DNA REPAIR ALTERATIONS IN CHILDREN WITH PEDIATRIC MALIGNANCIES: NOVEL OPPORTUNITIES TO IDENTIFY PATIENTS AT RISK FOR HIGH-GRADE TOXICITIES

CLAUDIA E. RÜBE, M.D., PH.D.,* ANDREAS FRICKE, PH.D.,* RUTH SCHNEIDER, M.D.,* KARIN SIMON, M.D.*, MARTIN KÜHNE, PH.D.,* JOCHEN FLECKENSTEIN, M.D.,* STEFAN GRÄBER, PH.D.,† NORBERT GRAF, M.D., PH.D.,‡ AND CHRISTIAN RÜBE, M.D., PH.D.*

*Department of Radiation Oncology, †Institute of Medical Biometrics, Epidemiology and Medical Informatics, and ‡Department of Pediatric Hematology and Oncology, Saarland University, Homburg/Saar, Germany

Purpose: To evaluate, in a pilot study, the phosphorylated H2AX (γH2AX) foci approach for identifying patients with double-strand break (DSB) repair deficiencies, who may overreact to DNA-damaging cancer therapy.

Methods and Materials: The DSB repair capacity of children with solid cancers was analyzed compared with that of age-matched control children and correlated with treatment-related normal-tissue responses (n = 47). Double-strand break repair was investigated by counting γH2AX foci in blood lymphocytes at defined time points after irradiation of blood samples.

Results: Whereas all healthy control children exhibited proficient DSB repair, 3 children with tumors revealed clearly impaired DSB repair capacities, and 2 of these repair-deficient children developed life-threatening or even lethal normal-tissue toxicities. The underlying mutations affecting regulatory factors involved in DNA repair pathways were identified. Moreover, significant differences in mean DSB repair capacity were observed between children with tumors and control children, suggesting that childhood cancer is based on genetic alterations affecting DSB repair function.

Conclusions: Double-strand break repair alteration in children may predispose to cancer formation and may affect children’s susceptibility to normal-tissue toxicities. Phosphorylated H2AX analysis of blood samples allows one to detect DSB repair deficiencies and thus enables identification of children at risk for high-grade toxicities. © 2010 Elsevier Inc.

DNA repair, DNA double-strand breaks, Normal tissue toxicity, Predictive assay, Childhood cancer.

INTRODUCTION

Over the past decades, increasingly complex multimodality treatment protocols have led to tremendous improvements in the survival of children diagnosed with cancer. All cancer therapies developed to date are associated with early and late adverse effects, also known as normal-tissue toxicities. Normal-tissue responses show considerable variability among patients, whereas treatment-associated complications are not only related to the specific therapy used but may particularly be determined by the patient’s individual genetic predisposition (1–3). Most convincing evidence suggests that genetic alterations in proteins participating in the DNA damage response determine the individual risk of developing severe treatment-related side effects (4–6).

Deoxyribonucleic acid double-strand breaks (DSBs) are the most deleterious of all DNA lesions. If un repaired, DSBs can lead to loss of chromosome segments and threaten the cell’s survival. Double-strand breaks are generated naturally, for example by metabolic by-products of cellular respiration, but are also produced to a great extent when cells are exposed to DNA-damaging agents, such as ionizing radiation and certain chemotherapeutics (7, 8). Cells have evolved groups of proteins that function in signaling networks that sense DSBs, arrest the cell cycle, and activate the DNA repair pathways nonhomologous end-joining and homologous recombination (9). The ataxia–telangiectasia mutated (ATM) protein kinase is a critical component in these pathways and integrates the cellular response to DSBs by phosphorylating...
H2AX foci approach to identify patients with DSB repair deficiencies based on subtle heterogenous mutations in DNA damage response genes are expected to be more common in the human population.

In a clinical trial with children suffering pediatric malignancies, we evaluated the potential of the γH2AX foci approach to identify patients with DSB repair deficiencies, who may overreact to DNA-damaging cancer therapy. For this purpose, we applied the highly sensitive γH2AX foci approach to analyze DSB repair in blood lymphocytes after external irradiation of blood samples. This method is based on the detection of specific histone modifications, the phosphorylation of histone H2AX molecules, occurring on induction of DSBs (16). Phosphorylated H2AX, designated γH2AX, extends to megabase chromatin regions around the lesion and can be visualized by immunofluorescence microscopy as discrete nuclear foci reflecting sites of DSBs. The kinetic of γH2AX foci loss strongly correlates with the time course of DSB repair (17). In recent experimental studies, we could show that γH2AX foci analysis of blood lymphocytes provides precise information about the genetically defined DSB repair capacity, shown to be valid for different and complex organs in a given individual (18, 19). Moreover, previous clinical studies established the γH2AX foci approach as a sensitive technique to quantify the repair of radiation-induced DSBs in blood lymphocytes from individuals undergoing computed tomography examination (20). In that study, γH2AX analysis allowed us to verify a substantial DSB repair defect in a patient previously displaying severe side effects after radiotherapy, sustaining the close relationship between DSB repair deficiency and pronounced clinical radiosensitivity.

First, we analyzed blood samples of children with ataxia–telangiectasia (ATM−/− homozygote) and their heterozygous parents (ATM+/− heterozygote) to evaluate the feasibility of the γH2AX foci approach in the clinical setting to verify not only pronounced but also subtle, genetically defined DSB repair deficiencies. Subsequently, we investigated the DSB repair capacity of children with different solid tumors and monitored their treatment-associated normal-tissue responses. The primary objective was to evaluate the clinical potential of the γH2AX foci approach to identify patients with DSB repair deficiencies, as a screening tool in predictive testing for normal-tissue toxicities.

**METHODS AND MATERIALS**

**ATM−/− homozygote and ATM+/− heterozygote probands**

Three different families with 4 ATM−/− homozygote adolescents and 5 ATM+/− heterozygote parents were analyzed and compared with normal ATM+/+ individuals. The precise ATM gene mutations were previously identified and are listed in Table 1 (21).

**Patients**

Children with histologically confirmed solid tumors who received chemotherapy and/or radiotherapy in our departments between June 2006 and June 2008 were included in this study (n = 23; listed in Table 2) (sole exception: the patient HNEE was treated at Justus-Liebig-University Giessen and included on diagnosis of her third cancer). Exclusion criteria were previous radio-/chemotherapy within the last 3 months. Treatment-associated normal-tissue toxicities were documented according to the Registry for the Evaluation of Late Side Effects after Radiation in Childhood and Adolescence protocol (22). Age-matched healthy children served as controls (n = 24). Protocol procedures were approved by the local ethics committee, and all children and their parents provided written informed consent.

**Irradiation**

Whole-blood samples or isolated blood lymphocytes, respectively, were irradiated with 1 Gy or 2 Gy (X-ray: 90 kV, 19 mA; dose rate: 1 Gy/min). Repair kinetics were evaluated in isolated lymphocytes at 0.5, 2.5, 5, 8, and 24 h after irradiation.

**Immunofluorescence**

Lymphocyte separation was performed according to the manufacturer’s instructions (Percoll; PAA, Pasching, Austria). Isolated lymphocytes were fixed in formaldehyde, washed in Tween 20, and spotted onto coverslips. Samples were fixed in methanol, permeabilized in acetone, and incubated with anti-γH2AX antibody (Upstate, Charlottesville, VA) followed by Alexa Fluor 488–conjugated goat antimouse secondary antibody (Invitrogen, Karlsruhe, Germany). Afterward, samples were mounted in Vectashield with 4’,6-
<table>
<thead>
<tr>
<th>ID</th>
<th>Sex</th>
<th>Age at diagnosis (y)</th>
<th>Cancer histology</th>
<th>Cancer predisposition</th>
<th>DNA-damaging chemotherapy</th>
<th>Chemo-induced side effects</th>
<th>Radiotherapy (Gy)</th>
<th>Radiation-induced side effects</th>
<th>Abnormal DSB repair</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAEA</td>
<td>M</td>
<td>13</td>
<td>Hodgkin’s disease</td>
<td>—</td>
<td>CPM, DOX, PCB, VP-16</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>AITB</td>
<td>M</td>
<td>9</td>
<td>Glioblastoma</td>
<td>Turcot <em>PMS2</em> mutation</td>
<td>CCNU, TMZ</td>
<td>59.4 (1 × 1.8)</td>
<td>Grade 2 (skin)</td>
<td>Slightly impaired</td>
<td>—</td>
</tr>
<tr>
<td>BHJA</td>
<td>F</td>
<td>12</td>
<td>Chondrosarcoma</td>
<td>—</td>
<td>DACT, DOX, IDA, IFO, TFF, VP-16</td>
<td>50.4 (1 × 1.8)</td>
<td>Grade 0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>CRDL</td>
<td>M</td>
<td>9</td>
<td>Synovial sarcoma</td>
<td>—</td>
<td>DACT, DOX, IFO</td>
<td>44.8 (1 × 1.6)</td>
<td>Grade 0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>HNEE</td>
<td>F</td>
<td>5</td>
<td>Nephroblastoma</td>
<td>Multiple cancer, Beckwith-Wiedemann?</td>
<td>DOX, IFO</td>
<td>15.0 (1 × 1.5)</td>
<td>Grade 3 (lung radiation pneumonitis)</td>
<td>Impaired</td>
<td>—</td>
</tr>
<tr>
<td>HNLS</td>
<td>M</td>
<td>3</td>
<td>Neuroblastoma</td>
<td>—</td>
<td>CDDP, DOX, DTIC, IFO, VP-16</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>KKKA</td>
<td>F</td>
<td>16</td>
<td>Granulosa cell tumor</td>
<td>—</td>
<td>CDDP, IFO, VP-16</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>KNNA</td>
<td>F</td>
<td>12</td>
<td>Astrocytoma</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>KZBA</td>
<td>F</td>
<td>21</td>
<td>Osteosarcoma</td>
<td>—</td>
<td>CDDP, DOX, IFO, MTX, VP-16</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>LTJE</td>
<td>F</td>
<td>6</td>
<td>Desmoplastic small round-cell tumor</td>
<td>—</td>
<td>CBDCA, CPM, IDA, TFF, TOPO, VP-16</td>
<td>55.8 (1 × 1.8)</td>
<td>Grade 1 (skin)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>LNEY</td>
<td>F</td>
<td>4</td>
<td>Medulloblastoma</td>
<td>Fanconi anemia subtype FA-N <em>PALB2</em> mutation</td>
<td>CPM, MTX (intrathecal)</td>
<td>54.0 (1 × 1.8)</td>
<td>—</td>
<td>Impaired</td>
<td>—</td>
</tr>
<tr>
<td>MRSA</td>
<td>F</td>
<td>6</td>
<td>Pontine glioma</td>
<td>—</td>
<td>—</td>
<td>54.0 (1 × 1.8)</td>
<td>Grade 1 (skin)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MRCE</td>
<td>F</td>
<td>4</td>
<td>Nephroblastoma</td>
<td>—</td>
<td>DACT, DOX</td>
<td>54.0 (1 × 1.8)</td>
<td>Grade 1 (skin)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>NSNE</td>
<td>F</td>
<td>7</td>
<td>Astrocytoma</td>
<td>—</td>
<td>CCNU, CDDP, IFO, MTX, VP-16</td>
<td>59.4 (1 × 1.8)</td>
<td>Grade 2 (skin)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PNSH</td>
<td>F</td>
<td>17</td>
<td>Hodgkin’s disease</td>
<td>—</td>
<td>BLM, DOX, DTIC, IFO, PCB, VP-16</td>
<td>30.6 (1 × 1.8)</td>
<td>Grade 0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>SAAO</td>
<td>M</td>
<td>6</td>
<td>Astrocytoma</td>
<td>—</td>
<td>CPM, CBDCA, MTX (intrathecal + i.v.), VP-16</td>
<td>60.0 + 40.0 + 45.0 (2 × 1.0)</td>
<td>Grade 1 (skin, mucosa, GI tract)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>STFO</td>
<td>M</td>
<td>9</td>
<td>Medulloblastoma</td>
<td>—</td>
<td>CBDCA, CDDP, IFO, CPM, VP-16</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>SRKN</td>
<td>F</td>
<td>16</td>
<td>Malignant teratoma</td>
<td>—</td>
<td>CBDCA, CDDP, IFO, CPM, VP-16</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>SSCL</td>
<td>F</td>
<td>2</td>
<td>Astrocytoma</td>
<td>—</td>
<td>CBDCA, CDDP, CPM</td>
<td>—</td>
<td>—</td>
<td>Impaired</td>
<td>—</td>
</tr>
</tbody>
</table>

(Continued)
diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA).
For double labeling, samples were incubated with anti-γH2AX (Upstate) and anti-53BP1 (Bethyl, Montgomery, TX) antibodies, followed by Alexa Fluor 488–conjugated and Alexa Fluor 568–conjugated secondary antibodies (Invitrogen).

**γH2AX foci analysis**

Using an E600 epifluorescent microscope (Nikon, Düsseldorf, Germany), γH2AX foci per cell were counted by eye until at least 40 cells or 40 foci were scored for each data point. For every individual, blood samples were analyzed by two different investigators who were blinded to patient identity.

**Statistical analysis**

To evaluate potential differences in DSB repair capacity, statistical comparisons were performed at each time point (0.5, 2.5, 5, 8, and 24 h after irradiation) by Kruskal-Wallis and Mann-Whitney tests, using SPSS statistical software (SPSS, Chicago, IL). The criterion for statistical significance was $p \leq 0.05$.

**RESULTS**

The DSB repair capacity of ATM−/− homozygote and ATM+/− heterozygote probands was evaluated to test the sensitivity and reliability of the γH2AX foci approach in the clinical setting to identify DSB repair deficiencies. Whole-blood as well as isolated blood lymphocytes were irradiated with 1 Gy or 2 Gy, respectively. This low dose range was chosen to avoid radiation-induced apoptotic processes in blood lymphocytes, which might falsify results (23, 24). At defined time points after irradiation, samples were fixed, stained for γH2AX, and examined by fluorescence microscopy for enumeration of γH2AX foci per cell (Fig. 1). To provide another independent marker of unrepaired DSBs, we performed immunofluorescence analysis for p53-binding protein 1 (53BP1). 53BP1 has been shown to be recruited to sites of DSBs within minutes after exposure to ionizing radiation and forms radiation-induced foci, which colocalize with γH2AX (25, 26). In Fig. 2 the colocalization between γH2AX and 53BP1 can be clearly demonstrated, underscoring that counting of γH2AX foci can be used to analyze DSB repair.

The time course of γH2AX foci loss in blood lymphocytes of ATM−/− homozygote, ATM+/− heterozygote, and apparently normal individuals (ATM+/+) after exposure to 1 Gy is shown in Fig. 3. Irrespective of the mode of sample irradiation (whole-blood vs. purified lymphocytes), DSB repair kinetics were largely similar within the test groups. Lymphocytes from healthy individuals (ATM+/+) exhibited a rapid decrease in foci number within the first hours after irradiation, and only low levels of damage were observed at 8 h (1 Gy: 1.4 ± 0.2 foci per cell) and 24 h after irradiation (1 Gy: 1.0 ± 0.2 foci per cell; 2 Gy: 2.4 ± 0.4 foci per cell). In contrast, ATM-deficient lymphocytes of ATM−/− homozygotes showed a clearly slower decline, with considerably increased γH2AX foci numbers at 8 h (1 Gy: 3.7 ± 0.3 foci per cell) and 24 h (1 Gy: 3.2 ± 0.1 foci per cell; 2 Gy: 6.4 ± 0.7 foci per cell) after irradiation, with approximately 2 (1 Gy)
or 4 excess foci per cell (2 Gy) compared with controls. The time course for lymphocytes from ATM<sup>−/−</sup> heterozygotes showed an intermediate pattern of γH2AX foci disappearance after irradiation. Significantly, ATM<sup>+</ sup>− heterozygous individuals showed slightly elevated levels of remaining foci at 8 h (1 Gy: 2.6 ± 0.2 foci per cell) and 24 h (1 Gy: 1.9 ± 0.2 foci per cell; 2 Gy: 4.2 ± 0.3 foci per cell) after irradiation, with approximately 1 (1 Gy) or 2 excess foci per cell (2 Gy) compared with controls. Tests of significance for differences in mean values showed that values for all 8-h and 24-h samples from ATM<sup>−/−</sup> homozygote and ATM<sup>+</ sup>− heterozygote probands were significantly different from corresponding values for normal individuals (p ≤ 0.043). Collectively, these data indicate that analyzing the kinetics for γH2AX foci loss can be used to precisely assess the DSB repair capacity in blood samples of individuals; even slight impairments caused by heterogeneous mutations were detected reliably.

Subsequently, we analyzed the DSB repair of children with pediatric malignancies compared with that of age-matched control children. Childhood leukemia was excluded from this study because proliferating lymphoblasts in peripheral blood may falsify results. In the majority of cases blood samples were obtained at diagnosis, thus before any DNA-damaging chemo-/radiotherapy was applied. The characteristics of the 23 patients eligible for this study are summarized in Table 2. The mean age of the children with tumors was 9.9 years, compared with 9.7 years for control children. Of the 23 patients, 14 were female and 9 male. Most patients suffered from brain tumors, followed by soft-tissue and bone sarcomas, as well as embryonal tumors.

Whole-blood samples of children with tumors and control children were irradiated with 1 Gy or 2 Gy, respectively, and DSB repair kinetics were evaluated by γH2AX foci analysis at 0.5, 2.5, 5, and 8 after irradiation (Fig. 4). Strikingly, the average numbers of γH2AX foci for the children with tumors were significantly higher at 8 h after irradiation (1 Gy: 2.2 ± 0.1; 2 Gy: 3.8 ± 0.2) compared with values obtained for healthy controls (1 Gy: 1.8 ± 0.1; 2 Gy: 2.8 ± 0.2; p ≤ 0.001), indicating a slightly impaired DSB repair capacity in children with malignancies. Significantly, none of the healthy control children exhibited an impaired DSB repair capacity. In contrast, 4 children with solid cancers (AITB, HNEE, LNEY, and SSCL; Table 2) revealed clearly elevated...
H2AX foci at 5 h and 8 h (1 Gy: 3.0 ± 0.3; 2 Gy: 4.8 ± 0.5), with at least 1 (1 Gy) or 2 excess foci per cell (2 Gy) compared with corresponding average values of healthy controls. In the following paragraphs, these children are discussed in more detail, because their individual characteristics and their clinical courses seem likely to correlate with impaired DSB repair capacity.

Already at diagnosis of glioblastoma, attending pediatric oncologists were aware that the 9-year-old boy AITB suffered from Turcot syndrome caused by a homozygous mutation in the DNA mismatch repair (MMR) gene PMS2 (exon 11: c.1768delA) (27). Turcot syndrome is a rare inherited disorder in which central nervous system tumors are associated with colonic polyposis and cancer (28). Two siblings of AITB with identical PMS2 mutations had already died of glioblastoma, as previously reported (29). During the course of radiotherapy AITB suffered from moderate radiation-induced skin reactions, consistent with his only slightly impaired DSB repair capacity.

The girl HNEE, a fraternal twin born from in vitro fertilization, developed multiple embryonal tumors in early infancy: adrenocortical carcinoma at 2 years, rhabdomyosarcoma at 3 years, and pulmonal metastasized nephroblastoma at age 5 years. Genetic testing for the presence of Li-Fraumeni syndrome, an autosomal-dominantly inherited cancer predisposition syndrome caused by germline mutations in the p53 tumor-suppressor gene, revealed no pathologic finding. Moreover, HNEE (with the phenotypic anomaly of

![Image of immunofluorescence double staining of γH2AX (green) and 53BP1 (red) in blood lymphocytes of ATM−/− homozygotes, ATM+/− heterozygotes, and normal ATM+/+ individuals, analyzed at 8 h after irradiation with 2 Gy. Deoxyribonucleic acid was counterstained with 4',6-diamidino-2-phenylindole (blue), and images were merged to determine colocalization (yellow). By double labeling, the colocalization between γH2AX and 53BP1 can be clearly shown in all samples, underscoring that γH2AX can be used to analyze DSB repair. Original magnification, ×600.](image-url)
macroglossia) was tested for Beckwith-Wiedemann syndrome, an imprinting disorder resulting from mutations or epigenetic events affecting critical regions on chromosome 11p15.5 (30). Even though no evidence for hypomethylation of imprinting control regions KCNQ10T1, H19, and IGF-2 were found (performed by Dr. Dirk Prawitt), ongoing research supports the hypothesis that HNEE suffered from an epigenetic disorder with a more generalized imprinting defect (31). Because of pulmonal metastasis, HNEE received whole-lung irradiation with 15 Gy. Two months later HNEE suffered severe radiation pneumonitis requiring corticosteroids and intermittent oxygen supply. Patient HNEE died at age 5 years of a histologically non-confirmed pelvic tumor.

The girl LNEY was diagnosed with a medulloblastoma at age 4 years. After the first course of chemotherapy with systemic cyclophosphamide, LNEY suffered prolonged aplasia with septic events. Specific clinical stigmata (growth retardation, microcephaly, microphthalmia, bifurcated anus) raised suspicion for the presence of Fanconi anemia, a chromosomal instability disorder characterized by growth retardation, congenital malformations, progressive bone marrow failure, cancer predisposition, and cellular hypersensitivity to DNA cross-linking agents (32). Because of serious overreaction and a suspected diagnosis of Fanconi anemia, a modified chemotherapy with intrathecal methotrexate was applied, and only the tumor bed in the fossa posterior was irradiated with reduced dose, whereas prophylactic radiotherapy of the craniospinal axis was omitted. Three months after irradiation LNEY developed acute-onset nontraumatic paraparesis without fever or meningeal signs. Computed tomographic and magnetic resonance imaging revealed acute spinal cord necrosis but no spinal or meningeal dissemination of the tumor. Cerebrospinal fluid and other investigations were negative. Despite intensive care measures, the neurologic symptoms progressed to severe flaccid quadriparesis, and the child died within 2 days as a result of respiratory paralysis. Genetic analysis of DNA samples revealed a biallelic
mutation in PALB2 (2393_2394insCT; 3350+4A/G) causing the new FA-N subtype of Fanconi anemia, as previously reported (33).

The patient SSCL, a 2-year-old girl with astrocytoma, showed abnormal early carboplatin-related hypersensitivity reactions, with severe bronchospasm and urticaria (34). Carboplatin allergies are generally not related to deficiencies in DNA damage repair. However, because of these hypersensitivity reactions SSCL received only reduced doses of chemotherapy and no radiotherapy; thus, severe normal-tissue toxicities were not expected. The underlying molecular pathomechanism for deficient DSB repair is still unknown.

Collectively, 1 child with slightly impaired DSB repair capacity exhibited moderate normal-tissue responses, but at least 2 of 3 children with DSB repair deficiencies developed high-grade toxicities after treatment with DNA-damaging agents.

DISCUSSION

Major advances in pediatric cancer therapy have resulted in substantial improvements in survival. However, growing concern has emerged about severe normal-tissue toxicities associated with complex multimodality treatment strategies, compromising the clinical outcome of affected children.

In this pilot study, the highly sensitive γH2AX foci analysis was evaluated to identify patients with an impaired DSB repair capacity as the determining factor for high-grade normal-tissue toxicities. First, the feasibility of the γH2AX foci approach to detect even subtle, genetically determined DSB repair deficiencies was confirmed by testing blood samples of ATM−/− homozygote and ATM+/− heterozygote probands (35). Subsequently, the individual DSB repair capacity of children with different solid tumors was analyzed compared with that of healthy control children and correlated with their treatment-related acute and late side effects. Whereas all healthy control children exhibited proficient DSB repair, 3 of 23 children with tumors revealed clearly impaired DSB repair capacities, and the underlying genetic background could be discovered to some extent. Although none of the repair-proficient children with tumors developed high-grade toxicities, 2 of the repair-deficient children with tumors suffered unexpected serious adverse events—life-threatening radiation pneumonitis and lethal spinal cord necrosis. These data suggest that γH2AX analysis detecting DSB repair deficiencies may provide a novel opportunity to identify children at risk for high-grade toxicities.

Little is known about the etiology of childhood cancers. A variety of environmental toxicants present before conception (affecting parents’ germinal cells), during pregnancy, and after birth have long been thought to be important determinants of childhood malignancies, mainly owing to the physiologic immaturity of infants (36). However, it is now well accepted that genetic alterations in proteins participating in the DNA damage response play a significant role in determining the individual’s cancer susceptibility (37–40). In this study we observed statistically significant differences in the mean DSB repair capacity between children with tumors and healthy control children, even if the children with tumors with striking DSB repair deficiencies (AITB, HNEE, LNEY, and SSCL) were disregarded from statistical analysis (Fig. 4; $p \leq 0.028$). Moreover, the spontaneous rate of γH2AX foci was slightly higher in children with tumors compared with healthy control children (0.095 ± 0.014 vs. 0.053 ± 0.005; $p = 0.0016$). These findings suggest that childhood cancer in general might have a genetic basis affecting DSB repair capacity. According to recently published large
epidemiologic studies, childhood cancer survivors have a persistent and high risk for a second primary cancer throughout their lives (41). This increased risk for second primary cancer among former childhood cancer patients is most likely the late sequelae of the carcinogenic effects of administered radio- and chemotherapy, but it may also be related to the children’s increased genetic susceptibility for cancer formation. The DSB repair capacity plays a fundamental role in maintaining genomic integrity. Our data suggest that children with malignancies have slightly reduced DSB repair capacities and thus are probably inherently more sensitive to carcinogenesis.

In 2 of the 4 repair-deficient children with tumors, gene mutations affecting regulatory factors involved in different DNA repair pathways could be identified to cause distinct cancer susceptibility syndromes. PMS2 mutations associated with Turcot syndrome are known to compromise the DNA MMR. Moreover, experimental studies indicate that the repair of DSBs is frequently impaired in MMR-deficient cells, leading to slightly increased radiosensitivity (42, 43). This may explain our finding of slightly impaired DSB repair capacity in the patient AITB, whereas the aberrant DSB repair may be an additional mechanism contributing to genomic instability and tumorigenesis.

PALB2 mutations have recently been shown to cause a new subtype of Fanconi anemia and predispose to childhood malignancies (33). Previous experimental studies revealed that PALB2 colocalizes in nuclear foci with BRCA1, BRCA2, and partially with γH2AX after ionizing irradiation, and thus seems to participate in the DNA damage response (44–46). Although BRCA2 promotes DSB repair mainly by homologous recombination, BRCA1 is involved in multiple DNA repair pathways, including nonhomologous end-joining (47, 48). Furthermore, BRCA1 and BRCA2 are involved in regulating cellular responses to DNA cross-links induced by certain chemotherapeutics, such as cyclophosphamide (45, 49). Therefore, it seems likely that LNEY, because of her genetically determined DSB repair defect causing hypersensitivity to cross-linking agents, suffered severe treatment-related toxicities, namely spinal cord necrosis. Neurologic complications associated with systemic and/or intrathecal chemotherapy generally become manifest during or shortly after treatment (50, 51). However, cumulative effects of prior intrathecal doses and preceding courses of systemic chemotherapy in combination with the genetically defined DSB repair defect may have led to this fatal outcome several months after initiation of therapy. Although the exact cause of spinal cord necrosis was not proven by autopsy (postmortem examination was declined), no alternative explanations could be elucidated.

The causative genetic or epigenetic defect of the patient HNEE with presumed Beckwith-Wiedemann syndrome is still not identified, even though multiple malignancies in her early infancy are most indicative of the presence of the cancer susceptibility syndrome (52). Recent studies reported a close association between Beckwith-Wiedemann syndrome and assisted reproductive technologies, such as in vitro fertilization, caused by disturbed genomic imprinting (53). Children with Beckwith-Wiedemann syndrome have an increased risk for development of embryonal tumors. Considerable evidence suggests that aberrations in DNA methylation and histone modifications affecting the configuration of chromatin may have an important impact on DSB repair capacity (54).

Over the past years γH2AX foci formation has been shown to be a reliable surrogate for DSBs, and the γH2AX foci approach has been used extensively to measure induction and repair of DSBs. Despite numerous advantages of γH2AX as a marker of DSBs, the method has certain limitations that should be carefully considered in the interpretation of results. γH2AX analysis allows the uncovering of repair deficiencies in nonhomologous end-joining, the dominant DSB repair pathway in mammalian cells. However, this approach is not suitable to detect impairments in homologous recombination, which is restricted to the S–G2 phase of the cell cycle. Moreover, in contrast to physical methods of DSB detection, such as pulsed-field gel electrophoresis, scoring γH2AX foci does not follow the actual fate of physical DSBs but rather registers cellular metabolic activities initiated to facilitate and optimize DSB repair. Hence, discrepancies between the actual DSB removal (as measured by physical changes in size-dependent properties of DNA molecules) and the γH2AX foci decay (based on biochemical modifications) have been reported in different cell systems, leading to some disparities in the measurements of DSB repair kinetics (discussed by Kinner et al.[55]). Furthermore, “repair” in the context of these experiments does not imply that the DNA molecule is restored to its original state. Deletions or deletions of bases around the DSB, as well as mis-rejoining events, remain undetected by the γH2AX foci approach. Accordingly, γH2AX foci analysis does not provide any information concerning the fidelity of the DSB rejoining process.

However, our data suggest that the γH2AX foci approach may serve as a predictive assay in the routine screening of cancer patients to identify those individuals at greatest risk for the development of adverse effects resulting from radiotherapy or chemotherapy. The decisive advantage of the γH2AX foci approach is that the functional integrity of the DSB repair is screened, independent of the underlying molecular background. Therefore, a broad range of genetic and epigenetic variations affecting DSB repair functions can be detected, determining the individual sensitivity to DNA-damaging agents. Because blood samples are easily accessible before cancer therapy and γH2AX analysis can be performed within 24 h, simple blood tests, adequate for diagnostic routine, allow pretherapeutic testing for DSB repair deficiencies in the clinical setting. The results of this pilot study suggest that γH2AX analysis allows identification of patients genetically predisposed to develop severe normal-tissue toxicities. However, because the number of patients is small, larger trials are needed before definite conclusions can be drawn.
REFERENCES


