Letter to the Editor

No evidence for FANCF gene silencing in head-and-neck squamous cell carcinomas

To the Editor,

Fanconi anemia (FA) is a recessively inherited genetic disorder with a strong predisposition to malignancies, in particular acute myeloid leukemia (AML) and squamous cell carcinoma (SCC) [1,2]. Thirteen FA genes are known today (FANCA, -B, -C, D1/BRCA2, -D2, -E, -F, -G, -I, -J/BRIP1, -L, -M and -N/PALB2) [3–6], whose products act in the ‘FA/BRCA pathway’ to protect the genome against spontaneous as well as cross-linker-induced chromosomal breakage and rearrangements. Genomic instability is thought to be the underlying cause of cancer predisposition in FA patients as well as in other chromosomal instability and premature-ageing diseases [7]. It has been hypothesized that FA gene defects might also play a role in sporadic cancer [5,8]. In sporadic malignancies, loss of DNA stability and tumor suppressor gene function is often based on (a combination of) DNA sequence alterations, deletions, and promoter methylation. For FANCF, methylation of the promoter sequence has been reported for a proportion of several types of cancer, including leukemia [9,10] and cancer of the breast [11,12], ovary [13–16], bladder [17], cervix [18], testis [19], lung [20], and head-and-neck [20]. All of these studies were based on a methylation-specific PCR assay (‘MSP’), described in detail by Taniguchi et al. [15] and discussed in more general terms by Derks et al. [21].

Using the recently developed method of ‘methylation-specific multiplex ligase-mediated probe amplification’ or ‘MS-MLPA’ [22] we recently carried out a survey of FANCF promoter methylation in a panel of 25 cell lines derived from squamous cell carcinomas of various origins including the head-and-neck. We were unable to ascertain any convincing example of FANCF silencing, except in CHRF-288 leukemia cells, which were used as positive controls [10].

Because cell lines, due to culturing-artifacts, may not in every respect represent the initial tumor, we analyzed archival formalin-fixed paraffin-embedded HNSCC tumor material to retrospectively examine the possible relevance of FANCF silencing for tumor response to treatment regimens containing cisplatin. Twenty-two tumors from patients treated with cisplatin-containing chemoradiation were analyzed using MSP. Eleven tumors had shown sensitivity towards this treatment regimen; this subgroup was expected to be enriched for cases with FANCF methylation. However, no FANCF promoter methylation was observed in any of the 22 tumors, while formalin-fixed paraffin-embedded CHRF-288 cells, which served as a positive control, clearly showed methylation (Fig. 1). Our results represent a marked difference from those previously reported (0 positives out of 22 tested versus the reported 13 positives out of 89 tested; \(p < 0.07\), 2-tailed Fisher’s exact test).

We next analyzed FANCF promoter methylation using an MS-MLPA test (MRC-Holland, Amsterdam, The Netherlands). This test has the advantage over MSP that methylation is determined at multiple promoter sites simultaneously, while at the same time deletions can be observed. However, this screen did not reveal any case of FANCF promoter methylation or homozygous deletion. Thus, so far our results have failed to provide evidence for FANCF inactivation underlying a favorable response to cisplatin-containing treatment regimens of head-and-neck SCCs.

The discrepancy between our results and those published earlier may be explained, at least in part, by experimental details that could critically influence the results to be obtained with MSP (see [21] for a review of the MSP method).

First, Marsit et al. [20], who followed the PCR conditions as described by Taniguchi et al. [15], used 44 PCR cycles to amplify the (bisulphite-modified) promoter sequences, whereas we used 34 cycles, which is in compliance with Derks et al. [21], who recommend not to exceed 35 cycles. The use of too many cycles may carry a risk of producing false-positive amplification products.

Second, since the yields of microdissected material may be low and highly variable, we used carrier
Letter to the Editor

Fig. 1. Bisulphite concentration-dependent variation in results obtained by MSP. MSP carried out on DNA isolated from FANCF-methylated CHRF-288 cells, and from unmethylated HSC93 wild type control cells (both formalin-fixed and paraffin embedded), after treatment with various concentrations of sodium bisulphite. An amplification product was generated with the M primers using methylated DNA even when the bisulphite concentration was down to 10% of the prescribed concentration (EZ DNA methylation kit, Zymo research). Using unmethylated DNA as a template, the PCR does not generate an amplification product when reducing the bisulphite concentration to 50%. Unmethylated DNA treated with 10% and 25% of the prescribed concentrations of sodium bisulphite, however, appeared to produce a PCR product with the M primers that will be interpreted as methylation-positive, but in fact is false-positive and due to suboptimal bisulphite treatment of the DNA.

DNA to standardize the amount of template for bisulphite treatment and to facilitate precipitation, as recommended [21]. Whether carrier DNA has been used in the reported studies has not been specified.

Third, bisulphite is known to be chemically unstable and may thus be a variable component of the reaction mixture if not used freshly-made. The MSP assay essentially relies on the conversion of unmethylated cytosine residues into uracil by sodium bisulphite treatment and the use of primers that specifically amplify either unmethylated (U) or methylated (M) DNA. However, DNA isolated from formalin-fixed paraffin-embedded specimens is often of relatively poor quality, which might interfere with efficient bisulphite modification of unmethylated cytosines. We hypothesized that incomplete conversion of cytosine bases could cause false-positive results by PCR amplification, as most amplification protocols allow few mismatches in the annealing primers. We mimicked suboptimal conditions by applying a range of bisulphite concentrations and determined the amplification of methylated and unmethylated promoter region of FANCF using primers identical to those used in published studies [15,20]. A concentration-dependent result of the PCR reaction was observed for the amplification of methylated and unmethylated DNA with M and U primers, respectively (Fig. 1). Remarkably, a PCR product was generated with unmethylated DNA using M primers at bisulphite concentrations that were 10% and 25% of the prescribed concentration. Moreover, when 10% of bisulphite was used for conversion, only the M- and no U-PCR product was generated with unmethylated control DNA, in which case the sample was to be scored as fully methylated for FANCF.

Our results revealed that the method as often applied in the assessment of FANCF promoter methylation, is liable to produce false-positive results when suboptimal conditions are used. Suboptimal conditions might exist particularly when using archival formalin-fixed paraffin-embedded material. We suggest that the discrepancy between our results and those reported by Marsit et al. [20] may be partly explained by this effect and that the occurrence of FANCF silencing in head-and-neck cancer may in fact be less frequent than suggested, if existing at all.

In conclusion, the molecular basis underlying a favorable response of HNSCC patients to cisplatin treatment is not related to silencing of FANCF. In addition, the reported MSP-results that have led to conclusions regarding FANCF promoter methylation in tumor samples of various origins need to be considered with great caution in view of the possibility for the MSP assay to produce false-positive results.
Abstract

Hypermethylation of the Fanconi anemia gene, \textit{FANCF}, has been reported to occur in an appreciable proportion of malignancies, including 15\% of head-and-neck squamous cell carcinomas. This feature is claimed to be a useful tumor marker predicting a favorable response to cisplatin-based treatment protocols, since cells with a defective FA/BRCA pathway are hypersensitive to cross-linking agents, such as cisplatin. We examined the promoter methylation status of \textit{FANCF} in paraffin-embedded oral tumor material from a cohort of 22 patients, of whom 11 (50\%) had responded favorably to a cisplatin-containing treatment regimen. No evidence for silencing of \textit{FANCF} was obtained in any of these tumors. This result is in clear contradiction with published data. We provide evidence that the methylation-specific PCR method as used routinely to probe for \textit{FANCF} methylation is flawed and liable to produce false-positive results. We suggest that the discrepancy between our results and those previously reported may be partly explained by this effect and that the occurrence of \textit{FANCF} silencing in head-and-neck cancer may in fact be less frequent than suggested, if existing at all.

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