Am. J. Hum. Genet. 67:759–762, 2000

Report

Complementation Analysis in Fanconi Anemia: Assignment of the Reference FA-H Patient to Group A

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Fanconi anemia (FA) is an autosomal recessive disorder with diverse clinical symptoms and extensive genetic heterogeneity. Of eight FA genes that have been implicated on the basis of complementation studies, four have been identified and two have been mapped to different loci; the status of the genes supposed to be defective in groups B and H is uncertain. Here we present evidence indicating that the patient who has been the sole representative of the eighth complementation group (FA-H) in fact belongs to group FA-A. Previous exclusion from group A was apparently based on phenotypic reversion to wild-type rather than on genuine complementation in fusion hybrids. To avoid the pitfall of reversion, future assignment of patients with FA to new complementation groups should conform with more-stringent criteria. A new group should be based on at least two patients with FA whose cell lines are excluded from all known groups and that fail to complement each other in fusion hybrids, or, if only one such cell line were available, on a new complementing gene that carries pathogenic mutations in this cell line. On the basis of these criteria, the current number of complementation groups in FA is seven.

Fanconi anemia (FA) is an autosomal recessive chromosomal breakage disorder with diverse clinical symptoms including progressive bone marrow failure and increased cancer risk (Auerbach et al. 1998 [MIM 227650]). Cells from patients with FA are hypersensitive to cross-linking agents, such as diepoxybutane and mitomycin C (MMC); this hypersensitivity has been exploited to assess genetic heterogeneity through complementation analysis. Eight complementation groups have been reported (Joenje et al. 1997), each of which is thought to be related to a distinct FA gene. Four FA genes—*FANCA* (Fanconi Anaemia/Breast Cancer Consortium 1996; Lo Ten Foe et al. 1996), *FANCC* (Strathdee et al. 1992), *FANCF* (de Winter et al. 2000), and *FANCG/XRCC9* (de Winter et al. 1998)—have been identified thus far, whereas genetic map locations have been determined for *FANCD* (Whitney et al. 1995) and *FANCE* (Waisfisz et al. 1999*c*). Attempts to clone or map the genes for the remaining groups—B and H—have thus far failed.

Complementation group H is unique because it is represented by a single cell line, EUFA173. This cell line was defined as "H," because of its capacity to complement the cross-linker hypersensitivity of all other groups in fusion hybrids; in addition, EUFA173 cells failed to be complemented by transfection with expression plasmids containing cDNAs for FANCA or FANCC (Joenje et al. 1997). We were recently led to re-examine this assignment, because two of us (A.D. and I.G.-H.) found evidence for correction of its MMC sensitivity after transduction with a retroviral vector containing the FANCA cDNA, suggesting that EUFA173 might in fact be FA-A (fig. 1). We subsequently screened the *FANCA* gene for mutations and found this cell line to be a compound heterozygote for two novel mutations: a missense mutation in exon 29 (2852G→A; Arg951Gln) and a mutation that removes exons 17–31 from the open reading frame (E17–31del) (Fanconi Anemia Mutation Database; Gen-

Received December 23, 1999; accepted for publication June 29, 2000; electronically published August 8, 2000.

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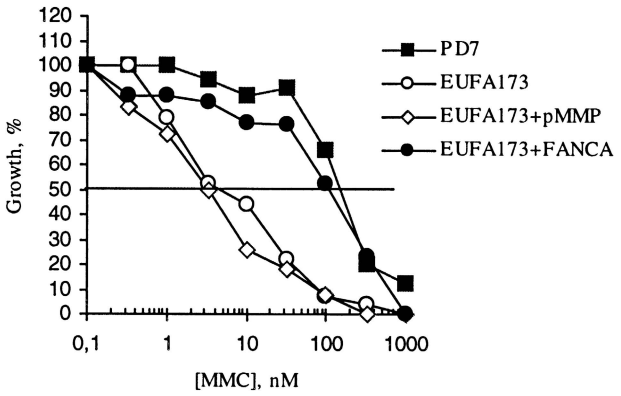

Figure 1 Correction of MMC hypersensitivity of EUFA173 cells after retroviral transduction of FANCA cDNA. EUFA173 lymphoblasts were infected with retroviral supernatants carrying either the FANCA cDNA or the empty vector pMMP, essentially according to the study by Pulsipher et al. (1998). After puromycin selection, cells were tested for growth inhibition by MMC. A normal lymphoblastoid cell line (PD7) and the uninfected EUFA173 cells were also included in the assay. Results shown are representative of multiple independent MMC survival assays performed with cells from three separate retroviral infections.

Bank). The latter mutation may be assumed, on the basis of its severity, to be pathogenic. The missense mutation changes an amino acid residue that is conserved in the mouse (Van de Vrugt et al. 2000), whereas this alteration was not detected in 96 control chromosomes. In addition, sequencing of the entire *FANCA* open reading frame did not reveal any further alterations. Moreover, western blotting experiments had previously shown the presence of a full-length FANCA protein in extracts from EUFA173 cells (Waisfisz et al. 1999*a*), which essentially excludes splice-site mutations as well as promoter-inactivating mutations. Therefore, the Arg951Gln mutation is most probably pathogenic. Altogether, these results indicate that EUFA173 should indeed be reassigned to complementation group A. Consequently, the current number of FA complementation groups should be corrected from eight to seven. Reports describing certain cellular features as specific for FA group H should be reinterpreted accordingly (Yamashita et al. 1998; Carreau et al. 1999; Garcia-Higuera et al. 1999; Waisfisz et al. 1999*a*).

To explain the discrepancy between the results obtained with retroviral transduction and our earlier results, we scrutinized the original cell-fusion and transfection data that indicated EUFA173 cells to be classified as non-A. Five independent fusions between EUFA173 and the reference group A cell line HSC72OT had been evaluated for MMC-induced growth inhibition (table 1). Only one of the hybrids was not complemented (indicating identity with the FA-A fusion partner), whereas four hybrids were complemented (indicating nonidentity with group A). On the basis of this result, in combination with the apparent lack of complementation by FANCA cDNA (discussed below), we concluded that EUFA173 was non-A. In retrospect, the single sensitive hybrid was apparently more meaningful than the four resistant hybrids. Given the new transduction results, resistance in those hybrids presumably had resulted from phenotypic reversion rather than from genuine complementation. Reversion in the four resistant hybrids might have occurred through the generation of a wild-type *FANCA* allele resulting from intragenic recombination or gene conversion (see Lo Ten Foe et al. 1997) or through a sequence alteration, in *cis*, affecting the missense mutation in EUFA173 (see Waisfisz et al. 1999*b*). The reference group A cell line HSC72OT is homozygous for a deletion of exons 18–28 (results not shown), which does not overlap with the Arg951Gln missense mutation in EUFA173, making homologous recombination between these two mutations theoretically possible (fig. 2). We tested this hypothesis by amplifying a fragment from cDNA isolated from the reverted hybrid cells, using one primer specific for a region in the overlapping deletions and the other downstream of the missense mutation (fig. 2). In the case of mitotic recombination or gene conversion, the missense mutation was predicted to be absent from this fragment. However, sequencing showed the mutation still to be present, implying that mitotic recombination could not explain the reversion. In addition, no other sequence alterations were noted in the amplified fragment, indicating that there were no nearby in *cis* alterations that could functionally compensate for the missense mutation (Waisfisz et al. 1999*b*). To check whether the MMC-resistant phenotype was caused by a defect in the bioactivation of MMC, we used cisplatin, a cross-linking agent that does not depend on cellular metabolism, to assess growth inhibition of the resistant hybrid cells. The results (not shown) showed a complete cross-resistance to cisplatin—that is, equal to the sensitivity of wild-type cells—indicating that the re-

Table 1

MMC Sensitivity of EUFA173 × HSC72OT Fusion Hybrids

Fusion No.	IC_{50}[a] (nM)	Result
1	2.5	A
2	20	non-A
3	35	non-A
4	38	non-A
5	45	non-A

NOTE.—HSC72OT cells are the reference group A cells HSC72, marked with ouabain resistance ("O") and thioguanine resistance ("T"; this marker provides sensitivity to medium containing hypoxanthine, aminopterine, and thymidine [HAT], because of a mutation in *HPRT*) (Duckworth-Rysiecki et al. 1985).

[a] IC_{50} values >10 nM indicate a complemented phenotype (Joenje et al. 1995).

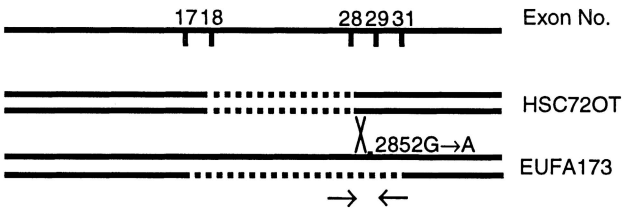

Figure 2 Four *FANCA* alleles in the fusion hybrid from EUFA173 and HSC72OT cells, with the mutations indicated (2852G→A [Arg951Gln] and E17–31del in EUFA173; and E18–28del [homozygous] in HSC72OT [dotted regions are deletions; drawing is not to scale]). Either mitotic recombination at the "X" or a gene-conversion event would predict the generation of a wild-type allele, which would explain the reverted phenotype of the hybrid cells. PCR primers were chosen as indicated by the arrows, allowing specific amplification of a 200-bp fragment (nucleotides 2748–2947) predicted to have lost the missense mutation after recombination.

version was not due to a mutation affecting the MMC-bioactivation pathway. We entertain two possibilities that remain to explain the phenotypic reversion of the fusion hybrids: first, a secondary in-*cis* alteration compensating the Arg951Gln mutation relatively far away from the primary missense mutation—that is, outside the amplified fragment described in figure 2; and, second, a mutation, in a modifier gene, that compensates for the FA defect in *trans*. We are currently trying to address these possibilities.

We have previously found lack of complementation in EUFA173 cells by FANCA cDNA in the episomal Epstein-Barr virus–derived plasmid pDR2 (Joenje et al. 1997). We have now repeated those experiments and—depending on the time after transfection—have found different results

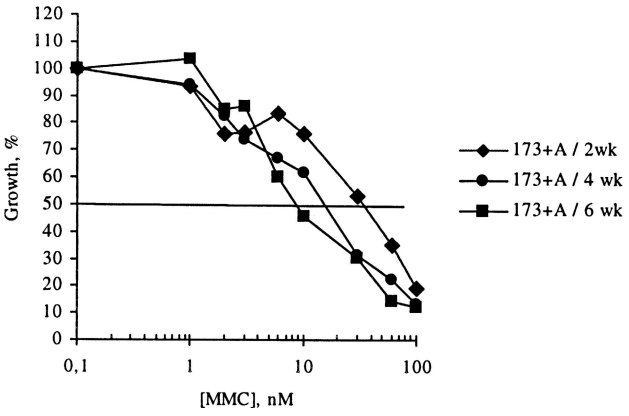

Figure 3 Loss of complementation in EUFA173 cells, as a function of time after transfection with FANCA cDNA. Cells were transfected with the episomal vector pDR2 containing the FANCA cDNA (Kruyt et al. 1996) and were grown in the continued presence of hygromycin (200 μg/ml), to select for plasmid-containing cells. Growth inhibition by MMC was tested at 2, 4, and 6 wk after transfection, as indicated.

(fig. 3). After 2, 4, and 6 wk of growth in the presence of hygromycin (used to select for cells containing the plasmid), the IC_{50} values dropped from 32 to 24 to 9 nM MMC, respectively, indicating progressive loss of complementation (IC_{50} values <10 nM indicate absence of complementation). This result explains why a similar experiment performed previously failed to show complementation. Meanwhile, we have obtained evidence for growth inhibition resulting from overexpression of FANCA in combination with hygromycin selection (F. A. E. Kruyt and H. Joenje, unpublished data), which might explain progressive loss of complementation in a transfected culture: if, in a minority of cells, FANCA expression were to be uncoupled from the hygromycin resistance (e.g., by intraepisomal recombination or by integration of the resistance marker into the genomic DNA), continued selection with hygromycin would give FANCA-nonexpressing cells a proliferative advantage over FANCA-expressing cells, finally resulting in elimination of complemented cells from the cell population. In retroviral transduction experiments with high virus titers, gene-transfer efficiencies are typically much higher than those obtained with direct transfection of DNA. After high-titer viral transduction, selection is therefore hardly necessary, so that loss of complementation is not expected to pose a serious problem. Hence, retroviral transduction appears to be a preferred method to classify patients with FA by gene-mediated functional complementation (see Pulsipher et al. 1998). However, cell-fusion experiments will continue to serve as a generally reliable method to assign cell lines to groups for which the gene has not been identified. In spite of the overall success obtained with this approach, the present findings illustrate that, occasionally, "complemented" fusion hybrids may result from reversion to MMC resistance rather than from phenotypic correction by complementation. To avoid future misassignments of cell lines from patients with FA to new complementation groups, more-stringent criteria should be adopted—that is, identification of at least two patients whose cell lines have been excluded from all known groups and that fail to complement each other in fusion hybrids. However, ultimate proof for the correct assignment of a patient with FA to a new complementation group will require both identification of a complementing gene for that group and demonstration of pathogenic mutations in this gene.

Acknowledgment

We thank H. Hanenberg for communicating his result on retroviral complementation of EUFA173 cells.

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Fanconi Anemia Mutation Database, http://www.rockefeller.edu/fanconi/mutate/ (for mutations and polymorphisms in human *FANCA*)

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/GenbankOverview.html (for human cDNA of *FANCA* [accession number X99226] and nucleotide sequences of all intron-exon boundaries [accession number AC005567])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim/ (for FA [MIM 227650])

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