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Induction of BCR-ABL Fusion Genes by *in vitro* X-irradiation

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The Philadelphia chromosome consists of a reciprocal translocation between the ABL oncogene at chromosome 9q34 and the BCR gene at chromosome 22q11, resulting in the expression of chimeric BCR-ABL mRNAs specific to chronic myelogenous leukemia (CML). Presence of the fusion gene can be detected with high specificity and sensitivity by means of reverse transcription and polymerase chain reaction. Using this assay, it was possible to detect BCR-ABL fusion genes induced among HL60 cells after 100 Gy of X-irradiation *in vitro*. In total, five fusion gene transcripts were obtained among 10^8 cells examined. These fusion genes contained not only CML-specific BCR-ABL rearrangements, but also other forms of BCR-ABL fusions. These latter genes had junctions of BCR exon 4/ABL exon 2 intervened by a segment of DNA of unknown origin, BCR exon 5/ABL exon 2, and BCR exon 4/ABL exon 2. The results appear to be direct evidence for the induction of the BCR-ABL fusion gene by X-irradiation. In terms of leukemogenesis, it appears that only those cells bearing certain CML-related BCR-ABL fusion genes are positively selected by virtue of a growth advantage *in vivo*.

Key words: BCR-ABL — CML — X-irradiation — Ph[1]

The Philadelphia chromosome (Ph[1]) is well known as a specific human chromosomal abnormality strongly associated with chronic myelogenous leukemia (CML).[1] It consists of reciprocal translocations between the BCR gene at chromosome 22q11 and the ABL gene at chromosome 9q34.[2] The breakpoints on chromosome 22 in the majority of cases with CML are clustered within a small DNA segment designated as the BCR region, or more specifically, fall within the second or the third intron of the BCR region.[3] The breakpoints in the ABL gene on chromosome 9 are scattered around exon 1a in a region of up to 200 kb or more.[4,5] However, the chimeric mRNA does not contain ABL exon 1a, regardless of the breakpoints in the ABL gene. Thus, only two types of mRNA are produced. One has a junction between BCR exon 2 and ABL exon 2, and the other has a junction between BCR exon 3 and ABL exon 2 in the transcript.[3,6] To detect BCR-ABL fusion genes, it is not possible to use the polymerase chain reaction (PCR) technique directly on DNA samples because of the wide range of the breakpoints. However, this difficulty can be overcome by the use of reverse transcription PCR (RT-PCR), taking advantage of the shorter length of the chimeric BCR-ABL mRNA devoid of large introns.

With respect to the etiology of human leukemia, exposure to ionizing radiation has been considered as one of the risk factors.[7] Even though it has been well recognized that chromosomal aberrations such as deletions or rearrangements are induced by ionizing radiation,[8] there has been no direct evidence concerning the induction of specific chromosomal aberrations such as the Philadelphia translocation due to the lack of appropriate detection systems. In this study, using the RT-PCR technique, we investigated whether BCR-ABL fusion genes were inducible by X-ray exposure.

For RT-PCR assay, first RNA was isolated from cultured cells by pelleting through a CsCl step gradient.[9] Then three micrograms of total RNA was dispensed in 20 μl of reverse transcription buffer (50 mM KCl, 50 mM Tris-Cl pH 8.4, 2.5 mM $MgCl_2$, and 100 μg of bovine serum albumin/ml) containing 2.5 pmol of primer D, 1 mM dNTPs, and 100 U of MMLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD), and incubated at 37°C for 1 h. The reaction mixture was diluted with 15 μl of PCR buffer (50 mM KCl, 50 mM Tris-Cl at pH 8.4, 1.5 mM $MgCl_2$, and 100 μg of bovine serum albumin/ml) containing 2.5 pmol of primer A and 0.5 U of *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT), and PCR amplification was performed by 35 thermal cycles as follows: 30 s at 94°C (denaturation), 1 min at 55°C (annealing), and 1 min at 72°C (polymerization). Using 1/100th of the products as templates, another 35 cycles of PCR amplification were performed in 25 μl of the same PCR buffer with a pair of internally located primers, B and C (2.5 pmol). The PCR products were detected by polyacrylamide gel (8%) electrophore-

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sis. PCR primers based on published sequence information[10] were synthesized on a DNA synthesizer (Applied Biosystems, Foster, CA), and their sequences are given below. Fig. 1 shows the approximate locations of the oligonucleotides in the two forms of chimeric BCR-ABL mRNA.

Primer A: 5′-CTGACCAACTCGTGTGTGAAAC-3′
Primer B: 5′-TGTGTGAAACTCCAGACTGTCC-3′
Primer C: 5′-ACGAGCGGCTTCACTCAGACC-3′
Primer D: 5′-TTTCCTTGGAGTTCCAACGAGC-3′

The feasibility and specificity of the RT-PCR assay to detect the chimeric BCR-ABL mRNAs in Ph¹-positive CML cells is shown in Fig. 2. K562, BV173, and KCL22 are Ph¹-positive cell lines derived from CML.[11-14] HL60 is a Ph¹-negative cell line,[15] and Daudi is a Ph¹-negative cell line established from Burkitt's lymphoma.[16] All of these cell lines were obtained from the Japanese Cancer Research Resources Bank, Tokyo. The amplified products of 189 bp from KCL22 and BV173 cells represent the junction between BCR exon 2 and ABL exon 2. Nonspecific products were not observed from the RNAs of the Ph¹-negative cell lines HL60 and Daudi.

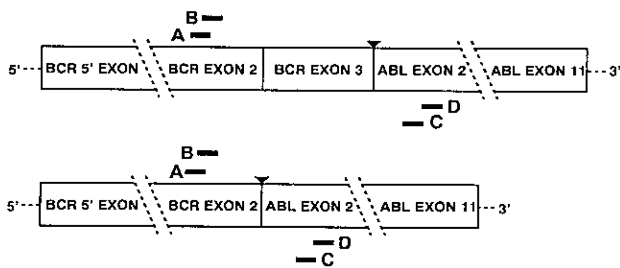

Fig. 1. Two forms of chimeric BCR-ABL mRNA structures specific to CML and the location of the oligonucleotide primers. The arrowheads indicate the junction between the BCR and ABL exons.

To determine the sensitivity of the RT-PCR assay, a dilution experiment was carried out as shown in Fig. 3. Ph¹-positive K562 cells were mixed with Ph¹-negative Daudi cells with ratios varying from 1:10 to 1:10^6 and about 100 μg of total RNA was extracted from the mixture of 10^7 cells. Subsequently, 1/100th of the RNAs was subjected to RT-PCR amplification. The 189-bp products could be detected even from one K562 cell among 10^6 Daudi cells. No difference in the intensities of the bands was observed, probably because the product levels reached a plateau after two rounds of PCR amplifications.

For detection of BCR-ABL fusion genes, RT-PCR was initially applied with a combination of Southern blot hybridization.[17] The detection limit of the assay was 10 pg of total RNA from Ph¹-positive K562 cells with one round of PCR using a pair of primers. In the present study, two rounds of PCR amplification were performed using an additional pair of internally located primers for the second round in order to reduce artifactual products and to increase the sensitivity. As a consequence, only the BCR-ABL transcripts were amplified and the detection limit was improved by a factor of approximately ten. Less than 1 pg of total RNA from the K562 cells could

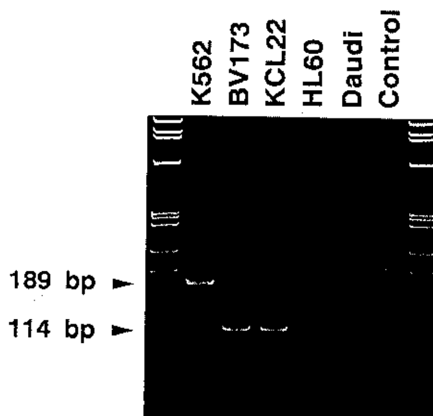

Fig. 2. RT-PCR analysis of cell lines. 189 bp product from Ph¹-positive K562 cells indicates the junction between BCR exon 3 and ABL exon 2, and 114 bp product from BV173 and KCL22 indicates the junction between BCR exon 2 and ABL exon 2. HL60 and Daudi are Ph¹-negative cell lines and the control did not contain DNA.

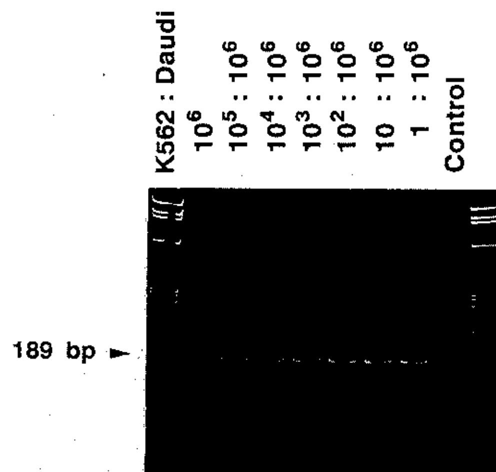

Fig. 3. Sensitivity of the RT-PCR assay. Ph¹-positive K562 cells were mixed with Ph¹-negative Daudi cells with ratios varying from 1:10 to 1:10^6 and RNAs were extracted and subjected to RT-PCR amplification. BCR-ABL fused genes could be detected even from RNA samples corresponding to 1 K562 cell in 10^6 Daudi cells.

be detected by RT-PCR alone without further analysis such as Southern blot hybridization.

Based on these results, the inducibility of the BCR-ABL translocation after X-irradiation was examined using HL60 Ph[1]-negative cells. Actively growing HL60 cells were irradiated with 100 Gy in a 10 cm dish at room temperature (approximately 15°C) and were incubated for 48 h under 5% CO_2 and 95% air at 37°C in RPMI 1640 medium supplemented with 10% FCS, 1% L-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin prior to RNA extraction. The X-ray generator (Shimadzu WSI-250S, Kyoto) was operated at 220 kVp, 8 mA with a 0.5 mm Al and 0.3 mm Cu filter, at a dose rate of about 2.8 Gy/min. Following irradiation in triplicate, total RNA from the 10^8 irradiated cells was analyzed by 41 independent RT-PCRs. Five of the 41 samples showed positive bands. Re-electrophoresis of the five positive samples in a single gel demonstrated that these five bands all differed in size, as shown in Fig. 4. Direct sequencing of the products was performed to clarify the junction sites. Using 1/100th of the second PCR products, 35 cycles of asymmetric PCR were performed in 25 μl of the same PCR buffer with an uneven molar ratio of the primers B and C (0.5 pmol:50 pmol or 50 pmol:0.5 pmol). The products of asymmetric PCR were purified by precipitation with ethanol and isopropanol in the presence of ammonium acetate. The purified DNA was used as a template for sequencing after being denatured at 95°C for 5 min, and annealed at 65°C for 10 min with 1 pmol of primers B or C labeled at their 5′ ends with [γ-^{32}P]ATP by T4 polynucleotide kinase. The nucleotide sequencing was performed using a Sequenase version 2.0 kit (U.S. Biochemical, Cleveland, OH), which uses the dideoxy chain termination method of Sanger *et al.*[18]

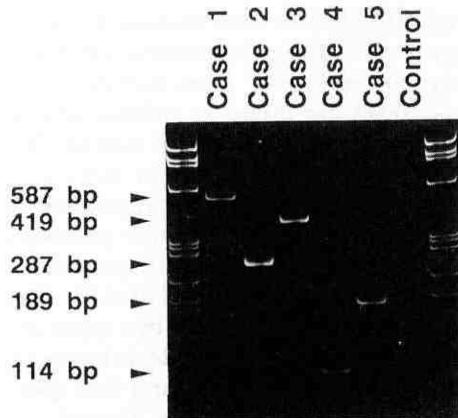

Fig. 4. Results for the inducibility of BCR-ABL fused genes by X-irradiation. Total RNA was extracted from HL60 cells cultured for 48 h after 100 Gy of X-irradiation. Five positive bands were identified among a total of 1×10^8 irradiated cells. Re-electrophoresis of the five samples side-by-side in a single gel demonstrated that these five positive bands all differed in size.

The sequences of the junction sites are shown in Fig. 5. Schematic illustrations of these fusion products are shown in Fig. 6. In case 1, a segment of DNA whose origin is not clear at present was found to intervene

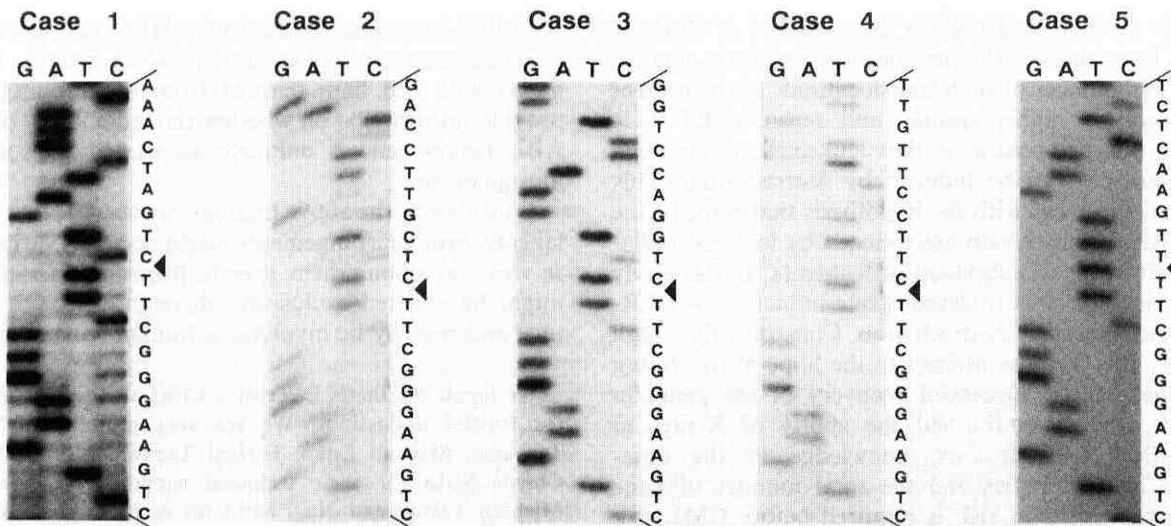

Fig. 5. Direct sequencing of positive cases was performed to clarify the junction sites. The arrowheads indicate junctions between unknown sequence and ABL exon 2 in case 1, BCR exon 4 and ABL exon 2 in case 2, BCR exon 5 and ABL exon 2 in case 3, BCR exon 2 and ABL exon 2 in case 4, and BCR exon 3 and ABL exon 2 in case 5.

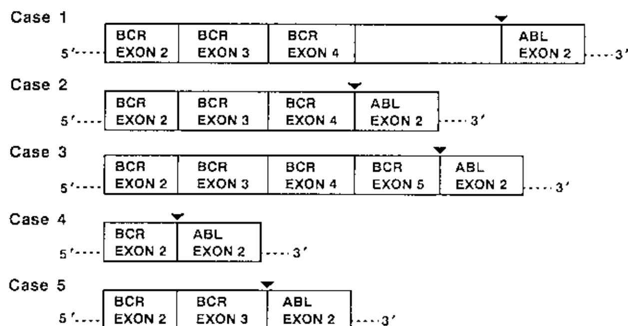

Fig. 6. Schematic illustration of the induced BCR-ABL fusion genes. The blank indicates an unknown sequence. The arrowheads indicate junction sites. These five BCR-ABL fusion genes were different from each other and contained not only CML-specific fusion genes, as in cases 4 and 5, but also other types of fusion genes, as in cases 1, 2, and 3.

between BCR exon 4 and ABL exon 2. A homology search of the Gen Bank^{R} DNA database using this unknown upstream sequence from the junction site did not reveal any known sequences with more than 60% homology. In the remaining four cases, BCR exon 2, 3, 4, or 5 was found to be fused to ABL exon 2, respectively. In the control experiments, analysis of the same number of non exposed HL 60 cells (1×10^{8} cells) showed no positive signals for BCR/ABL fusion genes (data not shown).

It is well known that most human CML cells are associated with the Philadelphia translocation, which seems to play a crucial role in the development of human leukemia.[1] In addition, risks of leukemia, including CML, are elevated in atomic-bomb survivors.[7] Although epidemiologic data indicate that radiation exposure results in an increased risk for development of CML, it has not been shown whether ionizing radiation acts directly as an inducer of such translocations. In the present study, using a highly specific and sensitive RT-PCR assay, it was demonstrated that leukemia-specific gene rearrangements can be induced by X-irradiation. This finding is consistent with the hypothesis that some of the BCR-ABL fusions which are induced by ionizing radiation result in the development of leukemia. In this study the primary goal was to demonstrate induction of BCR-ABL fusion genes by X-irradiation. Consequently a high dose, i.e., 100 Gy, was utilized in the hope of increasing the probability of successful recovery of the genes in question. Having established the ability of X-rays to induce BCR-ABL fusions, knowledge of the dose-response characteristics and the total number of bone marrow stem cells at risk is required before CML risk assessment in radiation-exposed people can be made.

Due to the ultra-sensitivity of this assay, extreme care was necessary to avoid contamination. Because control samples from non-irradiated HL60 cells were all negative and at least three of the five chimeric mRNA were of atypical sizes not usually seen in CML or ever detected in our institute, we are convinced that these results were not due to contamination. Furthermore, for the same reasons, we speculate that translocation of BCR and ABL genes might occur at nonselective sites in the BCR and ABL alleles of cells irradiated by ionizing radiation. Previous cytological studies have shown that various chromosome breaks are observed among X-ray-irradiated human lymphocytes. According to these results such breakpoints were scattered over the entire chromosomes with their distribution patterns differing from report to report.[19–23] Even though the possibility that certain genes are prone to undergo rearrangement cannot be excluded, the cytological multiplicity of chromosomes involved and our molecular analysis of various translocations induced *in vitro* suggest that radiation-induced translocation is not a unique phenomenon that occurs only at specific regions of the genome.

According to the reports that BCR-ABL fusion genes in CML are transcribed only into two types of chimeric mRNA,[3,6] it might be reasonable to consider that atypical transcripts are not functional and that cells carrying these transcripts are silent or are aborted during cell proliferation. In fact, it was found that in cases 2 and 3, ABL codons are out of frame, resulting in stop codons appearing soon after the junction sites. However, further investigation is needed, considering the fact that other types of correctly framed fusion genes cannot be detected with primers used in this experiment. The altered biological functions of BCR-ABL fusion gene, which is important in the deregulation of kinase activity of ABL proteins,[24,25] will clearly need to be studied as well.

In this experiment we have used HL60 cell line because it is derived from leukemia of myeloid lineage. Experiments with cell lines derived from other lineages will provide information on whether the inducibility of BCR-ABL fusion gene is uniquely associated with myeloid lineage or not.

Considering the above findings, we suspect that miscellaneous gene rearrangements might occur at various sites *in vivo*, and among them, specific forms of rearrangement might be selected exclusively through a growth advantage and thereby be involved in human leukemogenesis.

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