Germline and Somatic Mutations of the INK4a-ARF Gene in a Xeroderma Pigmentosum Group C Patient

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Xeroderma pigmentosum is an inheritable autosomal recessive DNA repair deficient syndrome characterized by a high predisposition to skin cancers. An elevated proportion of tumors from xeroderma pigmentosum patients harbor ultraviolet-induced mutations (CC:GG > TT:AA tandem transitions) of the p53 and/or the INK4a-ARF genes. Here, we report the clinical and molecular features of a 12 y old xeroderma pigmentosum patient who, in addition to severe cutaneous clinical symptoms, also had three unusual tumors, a mediastinal lymphoblastic lymphoma, an atypical fibroxanthoma, and an epithelioid hemangiomma. Single strand conformation polymorphism and sequencing analysis of the p53 and INK4a-ARF genes were carried out in DNA from normal skin and different tumors (four actinic keratoses, two microinvasive squamous cell carcinomas, one basal cell carcinoma, and one atypical fibroxanthoma) from the patient. After characterization of the xeroderma pigmentosum C complementation group, we found unexpectedly that this patient also carried a germline mutation of the INK4a-ARF locus affecting the p16INK4A reading frame. Three different somatic mutations that all harbor the signature of ultraviolet light (two of p16INK4A and one of p53) were also detected in the basal cell carcinoma. We hypothesize that the germline mutation of p16INK4A, in association with the nucleotide excision repair defect, could explain the patient’s unusual phenotype. Furthermore, this study confirms that concomitant somatic mutations of INK4a-ARF and p53 occur in some xeroderma pigmentosum associated tumors, and seem to accumulate during tumor progression rather than the initiation step.

this locus are common in SCC lines and in SCC (Loughran et al., 1994).

-p63\textsuperscript{in} is inactivated during keratinocyte immortalization (Loughran et al., 1994; Munro et al., 1999). Moreover, nulligous INK4a-ARF (\textsuperscript{-/-}) or specifically ARF (\textsuperscript{-/-}) mice are susceptible to the development of SCC (Serrano et al., 1996; Kamijo et al., 1997). UV-induced INK4a-ARF mutations have been detected in 12.5% of sporadic epithelial skin tumors (Soufir et al., 1999). XP skin carcinomas show a higher frequency of INK4a-ARF mutations (29% of XP associated skin tumors) that are often multiple and associated in the same tumors with p53 mutations (Soufir et al., 2000b). Little is known, however, about the timing of INK4a-ARF mutation during the multistep process of skin carcinogenesis. To gain insight into the role of this locus in skin carcinogenesis, we performed a molecular analysis of skin tumors, of different histologic nature or stage, arising in a single XP patient who displays an unusual phenotype. Strikingly, we found that this patient, in addition to belonging to the XPC group, carries a germline mutation on the p63\textsuperscript{in} gene. This is the first report of a XPC patient presenting a p63\textsuperscript{in} germline mutation. Among the tumors, the only BCC carried somatic mutations in INK4a-ARF and p53, suggesting that these modifications occurred late in tumor progression rather than the initiation stage.

MATERIALS AND METHODS

DNA repair analysis and complementation test Unexposed XP skin biopsies were cultured to establish diploid fibroblast lines and examined for DNA repair level (unscheduled DNA synthesis, UDS) after UV irradiation as described previously (Sarasin et al., 1992). In order to confirm at the molecular level the diagnosis of XP, and to determine which of the seven XP genes involved in NER is deficient in our patient, we carried out genetic complementation of his skin diploid fibroblasts using infectious retroviral particles expressing the wild-type XP cDNA (Carreau et al., 1995; Zeng et al., 1997). The level of DNA repair of the patient was quantified by incorporation of thymidine (UDS) in the DNA of his skin fibroblasts after increasing UVC doses.

Analysis of the XPC group by western blotting analysis Protein was extracted in 8 M urea buffer as described (Sybert et al., 1985). Twenty micrograms of protein was run through a 6% acrylamide-glycerol at room temperature or without glycerol at

Tumor samples, normal skin, and DNA extraction Eight skin neoplasms (four actinic keratosis, two microinvasive SCC, one basal cell carcinoma, and one atypical fibroxanthoma) from the XP patient were collected following surgical resection. One part was immediately fresh frozen in liquid nitrogen and stored at -80°C. A second part was formalin fixed and paraffin embedded, and histopathologic assessment was performed. A biopsy was also obtained of nonsun-exposed normal skin of this XP patient. DNA was prepared from frozen samples as previously described (Daya-Grosjean et al., 1993).

Single strand conformation polymorphism (SSCP) analysis Coding sequence and flanking intronic sequences of exon 1\textsubscript{a}, exon 1\textsubscript{b}, and exon 2 of the INK4a-ARF gene were analyzed by polymerase chain reaction (PCR)-SSCP. Primer sequences for exons 1\textsubscript{a} and 2 were previously described (Soufir et al., 1999, 2000a). Exon 1\textsubscript{b} was analyzed through two overlapping PCR products generated by using the following primers: P1F1 \textsuperscript{5’}-TCAAGGAGGGCAGGGTTGC-3’; P1R1 \textsuperscript{5’}-GCCGCGGGA TGTTGAACCA-3’ (PCR: product 245 bp); P2F2 \textsuperscript{5’}-GCCGGGAGT GTGGAATT-3’ and P2R2 \textsuperscript{5’}-CAGCGGCTTATCTC-3’ (PCR product 287 bp). Exons 4-9 of p53 gene were amplified using the same conditions as previously described (Soufir et al., 1999). Total PCR reaction volume was 20 \muL, including 50–100 ng of genomic DNA template, 30 pmol of each primer, 0.5 U (international unit) of Taq polymerase (Gibco-BRL) and 0.1 \muL of \(\left[\textsuperscript{32}P\right]\) deoxyctydine triphosphate (Amersham). Final MgCl\textsubscript{2} concentration was 1.5 mM for all PCR reactions. All reactions were supplemented with 10% dimethyl sulfoxide for optimal PCR amplification. PCR was processed with an annealing temperature of 60°C for the three studied exons of the INK4a-ARF gene, and 55°C for exons 4–9 of p53. For SSCP, PCR products were migrated on two 0.1 × TBE Hydrolink MDE gels (FMC Bioproducts, Têbù, France), with either 8% glycerol at room temperature or without glycerol at +4°C. Gels were run at 8 W, either 14 h at room temperature or 12 h at 4°C, dried, and exposed to autoradiography. The entire procedure was repeated at least twice for each sample. Shifted bands were cut out of the gel, and amplified using the primers mentioned above.

Sequence analysis Products from 50 \muL PCR reactions from reamplified shifted bands and tumoral genomic DNA were purified using a Microcon 100 (Amicon, Millipore, France) and sequenced using dye-labeled terminator on an automated sequencer 310 (Applied Biosystem, Applera, France) according to the manufacturer’s instructions.

RESULTS

Clinical presentation A 12 y old boy from Algeria had typical clinical features of XP (i.e., pronounced photosensitivity from the age of 3 y, erythema of photo-exposed skin, poikiloderma, cutaneous xerosis, photophobia, and bilateral conjunctivitis). Starting at the age of 4, multiple skin neoplasms appeared progressively on sun-exposed areas (actinic keratosis, SCC, and BCC).

At the age of 7, the patient presented with acute respiratory syndrome leading to the discovery of a T cell lymphoblastic lymphoma involving the anterior mediastinum, thymus, pericardium, and left pleura. Blood analysis showed 40 × 10\textsuperscript{9} per ml lymphocytes with 50% blasts. Immunophenotyping revealed that these lymphocytes were CD1a\textsuperscript{+}, CD4\textsuperscript{+}, CD7\textsuperscript{+}, CD8\textsuperscript{+}, HLA-DR\textsuperscript{+}, and weakly CD3\textsuperscript{+}. All B markers were negative. A bone marrow analysis showed low cell counts without blasts. Highly carcinogenic drugs (i.e., antracyclins, alkylating agents, or cyclophosphamide) were considered contraindicated in this patient and a milder treatment regimen, including corticosteroids, Aracytin and Purinethol was started and well tolerated. The patient did not receive radiotherapy. After the initiation cure followed by several consolidation cures, the patient was considered in complete remission and has been stable since 1997.

At the age of 10, this patient had two unusual tumors: an atypical fibroxanthoma localized on the left ear and an epiteliod hemangiomia of the neck that were surgically removed.

DNA repair deficiency A near to wild-type recovery of UV cell survival was observed solely after transduction of the XPC cDNA (Fig 1). In contrast, transduction of other XP cDNA (as exemplified in Fig 2 using the XPD cDNA) did not result in the recovery of UV cell survival (Fig 1). The level of repair defect has been quantified by UDS after UV irradiation of the diploid fibroblasts of the patient, as previously described (Sarasin et al., 1992). As shown in Fig 2, the UDS level is only 10–15% of our control normal fibroblast lines and similar to a control XPC fibroblast line.

This allowed us to conclude that our patient belongs to the XPC group. The patient's fibroblasts complemented by the wild-type XPC gene exhibit normal UDS (not shown).

Recovery of XPC protein expression in stable XPC-transduced XPC keratinocytes Our complementation data (Figs 1 and 2) strongly supported the apparenance of XP208VI to the XPC group. To support further these functional observations, protein extracts were prepared from WT (198VI), XP208VI, and XPC-transduced XP208VI fibroblasts. A
Figure 1. Genetic complementation and UV cell survival of the patient's skin fibroblasts. Relative cell survival is indicated as the ratio of the number of colonies in irradiated to nonirradiated cells. UV doses are indicated. Note that transduction of the patient's fibroblasts (XP208 VI) by the XPC cDNA (XP208VI+LXPCSN) but not by XPD cDNA (XP208 VI+LXPDSN) leads to near normal UV cell survival (198 VI), thus assigning the patient to the XP complementation group C.

keratinocyte strain, XPI48VI, known to express a truncated XPC protein and its XPC-transduced counterpart (Arnaudeau et al, 2002), were used as control. Protein were processed for western blot analysis using the anti-XPC rabbit anti-serum (Fig 3). The apparently full length XPC protein (125 kDa) was detected in a WT control cell (198VI). In contrast, the XPC protein was neither detected in extracts from XP208VI nor, as already shown, in extracts from the XPC control cell strain XPI48VI (Arnaudeau et al, 2002). In both XP cell strains (XP208VI, XPI48VI) the absence of XPC protein detection indicated the absence of the XPC epitope recognized by the anti-serum raised against the carboxyterminal part of the XPC protein. On the contrary, XPC-transduced cells, i.e., XP208VI+XPC and XPI48VI+XPC, expression of the apparently full length XPC protein was recovered as compared with that detected from WT 198VI extracts. As in most other XPC patients studied to date (Chavanne et al, 2000), the absence of the full length XPC protein in XP208VI cells could be considered as a definitive assignment to the XPC complementation group.

Figure 3. Absence of the full length XPC protein in XP208VI patient cells. Western blot analysis was carried out using protein extracts from XP208VI cells before XP208VI (XPC) and after retroviral transduction (XP208VI+XPC). 198VI (WT control) is a normal fibroblast strain. XPI48VI (XPC) is a keratinocyte strain known to belong to the XPC complementation group. XPI48VI+XPC are XPI48VI cells following retroviral transduction of XPC (Arnaudeau et al, 2002). Note that XPC is not detected in extracts from XPI48VI and XP208VI (studied patient), whereas the full length protein is re-expressed in transduced cells as in XP98 WT control. The same membrane was stripped and then rehybridized using anti-β-catenin monoclonal to control that similar amounts of protein were present in this area of the membrane. The star indicates a nonspecific band found in any condition.

Mutational analysis of the INK4a-ARF locus SSCP analysis revealed abnormal bands for exon 1a only in one tumor (sample 7), the BCC. In exon 2, a common abnormal migration pattern was observed from all DNA analyzed (tumoral, normal skin, and normal fibroblast cell line), with an additional bandshift in tumor sample 7, the BCC. No abnormal band was seen for exon 1β of p14ARF DNA sequencing of abnormal bands revealed three mutations located within the cDNA sequence (Table I and Fig 4). One was characterized as a germline mutation, as it was present in DNA from all tumoral samples as well as from the patient's normal skin and fibroblast cell line. This germline mutation was a G>T transversion resulting in a mis-sense mutation in the p16INK4a reading frame (Ala127Ser). In contrast, this mutation had no consequence on p14ARF, as it lies outside its coding sequence (Table I). The two other alterations are somatic mutations of the BCC (tumor 7), which are characteristic UV-induced mutations occurring at dipyrimidic sites. One was a CG>TA transition located in the 5′ untranslated sequence of p16INK4a cDNA, possibly corresponding to a regulatory mutation. The second was a tandem CCG>TTAA transition and corresponds to a known mutational hotspot in XP-associated skin tumors (Soufir et al, 2000b). This mutation also affects the p14ARF reading frame, at a codon conserved between mouse and human cDNA. The nucleotide composition of this codon (CCC) makes it a prime target for mutagenesis by UV.

Figure 2. Severe alteration of DNA repair capacity of the patient's fibroblasts revealed by UDS after UVB irradiation. Number of autoradiographic grains over nuclei of the patient's and control fibroblasts were plotted as a function of the UVB dose. Note that the patient's fibroblasts revealed by UDS after UVB irradiation.

Table I

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Sample</th>
<th>Exon</th>
<th>Type</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>G&gt;T</td>
<td>7</td>
<td>2</td>
<td>Mis-sense</td>
<td>Ala127Ser</td>
</tr>
<tr>
<td>CG&gt;TA</td>
<td>7</td>
<td>1a</td>
<td>Mis-sense</td>
<td></td>
</tr>
<tr>
<td>CCG&gt;TTAA</td>
<td>7</td>
<td>1a</td>
<td>Somatic</td>
<td></td>
</tr>
</tbody>
</table>

Mutational analysis of the p53 gene SSCP analysis revealed a unique bandshift in exon 7 of the p53 gene, in BCC 7, corresponding to a tandem CCG>TTAA transition that resulted in a mis-sense mutation at codon 248 of the p53
Table I. p16\textsuperscript{INK4a}, p14\textsuperscript{ARF}, and p53 mutations characterized in XPC skin epithelial tumors

<table>
<thead>
<tr>
<th>Samples analysed</th>
<th>Histological Diagnosis</th>
<th>INK4a-ARF Sequence change</th>
<th>p16\textsuperscript{INK4a} aa change</th>
<th>p14\textsuperscript{ARF} aa change</th>
<th>p53 sequence change</th>
<th>p53 aa change</th>
<th>Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin tumors</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>microinvasive SCC</td>
<td>tcGca &gt; tcTca</td>
<td>Ala127Ser</td>
<td></td>
<td></td>
<td></td>
<td>lip</td>
</tr>
<tr>
<td>2</td>
<td>AK</td>
<td>tcGca &gt; tcTca</td>
<td>Ala127Ser</td>
<td></td>
<td></td>
<td></td>
<td>forehead</td>
</tr>
<tr>
<td>3</td>
<td>AK</td>
<td>tcGca &gt; tcTca</td>
<td>Ala127Ser</td>
<td></td>
<td></td>
<td></td>
<td>ear</td>
</tr>
<tr>
<td>5</td>
<td>microinvasive SCC + AK</td>
<td>tcGca &gt; tcTca</td>
<td>Ala127Ser</td>
<td></td>
<td></td>
<td></td>
<td>vertex</td>
</tr>
<tr>
<td>6</td>
<td>AK</td>
<td>tcGca &gt; tcTca</td>
<td>Ala127Ser</td>
<td></td>
<td></td>
<td></td>
<td>vertex</td>
</tr>
<tr>
<td>7</td>
<td>nodular BCC</td>
<td>tcGca &gt; tcTca</td>
<td>Ala127Ser</td>
<td></td>
<td></td>
<td>Ala128 Ala/</td>
<td>right eye</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gcCCgt &gt; gcTTgt</td>
<td>Prol41Leu</td>
<td></td>
<td></td>
<td>Arg129Cys</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Atypical fibroxanthoma</td>
<td>gcGgg &gt; gtgg (−69 atg)</td>
<td>5’UTR, none</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal skin</td>
<td></td>
<td>tcGca &gt; tcTca</td>
<td>Ala127Ser</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibroblastline</td>
<td></td>
<td>tcGca &gt; tcTca</td>
<td>Ala127Ser</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4. Germline and somatic mutations of the INK4a-ARF gene. Arrows above the automated sequence traces indicate the positions of mutated bases. The wild-type sequence and the nucleotide changes, and the coding effect of the changes on p16\textsuperscript{INK4a} and p14\textsuperscript{ARF} are listed in Table I. The sequence is shown 5’→3’ for the coding strand. The sequence is written 5’→3’ for the coding strand; the mutated base is written in upper case letters. The text in bold indicates nucleotide and amino acid changes. The text in italics, indicates the germline p16\textsuperscript{INK4a} mutation. Mutations indicated by an asterisk are predicted to be UVB-induced mutations. Mutations on conserved residues of p14\textsuperscript{ARF} are underlined.

gene (Table I). This mutation is a p53 hotspot found predominantly in skin cancers from XP patients (Beroud and Soussi, 1998).

**DISCUSSION**

To gain insight into the role of the INK4a-ARF and p53 genes in XP skin carcinogenesis, we performed a molecular analysis of the INK4a-ARF and p53 genes in skin tumors with different characteristics and stages arising in a single XP patient that displayed a particularly severe phenotype.

Remarkably, our XP patient developed three unusual tumors. To the best of our knowledge, non-Hodgkin lymphoblastic mediastinal lymphoma has never been described in a XP patient (Kraemer et al, 1987). Epithelioid hemangioendothelioma arising in the skin is extremely rare, and was also not previously described in XP patients (Kraemer et al, 1987). Atypical fibroxanthoma is a pleomorphic tumor usually classified as a distinct subtype of malignant fibrous histiocytoma that usually occurs on actinically damaged skin in the elderly, only rarely reported in XP patients (Patterson and Jordan, 1987; Dilek et al, 2000).

Unexpectedly, this patient was found to carry a p16\textsuperscript{INK4a} germline mutation (Ala127Ser) characterized by its presence in normal skin and a fibroblast cell line as well as in all the tumor samples analyzed. Several arguments convey toward a pathogenic effect of this mutation. First, this mutation is located in the fourth ankyrin domain of the protein, a domain crucial in p16\textsuperscript{INK4a} function. Secondly, this mutation was reported as a germline mutation in patients that developed different types of cancer: melanoma (Gruis et al, 1995); lung cancer (Okamoto et al, 1994); and primary human esophageal tumors (Zhou et al, 1994; Suzuki et al, 1995). Furthermore, in Suzuki et al (1995), this mutation was associated with loss of the normal remaining allele in the esophageal tumor. Alal27Ser was also characterized as an acquired somatic mutation in a SCC of the bladder (Gonzalez-Zulueta et al, 1995). Some functional studies did not find any effect of this variant on CDK4 binding (Koh et al, 1995; Ruas et al, 1999). A recent study, however, reported that testing of p16\textsuperscript{INK4a} interactions with CDK in protein binding assays is an unreliable predictor of mutant p16\textsuperscript{INK4a} function in cells (Becker et al, 2001). Indeed, in one study, this Alal27Ser allele was intermediate in its ability to cause growth cell arrest (Koh et al, 1995). Altogether, these data indicate that Alal27Ser may be a p16\textsuperscript{INK4a} germline mutation rather than a polymorphism. In addition, our hypothesis that abrogation of p16\textsuperscript{INK4a} could probably have contributed to the patient’s particularly severe phenotype is reinforced by previous observations. First, germline defects of the INK4a-ARF locus might predispose to hematologic malignancies: (i) a germline mutation of p16\textsuperscript{INK4a} that was recently shown to predispose a patient to multiple myeloma, which is another hematopoietic disorder (Dilworth et al, 2000), and (ii) mice nullizygous or heterozygous for the INK4a-ARF locus are highly prone to lymphoid malignancies as well as reticulosarcomas (Kamijo et al, 1997). Second, inactivation of the INK4a-ARF locus occurs commonly in human lymphoid disorders (Ruas and Peters, 1998) as well as in malignant fibrous histiocytoma, a tumor histologically related to atypical fibroxanthoma (Brinck et al, 1998; Simons et al, 2000).

Three somatic mutations were also detected, one in the p53 gene, and two in the INK4a-ARF gene, that were all found in only one of eight (12.5%) skin tumors. This is an overall low mutation frequency. This contrasts with the high frequency of p53
mutations detected in normal skin, precancerous, and cancer lesions arising in XP patients (Williams et al., 1998). This discrepancy may have several explanations. First, it should be noted that not all but one tumor (85%) were small lesions; i.e., either precancerous lesions (actinic keratinosis in 57% of cases) or microinvasive SCC (28% of cases). In line with this, in a previous study, XP skin tumors less than 8 mm in diameter were shown to harbor a significantly lower frequency of p53 mutation than larger tumors from the same XP patients (Matsumura et al., 1995). Additionally, our tumor was not microdissected and this could account for a lower sensitivity of mutation detection. Finally, in our particular case, the presence of a p16NK4A germline mutation could have decreased the need for additional p53 mutations.

Moreover, we previously detected INK4a-ARF mutations in only one of six sporadic SCC of early grade (Bowen diseases or actinic keratosis; Soufr et al., 1999), which correlate with our present results and seem to signify that such mutations occur in the late stage of tumor development.

All three mutations detected in the BCC were characteristic of UV-induced lesions, occurring at dipyrimidinic sites, and located in two cases on the nontranscribed strand of the gene. The third mutation is due to an unrepairable DNA lesion that lies outside the transcribed sequence and therefore is not preferentially repaired. This mutational pattern is highly characteristic of XPC patients, proficient in preferential repair of active transcribed genes (Gigli et al., 1998; Soufr et al., 2000b). It is important to note that mutations of the three genes (p53, p16NK4A, and p14ARF) occurred in the same tumor. This association was previously found in XP tumors (Soufr et al., 2000b), and is probably related to the high genetic instability linked to the DNA repair defect in these patients. The accumulation of unrepaired mutations leads to the preferential selection of tumoral clones sharing inactivation of multiple tumor suppressor pathways.

In conclusion, we show for the first time that the coexistence of germline inactivation of a DNA repair gene and a cell cycle regulator gene that may have acted in concert to generate cancer proneness in a patient.

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