Short Communication

Curcumin-Mediated Reversal of *p15* Gene Promoter Methylation: Implication in Anti-Neoplastic Action against Acute Lymphoid Leukaemia Cell Line

(curcumin / apoptosis / DNA methyltransferase 1 / cell cycle)

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Abstract. Curcumin has been documented to exert anticancer effects by interacting with altered proliferative and apoptotic pathways in cancer models. In this study, we evaluated the potential of curcumin to reverse promoter methylation of the *p15* gene in Raji cells and its ability to induce apoptosis and genomic instability. Anti-neoplastic action of curcumin showed an augmentation in reactive oxygen species (ROS) and cell cycle arrest in G1 phase. Subsequently, curcumin-exposed Raji cells showed structural abnormalities in chromosomes. These observations suggest that curcumin also causes ROS-mediated apoptosis and genomic instability. The treatment of Raji cell line with 10 μM curcumin caused hypomethylation of the *p15* promoter after six days. Hypomethylation of *p15* was further found to be favoured by down-regulation of DNA methyltransferase 1 after 10 μM curcumin treatment for six days. Methylation-specific PCR suggested demethylation of the *p15* promoter. Demethylation was further validated by DNA sequencing. Reverse-transcription PCR demonstrated that treatment with curcumin (10 μM) for six days led to the up-regulation of *p15* and down-regulation of DNA methyltransferase 1. Furthermore, curcumin-mediated reversal of *p15* promoter methylation might be potentiated by down-regulation of DNA methyltransferase 1 expression, which was supported by cell cycle analysis. Furthermore, curcumin acts as a double-pronged agent, as it caused apoptosis and promoter hypomethylation in Raji cells.

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Abbreviations: ALL – acute lymphoid leukaemia, aza – 5-aza-2'-deoxycytidine, MSP – methylation-specific PCR, ROS – reactive oxygen species.

Introduction

Cancer is a multi-factor, multi-gene-related complex process and DNA methylation plays a major role in tumorigenesis (Wajed et al., 2001). DNA methylation is catalysed by DNMTs (DNA methyltransferases). Among different DNMTs, DNMT1 is responsible for maintaining the methylation pattern during cell division from one generation to the next generation. Overexpression of DNMTs in previous studies with leukaemia samples suggested their role in molecular pathogenesis (Mizuno et al., 2001). The frequent gene silencing by promoter hypermethylation has been implicated in the development of malignancies including lymphoma (Wong et al., 2000; Chim et al., 2003; Zhang et al., 2007). Since epigenetic alterations do not change DNA sequences (Chen et al., 2009), it is proposed that epigenetic changes are dynamic identities and can be reversed. Presently, several synthetic drugs that can reduce DNA methylation are under trial. Therapy to restore silenced gene expression is being tested for anticancer potential (Andreoli et al., 2013). However, these synthetic chemicals are very toxic. Therefore, several natural plant products are being tested for epigenetic reversal (Reuter et al., 2011). One of the natural products, curcumin, has been shown to possess a variety of biological functions including anti-tumour activity and may target all steps of cancer development, including tumour initiation to progression. It has been investigated for its anti-neoplastic action against a variety of malignancies (Aggarwal et al., 2003). However, the mechanism by which curcumin inhibits cancer cell proliferation needed additional clarifications. Only a few studies have reported the effect of curcumin on DNA methylation. We reported reversal of promoter hypermethylation and reactivation of the *RARb* gene in a cervical cancer cell line by curcumin (Jha et al., 2010). Recently it has been shown that curcumin can modulate DNA methylation in acute myeloid leukaemia (Yu et al., 2013).

The *p15* gene, a target of cyclin-dependent kinase inhibitor, is aberrantly methylated in several human neoplasms, especially among hematopoietic malignancies

(Herman et al., 1997; Chim et al., 2003). *p15* has been postulated to be a tumour suppressor gene modulating pRb phosphorylation (Stone et al., 1995). Among solid and soft tissue tumours, *p15* promoter hypermethylation has occasionally been found in plasmacytoma, brain lymphoma, non-Hodgkin's lymphoma, Burkitt's lymphoma, and mantle cell lymphoma (Gronbaek et al., 1998; Wong et al., 1998; Zhang et al., 1998). In an attempt to study the methylation pattern of promoters of tumour suppressor genes in acute lymphoid leukaemia (ALL) patients of North India, we observed hypermethylation of the *p15/INK4b* promoter in 39 out of 125 biopsies (31.2 %). The difference in methylation of *p15/INK4b* ($P = 0.000$) was statistically highly significant when compared with control samples (data communicated). Therefore, the *p15* gene might provide an appropriate model for reversal of hypermethylation studies in ALL. To this aim, the present study was undertaken to examine some of the so far unexplored consequences of curcumin effects on the reversal of promoter methylation and reactivation of the *p15* gene in ALL.

Material and Methods

The Raji cell line (ALL) was procured from the National Centre for Cell Sciences (Pune, India). Tissue culture medium RPMI-1640 was obtained from Sigma-Aldrich Chemicals (Pvt) Ltd. (St. Louis, MO) and foetal bovine serum (FBS) was obtained from HiMedia (Mumbai, India). Curcumin was purchased from Acros Organics (Fair Lawn, NJ). 3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from HiMedia. 5-aza-2'-deoxycytidine, trizol, propidium iodide (PI), and 2,'7'-dichlorofluorescein diacetate (DCFH-DA) reagents were obtained from Sigma-Aldrich.

Cell culture and treatment

Raji cells were cultured according to the protocol optimized earlier in our lab. Briefly, the Raji cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS in 5% CO_2 at 37 °C. The cells were re-supplemented with fresh medium and test compounds every 48 h. All the treatments for reversal of promoter methylation analysis were carried out for six days since the DNA methylation pattern was transferred from one generation to the next generation and maintained by DNMTs, and therefore it was necessary to analyse its effect in a time-dependent manner. Treatment concentrations were kept low, i.e. 5 μM and 10 μM, in order to find the minimum effective concentrations and to make cells survive for longer duration. All other treatments were carried out using standard concentration at IC_{50} (20 μM) and below IC_{50} (10 μM).

Cytotoxicity of chemopreventive agent

The cytotoxic effect of curcumin was studied on Raji cells by the MTT method (Heckenkamp et al., 1999). Briefly, the cells were cultured in 96-well plates at a density of 1×10^4 cells per well with or without different concentrations of curcumin (0–35 μM). After incubation for 48 h, MTT dissolved in PBS was added to each well at a final concentration of 5 mg/ml and then incubated at 37 °C and 5% CO_2 for 2 h. The water insoluble dark blue formazan was dissolved in DMSO. The optical density was measured by a microplate reader (Bio-Rad, Berkeley, CA) at a wavelength of 570 nm.

Morphological study

To determine the dose-dependent morphological changes in Raji cells, the cells were treated with different concentrations of curcumin and observed through a phase-contrast microscope (Nikon, Tokyo, Japan) after 48 h of treatment with curcumin at 10 μM or 20 μM. Untreated Raji cells were taken as a control.

Acridine orange/ethidium bromide staining

To study the effect of curcumin on apoptosis induction, Raji cells were grown in the presence of 10 μM or 20 μM of curcumin for 48 h and counted using a haemocytometer and then observed under a fluorescence microscope (Nikon) after staining with acridine orange/ethidium bromide (10 μg/ml).

DNA fragmentation assay

The amount of 1×10^6 cells /ml was treated with curcumin at 10 μM or 20 μM for 48 h along with a proper control (without treatment). Cellular DNA of the treated cells was extracted from the cells according to the Gong's modified method (Gong et al., 1994). Briefly, curcumin-treated and untreated cells were washed twice in cold PBS (10 mM) and re-suspended in hypotonic lysis buffer (5 mM Tris, 20 mM EDTA, pH 7.4) containing 0.5% Triton X-100 for 30 min at 4 °C. The lysate was centrifuged at 13,000 *g* for 15 min at 4 °C. DNA was extracted from the supernatant with equal volume of phenol-chloroform-isoamylalcohol, precipitated by addition of two volumes of absolute ethanol, 0.1 volume of 3 mM sodium acetate and treated with RNAse A (500 U/ml) at 37 °C for 3 h. The pattern of DNA fragmentation was analysed in 1.5% agarose gel.

Chromosome preparations

Raji cells were seeded at 1×10^6 cells/ml and treated with curcumin at different concentrations (10 μM, 20 μM) for 48 h. Cells without treatment were taken as a control. Chromosomes were prepared after 48 h following 2 h colcemid treatment (0.1 μg/ml). G banding was done using 0.005% trypsin and chromosomes were analysed under a light microscope. Comparative mitotic indices of the slides were calculated by considering the average values of the ratio of metaphase plate vs. the number of cells in five different areas of slides at 10× magnification.

Reactive oxygen species (ROS) estimation

Intracellular ROS accumulation in Raji cells was measured and quantified with DCFH-DA dye. Raji cells

(1×10^6 cells/ml) were treated with curcumin at a concentration of 10 μM and 20 μM for 48 h and 72 h, respectively. Harvested cells were incubated with DCFH-DA dye followed and investigated by flow cytometric analysis. DCFH-DA crosses cell membranes and its hydrolysis with nonspecific esterases generates non-fluorescent DCFH. This DCFH in the presence of ROS is further oxidized to DCF, which is readily detected by flow cytometry. The fluorescence is equal to the amount of intracellular ROS generated. Cells without treatment were secured as a control.

Cell cycle analysis

Cell cycle analysis was carried out using propidium iodide as described previously (Krishan, 1975). Briefly, 1×10^6 cells /ml were treated with or without curcumin at a concentration of 10 μM or 20 μM and then washed with chilled PBS twice. Further, cells were fixed with 70% ethanol for 40 min. After that, cells were washed again with PBS and resuspended in 500 μl of staining solution containing propidium iodide, detergent and RNAse as described previously.

Methylation-specific PCR

The effect of curcumin on the promoter methylation of *p15* gene in the Raji cell line was assessed by methylation-specific PCR (MSP) (Lee et al., 2005). The Raji cell line was exposed to 5 μM or 10 μM curcumin for six days before conducting MSP for the tested gene. DNA extracted from the cell line was modified with sodium bisulphite and MSP was carried out using specific primers for methylation and non-methylation of the tested gene (Herman et al., 1996).

RT-PCR

RNA was isolated from the treated and untreated cells after 72 h and 6 days using the trizol reagent (Sigma-Aldrich). An equal amount of RNA was used to synthesize cDNA after DNAse treatment using the Revert Aid first-strand cDNA synthesis kit (Fermentas, Glen Burnie, MD). RT-PCR was carried out to check the alteration in the level of mRNA expression after the treatment with curcumin and 5-aza-2'-deoxycytidine using primers as given in Table 1.

Results and Discussion

Anticancer drugs have demonstrated distinct mechanisms of action/effects on different types of cancer cells. Zwaan et al. (2000) demonstrated that several drugs showed different cellular resistance in the case of AML and ALL patients. The cellular drug resistance has also shown to correlate with both the cell lineage and the type of genetic rearrangement (Palle et al., 2005). Since different types of cancer and different cells within a tumour can behave very differently to cancer therapy, and cancer drugs are supposed to be tissue specific and cell specific, it is relevant to explore the curcumin behaviour in the acute lymphoid leukaemia model. Raji cells (ALL) were chosen for the study as the *p15* gene promoter is methylated in this cell line. The effect of curcumin was studied extensively in terms of genomic instability, apoptosis, cell cycle, ROS generation, and its association with the reversal of promoter methylation and reactivation of the *p15* gene.

Curcumin exhibits an apoptotic and growth inhibitory effect

The dose kinetics of curcumin was evaluated by MTT assay (Fig. 1A). The cytotoxicity of curcumin was significantly increased in a dose-dependent manner, whereas the IC_{50} dose of curcumin was 20 μM ± 2.0. It was also observed that curcumin caused apoptosis as observed by acridine orange/ethidium bromide (AO/EB) staining, which showed nuclear changes and apoptotic body formation (Fig. 1C). Subsequently, DNA fragmentation was observed after treatment with 20 μM curcumin (Fig. 1B).

Curcumin caused ROS-mediated apoptosis and genomic instability

The ROS content inside the cells was estimated by flow cytometry as described in Material and Methods. The level of florescence is indicative of the presence of oxidative stress inside the cell. ROS have been shown to be involved in cell proliferation and apoptosis. In order to measure the capacity of curcumin to induce ROS, we used DCFH-DA (Fig. 2A, B). Exposure of Raji cells

Table 1.

Sequence (5′-3′)		Anneal. temperature (°C)	Extent (bp)	Reference
p15	MF: GCGTTCGTATTTTGCGGTT MR: CGTACAATAACCGAACGACCGA	62 °C	148	Herman et al., 1996
p15	UF: TGTGATGTGTTTGTATTTTGTGGTT UR: CCATACAATAACCAAACAACCAA	59 °C	154	Herman et al., 1996
p15	F: TGGGGGCGGCAGCGATGAG R: AGGTGGGTGGGGGTGGGAAAT	63 °C	451	Cameron et al., 1999
β-actin	F: GTGGGCCGCTCTAGGCACCA R: GGTTGGCCTTAGGGTTCAGGGGGG	56 °C	245	Heyer et al., 2002
DNMT1	F: ACCGCTTCTACTTCCTCGAGGCCTA R: GTTGCAGTCCTCTGTGAACACTGTGG	56 °C	335	Minami et al., 2010

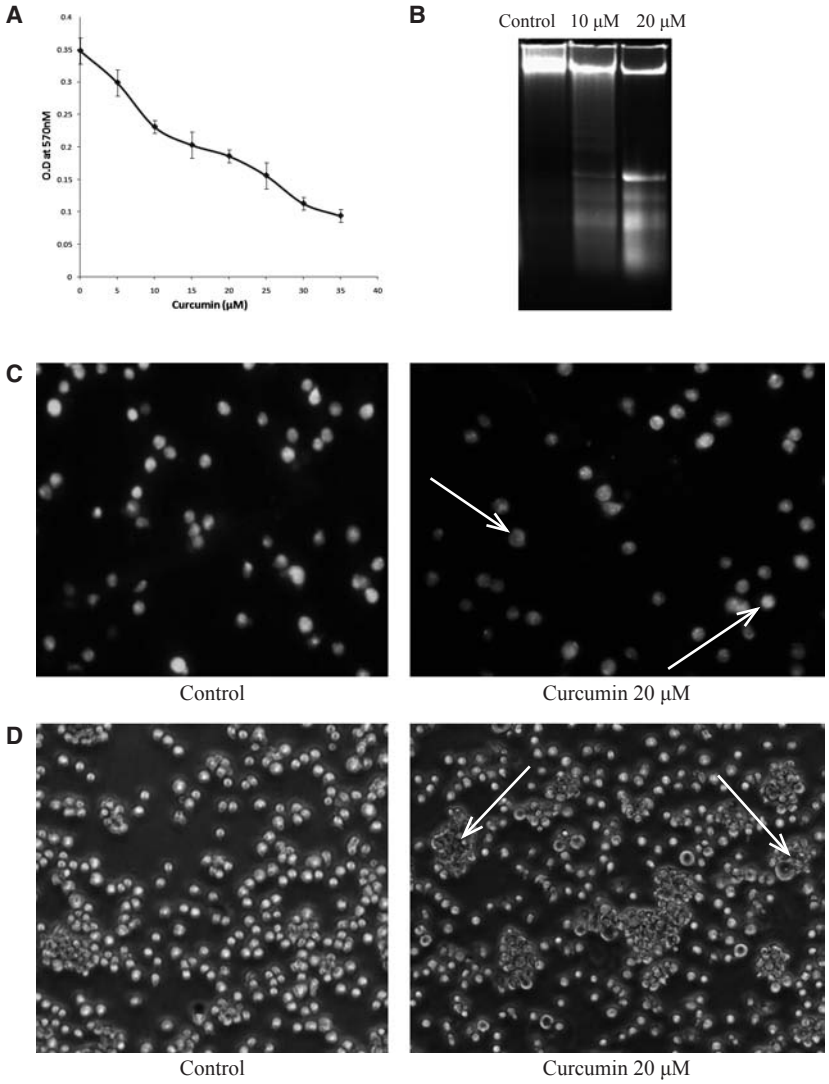

Fig. 1. Effect of curcumin on Raji cell viability and cytotoxicity. (A) MTT assay to estimate cytotoxicity after curcumin treatment. (B) DNA fragmentation assay of Raji cells after 48 h treatment with curcumin. (C) Acridine orange/ethidium bromide staining of Raji cells. (D) Morphological studies of Raji cells after the treatment with curcumin under a phase-contrast microscope. Arrows correspond to apoptotic cells.

to curcumin at 10 μM and 20 μM for 48 h and 72 h, respectively, caused significant ROS formation, whose amounts increased in a dose- and time-dependent manner. Culture of Raji cells without curcumin for 72 h was depicted by fluorescence of 2.97. As compared to the control experiment, when the cells were cultured with curcumin at 10 μM and 20 μM (Fig. 2Bb,c), the level of ROS increased to 18.63 and 30.19 after 72 h, respectively. Treatment at 10 μM and 20 μM showed 6.42-fold and 10.4-fold increase in the ROS content after 72 h, respectively. The shift in the peak corresponds to concentration-dependent ROS generation after the treatment with curcumin (Fig. 2). The significant extent of ROS production corresponds to ROS-mediated apoptosis in Raji cells. This report is in agreement with earlier investigation showing ROS production by curcumin *in vitro* (Sanchez et al., 2010).

After that, we analysed the effect of curcumin on genomic stability in order to assess the ROS contribution to the genomic instability. Chromosome analysis was successfully achieved after G banding**.** The results were compared between treated and non-treated cells (Fig. 3). Curcumin (20 μM) treatment resulted in breakage of chromosome arms. The comparative mitotic index of control slides was found to be 0.35, while for curcumin (20 μM) 0.19 was observed. Subsequently, the frequency of aberrant metaphases (N = 20) was found for the control: 0.0 (N = 25) and for curcumin: 0.24 (N = 25). It appears that curcumin caused genomic instability in Raji cells. Since ROS was supposed to have a destructive effect on DNA and protein (Simon et al., 2000), ROS generation after curcumin treatment appeared to be associated with chromosomal abnormality or genomic instability in the Raji cells. Previously, curcumin has been reported to cause structural abnormality in chromosomes of the CHO cell line (Antunes et al., 1999).

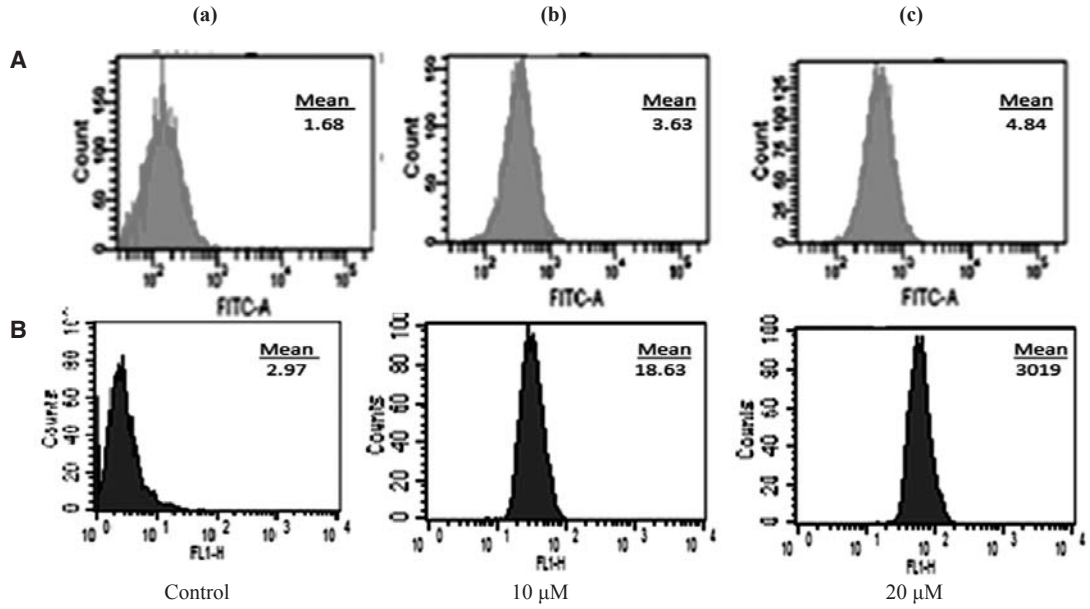

Fig. 2. ROS estimation in Raji cells after 48 h (A), 72 h (B) with (a) control, (b) 10 μM curcumin, (c) 20 μM curcumin. The treatment shows an augmentation in the ROS content in a concentration- and time-dependent manner.

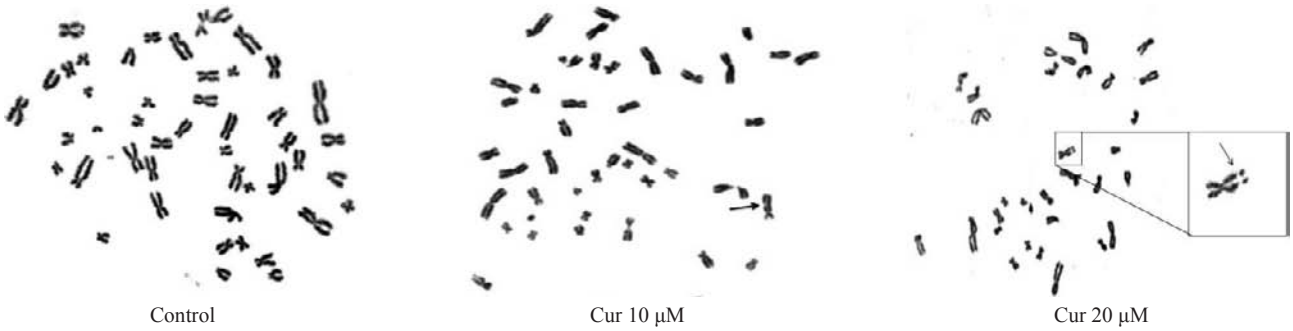

Fig. 3. Representative metaphase plates showing chromosome breaks following curcumin treatment at 10 μM and 20 μM.

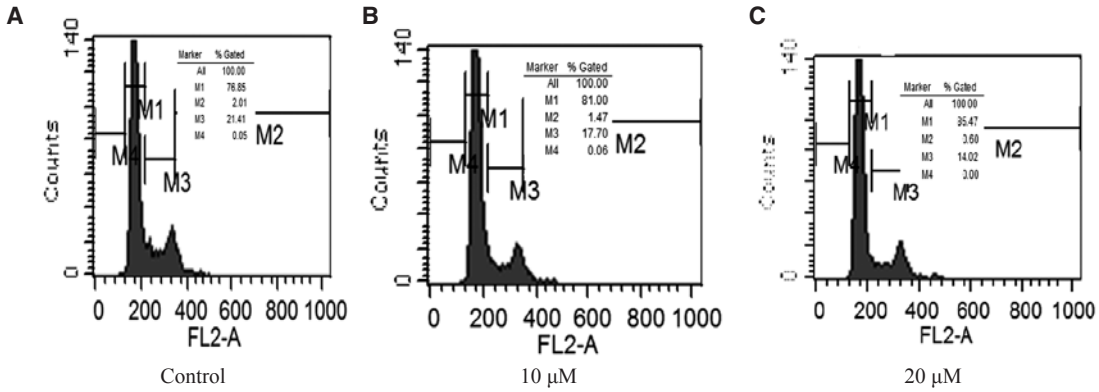

Fig. 4. Effect of curcumin on cell-cycle distribution in Raji cells. Cells were treated with curcumin (0, 10 μM, 20 μM) for 72 h. Cell-cycle distribution was determined by flow cytometry. Representative sets of histograms for Raji cells (A) untreated, (B) 10 μM and (C) 20 μM curcumin-treated cells. Percentages of cells in Sub G1, G0/G1, S, and G2/M phase are shown as insets for each experiment. Curcumin caused G1 phase arrest.

Curcumin caused cell-cycle arrest

Raji cells were treated with 10 μM or 20 μM curcumin for 48 h and 72 h. Beside induction of apoptosis, curcumin also caused cell-cycle arrest, suggesting curcumin as a dual agent. Curcumin treatment resulted in cell-cycle arrest in G1 phase and depletion in S-phase fraction (Fig. 4). These results are in agreement with earlier studies on Raji cells with curcumin (Sun et al., 2005).

Curcumin caused up-regulation of p15 and down-regulation of DNMT1

In order to elucidate curcumin anti-neoplastic action, we checked the expression of *p15* after treatment with curcumin at 10 μM concentration for 72 h. We observed up-regulation in expression of the *p15* gene (Fig. 5). Since the *p15* gene has been reported to be methylated in the Raji cell line, to find out whether these changes are accompanied by promoter demethylation we extended our treatment for six days. For further experiments we found 5 μM and 10 μM as minimum effective concentrations in comparison to 10 μM and 20 μM previously reported in AML. These studies suggested higher sensitivity of ALL toward curcumin as compared to AML (Yu et al., 2013). Despite previous reports regarding the molecular mechanism of curcumin in epigenetic regulations, there are few reports demonstrating that it might display an inhibitory action on DNMT1. To further correlate curcumin-mediated reversal of promoter methylation of the *p15* gene and DNMT1 inhibition, gene expression analysis was carried out after treatment of Raji cells with curcumin (5 μM, 10 μM) for six days. The mRNA levels of *p15* and *DNMT1* genes in Raji cells were examined by reverse transcription PCR with and without exposure to curcumin. Treatment with curcumin at 10 μM resulted in nearly 15-fold up-regulation of the *p15* mRNA. Further, to examine whether curcumin-mediated reversal of promoter methylation had any correlation with *DNMT1* expression, its expression level was examined in the same samples.

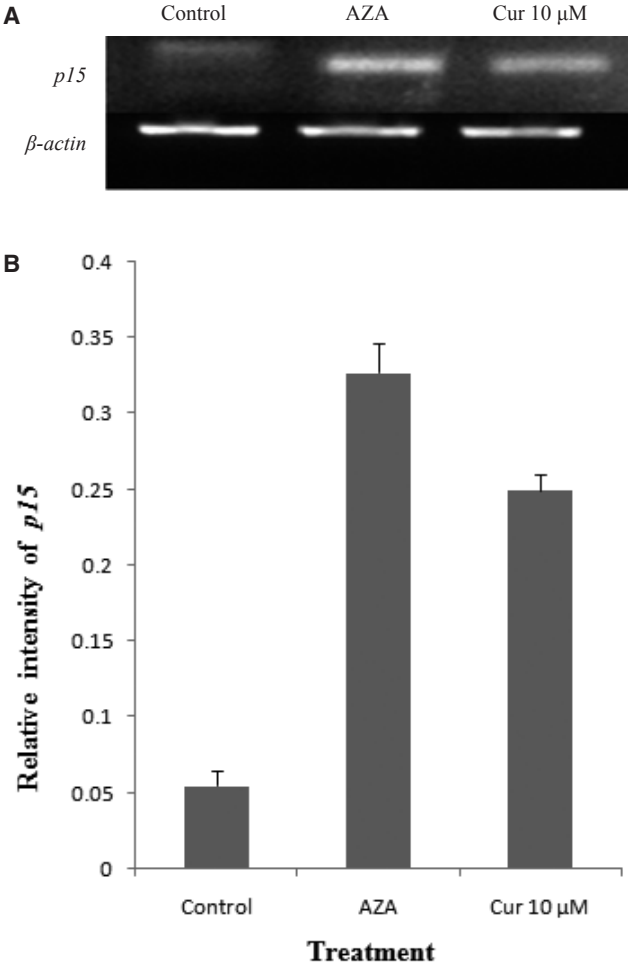

Fig. 5. Up-regulation of *p15* gene expression after 72 h treatment with curcumin at 10 μM. (A) Semi-quantitative PCR revealed up-regulation of *p15* expression at 10 μM. (B) Densitometric analysis of relative band intensity for the *p15* gene.

The expression of *DNMT1* was found to be down-regulated in a time- and concentration-dependent manner (Fig. 6), while no change in expression was observed with 5-aza-2'-deoxycytidine. Current demethylating drugs, such as 5-aza-2'-deoxycytidine, exert their inhibitory effect by covalent binding to DNMT1, which leads to irreversible inhibition of the DNMT1 activity, causing hypomethylation of the genomic DNA (Christman, 2002). As previously described in the literature, demethylation may be favoured via binding with the catalytic domain of DNMT1 or depletion of DNMT1 protein levels (Loriot et al., 2006). Molecular docking analysis revealed that curcumin covalently blocks the catalytic site of DNMT1 to exert its inhibitory effect (Zhongfa et al., 2009). During our investigation we observed that curcumin also led to the down-regulation of the *DNMT1* mRNA level. Melki et al. (1998) reported that leukaemia patients show 4.4-fold higher expression than normal control. This higher activity of *DNMT1* could be responsible for hypermethylation of the *p15* gene. This suggests that in addition to direct inhibition, down-regulation of *DNMT1* expression by curcumin could be an additional factor favouring reversal of promoter methylation and up-regulation of the *p15* gene. Cell-cycle analysis revealed G1 arrest. This arrest might be associated with early hypomethylation of the *p15* gene promoter. Such reversal of promoter methylation may potentiate curcumin-associated anticancer properties. Generation of ROS is accompanied by a significant decrease in the *DNMT1* mRNA level. This suggested that there might be an interplay between ROS generation and *DNMT1* down-regulation. These observations also emphasized the role of ROS in epigenetic changes, and further study is needed to elucidate the exact interplay between ROS and DNA methylation.

Reversal of p15 methylation by curcumin

p15 is hypermethylated in cancer cell lines and has been considered as a methylation marker for tumorigenesis (Cameron et al., 1999). The *p15* promoter was found to be methylated in the Raji cell line. Methylation-specific analysis showed that curcumin decreased the methylation of the *p15* gene. Both the appearance and enhanced intensity of non-methylation-specific amplified product and correspondingly decreased intensity of the methylation-specific amplified product in the gel were observed. The decrease in methylation-specific amplified product intensity after six days confirmed reversal of *p15* gene promoter methylation (Fig. 7). Similar results after 5-aza-2'-deoxycytidine (a positive con-

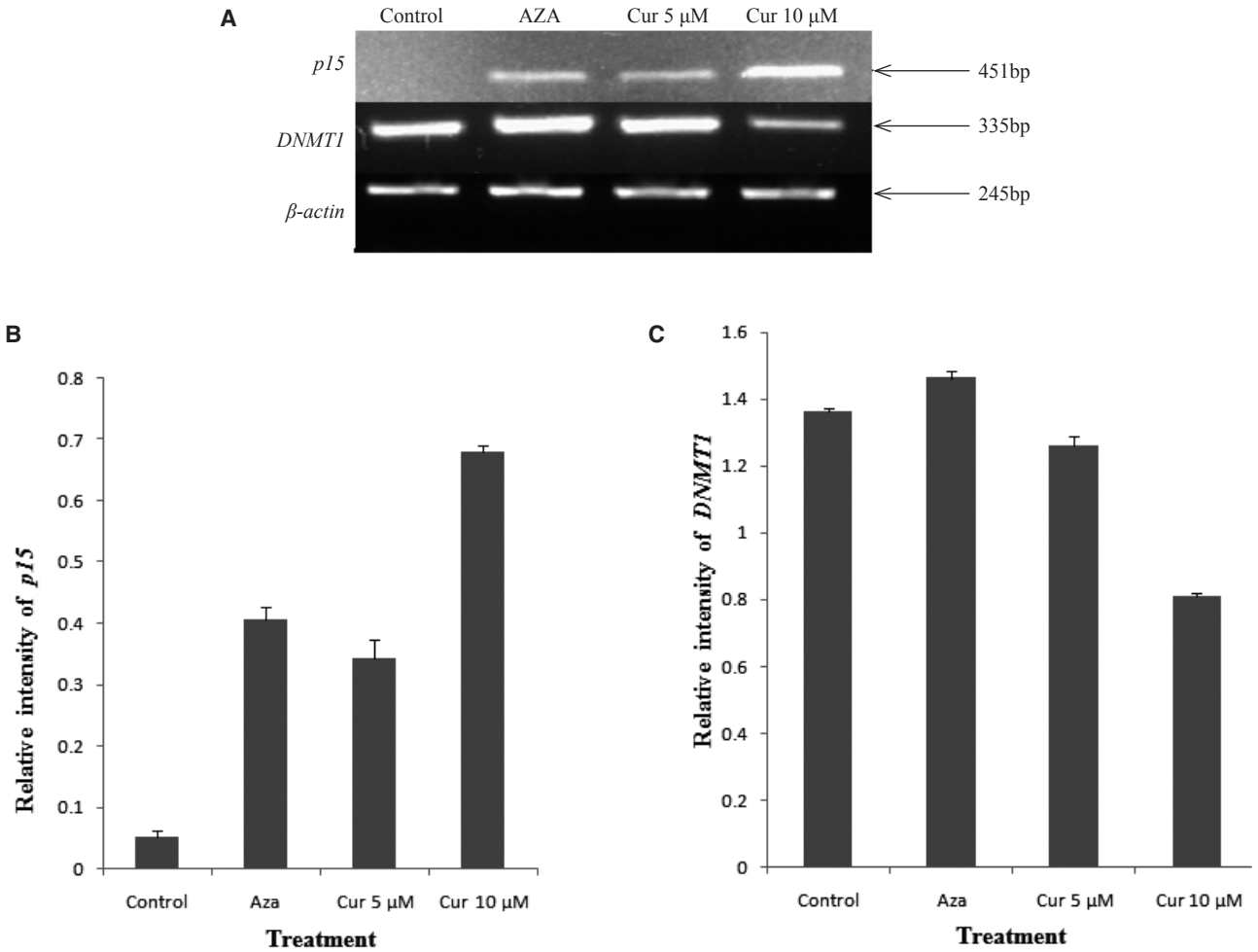

Fig. 6. Alterations in levels of mRNA expression of *p15* and *DNMT1* genes after 6-day treatment of Raji cells with 5 μM and 10 μM curcumin and 20 μM 5-aza-2'-deoxycytidine; mRNA expression was determined using RT PCR. (A) Semi-quantitative PCR demonstrated up-regulation of the *p15* gene and down-regulation of *DNMT1* after treatment with curcumin and 5-aza-2'-deoxycytidine; representative agarose gel from one of three independent similar experiments. Densitometric analysis of relative band intensity for *p15* (B) and *DNMT1* (C) genes with control, aza, curcumin 5 μM, 10 μM respectively. The relative band intensity was calculated as sample average band intensity/β-actin band intensity.

trol) treatment confirmed reversal of *p15* gene promoter methylation. However, we did not observe complete disappearance of the methylation-specific amplified product at 10 μM treatment with curcumin for six days. Since there is no direct experimental evidence to show the epigenetic effect of curcumin in Raji cells, in this study we showed that curcumin was able to demethylate specific cell-cycle regulatory gene *p15* in the Raji cell line.

Conclusion

Based on this study we suggest that curcumin acts as a dual agent causing both apoptosis and reversal of promoter methylation of the *p15* gene in an ALL cell line. Taken together, the results of the present study would be an innovative quest to understand the molecular mechanism of curcumin as an epigenetic modifying anticancer therapeutic agent. Based on these observations, we could draw a possible conclusion that curcumin is a potent demethylating agent that restores the epigenetically silenced *p15* gene expression by down-regulating DNMT1 and could be a novel therapeutic strategy against ALL patients. Demethylation of the *p15* gene by curcumin would contribute to an effective regimen for cancer therapy.

Declaration of interest

None.

References

Aggarwal, B. B., Kumar, A., Bharti, A. C. (2003) Anticancer potential of curcumin: preclinical and clinical studies. *Anticancer Res.* **23**, 363-398.

Andreoli, F., Barbosa, A. J., Parenti, M. D., Del Rio, A. (2013) Modulation of epigenetic targets for anticancer therapy: clinicopathological relevance, structural data and drug discovery perspectives. *Curr. Pharm. Des.* **19**, 578-613.

Antunes, L. M., Araújo, M. C., Dias, F. L., Takahashi, C. S. (1999) Modulatory effects of curcumin on the chromosomal damage induced by doxorubicin in Chinese hamster ovary cells. *Teratog. Carcinog. Mutagen.* **19**, 1-8.

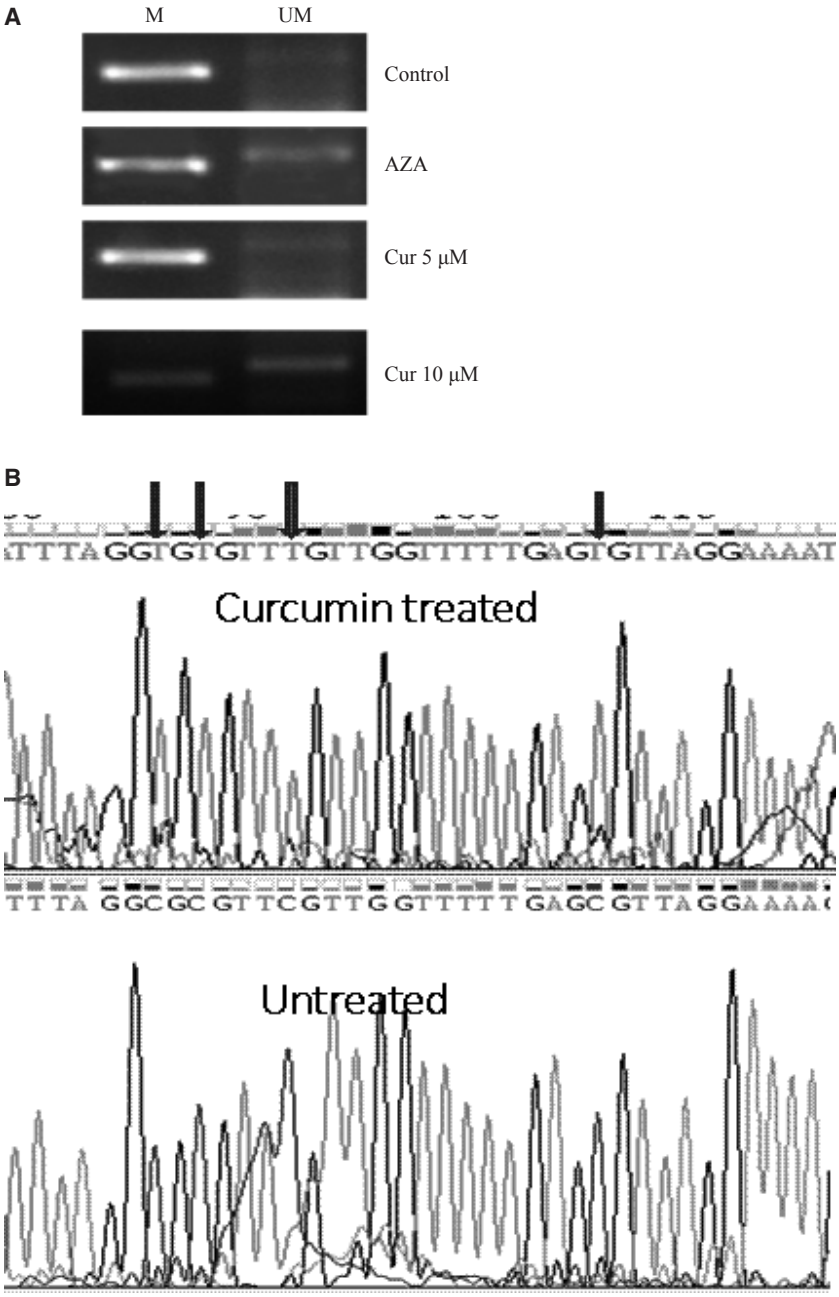

Fig. 7. (A) Curcumin reversed promoter methylation of *p15* and induced transcription activation. **M** shows methylation-specific amplified product of the control and after aza and curcumin treatment. **UM** shows unmethylation-specific amplified product treated with control, aza, and curcumin. Cells were treated with curcumin at 5 μM or 10 μM and with 20 μM of 5-aza-2'-deoxycytidine for six days. (B) Reversal of promoter methylation was confirmed by DNA sequencing; a comparison between curcumin-treated and untreated samples. Unmethylated cytosine after curcumin treatment followed by bisulphite modification was converted to thymine. Data are representative of one of three similar experiments.

Cameron, E. E., Baylin, S. B., Herman, J. G. (1999) *p15INK4B* CpG island methylation in primary acute leukemia is heterogeneous and suggests density as a critical factor for transcriptional silencing. *Blood* **94**, 2445-2451.

Chen, S. S., Raval, A., Johnson A. J., Hertlein, E., Liu, T. H., Jin, V. X. (2009) Epigenetic changes during disease progression in a murine model of human chronic lymphocytic leukemia. *Proc. Natl. Acad. Sci. USA* **106**, 13433-13438.

Chim, C. S., Wong, A. S., Kwong, Y. L. (2003) Epigenetic inactivation of INK4/CDK/RB cell cycle pathway in acute leukemias. *Ann. Hematol.* **82,** 738-742.

Christman, J. (2002) 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene* **21**, 5483-5495.

Gong, J., Traganos, F., Darzynkiewicz, Z. (1994) A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. *Anal. Biochem.* **218**, 314-319.

Gronbaek, K., Nedergaard, T., Andersen, M. K., thor Straten, P., Guldberg, P., Møller, P., Zeuthen, J., Ebbe Hansen, N., Hou-Jensen, K., Ralfkiaer, E. (1998) Concurrent dis-

ruption of cell cycle associated genes in mantle cell lymphoma: a genotypic and phenotypic study of cyclin D1, p16, p15, p53 and pRb. *Leukemia* **12**, 1266-1271.

Heckenkamp, J., Leszczynski, D., Schiereck, J., Kung, J., LaMuraglia, G. M. (1999) Different effects of photodynamic therapy and γ-irradiation on vascular smooth muscle cells and matrix: implications for inhibiting restenosis. *Arterioscler. Thromb. Vasc. Biol.* **19**, 2154-2161.

Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D., Baylin, S. B. (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl. Acad. Sci. USA* **93**, 9821-9826.

Herman, J. G., Civin, C. I., Issa, J. P., Collector, M. I., Sharkis, S. J., Baylin, S. B. (1997) Distinct patterns of inactivation of *p15INK4B* and *p16INK4A* characterize the major types of haematological malignancies. *Cancer Res.* **57**, 837-841.

Heyer, G., Saba, S., Adamo, R., Rush, W., Soong, G., Cheung, A., Prince, A. (2002) *Staphylococcus aureus agr* and *sara* functions are required for invasive infection but not inflammatory responses in the lung. *Infect. Immun.* **70**, 127-133.

Jha, A. K., Nikbakht, M., Parashar, G., Shrivastava, A., Capalash, N., Kaur J. (2010) Reversal of hypermethylation and reactivation of the *RARβ2* gene by natural compounds in cervical cancer cell lines. *Folia Biol. (Praha)* **56**, 195-200.

Krishan, A. (1975) Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. *J. Cell. Biol.* **66**, 188-193.

Lee, W. J., Shim, J. Y., Zhu, B. T. (2005) Mechanisms for the inhibition of DNA methyltransferases by tea catechins and bioflavonoids. *Mol. Pharmacol.* **68**, 1018-1030.

Loriot, A., De Plaen, E., Boon, T., De Smet, C. (2006) Transient down-regulation of DNMT1 methyltransferase leads to activation and stable hypomethylation of MAGE-A1 in melanoma cells. *J. Biol. Chem.* **281**, 10118-10126.

Melki, J. R., Warnecke, P., Vincent, P. C., Clark, S. J. (1998) Increased DNA methyltransferase expression in leukaemia. *Leukemia* **12**, 311-316.

Minami, K., Chano, T., Kawakami, T. (2010) DNMT3L is a novel marker and is essential for the growth of human embryonal carcinoma. *Clin. Cancer Res.* **16**, 2751-2759.

Mizuno, S., Chijiwa, T., Okamura, T., Akashi, K., Fukumaki, Y., Niho, Y. (2001) Expression of DNA methyltransferases *DNMT1*, *3A*, and *3B* in normal hematopoiesis and in acute and chronic myelogenous leukemia. *Blood* **97**, 1172-1179.

Palle, J., Frost, B. M., Forestier, E., Gustafsson, G., Nygren, P., Hellebostad, M., Jonsson, O. G., Kanerva, J., Schmiegelow, K., Larsson, R., Lönnerholm, G., (2005). Cellular drug sensitivity in MLL-rearranged childhood acute leukaemia is correlated to partner genes and cell lineage. *Br. J. Haematol.* **129**, 189-198.

Reuter, S., Gupta, S. C., Park, B., Goel, A., Aggarwal, B. B. (2011) Epigenetic changes induced by curcumin and other natural compounds. *Genes Nutr.* **6**, 93-108.

Sanchez, Y., Simo, G. P., Calvin, E., Blas, E. D., Aller, P. (2010) Curcumin stimulates reactive oxygen species production and potentiates apoptosis induction by the antitumor drugs arsenic trioxide and lonidamine in human myeloid leukemia cell lines. *J. Pharmacol. Exp. Ther.* **335**, 114-123.

Simon, H. U., Haj-Yehia, A., Levi-Schaffer, F. (2000) Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis* **5**, 415-418.

Stone, S., Dayananth, P., Jiang, P., Weaver-Feldhaus, J. M., Tavtigian, S. V., Cannon-Albright, L. (1995) Genomic structure, expression and mutational analysis of the *P15* (MTS2) gene. *Oncogene* **11**, 987-991.

Sun, C., Liu, X., Chen, Y., Liu, F (2005) Anticancer effect of curcumin on human B cell non-Hodgkin's lymphoma. *J. Huazhong Univ. Sci. Technolog. Med. Sci*. **25**, 404-407.

Wajed, S. A., Laird, P. W., De Meester, T. R. (2001) DNA methylation: an alternative pathway to cancer. *Ann. Surg.* **234**, 10-20.

Wong, I. H., Ng, M. H., Lee, J. C., Lo, K. W., Chung, Y. F., Huang, D. P. (1998) Transcriptional silencing of the *p16* gene in human myeloma-derived cell lines by hypermethylation. *Br. J. Haematol.* **103**, 168-175.

Wong, I. H., Lo, Y. M., Yeo, W., Lau, W. Y., Johnson, P. J. (2000) Frequent *p15* promoter methylation in tumor and peripheral blood from hepatocellular carcinoma patients. *Clin. Cancer Res.* **6**, 3516-3521.

Yu, J., Peng, Y., Wu, L. C., Xie, Z., Deng, Y., Hughes, T., He, S., Mo, X. K., Chiu, M., Wang, Q. E., He, X., Shujun Liu, S., Grever, M. R., Liu, Z. (2013) Curcumin down-regulates DNA methyltransferase 1 and plays an anti-leukemic role in acute myeloid leukemia. *PLoS One* **8**, e55934

Zhang, S. J., Endo, S., Ichikawa, T., Washiyama, K., Kumanishi, T (1998) Frequent deletion and 5'CpG island methylation of the *p16* gene in primary malignant lymphoma of the brain. *Cancer Res.* **58**, 1231-1237.

Zhang, Y. J., Wu, H. C., Shen, J., Ahsan, H., Tsai, W. Y., Yang, H. I. (2007) Predicting hepatocellular carcinoma by detection of aberrant promoter methylation in serum DNA. *Clin. Cancer Res.* **13**, 2378-2384.

Zhongfa, L, Zhiliang, X., William, J., Ryan, E. P., Shujun, L., Jianhua, Y., Pui-kai, L., Jiayuh, L., Jame, R. F., Marcucci, G. M., Chenglong, L., Chan, K. K. (2009) Curcumin is a potent DNA hypomethylation agent. *Bioorg. Med. Chem. Lett.* **19**, 706-709.

Zwaan, C. M., Kaspers, G. J., Pieters, R., Ramakers-Van Woerden, N. L, den Boer, M. L., Wünsche, R., Rottier, M. M., Hählen, K., van Wering, E. R., Janka-Schaub, G. E., Creutzig, U., Veerman, A. J. (2000) Cellular drug resistance profiles in childhood acute myeloid leukemia: differences between FAB types and comparison with acute lymphoblastic leukemia. *Blood* **96**, 2879-2886.