ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 07, Issue, 10, pp.16309-16317, October, 2017

ORIGINAL RESEARCH ARTICLE OPEN ACCESS

STRUCTURAL ANALYSIS OF RUNX1 PROTEIN AND ITS MUTANTS IN COMPLEX WITH RUNX3 PROMOTER SHOWS ALTERED CONFORMATIONS

[1]Jyoti Zack, [1]Laishram R. Singh, [1]Madhu Chopra, [1]Gurumayum Suraj Sharma, [1]Daman Saluja [2]Rukmani Pandey and *[3]Dharmendra Kumar Chaudhary

[1]Dr. B. R. Ambedkar Center for Biomedical Research, University of Delhi, Delhi-110 007, India
[2]Developmental Toxicology Laboratory, System Toxicology and Health risk Assessment Group, IITR Lucknow, India
[3]Assist Prof. Department of Nuclear medicine, BBAU Central University, Lucknow-226025, India

ARTICLE INFO

Article History:
Received 29th July, 2017
Received in revised form
14th August, 2017
Accepted 07th September, 2017
Published online 10th October, 2017

Keywords:
RUNX1, Acute myeloid leukemia,
DNA binding affinity,
Mutation.

ABSTRACT

Runt related transcription factor 1 also known as RUNX1 play a pivotal role in the regulation of the development of hematopoietic system in accordance with various transcriptional co-regulators. It is one of the most common targets of chromosomal translocations and mutations in its runt domain are frequently associated with leukemogenesis. Structural studies of RUNX1-DNA complexes provide details of direct contacts formed between runt domain of RUNX1 and DNA. The amino acid residues, Lys83 and Arg174, of RUNX1 directly interact with its binding site on the promoters. In present study, we have used a combined approach involving a detailed *in-vitro* and *in-silico* analysis of RUNX1 and its mutants for their structural and functional evaluation. CD spectroscopy and Tryptophan fluorescence of wild type and mutant full-length purified RUNX1 protein suggested an altered secondary and tertiary structure of mutant proteins. The mutant proteins also exhibited decrease in DNA binding as evident by Electrophoretic Mobility Shift Assays and binding kinetics using fluorescence spectroscopy. We observed that DNA binding affinity of mutated RUNX1 with RUNX3 promoter was about 5-7 fold lower than that of wild type RUNX1. These results suggest that both the point mutations (Lys83Glu/Arg174Gln) lead to a change in conformation of full-length RUNX1 protein which in turn affects its binding to DNA. Investigations of the molecular insights using *in-silico* approach suggest that this decrease in DNA binding could be due to changes in hydrogen bonding pattern, and lengths between wild type and mutant protein complexed with RUNX3 promoter.

Corresponding author

Citation: **Jyoti Zack, Laishram R. Singh, Madhu Chopra, Gurumayum Suraj Sharma, Daman Saluja Rukmani Pandey and Dharmendra Kumar Chaudhary. 2017**. "Structural analysis of runx1 protein and its mutants in complex with runx3 promoter shows altered conformations", *International Journal of Development Research,* 7, (10), 16309-16317.

INTRODUCTION

Hematopoiesis is a precisely regulated process that generates terminally differentiated cells in peripheral blood from immature progenitors in bone marrow with the cooperation of a number of hematopoietic genes. Acute myeloid leukemia-1 (AML1/PEBP2β/RUNX1/CBFA1) is a transcription factor essential for hematopoiesis (Huang, Crute *et al.* 1998, Lutterbach and Hiebert 2000). The other family members of RUNX1 include RUNX2, a factor essential for osteoblast differentiation (Otto, Thornell *et al.* 1997), (Komori, Yagi *et al.* 1997); and RUNX3, for anti-proliferation, apoptosis and other functions (Inoue, Ozaki *et al.* 2002, Levanon, Bettoun *et al.* 2002, Taniuchi, Osato *et al.* 2002, Woolf, Xiao *et al.* 2003, Wang, Jacob *et al.* 2010). RUNX1 is a modular transcription factor with several distinct functional domains; 5' negative regulatory domain (first 50 amino acids), runt homology domain (RHD) (50 to 177 amino acids) (Huang, Crute *et al.* 1998), 3' negative regulatory domain (178 to 290 amino acids), nuclear localizing region (167 to 183 amino acids), transactivation domain (291 to 453 amino acids) and VWRPY motif (extreme C-terminus) (Kagoshima, Shigesada *et al.* 1993, Tanaka, Kurokawa

et al. 1996, Zeng, van Wijnen *et al.* 1997, Kanno, Kanno *et al.* 1998, De Braekeleer, Douet-Guilbert *et al.* 2011). It is known that RUNX1 heterodimerizes with CBFβ and mediates DNA binding through a highly conserved runt domain of 128 amino acids, which is homologous to the Drosophila pair-rule protein runt (Kagoshima, Shigesada *et al.* 1993, Thiel, Giaimo *et al.* 2017). The structural study of partial protein containing mainly runt domain suggests that DNA binding site lies distinctly to the CBFβ interaction domain (Nagata and Werner 2001). RUNX1 is one of the common targets of chromosomal translocation t(8;21) (Hart and Foroni 2002), and one of the most frequent targets of somatic mutations which biases the patients to the development of leukemia. The heterodimerization domain of RUNX1 with CBFβ has centrally located six highly twisted beta sheets which are encircled by four alpha helices and one 3_{10} helix (Zhang, Lukasik *et al.* 2003). The mutation or changes in this domain is prophesied to disrupt protein-protein/protein-DNA interactions leading to altered function of RUNX1 protein. Crystal structure revealed that several amino acids including Arg80, Lys83, Asp174 and Arg177 were involved in the protein-DNA interaction. These amino acids are in fact, most frequently mutated in patients of acute myelogenous leukemia (AML), familial platelet disorder (FPD), cleidocranial dysplasia (CCD), and myelodysplastic syndrome (MDS) (Akamatsu, Tsukumo *et al.* 1997, Osato, Asou *et al.* 1999, Quack, Vonderstrass *et al.* 1999, Song, Sullivan *et al.* 1999, Imai, Kurokawa *et al.* 2000, Preudhomme, Warot-Loze *et al.* 2000, Garvie and Wolberger 2001, Michaud, Wu *et al.* 2002). Therefore, it is important to investigate how variability in amino acid sequence modifies conformation of RUNX1, and its binding to DNA leading to the predilection of leukemia. Previous studies indicated that most of the structural studies of DNA-RUNX1 interaction have been carried out using 8-10bp sequence of DNA constituting runt binding site (Tahirov, Inoue-Bungo *et al.* 2001), (Bravo, Li *et al.* 2001). It is therefore, essential to study the function of full-length protein with its natural promoter binding site so as to understand the effect of mutations *in-vivo*. The present study describes binding of RUNX1 on one of its target gene promoters (RUNX3) and effect of its most frequent mutants (Lys83Glu and Arg174Gln) on the secondary and tertiary structure of the protein. The purified proteins were also used for comparing binding affinity of native wild type (WT) and mutant RUNX1 with RUNX3 promoter using *in-vitro* gel retardation assays and binding kinetics. *In-silico* modeling supports altered conformation of mutant protein-DNA complex and provides molecular insights into these interactions.

MATERIALS AND METHODS

Molecular Dynamics of WT and mutant RUNX1-DNA complex

The crystal structure of heterodimeric ternary complex containing runt domain of RUNX1/CBFβ/DNA was derived from protein Data Bank; www. rcsb. org (PDB code: 1H9D) (Bravo, Li *et al.* 2001). The promoter sequence of RUNX3 gene, target of RUNX1, containing RUNX1 binding site, was selected to analyze protein-DNA interaction. Since crystal structure of complex contains a synthetic sequence of DNA, we have modified DNA sequence with that of RUNX3 promoter. The pdb file 1H9D was modified by deleting CBFβ, and the runt domain of RUNX1/DNA complex was energy minimized using AMBER 8.0 suites of program (Case, Cheatham *et al.* 2005). The amino acid substitutions, Lys83Glu (K83E) and Arg174Gln (R174Q) in runt domain were done using Insight II software (Accelrys Inc.: San Diego, CA). The runt-DNA complex was solvated with water as it provides room temperature conditions within sliced-off truncated octahedron box. To equilibrate the system and to maintain electroneutrality, counterions were added. The energy minimization of mutant-DNA complex was also performed using AMBER 8.0 software. The temperature was kept constant throughout the simulation runs i.e. 300K by weak coupling to an external temperature bath. The simulation was run for 500 nanoseconds using the AMBER 8.0 software with GROMACS96 force fields (Case, Cheatham *et al.* 2005). The interaction between runt domain of RUNX1 (or mutants) and RUNX3 promoter was studied by measuring changes in its interaction and conformation of the protein-DNA complex with respect to WT runt-DNA complex. All numbers given to amino acids refer to amino acid residues of full-length native protein. The Ramachandran plot of all the three complexes was created by Discovery Studio 2.5 (DS Modelling 2.5, Accelrys Inc.: San Diego, CA). The deviation between wild type runt-DNA complex and mutant runt (K83E/R174Q)-DNA complex was evaluated by its RMSD values. Furthermore, structural analysis of all the respective protein-DNA complexes was done by Hydrogen-bond plots which were prepared using, Discovery Studio 2.5 software.

Cloning and nuclear lysis of WT and mutant RUNX1 in mammalian expression vector

Full-length WT RUNX1 and mutant RUNX1 cDNA was amplified using RUNX1 cloned in pEF-Bos vector (gifted from Dr. Y. Ito and Dr. M. Osato, Singapore) as a template using a forward primer (5'CTTGTTGTGATGCG TATCCCCGTAG3') and reverse primer (5'GTAGGCCTCC ACACGGCCTCCT3'). The amplicons of WT and mutant RUNX1 were cloned in the mammalian expression vector (pEF6/V5-His TOPO vector) individually. HEK293 cells, obtained from NCCS Pune, were transiently transfected with pEF6/V5-His-TOPO vector containing WT RUNX1 or mutant RUNX1 using Lipofectamine 2000 reagent (Invitrogen) as described by the manufacturer. The transfected HEK293 cells were cultured under standard conditions using DMEM as nutrient media supplemented with 10% Fetal Bovine Serum and antibiotics. Following transfection, cells were incubated for 48 hr. in the same nutrient media and thereafter harvested by centrifugation (400Xg for 2 min.) in a minimal volume of ice-cold 1X PBS. Two washings were given to the cell pellet with PBS and then re-suspended in Buffer A (20 mM HEPES, 20% Glycerol, 10mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.1% TritonX) approximately 5 times the packed cell volume and incubated at 4^0C for 15 minutes for lysis. Nuclei were collected by centrifugation followed by lysis in Buffer B (20mM HEPES, 20% Glycerol, 500mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.1% TritonX, 0.1mM PMSF, 2mM DTT) at 4^0C for 1hr. The protein concentration was estimated using BCA kit.

Electrophoretic mobility shift assay (EMSA)

RUNX3 promoter sequences were PCR amplified in presence of α^{32}P dATP using primers (RUNX3 promoter: Fp 5'GGGTTGACACTAAGAAGGC3', Rp 5'CCTGGTAGT GTGGTTCTG3'). The probe was purified by nucleotide removal kit (Qiagen). Binding reaction mixture contained α^{32}P

radiolabelled probe, binding buffer (125mM HEPES pH 7.6, 50% Glycerol, 0.5mM PMSF, 0.5mM EDTA, and 5mM DTT), 2µg poly (dI-dC) and 2µg nuclear lysate protein. The protein-DNA binding was allowed to proceed at 4^0C for 30 minutes before fractionation on 6% native PAGE. A Competition assay was performed in the presence of 2 fold and 10 fold molar excess of the unlabelled specific probe (specific competition) or unlabelled non-specific probe (non-specific competition).

Cloning and purification of WT and mutant RUNX1 in bacterial expression vector

Cloning of respective RUNX1 from mammalian expression vector, pEF6/V5-His TOPO to a bacterial expression vector, pGEX-5X-3 was done using primers (FP: forPGX 5'TGTGGTGGGAATTCCCTTCTTG3', RP: revPGX 5'GCCCTCTAGGCTCGAGCGGCC3'). Ligation of the cDNA was carried out using PCR products which was then followed by ligation using T4 quick ligation kit (NEB, as recommended by manufacturer) directly in pGEM®-T Easy vector (Promega). The cloned gene of interest in pGEM®-T easy vector was digested with EcoRI and XhoI restriction enzymes, purified and subcloned into pGEX-5X-3 vector. *E.coli* BL21 (DE3) strain was transformed with recombinant plasmids containing desired genes and induced with IPTG. 250ml of induced cell pellet was suspended in 25ml of sonication buffer containing 30mM TrisCl pH 8.0, 300mM NaCl, 20% glycerol, and 1mM sodium orthovanadate. Lysis was done using 15 cycles of 30 seconds ultra-sonication of bacterial cells at 50% duty cycle on ice with gaps of 30 seconds between the consecutive cycles. The lysate was centrifuged at 10,000Xg for 40 min at 4^0C to obtain clear supernatant, which was used for affinity purification of GST-tagged recombinant protein over GST Uniflow resin (Clontech). Furthermore, GST tag was cleaved off by overnight incubation at 4^0C of Factor Xa along with elution buffer.

Fluorescence Spectroscopy

Tryptophan fluorescence (Typ) measurements were performed in Horiba spectrofluorometer equipped with peltier system. For the measurements (including GdmCl-treated controls), 2µM of each of the protein was diluted in a volume of 1ml using Tris buffer (pH 8.0), and incubated overnight. Around 500µl of the samples were put in fluorometer cuvette, and spectral measurements were made for each of WT and mutant proteins at 25^0C. For each sample, measurement was repeated for at least three times. Protein samples were excited at 280nm and emission was collected at 300-500nm. The excitation and emission slit width was set up at 5nm. For DNA binding experiments, DNA probe of RUNX3 for fluorometric experiments was synthesized by annealing complementary oligos for RUNX3 (ForRUNX3: 5'CTGTCCCTCAA CCACAGAACCA3' and RevRUNX3: 5'TGGTTCTGTGGT TGAGGGACAG3') at 95^0C for 5 minutes followed by slow ramping to bring down the temperature to 25^0C. Binding assays were performed with WT and mutant proteins by titrating with different concentrations of DNA probe ranging from 0.4µM to 4µM. Fluorescence intensities at 350nm were normalized to 1 and plotted as a function of [DNA probe], the molar concentration of DNA probe. The resulting curves were analyzed for the binding and dissociation constants using non-linear curve fit (hyperbola) of Origin software (version 7.0).

Circular Dichroism (CD) Spectroscopy

The secondary structure of purified RUNX1 (2µM) was determined by CD spectroscopy using Jasco J-810 Spectropolarimeter (163-900nm RANGE) equipped with a Peltier thermoelectric type temperature control system. The instrument is controlled by Jasco's Spectra Manager™ software. Each spectral measurement was made at least three times with at least 6 accumulations. The far-UV CD spectra of WT and mutant proteins were measured in Tris buffer, pH 8.0 and at 25^0C. All spectra obtained were subtracted from the contributions of buffers and necessary blanks. The protein concentration for far-UV CD study was 2µM.

RESULTS

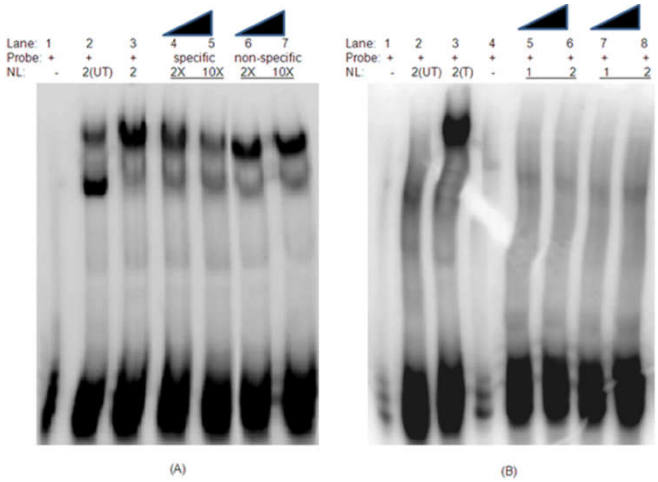
Mutant RUNX1 protein binds weakly to the promoter of its target DNA (RUNX3)

To investigate any difference in binding affinity of WT RUNX1 protein with RUNX3 promoter DNA fragment with that of mutant RUNX1, EMSA experiments were carried out. Initially binding of RUNX1 with RUNX3 promoter was established by increasing the concentration of nuclear lysate transfected with WT RUNX1. The competition assay confirmed the specific protein:DNA interaction (Figure 1(a)) as specific unlabelled probe competes with specific radiolabelled probe for its interaction with RUNX1 protein while cold non-specific probe did not compete with the interaction of RUNX1 protein and RUNX3 promoter. As is evident from Figure 1(b) (lanes 3 and 4), the nuclear lysates from cells expressing WT RUNX1 gave a discrete gel shift. However, cells expressing mutant RUNX1 (K83E) binds poorly even when 2µg protein of nuclear lysate was used (lanes 1 and 2 of Figure 1(b)). The mutant RUNX1 (R174Q), which also falls within the DNA binding domain, showed negligible binding with probe even when 3µg of nuclear lysate was used (Figure 1(b), lanes 7, 8). Thus our studies clearly indicate that similar to runt domain, full-length RUNX1 protein also binds to its target sequence effectively only when present as wild type. Any mutation in DNA binding domain of RUNX1 affects its binding to its target promoters (RUNX3). Previous studies using runt domain suggested about 10-fold decrease in binding constant of mutant runt domain as compared to that of wild type (Crute, Lewis *et al.* 1996). We therefore decided to purify full-length proteins (WT and mutant) and compare their binding constant with RUNX3 promoter. For this, we have intentionally cloned RUNX1 cDNA in bacterial expression vector pGEX-5X-3, expressed and purified (Figure 2). To further investigate the effect of the mutations (K83E and R174Q) on binding affinity to their target promoter sites, highly purified WT type and mutant RUNX1 protein (Figure) were titrated with a different (increasing) concentration of DNA (RUNX3 promoter, 2 fold and 10 fold). We then measured any change in tryptophan

Table 1. Binding constant of RUNX1 and its mutants with RUNX3 promoter

	Association constant (mM)	Fold Change
Binding of wild type RUNX1 with RUNX3 promoter	1.8×10^{-3}	1
Binding of RUNX1 (K83E) with RUNX3 promoter	2.6×10^{-2}	7
Binding of RUNX1 (R174Q) with RUNX3 promoter	3.5×10^{-2}	5

fluorescence intensity at 350nm. Figure 3 demonstrates spectra of WT and mutant proteins obtained by treatment with DNA-probe. We then plotted change in fluorescence at 350nm as a function of DNA concentration (Figure 4) and using a non-linear least square analysis as mentioned in material and methods, binding constants (association constant) of proteins were evaluated (Table 1). As shown in this table there is 5-7 fold decrease in binding affinity of mutants as compared to the wild type. Binding constant for R174Q is lower than that of K83E indicating that different mutations may lead to a different magnitude of binding affinity.



*Positive (+) and negative (-) signs denote the presence and absence of the components of the reaction.
* NL-nuclear lysate (in µg), UT-nuclear lysate of untransfected cells, T-nuclear lysate of transfected cells.

Figure 1. Gel shift assays to show binding of RUNX1 protein with RUNX3 promoter. (A) Competition assay to show the specific binding of RUNX1 protein with RUNX3 promoter. Lane 1: Probe alone, Lane 2: Binding of nuclear lysate of untransfected HEK cell line with labeled RUNX3 promoter, Lane 3: represents the specific binding of RUNX1 with labelled DNA probe. RUNX1 protein was from nuclear lysate of HEK293 transfected with wild type RUNX1, Lanes 4, 5: represent the decrease in band intensity with an increase in the concentration of specific cold probe, the concentration of hot probe remains the same, Lanes 6, 7: Binding of cold non-specific probe with RUNX1 protein in the presence of specific labeled probe. (B) Gel shift assay to show change in binding affinity of wild type RUNX1 (lane 3) vs. mutant RUNX1 (K83E) (lanes 5, 6) and RUNX1 (R174Q) (lanes 7, 8) in increasing concentration with labeled RUNX3 promoter. Lane 1: Probe alone, Lane 2: Binding of nuclear lysate of untransfected HEK cell line with labeled RUNX3 promoter.

Conformation of mutant proteins is different from that of WT RUNX1

We speculated that mutations might have altered conformations of mutant proteins resulting in changed affinity to DNA-probe. Therefore, we investigated the structural changes due to mutations and compare it with that of wild type protein using far-UV CD (a signature of secondary structural content). The two mutants have different secondary structural contents as compared to that of wild type (Figure 5). The mutant, K83E is nearly identical to that of WT, while R174Q has no appreciable CD signal indicating that this mutant has lost secondary structures. We also measured tryptophan-fluorescence that tells about the environment of tryptophan in wild type, and mutant proteins. The Fluorescence spectrum of R174Q is quite near (but not similar) to GdmCl-denatured wild type protein (Figure 6). We consider that the partial tertiary structure, as evident from fluorescence spectra, could be due to the formation of local structures in its unfolded state.

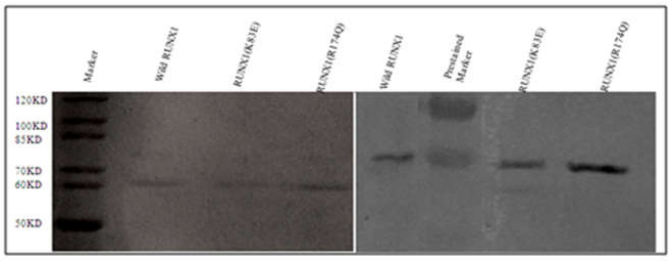


Figure 2. Coomassie stained SDS-PAGE and western blot Represents (A) coomassie stained SDS-PAGE and (B) western blot of purified GST tagged WT RUNX1 protein purified by GST Uniflow resin via affinity chromatography

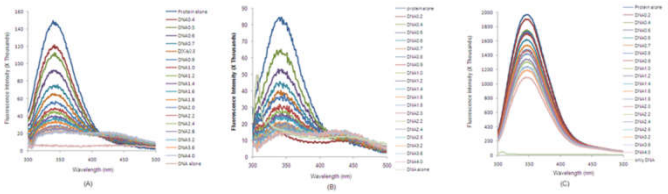


Figure 3. Net fluorescence spectrum The spectrum obtained by Trp fluorescence of (A) WT RUNX1, (B) RUNX1 (K83E), and of (C) RUNX1 (R174Q) protein titrated with DNA (RUNX3 promoter

In case of K83E although there is slight change in secondary structural content, the tertiary structure is as compact (since the spectra is nearly overlapping) as that of WT protein. This indicated that the substitution leaded to minor change in its secondary structure without affecting the tertiary structure. Taken together, these results indicate that the change in conformation of full-length protein due to point mutations might be responsible for observed decrease in binding to DNA (as was evident from electrophoretic mobility shift assays and kinetic studies).

In-silico analysis suggests altered structure of mutant RUNX1-DNA complex

The monomeric form of a complex containing runt domain of WT and mutant RUNX1 protein and promoter fragment of RUNX3 was modeled using AMBER 8.0 program as described under methods. Initially, minimization of energy of wild type runt domain with target promoter DNA (RUNX3 promoter) was carried out, followed by reviewing the interactions of mutant runt domains (K83E and R174Q) with RUNX3 promoter DNA (Figure 7). The interactions between RUNX1 (mutants) with RUNX3 promoter were studied by measuring changes in interaction and conformation of protein-DNA complex with respect to WT RUNX1. The Ramachandran plots depicted that the modeled structures are viable as most of the amino acids fall in the permissible zone (Figure 8). Several changes in hydrogen bonding of protein–DNA complexes were observed as depicted by differences in the distribution of hydrogen bonds between interface of macromolecular assembly, i.e., between protein and DNA. The number of hydrogen bonds also varied when we compared mutated complex with wild type complex. Some of the hydrogen bonds could not be shaped while new hydrogen bonds were observed between amino acid residues of mutant

runt domain and DNA probe. In the RUNX1-DNA complex, the DNA got inclined towards the runt domain. As is evident from modeled complex of wild type runt-RUNX3 promoter superimposed on that of mutant runt-RUNX3 promoter, several amino acids show differential interaction with DNA (Figure 9).

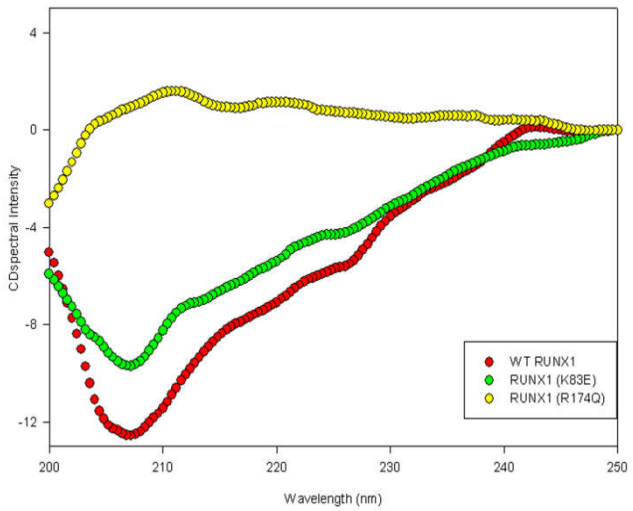


Figure 5. Comparative far UV spectrum of purified WT RUNX1, RUNX1 (K83E) and RUNX1 (R174Q) protein

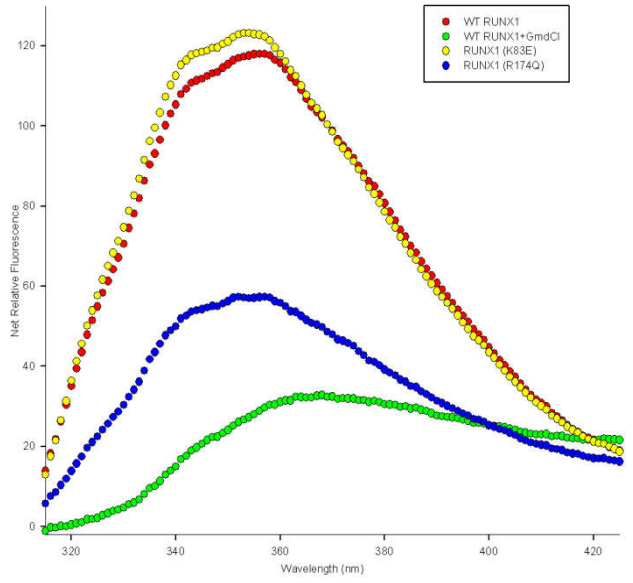


Figure 6. Fluorescence spectroscopy of WT RUNX1, RUNX1 (K83E), RUNX1 (R174Q) and GmdCl denatured wild type RUNX1

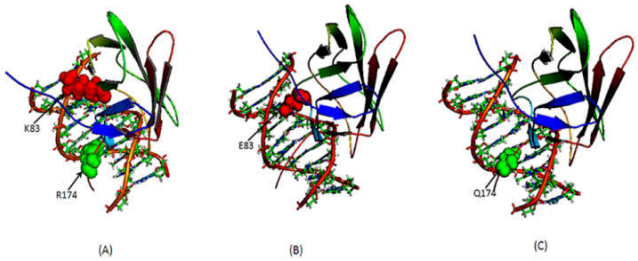


Figure 7. Three dimensional energy minimized model of the protein-DNA complexes. The stable structures obtained after energy minimization of runt domain of (A) WT RUNX1-RUNX3 promoter complex, (B) RUNX1 (K83E)-RUNX3 promoter complex, and of (C) RUNX1 (R174E)-RUNX3 promoter complex

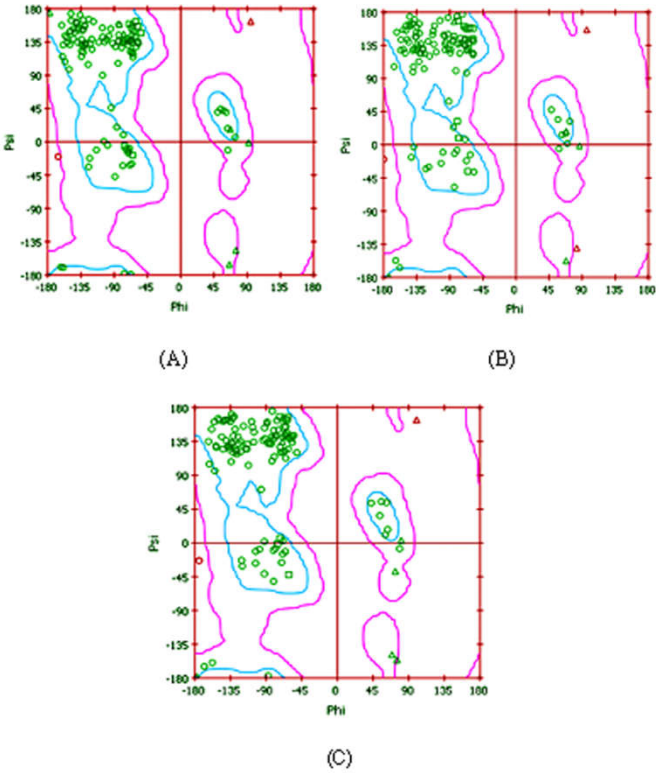


Figure 8. 2D graphical representation of the Ramachandran plots. Ramachandaran plots showing Φ and Ψ angles of the complexes of (A) runt domain of WT RUNX1-RUNX3 promoter, (B) runt (K83E)-RUNX3 promoter, and (C) runt (R174Q)-RUNX3 promoter

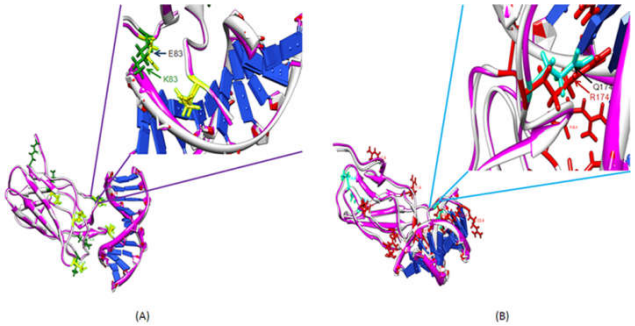


Figure 9. Energy minimized superimposed structures. (A) Superimposed structures of complexes of wild type runt-RUNX3 promoter (grey) and runt (K83E)-RUNX3 promoter (magenta) showing a change in conformation and gave RMSD value of 0.796 (SYBYL-X 1.1.1 software). The Lysine 83 of wild type runt was colored as green while Glutamic acid 83 of mutant runt was in yellow color. (B) Comparison of wild type runt-RUNX3 promoter complex and runt (R174Q)-RUNX3 promoter. The complexes of wild type runt-RUNX3 promoter (grey) and RUNX1 (R174Q)-RUNX3 promoter (magenta) gave RMSD value of 0.788 on superimposition (SYBYL-X 1.1.1). The substituted residue of wild type runt domain; Arginine was colored in red while Glutamine of runt (R174Q) was in cyan. All figures were generated using UCSF Chimera software.

A change in the angle of orientation of amino acids was observed when runt domain of mutant RUNX1 (K83E)-RUNX3 promoter complex was superimposed on runt domain of WT RUNX1-RUNX3 promoter complex (Figure 9(a)). The RMSD value was found to be 0.796. Similarly, on superimposing runt domain of mutant RUNX1 (R174Q)-RUNX3 promoter complex on runt domain of WT RUNX1-RUNX3 promoter complex, several changes were observed as shown in Figure 9(b). The RMSD value was found to be 0.788 as predicted by SYBYL-X 1.1.1

software suggesting a change in structure of the complex. To get a better insight into protein-DNA structure, we compared bond lengths and formation of hydrogen bonds between promoter DNA and side chains of amino acids of runt domain of WT and mutant RUNX1. Table 2 summarizes various interactions between RUNX3 promoters DNA with WT as well mutant runt domain of RUNX1 protein (Figure 10). As is evident, several bases (T2, G4, G6, G7, C11, A12, A13, C14 and C15) interact with wild type as well as with mutant protein; however, the interacting sites and bond length vary. The hydrogen atoms of Asn82 and Thr84 of wild type runt domain formed hydrogen bond with T2 and T3 respectively but this interaction was lost in mutant complexes. Similarly, Arg80 and Gln174 formed hydrogen bonds with T5 and G6 bases of DNA respectively uniquely in mutant (R174Q) while Arg177 formed hydrogen bond with T8 in case of mutant K83E but not in wild type and another mutant. We also observed that hydrogen bond lengths formed between several Arg residues and DNA were altered in mutant complexes when compared with wild type runt-DNA complex (Table 2). For example, arginine residues in WT RUNX1 protein present at 135, 177, 142 and 139 were engaged in formation of hydrogen bonds with O1 of T2, O6 of G7, O2 of C11 and O2 of C15 in wild type as well as in mutant runt domain but H-bond lengths varied among all these three complexes as shown in Table 2 and Figure 10.

Similarly due to a change in the conformation of mutant protein-DNA complex H-bond length of several other residues also got altered (Table 2). For example, H-bond length altered for an interaction of Lysine 83 with G4 in wild type complex (1.8 Å) in comparison to mutant complex (R174Q) (2.1 Å). In addition, T5 and T8 form unique interactions with mutant runt (R174Q) and (K83E) respectively. Figure 11 highlights some of the interactions which got altered in energy minimized complexes of mutant protein-DNA complexes in comparison to that of wild type protein-DNA complexes. A hydrogen bond plot (HB plot) generated by Discovery Studio 2.5 summarizes a change in hydrogen bond pattern between RUNX3 promoter and runt domain (wild type and mutant) (Figure 12). We envisage that although different hydrogen bonds are formed between mutant runt and DNA complex, they may be sufficient to provide only partial stability to the complex.

DISCUSSION

The effect of missense mutations on protein structure and folding has been largely investigated (Stenson, Ball *et al.* 2003), (Bross, Corydon *et al.* 1999). The generally held belief for the effect of missense mutations on protein folding is that these mutations affect protein folding by "trapping" the protein

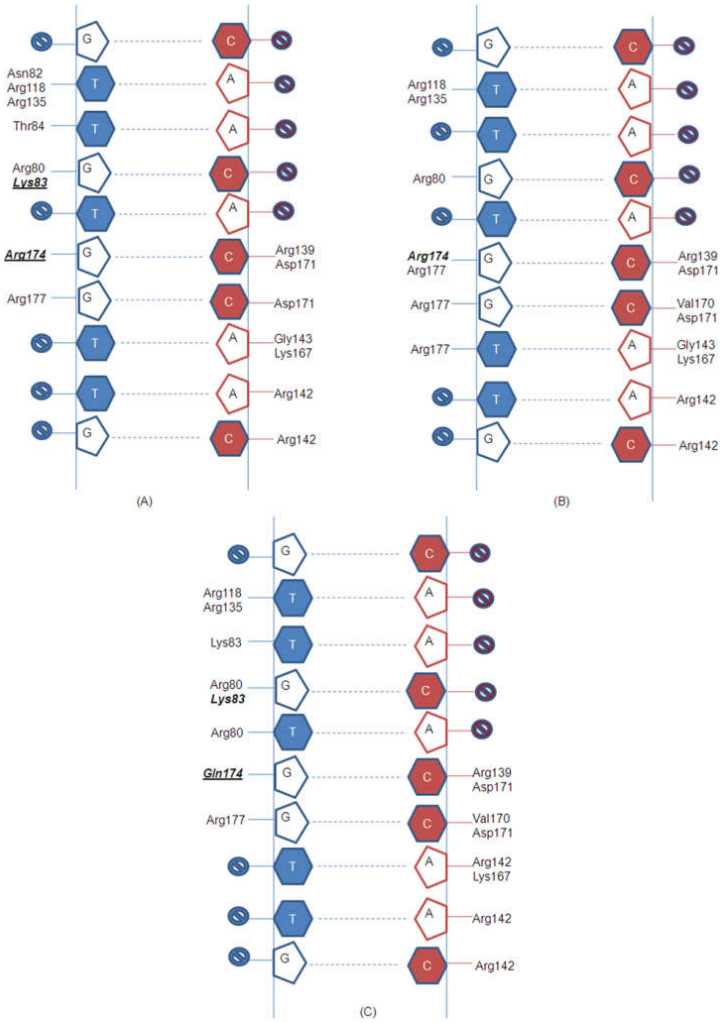


Figure 10. Interacting amino acid residues of runt domain of RUNX1 with bases of DNA (RUNX3 promoter). (A) Representative of amino acids of WT RUNX1 protein with bases of RUNX3 promoter. (B) Shows interaction of amino acid residues of RUNX1 (K83E) with bases of RUNX3 promoter. (C) Interaction of amino acid residues of RUNX1 (R174Q) with bases of RUNX3 promoter. *The complementary DNA chains are shown to be bonded together with dashed lines and the amino acids which are involved in hydrogen bond formation with the respective base pair are labeled accordingly with bold lines. The purines are indicated as empty boxes while pyrimidines are filled boxes.

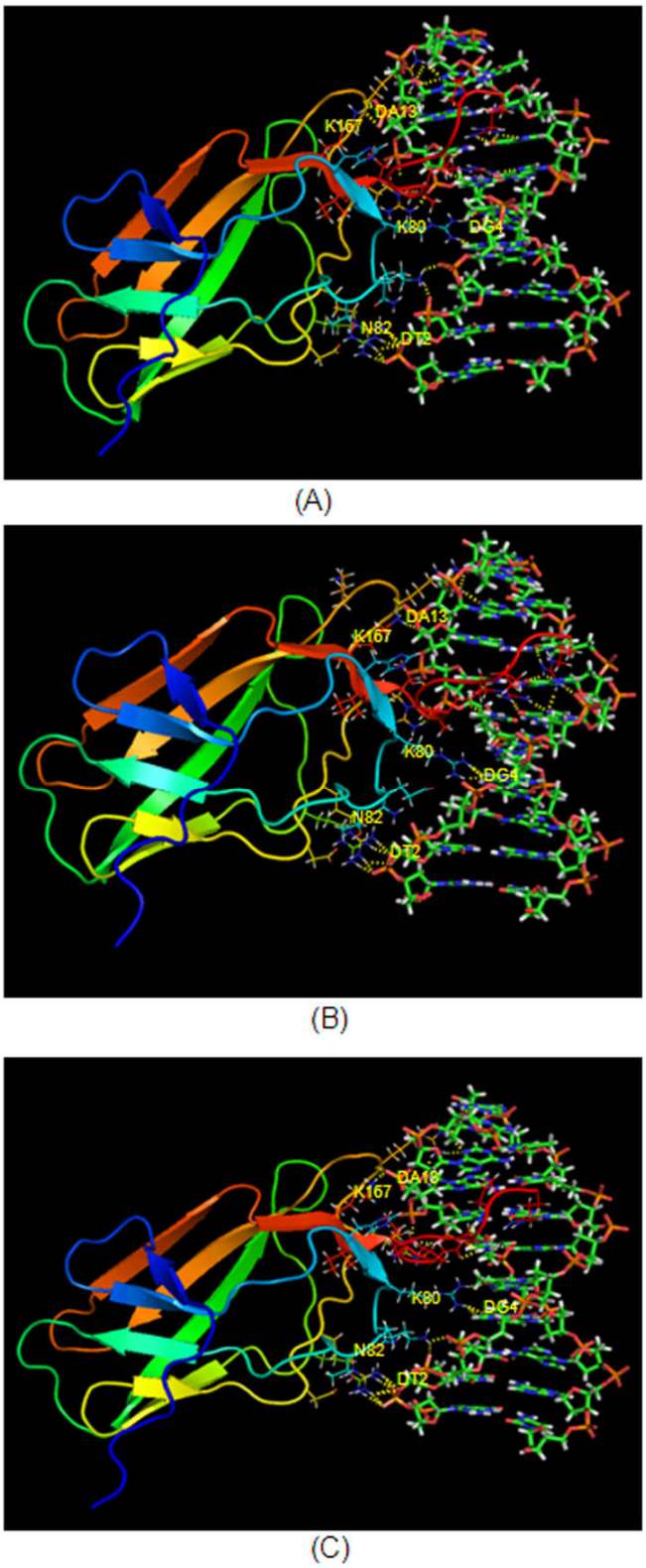


Figure 11. Energy minimized models showing protein-DNA interaction (Hydrogen bonds). (A) wild type runt-RUNX3 promoter. (B) runt (K83E) -RUNX3 promoter. (C) runt (R174Q)-RUNX3 promoter.

in a non-functional intermediate state, preventing it from folding into its lowest-free energy native state and consequently this lead to the formation of large molecular weight aggregates (thereby losing functional activity). Previous literature stated that there is well recognized role of RUNX1/CBFBeta in definitive hematopoiesis (Engel and Hiebert 2010). Our results on EMSA and kinetic binding experiments on two RUNX1 mutants (R174Q and K83E) suggest that mutations do not affect proteins to an extent so as to completely lose their activity.

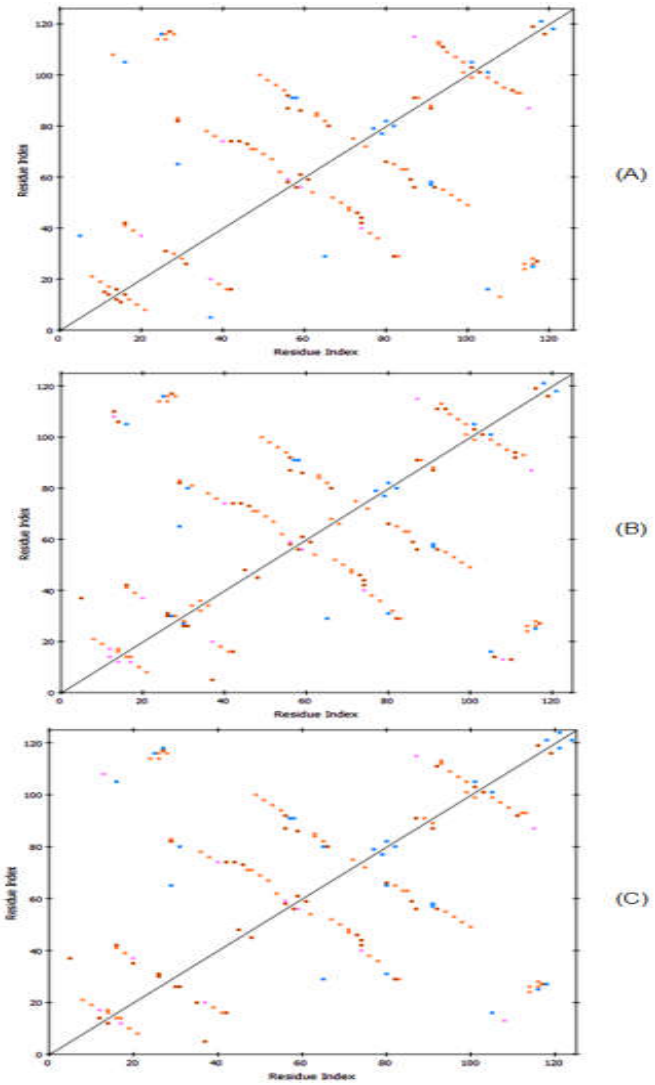


Figure 12. HB plot representation of wild type and mutant runt-RUNX3 promoter, Representative of HB plot of (A) wild type runt-RUNX3 complex, (B) runt (K83E)-RUNX3 complex, (C) runt (R174Q)-RUNX3 complex. All the plots were prepared using the software Discovery Studio 2.5 (DS Modelling 2.5, Accelrys Inc., San Diego, CA). Different colors of the dots indicate the various types of hydrogen bond interactions i.e. side chain-side chain (blue), main chain-main chain (orange), main chain-side chain (red) and multiple hydrogen bonds (pink)

Instead, there is 5-7 fold reduction in binding ability of mutants as compared to that of wild type. The absence of functional loss (of binding ability) to DNA of the two mutants (R174Q and K83E), and existence of mutant proteins in soluble form (not aggregate in the form of inclusion body) upon expression in bacteria indicate that mutations indeed, do not affect the folding pathway or kinetic. Therefore, the effect mutations might perhaps be in the structure of its native state. It might be possible that mutations have brought about a change in native state of the mutant proteins that in fact dictates in lowering the binding affinity of the mutant proteins with DNA. For this, we performed conformational characterization of the WT and the mutant proteins. It was observed that native WT RUNX1 protein revealed a typical spectrum suggesting the presence of prominent alpha helices. However, circular dichroism and Trp fluorescence spectrum of RUNX1 (R174Q) strongly suggested that protein is highly

disordered with complete loss of secondary structure and tertiary structure. Contradictorily, mutant RUNX1 (K83E) protein has a structure somewhat similar to that of WT RUNX1 but a mutation in R174 residue apparently has disrupted the structure of full-length protein. The results indicate that different mutations have different effects on the native state of proteins. Interestingly, neither the structurally disordered mutant (R174Q) nor the stable mutant (K83E) protein completely loses its ability to bind DNA. This might be due to the fact that mutations might have introduced changes in structure of native protein elsewhere leading to altered secondary structure or the environment of tryptophan, but not essentially perturbed the structure of DNA binding core of proteins. Thus both data on CD spectroscopy and fluorescence spectroscopy indicate that a change in conformation of full-length protein, due to point mutations, might be responsible for decrease in binding of DNA as was evident from electrophoretic mobility shift assays and kinetic binding studies. The data on null-CBFbeta mice and null RUNX1 mice leads to the same spectrum of abnormalities; in turn suggested that thers is pivotal role of both RUNX1 and CBFbeta during definitve hematopoiesis and there is interdependence corelation between RUNX1 and CBFbeta (Wang, Stacy *et al.* 1996).

We were further interested to investigate the key interactions lost or formed due to the mutations. For this, we modeled a binding between DNA and proteins by using Insight II. NMR and crystal structure of runt domain with DNA has shown that runt domain makes contacts in both the major and minor grooves of DNA using loops extending from one end of the barrel (Bravo, Li *et al.* 2001, Zhang, Lukasik *et al.* 2003). Arginines recognize 3 guanine residues in the consensus sequence of DNA, while Asp171 makes contacts with two cytosines situated in the complementary strand and Arg142 in loop L9 makes direct contact with two bases in the minor groove (Tahirov, Inoue-Bungo *et al.* 2001). The crystal structure of the uncomplexed runt domain as well as of full-length RUNX1 has not been determined till date and it is thus not possible to fully understand the effect of mutations on its binding with DNA and CBFβ. The present study describes the binding of RUNX1 on its target genes and the effect of the most frequent mutations (K83E and R174Q) on conformation of DNA-protein complex. The promoter sequence of RUNX3 gene, one of the targets of RUNX1, containing RUNX1 binding site, was selected for analyzing protein-DNA interactions. Ramachandran plots depicted that modeled structures of RUNX1 (WT and mutants) complexed with RUNX3 promoter were stable as most of the amino acids fall in the permissible zone, once again supporting our observations that runt domain retains its secondary structure. *In-silico* modeling suggests that both wild type and mutant runt domain inclines the promoter DNA towards protein. However, several amino acids showed differential interaction with DNA (RUNX3 promoter) in case of mutant runt domain. We also observed several differences in the distribution of hydrogen bonds between interfaces of macromolecular assemblies as well as in H-bond lengths between runt domain of WT RUNX1–DNA complex and mutant RUNX1-DNA complex. The amino acids that interrelate sequence specifically with the DNA binding site are located on L3, L12, L9, and β3 and include Arg80, Lys83, Arg142, Val 170, Asp171, Arg174 and Arg177. On the other hand amino acids Arg135, Arg139, Gly143 and Lys167 located on β9, L9, and β12 form non-specific interactions with phosphate backbone (Bartfeld, Shimon *et al.* 2002). Our *in-silico* experiments using natural promoter (RUNX3 promoter spanning consensus binding sequence) clearly suggests that although these amino acids (Arg80, Lys83, Arg142, Val 170, Asp171, Arg 174 and Arg 177) form interactions with their consensus binding site, other amino acids do interact outside this site. Infact, several new hydrogen bonds were observed between DNA and mutated RUNX1 apparently due to conformational change in the mutated RUNX1 protein (Table 2).

Within runt domain, Lys83 interact with Arg80, present in close proximity to it. Lys 83, which interacts with the sugar-phosphate backbone of DNA also binds to guanidinium group of Arg80, stabilizing its side chain conformation in both cases via water molecules (Tahirov, Inoue-Bungo *et al.* 2001). The mutation in Lysine leads toa conformational change in the full-length protein as suggested by our *in-silico* and *in-vitro* experiments. This substitution in Lys83 effects its interaction with Arg80 which in turn may lead to the decrease binding affinity of catalytic core of runt domain to DNA. Our *in-silico* results with R174Q mutant of RUNX1 support the earlier observations by Zhang *et al.* in 2003, wherein they suggested that R174Q mutations in FPD-AML patients disrupt a specific contact made by runt domain only to DNA. Using full-length RUNX1 (WT and mutant) as well as natural promoters (RUNX3 gene promoter), we have now clearly shown that the mutated RUNX1 protein not only has an altered conformation but also a lower binding affinity with its consensus-binding site. At present, it is not known whether amino acids present outside the runt domain also contribute to DNA binding or not. It is also possible that amino acids outside the runt domain may contribute to the stability of the conformation of runt domain to facilitate its interaction with DNA. In the absence of a structure of full-length RUNX1, it is difficult to check this hypothesis.

Acknowledgement

We are thankful to Dr. Y. Ito and Dr. M. Osato from Institute of Molecular and Cell Biology, National University of Singapore, Singapore for providing us the clones of RUNX1 inserted into pEF-Bos vector. We are also grateful to Dr. G. P. S. Raghava and Dr. Balwinder Singh from Institute of Microbial Technology, Chandigarh, India, for their advice and support in *in-silico* work.

REFERENCES

Akamatsu, Y., *et al.* 1997. "A simple screening for mutant DNA binding proteins: application to murine transcription factor PEBP2alpha subunit, a founding member of the Runt domain protein family." *Gene* 185(1): 111-117.

Bartfeld, D., *et al.* 2002. "DNA recognition by the RUNX1 transcription factor is mediated by an allosteric transition in the RUNT domain and by DNA bending." Structure 10(10): 1395-1407.

Bravo, J., *et al.* 2001. "The leukemia-associated AML1 (Runx1)--CBF beta complex functions as a DNA-induced molecular clamp." *Nat Struct Biol* 8(4): 371-378.

Bross, P., *et al.* 1999. "Protein misfolding and degradation in genetic diseases." *Hum Mutat* 14(3): 186-198.

Case, D. A., *et al.* 2005. "The Amber biomolecular simulation programs." *J Comput Chem* 26(16): 1668-1688.

Crute, B. E., *et al.* 1996. "Biochemical and biophysical properties of the core-binding factor alpha2 (AML1) DNA-binding domain." *J Biol Chem* 271(42): 26251-26260.

De Braekeleer, E., *et al.* 2011. "RUNX1 translocations and fusion genes in malignant hemopathies." Future Oncol 7(1): 77-91.

Engel, M. E. and S. W. Hiebert, 2010. "Proleukemic RUNX1 and CBFbeta mutations in the pathogenesis of acute leukemia." Cancer Treat Res 145: 127-147.

Garvie, C. W. and C. Wolberger, 2001. "Recognition of specific DNA sequences." Mol Cell 8(5): 937-946.

Hart, S. M. and L. Foroni, 2002. "Core binding factor genes and human leukemia." Haematologica 87(12): 1307-1323.

Huang, X., *et al.* 1998. "Overexpression, purification, and biophysical characterization of the heterodimerization domain of the core-binding factor beta subunit." J Biol Chem 273(4): 2480-2487.

Imai, Y., *et al.* 2000. "Mutations of the AML1 gene in myelodysplastic syndrome and their functional implications in leukemogenesis." Blood 96(9): 3154-3160.

Inoue, K., *et al.* 2002. "Runx3 controls the axonal projection of proprioceptive dorsal root ganglion neurons." *Nat Neurosci* 5(10): 946-954.

Kagoshima, H., *et al.* 1993. "The Runt domain identifies a new family of heteromeric transcriptional regulators." *Trends Genet* 9(10): 338-341.

Kanno, T., *et al.* 1998. "Intrinsic transcriptional activation-inhibition domains of the polyomavirus enhancer binding protein 2/core binding factor alpha subunit revealed in the presence of the beta subunit." *Mol Cell Biol* 18(5): 2444-2454.

Komori, T., *et al.* 1997. "Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts." Cell 89(5): 755-764.

Levanon, D., *et al.* 2002. "The Runx3 transcription factor regulates development and survival of TrkC dorsal root ganglia neurons." *EMBO J* 21(13): 3454-3463.

Lutterbach, B. and S. W. Hiebert 2000. "Role of the transcription factor AML-1 in acute leukemia and hematopoietic differentiation." Gene 245(2): 223-235.

Michaud, J., *et al.* 2002. "In vitro analyses of known and novel RUNX1/AML1 mutations in dominant familial platelet disorder with predisposition to acute myelogenous leukemia: implications for mechanisms of pathogenesis." Blood 99(4): 1364-1372.

Nagata, T. and M. H. Werner, 2001. "Functional mutagenesis of AML1/RUNX1 and PEBP2 beta/CBF beta define distinct, non-overlapping sites for DNA recognition and heterodimerization by the Runt domain." J Mol Biol 308(2): 191-203.

Osato, M., *et al.* 1999. "Biallelic and heterozygous point mutations in the runt domain of the AML1/PEBP2alphaB gene associated with myeloblastic leukemias." Blood 93(6): 1817-1824.

Otto, F., *et al.* 1997. "Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development." Cell 89(5): 765-771.

Preudhomme, C., *et al.* 2000. "High incidence of biallelic point mutations in the Runt domain of the AML1/PEBP2 alpha B gene in Mo acute myeloid leukemia and in myeloid malignancies with acquired trisomy 21." Blood 96(8): 2862-2869.

Quack, I., *et al.* 1999. "Mutation analysis of core binding factor A1 in patients with cleidocranial dysplasia." Am J Hum Genet 65(5): 1268-1278.

Song, W. J., *et al.* 1999. "Haploinsufficiency of CBFA2 causes familial thrombocytopenia with propensity to develop acute myelogenous leukaemia." Nat Genet 23(2): 166-175.

Stenson, P. D., *et al.* 2003. "Human Gene Mutation Database (HGMD): 2003 update." Hum Mutat 21(6): 577-581.

Tahirov, T. H., *et al.* 2001. "Structural analyses of DNA recognition by the AML1/Runx-1 Runt domain and its allosteric control by CBFbeta." Cell 104(5): 755-767.

Tanaka, T., *et al.* 1996. "The extracellular signal-regulated kinase pathway phosphorylates AML1, an acute myeloid leukemia gene product, and potentially regulates its transactivation ability." Mol Cell Biol 16(7): 3967-3979.

Taniuchi, I., *et al.* 2002. "Differential requirements for Runx proteins in CD4 repression and epigenetic silencing during T lymphocyte development." Cell 111(5): 621-633.

Thiel, V. N., *et al.* 2017. "Heterodimerization of AML1/ETO with CBFbeta is required for leukemogenesis but not for myeloproliferation." Leukemia.

Wang, C. Q., *et al.* 2010. "Runx family genes, niche, and stem cell quiescence." Blood Cells Mol Dis 44(4): 275-286.

Wang, Q., *et al.* 1996. "The CBFbeta subunit is essential for CBFalpha2 (AML1) function in vivo." Cell 87(4): 697-708.

Woolf, E., *et al.* 2003. "Runx3 and Runx1 are required for CD8 T cell development during thymopoiesis." Proc Natl Acad Sci U S A 100(13): 7731-7736.

Zeng, C., *et al.* 1997. "Identification of a nuclear matrix targeting signal in the leukemia and bone-related AML/CBF-alpha transcription factors." Proc Natl Acad Sci U S A 94(13): 6746-6751.

Zhang, L., *et al.* 2003. "Structural and functional characterization of Runx1, CBF beta, and CBF beta-SMMHC." Blood Cells Mol Dis 30(2): 147-156.

****