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SNPs in 3′-UTR region of *MBL2* increases susceptibility to recurrent vulvovaginal infections by altering sMBL levels

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ABSTRACT

Recurrent vulvovaginal infections (RVVI), owing to their adverse health consequences, have become a serious dilemma worldwide. Low serum levels of Mannose-Binding Lectin (sMBL), a main component of innate immunity, was found to be associated with RVVI risk, though complete genetic bases are still elusive. To reveal unrecognised regulatory variants, 3′-UTR region of *MBL2* with six putative functional SNPs *i.e.* rs10824792, rs2120132, rs2120131, rs2165813, rs2099903 and rs2099902 was sequenced and genotyped in the present study for 109 RVVI cases and age matched healthy controls. sMBL levels were measured by enzyme-linked immunosorbent assay. The homozygous CC genotype of rs10824792 polymorphism was found to be conferring risk (OR = 2.94) of developing RVVI. Significantly high frequency of corresponding CC genotype was found in Vulvovaginal Candidiasis (VVC) and Mixed Infections (MI) relative to controls. Significantly insufficient sMBL levels were observed in RVVI and its types (Bacterial Vaginosis, VVC and MI) than controls. sMBL levels varied for rs10824792 SNP as expected from the genetic analyses. Six marker haplotype analyses have shown *C*TTGCT, the haplotype containing only risk allele of rs10824792, conferred risk of RVVI and its types by lowering sMBL levels. In conclusion, a 3′-UTR SNP *i.e.* rs10824792 was identified as novel associated genetic marker for contributing low sMBL levels and RVVI risk. Our findings contribute to the novel future research directions for the development of emerging MBL substitution as effectual therapy for RVVI.

1. Background

Vulvovaginal infections (VVI), owing to their adverse health consequences, have become a serious dilemma worldwide. Its three main types that commonly affect reproductive age women are Bacterial Vaginosis (BV), Vulvovaginal Candidiasis (VVC) and Trichomoniasis (TV) (Sherrard et al., 2011). The cases of mixed VVI (MI) in > 20% of women have also been reported (Sobel et al., 2013; Kalia et al., 2015). An abnormal vaginal discharge is the characteristic and commonly complained symptom of VVI (Workowski and Bolan, 2015). National Family Health Survey 2 reported 30 percent prevalence of vaginal discharge in India with 29.9 percent of its prevalence in Delhi (National Family Health Survey, 1999). However, the major concern for researcher is the repeated episodes of common VVI, collectively stated as recurrent VVI (RVVI) (Powell and Nyirjesy, 2014). Untreated RVVI will not just affect the female reproductive health but may also result in many co-morbid conditions and adverse pregnancy outcomes (Atashili et al., 2008; Durugbo et al., 2015; Rose et al., 2017). Fall in *Lactobacilli* and predominance of either normally inhabiting or sexually transmitted opportunistic pathogens has been suggested as the basis for RVVI pathogenesis (Powell and Nyirjesy, 2014). Besides this, excessive antibiotics use, contraceptives, sexual activity, immune-suppression, ethinicity are the other suggested risk factors for RVVI (Gonçalves et al., 2016). However, development of RVVI in women lacking any of these recognized pre-disposing factors, suggest the involvement of host immune components that are instrumental in elimination of causative agents of RVVI (Bradford et al., 2013).

Human Mannose-Binding Lectin (MBL), a liver derived 32 kDa acute phase protein, is an important component of innate immune system, encoded by *MBL2* mapped to 10q21.1 (Turner, 2003). MBL has been suggested as a prototypical pattern recognition receptor that binds to specific sugars on pathogen's surface, subsequently leading to pathogen elimination by opsonisation, complement activation and/or phagocytosis (Cedzyński and Kilpatrick, 2018). Altered serum MBL levels (sMBL) have been reported as predisposing factor to a variety of infectious diseases (Eisen and Minchinton, 2003). Single nucleotide

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polymorphisms (SNPs) present in exon 1 (codon 52, 54 and 57) and promoter (L/H, Y/X and P/Q) region of *MBL2* have been documented to affect sMBL levels. These SNPs interfere in MBL structure and its transcription, significantly affecting its functional capacity (Jüliger et al., 2000; Larsen et al., 2004). Literary evidences have shown linkage between these polymorphisms leading to seven standard secretor haplotypes including HYPA, LYQA and LYPA that are associated with high sMBL levels and LXPA, LYPB, LYQC and HYPD that are associated with low sMBL levels (Madsen et al., 1998).

Sporadic reports have documented the role of *MBL2* Exon 1 polymorphisms in RVVI in various populations. Five studies have found an association of codon 54 polymorphism with increased RVVI risk (Babula et al., 2003; Liu et al., 2006; Giraldo et al., 2007; Donders et al., 2008; Wojitani et al., 2012). Three studies have demonstrated a lack of association between structural polymorphisms in various RVVI (De Seta et al., 2007; Milanese et al., 2008; Velazquez-Hernandez et al., 2017). Only five studies have assessed the status of MBL levels in either vaginal fluid or serum of RVVI cases globally and have indicated the major involvement of MBL in susceptibility to RVVI (Babula et al., 2003; Liu et al., 2006; Milanese et al., 2008; Henić et al., 2010; Ghazanfari et al., 2017).

Recently, we first time reported the predisposing role of combined *MBL2* promoter and codon 54 polymorphisms with low sMBL levels in RVVI and its types (Kalia et al., 2017). However, our study, in consonance with others, found that measured sMBL levels do not fully correlate with the 'secretor haplotypes' suggesting the presence of other regulating variants in *MBL2* that might be altering sMBL levels (Madsen et al., 1998; Kalia et al., 2017; Bernig et al., 2005). Three studies have assessed the role of SNPs across the 3′-UTR region of *MBL2* in breast cancer, postoperative myocardial infarction and colon cancer (Bernig et al., 2007; Collard et al., 2007; Zanetti et al., 2012). Their findings indicated that 3′-UTR variants may modify sMBL levels possibly by generating miRNA binding sites with subsequent miRNA mediated mRNA degradation and reduced protein translation. Considering this, we hypothesized that SNPs in the 3′-UTR region of *MBL2* might affect susceptibility to RVVI by altering sMBL levels making the present study first approach towards it.

2. Materials and methods

2.1. Ethics statement

The present study was commenced after getting approval from the Institutional Ethics Committee (Approval no. 06/HG dated 02/01/2015) of Guru Nanak Dev University, Amritsar (Punjab), India, in accordance with Indian Council of Medical Research guidelines (ICMR, 2006) modified from Declaration of Helsinki (2004). Voluntary consent in written was attained from all the subjects.

2.2. Participants

The present study included 109 RVVI cases (mean age ± S.D., 29.22 ± 7.95 y) recruited from Department of Gynaecology and Obstetrics, Bebe Nanki Mother and Child Care Centre, Government Medical College, Amritsar (Punjab). These cases suggested by gynaecologist were pre-diagnosed with RVVI with minimum 4 documented recurrent experiences in a year by the clinicians (Powell and Nyirjesy, 2014). These cases complained of having frequent symptoms like discharge, vaginal fishy smell, burning, itching and pelvic pain. These cases were further classified into three main groups of RVVI *i.e.* BV (n = 56), VVC (n = 28) and MI (n = 25) in laboratory. The control group consisted of 109 age-matched healthy women (mean age ± S.D., 29.42 ± 7.70 y) without any recurrent vaginal infection complaints. Participants using immunosuppressive medications, under chemotherapy and having HIV infections or any other chronic conditions were excluded from the study. Majority of the participants (≈99%) had premenopausal status and a very low history of spontaneous abortions. Only 9.17% of the cases were using one or the other contraceptive methods comparative to no such controls.

2.3. Sample collection and processing

Two types of samples *i.e.* vaginal discharge and peripheral blood samples were collected in the present study and were carried to the laboratory for their respective processing. The vaginal discharge samples were collected by the clinicians from RVVI cases. These samples were processed on the basis of standard diagnostics tests given in European (IUSTI/WHO) guidelines on vaginal discharge management (Sherrard et al., 2011). The fine points of vaginal discharge sample collection, processing, diagnosis of various RVVI types have been reported previously (Kalia et al., 2015). The peripheral blood samples (5 ml) were collected from cases as well as healthy controls with technical assistance. These blood samples were subjected to DNA and serum isolation following procedures reported previously (Kalia et al., 2017, 2018).

2.4. SNPs selection and genotyping

To identify 3′-UTR SNPs that could potentially be associated with RVVI risk, the 3′-UTR region of *MBL2* was selected with maximum number of previously identified putative functional SNPs (Kalia et al., 2016). This region contains six conserved SNPs including rs10824792, rs2120132, rs2120131, rs2165813, rs2099903 and rs2099902 with minor allele frequency (MAF) ≥ 0.27. These SNPs were predicted to functional and affecting miRNA binding site by SNPinfo (FuncPred) (http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.html). Moreover, SNPs including rs2099902, rs2099903 and rs2165813 also fell within the seed region of miRNA binding sites as predicted by PolymiRTS (http://compbio.uthsc.edu/miRSNP/) in our previous study (Kalia et al., 2016). Suitable primers were designed for the selected 3′-UTR region from human *MBL2* genomic sequence (NCBI Reference Sequence: NC_000010.11) using the online software Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). The best primer pair including forward-5´AAACA TCGTAAGACTACACACAAAAC3´ and reverse-5´GAATAGATATCCACT TGAGACAGCA3´ flanking 3′-UTR region of 552 bp was selected and custom-synthesized from Bioserve Biotechnologies (Hyderabad, India). Polymerase chain reaction (PCR) was performed in 20 µl reaction volumes containing template DNA (50 ng/µl), *Taq* buffer containing 15 mM $MgCl_2$ (1X), dNTPs (0.025 mM), forward and reverse primer (0.15 p mol/µl each) and *Taq* DNA polymerase (0.3 U). All these components of PCR except primers were procured from GeNei™, Merck (Bangalore, India). PCR was initiated by a 10-min denaturation step at 95 °C and completed by a 5-min extension step at 72 °C. The PCR involves 35 cycles of 30 s at 94 °C, 30 s at 57 °C and 45 s at 72 °C carried out in a thermocycler (Applied Biosystems, Life Technologies, USA). The PCR products were loaded on 1.5% agarose (Himedia, India) gel stained with ethidium bromide (SRL, India) (Fig. 1). Gel electrophoresis was carried out at 100 V and visualized using gel documentation system (AlphaImager MINI, ProteinSimple, USA). The genotypes were ascertained by Sanger sequencing (Fig. 1).

2.5. MBL quantification

The sMBL levels were quantified by enzyme linked immnosorbent assay (ELISA) kit (Ray Biotech, USA) according to manufacturer's instructions as reported previously (Kalia et al., 2017).

2.6. Statistical analysis

Genetic Association Study (GAS) power calculator (http://csg.sph.umich.edu/abecasis/gas_power_calculator/) was used to calculate optimal sample size for the present study to achieve a minimum adequate

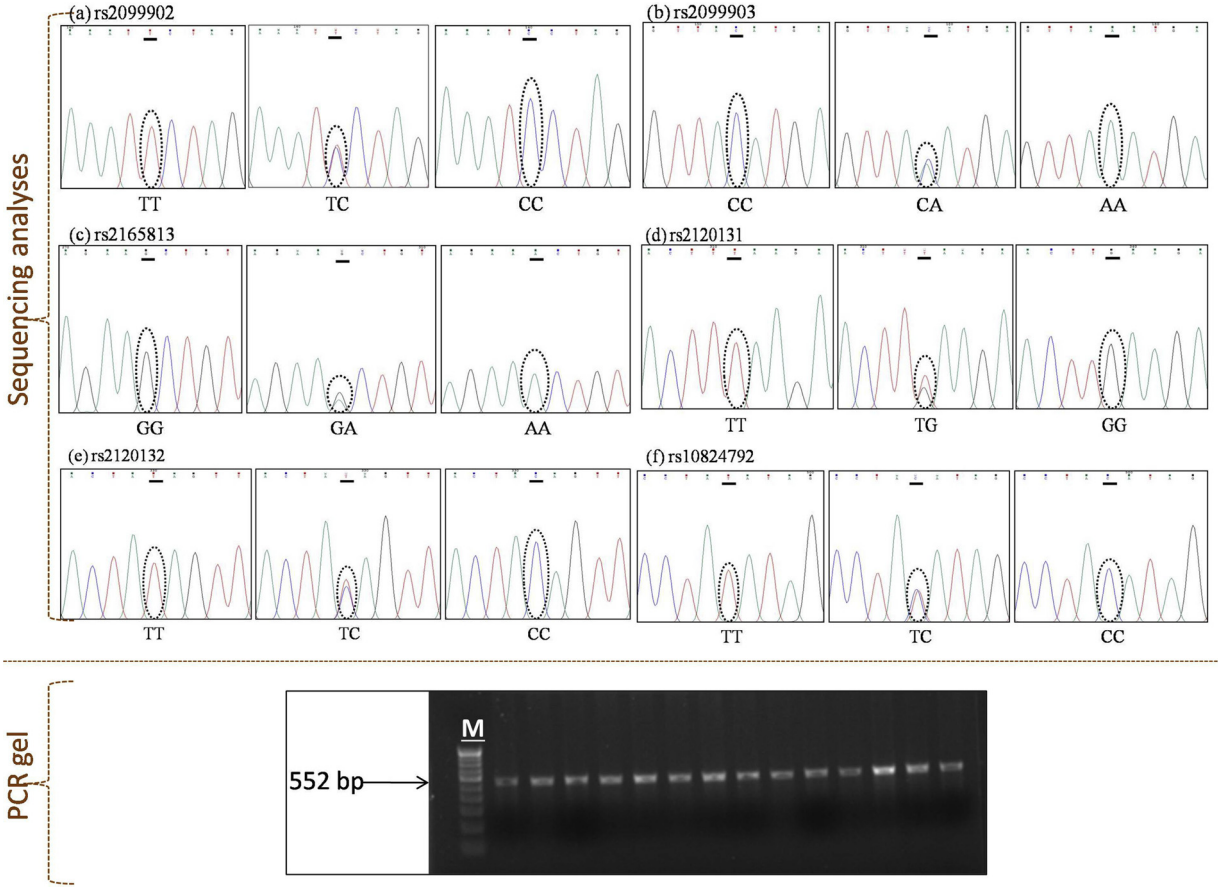

Fig. 1. The detected 3′-UTR polymorphisms of *MBL2*. Sequencing analyses of 3′-UTR region using forward primer indicating the presence of (a) rs2099902, (b) rs2099903, (c) rs2165813, (d) rs2120131, (e) rs2120132 and (f) rs10824792 SNPs. Agarose gel of purified PCR product used for sequencing M: 100 bp DNA ladder.

power of 80%. Conventions taken for one-stage sample design were 5% error rate ($\alpha = 0.05$), national prevalence of abnormal vaginal discharge (30%), MAF of 0.27 or 27% and odds ratio (OR) of 1.5. The sample size of 105 cases and 105 controls was calculated. The final sample size attained in the present study was 109 for cases and 109 for controls with the final study power of 82%. Manual counting was performed to calculate allelic and genotypic frequencies of *MBL2* SNPs. Odds ratio statistics to compare genotype/haplotype frequencies between cases and controls was applied using MedCalc software v 9.3.9.0 (MedCalc Software, Ostend, Belgium). The genotype/haplotype with highest frequency in total participants was selected as reference (OR = 1). Hardy-Weinberg equilibrium (HWE) and inheritance models were determined using SNPStats (https://www.snpstats.net/snpstats/start.htm). The best inheritance model for each variant was determined based on lowest value of Akaike's Information Criterion (AIC) and Bayesian Information Criterion (BIC) provided by SNPStats. Linkage disequilibrium (LD) analysis was performed with Haploview v 4.2 (http://www.broad.mit.edu/mpg/haploview/). Haplotypes were constructed from genotype data by PHASE software v 2.1.1. (http://stephenslab.uchicago.edu/phase/download.html). Kruskal-Wallis test was performed to compare sMBL levels for more than two categories. Comparison of sMBL levels between two categories was done by Mann–Whitney U –test. All these statistical analyses were carried out by the SPSS v 16.0 (SPSS Inc., Chicago, IL) unless stated. $p \leq 0.05$ was considered statistically significant. Bonferroni's correction was applied for multiple comparisons.

3. Results

3.1. Genetic analyses of 3′-UTR variants in RVVI relative to controls

The frequency distribution of *MBL2* 3′-UTR polymorphisms showed rs10824792_C, rs2120132_C, rs2120131_G, rs2165813_A, rs2099903_A and rs2099902_C to be minor alleles in the representative North Indian population of the present study (Supplementary Table 1). All the studied *MBL2* polymorphisms were found to lie in HWE ($p > 0.05$). The recessive inheritance model was found to be the best genetic model for all the polymorphisms (Table 1). Of which, only two SNPs *i.e.* rs10824792 and rs2099903 were found to be associated with RVVI risk (Table 1). However, after correction for multiple comparisons, only the homozygous 'CC' genotype of rs10824792 SNP was found to be significantly more frequent in RVVI cases relative to controls (OR = 2.94).

3.2. Genetic analyses of rs10824792 polymorphism in RVVI types relative to controls

Considering the association of recessive mode of inheritance of rs10824792 polymorphism, the frequency of its genotypes was compared between RVVI types and controls (Table 2). The homozygous CC genotype was found to be significantly more frequent in VVC and MI comparative to controls even after correction for multiple comparisons ($P < 0.05$).

3.3. Linkage disequilibrium and r^2 statistics

All the studied SNPs were found in strong LD with each other ($D' > 0.95$), with some SNPs exactly in complete LD *i.e.* $D' = 1$ (Fig. 2a).

Table 1
Distribution of genotypic frequencies along with inheritance models of *MBL2* 3′-UTR variants in RVVI cases and controls.

Genetic Models	Genotypes	RVVI Cases (N = 109) Freq (%)	Controls (N = 109) Freq (%)	AIC	BIC	#OR (95% CI)	#*p* (p_c)
rs10824792							
Codominant	T/T	32 (29.41)	31 (28.42)	299.3	312.9		
	T/C	46 (42.21)	65 (59.61)				
	C/C	31 (28.42)	13 (11.91)				
Dominant	T/T	32 (29.41)	31 (28.42)	308.2	318.3		
	T/C-C/C	77 (70.61)	78 (71.62)				
Recessive	T/T-T/C	78 (71.62)	96 (88.11)	**298.7**	**308.9**	1.00	
	C/C	31 (28.42)	13 (11.91)			2.94 (1.44-5.99)	0.002** (0.012*)
Overdominant	T/T-C/C	63 (57.81)	44 (40.42)	301.5	311.7		
	T/C	46 (42.21)	65 (59.61)				
Log-additive	—	—	—	305.4	315.5		
rs2120132							
Codominant	T/T	59 (54.12)	62 (56.92)	308.4	321.9		
	T/C	43 (39.51)	44 (40.41)				
	C/C	7 (6.42)	3 (2.82)				
Dominant	T/T	59 (54.11)	62 (56.91)	308.0	318.2		
	T/C-C/C	50 (45.92)	47 (43.12)				
Recessive	T/T-T/C	102 (93.61)	106 (97.21)	**306.4**	**316.3**	1.00	
	C/C	7 (6.42)	3 (2.82)			2.45 (0.62-9.77)	0.18 (1.08)
Overdominant	T/T-C/C	66 (60.51)	65 (59.64)	308.2	318.3		
	T/C	43 (39.51)	44 (40.42)				
Log-additive	—	—	—	307.5	317.7		
rs2120131							
Codominant	T/T	60 (55.12)	62 (56.92)	308.4	322.0		
	T/G	42 (38.52)	44 (40.41)				
	G/G	7 (6.41)	3 (2.82)				
Dominant	T/T	60 (55.12)	62 (56.91)	308.1	318.2		
	T/G-G/G	49 (45.00)	47 (43.12)				
Recessive	T/T-T/G	102 (93.60)	106 (97.21)	**306.4**	**316.6**	1.00	
	G/G	7 (6.40)	3 (2.81)			2.45 (0.62-9.77)	0.18 (1.08)
Overdominant	T/T-G/G	67 (61.50)	65 (59.64)	308.1	318.3		
	T/G	42 (38.50)	44 (40.41)				
Log-additive	—	—	—	307.7	317.8		
rs2165813							
Codominant	G/G	59 (54.10)	62 (56.90)	308.4	321.9		
	G/A	43 (39.50)	44 (40.40)				
	A/A	7 (6.40)	3 (2.80)				
Dominant	G/G	59 (54.10)	62 (56.90)	308.0	318.2		
	G/A-A/A	50 (45.90)	47 (43.10)				
Recessive	G/G-G/A	102 (93.60)	106 (97.20)	**306.4**	**316.6**	1.00	
	A/A	7 (6.40)	3 (2.80)			2.45 (0.62-9.77)	0.18 (1.08)
Overdominant	G/G-A/A	66 (60.50)	65 (59.60)	308.2	318.3		
	G/A	43 (39.50)	44 (40.40)				
Log-additive	—	—	—	307.5	317.7		
rs2099903							
Codominant	C/C	58 (53.20)	62 (56.90)	305.2	318.7		
	C/A	42 (38.50)	45 (41.30)				
	A/A	9 (8.30)	2 (1.80)				
Dominant	C/C	58 (53.20)	62 (56.90)	307.9	318.0		
	C/A-A/A	51 (46.80)	47 (43.10)				
Recessive	C/C-C/A	100 (91.70)	107 (98.20)	**303.2**	**313.3**	1.00	
	A/A	9 (8.30)	2 (1.80)			4.80 (1.01-22.81)	0.02* (0.12)
Overdominant	C/C-A/A	67 (61.50)	64 (58.70)	308.0	318.2		
	C/A	42 (38.50)	45 (41.30)				
Log-additive	—	—	—	306.6	316.7		
rs2099902							
Codominant	T/T	59 (54.11)	62 (56.91)	307.1	320.7		
	T/C	43 (39.52)	45 (41.32)				
	C/C	7 (6.41)	2 (1.81)				
Dominant	T/T	59 (54.12)	62 (56.92)	308.0	318.2		
	T/C-C/C	50 (45.91)	47 (43.11)				
Recessive	T/T-T/C	102 (93.62)	107 (98.22)	**305.1**	**315.3**	1.00	
	C/C	7 (6.41)	2 (1.81)			3.66 (0.74-18.05)	0.08 (0.48)
Overdominant	T/T-C/C	66 (60.52)	64 (58.72)	308.1	318.3		
	T/C	43 (39.51)	45 (41.31)				
Log-additive	—	—	—	307.3	317.4		

Akaike's Information Criterion (AIC) and Bayesian Information Criterion (BIC), Bold values indicate low AIC & BIC value, # Best inheritance model, p = crude p-value, p_c = p-value after Bonferroni correction,* indicates ($p \leq 0.05$), ** indicates ($p \leq 0.01$).

Table 2
Distribution and comparison of rs10824792 polymorphism in RVVI types with controls.

Genotypes	No.(%) of Controls	No. (%) of RVVI categories			BV vs Controls		VVC vs Controls		MI vs Controls	
	Controls (n = 109)	BV (n = 56)	VVC (n = 28)	MI (n = 25)	OR (95% CI)	p (pc)	OR (95% CI)	p (pc)	OR (95% CI)	p (pc)
rs10824792										
T/T-T/C	96 (88.07)	43 (76.78)	19 (67.85)	16 (64.00)	1		1		1	
C/C	13 (11.91)	13 (23.21)	9 (32.12)	9 (36.12)	2.23 (0.95 - 5.21)	0.063 (0.189)	3.49 (1.31 - 9.34)	0.012* (0.036*)	4.15 (1.52 - 11.30)	0.005** (0.015*)

OR = Odds Ratio; CI = Confidence Intervals; * indicates significant values ($p \leq 0.05$); **indicates highly significant values ($p \leq 0.01$). p = crude p-value, pc = p-value after Bonferroni correction.

Genotypes of all the SNPs expect rs10824792 were found to be strongly correlated ($r^2 > 0.96$) with some SNPs *i.e.* rs2120132, rs2120131 and rs2165813 exactly approaching to 1 ($r^2 \approx 1$) due to strong LD and similar MAF between them (Fig. 2b). This implies the state of perfect predictability *i.e.* SNPs including rs2120132, rs2120131 and rs2165813 can be considered as the tag SNPs for each other. However, rs10824792 SNP, although in complete LD with other SNPs, had $r^2 < 0.8$ due to its very high MAF = 0.451 than the other SNPs.

3.4. Haplotypes analyses in RVVI and its types relative to controls

Based on the strong LD and r^2 statistics, three common haplotypes *i.e.* TTTGCT (3′H-1), CCGAAC (3′H-2) and CTTGCT (3′H-3) having frequency ≥ 0.05 were observed in RVVI cases and controls of the present study (Table 3). The six markers of these haplotypes correspond to rs10824792, rs2120132, rs2120131, rs2165813, rs2099903 and rs2099902 SNPs respectively. The comparison of these haplotypes between various case-control groups showed significantly high frequency of 3′H-3 haplotype in VVC cases than controls. However, this statistical significance was lost after correction for multiple comparisons. Some rare haplotypes *i.e.* TTTGAT (3′H-4), CCGACT (3′H-5), CTTGAT (3′H-6), CCTAAT (3′H-7) and CTTGCC (3′H-8) having very low frequency < 0.05 were also observed in present study. However, due to their absence in either case or control group, their statistical comparison could not be made, hence not included in the analysis.

3.5. Mean sMBL levels in cases and controls

The previously measured sMBL levels were segregated in cases and controls groups of the present study (Kalia et al., 2017). sMBL levels significantly (p = 0.000) varied between cases and controls, as determined by the Kruskal–Wallis test (Fig. 3). Low sMBL levels were observed in RVVI (700.73 ± 438.81 ng/ml), its subtypes *i.e.* BV (693.65 ± 375.28 ng/ml), VVC (359.34 ± 134.20 ng/ml) and MI (742.78 ± 321.67 ng/ml) as compared to controls (1034.4 ± 497.70 ng/ml).

3.6. Association of rs10824792 genotypes with sMBL levels

Analysis of genotype-phenotype association of rs10824792 polymorphism in cases and controls, revealed no significant difference (Table 4). However, low sMBL levels were observed in homozygous recessive genotype of this polymorphism than reference genotype in cases and controls.

3.7. Association of MBL2 3′-UTR variants haplotypes with sMBL levels

Association of sMBL levels with respect to common 3′-UTR haplotypes in cases and control groups was investigated (Table 5). For this individual haplotype pairs were reconstructed for cases and controls. An overall trend for low sMBL serum levels was observed among carriers of the 3′H-3 haplotype. Thus, it is justifiable to ease the interpretation by pooling the individuals with at least one copy of 3′H-3 haplotype as 3′H-3 carriers, while pooling the rest haplotypes as others. Significantly low sMBL levels were observed in pooled 3′H-3 carriers (513.16 ± 326.57 ng/ml) than pooled others (682.96 ± 320.22 ng/ml) in RVVI, even after correction for multiple comparisons (P = 0.005). Among RVVI types, the same difference was found to be significant for BV and MI. However, the statistical significance for MI was lost after correction for multiple comparisons.

4. Discussion

Different studies have suggested the direct role of MBL in providing

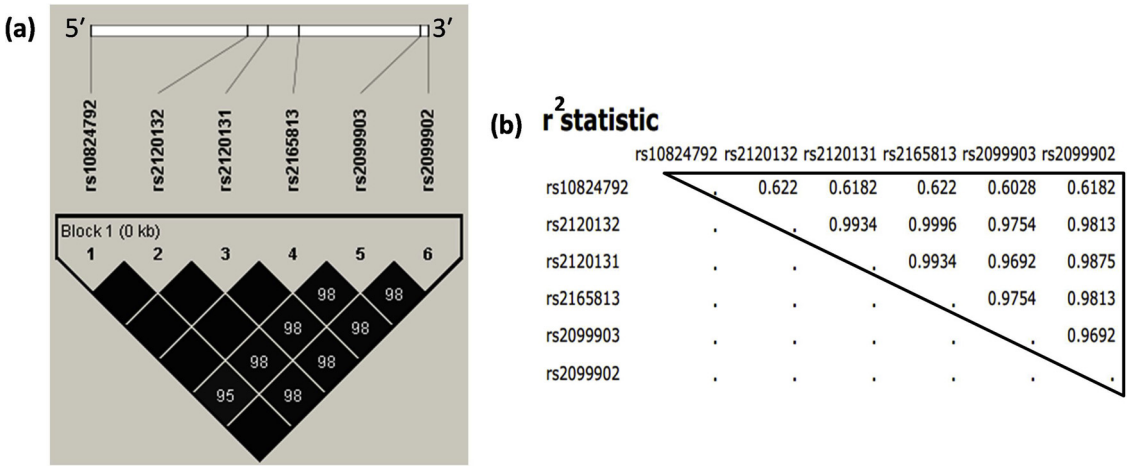

Fig. 2. The LD pattern **(a)** and r^2 statistics **(b)** of *MBL2* 3′-UTR variants in all subjects. D' is scaled between white diamond: D' = 0 *i.e.* complete Linkage equilibrium, shades of grey: 0 < D' < 1, Black diamond: D' = 1 *i.e.* complete LD. Numbers in diamonds are D′-value expressed as percentile. r^2 is scaled between -1 to +1.

Table 3
Distribution and comparison of *MBL2* 3′-UTR haplotypes in cases and controls.

Haplotypes	Controls No. (%) (N = 218)	Total RVVI Cases (N = 218)	No. (%) Clinical categories of RVVI			RVVI vs Controls		BV vs Controls		VVC vs Controls		MI vs Controls	
			BV (N = 112)	VVC (N = 56)	MI (N = 50)	OR (95% CI)	p (p_c)	OR (95% CI)	p (p_c)	OR (95% CI)	p (p_c)	OR (95%CI)	p (p_c)
TTTGCT (3′H-1)	127 (58.21)	108 (49.54)	63 (56.21)	22 (39.21)	23 (46.12)	1		1		1		1	
CCGAAC (3′H-2)	49 (22.42)	56 (25.61)	27 (24.11)	15 (26.71)	14 (28.21)	1.34 (0.84 - 2.13)	0.20 (1.6)	1.11 (0.63 - 1.94)	0.71 (5.68)	1.76 (0.84 - 3.68)	0.12 (0.96)	1.57 (0.75 - 3.31)	0.22 (1.76)
CTTGCT (3′H-3)	41 (18.81)	49 (22.47)	19 (16.91)	17 (30.31)	13 (26.01)	1.40 (0.86 - 2.28)	0.17 (1.36)	0.93 (0.50 - 1.74)	0.83 (6.64)	2.39 (1.16 - 4.93)	0.01* (0.08)	1.75 (0.81 - 3.76)	0.15 (1.20)

Global p-value for case/control haplotype association was 0.2; p = crude p-value, pc = p-value after Bonferroni correction;* $p \leq 0.01$.

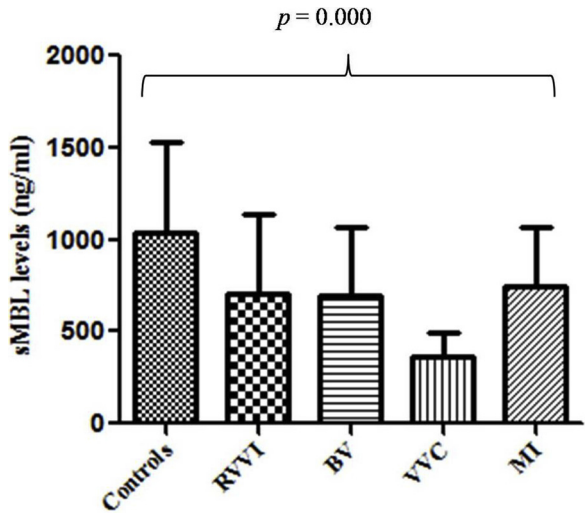

Fig. 3. Serum MBL levels in cases and controls. sMBL Levels (ng/ml) represented as Mean ± Standard Deviation (Kruskal–Wallis, P = 0.000).

defence against RVVI pathogens (Ip and Lau, 2004; Pellis et al., 2005; Van Asbeck et al., 2008; Li et al., 2012; Chatterjee et al., 2015). Complementary evidences recommended that the chances of acquiring RVVI will be more in cases with insufficient sMBL levels (Babula et al., 2003; Liu et al., 2006; Kalia et al., 2017). Therefore, the emerging MBL substitution therapy could possibly be the future treatment strategy for RVVI (Keizer et al., 2014). As sMBL levels are genetically determined, complete phenotype-genotype association is mandatory to reveal unrecognised regulatory variants of *MBL2* that might be associated with RVVI risk (Kalia et al., 2016; Keizer et al., 2014). The 3′-UTR variations have been shown to profoundly affect target protein expression by generating miRNA binding sites (Ryan et al., 2010). Thus, the purpose of our study was to identify, SNPs in the 3′-UTR region of *MBL2* that might be contributing RVVI risk by altering sMBL levels.

The study revealed the association of rs10824792, the 3′-UTR variant of *MBL2* with RVVI risk. The best fit model for this variant represented its minor allele as recessive, suggesting that the homozygous minor allele carriers of this polymorphism will be at higher risk of developing RVVI. The recessive nature of the minor allele of rs10824792 SNP has also been documented earlier (Segat et al., 2010). No study, till date, has evaluated the role of *MBL2* 3′-UTR polymorphisms in association with RVVI or its types. However, rs10824792 polymorphism has earlier been reported to be associated with breast cancer as well as in modulating immune response to immunotreatment in HIV patients (Bernig et al., 2007; Segat et al., 2010). Moreover, there are studies that have also demonstrated the lack of association of rs10824792 with Nasopharyngeal carcinoma, severe asthma with fungal sensitization, allergic bronchopulmonary aspergillosis (ABPA), colorectal cancer, fever during neutropenia and hepatitis C virus infection (Overton et al., 2016; Brown et al., 2008; te Poele et al., 2012; Lu et al., 2013; Moumad et al., 2013; Overton et al., 2017).

To avoid misclassify the effect of single variants it is notable that association studies on the basis of haplotypes turned out to be more powerful than individual SNP analyses. Therefore, *MBL2* 3′-UTR haplotypes distributions and their comparison among cases-control groups were evaluated. Though not significant, 3′H-1 (TTTGCT), the major allele haplotype was prevalent in controls than cases, while, 3′H-2 (CCGAAC), the minor allele haplotype along with 3′H-3 (*C*TTGCT), the haplotype containing only risk allele of rs10824792 were prevalent in RVVI than controls. This suggests rs10824792 SNP as a casual marker for the observed high prevalence of both 3′H-2 and 3′H-3 haplotypes in RVVI than controls, in consonance with the single variant analyses of the present study. So far, only two case-control reports have documented the role of *MBL2* 3′-UTR haplotypes in association with cancer (Bernig et al., 2007; Zanetti et al., 2012). 3′-UTR haplotype analyses of *MBL2* by Bernig et al., (Bernig et al., 2007) showed significantly high frequency of TATAAC haplotype in controls than in cases with breast cancer among African American women suggesting a protective effect. This haplotype symbolises rs10082466, rs10824792, rs12254557, rs2120132, rs2099902 and rs10450310 SNPs respectively, containing major allele of SNPs investigated by the present study (marked by

Table 4
Distribution of sMBL levels in cases and controls, stratified on the basis of the recessive model genotypes of rs10824792.

Genotypes	Controls		RVVI		BV		VVC		MI	
	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD
rs10824792										
T/T-T/C	96	1046.1 ± 514.67	78	742.13 ± 469.14	43	748.19 ± 433.64	19	692.58 ± 647.7	16	785.83 ± 307.49
C/C	13	936.86 ± 350.07	31	593.65 ± 336.03	13	622.57 ± 345.59	9	393.70 ± 84.3	9	656.69 ± 349.31
p	†	0.660	†	0.158	†	0.415	†	0.555	†	0.308
p_c		3.3		0.79		2.075		2.775		1.54

†Mann–Whitney U –test; sMBL Levels represented as Mean ± Standard Deviation (Mean ± SD), p = crude p-value, pc = p-value after Bonferroni correction.

Table 5
Distribution of sMBL levels in observed diplotypes of *MBL2* 3′-UTR common haplotypes in cases and controls.

Haplotypes pairs	Controls		RVVI		BV		VVC		MI	
	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD	n	Mean ± SD
3′H-1/3′H-1	31	1232.60 ± 603.95	31	740.92 ± 321.81	21	601.57 ± 215.70	3	856.69 ± 497.87	7	982.68 ± 332.21
3′H-1/3′H-2	36	913.11 ± 359.08	24	614.76 ± 355.24	11	629.97 ± 207.31	9	308.66 ± 128.94	4	582.68 ± 90.04
3′H-1/3′H-3	29	897.59 ± 375.80	21	606.61 ± 414.98	9	345.41 ± 133.30	7	288.50 ± 144.53	5	570.08 ± 71.89
3′H-2/3′H-2	2	1234.6 ± 463.23	7	660.16 ± 66.10	4	663.52 ± 52.53	1	560.63 ± 0.00	2	869.29 ± 169.26
3′H-2/3′H-3	8	719.16 ± 240.82	18	455.25 ± 203.48	8	523.80 ± 284.62	4	344.36 ± 44.93	6	425.83 ± 108.63
3′H-3/3′H-3	2	1227.7 ± 140.33	5	329.13 ± 78.47	1	220.47 ± 0.00	3	365.35 ± 66.66	1	138.58 ± 0.00
Pooled others *vs* 3′H-3 carriers										
Others	69	1066.0 ± 506.27	62	682.96 ± 320.22	36	617.13 ± 198.86	13	454.51 ± 331.25	13	842.16 ± 306.07
3′H-3 carriers	39	877.91 ± 356.79	44	513.16 ± 326.57	18	417.75 ± 227.88	14	320.93 ± 109.46	12	565.93 ± 281.19
p	†	0.07	†	0.001***	†	0.004**	†	0.369	†	0.014*
p_c		0.35		0.005**		0.02*		1.845		0.07

† Mann–Whitney U–test; p = crude p-value, pc = p-value after Bonferroni correction; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001; sMBL Levels (ng/ml) represented as Mean ± Standard Deviation (Mean ± SD), Pooled 3′H-3 carriers (3′H-1/3′H-3, 3′H-2/3′H-3, 3′H-3/3′H-3), Pooled others (3′H-1/3′H-1, 3′H-1/3′H-2, 3′H-2/3′H-2).

underline). Another analysis of 3′-UTR haplotypes by Zanetti et al., (Zanetti et al., 2012) showed that the CGGT haplotype (symbolising rs10082466, rs2120132, rs209902, rs10450310 SNPs respectively), containing minor allele of SNPs investigated by the present study, was associated with an increased risk (OR: 2.1) of colon cancer in African Americans. Taken together, the genetic analysis of the present study as well as literary evidences suggested *MBL2* 3′-UTR variants as potential molecular determinants of disease risk.

The rs10824792 polymorphism was previously predicted to be affecting miRNA binding sites (Kalia et al., 2016), which could be the possible functional implication of the observed low sMBL levels in homozygous recessive genotype of rs10824792 polymorphism than reference genotype in the present study. However, the observed difference was not significant, which may be due to the observed low frequency of homozygous recessive genotype or the possibility of other regulating variants of *MBL2*. Thus, to reach at definite conclusion, further association of haplotypes with sMBL levels was carried out. The carriers of 3′H-3, the haplotype containing only risk allele of rs10824792, were found to have significantly low sMBL levels than the individuals with other 3′-UTR haplotypes, in RVVI and BV. Of note, studies have investigated the association of various *MBL2* 3′-UTR haplotypes with risk of colon and breast cancers (Bernig et al., 2005, 2007; Zanetti et al., 2012). Though the association was only borderline significant, these reports have indicated lower sMBL levels in carriers of the 3′-UTR haplotypes conferring diseases risk (Bernig et al., 2007; Zanetti et al., 2012).

The other 3′-UTR variants evaluated in the present study *i.e.* rs2120132, rs2120131, rs2165813, rs2099903 and rs2099902 showed no association with susceptibility to RVVI and its types. However, rs2099903 variant was found to be associated with susceptibility to ABPA, rs2099902 variant was found to be associated with increased risk of colon cancer (African American) and bladder cancer, while, rs2120132 conferred susceptibility to colon cancer (Zanetti et al., 2012; Overton et al., 2016; Andrew et al., 2015). No study till date has evaluated the role of rs2165813 SNP in any clinical relevant scenario. The frequency distribution of all the studied 3′-UTR variants was reported for the first time by the present study in North Indian population. This distribution was compared with all the modern human populations of the 1000 Genomes Project Phase 3 (Supplementary Table 1). The prevalence of the studied SNPs was found to be in consonance with all the populations except African, leading to skew in the global frequency of rs10824792 polymorphism. The possible reason could be weak LD, large population substructure and high level of genetic diversity that was found in African population relative to non-Africans (Campbell and Tishkoff, 2008).

In conclusion, our study supports the hypothesis that genetic variations in 3′-UTR region of the *MBL2* increases susceptibility to RVVI by altering sMBL levels. A 3′-UTR SNP *i.e.* rs10824792 was identified as associated genetic marker for contributing low sMBL levels and RVVI risk. Though, these preliminary findings require necessary functional validations, still the present study is important in suggesting novel SNP that may be involved in RVVI pathogenesis and thus contributes to the novel future research directions for effectual diagnostics and prophylaxis development for RVVI. Such studies in larger datasets are needed to validate the medicinal possibilities of MBL for RVVI and its types in different populations.

Author's contributions

NK reviewed the literature, was involved in design, performing experiments, analysis, interpretation, and drafted the manuscript. SS participated in sample collection and RVVI diagnosis. MK, JS, SS contributes in the experimental design, data analysis, manuscript editing and supervision. All authors read and approved the final manuscript.

Conflict of interest

The authors have declared no conflict of interest

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2018.10.009.

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