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CASE REPORT

A *de novo* germline *MLH1* mutation in a Lynch syndrome patient with discordant immunohistochemical and molecular biology test results

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Abstract

We describe a patient with a *Homo sapiens* mutL homolog 1 (*MLH1*)-associated Lynch syndrome with previous diagnoses of two distinct primary cancers: a sigmoid colon cancer at the age of 39 years, and a right colon cancer at the age of 50 years. The mutation identified in his blood and buccal cells, c.1771delG, p.Asp591Ilefs*25, appears to be a *de novo* event, as it was not transmitted by either of his parents. This type of *de novo* event is rare in *MLH1* as only three cases have been reported in the literature so far. Furthermore, the discordant results observed between replication error phenotyping and immunohistochemistry highlight the importance of the systematic use of both pre-screening tests in the molecular diagnosis of Lynch syndrome.

Key words: Lynch syndrome; *Homo sapiens* mutL homolog 1; *De novo* mutation; Replication error phenotype; Immunohistochemistry

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INTRODUCTION

Lynch syndrome or hereditary non-polyposis colorectal cancer syndrome (MIM#120435)[1] is a familial form of cancer mainly involving the colon, rectum or endometrium and, more rarely, the small bowel, urinary tract, ovaries, stomach, brain or skin. The pathology is transmitted in an autosomal dominant mode of inheritance with an incomplete penetrance. It is linked to mutations in the genes of the DNA mismatch repair system (MMR), including *Homo sapiens* mutL homolog 1 (*MLH1*), *Homo sapiens* mutS homolog 2 (*MSH2*), and to a lesser extent,

Homo sapiens mutS homolog 6 (*MSH6*) and PostMeiotic Segregation increased 2 (*PMS2*). International scientific community defined the clinical and familial criteria which had to be fulfilled to consider a likely diagnosis of Lynch syndrome, and thereby the value of a mutation screening of *MMR* genes in a patient. The initial recommendations following the Amsterdam (1991)[2] and Amsterdam Ⅱ (1999) criteria[3] were too restrictive. New criteria were therefore proposed at the Bethesda conference in 2004[4], in order to increase the sensitivity of mutation carrier detection. These recommendations include an age at diagnosis younger than 50 years, or younger than 60 years when the tumor has a microsatellite instability-high phenotype (MSI-high), which is the hallmark of tumors linked to a defective *MMR* gene system[5,6].

Here we report on the unique case of a *de novo* mutation encountered by our Oncogenetic Laboratory through its diagnostic activity in Lynch syndrome. In our molecular diagnostic strategy, replication error (RER) phenotyping is performed systematically before sequencing of the *MMR* genes whenever tumor tissue is available, and this is done concomitantly with immunohistochemistry in order to direct the sequencing as far as possible. Sequencing of either *MLH1*, *MLH2* or both genes is initiated each time a tumor is found to be MSI-high or -low (low microsatellite instability: one system with replication error), even when the immunohistochemistry results are ambiguous or show no extinction of a MMR protein. The sequencing step is also initiated under three specific circumstances: (1) the finding of an microsatellite stable (MSS) phenotype with validated Amsterdam criteria; (2) when no tumor material is available; and (3) in the case of a discrepancy between the immunohistochemistry results and the RER phenotype.

Whenever necessary, DNA samples are sent to another laboratory within the French MMR network (Groupe Génétique et Cancer MMR) for further testing of *MSH6*.

CASE REPORT

The patient is a 54 year-old man who had previously presented two distinct primary cancers, a sigmoid colon cancer at the age of 39 years, and a right colon cancer at the age of 50 years. He is the sixth of a family of 10 siblings, three of whom have had polyps removed: two brothers and one sister, at the ages of 38, 42 and 38 years respectively. A fourth sibling, another of his sisters, was diagnosed with cervical cancer at 45 years of age, but it is not in the tumor spectrum for Lynch syndrome. Among the older generations of the patient's family, the maternal grandfather died of colon cancer at the age of 84 years and the mother's medical records report an ovarian cyst and ovariectomy (Figure 1).

The right colon tumor had been removed by a colectomy covering 30 cm of the right colon and 5 cm of the ileum. The tumor measured 7 cm × 5 cm × 1.5 cm and was located in the caecum, invading the terminal ileum. The pathology examination revealed a grade 3 stage for the poorly differentiated, burgeoning and stenosing adenocarcinoma, and cancer staging was classified as pT-3N0Mx. Immunochemistry and molecular biology analyses were performed on formalin-fixed paraffin-embedded material from this tumor.

The immunohistochemistry analysis for the MMR proteins MLH1, MSH2 and MSH6[7] was performed but did not demonstrate a loss of expression, albeit a very weak expression was detected for MLH1 and MSH2.

RER phenotyping was then performed and showed an instable phenotype for four of the five markers routinely tested in our laboratory (BAT25, BAT26, NR21, NR22 and NR24)[8,9], with stability noted only for the NR24 system. The tumor was thus classified MSI-high, and the patient was considered for mutational analysis of *MMR* genes, according to the Bethesda criteria.

In keeping with routine practice at our laboratory, we started with the search for large rearrangements using a commercial Multiplex Ligation-dependent Probe Amplification (MLPA®) kit for *MLH1* and *MSH2* (P003-B1 kit, MRC-HOLLAND, Amsterdam, The Netherlands)[10]. This revealed the isolated loss of *MLH1* exon 16. In such cases of single exon deletion or duplication, we systematically verify the hybridizing sequence of the MLPA probes to exclude a false result caused by a single nucleotide polymorphism (SNP). However, in the present case, the sequence analysis showed a single nucleotide deletion of a G in position 1771 (NM_000249.3: c.1771delG, p.Asp591Ilefs*25). This mutation had previously been described in a Taiwanese Lynch family[11]. It had been classified as deleterious because of the occurrence of a premature stop codon at position 615, which disrupts the interacting protein domains to *Pms*2 and exonuclease 1[12].

From this point on, we set out to investigate the status of the mutation within the tumor. Finding the mutation in a homozygous or hemizygous state would have meant that the second hit in the carcinogenetic process was a loss of heterozygosity, and that the mutation was the first hit. Unfortunately, the poor quality of the DNA extracted from the Formaldehyde-fixed Paraffin-embedded material did not enable us to get readable sequences. We confirmed the presence of mutation c.1771delG (p.Asp591Ilefs*25) in a second independent sample (DNA extracted from a buccal swab spotted onto a FTA paper). Once the mutation had been confirmed, a presymptomatic test was offered to the patient's family. In this context, we tested both parents but did not identify any parental origin for the mutation as both parents tested negative for the mutation, in both blood and buccal cell samples. We then performed the complete sequencing of *MLH1* to exclude any other mutational event that could have been the true cause of the patient's personal and familial history of cancer, but we did not identify anything else.

These results led us to question the patient's paternity, given that a meta-analysis performed in 2005 reported false paternity in a median of 3.7% of births[13]. Nevertheless, a genetic fingerprinting kit (AmpFℓSTR® SGM

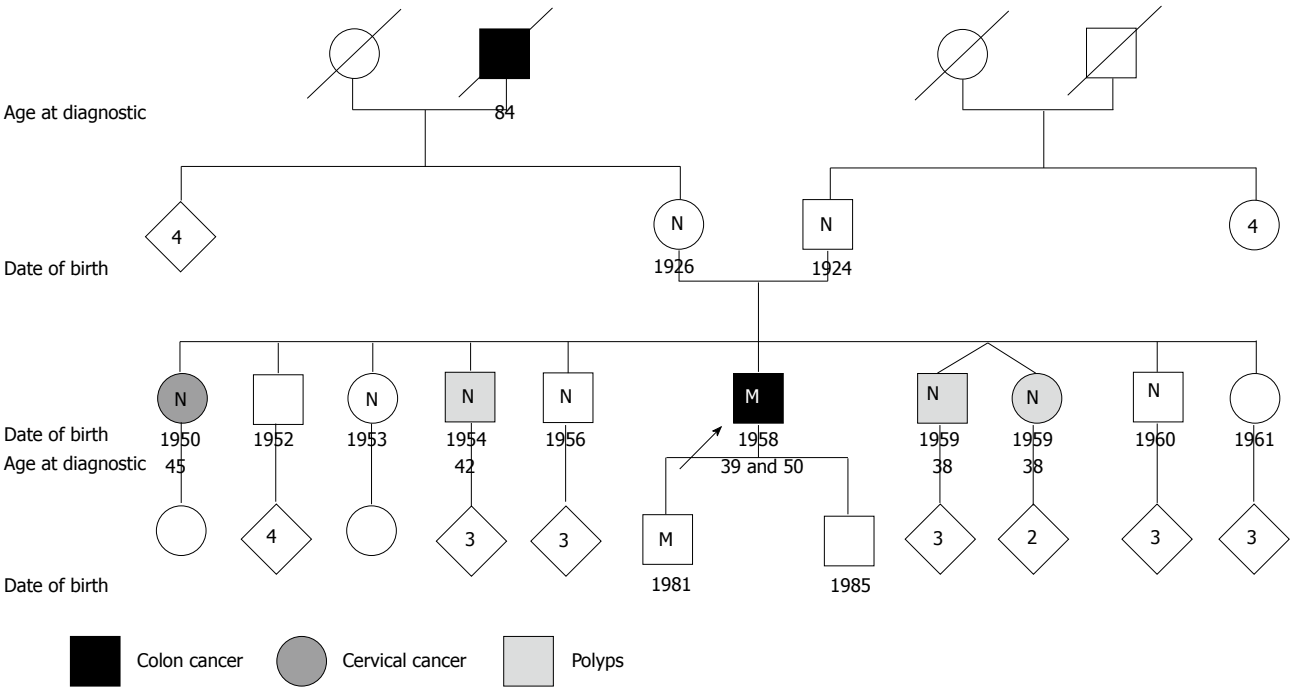

Figure 1 Pedigree. Family tree shows the segregation and clinical manifestations in the family with *de novo Homo sapiens* mutL homolog 1 mutation. N: Normal carrier status; M: Mutated carrier status.

Plus®; Applied) confirmed the paternal and maternal status of the supposed parents without ambiguity.

In order to further investigate the possibility of a mosaicism, we performed a haplotyping study using two frequent SNP within the *MLH1* gene (rs1800734 and rs2241031) chosen for their high heterozygosity frequencies and their belonging to different ancestral haplotypes. However, the lack of proband informativity meant that this analysis did not enable us to identify the maternal or paternal origin of the chromosome harboring the mutation. However, because we had tested two different types of tissues of different origins, i.e., mesodermic for blood cells and ectodermic for buccal cells, we were able to exclude mosaicism with quite a high level of confidence. We were finally able to conclude that the Lynch syndrome-causing mutation observed in our patient was a *de novo* event.

Up to now, we have been able to test seven of the patient's nine siblings and have not identified any of them as a carrier of the mutation, even those who have had polyps or cervical cancer. This could be considered as a further argument for a *de novo* mutation, since it does not follow the Mendelian transmission ratio of 1:2 and it is not concordant with polyp antecedents.

Finally, a presymptomatic test in our patient's 30 year-old son revealed the presence of mutation c.1771delG (p.Asp591Ilefs*25) in a heterozygous state, thereby highlighting the transmission (and conservation) of this *de novo* mutation by the proband.

DISCUSSION

Here we report on a French Lynch family in whom we have identified a frame-shift mutation that induces a premature stop codon in a crucial part of the *MLH1* gene. This mutation has never been reported in the open access *MMR* mutation database (INSIGHT, Newfoundland *etc.*), or in the French MMR network database (unpublished data). Moreover, we have never found this mutation in 592 chromosomes of patients of similar geographic origin who have been tested in our laboratory as part of routine Lynch syndrome screening.

In contrast with certain other genes, such as *NF1,* which exhibit a *de novo* mutation rate of about 50%, this event in *MLH1* is relatively rare (1% to 5%) according to the study recently published by Win *et al*[14]. To our knowledge, a *de novo* point mutation in *MLH1* has only been described three times until now. The first occurrence was a c.2101C>T (p.Gln701X) mutation in exon 18, which was detected in a 35 year-old man[15]. The second was a c.666dupA (p.Asn222Lysfs*4) mutation in exon 8 found in a 31 year-old man[16]. The third mutation was a nonsense mutation in exon 13, c.1459C>T (p.Arg487X) identified in a 36 year-old patient[14]. In all three cases the patients had no family history of colorectal cancer and seemed to develop cancer younger than inherited mutation carriers. In addition to these three single nucleotide mutations, two large deletions have already been published, one of the entire *MLH1* gene and one of exon 15, once again in a young man without any family history of cancer[14,17]. *De novo* mutations seem to be more frequent in *MSH2*, for which four different mutations have already been described[14,18,19], including the recurrent mutation c.942+3A>T. The latter can even be considered as a kind of mutation hotspot, as it has been proved to occur *de novo* with a relatively high frequen-

cy[20]. The nucleotide implicated in this mutation is part of the BAT26 homopolymer containing 26 adenines. This particular context is hypothesized to be responsible for misalignment during replication or recombination. Even though the *de novo MLH1* mutation described here arose in two families of different ethnic origin, a similar explanation cannot be considered.

This case report confirms the relevance of preceding *MMR* gene sequencing by the combination of the two prescreening tests (RER phenotyping and immunohistochmistry) in the molecular diagnostic strategy in Lynch syndrome, especially for young patients without familial antecedents. Indeed, it is worth noting that most of these patients would not have been considered as candidates for mutation analysis according to the Amsterdam I and II criteria; this might be the reason why *de novo* events in *MLH1* were not described prior to the implementation of the Bethesda criteria. It is also interesting to point out the discordance between immunohistochemistry and RER phenotyping results for our patient's tumor, which confirms the benefit of a dual approach for the screening of Lynch syndrome patients. Indeed, MSI-high phenotypes with conservation of protein expression have already been described and can easily be explained when they concern a missense mutation that does not occur in the epitope of the antibody used. Inversely, extinction of a protein associated with an MSS or MSI-low phenotype can also be encountered, especially when the *MSH6* gene is affected[21]. In glioblastoma, in the context of Turcot syndrome, changes in microsatellite profiles have also been described as more subtle than those in colorectal tumors[22] and thus have to be considered very carefully.

In conclusion, the frequency of *de novo* mutations in *MMR* genes may be higher than actually observed in diagnostic laboratories because once a mutation is identified, the parents of the proband are not systematically analyzed in routine practice. This may be because they are deceased, as the average age at molecular diagnosis of our index cases is 53 years, or because they do not wish to be tested

Moreover, we show here that the combined use of molecular biology and immunohistochemistry should be recommended when screening patients with suspected Lynch syndrome. This combined strategy should help to avoid missing a tumor linked to a deficiency in a *MMR* gene, and also to orientate the subsequent sequencing to one of these genes in a more precise and therefore cost-effective manner.

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