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Unusual long target duplication by insertion of intracisternal A-particle element in radiation-induced acute myeloid leukemia cells in mouse

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Abstract Retrotransmission into the IL-3/GM-CSF gene locus by the retrotransposon intracisternal A-particle (IAP) had been observed in distinct tumor cell lines. We analyzed the locus in genomes from 7 different myeloid leukemia cell strains which were originally generated by whole-body X-irradiation of the inbred C3H/He mice at a dose of 3 Gy and maintained by in vivo passage. In one leukemia cell strain out of 7 such cases, RFLP of an allele of the interleukin-3 gene was found. Sequence analysis after cloning from the genomic library showed that a type IΔ2 IAP element was inserted in the region upstream of the IL-3 gene in the head-to-head orientation. This suggests that the locus in myeloid cells is sensitive for integration of IAP elements. Additionally, an unusual long target duplication of 82 bp, 14-fold larger than normal one, was found at the junction of the element. This suggests the possibility of a radiation-induced integration mechanism which is distinct from normal retrotransmission.

Key words: Retrotransposon; Intracisternal A-particle; Myeloid leukemia; Target duplication; Interleukin-3 gene; Genomic DNA cloning

1. Introduction

The intracisternal A-particle (IAP), believed to be a defective provirus, is the most studied retrotransposon in the mouse (see reviews in [1,2]). Like a retrovirus, the integrated form of IAP in the mouse genome has *gag-pol-env* genes between two direct repeats of a retroviral long terminal repeat (LTR) sequence. The copy number of the IAP element is approximately 1000 per haploid genome, which is probably the result of accumulation by retrovirus-like integration and maintenance by vertical transmission in germline cells. In the case of somatic cells, the most commonly observed novel insertion of the element is limited in several tumor cells which possess unique functions due to corresponding gene rearrangement. It is possible that activation or inactivation of several genes by novel integration of the IAP element contributes to tumorigenicity of the cells [1,2]. However, the large copy number prevents determination and isolation of the IAP-inserted loci which are tumor specific.

Interestingly, integration of the IAP element into the interleukin (IL)-3/granulocyte macrophage colony-stimulating factor (GM-CSF) gene locus with gene activation has been found frequently in distinct tumor cell lines [3–8]. If the IL-3/GM-CSF locus has a tendency to accept IAP elements, several tumor cell with gene rearrangements of IL-3 by retrotransmission should be detected. To confirm this supposition as well as to study the chronic effects of radiation, we used radiation-induced acute myeloid leukemia cells from the C3H/He mouse for analysis. Approximately 20–30% of whole-body X-irradiated C3H/He mice develop acute myeloid leukemia within 2 years with chromosomal aberrations [9,10]. Each leukemia cell strain from different individuals should be an 'independent' leukemia since they became tumorigenic by separate processes. In leukemia cells, not only tumor-specific but also radiation-specific events may be recorded in the genome.

We analyzed this locus and found integration of the IAP element in the IL-3 gene in one leukemia cell strain from a total of 7 independent leukemias. Additionally, an unusual long direct repeat of mouse DNA of 82 bp in length was generated at the insertion site of the IAP element, although the target duplication is no more than 6 bp in length in all the reported sequences suggesting a retroviral-like integration mechanism [3,5,6,8,11–19]. The long target duplication may reflect a specific event which occurs during radiation-induced leukemogenesis.

2. Materials and methods

2.1. Leukemia cells and DNA preparation

Radiation-induced myeloid leukemia cells were originally prepared by Yoshida et al. [9]. In brief, 6-week-old C3H/He mice inbred in our institute received whole-body X-irradiation at a dose of 3 Gy. Leukemia cells were isolated from leukemic individuals and were characterized histochemically [9]. After 5 cycles of in vivo passage for stabilization of histochemical phenotype [9] and chromosome number [10], leukemic cells were stored in liquid nitrogen. The frozen cells were thawed, washed with saline and injected intravenously ($1–10 \times 10^4$ cells per mice) into 6-week-old male C3H/He mice, and after 2–4 weeks, proliferated leukemia cells were isolated from the spleen of the leukemia mice. Total DNAs from the cells were prepared by the standard method [20].

2.2. Probes and the labeling

Probes for IL-3 cDNA corresponded to nucleotides (nt) 104–296 of the previously reported sequence (Genbank K01850). GM-CSF cDNA was a gift from Kirin Beer Inc., and cDNAs for IL-1α and IL-1β were from Yakult Central Institute. CDNAs for IL-3, IL-4, IL-5, IL-6 and IL-7 were supplied by the Japanese Association of Immunology. These cDNAs were labeled with [α-^{32}P]dCTP (NEN; 3000 Ci/mmol) using a random primer labeling kit (BRL).

2.3. Polymerase chain reaction (PCR)

Four primers used in the study are as follows: the J1F primer (5′-GAAGGCTCCTGTGGCTTCTT-3′) corresponding to nt −22 to −3 of the putative start site for transcription of the IL-3 gene at sense direction, the J1R primer (5′-AAGAAGCCACAGGAGCCTTC-3′) having complemental sequence to the J1F primer, the IAPF2 primer (5′-ATATGGGTGGCCTATTTGCT-3′) corresponding to the IAP element at sense orientation and the IAPR1 primer (5′-GGTTTCGGC-ACCAATTGTTA-3′) corresponding to the IAP element at antisense orientation. Twenty ng of total genomic DNA with these primers and

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The sequences reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the following accession numbers D63766 and D63767.

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Taq DNA polymerase (Perkin-Elmer) was amplified for 30 cycles (94°C; 1 m; 55°C; 1 m; 72°C; 5 m) using a thermal cycler (Perkin-Elmer model 480).

2.4. Southern blot hybridization

According to the standard method [20], 10 μg of total DNA was digested by an appropriate restriction enzyme, electrophoresed and transferred onto nylon membranes. The blots were prehybridized at 65°C for 1 h in 5 × SSC buffer containing 2 × Denhardt's solution, 10% SDS, 50 mM sodium phosphate, 50 mM Tris-HCl (pH 7.5) and 100 μg/ml sonicated salmon sperm DNA. Hybridization was performed by incubation at 65°C overnight after addition of radioactive probe. The membranes were washed twice with 2 × SSC buffer containing 5 mM sodium phosphate and 1 mM EDTA at 65°C for 30 min, and radioactive bands were visualized by the BAS 2000 system (Fuji Film Co., Japan) after exposure of the Imaging Plate.

2.5. Construction of genomic library, cloning and sequencing

Total DNAs from leukemia cells and from the liver of C3H/He mice were partially digested with *Mbo*I, size-fractionated by sucrose gradient centrifugation, ligated into lambda EMBL3 vector DNA, incorporated into phage particles using Giga pack gold (Strategene), and introduced into *E. coli* P2–392 cells [20]. After amplification of the library containing 10^7 independent clones, 2×10^6 clones were screened by the plaque hybridization method [20] using IL-3 cDNA as a probe. Ten IL-3-positive clones were selected and purified by 4 cycles of single-plaque isolation. The inserted DNA fragments were subcloned using the plasmid vector pBluescript KS+ (Strategene), and the nucleotide sequences were then determined using a SequenaseII sequencing kit (Strategene).

3. Results

3.1. IL-3 gene rearrangement in radiation-induced myeloid leukemia in C3H/He mice

We used 7 independent populations of myeloid leukemia cells which were originally isolated from different leukemic individuals after whole-body irradiation of C3H/He mice. Fig. 1 shows data from Southern blot hybridization of total DNA from the leukemia cells using IL-3 cDNA as a probe. Of 7 leukemia cell populations, one (L-8028) harbored an RFLP of one allele of the IL-3 gene. No such rearrangement was found in genes for GM-CSF in any of the 7 leukemia cell populations. Remarkable expression of IL-3 or GM-CSF mRNA was not observed by Northern blot analysis (data not shown) in all the leukemia cell populations. Additionally, no rearrangements in genes for IL-1α, IL-1β, IL-2, IL-4, IL-5 and IL-6 were found in the genome of the L-8028 cells (data not shown).

3.2. Cloning and sequencing of rearranged region of the IL-3 gene

We constructed a genomic DNA library from L-8028 cells and isolated 2 independent clones possessing a rearranged IL-3 allele and 8 clones corresponding to the normal IL-3 allele from 2×10^6 clones. There was no difference between the normal allele of the leukemic cell and the germline configuration. Sequence and restriction analyses showed that the IAP element was inserted into the 5′-region of the IL-3 gene in head-to-head orientation as shown in Fig. 2. Unlike other reported target sites in the IL-3 gene, the insertion site was close to the promoter region of IL-3. The IAP element is 5.4 kb in length, categorized as type IΔ2, which is a subtype probably derived from the full-length IAP element by deletion. Nucleotide sequence analysis showed the 5′ side of the inverted repeat sequence was not present in the 5′-LTR sequences in this case, although inverted repeats of 3–4 bp have been reported at both ends of the LTR in most IAP elements (Fig. 3). Since this

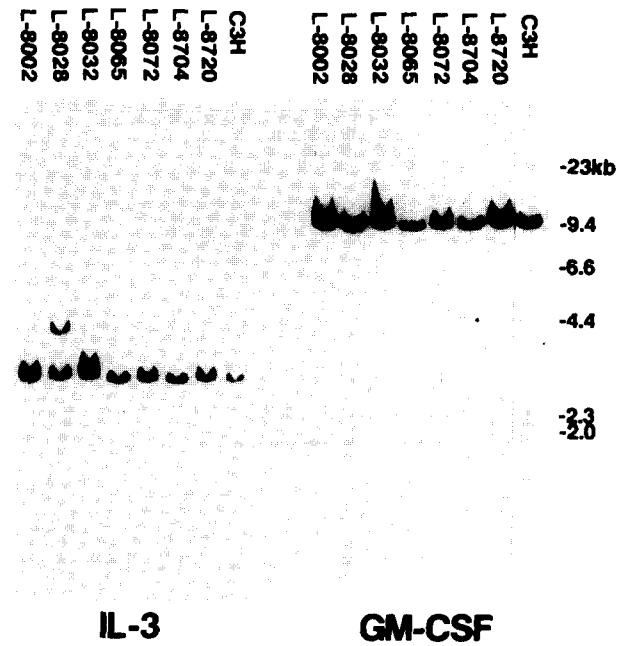

Fig. 1. Southern blot analysis of genomes from radiation-induced myeloid leukemia cells. Ten μg of *Bam*HI-digested DNA from liver of C3H/He and leukemia cells of different-origin (printed at the top of each lanes) were electrophoresed, blotted and hybridized with IL-3 cDNA (left panel) and GM-CSF cDNA (right panel).

incomplete inverted repeat was found only in the 5′-LTR, the defect should have occurred during or after integration. Additionally, complete identity of both LTRs suggest that the insertion event occurred during tumorigenesis in somatic cells. Except the polymorphic structure within the R-region [14], the LTR sequence was similar to other reported data such as the germline-derived LTR from MIA14 [14] at 82% identity, IL3/IAP from WEHI-3B [3] at 90% identity and that from FL5.12 [7] at 99.7% as shown in Fig. 3.

3.3. Unusual long target duplication surrounding IAP element

In the normal IL-3 allele, a single *Apa*I restriction site was present in the promoter region of the IL-3 gene (Fig. 2), as in the nucleotide sequence data from Genbank X02732 and K03233. However, the restriction map of the IL-3 gene locus in the rearranged allele showed the presence of two ApaI restriction sites close to junctions of the IAP element (Fig. 2). Sequence analysis of the junctions revealed that the IAP element lay between two unusual long direct repeats of the IL-3 gene 82 bp in length (Fig. 4a–c). The presence of an 82 bp target duplication in the IL-3 gene locus was further confirmed by allele-specific PCR amplification using L-8028 genomic DNA. Using a forward primer corresponding to the target duplication of the IL-3 gene at sense orientation (Fig. 4a, J1F) and a backward primer corresponding to the IAP element at sense orientation (Fig. 4a, IAPF2), DNA amplification of junction with 3′-LTR was observed only in the leukemia L-8028 (Fig. 4d, lane 3) but not in the germline (Fig. 4d, lanes 1 and 2). Similarly, the leukemia-specific amplification of junction with 5′-LTR was detected using a forward primer (Fig. 4a, IAPR1) corresponding to the IAP element at antisense orientation and a backward primer (Fig. 4a, J1R) having sequence complement

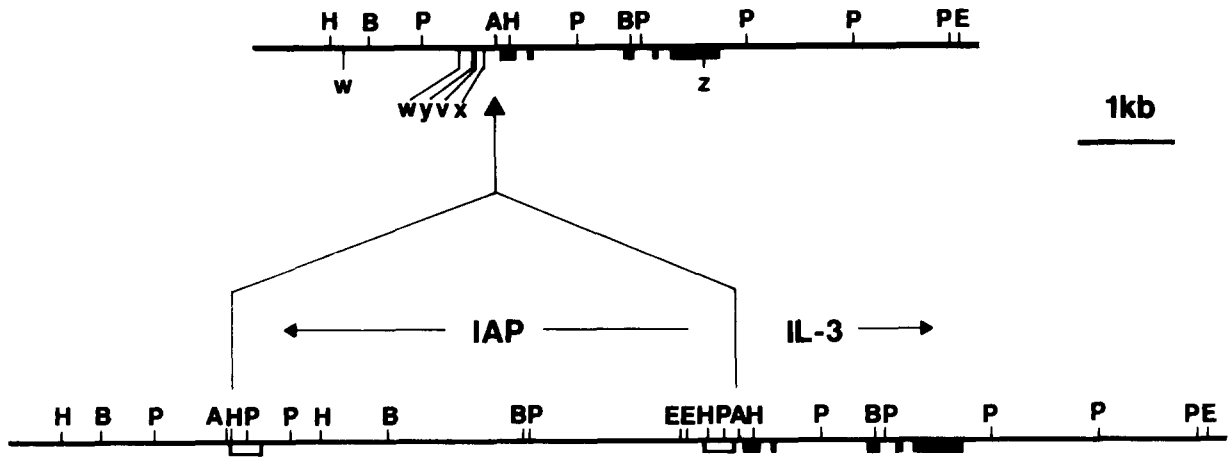

Fig. 2. Restriction map of IL-3 allele in L-8028 leukemia cells. Restriction maps of normal (upper line) and abnormal (lower line) IL-3 alleles were constructed of genomic clones from L-8028 leukemia cells and C3H/He liver cells. Closed boxes correspond to putative exons of the IL-3 gene, and open boxes represent LTR sequences of the IAP element. Restriction sites above both lines indicated are: E = *Eco*RI, B = *Bam*HI, H = *Hin*dIII, P = *Pst*I, A = *Apa*I. Insertion sites found in other previously published cell lines are indicated under the upper line as follows: v, [3]; w, [5]; x, [6]; y, [7]; z, [8].

to the J1F (Fig. 4d, lane 6). Thus, the long target duplication is present in an allele of the IL-3 gene in L-8028 leukemia cells.

4. Discussion

Integration by retrotransposon IAP element has the capacity to contribute to malignancy by activation or inactivation of genes, since at least some levels of IAP message are always present in normal cells [1,2]. Indeed, several established tumor cell lines have tumorigenic features generated at least in part by IAP element-derived activation of limited number of genes for c-mos [12], IL-3 [3,5–8], GM-CSF [4,5,6], IL-6 [16], IL-5 [21], IL-6 receptor [22], IL-2 receptor [23] and hox 2.4 [24]. Heidemann determined the efficiency of retrotransmission by IAP element as 1.5×10^{-6} per cell per generation in a teratocarcinoma cell line [25]. However, the large copy number (1000 copies per haploid genome) of the IAP element in the mouse genome strongly prevents further analyses such as determination and isolation of novel tumor-specific integration sites.

Activation of the IL-3 gene by integration of an IAP element in WEHI-3B cells causes factor-independence which contributes to malignancy [3]. Similar integration events by IAP elements at different sites in the IL-3/GM-CSF locus were found in factor-independent sublines from several cell lines including D35 myeloid [4], FDC-P1 myeloid [5], WEHI-274 myeloid [6], PB-3c mast cell [7] and FL-5.12 lymphoid [8]. Because most of these sublines produce a large amount of IL-3 or GM-CSF mRNA, they are probably selected from IL-3/GM-CSF-dependent population with dominant phenotype of factor-independence. Frequency of in IL-3/GM-CSF-independent clones which included IAP-transposed one at $4–70 \times 10^{-8}$ had been estimated in D35 cell line by Stocking et al.[4].

We found the IAP transposition into IL-3 gene in one strain (L-8028) out of 7 in vivo-passaged leukemia strains (Figs. 1, 2 and 3) which are not selected as factor-independent cells. Stimulation of IL-3 mRNA expression was not observed in the L-8028 cells unlike WEHI-3B cells, although IAP-LTR possessed trascriptional activity. It may be due to the excessively short distance between integration site and the promoter of the IL-3 gene (GC-rich region, Fig. 4a) in the cell, as compared with above-mentioned cell lines. The IAP-transposed IL-3 gene was found in one leukemia strain, even though we tested only 7 strains. This may suggest that the transposition into the IL-3 gene locus is a frequent event in some kind of cell types. The

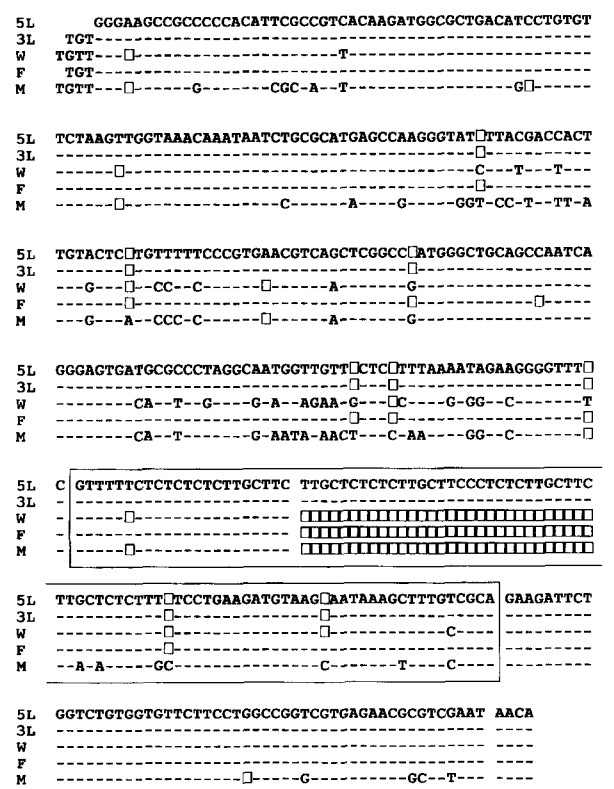

Fig. 3. Sequence comparison of IAP LTRs. Nucleotide sequence of 5′-LTR at the IL-3 allele from L-8028 cells (5L) was compared with the 3′-LTR from the allele (3L) and other LTRs containing IL-3-IAP from WEHI-3B (W)[2], IL3-IAP from FL5.12 (F)[8] and germline IAP of MIA14 (M)[14]. Comparisons to 5L are denoted as follows: – = same sequence; N = different sequence; □ = absent sequence. The R-region in the LTRs is boxed.

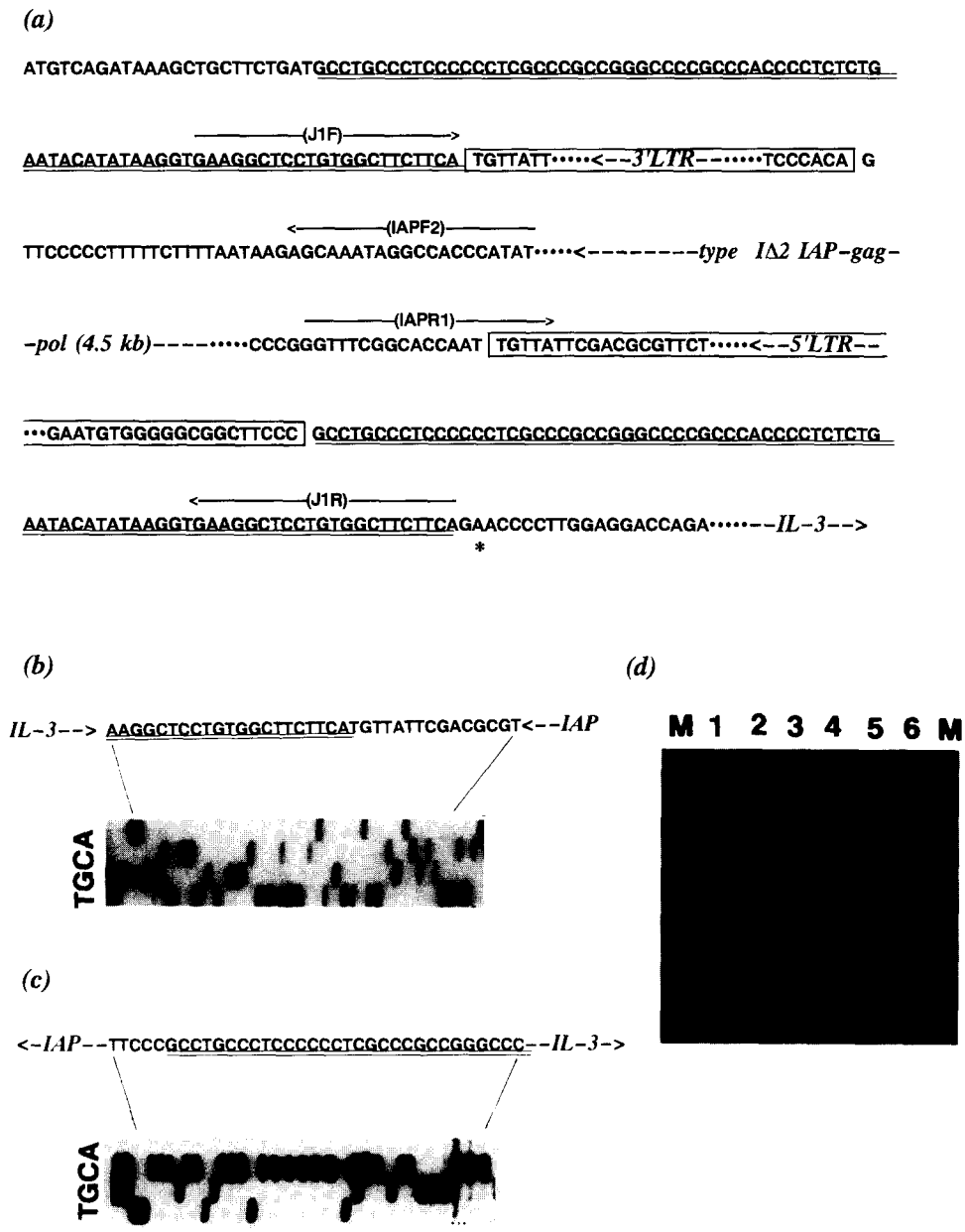

Fig. 4. Analyses of nucleotide sequence at junction of IAP element and IL-3 gene. (a) IAP element sequence is boxed and unique long target duplication sequences are double underlined. Four arrows above the line of sequence shows PCR primers (J1F, IAPF2, IAPR1 and J1R) for Fig. 4d. The asterisk in the bottom line of sequence indicates putative transcription start site. (b) Part of the nucleotide sequence gel showing the junction of the 3'-LTR/IL-3 gene. (c) Part of the sequence gel showing the junction of the 5'-LTR/IL-3 gene. (d) Electrophoresis of DNA after an amplification by PCR. Different lots of genomic DNA from C3H/He liver (lanes 1, 2, 4 and 5) and from L-8028 (lanes 3 and 6) were used for PCR with a set of the J1F and the IAPF2 primers (lanes 1–3), and a set of the J1R and the IAPR1 primers (lanes 4–6). The M is ϕX174DNA/*Hae*III as a molecular length standard.

presence of such a sensitive locus can be proposed based on recent evidence of insertions of IAP elements into the *agouti* locus in germline cells in three different hair color mutant mice [18,19]. At present, we cannot exclude possibility that we could accidentally found the integrated leukemia without selection. To estimate the sensitivity to the transposition in the IL-3/GM-CSF locus, further studies using numerous leukemia strains by wide-range analysis such as pulsed-field gel electrophoresis should be required.

We also found a unique long target duplication at the insertion site (Fig. 4). With the exception of one case with no target duplication [11], all junction sequences published have a 6 bp target duplication suggesting that the insertion was due to a retroviral integration mechanism [3,5,6,8,11–19]. In addition, an incomplete inverted repeat was found only in the 5'-LTR (Fig. 3). No supporting mechanisms nor speculations have yet to suggest that such features may be the recombination after integration.

An alternative mechanism to explain these observations is that both ends of the IAP cDNA with a small deletion bind to the 82 nucleotide cohesive ends. These DNA ends may be generated by artificial reactions induced by ionizing radiation

via superoxide. Gene rearrangement is still not well understood at the molecular level in radiation-induced tumor cells. However, speculative data were presented that X-irradiation increased in the level of message for VL30-retro element which is also a member of the mouse retrotransposon family [26]. If IAP message is increased with VL30 mRNA, then frequency of incorporation can be increased. Additionally, if a limited number of loci are chemically sensitive and subsequent DNA ends behave as acceptors for such elements, the insertion into these loci may be found. Further analyses of radiation- and nonradiation-induced tumors with regard to IAP integration should be performed to reveal the detailed mechanisms of retrotransmission and tumorigenesis.

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