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Original Article

GENETIC ASSOCIATION OF SIRTUIN 3 INTRON 5 VNTR POLYMORPHISM WITH THE DEVELOPMENT OF CHRONIC MYELOID LEUKEMIA: A CASE-CONTROL STUDY

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Abstract: ***Drug resistance and recurrence are the current clinical challenges in the management of Chronic Myeloid Leukaemia (CML) associated with emerging BCR-ABL kinase site mutations, increasing genomic instability, altered mitochondrial/cellular metabolism and survival of quiescent leukemic stem cells. Sirtuin3 (SIRT3), a mitochondrialdeacetylase is a regulator of mitochondrial metabolism, oxidative stress, and ROS production affecting diverse cellular functions. Altered expression of SIRT3, and was shown to correlate with poor differentiation, metastasis and poor prognosis. The current case-control study is an attempt to explore the association of SIRT3 VNTR polymorphism in intron 5 with the development and progression of CML. In the present case-control study on 297 CML cases and 321 normal healthy controls, genomic DNAs isolated from peripheral blood samples were analysed by polymerase chain reaction for genotyping of SIRT3 Intron 5 VNTR polymorphism. The association was assessed statistically with respect to epidemiological and clinical phenotypes. The results revealed significant allelic distribution (p =0.03). The risk of CML development was found to be elevated for individuals carrying lower repeat allele [0R(CI):1.59(1.14-2.22); p= 0.006] and 0R/0R [OR(CI):1.85(0.03-3.31); P=0.03] genotype. The influence of zero repeat on altered expression of SIRT3 might confer increased susceptibility to CML. Hence, the VNTR polymorphism in the intron 5 region of SIRT3 gene could serve as a prognostic marker as well as an important marker in CML development. Further studies are warranted to study the prognostic significance of this SIRT3 polymorphism*.

Keywords: CML, VNTR polymorphism, Sirtuin 3

***Dr. A. Sandhya, Assistant Professor:** Her major scientific area of interest is human molecular genetics with a focus on genetic screening of functional variants for prognostic significance in breast cancer and chronic myeloid leukemia. She reported Screening of FLT3-ITD mutations in CML; Association of XRCC1 gene polymorphisms with CML development; Genetic association of PTEN, AKT1 and PIK3CA with CML development indicating the significance of PI3K pathway. Recently, with the support of International visiting fellowship (2019-20), carried out preliminary work on synthetic lethality based therapeutic approaches to treat metastatic and chemo-resistant ovarian cancer.

INTRODUCTION

Chronic myeloid leukaemia (CML) is a type of leukaemia with characteristic BCR/ABL fusion gene produced due to reciprocal translocation t (9;22) (q34; q11) event [1] generating a tyrosine kinase fusion protein which aberrantly activates the downstream signalling pathways, transforming the normal stem cells to leukemic stem cells (LSC) [2]. Development and use of BCR/ABL targeted drugs Tyrosine Kinase Inhibitors (TKIs), specifically the frontline TKI Imatinib mesylate (IM), had revolutionized the treatment of CML and increased the life span of patients. However, the maintenance of LSC and CML progression involve BCR/ABL dependent as well as independent mechanisms which might lead to drug resistance and recurrence of the disease upon treatment with TKI for longer duration [3,4].

TKI resistance could be due to primary or secondary mutations possibly affecting genomic instability, kinase activity, altered drug efflux or metabolism, etc. andmechanisms underlying are being studied extensively [5,6]. Recent studies suggested the role of mitochondrial metabolism in Imatinib resistance pointing to putative dysregulation of several genomic, epigenetic and metabolic regulators in BCR/ABL-positive cells [7].

Sirtuin 3 (SIRT3), a member of NAD dependant class III histone deacetylases (HDACs) is a mitochondrial deacetylase and one of the critical regulators of metabolic homeostasis that regulates the global acetylation landscape of mitochondrial proteins and oxidative stress/energy metabolism [8,9]. SIRT3 targets components of electron transport chain regulating ATP production [10,11,12]. Knock out studies demonstrated that deficiency/loss of SIRT3 compromises Hematopoietic Stem Cell (HSC) function and may drive HSCs out of quiescence and reduce their survival [13]. The fact that SIRT3 is highly expressed in young HSCs and it is suppressed with aging further provides an insight to its role in leukemic origin. Depending on the cell-type and tumour-type, SIRT3 may function as either oncogene or tumour suppressor on influencing cell death by targeting a series of key modulators and their relevant pathways in cancer [14]. Targeting SIRT3 would be a promising therapeutic strategy for the discovery of more activators or inhibitors in cancer. However, there are limited studies on SIRT3 gene variants or its dysregulation in cancer and indeed no reports in leukaemia. Hence, it will be interesting to explore the association of functional gene variants of SIRT3 and their role in the development of CML.

SIRT3 gene mapped to Chr 11p15.5, and its gene variants have been shown to be associated with metabolic disorders such as insulin resistance [15], obesity [16], and hepatic steatosis [17] and, human longevity [18,19]. This gene has a bidirectional promoter sharing with the PSMID gene have binding sites for sp1, zf5, nfkb/GATA1 factors [20]. A VNTR polymorphism in SIRT3, about a 72 bp repeat copy number variation in the enhancer element of intron 5 was reported to be associated with longer survival and lack of SIRT3 was found to be detrimental to longevity. This enhancer element has the capability of binding with a greater number of transcriptional factors with increased repeats Different alleles of this functional polymorphism are able to modulate the gene expression of a reporter gene in an allele specific way. Therefore, the present study is planned to analyse the genetic association of this VNTR polymorphism in CML in order to evaluate its role in the development and prognosis of CML.

MATERIALS AND METHODS

For the present case-control genetic association study, 297 clinically confirmed ph+ve CML cases and 321 healthy control samples were included. The patients were recruited from Nizams Institute of Medical Sciences (NIMS), Hyderabad during 2010-2018 while, ethnically matched normal healthy individuals without family history of cancers (served as control group) were recruited from various locations. The study was approved by the ethical committee of the Department of Genetics, Osmania University and NIMS, Hyderabad.

After obtaining informed consent from each case and control, 5ml of peripheral blood samples in EDTA vacutainers were collected for genotype analysis. The history of the patients pertaining to the clinical data such as the phase of the disease, haematological and cytogenetic response of the patients to Imatinib Mesylate were noted from the tumour registry of NIMS and their epidemiological data was recorded for genotype-phenotype comparisons to assess the association for confounding variables.

Genomic DNA [21] was isolated from peripheral blood leucocytes by rapid non enzymatic/salting out

method and estimated by nanodrop (Thermo Fisher Nanodrop Lite). Polymerase chain reaction was performed on isolated DNA using sequence specific primers to amplify the VNTR region at intron 5 of SIRT3.The forward primer sequence is 5'TTCCTGAAGCTGGGTACA3' and reverse is 5'CATTCACCTTCCCAAAGTGG3.The amplification mixture (10 ìL) is composed of; 10xPCR buffer, 25mM $MgCl_2$, 25mM dNTP mix, 25pM of each forward and reverse primers,0.25-0.5 U of taq DNA polymerase and 1ìL genomic DNA (50ng). PCR cycle program was 5 min at 95°C, 32 cycles of 40 sec at 95°C, 40 sec at 52.9°C, and 1 min at 72°C, and the terminal extension was 5min at 72°C. Amplified PCR products were electrophoresed on 2.5% agarose gel containing 0.5 ìg/ mL ethidium bromide and visualized on a UV transilluminatorand the gel picture was captured on GelDoc (Bio-Rad) (Fig 1). Based on the sizes of the PCR product, VNTR alleles were noted and samples were genotyped. The VNTR repeat size was 72bp. Samples with zero VNTR repeats (0R) corresponded to PCR product size of 421bp. With each repeat number increase, the respective band sizes were observed to be 493bp (1R), 565bp (2R), 637bp (3R) and so on.

The allele and genotype frequencies were calculated for CML cases and controls. The data was stratified with respect to gender, age at onset, clinical phase and treatment response (haematological, cytogenetic and molecular response) for appropriate statistical comparison. The chi-square, odds ratios and p values were calculated using SPSSv26 and MEDCALC online software tools. p value of 0.05 is considered significant.

RESULTS

The genotyping analysis of 297 CML patients and 321 controls revealed in total 6 VNTR alleles (0R, 1R, 2R, 3R, 4R and 9R) and 12 genotypes. Globally, we are the first to identify and report 9R allele in SIRT3 gene at intron 5 for the 72 bp VNTR repeat polymorphism, though it was found to be rare with a frequency of 0.16. This novel variant allele (9R) was observed in CML patient as 2R/9R genotype.

Fig 1. Gel picture showing 13 different genotypes of SIRT3 VNTR polymorphism

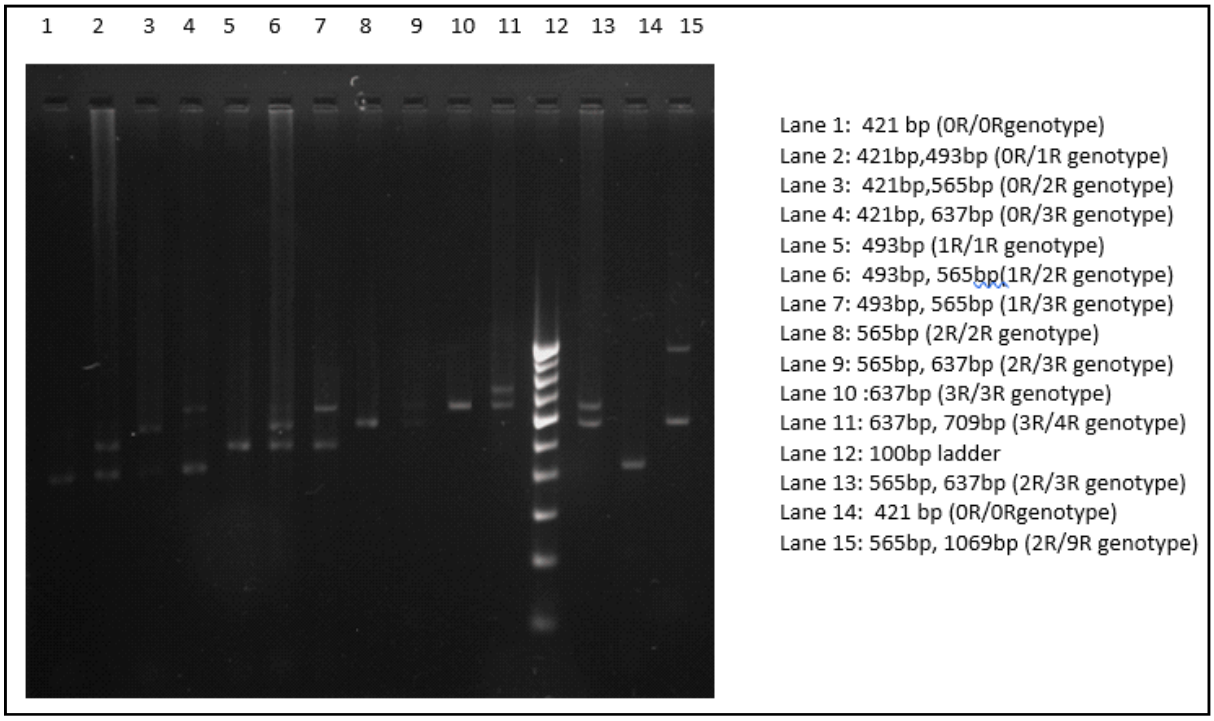

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Table 1: Frequency distribution of genotypes of SIRT3 Intron 5 VNTR polymorphism among CML cases and controls- Bold indicates the frequency, odds ratio and the p value of significant genotype. * indicates significance at p<0.05

	Cases (n=297)		Controls (n=321)		Odds Ratio (95%CI)	Odds ratio p value	Chi square test value (p)
Genotype	n	%	n	%			
2R/2R	75	25.25	95	29.59	Reference		12.26 (0.34)
0R/0R	38	12.79	26	8.09	1.85 (1.03-3.31)	0.03*	
0R/1R	16	5.38	16	4.98	1.26 (0.59-2.69)	0.54	
0R/2R	19	6.39	13	4.04	1.85 (0.85-3.98)	0.11	
0R/3R	7	2.35	4	1.24	2.21 (0.62-7.85)	0.21	
1R/1R	47	15.82	58	18.06	1.02 (0.62-1.67)	0.91	
1R/2R	25	8.41	28	8.72	1.13 (0.6-2.09)	0.69	
1R/3R	5	1.68	9	2.8	0.7 (0.22-2.18)	0.54	
2R/3R	21	7.07	17	5.29	1.56 (0.77-3.17)	0.21	
3R/3R	42	14.14	55	17.13	0.99 (0.60-1.63)	0.96	
3R/4R	1	0.33	0	0	3.79 (0.15-94.4)	0.41	
2R/9R	1	0.33	0	0	3.79 (0.15-94.4)	0.41	

Table- 2: Allelic frequency distribution of SIRT3 Intron 5 VNTR polymorphism among CML cases and controls. -Bold indicate the frequency, odds ratio and the p value of significant allele. * indicates significance at p<0.05, -** indicates significance at p<0.01

	Cases (n=297)		Controls (n=321)		Odds ratio (95%CI)	Odds ratio p value	Chi square value (p)
Alleles	n	%	n	%			
2R	216	36.36	248	38.62	Reference		11.76 (0.03*)
0R	118	19.86	85	13.23	1.59 (1.14-2.22)	0.006**	
1R	140	23.56	165	25.70	0.97 (0.72-1.30)	0.85	
3R	118	19.86	138	21.49	0.98 (0.72-1.35)	0.9	
4R	1	0.16	0	0	3.44 (0.13-84.97)	0.44	
9R	1	0.16	0	0	3.44 (0.13-84.97)	0.44	

Table 3: Frequency distribution of SIRT3 Intron 5 VNTR polymorphism with regard to genotype groups among CML cases and controls, Bold indicates the frequency, odds ratio and p value of significant genotype group. -* indicates significance at p<0.05

Genotype Groups	Cases (n=297) n (%)	Controls (n=321) n (%)	Oodds ratio (95% CI)	p - Value	χ^2 - p Value
Group 1 2R/2R	75 (25.25)	95(29.59)	Reference		
Group 2 2R/3R,3R/3R,4R/3R, 9R/2R,1R/2R,1R/3R	95 (31.98)	109(33.95)	1.10 (0.73-1.66)	0.63	0.19
Group 3 1R/1R, 0R/1R,0R/2R,0R/3R	89 (29.96)	91(28.34)	1.23 (0.81-1.88)	0.31	
Group 4 0R/0R	38 (12.79)	26(8.09)	1.85 (1.03-3.31)	0.03*	

Table 4: Distribution of genotype groups of SIRT3 Intron 5 VNTR polymorphism with respect to Gender

Genotype Groups	Males (n=180) n (%)	Females (n=117) n (%)	Odds ratio (95% CI)	p - value	χ^2 - pvalue
Group 1	43 (23.88)	32 (27.35)	Reference	–	0.46
Group 2	61(33.88)	34 (29.05)	1.33(0.71-2.48)	0.36	
Group 3	50(27.77)	39 (33.33)	0.95(0.51-1.77)	0.88	
Group 4	26 (14.44)	12 (10.25)	1.61(0.70-3.67)	0.25	

The genotype distribution of this polymorphism among the CML cases did not deviate from that of controls (Chi-square value = 12.26; p=0.34). The genotype 2R/2R was the most common type among controls (29.59%) as well as CML cases (25.25%) and hence was considered as reference genotype. Comparison of results with respect to this genotype revealed significant elevation of 0R/0R genotype among

Table 5: Distribution of genotype groups of SIRT3 Intron 5 VNTR polymorphism with regard to age at onset of CML

Genotype Groups	20-40 years (n=144) n (%)	> 40 years\ (n=125) n (%)	Odds ratio (95% CI)	p- value	<20 years (n=28) n (%)	Odds ratio (95% CI)	p-Value	χ^2 - p Value
Group 1	20(13.88)	49(39.20)	Reference	--	6(21.42)	Reference	--	0.18
Group 2	45(31.25)	37(29.60)	2.97 (1.51-5.8)	0.0016	13(46.42)	1.03 (0.34-3.12)	0.94	
Group 3	53(36.80)	30(24.0)	4.32 (2.17-8.59)	<0.0001	6(21.42)	2.65 (0.76-9.1)	0.12	
Group 4	26(18.05)	9(7.20)	7.07 (2.82-7.7)	<0.0001	3(10.71)	2.6 (0.57-11.6)	0.21	

Table 6: Distribution of genotype groups of SIRT3 Intron 5 VNTR polymorphism with regard to clinical phase of CML

Genotype Groups	Chronic (n=276) n (%)	Advanced (n=21) n (%)	odds ratio (95% CI)	p- value	χ^2 - p value
Group 1	68 (24.63%)	7(33.33%)	Reference		0.70
Group 2	90 (32.6%)	5(23.8%)	1.85 (0.56-6.08)	0.30	
Group 3	82(29.71%)	7(33.33%)	1.20 (0.40-3.60)	0.73	
Group 4	36(13.04)	2 (9.52%)	1.85 (0.36-9.38)	0.45	

Table-7: Distribution of genotype groups of SIRT3 Intron 5 VNTR polymorphism with regard to with respect to hematological response

Genotype Groups	Major (n=169) n (%)	Poor (n=96) n (%)	Odds ratio (95% CI)	p - value	χ^2 - p value
Group 1	42 (24.85%)	27(28.12%)	Reference		0.62
Group 2	51(30.17%)	31(32.29%)	1.05(0.54-2.04)	0.86	
Group 3	51(30.17%)	29(30.2%)	1.13(0.58-2.19)	0.71	
Group 4	25(14.79%)	9(9.37%)	1.78(0.72-4.4)	0.2	

Table 8: Distribution of genotype groups of SIRT3 Intron 5 VNTR polymorphism with respect to cytogenetic response.

Genotype Groups	Major (n=145) n (%)	Poor (n=117) n (%)	Odds ratio (95% CI)	p- value	χ^2 - p value
Group 1	35 (24.13%)	29(24.78%)	Reference		0.8
Group 2	50 (34.48%)	34(29.05%)	1.21(0.63-2.35)	0.55	
Group 3	41(28.27%)	38(32.47%)	0.89(0.46-1.73)	0.73	
Group 4	19(13.1%)	16(13.67%)	0.98(0.43-2.25)	0.96	

Table 9: Distribution of genotype groups of SIRT3 Intron 5 VNTR polymorphism with respect to molecular response.

Genotype Groups	Responders (n=110) n (%)	Non-Responders (n=79) n (%)	Odds ratio (95% CI)	p - value	χ^2 - p value
Group 1	29 (26.36%)	21(26.58%)	Reference		0.98
Group 2	33 (30.0%)	25 (31.64%)	0.9 (0.44-2.05)	0.90	
Group 3	33 (30.0%)	22(27.84%)	1.08 (0.49-2.36)	0.83	
Group 4	15(13.63%)	11(13.92%)	0.98 (0.37-2.5)	0.97	

patients (12.79% vs. 8.09% in controls) with an odds ratio of 1.85 (95%CI: 1.032-3.31) (p=0.03) indicating 0R/0R as risk conferring genotype for CML (Table 1). Interestingly, the frequencies of 0R carrier group (0R/0R, 0R/1R, 0R/2R and 0R/3R) were also found to be significantly elevated among cases as compared to controls (26.9% vs 18.3%) with OR ratio of 1.71(95%CI: 1.09-2.7) (p =0.01) exhibiting dominant nature of 0R allele to confer relatively higher risk in its genotypes. Although this difference was insignificant forindividual 0R heterozygotes, the combined 0R heterozygote frequency among cases wasfound to be moderately significant with an OR of 1.61(95% CI: 0.93-2.78). (Table 1)

Further, the allelic distribution revealed significant heterogeneity (chi-square=11.76; p= 0.03). The rare (4R) and novel (9R) alleles were found in only 1 patient each. Apart from these, the 0R allele was found to be the least common showing significant elevation among cases (19.86% vs. 13.23%) indicating 0R allele as risk conferring allele [OR(CI): 1.59 (1.14-2.22); p=0.006]. Surprisingly, other low repeat allele (1R) did not show significant variation in allelic distribution (Table 2).

To understand the probable dominant risk nature of 0R repeat allele or lower repeat alleles relative to 2R (the wild type and the most common allele), the data was stratified into four genotype groups, i.e., 2R/2Rthe reference group (group 1), high repeat group (group 2- 2R/3R, 2R/9R, 3R/3R, 3R/4R, 1R/2R, 1R/3R), the low repeat group (group 3- 0R/1R, 0R/2R, 0R/3R, 1R/1R) and 0R/0Rthe risk group (group 4). The genotype group distribution revealed significant variation in the frequency for only group 4emphasizing that 0R allele may pose relatively greater risk and is dominant over other alleles [OR (CI): 1.85(1.03-3.31), p=0.03)]. Further, the odds ratios showed a declining trend for risk from this group 4 to low repeat group (group 3) [OR (CI):1.23(0.81-1.88)] to high repeat group (group 2) [OR (CI): 1.10(0.73-1.66)] (Table 3).

With regard to epidemiological risk variables, the genotype distribution was found to be insignificant for gender. However, the 0R/0R risk genotype and group 2 genotype frequencieswere slightly elevated in males suggesting a possible predominant genotype specific risk for CML in males (Table 4). Age wise distribution indicated significant risk for individuals carrying low repeat genotypes (group 3 and 4) withage range 20-40 years (36.8 % and 18.05% respectively) while group 2 genotype carriers with young age (46.42% in <20 years). Statistically, the odds ratios showed significant risk for all the comparative genotype groups [group 2- OR: 2.97(1.51-5.8); group 3- OR: 4.32(2.17-8.59); group 4 OR: 7.07(2.82-7.7)] among cases with the age group 20-40 years. It is also evident that 0R/0R risk group 4 showed highest odds value confirming strong risk forage at onset of 20-40 years in individuals with this genotype. Interestingly, among 28 CML cases < 20 years, 13 belonged to group 2 (46.42%) indicating early onset is strongly associated with group 2 genotypes (Table 5).

Comparison with clinical variables such as clinical phase and drug response (haematological, cytogenetic and molecular response) did not reveal any significant association with any of the genotype groups suggesting that the repeat variation of this polymorphism may not influence progression or drug response in CML cases (Tables 6-9). Further, the survival analysis also did not reveal any genotype specific association with regard to the 0R/0R risk genotype. These results indicate strong association of 0R allele with the development of CML but not progression or drug response.

DISCUSSION

Haematopoiesis is a tightly regulated system consisting of HSC progenitors with high self-renewal capability and multilineage potential as well as series of fully differentiated cell types with short life span required to be replenished continuously. HSC aging can lead to decline in immune competence, onset of anaemia etc., posing a risk for several haematological malignancies. HSC aging can display skewed myeloid cell differentiation and expansion due to intrinsic events like DNA damage, genomic instability, epigenetic alterations, and telomere attrition, mitochondrial dysfunction, cell senescence pathways, etc.

CML accounts for 20% of all adult leukaemia's the incidence being more in the age group 45-55 years with male preponderance is associated with single specific reciprocal t(9;22) (q34; q11) translocation (9-22).The fusion gene of this translocation is BCR/ABL which codes for a tyrosine kinase enzyme leading to abnormal regulation of cell growth and survival transforming the normal stem cells to LSCs and is responsible for the development of CML. Several signalling pathways of cell proliferation, apoptosis and cell survival were modulated in integrative manner there by dynamically altering the CML phenotype. Although different lines of therapies against tyrosine kinases were successful, failure in overcoming relapse of the disease was a big challenge in treatment of the disease.

Several studies were being carried out to analyse the underlying mechanism for recurrence. Kinase inhibitors were found to be ineffective in completely eradicating quiescent cells which gain self-renewal capacity. BCR-ABL can induce ROS production that promotes oxidative damage leading to genomic instability affecting several genes responsible for regulation of proliferation, growth and survival during

the progression of leukaemia. Maintaining the balance between ROS and antioxidants was regulated by several proteins of which Sirtuin 3 was known to maintain the mitochondrial integrity further impacting genomic stability by its acetylation and deacetylation action on antioxidant enzymes such as Manganese superoxide dismutase (MnSOD) that catalyses the conversion of highly reactive superoxide radicals to H_2O_2.

SIRT3 harbours multiple copy number variations whose functional relevance remains unexplored. A 72 bp repeat variation located in intron5 of Sirtuin 3 gene at enhancer element with bidirectional promoter sharing with the PSMID gene and have binding sites for sp1, zf5, nfkb/GATA1 factors. Recently, this VNTR polymorphism was found to be significantly associated with breast cancer [23]. In leukemic origin and progression, as repeat variations were reported to be related to genomic instability events such as secondary translocations, it was pertinent to study the association of this functional variant in CML.

The present case-control study of this polymorphism revealed significant association of 0R0R genotypewith the development of CML but not progression and drug response. Further, the results indicated that 0R allele may pose relatively greater risk as compared to the wild type 2R alleleand is dominant over other alleles. The 0R variant due to lack of repeat sequences at the enhancer site possibly could affect transcriptional and splicing efficiency at this SIRT3 locus, there by owing to altered functional activity in leukemic cell.

The genotypic distribution with regard to epidemiological and clinical variables showed association of 0R/0R risk genotype with male gender and age range 20-40 years. However, lack of association was found with respect to clinical variables, drug response and survival status. These findings indicate that SIRT3 intron 5 VNTR polymorphism could be an important marker for predicting risk of CML onset but not for prognosis.

CONCLUSION

Our study is the first of its kind to analyse SIRT3 intron 5 VNTR polymorphism in CML and to report strong association of 0R repeat allele with CML development. Albeit the prognostic significance of this polymorphism cannot be ruled out from our study and need to be studied in a systematic manner. As this variant is a regulator of gene expression, the potential of this polymorphism as a marker can be analysed by screening this polymorphism on large scale in different leukaemias. Future in vitro studies are warranted on functional implications of this variant in leukaemia transformation and progression. Further, expression analysis of SIRT3 gene in CML cases not only at diagnosis but also during treatment follow up may provide further clues about its role in CML recurrence and therapeutic resistance.

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