Novel mutation in \textit{POLH} gene responsible of severe phenotype of XP-V

\textbf{Methods.} In the present study, three patients that manifest severe XP-V phenotype were investigated. Linkage study by the homozygosity mapping strategy using microsatellite markers flanking \textit{POLH} gene and mutation screening were conducted. We assessed the morbid effect of the mutation by bioinformatic tools and molecular assays.

\textbf{Results.} Haplotype analysis showed the presence of a common haplotype for two patients and a common genotype for the closest marker to the gene for all patients. Molecular analysis revealed the presence of a homozygous mutation c.660+1G>A for all patients.

\textbf{Conclusion.} The analysis supports the pathological effect of the recently identified c.660+1G>A mutation in \textit{POLH} gene and unravels the molecular mechanism by which c.660+1G>A mutation acts.

\textbf{KEY WORDS:} XP-V; severe phenotype; splice site mutation; \textit{POLH} gene; Tunisian patients.

\section*{Introduction}

\textit{Xeroderma pigmentosum} (XP: OMIM 278700-278780) means dry and pigmented skin. It is a rare genodermatosis inherited as an autosomal recessive trait. XP is characterized by a high photosensitivity of sun-exposed skin (1), ocular abnormalities (2), and neurological alterations (3). XP is also genetically heterogeneous. Indeed, seven complementation groups of classic XP (XP-A to XP-G) and a variant form (XP-V) were reported (4). The classic forms are manifested in patients deficient in the Nucleotide Excision Repair (NER) system (3), while XP-V is manifested in patients who are deficient in the Translesion Synthesis (TLS) (5).

In Tunisia, XP is present at a higher frequency than that in USA and Japan (1/10000 against 1 per million and 1/22000 respectively) (6-10) and the disease is classified into three clinical forms: severe, intermediate and moderate (7). Nevertheless, only patients belonging to the XP-V and XP-F group have been reported so far (11, 12). XP-V is characterized by mild dermatological manifestations, absence of neurological abnormalities and late onset of skin cancers. Approximately 20% of XP patients belong to XP-V complementation group and bare mutations in \textit{POLH} gene (13). \textit{POLH} gene contains 11 exons and encodes an enzyme called polymerase \textit{η} (pol \textit{η}). This protein encompasses two domains: a catalytic domain that recognizes cyclobutane pyrimidine dimers (CPD) in DNA and ensures replication at the injured site and a
binding domain that binds the enzyme to DNA (14). In XP-V patients who are lacking for pol η, CPD is bypassed by other TLS polymerases that are more prone to misincorporation. Once such mismatched CPDs occur, they are more easily recognized by NER than normal CPDs, thereby promoting fixation of mutations (15). The expression of pol η can be quantified by immune-precipitation and western blot (16) but molecular investigation remains the best method to study XP-V.

In the present study, we report on genetic investigation of POLH gene in three Tunisian patients.

Patients and methods

Three patients of consanguineous families XP27, XP69 and XP67 were evaluated at the Dermatology Department of Habib Thameur Hospital in Tunis and classified as XP-V on the basis of their clinical features (Figure 1A). These patients originated from Jendouba and Zaghouan, located in Northern Tunisia. Their average age is 28 years. After signing an informed consent, all available members were interviewed with a questionnaire including a pedigree drawing in order to link closely related families. Among the three families, two were related (XP27 and XP69).

Genomic DNA was isolated from peripheral blood leukocytes using *salting out* method (17). This technique is based on differential precipitation between nucleic acids and proteins (18). Homozygosity by descent for POLH gene (NG_009252.1) was performed using two polymorphic microsatellite markers D6S1582 and D6S271 (Figure 1B). This strategy assumes that an individual born to related parents is homozygous not only for the mutation but also for genetic markers surrounding the mutation location. Mutations were screened by direct sequencing of exons 5, 9, 10 and 11 for XP27. The electropherogram analysis revealed a new splice site mutation at the donor site of intron 5: c.660+1G>A in the POLH gene. This mutation was subsequently confirmed for the remaining patients.

Table 1 - Clinical features of XP-V patients.

<table>
<thead>
<tr>
<th>Family code</th>
<th>Number of patients</th>
<th>Patient code</th>
<th>Age (years)</th>
<th>Consanguinity</th>
<th>Age at onset of erythema and pigmented macules</th>
<th>Basal cell carcinoma (BCC)</th>
<th>Squamous cell carcinoma (SCC)</th>
<th>Other tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>XP27/2</td>
<td></td>
<td>XPV27-1*</td>
<td>29</td>
<td>1st degree</td>
<td>5</td>
<td>20 (8)</td>
<td>17 (14)</td>
<td>keratoachontoma</td>
</tr>
<tr>
<td>XP69</td>
<td></td>
<td>XPV69-1*</td>
<td>38</td>
<td>1st degree</td>
<td>5</td>
<td>14 (&gt;5)</td>
<td>116 (&gt;5)</td>
<td>-</td>
</tr>
<tr>
<td>XP67/1</td>
<td></td>
<td>XPV67-1*</td>
<td>20</td>
<td>1st degree</td>
<td>5</td>
<td>14</td>
<td>ND</td>
<td>Actinic keratosis</td>
</tr>
</tbody>
</table>

In order to identify the impact of this genetic alteration on the transcription, total RNA was isolated from leukocytes using trizol-chloroform purification and isopropanol precipitation (19). Quality of the cDNA was assessed by comparison to a housekeeping gene, GAPDH (20).

Results

In this study, we report on clinical and genetic investigations of XP-V in two unrelated Tunisian families. Patients are born to first degree parents. They manifest the disease in early childhood. Erythema and pigmented macules appeared at the age of 5 years. All patients have developed more than 10 tumors up to now (Table 1). No neurological abnormality was noted.

In order to confirm clinical diagnosis and homozygosity by descent for POLH gene, patients and their family members whenever available were genotyped with D6S1582 and D6S271 microsatellite markers. Both XP27 and XP69 patients share the same haplotype at a homozygous state [143-190] (Figure 1C) whereas XP67 patient presented a different haplotype [151-190] (Figure 1C). It is noteworthy that all affected individuals share a common allele [190] for D6S271 marker.

Mutation screening was performed by direct sequencing of exons 5, 9, 10 and 11 for XP27. The electropherogram analysis revealed a new splice site mutation at the donor site of intron 5: c.660+1G>A in the POLH gene. This mutation was subsequently confirmed for the remaining patients. Exon 5 was sequenced for available healthy relatives. XP69-2 and XP67-2 displays a heterozygous genotype (Figure 1D).

The c.660+1G>A mutation was predicted to abolish the donor splice site of exon 5 using the BDGP while the analysis by Splice-site Analyzer Tool showed that the value of donor splice site has decreased by 17-18%. HSF has predicted the abolition of the donor splice site caused by a sharp decline of its score (32%) as well as the abolition of the entire ribonucleoprotein binding site (hnRNP).

To assess the impact of c.660+1G>A mutation, all the POLH gene cDNA sequence was amplified by RT-PCR. Results showed the absence of the corresponding mRNA in all patients. However, the GAPDH housekeeping gene was amplified successfully.
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Discussion

XP is among the most severe and debilitating genodermatoses. It is a highly heterogeneous disease at clinical and genetic level. Clinical investigation has shown that our patients exhibited the variant form of XP. Homozygosity by descent study revealed that patients are linked to POLH gene and shared the same genotype [190-190] for D6S271, the closest marker to POLH gene. Mutation analysis revealed that patients share a common founder mutation c.660+1G>A. XP-V group is characterized by middle manifestations compared to other forms of XP. The median age at onset of the first clinical signs is four years unlike XPA and XPC groups which is less than 12 months (9, 21, 22). The first skin cancer usually appears at age 45 (16). Patients reported in this study have developed their first clinical signs at age of 5 but they have manifested their first tumors since the age of 14. Furthermore, XP27 family contains 7 affected members; among them, two patients, XP27-1* and XP27-2* died at age of 20 and 29, respectively (Figure 1A). Therefore, the phenotype of XP-V patients seems to be severe, suggesting a deleterious effect of c.660+1G>A mutation.

To predict the impact of this mutation, we have used bioinformatic tools that have shown two different interpretations. For the new donor splice site, Splicing Analyzer Tool predicted a relatively low score of 60% compared to the wild type sequence (77%). This difference could influence the binding of U1 and U6 splicing factors to the donor site and promotes them to another consensus sequence with a higher score. Consequently, various mRNA species would likely be synthesized: aberrant mRNA would be predominantly expressed with a small amount of the normally spliced mRNA. In this case, XP-V phenotype would be moderate (23). In the literature, mild clinical features have been reported in individuals with an XP-A or XP-C splicing mutation resulting in 5% of normal residual mRNA (23, 24). Nevertheless, this was not the case for our patients as we showed total absence of mRNA and severe presentation of the disease.
In contrast, BDGP and HSF predicted the abolition of the donor site. A mutation that occurs in a splicing junction might create a cryptic splice site, generate one or more exon skipping or both (25). In both situations, the altered transcript can produce an aberrant protein if the reading frame is conserved, or more often leads to a premature termination codon (PTC) that will activate the Nonsense Mediated Decay mechanism (NMD) and hence, reduce the accumulation of potentially toxic truncated proteins (26). Moreover, the PTC must be located before the fiftieth nucleotide upstream of the last exon-exon junction to eliminate aberrant mRNAs, which is the case for c.660+1G>A mutation (27).

To check whether the NMD was involved in post-transcriptional aberrant mRNA regulation for patients bearing the c.660+1G>A mutation, RT-PCR experiments were performed. The amplification of PLOH gene cDNA showed the absence of mRNA expression in all patients, although we succeeded in amplifying cDNA of the GAPDH housekeeping gene. This mutation probably alters the transcription stability of the PLOH gene. Nevertheless, the hypothesis of mRNA degradation by the NMD system could not be ruled out. The NMD is a widespread quality control mechanism in eukaryotic cells. Approximately, 30% of human genetic diseases are caused by transcripts containing PTCs. These transcripts are potential targets of NMD. For monogenic diseases, NMD has effects on the phenotype or the mode of inheritance (28). Indeed, incorrect splicing of downstream genes may inhibit expression through this mechanism, thus resulting in a total absence of pol η protein.

The lack of pol η in our patients would promote the accumulation of mutations in tumor suppressor genes and oncogenes and could explain the early onset of tumors in XP-V patients. This hypothesis is consistent with clinical features exhibited by our patients. In fact, their severe phenotype could result from overexposing to sun and absence of UV protection, thereby promoting further accumulation of mutations and by consequence, developing skin cancers. Molecular investigation of XP27, XP69 and XP67 families allowed identification of the genetic basis of the severe form of XP-V in Tunisian population. Hence, members of these families will benefit from genetic counseling. The identification of c.660+1G>A mutation at a heterozygous state in XP69-2 will provide her the possibility to perform prematernal counseling particularly if her partner is of the same family.

In our study, we have reported on a novel splice site mutation, c.660+1G>A, in the donor site of intron 5 in PLOH gene. This mutation likely results in the degradation of the mutant mRNA by the NMD pathway and therefore could explain the severe clinical phenotype.

Acknowledgements

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