

Etoposide-induced apoptosis results in chromosome breaks within the *AF9* gene: Its implication in chromosome rearrangement in leukaemia

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ABSTRACT

Treatment with etoposide (VP-16) has been associated with translocation of the mixed lineage leukaemia (*MLL*) gene seen in treatment-related acute myeloid leukaemia (t-AML). Among the different partner genes, *AF9* is the most common partner gene of *MLL*. *AF9* shares similar structural element with the *MLL* gene. Various mechanisms of translocation have been proposed for the *MLL* gene, including apoptosis, particularly the apoptotic nuclease. In the current study, we show that VP-16 induced cleavage of the *AF9* gene in both leukaemic cells and cultured normal blood cell. All the breakpoints were mapped within the BCR1 of the *AF9* gene. *AF9* cleavages in leukaemic cells were abolished by pre-treatment with caspase inhibitor (Z-DEVD-FMK), suggesting the involvement of caspase-activated DNase (CAD). The absence of *AF9* cleavage in K562 cells further supported the involvement of apoptosis. However, *AF9* cleavages in cultured normal blood cell were not eliminated by caspase inhibitor. The possible role of CAD and other apoptotic nucleases/effectors that could be involved in *AF9* translocation are discussed.

Keywords: Etoposide (VP-16); Apoptosis; *AF9*; Chromosome Breaks; Leukaemia

1. INTRODUCTION

Etoposide (VP-16) is an epipodophyllotoxin that targets DNA topoisomerase II [1]. It stabilises the enzyme-DNA cleavage complex by inhibiting the religation reaction of topoisomerase II [2]. Etoposide exerts its anticancer effects by “poisoning” topoisomerase II, resulting in DNA cleavages [1]. Clinically, etoposide is widely used as an effective anticancer drug. However, about 2% - 12% of the patients eventually developed treatment-related acute

myeloid leukaemia (t-AML) [3]. The major chromosome translocation found in this group of patients involves the mixed lineage leukaemia or myeloid lymphoid leukaemia (*MLL*) gene at 11q23 [4,5]. Translocation of the *MLL* gene is not only found in t-AML but in *de novo* leukaemia as well [6]. In both cases, mapping of the chromosomal breakpoints in *MLL* revealed that almost all of the breakpoints clustered within an 8.3-kb region known as the breakpoint cluster region (BCR) [6]. Interestingly, this region contains a weak- and a high-affinity Scaffold Attachment Region (SAR) [6].

Unlike many other genes, the *MLL* gene rearranges with more than 66 different partner genes [7]. Among them the *AF9* gene located at 9p22 is one of the most common partner genes [8]. The *AF9* gene is more than 100 kb in size and contains two patients' BCR, namely BCR1 at intron 4 and BCR2 that spans introns 7 and 8 [8,9]. There is also a co-localising *in vivo* DNA topoisomerase II cleavage site and an *in vitro* DNase I hypersensitive site in intron 7 in BCR2. *AF9* also contains two SARs, located centromeric to the topoisomerase II site, bordering the BCR1 and BCR2 [10]. The *AF9* BCR was shown to have similar structural elements as the *MLL* BCR [10].

Various mechanisms have been proposed to mediate the event of chromosome translocation in patients treated with VP-16. These includes *Alu* repeats [11], V(D)J recombination [12] and DNA topoisomerase II [13]. However, more recently, the apoptotic nuclease was suggested to play a key role in the initial event of chromosome translocation, that is the breakage of the chromosome [14].

Apoptosis is a naturally occurring programmed cell death process. The apoptotic nuclease, caspase-activated DNase (CAD) is normally inhibited by the inhibitor of CAD (ICAD). During apoptosis induction, caspase cleaves ICAD, releasing the activated CAD. CAD then cleaves the chromosomal DNA resulting in the formation of high molecular weight (HMW) DNA and nucleosomal

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DNA ladder [15]. Although apoptosis is a cell death process, however, cells have the potential to recover upon DNA repair [16]. It is highly possible that during the recovery, different fragments of the chromosomes are joined and resulted in chromosome translocation. This is supported by the observation that etoposide stimulated the formation of *MLL* fusion product in CAD cDNA-complemented mouse embryonic fibroblasts (MEFs), but not in the CAD knock-out MEF [17].

In the current study, *AF9* gene was chosen because it is the most common translocation partner of the *MLL* gene [8], and *AF9* also has similar structure as the *MLL* gene [10]. We would like to hypothesise that during VP-16-induced apoptosis, chromosome cleavages occur in various parts of the genome including the *AF9* gene, which upon erroneous DNA repair, the cells survive with chromosome rearrangement. Thus in the current study we aimed to investigate the role of apoptotic nuclease in VP-16-induced chromosome breaks within the *AF9* gene. An understanding of the chromosome break event within this gene is important because this is the initial step of chromosome translocation.

2. MATERIALS AND METHODS

2.1. Cell Lines and Materials

CEM leukaemic cell line was purchased from American Type Culture Collection (ATCC). K562 was a generous gift from Prof. Dr. Leroy F. Liu of University of Medicine and Dentistry of New Jersey, New Jersey, USA. K562 is a cell line derived from chronic myeloid leukaemia (CML) patient and belongs to the granulocytic series of cell [18]. K562 cells exhibits delayed apoptotic features such as cleavage of caspase substrate, cytochrome c release, DNA fragmentation and apoptotic morphological changes [19]. Etoposide (VP-16) was purchased from SIGMA. DNA Polymerase I, Large (Klenow) fragment, T4 DNA Ligase and all the restriction enzymes were purchased from New England Biolabs (NEB, England). Phytohaemagglutinin (PHA) was purchased from Gibco, USA. PCR primers were obtained from First Base Laboratories. Phusion DNA Polymerase was purchased from Finnzymes, Finland. Caspase-3 inhibitor II (Z-DEVD-FMK) was purchased from Calbiochem, USA. Blood and cell culture DNA Mini-Prep kit, and QIAquick Gel Extraction Kit were obtained from QIAGEN, Germany. DYEnamic ET Terminator Cycle Sequencing Kit was purchased from Amersham, UK.

2.2. Cell Culture and Apoptosis Induction Assay

Cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-gluta-

mine. Cells were constantly grown at 37°C with 5% CO₂. CEM and K562 cells at a density of 1 × 10⁶ cells/ml were treated with 50 µM of VP-16 to induce apoptosis. Treatment with 0.1% DMSO as solvent control was included. All treatments were performed at 37°C for 5 hours otherwise mentioned. Cells were collected for genomic DNA extraction using QIAGEN Blood and Cell Culture DNA Mini-Prep according to manufacturer's protocol. The purified DNA was then digested with 100 U of *Bam*H I in a 150 µl reaction at 37°C for 16 hours.

2.3. Modification of Digested DNA as Template for Inverse Polymerase Chain Reaction (IPCR)

Figure 1 shows a summary of DNA modification as well as the IPCR. The *Bam*H I-digested genomic DNA consists of a mixture of intact *Bam*H I *AF9* fragments and cleaved *AF9* fragments. *Bam*H I digestion generates sticky ends while the ends of the cleaved *AF9* fragments are believed to be either a 1-base 5' overhang or a blunt end [20]. Therefore, Klenow fill-in was carried out to generate fragments with blunt ends. Two µg of DNA was used as template for Klenow fill-in following standard protocol [21]. Subsequently ligation/cyclisation with 2000 U of T4 DNA Ligase was performed in a 500 µl reaction at 16°C for 16 hours. Ethanol precipitation was carried out with 2 volumes of cold absolute ethanol and 0.1 volume of 3 M sodium acetate, pH 5.2. DNA pellet was washed with 70% ethanol and briefly air-dried, then dissolved in TE buffer, pH 8.0. The dissolved DNA was divided into two, one digested with 20 U of *Kpn* I and the other digested with 20 U of *Nde* I. *Kpn* I digestion was performed to linearise the cyclised DNA whereas *Nde* I digestion was performed to eliminate amplification of the intact *Bam*H I *AF9* fragment, thus eliminating its competition with the cleaved *AF9* fragment during IPCR. Digested DNA was purified using QIAGEN QIAquick Gel Extraction Kit according to manufacturer's protocol.

2.4. Nested IPCR and DNA Sequencing

Nested IPCR was performed with 200 ng of template DNA (*Kpn* I- or *Nde* I-digested), 0.5 mM of each primer, 200 µM of each dNTP, 1× Phusion buffer HF (containing 1.5 mM MgCl₂) and 0.4 U of Phusion Polymerase. IPCR cycle condition for the first round was: 30 seconds of 98°C for 1 cycle, followed by 30 cycles of 98°C for 10 seconds, 69°C for 30 seconds, 72°C for 15 seconds, followed by a 1 cycle of 72°C for 10 minutes. Second round IPCR was performed with 2 µl of 5-times diluted first round IPCR product with similar cycle condition, except that the annealing temperature was 57°C. The primers used during the first round of IPCR were 5'-ATTCTA-GACCCCAAAAATTCTCAG-3' and 5'-CTCTTAATGCCACTGCCATGA-3', while the primers used in the

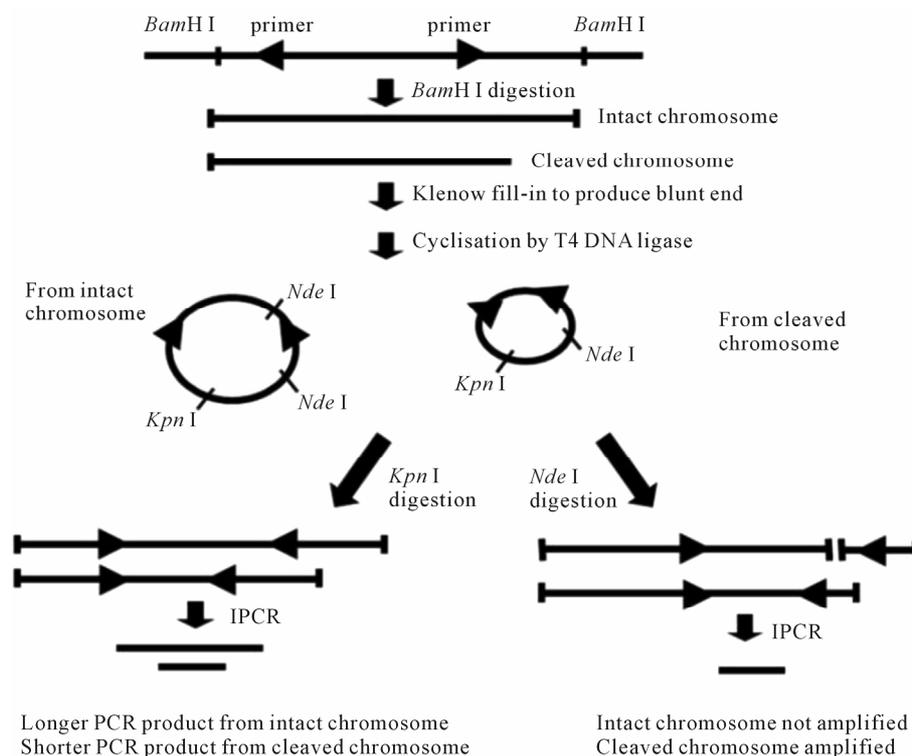


Figure 1. Flow chart showing DNA modification and IPCR. The arrow heads indicate the forward and reverse primers that were designed in opposite direction. *Bam*H I digestion yielded a mixture of intact chromosome and cleaved chromosome. Klenow fill-in produced blunt ended chromosome fragments which were then cyclised by T4 DNA ligase. The intact chromosome will become a large circle while the cleaved chromosome will become a smaller circle and lost one of the *Nde* I sites. Upon cyclisation, the primers are now in correct orientation for amplification. *Kpn* I digestion cleaved both circles outside the amplification region, thus merely linearise the molecule. *Nde* I digestion linearise the small circle but also cleave within the amplification region of the large circle, thus preventing amplification of the intact chromosome. This enhances the amplification of the cleaved chromosome.

second round were 5'-ATTGGTGTCAATCAAATGC-3' and 5'-CATATCCTTTTCATACCTGG-3'. IPCR products were then analysed on 1.5% agarose gel in 0.5× TBE buffer. Amplification of the intact *Bam*H I *AF9* fragment was expected to produce a 950 base pairs IPCR product. IPCR products smaller than 950 base pairs, which were expected to be the amplification of cleaved *AF9* fragments were excised and purified using QIAGEN QIAquick Gel Extraction kit according to manufacturer's protocol. These IPCR products were cloned and sequenced using DYEnamic ET Terminator Cycle Sequencing Kit according to manufacturer's protocol. Sequencing data was analysed using SeqMan DNASTAR software (Lasergene, USA) and compared with the published *AF9* gene sequence (GenBank accession No. AC000007).

2.5. Caspase Inhibition Assay in Leukaemic Cells

CEM cells at 1×10^6 cells/ml were pre-treated with 50

μ M of caspase-3 inhibitor II (Z-DEVD-FMK) at 37°C, 5% CO₂ for 1 hour. DMSO was included as solvent control. Subsequently the cells were co-treated with either 50 μ M of VP-16 or 0.1% DMSO for 5 hours. Genomic DNA was extracted and purified for use as DNA template for IPCR as described before.

2.6. VP-16-Induced Chromosome Breaks in Cultured Normal Blood Cell and Its Caspase Inhibition Assay

Venous blood from a normal volunteer was collected in a lithium heparin tube. One ml of the peripheral whole blood was cultured in 5 ml of RPMI 1640 medium containing 20% heat-inactivated foetal bovine serum, 200 units/ml penicillin, 200 μ g/ml streptomycin, 2 mM L-glutamine, and 2% phytohaemagglutinin (PHA) (M-form) [22]. Whole blood cultures were incubated at 37°C for 48, 54, 60, 66 and 72 hours in the presence of PHA. Cells were then treated with 50 μ M of VP-16 for 5 hours. Subsequently genomic DNA was extracted and modified

for IPCR analysis. For caspase inhibition assay, after the cells have been cultured in the presence of PHA for each time point (60, 66 and 72 hours), cells were pre-treated for 1 hour with caspase inhibitor. Subsequently, they were either co-treated with 50 μ M of VP-16 or 0.1% DMSO for 5 hours. Cells were then collected and processed for IPCR.

3. RESULTS

3.1. VP-16 Induces Cleavage of the *AF9* BCR

CEM cells were treated with VP-16 and the extracted genomic DNA was modified and processed for IPCR analysis. IPCR primers were designed to detect chromosome breaks within the BCR1 (intron 4 telomeric end) of *AF9*. As shown in **Figure 2**, with *Kpn* I digestion, only the intact *AF9* gene was amplified, represented by the 950 base pairs bands (lanes 2-4). IPCR band less than 950 base pairs representing cleaved *AF9* was not detected. This could be due to the competition between the intact chromosome and the cleaved chromosome (which is in a very small quantity) for the amplification process. Thus, *Nde* I was used to eliminate the amplification of the intact chromosome while at the same time to linearise the cyclised cleaved fragment as shown in **Figure 1**. Amplification of the intact *AF9* was not eliminated but reduced (**Figure 2**, lanes 5-7), and at the same time smaller IPCR products of sizes 180 base pairs and 730 base pairs were detected upon VP-16 treatment (**Figure 2**, lane 7). DNA sequencing shows that the 180 base pairs was an amplification product of an *AF9* cleavage fragment that carries a breakpoint at coordinate 26805 (GenBank accession No. AC000007), while the 730 base pairs corresponds to amplification of a cleaved *AF9* fragment that carries a breakpoint at coordinate 26235 (GenBank accession No. AC000007).

3.2. Caspase Inhibitor Eliminates VP-16-Induced Cleavage of the *AF9* BCR

VP-16 is known to induce apoptosis [14], thus in order to investigate if this VP-16-induced *AF9* cleavage was mediated by the apoptotic nuclease CAD, caspase inhibition assay was performed. CAD usually exists as a complex with its inhibitor, ICAD. It is only upon apoptosis activation, when caspase cleaves ICAD, then CAD will be released and be activated to cleave the genomic DNA [23]. Thus by inhibiting the caspase, CAD will not be activated and would prevent cleavage of the *AF9* BCR. As shown in **Figure 3(a)**, in the absence of caspase inhibitor, treatment with VP-16 resulted in cleavage of the *AF9* BCR producing a 420 base pairs band (lane 5). Indeed, pre-treatment with caspase inhibitor (Z-DEVD-FMK), and later co-treatment with VP-16 prevented VP-16-

induced cleavage (**Figure 3(a)**, lane 8). To ensure that the non-amplification observed in other samples were not due to template DNA degradation, IPCR with *Kpn* I-digested template DNA was performed, and amplification of the intact *AF9* was observed as a 950 base pairs band (**Figure 3(b)**, lanes 2-8).

3.3. K562 Cells are Resistant to VP-16-Induced Cleavage

K562 cells have been shown to be more resistant to apoptosis induction compared with HL-60 cells [19]. Therefore K562 cells were used in this study to investigate if VP-16-induced *AF9* cleavage would be delayed. As shown in **Figure 4**, with *Kpn* I digestion, amplification of the intact *AF9* fragment was detected (lanes 2-8). However, with *Nde* I digestion, amplification of the intact *AF9* fragment was eliminated, but there was no detection of the cleavage fragment (lanes 10-15). Note that the treatment times for K562 (5, 7 and 24 hours) were much longer as compared with that of CEM (5 hours) as presented before. Even with the extended treatment time, VP-16 failed to induce *AF9* cleavage in K562 cells, further supporting *AF9* cleavage was mediated through apoptosis.

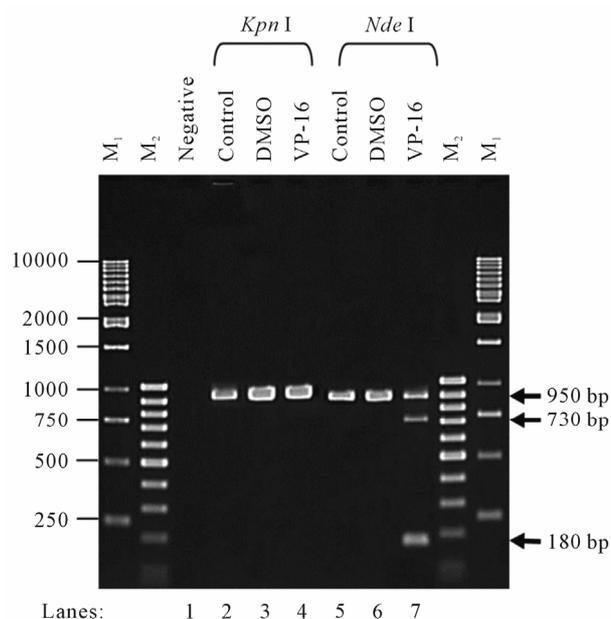


Figure 2. VP-16 induces *AF9* cleavage. CEM cells were treated with 50 μ M of VP-16 (lanes 4 and 7), 0.1% DMSO (lanes 3 and 6), or left untreated as cell control (lanes 2 and 5). The cells were then lysed, genomic DNA was extracted and modified for IPCR as described in Materials and Methods. The modified DNA was either digested with *Kpn* I (lanes 2-4) or *Nde* I (lanes 5-7) prior to IPCR. Lane 1 is a negative control, which does not contain DNA template. The side arrows show the 950 bp, 730 bp, and 180 bp DNA fragments. M₁: 1 kb DNA ladder. M₂: 100 bp DNA ladder.

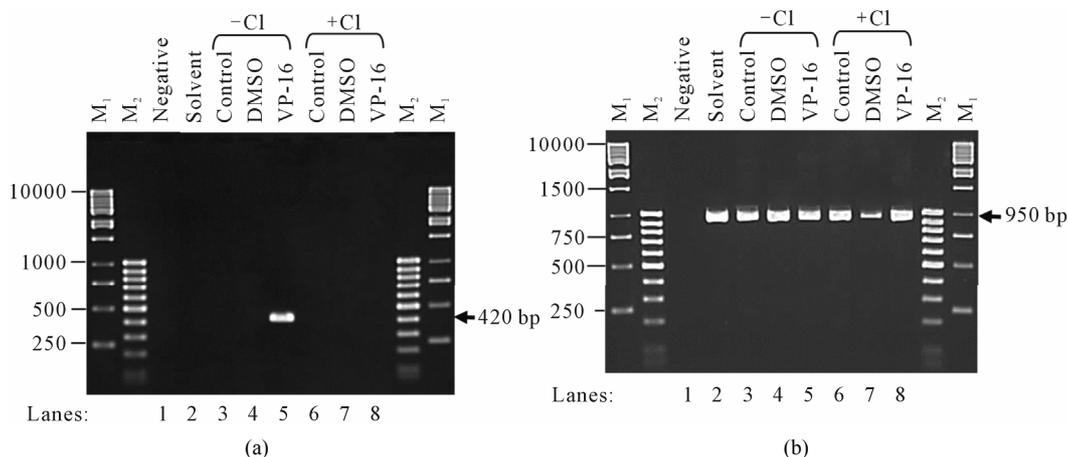


Figure 3. Caspase inhibitor abolishes VP-16-induced *AF9* cleavage. CEM cells were either pre-treated with caspase inhibitor Z-DEVD-FMK for 1 hr (lanes 6-8) or left untreated (lanes 3-5). The cells were then either co-treated with VP-16 (lanes 5 and 8), DMSO (lanes 4 and 7), or left untreated (lanes 3 and 6) to serve as cell control. DMSO was included as a solvent control for Z-DEVD-FMK pre-treatment (lane 2). Genomic DNA was extracted, processed and digested with *Nde* I (panel (a)) or *Kpn* I (panel (b)) prior to nested IPCR. A negative control containing no DNA template was included as well (lane 1). The side arrows indicate the 950 bp and 420 bp DNA fragments. M_1 : 1 kb DNA ladder. M_2 : 100 bp DNA ladder.

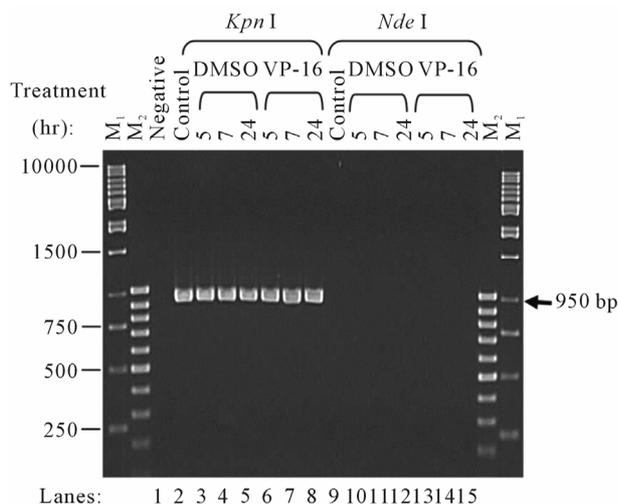


Figure 4. VP-16 does not induce *AF9* cleavage in K562 cells. K562 cells were treated with DMSO (lanes 3-5 and 10-12) or VP-16 (lanes 6-8 and 13-15) for 5 hr (lanes 3, 6, 10, and 13), 7 hr (lanes 4, 7, 11, and 14), and 24 hr (lanes 5, 8, 12, and 15). Genomic DNA was extracted, processed, and digested with either *Kpn* I (lanes 2-8) or *Nde* I (lanes 9-15). Nested IPCR was performed. Untreated K562 cells were included as control (lanes 2 and 9). Lane 1 is a negative control that does not contain DNA template. The side arrow shows the 950 bp DNA fragment. M_1 : 1 kb DNA ladder. M_2 : 100 bp DNA ladder.

3.4. VP-16 Induces *AF9* Cleavage in Cultured Normal Blood Cells

Our results using CEM cells suggest that VP-16 indeed induced chromosome breaks within the *AF9* BCR. As CEM is a leukaemic cell line, it brings to the question that if the same thing is happening in normal blood cell.

To answer this question, peripheral whole blood from a normal volunteer was used to study VP-16-induced *AF9* cleavage. For this purpose, the whole blood microculture was performed in the presence of phytohaemagglutinin (PHA) (M-form), which is a strong mitogen. Exposure of fully differentiated lymphocytes in the whole blood to PHA will transform these cells into mitotically active cells. It is crucial that the cells are mitotically active as VP-16 targets DNA topoisomerase II that is highly expressed in proliferating cells [24]. The whole blood culture was performed in the presence of PHA for specific periods of time and then treated with VP-16 as described in materials and methods. As shown in **Figure 5(a)**, with *Kpn* I digestion, only amplification of the intact *AF9* was detected (lanes 2-6). However, with *Nde* I digestion, three smaller IPCR bands of 740 base pairs, 180 base pairs and 240 base pairs were detected (**Figure 5(b)**). They were detected in cells cultured in the presence of PHA for 48 hours, 66 hours and 72 hours respectively (**Figure 5(b)**, lanes 2, 5 and 6). Sequencing results reveal that the 740 base pairs band was derived from cleavage of *AF9* at coordinate 26335 while the 180 base pairs and 240 base pairs bands were amplification products of *AF9* cleavage at coordinates 26817 and 26756 respectively (GenBank accession No. AC000007).

3.5. Caspase Inhibitor Does Not Inhibit VP-16-Induced *AF9* Cleavage in Cultured Normal Blood Cells

As discussed earlier, VP-16-induced *AF9* cleavage in CEM leukaemic cells was caspase-dependent. Thus, we would like to know if VP-16-induced *AF9* cleavage in

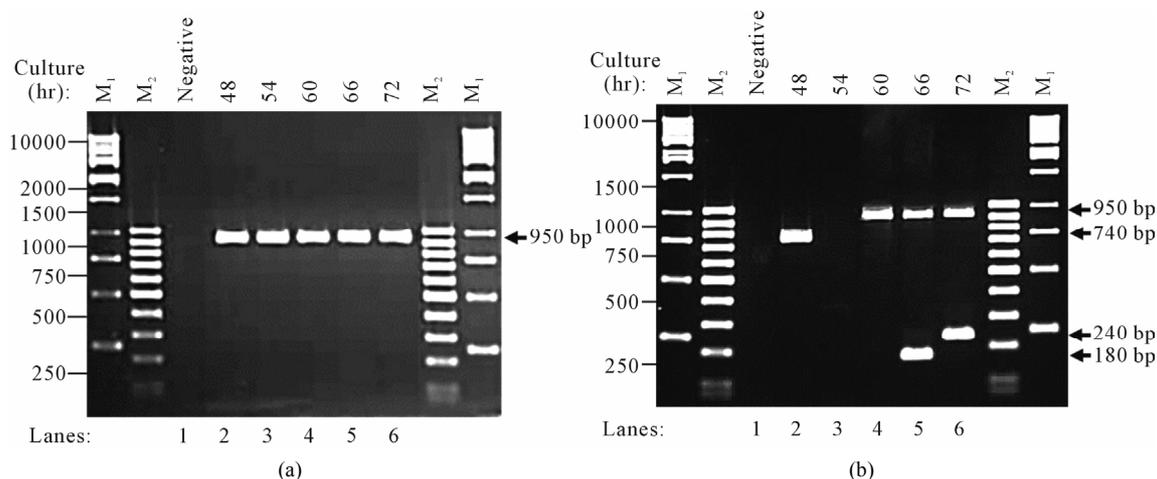


Figure 5. VP-16 treatment of cultured normal blood cells results in *AF9* cleavage. Blood cells from a normal volunteer were cultured in the presence of PHA for 48 hr (lane 2), 54 hr (lane 3), 60 hr (lane 4), 66 hr (lane 5), and 72 hr (lane 6). The cells were then treated with VP-16 for 5 hr. Genomic DNA was extracted and processed as described in Materials and Methods. DNA was digested with either *Kpn* I (panel (a)) or *Nde* I (panel (b)) followed by IPCR. Lane 1 is negative control, which does not contain DNA template. The side arrows show the 950 bp, 740 bp, 240 bp, and 180 bp DNA fragments. M₁: 1 kb DNA ladder. M₂: 100 bp DNA ladder.

cultured normal blood cell is also caspase-dependent. As shown in **Figure 6**, in the absence of caspase inhibitor, VP-16 treatment resulted in cleavage of the *AF9* BCR, as indicated by IPCR bands of sizes smaller than 950 base pairs (lanes 6 and 8). However, in contrast to that observed in the CEM leukaemia cells, these breaks formation was not inhibited by caspase inhibitor (lanes 14). Interestingly, treatment with 0.1% DMSO also resulted in *AF9* cleavages that were not inhibited by caspase inhibitor (lanes 3, 10 and 11).

4. DISCUSSION

Treatment with etoposide (VP-16) has been correlated with translocation of the *MLL* gene observed in t-AML patients [4,5]. Multiple mechanisms of translocation had been proposed for the *MLL* gene [11-13,25]. Among them, the involvement of apoptosis, particularly the apoptotic nuclease, CAD was proposed [14]. Apoptosis is a natural cell death pathway, however, it has been suggested that apoptotic cells may survive apoptosis and eventually survive with chromosome rearrangements [16]. Since the *AF9* gene has similar structure as the *MLL* gene [10], we intended to investigate if VP-16-induced apoptosis does lead to cleavage of the *AF9* gene. Indeed, our results show that VP-16 induced chromosome breaks within the *AF9* breakpoint cluster region (BCR). The breakpoints identified in this study clustered with those mapped in patients as well as those obtained from cell lines carrying t(9, 11) [9,26-28]. Interestingly, treatment of TK6 cells with anti-CD95 antibody, a known apoptosis inducer, also resulted in cleavage of the *AF9* BCR [29]. The breakpoints obtained in this study

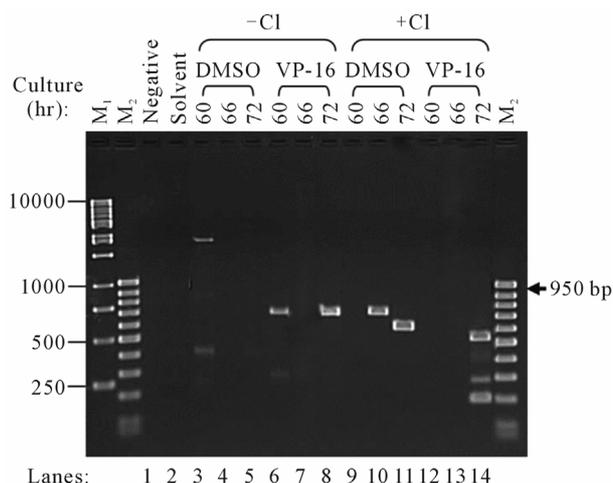


Figure 6. Caspase inhibitor does not inhibit VP-16-induced *AF9* cleavage in cultured normal blood cells. Blood cells from a normal volunteer were cultured in the presence of PHA for 60 hr (lanes 3, 6, 9, and 12), 66 hr (lanes 4, 7, 10, and 13), and 72 hr (lanes 5, 8, 11, and 14). The cells were then either pre-treated with caspase inhibitor for 1 hr (lanes 9-14) or left untreated (lanes 3-8). Subsequently, the cells were co-treated with DMSO (lanes 3-5 and 9-11) or VP-16 (lanes 6-8 and 12-14) for 5 hr. DMSO was included as a solvent control for caspase inhibitor pre-treatment (lane 2). Genomic DNA was extracted, processed and digested with *Nde* I prior to nested IPCR. Lane 1 is a negative control, which does not contain DNA template. The side arrows show the 950 bp DNA fragment. M₁: 1 kb DNA ladder. M₂: 100 bp DNA ladder.

are in very close proximity to those identified in the TK6 cells. From this comparison, it seems that cleavage of the *AF9* BCR is independent of cell line/patients and the type of stimuli. However, it is worth noticing that, both

VP-16 and anti-CD95 are apoptosis stimuli. Similar results were observed for the *MLL* BCR cleavage, whereby various chemotherapeutic drugs (VP-16, 5-fluorouracil, methotrexate, vinblastine and camptothecin) induced apoptosis as well as cleavage of the *MLL* BCR [14].

VP-16 targets DNA topoisomerase II and results in DNA double stranded breaks [30]. This DNA damage may induce the apoptotic signalling pathway [31], whereby the caspase cascade is activated. CAD is normally inhibited by ICAD. During apoptosis, caspase 3 cleaves ICAD, thus releasing the activated CAD, which eventually cleaves the chromosomal DNA [23,32]. In our study, Z-DEVD-FMK was used to investigate if the VP-16-induced *AF9* cleavage was mediated by CAD. Z-DEVD-FMK is a potent, cell-permeable and irreversible inhibitor of caspase 3, 6, 7, 8 and 10. Our results in leukaemic cell line indicated that CAD was most likely responsible for the *AF9* cleavage. This is in line with other reports in leukaemic cell line [14] and in nasopharyngeal carcinoma cell line as well [33]. In apoptotic cells, CAD associates with the nuclear matrix [34], thus allowing it to cleave the base of the chromatin loops attaching to the nuclear matrix or scaffold, generating high molecular weight (HMW) DNA during early stage apoptosis [15,35]. This is again supported by our results in K562 cells, where *AF9* cleavage was absent despite long period of VP-16 treatment. K562 is known to exhibit delayed apoptosis induction. This delay resulted from a delay in the signaling cascade upstream of cytochrome c release and caspase activation [19]. Taken together, it seems that VP-16-induced cleavages of the *AF9* were most likely to be mediated by CAD.

Our results showed that normal blood cell also exhibited *AF9* cleavage upon VP-16 treatment. However, these cleavages were not inhibited by caspase inhibitor. This contradictory observation could be due to a few possibilities. Firstly, normal blood cells and leukaemic cells may have different capacity of uptake for the caspase inhibitor. Although the uptake of caspase inhibitor has not been reported, it is known that the uptake capacity of methotrexate is different between leukaemic cells and normal leucocytes [36]. In addition, uptake of nitrogen mustard and choline were shown to be higher in leukaemic lymphoid cells compared with normal lymphoid cells [37]. Secondly, the normal blood cells might have died via the caspase-independent pathway as demonstrated by others [38,39]. This is seconded by the observation that mouse embryonic fibroblast (MEF) from caspase 3-deficient mice and CAD-deficient chicken DT40 cells were able to generate HMW DNA during apoptosis induction [40,41]. Other than CAD, the other possible candidate is Endonuclease G (Endo G). Endo G is an evolutionary conserved endonuclease that usually localised in the intermembrane space of mitochondria,

but released during apoptosis and results in caspase-independent DNA degradation [42,43]. However, based on its cleavage preferences, Endo G most likely does not work alone. It may work with DNase-I-like enzymes together with exonucleases *in vivo* for apoptotic DNA processing [44]. In addition, apoptosis-inducing factor (AIF) is another enzyme that is released from mitochondria during apoptosis and cause chromatin condensation and large scale DNA fragmentation [41]. DNA topoisomerase II is another possible player. It was proposed to be directly involved in the *MLL* BCR cleavage [45]. Poisoning of topoisomerase II by VP-16 resulted in chromatin loop excision [46]. This is very logical as topoisomerase II is one of the major proteins found in the nuclear scaffold [47]. Interestingly, CAD interacts with topoisomerase II and enhances topoisomerase II's decatenation activity *in vitro* [48].

Our sequencing analysis revealed that all the breakpoints are mapped inside the BCR1 within close proximity to one of the MAR/SAR. MAR/SAR sequences are abundantly found at the base of chromatin loop structures [49], and excision of the chromatin loops forming HMW DNA occurs during early apoptosis [35]. Thus our results indicate a possibility that the apoptotic nuclease(s) could be cleaving the base of the chromatin loop structure during early apoptosis. This is a crucial observation, as we hypothesise that apoptotic cells escape the cell death pathway and rescued as cells carrying chromosome rearrangements, thus it is logical that only those at early apoptosis could be rescued.

In conclusion, our results suggest that apoptotic nuclease(s) could be involved in VP-16-induced *AF9* cleavage, and thus contributing to chromosome translocation in t-AML.

5. ACKNOWLEDGEMENTS

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