AML1 Gene Rearrangements and Mutations in Radiation-Associated Acute Myeloid Leukemia and Myelodysplastic Syndromes

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INTRODUCTION

A large body of epidemiological evidences has established the leukemogenic potential of ionizing radiation. However, little is known about the molecular mechanisms by which radiation induces the leukemia. Several studies have suggested a link between AML1 gene rearrangements and radiation-induced acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS). Fifty-three AML samples were analyzed for the presence of AML1 abnormalities using fluorescent in-situ hybridization (FISH) and reverse transcription polymerase chain reaction (RT-PCR). Of these patients, 24 had experienced radiation exposure due to the Chernobyl accident, and 29 were non-irradiated spontaneous AML cases and served as controls. AML1/ETO translocations were found in 9 of 29 spontaneous AML but only in 1 of 24 radiation-associated AML cases. This difference between translocation frequencies is statistically significant in the age-unstratified cohorts (p = 0.015). Following age stratification, the difference becomes less pronounced but remains on borderline significance (p = 0.053). AML1 mutation status was assessed in 5 clean-up workers at Chernobyl NPP with MDS, or AML following MDS, by direct sequencing of genomic DNA from the coding region (exon 3 through 8). In one patient who developed MDS following an acute radiation syndrome, a hexanucleotide duplication of CGGCAT in exon 8 was found, inserted after base position 1502. Our results suggest that AML1 gene translocations are infrequent in radiation-induced leukemogenesis but are consistent with the idea that radiation may contribute to the development of MDS through AML1 gene mutation.

The AML1 gene encodes a DNA-binding subunit of the heterodimeric core-binding factor (CBF). CBF binds to the transcriptional regulatory regions of multiple genes, some of which are essential for the induction of myeloid cell differentiation in the bone marrow. The AML1 gene is the most common target for chromosomal translocations in spontaneous human leukemia, consistent with a key regulatory role in hematopoiesis. Hromas et al. described novel translocations of the AML1 gene, detected in 3 patients who developed the disease after exposure to nuclear explosions. This was taken to indicate a key role of AML1 gene disruption in radiation-induced leukemogenesis. However, the history of exposure to ionizing radiation and radiation aetiology of leukemia in this study has been questioned.

Non-translocation-associated mutations of AML1 have been recently reported as a new mechanism of gene deregulation both in AML and MDS. AML1 mutations predominate in AML M0 by French-American-British (FAB) classification, but are rare in other types of AML or MDS developed de novo. Rousnier et al. reviewed 3 cases of AML1 mutations among 169 MDS patients. Harada et al. described a high frequency (46%) of AML1 point mutations.
in 13 MDS patients, who survived the atomic bomb explosions in Hiroshima.\textsuperscript{3} The aim of the present study was to establish if the AML1 gene rearrangements/mutations are of importance in the pathogenesis of radiation-associated AML and MDS. We have examined 24 samples of AML and 3 samples of MDS arising in patients exposed to ionizing radiation as a consequence of the Chernobyl accident in 1986. The presence of AML1 gene rearrangements was studied by fluorescent in-situ hybridization (FISH) and reverse transcription polymerase chain reaction (RT-PCR). Genomic sequence of AML1 exon 3 through 8 was determined by direct sequencing.

The results of epidemiological study conducted in Russia shown a significantly increased leukaemia incidence among clean-up workers at the Chernobyl NPP.\textsuperscript{12} There is no support to date for the notion that ionizing radiation exposure related to the Chernobyl accident affected leukaemia risk in inhabitants of contaminated areas. However, inhabitants of contaminated areas were exposed to considerably lower doses than clean-up workers, and epidemiological studies\textsuperscript{13,14} were not large enough to quantify the risk to a useful degree of precision. But at doses as low as 1.5 mSv, an increase in leukaemia risk is suggested\textsuperscript{15} in children under age 5 years who were exposed in Nordic countries to fallout from nuclear weapons testing. These results were consistent with the case-control study\textsuperscript{16} of leukaemia in Utah associated with fallout from the Nevada nuclear test site as well as with pooled studies of nuclear workers in the United States, Canada, and the United Kingdom.\textsuperscript{17} We, therefore, included in our study both clean-up workers and inhabitants of contaminated areas.

MATERIALS AND METHODS

Patients

For the FISH and RT-PCR study we used bone marrow or peripheral blood samples preserved with informed consent from 53 unselected adult AML patients, initially diagnosed between 1997 and 2004. Of these patients, 24 had experienced radiation exposure due to the Chernobyl accident, and 29 developed spontaneous AML, which served as controls. In the group of patients with radiation-associated AML there were 11 clean-up workers, which were involved in recovery operations in 1986–1987 and 13 patients either evacuated from the Chernobyl exclusion zone or had their residence in Ukrainian territories with high contamination from radionuclide fallout. Details of patients’ age, sex, and FAB type are given in Table 1.

AML1 gene sequencing was performed on two samples of AML following MDS (case 1 and 3) and three samples (case 54, 55 and 56) from male clean-up workers with MDS not listed in Table 1. According to FAB classification, cases 54, 55 and 56 were refractory anaemia (RA) diagnosed at an age of 50 and 59 years and refractory anaemia with excess blasts

<table>
<thead>
<tr>
<th>Pts No.</th>
<th>Sex/age (yr)</th>
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<td>M6</td>
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<td>24</td>
<td>M/42</td>
<td>Victim</td>
<td>M5b</td>
<td>–</td>
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Total in group of radiation-associated AML: 1/24 (4.2%)
in transformation diagnosed at an age of 33 years. All cases were initially diagnosed between 1996 and 2002.

Individual dosimetry was only available for two patients, one of whom survived 1st degree acute radiation syndrome (case 54, 1.7 Sv) and the other survived 2nd degree acute radiation syndrome (case 7, 2.5 Sv), respectively. The approximate dose range estimation for the remaining patients was derived using the published data for the respective cohorts.  

The average effective dose from external irradiation was estimated to be 130 mSv for clean-up workers (cases 1–6, 8–11, 55–56) and 15 mSv for evacuees from the 30-km zone around the Chernobyl reactor (cases 13, 14, 16, 22). Effective doses for patients who lived in highly contaminated areas (cases 12, 15, 17–21, 23, 24) were calculated using dosimetry data from local indoor and outdoor measurements. The estimated individual values for these cases varied from 6 to 31 mSv with 12 mSv as the average.

FISH studies

Mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation and shock-frozen at −70°C or immediately fixed on slides and stored at −20°C. Interphase FISH was performed using the dual-colour AML1/ETO probe (Vysis, Downers Grove, USA) according to the manufacturer’s instructions. After hypotonic treatment in 0.075 M KCl, cells were fixed in methanol/acidic acid (3:1), dropped on slides, air dried, and held at 37°C. Slides were treated with 0.1 mg/ml pepsin in 0.01 M HCl for 2–3 min at 37°C, then washed in PBS and 0.05 M MgCl2/PBS at room temperature (RT), post-fixed in 1% paraformaldehyde/PBS, and dehydrated through an ethanol series. After denaturation for 4 min at 73°C in 70% formamide/2x SSC, slides were dehydrated in an ethanol series. The probe mixture was denatured at 73°C for 5 min, added to denatured cells at 45–50°C and covered. After overnight hybridization in a humidified chamber at 37°C, slides were washed in 50% formamide/2 x SSC, 2 x SSC, 0.2 x SSC at 42°C, and 100 mM sodium phosphate pH 8.0/0.1% NP-40 at RT. Cells were counterstained with 4',6-diamidino-2-phenyindole solution (150 ng/ml in 2x SSC) and examined using a Zeiss Axiosplan fluorescence microscope (Zeiss, Jena, Germany) with image processing software ISIS3/V.3.04 (Metasystems, Altlußheim, Germany). At least 100 nuclei were analyzed for each case. A cut-off value of positive case was set at 10% of nuclei showing an abnormality.

AML1/ETO fusion gene detection by RT-PCR

Total RNA was isolated immediately after collection of bone marrow or peripheral blood by the guanidinium thiocyanate phenol-chloroform extraction method. cDNA was synthesized from 1 µg of total RNA, using random hexamer primers and M-MLV reverse transcriptase (Fermentas, Vilnius, Lithuania) and following the protocol supplied by the manufacturer. Nested RT-PCR amplifications were performed for AML1/ETO fusion transcripts using the following primer sets: 5’-AGCCATGAAAGACAGGTTGC-3’ (AML1 outer primer), 5’-AGCTTTACCTGACCATCACT-3’ (AML1 inner primer), 5’-TCACAAATGGCTTAGGAAATGGC-3’ (ETO outer primer), 5’-AATCGTTTCTGGAGCTCCT-3’ (ETO inner primer).

Briefly, in a volume of 25 µl, 3 µl of the first-strand cDNA mixture was added to a mixture containing 1x PCR buffer, 2.5 mM MgCl2, 0.2 mM each dNTP, 75 nM concentration outer primers, and 1.5 U Taq polymerase (Fermentas, Vilnius, Lithuania) and following the protocol supplied by the manufacturer. Nested RT-PCR amplifications were performed for AML1/ETO fusion transcripts using the following primer sets: 5’-AGCCATGAAAGACAGGTTGC-3’ (AML1 outer primer), 5’-AGCTTTACCTGACCATCACT-3’ (AML1 inner primer), 5’-TCACAAATGGCTTAGGAAATGGC-3’ (ETO outer primer), 5’-AATCGTTTCTGGAGCTCCT-3’ (ETO inner primer).
gel, the RT-PCR products were visualized under UV illumination. Selected primers for amplification of AML1/ETO fusion gene yielded a 220-base pair (bp) product in positive cases.

Identification of AML1 mutations

Genomic DNA was extracted from bone marrow or peripheral blood samples preserved frozen at −70°C using the QIAamp DNA extraction kit (Quiagen, Hilden, Germany). The following intronic primer sets were used to amplify exons 3 through 8 of the AML1 gene: 5'-GTAATAAAGGCCCCCTGAACGTTGATG-3' (intron 2 forward), 5'-GTACCCCTGA- AAGCCGATGGCCAG-3' (intron 3 reverse), 5'-CTAAAAGTGTAATACACATCCCCTGATGCTG-3' (intron 3 forward), 5'-GAAATGTGGGTGTGGCAGAAACTG-3' (intron 4 reverse), 5'-CAACAGATATGTTGAGGGCCACCA-3' (intron 4 forward), 5'-GAGGGTGTTACCAGCCCCCAAG-3' (intron 5 reverse), 5'-AAAGCCCCAGTTTTAGGAAATCCAC-3' (intron 5 forward), 5'-CTGGAAAGGTGTGTGCACATG-3' (intron 6 reverse), 5'-AAGACATTITTTTAATATATTTCACCA-3' (intron 5 forward), 5'-CCCTCATGTCTCCTGGACCTCCACC-3' (intron 6 forward), 5'-CAGGAAGCTGCTGGCTCCGCAA-3' (intron 7 reverse), 5'-CTCCACACGGCCCTCCTCCAG-3' (exon 8 reverse).

Briefly, each PCR reaction was done in a final volume of 24 μl, about 20 ng DNA, 5 pmol of each primer, 0.35 mM each dNTP, and 1 U Taq polymerase (Amersham Pharmacia) using the buffer as supplied by the manufacturer. Cycling conditions on a GeneAMP 9700 were as follows: initial denaturation at 94°C for 4 min, 32 cycles of 94°C for 1 min, 60°C for 45 sec and 72°C for 90 sec, and a final extension step of 72°C for 90 sec. 20 μl of the reaction products were electrophoretically separated on 2% agarose gel with ethidium bromide staining. Single bands of each amplification product were cloned in TOPO-TA vector and used to transform TOP10 bacteria (all Invitrogen GmbH, Germany). Sequencing of the inserted PCR products belonging to the spontaneous cohort (cases 28, 33, 37 and 50) had an in frame hexanucleotide duplication of CGGCCAT instead of CGCGCAT for position 1502. This in-frame duplication would be translated into an extra Glycine and Isoleucine in exon 8.

RESULTS

The presence of AML1 gene abnormalities were examined by FISH using dual-colour AML1/ETO probe in 37 AML samples (Table 1). In contrast to the study of Hromas et al.,2,6 we did not find any alteration of the AML1 locus by this assay in radiation-associated cases. Clear signs of AML1/ETO translocations, however, were detected in 4 cases of the control group. In another case from the control group (case 49), the majority of cells showed three AML1 signals, but a normal number of ETO signals, indicative either of AML1 translocation with an undetermined fusion partner or AML1 duplication. The cytogenetic rearrangements involving the AML1 locus were found to be significantly less frequent in radiation-associated AML (0/21) than in spontaneous cases (5/16, P = 0.01 two-tailed Fisher’s exact test). The further analysis was restricted only to AML1/ETO translocation detection. Twelve additional cases could be screened for AML1/ETO fusion products using RT-PCR, and in 17% of all samples FISH and RT-PCR could be performed in parallel. There were no discrepant results between these two assays. Taking the FISH and the RT-PCR data together, AML1/ETO translocation was found in only 1 (4.2%) of 24 radiation-associated AML. In contrast, AML1/ETO translocations were identified in 9 (31%) of 29 spontaneous AML cases. This difference in translocation frequencies is statistically significant using two-tailed Fisher’s exact test (P = 0.015). To minimize a potential confounding effect caused by differences in the age distributions, we age-stratified both cohorts and repeated the test. For this purpose, 5 cases representing the oldest in the radiation-associated group together with 10 cases representing the youngest in the spontaneous group were removed from the cohorts. This reduced both groups sizes to 19 cases each, with mean ages of 48.7 years in the radiation-associated group and 47.8 years in the spontaneous group. In these age-stratified subgroups remain 4 cases with an AML1/ETO translocation, all belonging to the spontaneous cohort (cases 28, 33, 37 and 45, resp.). Fisher’s exact test now yields still borderline significance (P = 0.053).

AML1 mutation analysis was performed in 5 patients with MDS, or AML following MDS, by direct sequencing of PCR amplified exons. All suspicious sequences were re-evaluated by two independent investigators. No point mutations were found in exon 3 through 8 of the AML1 gene, but one patient who survived Acute Radiation Syndrome following emergency service after the Chernobyl accident (case 54) had an in frame hexanucleotide duplication of CGGCCAT in exon 8 (following position 1502). This in-frame duplication would be translated into an extra Glycine and Isoleucine inserted into C-terminal region of the protein (Fig. 1).
DISCUSSION

Fifty-three AML samples at diagnosis were analyzed for the presence of AML1 abnormalities using FISH and RT-PCR. Selection bias can be excluded in this analysis because all available AML patients were enrolled in the study and blindly checked for the presence of AML1 rearrangements. Whereas the spectrum of AML types as classified by the FAB system was similar among the radiation-associated and spontaneous cases, the frequency of gross chromosomal rearrangements at the AML1 locus differed significantly. Nine AML1/ETO positive cases were found among 29 spontaneous AML patients, only a single one with this translocation was present among 24 patients of the radiation-exposed group \((p = 0.015)\). A possible bias in this comparison could result from the different age distributions in the two groups. Whereas the mean age of spontaneous cases was 39.3 years, this value increases to 53.4 years in the radiation-associated groups. To correct for this difference, we repeated the significance test in age-stratified sub-cohorts, each comprising 19 cases (mean age 47.8 years in spontaneous and 47.8 years in radiation-associated sub-cohort). In these two sub-cohorts remained only 4 AML1/ETO positive cases, all appearing in patients without a preceding radiation exposure. Although the difference in translocation frequency drops after this age-stratification, it remains on the borderline of significance \((p = 0.053)\). This means that the lower than expected number of AML1/ETO translocations in radiation-associated cases can only in part be explained by the larger number of elder patients in this group. It is thus still reasonable to conclude that recurrent chromosomal translocations of the AML1 gene (characteristic both for de novo cases and secondary AML following treatment with topoisomerase II inhibitors) are less common among AML patients exposed to ionizing radiation during or after the Chernobyl accident. These patients rarely belong to the favourable AML category, which is characterized in spontaneous cases by an AML1/ETO translocation. There are, however several discords with other studies to comment. First, in a study of leukaemia cases among Japanese A-bomb survivors, translocations involving the AML1 gene were detected.22) Since it is impossible to find an unambiguous marker for the radiogenic origin of the leukaemia, heterogeneity among the cases in terms of aetiology might be one reason for the different findings. Another difference to the Japanese study is the dose range to which the patients were exposed. The higher dose experienced by the A-bomb survivors might trigger a leukaemogenic mechanism different from the one in our patients. Second, formation of AML1/ETO fusion genes was also reported in irradiated cells in-vitro.23) The discrepancy with this observation could be interpreted, with the exception of case 7, by relatively low radiation dose of AML patients in our study. Higher doses are required to cause chromosomal translo-
tions resulting from radiation-induced double-strand DNA breaks. We assume that other molecular mechanisms, rather than gene translocations could contribute to radiation leukaemogenesis in the low doses range.

Among 5 patients with MDS, or AML following MDS, mutation in the AML1 gene was found in one case. This patient developed MDS RA 10 years after acute radiation syndrome caused by an estimated whole-body exposure to 1.7 Gy. The excess relative risk per Gy derived from epidemiological data on radiation-associated leukaemia indicates that the MDS in this patient has a probability of about 90% of being radiation-induced. Although non-neoplastic tissue was not available from this patient, we can assume that the duplication described here is disease-associated rather than constitutional. Neither of the available genome databases lists this particular alteration as a constitutional AML1 gene polymorphism in the mRNA sequence (NM_001754) or in the entire genomic sequence (NT_011512.9). The patient survived 65 months from diagnosis of MDS. The disease was characterized by hypocellular bone marrow, anaemia and thrombocytopenia at presentation. The patient quickly became transfusion dependent and was unsuccessfully treated with steroids, granulocyte-macrophage colony-stimulating factor, cyclosporine A and a pentoxifylline-ciprofloxacin-dexamethasone combination. According to the criteria of Cheson et al., he showed a minor erythroid response to erythropoietin, and a minor neutrophil response to amifostine treatment.

The frequency and type of mutation we found is at variance with those described by Harada et al. In contrast to this earlier study, we found an intragenic duplication in the AML1 gene, rather than change of a single nucleotide in radiation-associated case. Interestingly, AML1 point mutations in MDS arising among atomic bomb survivors were found in the DNA-binding Runt homology domain, whereas mutations in the carboxy (C)-terminal region were considered to be strongly correlated with a spontaneous aetiology. The AML1 mutation in our study was localized to the C-terminal transcriptional activation domain, but was present in the MDS case with the highest probability of being radiation-induced. This case is typified by a higher radiation-dose and in particular a much shorter latency time (10 years) compared to 50–55 years in the atomic bomb victims. In spite of some differences to the data of Harada et al., our study does not contradict the model that radiation may contribute to leukaemia development through AML1 gene mutation. It is possible that secondary and spontaneous AML and MDS have distinct etiological pathways. It would be intriguing to define the molecular fingerprints describing the radiation aetiology of AML and MDS cases, and to provide insights into the role of AML1 gene mutations in radiation-induced leukaemogenesis.

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REFERENCES


