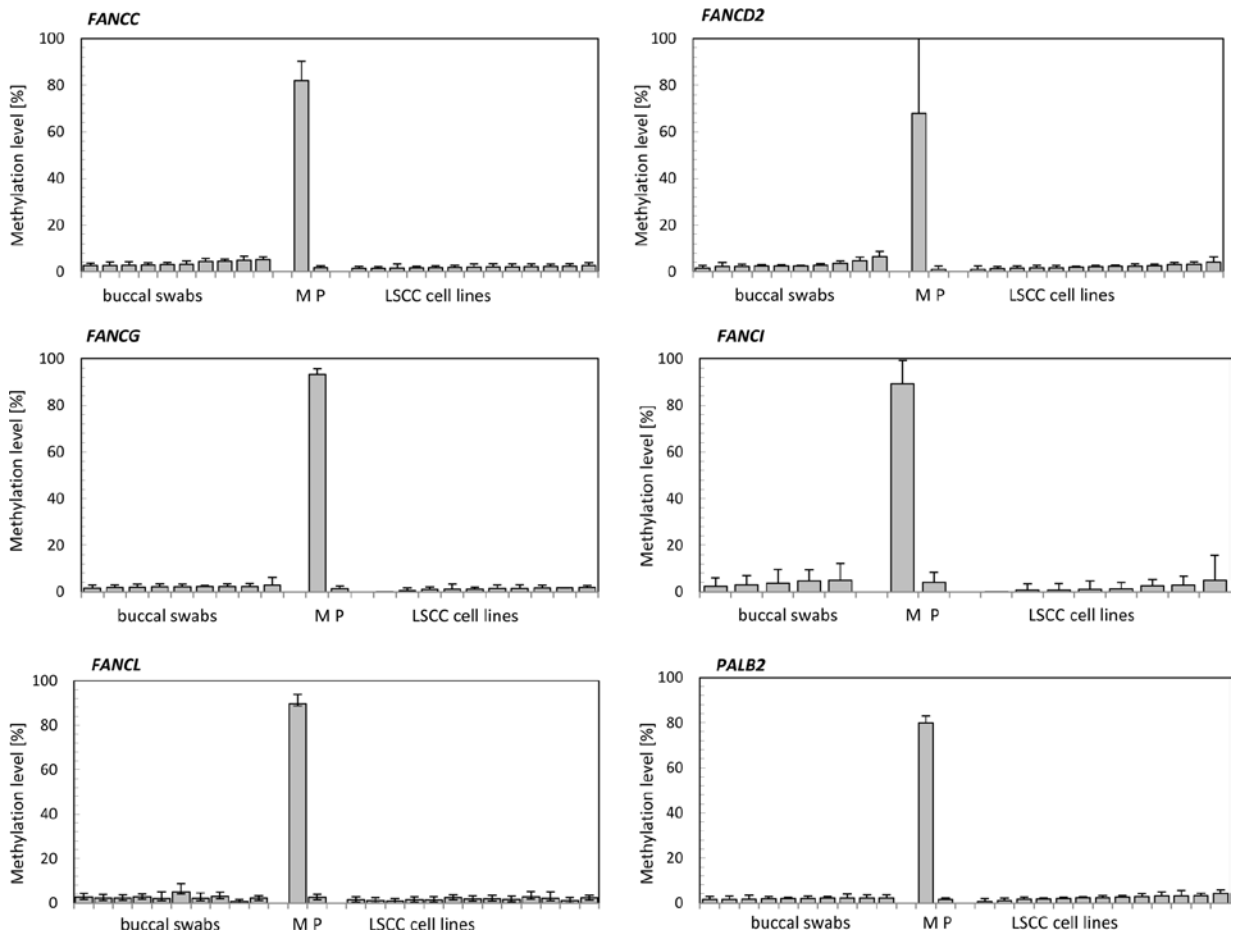

Figure 4. Pyrosequencing analysis results for *FANCC*, *-D2*, *-G*, *-I*, *FANCL* and *FANCN*/*PALB2*. No significant methylation level alterations were demonstrated between studied groups and control group; M, methylated control; P, pooled blood DNA; Different proportions of samples shown here were analyzable by pyrosequencing.

related mutations (37). Genetically inherited, cancer-related syndromes like FA provide an opportunity to investigate cancer biology. Thus, in the last three decades Fanconi anemia has been extensively studied. FA patients are susceptible to acute myeloid leukemia, gynecological and also aerodigestive tract tumors, in particular squamous cell carcinomas with the high risk of developing aggressive forms of head and neck carcinoma (15).

Fanconi anemia pathway genes were previously shown to be deregulated in various cancers including HNSCC (31,32,38). Thus, in the current study 14 Fanconi anemia/*BRCA* pathway genes were subjected to DNA methylation profiling in laryngeal squamous cell carcinoma cell lines. Thereafter, 64 primary LSCC tumors and the reference group of DNA samples obtained from epithelial cells of the oral cavity from 10 healthy donors were analyzed. Twenty peripheral blood samples were added as additional reference control.

Four out of 14 analyzed genes (*FANCA*, *FANCB*, *BRCA1* and *BRCA2*) showed altered methylation patterns in LSCCs samples compared to the control group.

We demonstrate a recurrent reduction of DNA methylation in the region analyzed for the *FANCA* gene (Table I) in both, cell lines and primary tumors (Fig. 2A). *FANCA* has been previously screened in various cancers for genetic and epigenetic alterations. Moreover, in the array-based analysis of Bauer *et al* (39) the region 16q23-q24 harboring the FANCA gene showed a significant gain associated with lower survival rates in HNSCC patients. Hypomethylation described in this study may be an alternative mechanism to gene amplification potentially leading to an increased gene activity.

FANCB located on chromosome X showed a gender specific methylation pattern, with higher level in females. In some samples obtained from male donors elevated methylation level was detected. Nevertheless this was in line with the pattern shown for epithelial cells from healthy control male donors (Fig. 1B). Evidently, the observed phenomenon is not cancer-specific.

Recently Wreesmann *et al* (32) showed recurrent down-regulation of *FANCB* in HNSCC samples (cell lines as well as oral epithelial carcinoma) compared to normal mucosa. Though, no evidence for aberrant methylation was found in this report. Smith *et al* (33) showed *BRIP1* and *FANCB* to be higher and more frequently methylated compared to normal mucosa. The authors suggest that *FANCB* inactivation play important role in HNSCC pathogenesis as the hypermethylation was detected almost exclusively in cancerous samples.

BRCA1 and *BRCA2* (breast/ovarian cancer susceptibility genes) are also involved in *FA*/*BRCA* DNA damage response pathway (9,10). In the current study the control group of *BRCA1* showed high DNA methylation levels in contrast to 46% of

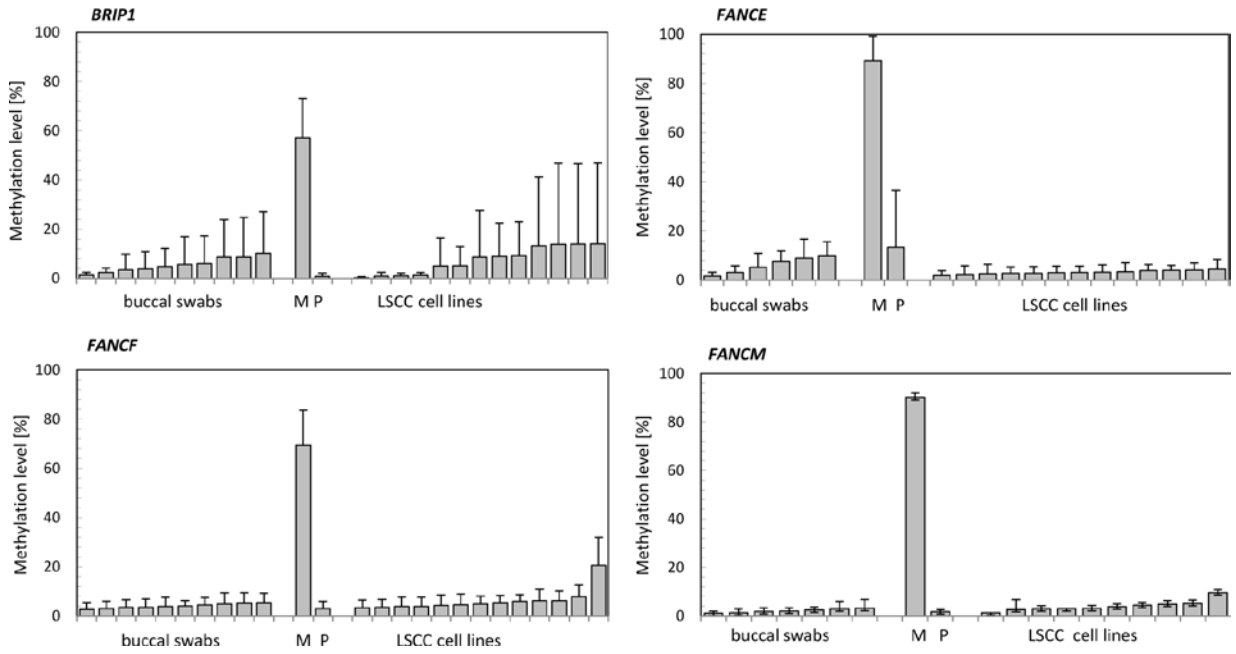

Figure 5. Pyrosequencing analysis results for *FANCE*, *-F -M* and *FANCJ/BRIP1*. No significant methylation level alterations were demonstrated between studied groups and control group; M, methylated control; P, pooled blood DNA; Different proportions of samples shown here were analyzable by pyrosequencing.

Table III. Characteristics of patient group.

Characteristic	No. of cases (%)
Total patients	64 (100)
Age (years)	
Mean	57.6
Range	39-79
Gender	
Female	3 (4.6)
Male	61 (95.4)
Primary tumor site	
Larynx	64 (100)
T classification	
T1	0 (0)
T2	8 (12.5)
T3	37 (57.8)
T4	19 (29.7)
N grading	
N0	43 (67.2)
N1	13 (20.4)
N2	7 (10.9)
N3	1 (1.5)
Metastasis	
M0	64 (100)
Histologic grading	
G1	24 (37.5)
G2	32 (50.0)
G3	3 (4.7)
No data	5 (7.8)

TNM classification (tumor, node, metastasis), cancer progression staging system; G, histological grading of tumor.

LSCC cell lines that appeared to be hypomethylated. This was true also for a few LSCC primary tumors (Fig. 2A). *BRCA1* and *BRCA2* methylation levels have been investigated in a wide subset of cancers. The genes were frequently reported to be hypermethylated in breast tumors (40) as well in ovarian (41) and lung cancers (42).

BRCA2 (*FANCD1*) showed hypermethylation in all 13 cell lines whereas oral epithelium controls demonstrated a pattern with a mean 60% methylation level. The observation was confirmed in the primary LSCC samples where mean value oscillated around 66%. In contrast, peripheral blood samples had a mean methylation level of 25% (Fig. 2B) that is probably a tissue specific pattern, distinct from the oral epithelium. In other studies hypermethylation of *BRCA2* was not detected either in oral cancers or in HNSCC samples (31).

Besides, previous studies on HNSCC tumors pointed also in other Fanconi genes to aberrant DNA methylation patterns. For example *FANCF* hypermethylation has been detected in 15% analyzed cases (31) and was associated with smoking habit as the established risk factor in HNSCC. Gasco *et al* (34) detected hypermethylation in regulatory elements of *FANCC*, *FANCD2*, *FANCF*, *FANCG*, *FANCL* genes in HNSCC that correlated with the gene expression pattern. In addition they showed that individuals with methylation in more than one FA gene have a better response to chemo-therapy. In the current study we found no evidence of aberrant methylation in *FANCC*, *FANCD2*, *FANCF*, *FANCG*, *FANCL*. This might be due to different regions analyzed and the method used. Most of previously mentioned studies were based on MS-PCR which is on one hand relatively specific, but on the other provides only qualitative data with a considerable risk of false positive results caused by mis-priming (43). Pyrosequencing used in our study offers significant advantages, over conventional

methods as quantitative and highly reproducible data are obtained (44).

In conclusion, we report recurrent DNA methylation alterations in *FANCA*, *BRCA1* and *BRCA2*. In addition DNA methylation variations in *FANCB* were shown to be a gender-specific event. These data support the assumption that the Fanconi anemia/BRCA pathway is disrupted in LSCC, contributes to LSCC carcinogenesis and is of clinical importance.

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