Internal tandem duplication of the Flt3 gene in acute myeloid leukemia with a probable radiation etiology

MICHAEL ROSEMANN[1*], SERGIJ KLYMENKO[2*], VLADIMIR BEBESHKO[2], DIMITRY BAZYKA[2], IRYNA DMYTRENKO[2], KLAUS R. TROTT[3] AND MICHAEL J. ATKINSON[1]

[1]Institute of Pathology, National Research Center for Environment and Health, Ingolstaedter Landstrasse 1, 85764 Neuherberg, Germany

[2]Department of Haematology, Research Centre for Radiation Medicine, Melnikova 53, 04050 Kyiv, Ukraine

[3]Gray Cancer Institute, P.O. Box 100, Mount Vernon Hospital, Northwood Middlesex HA6 2JR, United Kingdom

*MR and SK contributed equally to this study

Author for correspondence: Dr. Michael Rosemann, GSF Institute of Pathology, Ingolstaedter Landstrasse 1, 85764 N E U H E R B E R G, Germany, e-mail rosemann@gsf.de

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Introduction

The leukaemogenic potential of ionising radiation is well documented. An excess of acute myeloid leukemia (AML) has been reported following accidental or therapeutic incorporation of alpha-particle emitters [1, 2,] or exposure to external gamma irradiation [3]. However, the high incidence of spontaneous AML makes it difficult to unequivocally establish causality in irradiated cohorts. As a consequence, there are uncertainties regarding the extent of the excess AML risk as a potential late effect of the Chernobly accident [4, 5, 6]. It would be of great value to find molecular alterations that distinguish between AMLs with a radiogenic or a spontaneous etiology. Such a radiation fingerprint has already been proposed for some solid tumor types, but it is uncertain if these differences represent different etiological mechanisms, or if they result from a bias in tumor selection [7,8].

Intragenic alterations of the Flt3 gene have been observed in up to one third of primary and secondary therapy-related AMLs [9,10,11], making it the most common single-gene mutation in AML. The tyrosine kinase encoded by Flt3 kinase is affected either by amino acid substitution in the cytoplasmic activation loop or by an elongation of the juxtamembrane domain. The latter - resulting from internal tandem duplications (ITD) in the related coding sequence - are suggested to cause constitutive activation of the kinase activity, thereby conferring IL3-independent growth of myeloid precursors [12,13]. The presence of Flt3 ITD mutations in AML has been associated with a poorer prognosis [9, 10,14].

In the present study we have analysed the presence of Flt3/ITD mutations in a cohort of 33 radiation-associated and spontaneous AMLs. Twenty-one of the patients had worked or resided in or near to the Chernobyl nuclear plant during the 1986 accident, and thus received increased doses of gamma radiation. A series of 12 AMLs from unexposed patients diagnosed at the same clinic served as a control cohort, to determine if qualitative / quantitative differences in Flt3 mutations exist in radiation-associated AML.

Patients and Methods

Patients

The 33 AML cases included in this study were initially diagnosed with AML at the Kiew Institute for Radiation Medicine after 1986. Since individual exposures were not consistently monitored retrospective dosimetry for clean-up workers (liquidators) and the rural population served as the framework to classify a tumor as being radiation-associated or spontaneous [15,16,17]. Using these criteria 21 cases were classified as being radiation-associated, 12 of which arose in liquidators receiving an average dose of between 100 and 300 mSv [18]. The remaining 9 cases arose in inhabitants of nearby villages who had received individual doses between 1 and 70 mSv (fallout victims). Based upon an excess relative risk of leukemia of about 5 per Sv [19] we have estimated the probability of causality to be 44% for AML arising in the liquidators, suggesting that almost half of the tumors are indeed radiogenic, whilst slightly more than half are probably sporadic. A total of 12 cases had no recorded radiation exposure and served as the local control group.

Patient characteristics and AML subtypes are described in detail elsewhere [18,20]. In brief, the median age of all patients was 49 years at diagnosis, The following AML subtypes were diagnosed according to FAB standards: M0 (6%), M1 (18%), M3 (6%), M4 (33%), M5 (27%), M6 (0%), M7 (3%). The median percentage of blast cells at diagnosis was 66%. Distribution of AML subtypes, age, and blast cells showed no significant differences between exposed and non-exposed patients. A significant difference was found for the peripheral WBC counts: patients with radiation-associated AML had a median WBC of 41.4 * 10^6/ml, compared to only 12.2 * 10^6/ml in control patients.

Analysis of Flt3 status

DNA for molecular analysis was obtained from N2-snap-frozen mononuclear fractions of peripheral blood / marrow aspirates or dried blood smears. Genomic DNA was extracted using the Quiamp DNA extraction kit (Quiagen, Hilgen, Germany). Flt3 gene exon 13, intron 13 and exon 14 were amplified as a single fragment by PCR using primers 11F and 12R under conditions suggested by another group [21](Kiyoi et al 1997). A 32 cycle PCR amplification (94^{o}C 60sec, 64^{o}C 45sec, 72^{o}C 90sec) was performed with a GeneAMP 9700 (Applied Biosystems, Foster City, Ca, USA) in a final volume of 24ul, containing 20ng of template, 5pmol of each primer, 0.35 mM

dNTPs, 1U rTaq polymerase (Amersham Pharmacia, Freiburg, Germany) diluted in manufacturers buffer. After final extension at 72^{o}C for 90sec the PCR products were resolved by electrophoresis in 2% Agarose/TBE gels.

Sequencing

To characterize abnormal PCR fragments the individual bands were excised and purified using a DNA gel extraction kit (Quiagen, Hilden, Germany). The purified fragments were cycle sequenced using the BigDye system and an ABI377 sequencer (both Applied Biosystems, Foster City, Ca, USA) according to manufacturers protocols.

Results

Flt3/ITD mutations

As shown in Figure 1, internal tandem duplications of the Flt3 gene were present in 5 out of the 33 AML tumors studied. The frequency of ITD of 15.1% is close to the range reported in large studies on non-radiation associated disease in different populations, which reveal frequencies between 16.5% and 27% [9,10,14]. Flt3 ITD mutation frequency did not associate with radiation exposure, with 3/21 (14.2%) ITDs being present in radiation-associated tumors and 2/12 (16.7%) in controls. Of the 3 radiation-associated cases, two patients (#7 and #11) were former liquidators and one (#24) was an inhabitant of a contaminated area. Although sample size is relatively small, no affinity of Flt3 ITD for any particular AML subtype or percent of blast cells was obvious (Table 1).

The Flt3 abnormalities as shown here are mutually exclusive to cytogenetic alterations at the AML1 locus [18] and at the MLL locus [20], thus supporting the findings in another study [14].

Multiplicity of Flt3 ITDs

A number of different patterns of aberrant Flt3 amplification products was evident, with cases showing either 1, 2, or 3 additional bands (Figure 1). The presence of multiple Flt3 alleles was not equally distributed between the patient groups. In non-irradiated AMLs with Flt3 ITD only a single additional allele was present. In contrast, 2 cases of radiation-associated AML had Flt3 ITD with 2 and 3 additional bands, respectively.

Sequencing of the abnormal Flt3 bands revealed, that the duplications were always located between the primer binding sites. This confirms that multiple fragments amplified from an individual tumors represent independently mutated molecules, rather than additionally inserted primer binding sites. Whether the multiple Flt3 mutant alleles are present in the same cell, or if they result from individual clones, can not be answered here. This latter explanation may be formally discounted, however, as an earlier FISH study has reported no increase in the number of Flt3 signals in AML cells [14]. We thus interpret the Flt3 ITD mutations to be an indicator of clonal heterogeneity of each tumor.

Analysis of length and sequence variation of duplicated fragments

The length of the duplicated segments varied from 21 to 129bp, with the inclusion of additional sequences always maintaining the reading frame (Table 2). All fragments, including the different insertions in cases with multiple alleles, exhibited different 5´and 3´ break points, supporting the suggestion that each arises independently as a separate clone. The Flt3 ITD alterations in the radiation-associated AMLs exhibited the longest duplication events, each having a duplication of more than 66 nucleotides.

The in-frame alterations recorded here arise primarily from duplication of exon 13, and can be predicted to add from 7 to 43 extra amino acids to the protein. In two instances (case 11, fragment 3, and case 7, fragment 1), 6 and 3 of the inserted amino acids, resp., were derived from partial sequences of intron 13. Interestingly, alignment of all inserted residues revealed the inclusion of a common tetrapeptide (Arg-Glu-Tyr-Glu) in all cases. This consensus sequence has been observed in 94% of all cases of Flt3 ITD [22], 80% of acute promyelocytic leukemia [21] and only 42.8% of AMLs derived from MDS [23].

Discussion

Flt3 ITD mutations are one of the most prominent alterations in AML involving a single gene. They are a well-established prognostic marker for both a reduced overal survival [9,10] and an increased risk of relapse [14]. In the present study Flt3 ITDs were detected with a similar frequency in radiation-associated (3/21) and spontaneous AMLs (2/12). No preference for a particular AML subtype or percentage of blast cells was noticed among the Flt3 ITD positive cases. In terms of structure or complexity of

the alterations, however, radiation-associated tumors had a tendency to exhibit multiple and/or longer duplicated fragments. Multiple duplications were found in 2 of the 3 Flt3 positive radiation-associated AMLs, but in none of the spontaneous cases. The presence of the multiple Flt3 alleles most probably represent co-existent clones of leukemic cells [10], suggesting a higher degree of clonal evolution that may result from radiation-induced genomic instability [24,25,26].

In the radiation-associated AMLs there was also a trend towards longer duplicated fragments in Flt3 ITDs. The longest observed fragments of 66, 114 and 129bp were found in these tumors, whilst in spontaneous tumors the alterations were only 21 and 45bp long. Other investigators reported Flt3 ITD fragments longer than 100bp only in 0% - 7% of all positive cases [9,21,22]. Longer duplicated fragment lengths were found, however in a subset of secondary (therapy-related) AMLs [23]. Our findings are thus consistent with the idea that the longest duplicated fragments are associated with a radiation or therapy related etiology.

Although we find some indication of a higher frequency of multiple Flt3 ITD mutations and longer duplicated sequences in radiation-associated AML, it would be premature at the moment to consider Flt3 mutations to be a true radiation fingerprint. Indeed, the majority of the AMLs studied here still possessed normal Flt3 alleles, and this is independent of a potential radiation history. It is thus unlikely that the Flt3 mutation status alone can distinguish between spontaneous or radiation-induced AMLs. It will be interesting to determine, however if additional genetic, cytogenetic and histological factors can be added to the spectrum of markers, thereby increasing discrimination between radiation-induced and sporadic leukemias. Notwithstanding, the presence of Flt3 mutations in radiation-associated AMLs may have clinical prognostic relevance, as a greater number of mutant alleles has been suggested to be a predictor of relapse rate and disease free survival [10].

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case	patient group	Sex/Age	AML type	Blast cells
24	victim	F / 50	M4eo	45%
11	liquidator	M / 35	M5a	95%
7	liquidator	M / 66	M2	30%
53	control	M / 61	M1	80%
33	control	F / 36	M2	100%

Table 1

case #	patient group	Flt3 ITD allele	size [bp]	extra peptide sequence
11	liquidator	1	~ 24	n.d.
		2	48	GSSDNEYFYVDF**REYE**
		3	114	GKNGKEVTGSSDNEYFYVDF**REYE**YDLKWEFPRENLEF
7	liquidator	1	66	PPI**REYE**YDLKWEFPRENLEFG
24	victim	1	48	YFYVDF**REYE**YDLKWE
		2	129	KNGSQLQMVQVTGSSDNEYFYVDF**REYE**YDLKWEFPRENLEFG
53	control	1	21	VDF**REYE**
33	control	1	45	EYFYVDF**REYE**YDLK

Table 2

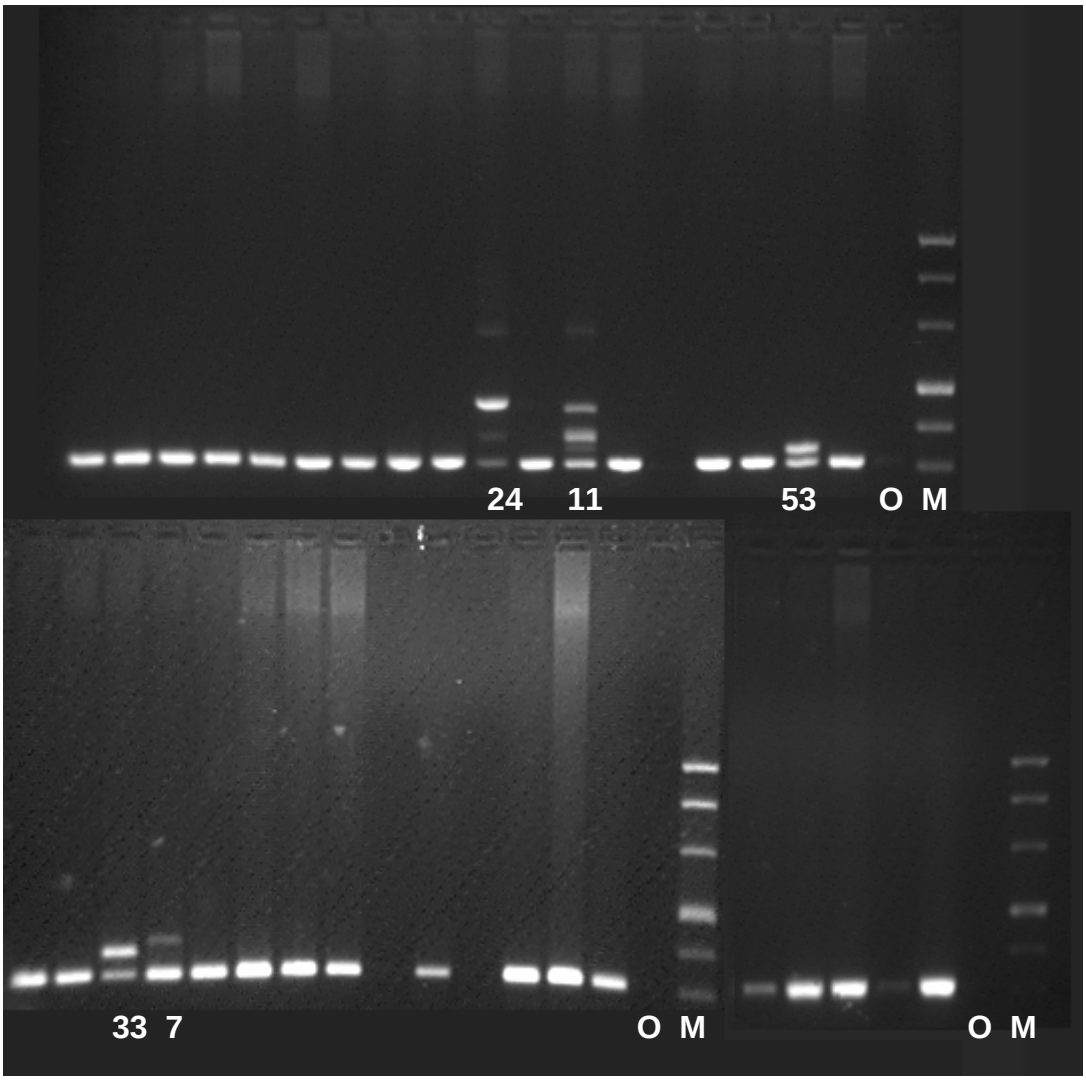

Fig. 1

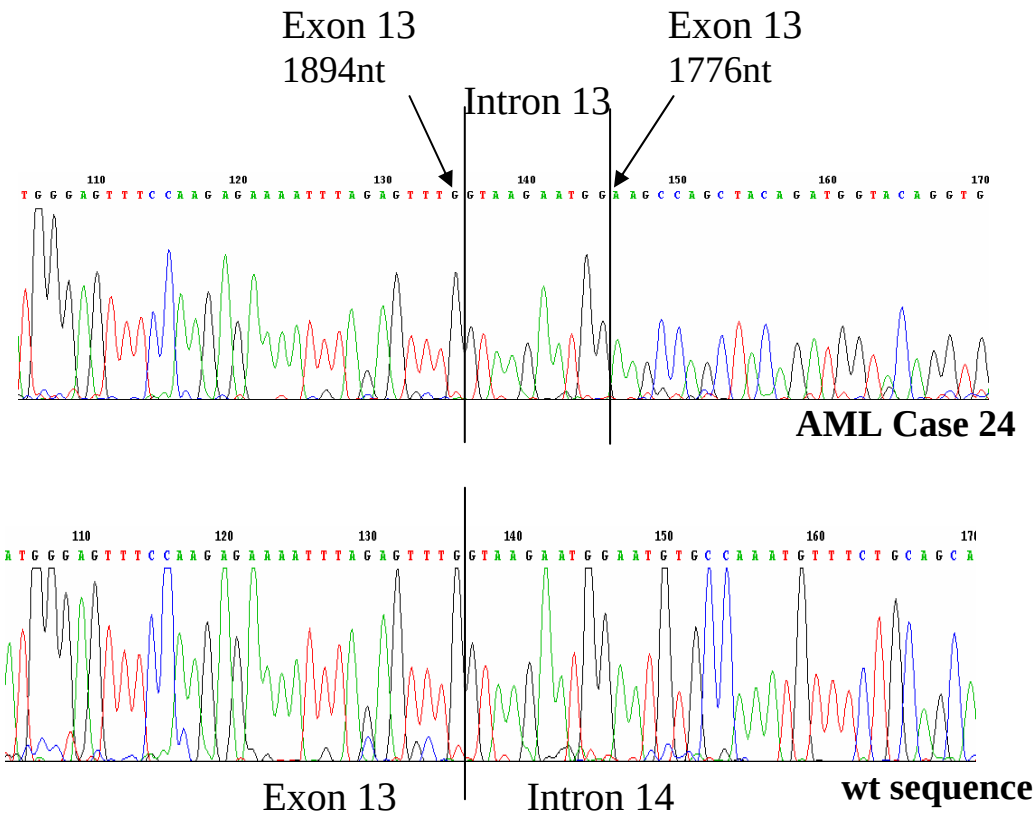
Exon 13
1894nt
Exon 13
1776nt
Intron 13
AML Case 24
Exon 13
Intron 14
wt sequence

Fig. 2

Figures

1 Flt3 exon 13 and 14 PCR from genomic DNA of Acute Myeloid Leukaemia. Case number and characteristics are given in table 1.

2 Sequence of a Flt3 allele carrying a 129 bp internal tandem duplication. (Case 24, 2nd. allele). This duplication involves the first 10bp of intron 13 and the last 119 bp of exon 13.

Tables

1 Tumor type and patient characteristic of AML cases showing Flt3 internal tandem duplications.

2 Multiplicity, length and inserted peptide sequence (predicted) of the Flt3 ITD duplications. The box shows the consensus tetra-peptide that was present in all analysed ITDs.