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Severe PATCHED1 Deficiency in Cancer-Prone Gorlin Patient Cells Results in Intrinsic Radiosensitivity

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Summary

Gorlin syndrome is a typical case of debated hypersensitivity to radiation, although it is well-recognized as a cancer-prone disorder. The present data reveal that only Gorlin cells presenting severe deficiency in PTCH1 gene expression exhibited significantly increased cellular radiosensitivity and DNA damage response gene expression (H2AX and 53BP1 foci assay). PTCH1

Purpose: Gorlin syndrome (or basal-cell nevus syndrome) is a cancer-prone genetic disease in which hypersusceptibility to secondary cancer and tissue reaction after radiation therapy is debated, as is increased radiosensitivity at cellular level. Gorlin syndrome results from heterozygous mutations in the PTCH1 gene for 60% of patients, and we therefore aimed to highlight correlations between intrinsic radiosensitivity and PTCH1 gene expression in fibroblasts from adult patients with Gorlin syndrome.

Methods and Materials: The radiosensitivity of fibroblasts from 6 patients with Gorlin syndrome was determined by cell-survival assay after high (0.5-3.5 Gy) and low (50-250 mGy) γ-ray doses. PTCH1 and DNA damage response gene expression was characterized by real-time polymerase chain reaction and Western blotting. DNA damage and repair were investigated by γH2AX and 53BP1 foci assay. PTCH1

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that the PATCHED1 protein had a direct role in regulating intrinsic radiosensitivity after both high and low radiation doses. PATCHED1 level may thus provide a prognostic screen for radiosensitive patients with PTCH1 heterozygous mutations.

Introduction

At least 15 different genetic disorders are now associated with increased cellular radiosensitivity (1). Gorlin syndrome is a typical case of debated cellular hypersensitivity, although it is well-recognized as a cancer-prone disorder. Gorlin syndrome is mainly caused by heterozygous mutations in the PTCH1 gene, which codes for the Sonic hedgehog (SHH) receptor (2, 3), because mutations in this gene have been described in around 60% of patients with a Gorlin phenotype (4, 5). We investigated the correlations between cellular radiosensitivity and PTCH1 gene expression in fibroblasts isolated from adult patients with Gorlin syndrome.

Gorlin syndrome, or basal cell nevus syndrome (BCNS), is an autosomal dominant inherited disease with prevalence varying from 1 in 57,000 to 1 in 256,000 and is characterized by developmental abnormalities and a predisposition to skin neoplasms and medulloblastoma, as reviewed by Lo Muzio (6). Because PATCHED1 is a repressor of the SHH signaling pathway through its interaction with the smoothened protein, it has been proposed to act as a tumor suppressor. Consequently, mutations of the second allele of PTCH1 result in tumor formation (7). To study molecular events and basal cell carcinoma (BCC) appearance associated with Ptc1 mutations, several murine models with heterozygous mutations, spontaneous or generated, have been described and are reviewed by Saran and Nitzki et al (7, 8). In addition to developmental pattern issues, individuals with PTCH1 mutations originally showed spontaneous medulloblastomas (9) and soft-tissue tumors such as rhabdomyosarcomas, with an incidence depending on the genetic background (10). After ionizing radiation (IR), Ptc1 heterozygous embryos exhibit a higher frequency of IR-induced developmental defects compared to their wild-type littermates (10), suggesting that Ptc1+/− mice are more sensitive to radiation. Since then, Ptc1 knockout mice have been useful models for molecular events involved in BCC development upon irradiation (11), as well as for adverse tissue reaction occurrence, such as cataract (12).

The first evidence that IR dramatically increases the incidence of tumors in patients with Gorlin syndrome was reported more than 40 years ago in patients with cancer (13), notably those treated for medulloblastoma, and this has since been the focus of several case reports (14-17). In particular, children with BCNS appear to be at high risk of developing multiple BCCs in irradiated areas, usually from 6 months to 3 years after radiation therapy (13, 18). Consequently, minimizing IR exposure and using nonionizing imaging modalities when possible is recommended (19). However, this susceptibility to IR-induced cancer is more controversial in adults, and several authors have reported patients who did not develop secondary BCC after multiple radiation therapy treatments (20, 21). In addition, patients with Gorlin syndrome may be prone to tissue reactions after radiation therapy, although this clinical aspect has been poorly documented. At the cellular level, data are again inconsistent, with some groups reporting decreased cell survival after radiation exposure (22-24) and others not reporting this (25-27).

In the present study, we show that only Gorlin cells presenting severe deficiency in PTCH1 gene and protein expression exhibited a significant increase in radiosensitivity, which suggests an explanation for the conflicting results so far reported concerning the response to radiation in cells from patients with Gorlin syndrome. Furthermore, our molecular data provide evidence for a direct role of the PATCHED1 protein in the regulation of intrinsic radiosensitivity.

Methods and Materials

Cells from patients with Gorlin syndrome

Nonimmortalized dermal fibroblasts from 6 adult patients with Gorlin syndrome (GM0-; age 27 to 58 years, 3 men and 3 women; see details in Table E1; available online at...
PTCH1 next-generation sequencing mutation screening

PTCH1 mutation screening was performed through 2 different next-generation sequencing procedures: exonic pyrosequencing with the Roche GS Junior System at CEA-CNRS (Evry, France) for transcripts NM_1083602 and NM_1083603 (PTCH1_M and L’ isoforms) and sequencing by synthesis following coding sequence capture on an Illumina Miseq benchtop sequencer (Illumina, San Diego, CA) at the Bergonié Institute (INSERM, Bordeaux, France) for transcript NM_000264 (PTCH1_L isoform) (Methods E1; available online at https://doi.org/10.1016/j.ijrobp.2018.05.057).

γH2AX and 53BP1 foci assays

For γH2AX (28), cells were irradiated with 3 Gy by using a 137Cs γ-ray source (dose rate, 1.02 Gy/min⁻¹), further cultured for the indicated times, fixed with 4% formaldehyde (paraformaldehyde) and permeabilized with 0.2% Triton X-100, followed by staining with 4’-6-diamidino-2-phenylindole dihydrochloride and γH2AX antibody. The average number of separate γH2AX foci was assessed on at least 300 cells using the Cellomics ArrayScan VTI (ThermoFisher). For 53BP1, cells were irradiated with 3 Gy by using an x-ray source (XRAD 320; Precision X-Ray, North Branford, CT), further cultured for the indicated times, fixed on coverslips (3% paraformaldehyde, 2% sucrose phosphate-buffered saline [PBS]) and permeabilized (20 mM HEPES pH 7.4, 50 mM NaCl, 3 mM MgCl₂, 300 mM sucrose and 0.5% Triton X-100). Coverslips were incubated with 5% goat serum PBS for blocking, before immunostaining with anti-53BP1 antibody in 2% goat serum goat serum PBS. Foci in 50 cells were counted using an Eclipse Ti-E inverted microscope (Nikon). (See Methods E4 for detailed foci assays; available online at https://doi.org/10.1016/j.ijrobp.2018.05.057).

RNA interference

Fibroblasts from healthy donors were stably transduced with lentiviral vectors (Vectalys SA., Toulouse, France). Transductions of sh-PTCH1-GFP and sh-scramble-GFP were performed on fibroblasts at ~40% confluence. Cells were incubated for 6 hours with lentiviral particles (multiplicity of infection at 20) in the presence of hexadimethrine bromide at 4 μg/mL. (Sigma). After 3 days, transduced cells (efficiency 60%-98%) were sorted by flow cytometry (MoFlo; Beckman Coulter, Brea, CA) according to green fluorescent protein fluorescence and amplified for 1 week before analysis.

Statistics

The Student test was used. Significance was assessed according to a normal distribution law, and means were considered significantly different if $Z_0 > 1 [Z_0 = (\text{mean } X - \text{mean } Y)/\sqrt{\sigma (\text{mean } X)^2 + \sigma (\text{mean } Y)^2}]$ where...
Fig. 1. A subpopulation of highly PTCH1-deficient Gorlin cells is radiosensitive. (A) Colony survival assays showed that GM0-1657, -1552, and -2138 cells exhibited significantly greater sensitivity to high doses than cells from 3 healthy donors (HNF 1-3 in black, averaged); 6 Gorlin cell strains (GM0-) were studied in 3 independent experiments, each with 3 to 6 replicates. (B) Parameters of the survival curves. HNF: mean for cells from 3 healthy donors. GM0-: cells from Gorlin patients. SF2 is the survival fraction after 2 Gy. D0 is the dose for which 37% survival was observed. (C) Colony survival assays showed hypersensitivity after low radiation doses for GM0-1657, -1552, and -2138; (dose rate, 50 mGy/min; survival values in Table E4; available online at https://doi.org/10.1016/j.ijrobp.2018.05.057); 3 independent experiments, each with 3 to 6 replicates. (D) PTCH1 mRNA levels (L isoform) measured in Gorlin cells by real-time quantitative polymerase chain reaction on 3 replicates and compared with the mean value for 3 normal cells, normalized to 100. (E) Representative image of
Results

Cells from 3 patients with Gorlin syndrome are radiosensitive

Colony survival assays showed that cells from only 3 patients with Gorlin syndrome (GM0-1657, -1552, and -2138) out of the 6 cell strains examined exhibited significantly greater sensitivity to medium and high doses (Fig. 1A and B) than cells from 3 healthy donors (HNF 1-3). Similar data were found after low doses (Fig. 1C and Table E4; available online at https://doi.org/10.1016/j.ijrobp.2018.05.057), within a window ranging from 50 to 250 mGy, suggesting a low-dose hypersensitivity response. Although a tendency to hypersensitivity between 100 and 250 mGy was observed for all Gorlin cells, survival reduction was significant only for the 3 cell strains sensitive to high doses (patients GM0-1657, -1552, and -2138).

Marked PTCH1 gene expression deficiency correlates with radiosensitivity

The radiosensitive cells (GM0-1657, -1552 and -2138) exhibited significantly less PTCH1 messenger RNA (mRNA) than cells from the 3 healthy donors (19%, 25%, and 21%, respectively; n = 6, P < .01) (Fig. 1D). Similarly, in protein level, PTCH1 expression was lower in the radiosensitive cells (22%, 25%, and 39% respectively, n = 3, P < .01) (Fig. 1E). Hedgehog signaling was also affected in these cells, notably with a reduced expression of the GLI2 transcription factor (Fig. E1; available online at https://doi.org/10.1016/j.ijrobp.2018.05.057).

Variable severity of PTCH1 mutations

Genetic studies (Table 1) revealed that cells from 5 patients with Gorlin syndrome exhibited at least 1 heterozygous mutation in the PTCH1 gene, but with different types and locations (Fig. 1F). Four of these mutations were not previously reported. For GM0-1657 and GM0-2138, the insertion and deletion events in exon 2 led to a premature stop codon in exon 3, which predicts nonsense-mediated mRNA decay for the mutated transcript or a strongly truncated protein that is probably rapidly degraded. For GM0-1575, -1725, and -2098, the observed mutations predicted more limited defects for the PATCHED1 protein. For 1 patient (GM0-1552), a variant was detected in the 5′UTR region, common to GM0-1575, 1725, and 2098, but no mutation was found in the coding regions of PTCH1.

Defective DNA damage response signaling in radiosensitive Gorlin cells

For the ATM/CHK2/P53 pathway, downregulations of protein expression were found at a basal level in the radiosensitive cells as compared to controls (Fig. E2; available online at https://doi.org/10.1016/j.ijrobp.2018.05.057) and after 2 Gy irradiation (Fig. 2A). Furthermore, activation by irradiation was impaired, notably for phospho-CHK2 and phospho-P53, and this deficiency was observed in all the radiosensitive cells (Fig. 2B), which may be due both to the reduced basal level and to an activation defect. For phospho-ATM, only GM0-1657 and -1552 were affected.

To monitor DNA double-strand break formation and repair, enumeration of γH2AX and 53BP1 foci was performed at 0, 0.25, 2, 6, 24, and 48 hours after 3 Gy irradiation and showed no difference between normal and Gorlin cells (Fig. 2C and D).

Induced PATCHED1 deficiency in normal cells results in increased radiosensitivity

PTCH1 expression was decreased in cells from healthy donors after infection with a lentiviral vector carrying a short hairpin RNA (shRNA) sequence targeting PTCH1, with a mean reduction of at least 60% for mRNA and around 50% for the protein (Fig. 3A, 3B, and 3F). Colony survival assays showed that cells from 3 different healthy donors transduced with sh-PTCH1 lentivector showed a significantly smaller survival fraction at 2 Gy, whereas cells receiving a scramble sh vector did not differ from controls (Fig. 3C and Fig. E3; available online at https://doi.org/10.1016/j.ijrobp.2018.05.057). Furthermore, irradiation between 0.5 and 3.5 Gy resulted in a survival curve close to that of the radiosensitive cells from patients with Gorlin syndrome (Fig. 3D). Moreover, induced PATCHED1 deficiency in cells from healthy donors resulted in increased sensitivity to low doses of IR (100 and 200 mGy, Fig. 3E and Fig. E4; available online at https://doi.org/10.1016/j.ijrobp.2018.05.057). Knock-down cells also showed defects in DNA damage response (DDR) pathways and notably decreased basal expression of CHK2 and P53 (Fig. 3F).
Investigate correlations between cellular radiosensitivity.

The present study, designed to investigate correlations between cellular radiosensitivity and Patched1 deficiency (29, 30). As a result, not all patients with A-T, including even homozygotes, are equally radiosensitive, testifying to the importance of defining target protein levels and activity for radiosensitive syndromes in each patient. To demonstrate the link between PATCHED1 and GLI2 regulation in cells from healthy donors resulted in reduced basal expression of DDR proteins, close to that found in the radiosensitive Gorlin cells. It can be hypothesized that PATCHED1 interacts directly with DDR proteins; this interaction is currently unverified and would deserve further studies. Also, a downstream member of the SHH pathway, affected by PTCH1 deficiency, may impair the integrity of the Golgi apparatus, which is involved in the normal function of GLI2; studies in the development of mouse embryos and NIH3T3 fibroblasts show that GLI2 regulation has a dominant influence on the overall SHH signaling dynamics (31). The downregulation of GLI2 that we show in the present study also strengthens this hypothesis.

Table 1. Genotyping analysis by next-generation sequencing

<table>
<thead>
<tr>
<th>Cell strain</th>
<th>Localization hg 19</th>
<th>Mutations and variants</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM0-2098</td>
<td>Chr9: 98221881 Intron 17</td>
<td>Splice site mutation (donor site +1) C&gt;G</td>
<td>Exon 18 skipping and stop codon in exon 19</td>
</tr>
<tr>
<td></td>
<td>Chr9: 98270646 5'UTR isoform L</td>
<td>Insertion T&gt; TGCC</td>
<td>Triplet insertion</td>
</tr>
<tr>
<td>GM0-1725</td>
<td>Chr9: 98268700 Exon 2</td>
<td>Missense mutation (c.383T &gt; L128P)</td>
<td>Protein structure modification</td>
</tr>
<tr>
<td></td>
<td>Chr9: 98270646 5'UTR isoform L</td>
<td>Insertion: T&gt; TGCC</td>
<td>Triplet insertion</td>
</tr>
<tr>
<td>GM0-1575</td>
<td>Chr9: 98218697 Intron 18</td>
<td>Splice site mutation (acceptor site -2) T&gt;G</td>
<td>Exon 19 skipping and stop codon in exon 20</td>
</tr>
<tr>
<td></td>
<td>Chr9: 98270646 5'UTR isoform L</td>
<td>Insertion: T&gt; TGCC</td>
<td>Triplet insertion</td>
</tr>
<tr>
<td>GM0-2138</td>
<td>Chr9: 98268800 Exon 2</td>
<td>Insertion (c.283 C&gt;CT )</td>
<td>Frameshift and stop codon in exon 3</td>
</tr>
<tr>
<td>GM0-1552</td>
<td>Chr9: 98270646 5'UTR isoform L</td>
<td>Insertion T&gt; TGCC</td>
<td>Triplet insertion</td>
</tr>
<tr>
<td>GM0-1657</td>
<td>Chr9: 98268792 Exon 2</td>
<td>Deletion GT&gt;G</td>
<td>Frameshift and stop codon in exon 3</td>
</tr>
<tr>
<td></td>
<td>Chr9: 98211572 Exon 22</td>
<td>Missense mutation (T4093A &gt; T1364S)</td>
<td>Protein sequence modification</td>
</tr>
<tr>
<td></td>
<td>Chr9: 98209213 Exon 23</td>
<td>Missense mutation (C4325T &gt; R1442Q)</td>
<td>Protein sequence modification</td>
</tr>
</tbody>
</table>

Five Gorlin cell strains harbor specific heterozygous mutations(s) in the PTCH1 gene, notably 2 splice site, 3 missense, and 2 frameshift mutations. Cells from 2 Gorlin patients (GM0-1657, -2138) present nonsynonymous mutations at the 5' end of the PTCH1 gene that predict higher protein deficiency as compared to the other cell strains. For 1 patient (GM0-1552), a variant was found in the 5'UTR region, which is common to GM0-1575, 1725, and 2098, but no mutations were detected in the coding regions of PTCH1. Locations are indicated using the human genome reference hg19.

Discussion

The radiosensitivity of patients affected by Gorlin syndrome is currently debated. The present study, designed to investigate correlations between cellular radiosensitivity and PTCH1 expression in the cells of patients with Gorlin syndrome, provides a possible explanation for the discrepant reported data. We show that the degree of PATCHED1 deficiency is variable among patient cells and that only severely decreased gene expression correlates with significantly increased radiosensitivity. Interestingly, we found that the increased sensitivity also affected cell response to low radiation doses, which had not been documented previously. Similarly to Gorlin syndrome, cell phenotypes from other radiosensitive syndromes are heterogeneous. For example, ATM genetic defects range from complete absence to some persistence of ATM kinase activity (29, 30). As a result, not all patients with A-T, including even homozygotes, are equally radiosensitive, testifying to the importance of defining target protein levels and activity for radiosensitive syndromes in each patient.

To demonstrate the link between PATCHED1 and radiation sensitivity at a molecular level, RNA interference-mediated PTCH1 deficiency was obtained in primary fibroblasts from different healthy donors, which systematically resulted in reduced cell survival after irradiation for both high and low radiation doses, suggesting that PTCH1 directly regulates cellular radiosensitivity.
A surprising finding of the present work concerns DNA damage and repair: PTCH1 defects seemed not to correlate with impaired double-strand break recognition, as revealed by the normal findings for γH2AX and 53 BP1 foci recruitment and kinetics. Based on these data, we propose that defective cell-cycle checkpoints and replication errors may be the key impaired processes in Gorlin cells, and this hypothesis is supported by the CHK2 anomalies consistently found both in patients and after shRNA-mediated PTCH1 downregulation.

Concerning genetic defects, for 2 radiosensitive patients with Gorlin syndrome, the mutations found in PTCH1 exon 2 result in stop codons, which explains the observed decrease in mRNA and protein expression. For one radiosensitive patient, the decreased mRNA and protein expression could not be explained by mutations in the coding regions of the PTCH1 gene. Several hypotheses can be suggested for this patient, notably mutations in other genes, such as those of the GLI family. Epigenetic mechanisms might also be involved, such as enhancer–related downregulation recently described in a case of genodermatosis (33) or inhibition by posttranscriptional regulatory networks such as LncRNAs or miRNAs (34, 35).

Databases report several enhancer elements associated with the PTCH1 gene, including 5 within the gene, and several long noncoding RNAs, which could all regulate its expression and deserve further investigation. The fact that no correlation has yet been demonstrated at the clinical level between genotypes and phenotypes in patients with Gorlin syndrome, as mutations in the same region or an identical mutation in 2 unrelated patients can lead to different clinical outcomes, supports the likely importance of epigenetic regulation for the PTCH1 gene.

Clinical consequences for radiation therapy and nuclear medicine can be extrapolated from the present findings. According to the Leiden Open Variation Database (36), at least 14% of patients with Gorlin syndrome with a mutation in the PTCH1 gene carry this mutation in the first 3 exons, and thus high gene expression deficiency and radiosensitivity can be expected in these patients, for whom the risk/
Fig. 3. PATCHED1 deficiency is directly associated with radiosensitivity. (A) PTCH1 messenger RNA levels measured by quantitative polymerase chain reaction after 3 independent infections of normal fibroblasts (HNF1 and HNF2: 2 healthy donors) with a lentiviral vector carrying either a short hairpin RNA (shRNA)-scramble sequence or a shRNA targeting PTCH1. Infected cells were compared with the mean value of the noninfected cells, normalized to 100. (B) Representative image of Western blot analysis with PATCHED1 antibody (L isoform) for normal fibroblasts (HNF1 and HNF2), either in control conditions or infected with lentiviral vectors (sh-scramble or sh-PTCH1). (C) Mean survival after 2 Gy was lower in the shRNA-PTCH1 condition than in the control condition (normalization to 100 vs the noninfected cells) but not in the shRNA-scramble condition; 3 independent experiments. (D) Mean survival was reduced after a range of radiation doses in sh-PTCH1 infected cells (gray) versus noninfected cells (HNF1, black), but not in the sh-scramble infected cells (dashed line); 6 flasks per dose, n = 1. (E) Colony survival assays showed that cells from healthy donor 1 (HNF1) transduced with sh-PTCH1 lentivector showed a reduced survival after low dose irradiation, whereas cells receiving control vector did not differ from controls. P = .0022. (F) Western blot analysis of basal levels of PATCHED1, CHK2, and P53 after PTCH1 knockdown in normal fibroblasts (HNF4), noninfected or infected with an sh-scramble or sh-PTCH1 vector (3 independent infections for each); a radiosensitive Gorlin cell strain (GM0-1552) was added for comparison, and actin was used as a loading control of total protein extracts. Significant at *P < .05 and **P < .01.
benefit ratio of IR for cancer therapy should be carefully evaluated. Because we found that the increased sensitivity also affected cell responses to low radiation doses, we suggest that repeated biomedical diagnostics using x-rays should also be carefully evaluated for these patients. A genetic test of blood samples to discriminate such at-risk patients might be developed and could be included in the development of systemic next-generation sequencing in the framework of personalized cancer genomic medicine. This approach might also be used for other genetic diseases associated with PTCH1 defects, such as microphthalmia or Hirschsprung syndrome.

In conclusion, the major finding of the present study is an inverse correlation PATCHED1 level and cellular radiosensitivity, affecting both high and low radiation doses. Furthermore, RNA interference-mediated PTCH1 deficiency in normal human cells resulted in reduced survival after irradiation, which directly links this gene to intrinsic radiosensitivity.

References