



## Short communication

# Homozygosity at variant *MLH1* can lead to secondary mutation in *NF1*, neurofibromatosis type I and early onset leukemia

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Received 25 June 2007; received in revised form 30 July 2007; accepted 1 August 2007

Available online 9 August 2007

## Abstract

Heterozygous germ-line variants of DNA mismatch repair (MMR) genes predispose individuals to hereditary non-polyposis colorectal cancer. Several independent reports have shown that individuals constitutionally homozygous for *MMR* allelic variants develop early onset hematological malignancies often associated to features of neurofibromatosis type 1 (NF1) syndrome. The genetic mechanism of NF1 associated to *MMR* gene deficiency is not fully known. We report here that a child with this form of NF1 displays a heterozygous *NF1* gene mutation (c.3721C>T), in addition to a homozygous *MLH1* gene mutation (c.676C>T) leading to a truncated MLH1 protein (p.R226X). The parents did not display NF1 features nor the *NF1* mutation. This new *NF1* gene mutation is recurrent and predicts a truncated neurofibromin (p.R1241X) lacking its GTPase activating function, as well as all C-terminally located functional domains. Our findings suggest that NF1 disease observed in individuals homozygous for deleterious *MMR* variants may be due to a concomitant *NF1* gene mutation. The presence of both homozygous *MLH1* and heterozygous *NF1* mutation in the child studied here also provides a mechanistic explanation for early onset malignancies that are observed in affected individuals. It also provides a model for cooperation between genetic alterations in human carcinogenesis.

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**Keywords:** Neurofibromatosis type 1; *MLH1*; *NF1*; Cooperative effect; Downstream mutation

## 1. Introduction

The classical neurofibromatosis type 1 (NF1) syndrome, also known as von-Recklinghausen's disease or peripheral neurofibromatosis, is an autosomal dominant disorder linked to *NF1* gene mutations of which 50% are new mutations [1]. In 1999, Ricciardone et al. [2] and Wang et al. [3] have identified a novel form of NF1 syndrome that is associated with homozygous germ-line

mutations of *MLH1* gene in two independent families. These initial observations have now been confirmed by others and extended to biallelic mutations of other DNA mismatch repair (MMR) genes, including *MSH2*, *MSH6* and *PMS2* [4–8]. Eight children (five girls, three boys) homozygous for *MMR* gene variants have been independently described [reviewed in ref. 7]. All of these children present with clinical features of NF1 that are often associated with hematological malignancies. Four of the families have been initially identified as hereditary non-polyposis colorectal cancer (HNPCC) families [2–4,6]. The fifth case did not have a familial history of cancer [5]. *MLH1* and *MSH2* genes were mutated in four and one families, respectively. Homozygous mutations of *MLH1* gene were demonstrated in five, and suspected

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in two children. The remaining child had a homozygous *MSH2* gene mutation. The novel form of NF1 is associated with early onset childhood malignancies, most of which are either leukemias or lymphomas [2,3,5]. All the parents of the affected children were heterozygous for the same MMR gene mutation, some of whom had developed colorectal cancers at the time of diagnosis, but none displayed NF1 disease and/or childhood malignancies.

NF1 with early onset childhood malignancies observed in these children is specifically associated with a homozygous MMR gene mutation. However, this association does not necessarily mean that homozygous MMR gene mutation is directly implicated in the development of this disease syndrome. NF1 which is not a known component of HNPCC syndrome [8] is not associated with MMR gene mutation [3]. However, it remains possible that a concomitant mutation affecting *NF1* gene is involved in this disease.

In this report, we further studied the archival genomic DNAs from a Turkish family that we have already reported for the status of *MLH1* [2]. In this HNPCC family, displaying a germ-line mutation in *MLH1* (p.R226X), there was a consanguineous marriage between two members, both heterozygous for the mutation (Fig. 1). All three children born from this marriage developed NF1 disease associated with early onset hematological malignancies and a homozygous *MLH1* mutation had been found in at least two siblings. The mother and father were heterozygous for *MLH1* muta-

tion and developed colorectal cancers at 26 and 33 years, respectively, but they did not display NF1 clinical features nor did they develop hematological malignancy [2]. We report here on the identification of a heterozygous *NF1* gene mutation in the archival DNA samples from child IV-4 who developed atypical chronic myeloid leukemia at 12 months, and displayed NF1 features with café au lait spots and fibromatous skin tumors [2].

## 2. Materials and methods

### 2.1. Patients and DNA samples

Informed consent was obtained from all participants in the study. The TF3 kindred was described previously [2]. Briefly, the focus of this investigation, child IV-4, developed atypical chronic myeloid leukemia at 12 months and displayed NF1 features with more than 10 abdominal café au lait spots ( $\geq 15$  mm) and two fibromatous skin tumors. Two siblings, IV-3 and IV-2, also developed leukemia/lymphoma and died in early childhood. Clinical signs of neurofibromatosis were confirmed in the sibling IV-3. The parents (III-5 and III-6) were both afflicted with colorectal cancer at an early age, 26 and 33 years old, respectively. Blood samples from the parents were collected in tubes containing EDTA. The DNAs of wt, FIII-6, MIII-5 and child IV-4 has been extracted from freshly collected blood samples and was then stored at  $-80^{\circ}\text{C}$ . The DNA for sibling IV-3 has been extracted from an archival bone marrow smear. Differential blood counting of the child IV-4 at the time the blood was drawn for DNA analysis displayed 14% blast cells [2].

### 2.2. PCR and SSCP

NF1 exons 16, 21, 22, 23-2 and 23a were amplified using specific primers that were described previously [9]. PCR was carried out in 25  $\mu\text{l}$  reaction volumes containing 1.5 mM  $\text{MgCl}_2$ , 10 pmoles of each primer, 200  $\mu\text{M}$  dNTP mix and 1 unit *Taq* DNA polymerase (Fermentas) and 50–100 ng genomic DNA. PCR conditions were an initial denaturation step at  $95^{\circ}\text{C}$  for 5 min followed by 30 cycles of 30 s at  $95^{\circ}\text{C}$ , 30 s at  $55^{\circ}\text{C}$ , 30 s at  $72^{\circ}\text{C}$  followed by a 10 min final extension step. Quality of PCR products was verified by agarose gel electrophoresis. For single strand conformation polymorphism (SSCP) analysis, PCR was done in the presence of 1  $\mu\text{Ci}$  [ $^{32}\text{P}$ ] dCTP (Amersham) per reaction and radiolabeled PCR products were denatured using SSCP gel loading buffer (95% formamide (Sigma), 10 mM NaOH (Carlo Erba), 0.25% bromophenol blue (Sigma) and 0.25% xylene cyanol (Sigma)). The DNA solution was heated at  $95^{\circ}\text{C}$  for 2 min and kept on ice for at least 10 min before loading onto a 6% [75:1] acrylamide gel on the EC 160 apparatus. Electrophoresis was performed at 45 W constant power at  $4^{\circ}\text{C}$  for 8 h. Phosphoimaging was performed using the Molecular Imager<sup>®</sup> System, GS-525 (Bio-Rad). The dried gel was placed into the cassette and a clean, erased screen was then placed over the gel. After exposure, the screen was scanned and the image was captured using Multi-Analyst software.

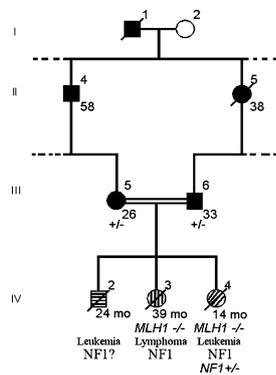


Fig. 1. Simplified pedigree of the HNPCC kindred TF3 with *MLH1* gene mutation. Filled symbols indicate colon cancer; shaded symbols indicate neurofibromatosis type 1. Numbers below symbols indicate age at diagnosis. ( $\pm$ ): Heterozygous *MLH1* mutation. The TF3 kindred was described previously [4]. Briefly, the focus of this investigation, child IV-4, developed atypical chronic myeloid leukemia at 12 months and displayed clinical signs of type I neurofibromatosis. Two siblings, IV-3 and IV-2, also developed leukemia/lymphoma and died in early childhood. Clinical signs of neurofibromatosis were confirmed in the sibling IV-3. The parents (III-5 and III-6) were both afflicted with colorectal cancer at an early age, 26 and 33 years old, respectively.

### 2.3. DNA sequence analysis

Sequence analysis was performed using DYEnamic ET Terminator cycle sequencing kit (Amersham) according to the recommended protocol with the following modifications: annealing temperature was 59 °C, and only 3 pmoles of primer were used for the reverse primer reaction. Automated sequence analysis was performed on the 310 Genetic Analyzer (ABI; Perkin-Elmer) as recommended by the manufacturer.

### 2.4. *TaqI* restriction digestion

Genomic DNAs from parents (MIII-5 and FIII-6), child IV-4 and a wild-type control were used to amplify *NF1* exon 22. The amplified products were analyzed by electrophoresis, and normalized by densitometric analysis using the BioRad Multi-Analyst software. Equal amounts of PCR products were used for *TaqI* digestion, which was performed at 65 °C under mineral oil. The restriction digestion products with undigested controls were resolved on a 4% NuSieve 3:1 agarose (FMC Bioproducts) prepared in 0.5× TBE, for 3 h at 8 V/cm. Densitometric quantification was performed on the undigested DNA fragments, the amount of the mutant allele resistant to restriction enzyme digestion was compared to the undigested PCR product.

## 3. Results and discussion

Archival genomic DNA from child IV-4 was subjected to a detailed *NF1* gene mutation analysis (Fig. 1). As *NF1* is a large gene, we applied a rapid screening technique based on SSCP analysis of selected *NF1* gene regions. The exons that contained microsatellites and CpG islands within the NF1 GAP-related domain (NF1-GRD) were targeted initially (Fig. 2).

Following the screening of exons 16, 21, 22, 23-2 and 23a, we obtained a band-shift with exon 22 in DNA from child IV-4 that was not present in parental DNAs. PCR products corresponding to other exons did not produce band-shifts (data not shown). An independently prepared PCR product for exon 22 was then used for direct DNA sequence analysis. We identified a C

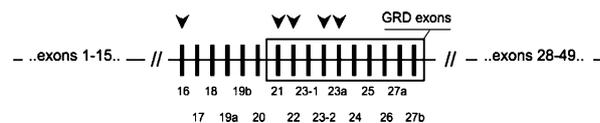


Fig. 2. Schematic representation of *NF1* exons used in mutation screening experiments. *NF1* exons selected for analysis were amplified from genomic DNA, and PCR products were further analyzed by SSCP and the presence of the mutation was then confirmed by direct DNA sequence analysis. Vertical lines represent *NF1* exons, transparent rectangle represents NF1-GRD exons, and arrowheads mark exons selected for analysis. Drawing is not to scale.

to T transition at nucleotide 3721 of *NF1* gene. The electropherogram profile obtained displayed both wild-type and mutant sequences, suggesting that child IV-4 had a unique heterozygous *NF1* mutation. Both father and mother displayed only wild-type *NF1* sequence at this site, indicating that child IV-4 had a new *NF1* gene mutation (Fig. 3a). This mutation is predicted to cause a substitution of arginine 1241 for a stop codon; (p.R1241X) at the protein level. The point mutation was verified by restriction enzyme digestion with *TaqI*, whose restriction site is lost as a consequence of the C to T transition. Restriction enzyme digestion experiments indicated that only 50% of the PCR product from child IV-4 was digested with *TaqI*, clearly confirming the presence of a heterozygous state in this exon. In contrast, PCR products from father, mother as well as from a control sample were completely digested by *TaqI*, as expected from the DNA sequence data (Fig. 3b).

To rule out the possibility of incomplete digestion from the mutant allele of child IV-4 sample, we amplified a longer DNA fragment using another intronic primer located 1438 bps upstream of the priming site of exon 22, harboring an additional *TaqI* restriction site. The PCR products (1770 bp) from both parents and both siblings (child IV-3 and child IV-4) were digested to completion with *TaqI*, as a result, the presence of the (c.3721C>T) heterozygous state was confirmed for child IV-4 by the loss of *TaqI* recognition site (see supplementary figure). This mutation was absent from a sister (child IV-3), further confirming that child IV-4 had a new *NF1* mutation.

Since the initial description of the association of neurofibromatosis features and early onset of childhood malignancies with homozygous mutations of *MLH1* gene [2,3], 14 cases of biallelic mismatch repair gene mutations (mostly homozygous, but also compound heterozygous) affecting the *MLH1*, *MSH2*, *MSH6* or *PMS2* genes have been described, as recently reviewed by Bandipalliam [8]. These cases are characterized by early onset hematological malignancies (mean age of diagnosis: 5.6 years), and gastrointestinal cancers (mean age of diagnosis: 12.2 years), as well as the distinct clinical features of type I neurofibromatosis [8]. More importantly, such clinical features have not been detected in the parents of these individuals [8], strongly suggesting that this particular form of neurofibromatosis is caused by mismatch repair deficiency leading to a mutation of *NF1* gene. Several studies provided convincing data supporting the hypothesis that MMR deficiency and neurofibromatosis may be associated with NF1 mutations. Wang et al. [10] have reported that *NF1* gene is often mutated in DNA mismatch repair-deficient cancer cell

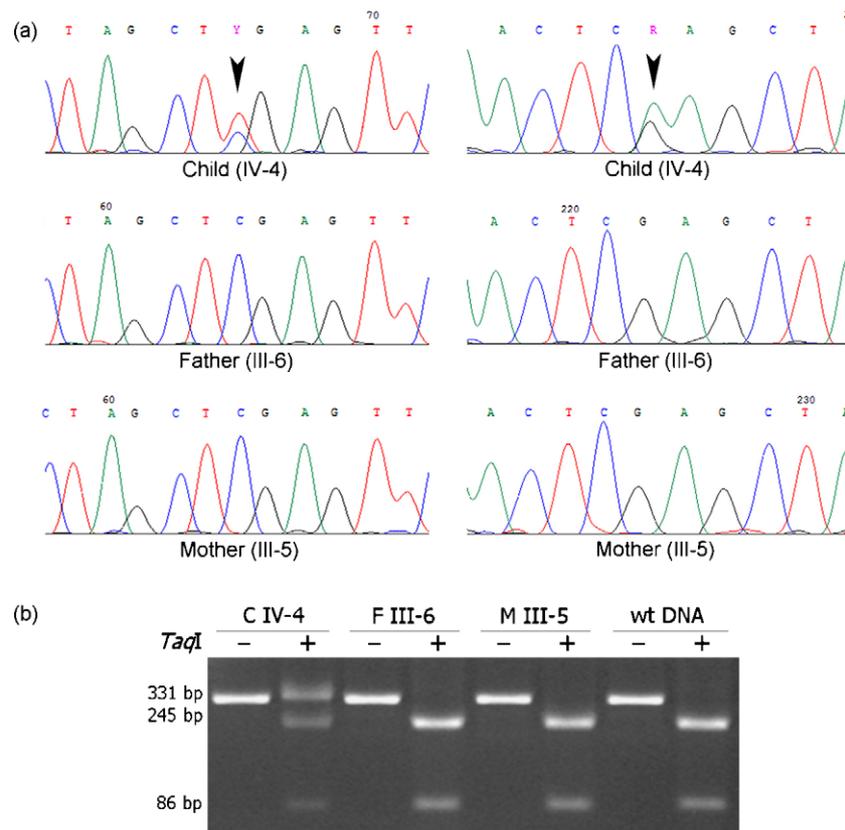


Fig. 3. Heterozygous *NF1* mutation in a *MLH1*-deficient child with neurofibromatosis type I and early onset leukemia. Genomic DNAs from parents (MIII-5 and FIII-6), child IV-4 and a wild-type control were used to amplify *NF1* exon 22 to be used for DNA sequence analysis and then for verification by restriction enzyme digestion. (a) A small stretch of the electropherogram flanking the mutation showing the nucleotide sequence obtained with forward and reverse primers (the left and right panels, respectively). Arrow heads indicate heterozygosity [T/C designated as Y] at nucleotide 3721. (b) PCR amplified DNA samples were analyzed by electrophoresis, and normalized by densitometric analysis using the BioRad multi-analyst software. Equal amounts of PCR products were used for *TaqI* digestion (lanes with “+” signs) as described in Section 2. The enzyme’s digestion products of wild-type sequence are 245 and 86 bp, while the c.3721C>T mutation will render the site unrecognizable by the enzyme. Lane C IV-4, child IV-4; F III-6, Father; M III-5, Mother; wt DNA, wild-type control. Data represents experiments repeated at least three times with freshly prepared PCR products.

lines, as well as in primary tumors exhibiting microsatellite instability. They have also shown a “mosaic” type of mutation of the murine *nf1* gene in 2 of 14 clones generated from *mlh1*-deficient mouse embryonic fibroblasts, providing additional evidence that *NF1* gene may be a mutational target in mismatch repair-deficient cells [10]. Moreover, leukemogenesis in *nf1* heterozygous mice was shown to be accelerated by *mlh1* deficiency [11]. However, to our knowledge, there is no previous data reporting potential *NF1* gene mutations in individuals with DNA mismatch repair gene mutations [7]. Thus, the molecular mechanism(s) of neurofibromatosis in individuals with biallelic mutations remained unknown. Here, we show that blood DNA from a child who developed atypical chronic myeloid leukemia at 12 months and displayed NF1 features with more than 10 abdominal café au lait spots (>15 mm) and two fibromatous

skin tumors displays a new c.3721C>T mutation at *NF1* gene. This is a recurrent mutation at a predicted CpG site, initially reported by Fahsold et al. [12], leading to a severely truncated neurofibromin protein (p.R1241X). The mutant protein is expected to have lost its negative regulatory function of ras oncogene because of the lack of GTPase activating as well as other C-terminally located functional domains [13]. To our knowledge this is the first demonstration of a *NF1* gene mutation in a MMR deficient patient with neurofibromatosis and early onset hematological malignancy. Our finding provides a plausible explanation for the genetic cause of neurofibromatosis observed in patients displaying this phenotype [8]. Thus, secondary *NF1* mutations may predispose MMR deficient individuals to neurofibromatosis and/or early onset malignancies, similar to classical NF1 syndrome [12].

Currently, the genetic mechanism of *NF1* mutation in MMR deficient individuals is unknown. *NF1* is known as an autosomally dominant condition. About 50% of cases result from new mutations, and some cases display germline mosaicism. Most of the new mutations occur on the paternally derived chromosome, whereas large deletions are usually of maternal origin [14]. The C to T mutation observed in the child IV-4 occurred at a CpG motif, such type of mutations are known to occur as a result of deamination of methyl cytosine which would lead to a T:G mismatch which is usually repaired by MMR system. As this mutation occurred in a MMR deficient condition, it may be secondary to this particular condition. It is unclear whether this mutation occurred post-zygotically in the parental chromosome, or rather somatically in the affected child. Such mutations appear to occur in *NF1* patients as some of them display a “mosaic” pattern [12,14]. If the same event took place in the child IV-4, this must have been a very early embryonic mutation, as the child already displayed many café-au-lait spots ( $\geq 15$  mm) and two fibromatous skin tumors at the age of 12 months, together with leukemia [2]. Although we were unable to test whether this particular mutation display “mosaicism”, we favor the hypothesis that the heterozygous *NF1* mutation detected in child IV-4 is a very early somatic mutation that occurred in the embryonic cells.

The co-existence of a homozygous MMR gene mutation with a tumor suppressor gene mutation (i.e. *NF1* mutation) at the individual level is also a new finding. This has been previously suggested as a potential mechanism involved in cancer predisposition of individuals carrying constitutional MMR gene mutations [2,3]. Accordingly, the loss of the wild-type MMR gene in pre-malignant cells results in genetic instability giving rise to a high rate of mutations eventually affecting critical tumor suppressor genes [15]. As already indicated, the *NF1* gene appears as a frequent target for mutations in MMR-deficient cells [10]. Therefore, the occurrence of the new *NF1* mutation identified here may have been facilitated by DNA mismatch repair deficiency in body cells due to inherited homozygous *MLH1* mutation in the affected child. However, this remains as an interesting, yet unproven hypothesis in the absence of a direct evidence for a cause–effect relationship for the co-occurrence of *MLH1* and *NF1* gene mutations in the same individual.

Cancer is a multistage process which requires the contribution of different genetic alterations. Mouse models have been successfully used to study carcinogenic effects resulting from combinations of genetic alterations. Such

studies have allowed developing several important concepts for tumorigenesis, such as cooperation between oncogenes, or oncogene-tumor suppressor gene interactions [16]. However, it is unknown how and to what extent such fundamental concepts apply to tumorigenesis processes in humans. Our observations provide a unique opportunity to address this issue. In the child we studied here, there was a homozygous *MLH1* mutation and a heterozygous *NF1* mutation. A similar situation has been created experimentally in mice homozygous for *mlh1* ( $-/-$ ) and heterozygous *nf1* ( $\pm$ ) mutations [11]. These double mutant mice displayed accelerated myeloid leukemogenesis when compared to mice carrying single gene mutations, indicating that two genetic alterations cooperated for the generation of hematological malignancies. In humans with classical *NF1* syndrome due to *NF1* mutations, hematological malignancies are detected, but only rarely (less than 5%; [17]), suggesting that *NF1* mutation alone has a moderate effect on hematological malignancy susceptibility. On the other hand, hematological malignancies are not a known component of tumors observed in HNPCC patients with heterozygous DNA mismatch repair gene mutations, including the parents of the child IV-4. In contrast, the child IV-4 with *MLH1* ( $-/-$ )/*NF1* ( $\pm$ ) developed leukemia at the age of 1 year. Thus, in line with the predictions based on data gathered using experimental mice models, multiple genes may also cooperate in humans for accelerated tumor susceptibility. Child IV-4 is not an isolated case; at least a dozen other children share exactly the same clinical features of early onset hematological malignancies and neurofibromatosis [8]. It will be interesting to know whether our gene alteration cooperation model is a general feature of this particular syndrome.

## Acknowledgements

We thank the TF3 family members for their cooperation. We also thank Yasemin Doruk and Bilge Özbayoglu for technical assistance.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mrfmmm.2007.08.003.

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