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HYPERMETHYLATION OF THE FANCC AND FANCL PROMOTER REGIONS IN SPORADIC ACUTE LEUKAEMIA

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ABSTRACT

Objective
Inactivation of the FA-BRCA pathway results in chromosomal instability. Fanconi anaemia (FA) patients have an inherited defect in this pathway and are strongly predisposed to the development of acute myeloid leukaemia (AML).
Studies in sporadic cancers have shown promoter methylation of the FANCF gene in a significant proportion of various solid tumours. However, only a single leukaemic case with methylation of one of the FA-BRCA genes has been described to date, i.e. methylation of FANCF in cell line CHRF-288.
We investigated the presence of aberrant methylation in 11 FA-BRCA genes in sporadic cases of leukaemia.

Methods
We analyzed promoter methylation in 143 AML bone marrow samples and 97 acute lymphoblastic leukaemia (ALL) samples using methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA).
Samples with aberrant methylation were further analyzed by bisulphite sequencing and tested for mitomycin C sensitivity using Colony Forming Units assays.

Results
MS-MLPA showed promoter methylation of FANCC in one AML and three ALL samples, while FANCL was found methylated in one ALL sample. Bisulphite sequencing of promoter regions confirmed hypermethylation in all cases.
In addition, samples with hypermethylation of either FANCC or FANCL appeared more sensitive towards mitomycin C in Colony Forming Units assays, compared to controls.

Conclusion
Hypermethylation of promoter regions from FA-BRCA genes does occur in sporadic leukaemia, albeit infrequently. Hypermethylation was found to result in hypersensitivity towards DNA cross-linking agents, a hallmark of the FA cellular phenotype, suggesting that these samples displayed chromosomal instability. This instability may have contributed to the occurrence of the leukaemia.
In addition, this is the first report to describe hypermethylation of FANCC and FANCL. This warrants the investigation of multiple FA-BRCA genes in other malignancies.

INTRODUCTION

The FA-BRCA pathway is thought to be essential for specific DNA repair processes[1,2]. Cells, which are defective for one of the FA-BRCA genes, display genomic instability and are hypersensitive to DNA cross-linking agents.
This cellular phenotype is associated with increased cancer risk as observed in patients with Fanconi anaemia (FA) [OMIM#227650]. These patients are characterized by a diversity of clinical symptoms including an increased risk to develop malignancies in particular myelodysplastic syndrome and acute myeloid leukaemia (AML), but also solid tumours.
In the past years a number of papers have reported on the potential role of acquired disturbances in the FA-pathway in sporadic cancers[3,4]. Hypermethylation of the FANCF promoter has been described to occur in various tumour types such as those of the ovaries[5], cervix[6], lungs and oral cavity[7]. Also in AML the occurrence of inherited and somatic abnormalities in the FA-BRCA genes has been studied[8-11]. Only in a small proportion of cases data was provided showing that these abnormalities were indeed associated with functional inactivation of the FA-BRCA pathway.
In an adult AML sample functional abnormality was suggested but in this case the molecular mechanism remained unidentified[10]. FANCF was found hypermethylated in the leukaemic CHRF-288 cell line resulting in hypersensitivity towards DNA cross-linking agents[9]. However, 36 additional AML patient samples appeared to be negative for FANCF hypermethylation suggesting that this is not a common event in leukaemia. This was recently substantiated by Meyers et al., who found no evidence for hypermethylation of either the FANCF or FANCB gene in a total of 33 AML and 48 acute lymphoblastic leukaemia (ALL) samples[12].
Here we report results from a study exploring aberrant methylation of 11 FA-BRCA genes (FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG, FANCJ/BRIP1, FANCL, and FANCM) in sporadic acute leukaemia, using the recently described Methylation Specific (MS)-MLPA[13] technique.

MATERIALS AND METHODS

Sample characteristics
Samples were obtained from the cell banks of the VU medical center, the AML-BFM Study Group and the DCOG. Snap frozen cell pellets of bone marrow samples obtained from newly diagnosed adult AML patients (n=119, median blast cell percentage was 66%, range 4-97%) together with 15 adult ALL (B-cell precursor (BCP)-ALL; n=10, T-cell ALL; n=5, median blast cell percentage of adult ALL samples was 85%, range 10-99%) samples were randomly selected.
Additionally, 20 paediatric AML samples with a complex karyotype, defined as 2 or more random aberrations, or loss of chromosome 5 or 7, or the long arms of these chromosomes, were studied, as well as 4 biphenotypic leukaemic samples.
For the analysis of 82 newly diagnosed paediatric ALL patients (BCP-ALL; n=67, T-cell ALL; n=15, median blast cell percentage of paediatric ALL samples was 94%, range 16-100%) cytospin slides were selected at random.
All specimens were collected with informed consent according to institutional guidelines and in accordance with the Helsinki Declaration of 1975. Control CD34+ cells were derived from healthy volunteers.

DNA isolation and treatment

Genomic DNA from cell pellets was isolated using QIAamp®DNA Blood Minikit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations. DNA from cytospin slides was isolated using phenol, chloroform, and isoamylalcohol (PCI; 25:24:1).

Methylated DNA was obtained by treating control genomic DNA with SssI CpG methyltransferase (M.SssI, New England Biolabs) according to the manufacturer's recommendations.

MS-MLPA

MS-MLPA reagents were kindly provided by MRC-Holland, Amsterdam, the Netherlands, (www.mlpa.com). Target DNA was diluted in TE buffer (10 mM Tris-HCl, pH 8.5, 1 mM EDTA) to a concentration of 100 ng/µl in a total volume of 5 µl and denatured for 10 min at 98°C. MS-MLPA was performed as previously described[13].

Probes were designed such that these were targeted to the CpG islands within the promoter regions of 11 FA-BRCA genes and contained a recognition site for the methylation-sensitive restriction enzyme HhaI.

The probe mix contained single probes directed to FANCA, FANCD2, FANCG, two probes directed to different sites of FANCB, FANCC, FANCD1/BRCA2, FANCE, FANCJ/BRIP1, FANCL, and FANCM, and three probes directed to different sites of FANCF.

In addition to the 11 FA-BRCA genes, probes were included for BRCA1, ATM, MLH1, XPA, WRN, BLM, and NBS1. For quantification of the levels of methylation seven control probes lacking HhaI sites were also included. Probe sequences are shown in table 1.

Sodium bisulphite sequencing

One µg of genomic DNA was converted by sodium bisulphite using the EZ DNA Methylation Gold kit™ (ZYMO Research Corporation, Orange, CA) to confirm the aberrant methylation status.

Bisulphite-treated genomic DNA was amplified by PCR using primers FANCC-F 5'-TTTTATATTTTGAATAGAATATGGAAGAAG-3' and FANCC-R 5'-CAATACATTCTAAAACCTAACTAAC-3' for FANCC, or FANCL-F 5'-TTAGTTTTGTGGATTTGAGGGTAAT-3' and FANCL-R 5'-TAAAACAAAAACAAAAAACACTAAC-3' for FANCL.

PCR products were subcloned, isolated, and analyzed by sequencing.

CFU assay and mitomycin C (MMC) sensitivity

To determine the number of Colony-Forming Units present in the total bone marrow of AML and ALL patients, samples were plated in duplicate in Methocult culture medium (Stemcell Technologies Inc., Vancouver, BC) at a concentration of 40.000, 100.000 and 400.000 cells/well. Colonies were counted after 7 days (37°C, 5% CO_2, full humidity).

MMC sensitivity was assessed by adding various concentrations of MMC (0, 5, 10, and 50 nM) to the cultures. Colonies were expressed as mean values from duplicate cultures and denoted as a percentage of the number of colonies in the wells without MMC.

Table 1. FA-BRCA probe mix

Size (bp)	Gene	Chr pos	Probe 1	Probe 2
130	*FLJ22301	01q44	GGTGAACCTGGCCACAGCTCACC	CTGGAACAGCCACAATGTCTGCCCCTTAGAGAAGAACCCATG
136	FANCF	11p15	GCGAAAGGAAGCGCGGAGACGTTCAT	GACTGGCATCATCTCGCACGTGGTTCCGGAAATTCTCGGTACATG
142	BRCA1	17q21	CCCCTTGGTTTCCGTGGCAACGGA	AAAGCGCGGGAATTACAGATAAATTAAAACTGCGACT
148	BRCA2	13q12.3	CGGGTTAGTGGTGGTGGTAGTGGGTT	GGGACGAGCGCGTCTTCCGCAGTCCCAGTCCAGCGTGGCATG
154	*MLH1	03p22.1	GGAGGGAGGCCTGAAGTTGATTCAGATCCA	AGACAATGGCACCGGGATCAGGGTAAGTAAAACCTCAAAGTAG
160	ATM	11q23	GCGGAGACCGCGTGATACTGGAT	GCGCATGGGCATACCGTGCTCTGCGGCTGCTTGGCCATG
166	MLH1	03p22.1	CGTTGAGCATCTAGACGTTTCCTTGGCTCT	TCTGGCGCCAAAATGTCGTTCGTGGCAGGGGTTATTC
175	*BRCA2	13q12.3	CAGCTGGCTTCAACTCCAATAATATTCAA	AGAGCAAGGGCTGACTCTGCCGCTGTACCAATCTCCTGTAAAAGAAT
184	FANCB	Xp22.31	CCAGACCCAGGCGTCCGCATT	GGATTGGGCGGTGCGGCGGCGCTTCTGTCCGCCTCATG
211	FANCB	Xp22.31	CCGGACCCGGAAGCCGCCTCT	GAGGCCCGGGGACCGAGCGCCCGTGTGCAGGCATG
220	FANCE	06p21.3	CGCGTCCGGTGTCGCCATGCC	GGGGCACGGGTGCCGCGCCGCCCCCTACTCTGGT
229	*RELA	11q13	GCTTGTAGGAAAGGACTGCCG	GGATGGCTTCTATGAGGCTGAGCTCTGCCCG
238	FANCF	11p15	CTGCTCTCTCTGCGCCTGCTGGA	GAACCGGGCCCTCGGGGATGCAGCTCGTTACCACCTGGCATG
247	FANCF	11p15	GTCGCCGTCTCCAAGGTGAAAGCGGAA	GTAGGGCCTTCGCGCACCTCATGGAATCCCTTCTGCAGCACCTCATG
256	FANCM	14q21.3	CTGCCGGAGACTCATGCGCATTAT	TAAGGCGTTACTCTAAATATGGTCCCCGGTGCGGTTCCGGCATG
265	XPA	09q22.3	GGCAGGCCACCCCGAGCCCCT	TAACTGCGCAGGCGCTCTCACTCAGAAAGGCCGCTGGGTGCATG
274	FANCA	16q24.3	CAGGGAGCCGCCGCCGGGGCT	GTAGGCGCCAAGGCCATGTCCGACTCGTGGG
283	*FANCD2	03p25.3	GGTGTGGCCAAGTGGGGATAAAGA	GAAGAGCAACATCTCTAATGACCAGCTCCATGCTCTGCTCCATG
292	FANCC	09q22.3	CGCCGGGGCTTCCGCGCGAAA	CCGGGAACACTGGAGCAGTACCGACTCCGCTCGCCTAGAA
301	BRCA2	13q12.3	CGGGAGAAGCGTGAGGGGACAGATTTGTG	ACCGGCGCGGTTTTTGTCAGCTTACTCCGGCCAAAAAAGA
310	BRIP1	17q23.2	CTAAGAACAGGCCTGCGCTCAAAGGA	GGTAAGGATAGGCTCCCTCCTCAGGTTTTCTGCCCCATG

Size (bp)	Gene	Chr pos	Probe 1	Probe 2
319	WRN	08p12	CTTGTACTCGGCAGCGCGGGAAT	AAAGTTTGCTGATTTGGTGTCTAGCCTGGATGCCTGGGCATG
328	FANCC	09q22.3	TGACACGTGTGCGCGCGCGCGGCT	CCACTGCCGGGCGACCGCGGGAAAATTCCAAAAAAACTCA
337	*ATM	11q23	CAGAGTCAGAGCACTTTTTCCGATGC	TGTTTGGATAAAAAATCACAAAGAACAATGCTTGCTGTTGTGGACTACATG
346	BLM	15q26.1	GGCGGGGTCGCCGTACAGCGC	CGGGAGGGACGCGTATCTCCAAAGCCCAATCAGAGTC
355	FANCL	02p14	GCGTCCGAGCGCCAGCGGACT	GCGCATGTGCAGGACCCAGCAGGTCTAGAGCTTTTCTGTGCATG
364	FANCM	14q21.3	CTGCTGGCAAAGGCGCCTTGG	AGCTGCCAGGGCTCTGAGGTCGCTCAGTTCCGGAGCTCATG
373	FANCD2	03p25.3	CGGTGATGGGCGAGCTTCTCTTCA	CCGGGGCGCAGTTGCTTCTCTCTGACGTCGCCTC
382	NBS1	08q21	CAACCACCAGGTGGCGGGCAA	GCGCCCAAGTCGCACTCCCGCCTCATCCAAGGCAGCCCATG
391	*NF1	17q11.2	CTCCCCTACCACTGGCCACTGTAACA	GTGGACGAACTCGCCACGGATCCGCAAGCCAAGTGCAGAAGCATG
400	FANCL	02p14	CCATGGCGGTGACGGAAGCGAGCCTGT	TGCGCCAGTGCCCCCTGCTTCTGCCCCAGAACCGGTCGCATG
409	FANCE	06p21.3	CACCGCCGCGTCAGGGACGGC	GCTGGAGTCCTCCGTTCCCCTCAGCCTCTGAGCT
418	WRN	08p12	GCAGCGAGGGCCTCCACGCAT	GCGCACCGCGGCGCGCTGGGCGGGGCTGGATCATG
427	XPA	09q22.3	CCCGGATGACAAGAGAGCAGGTAGTT	AGGCGGGTACTCCGTGTCCGCGCATACCCAGACTCCGCCATG
436	BRIP1	17q23.2	CTCGACTCCCAGCGCCCAATA	GCCCAGCGAGCTCGACCAATCACCCGCCAAGGCCCATG
445	*TNFRSF7	12p13	GAAAGTCCTGTGGAGCCTGCA	GAGCCTTGTCGTTACAGCTGCCCCAGGGAGG
454	FANCG	09p13	GCGGTAACTGGAGACGAGGGCGC	TCCGTAATCGTCCATCCTTTCTCTGCTCACTGTCGAGC
463	MLH1	03p22.1	CTGCTGAGGTGATCTGGCGCAGA	GCGGAGGAGGTGCTTGGCGCTTCTCAGGCTCCTCCTCTCATG
472	NBS1	08q21	CATGCCGACCGCAGGCGCCGT	ATCCGCGCTCGTCTAGCAGCCCCGGTTACGCGGTTGCCATG
481	*CASR	03q21	CCAGTGCCTGTAACAAGTGCCCAGATGACT	TCTGGTCCAATGAGAACCACACCTCCTGCATTGCCAAGGACATG

Indicated are the size of the expected PCR product in base pairs (bp), corresponding genes, chromosomal location, and probe sequences. In the probe sequences HhaI recognition sites are underlined. Control probes for genes lacking HhaI sites are indicated by asterisks.

RESULTS

MS-MLPA

Using MS-MLPA with probes directed against 11 FA-BRCA genes we first analyzed 119 unselected adult AML bone marrow samples and 20 paediatric AML samples selected on the basis of an FA-AML like karyotype (complex rearrangements see materials and methods). Aberrant promoter methylation of the FANCC gene was detected in a single adult patient with biphenotypic AML (figure 1). Methylation was also present in the relapse sample from this patient. To test whether FA-BRCA gene methylation was associated with the specific leukaemic phenotype, four additional biphenotypic AML samples, 15 adult ALL samples, and 82 paediatric ALL samples were analyzed. One adult ALL sample showed FANCL promoter methylation, while FANCC promoter methylation was found in three paediatric ALL samples. All four patients had been diagnosed as having BCP-ALL with a hyperdiploid phenotype. In the biphenotypic subgroup we did not detect additional methylated cases.

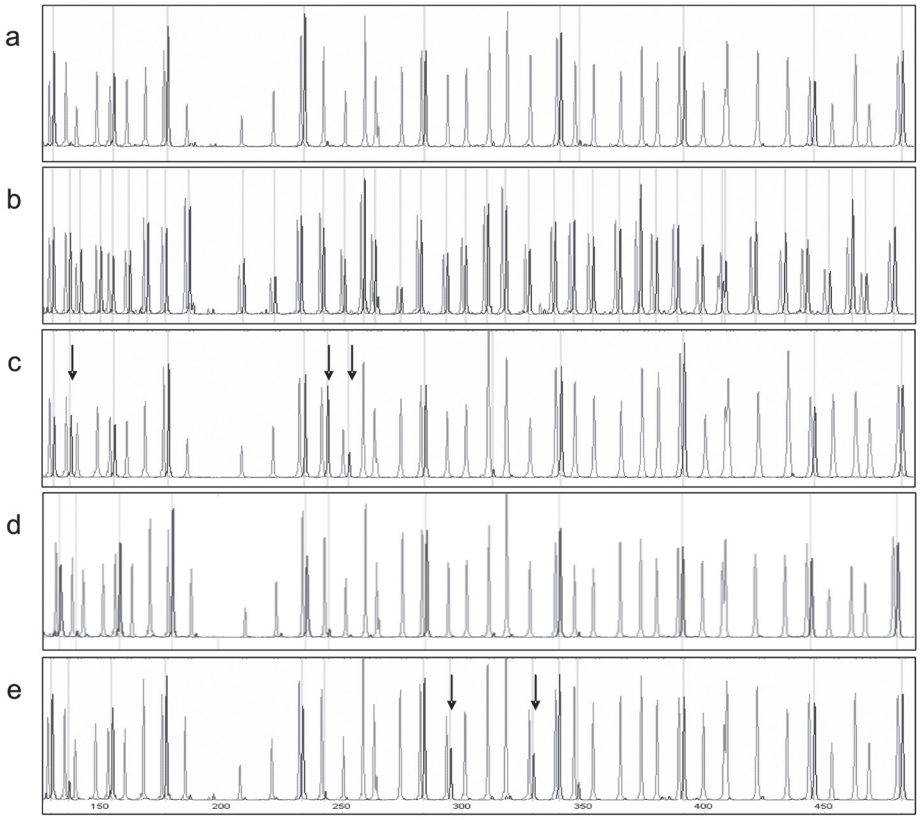

Figure 1. Detection of aberrant methylation by MS-MLPA. MS-MLPA products were analyzed by capillary electrophoresis (CE). Profiles in black correspond with the undigested samples and gray with digested samples. MS-MLPA profiles are from (a) control genomic DNA without aberrant methylation, (b) SssI CpG methyltransferase treated control genomic DNA showing methylation of all target sequences, (c) genomic DNA derived from the CHRF-288 cell line which shows FANCF methylation (gray peaks indicated by arrows), (d) genomic DNA from an AML sample without methylation, and (e) genomic DNA from an AML sample showing FANCC methylation (gray peaks indicated by arrows).

Sodium bisulphite sequencing

Sodium bisulphite sequencing confirmed hypermethylation in all cases (figure 2). Analysis of the FANCC promoter region from the adult biphenotypic AML sample (patient 1) at diagnosis and the corresponding relapse, showed equally dense methylation patterns. Similarly, FANCC hypermethylation was observed in the two paediatric ALL samples (patients 3 and 4). A third sample showed only partial methylation of the FANCC promoter region (patient 2). The methylation status of FANCL was determined in the adult ALL sample (patient 5) and showed dense methylation (figure 2b). No methylation of these genes was found in genomic DNA from control $CD34^{+}$ cells.

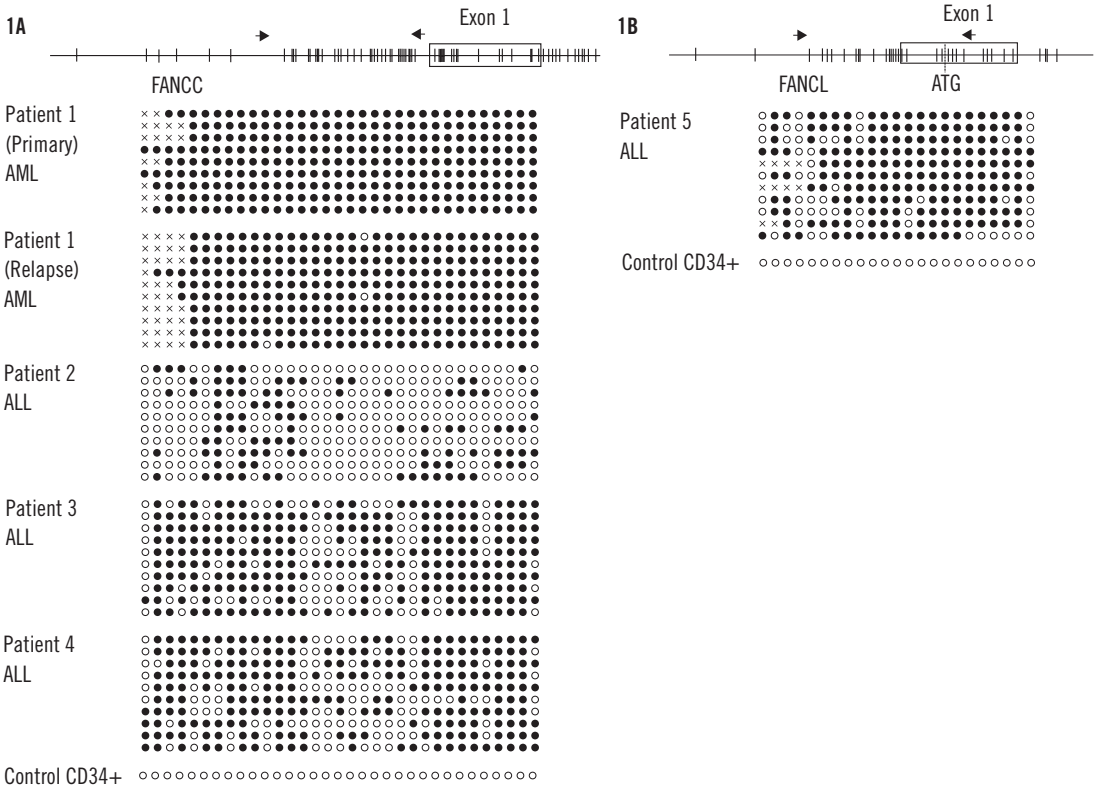

Figure 2. Bisulphite sequence analysis of the FANCC and FANCL promoter regions in primary leukaemic samples. (**A**) Upper part: schematic representation of the FANCC gene's promoter region. The 33 assessed CpGs are situated upstream of exon 1, between the arrows; the translation start is in exon 2 (not shown). In the lower parts of the figure each row represents an individually cloned and sequenced allele following sodium bisulfite DNA modification, black and open circles representing methylated and unmethylated CpGs, respectively. Sequence data for a number of CpGs were ambiguous, indicated by an x. Arrows indicate sequences complementary to the primers used. (Patient 1, adult diagnosed with biphenotypic AML, patients 2, 3 and 4, paediatric patients diagnosed with ALL). (**B**) Methylation status of the 23 assessed CpGs in the promoter region of the FANCL gene, represented as described above. The translation start of the FANCL gene is located in the first exon. (Patient 5, adult diagnosed with ALL)

Mitomycin C sensitivity

A CFU assay was performed to evaluate whether hypermethylation was associated with increased sensitivity towards mitomycin C (MMC), a hallmark of cells defective in the FA-BRCA pathway. From two paediatric ALL samples with FANCC hypermethylation no colonies were obtained in the CFU assay. The other four methylated samples were on average 6.9-fold more sensitive to MMC (median IC_{50} 4.6 nM) than controls (median IC_{50} 32.1 nM), see figure 3, an extent of hypersensitivity commonly observed in cells carrying biallelic FA gene defects.

This suggests that the observed hypermethylation in these samples indeed is associated with an FA-like cellular phenotype of DNA crosslinker sensitivity.

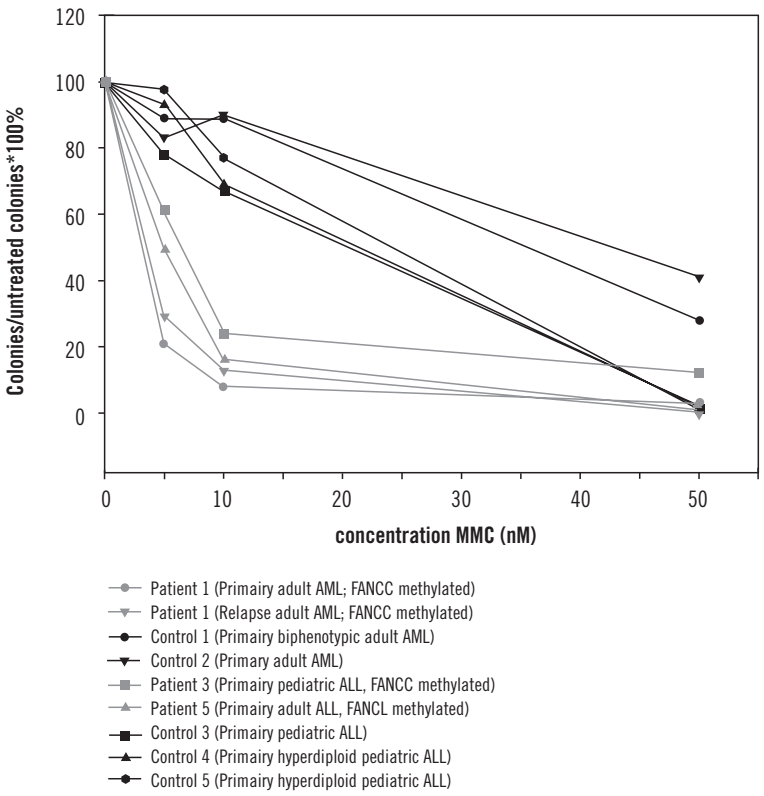

Figure 3. Mitomycin C (MMC) sensitivity in Colony Forming Units (CFU) assay. Number of colonies scored following a 7 day culture in Methocult culture medium with increasing concentrations (5, 10 and 50 nM) of MMC, expressed as percentage of the number of scored colonies in the untreated (0 nM MMC) fraction. Gray symbols, patients with FA-BRCA gene promoter methylation (patient 1, bifenotypic AML, FANCC methylated; patient 3, paediatric ALL, FANCC methylated; patient 5, adult ALL, FANCL methylated). Black symbols, controls (control 1, biphenotypic adult AML, control 2, primary adult AML, control 3, primary paediatric ALL, controls 4 and 5, primary hyperdiploid adult ALL).

DISCUSSION

This is the first report showing epigenetic alterations of the FANCC and FANCL promoter regions in malignant cells. Furthermore, we show here for the first time evidence for FA-BRCA gene hypermethylation in primary sporadic leukaemia samples.

We had anticipated to enrich for AML samples carrying a defect in the FA-BRCA pathway by selecting paediatric AML samples with cytogenetic abnormalities that are frequently found in FA patients[14,15]. However, no aberrant FA-BRCA gene methylation was detected in these samples using MS-MLPA for the detection of methylation in 11 FA-BRCA genes.

In a larger series of sporadic leukaemia samples, a relative higher incidence of FA-BRCA gene methylation was observed in ALL samples (4/97) compared to AML samples (1/143). This is somewhat surprising, since ALL is rarely observed in FA-patients[14] except for the FA subtype associated with a defect in the FANCD1/BRCA2 gene[16].

If methylation of the FA-BRCA genes is causally involved in the occurrence of sporadic leukaemia our data suggest that this accounts for a small proportion of these cases.

On the other hand the presented data may be an underestimate due to a number of reasons. First, MS-MLPA detects the presence of methylation only for a limited number of CpGs. Samples which are methylated but in which these specific CpGs are not will be missed. Second, silencing of the FA-BRCA pathway is predicted to be only necessary temporarily for the accumulation of tumourigenic alterations. Loss of FANCF methylation in vitro has been shown to occur[17]. Third, we have analyzed 11 of the 13 genes known to cause Fanconi anaemia. Not included were the recently identified FANCN/PALB2[18,19] and FANCI[20-22] genes.

In conclusion, FA-BRCA gene hypermethylation is observed in a small portion of primary sporadic acute leukaemia samples. These samples appeared hypersensitive to DNA cross-linking agents.

The data suggests that methylation of these genes resulted in chromosomal instability which may have contributed to the accumulation of oncogenic alterations eventually leading to leukaemia.

Since the role of crosslinking agents in the treatment of leukaemia's is limited in general, determining FA-BRCA gene hypermethylation may have clinical consequences, as such leukaemias are predicted to be particularly sensitive to regimens containing the crosslinking agents cyclophosphamide, cisplatin, or busulfan.

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