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Association of single nucleotide polymorphisms in *TNFA* and *CCR5* genes with Japanese Encephalitis: A study from an endemic region of North India

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ABSTRACT

TNFA, *IL1B*, *HMGB1*, *IL10*, *CXCL8*, *CCL2* and *CCR5* gene polymorphisms were investigated in 183 Japanese Encephalitis (JE) cases and 361 healthy controls from North India. Higher frequency of *TNFA* rs1800629 G/A, *CCR5* rs1799987 genotypes with A allele and lower frequency of combination lacking *TNFA* rs1800629 A, *CCR5* rs333 Δ32, and*CCR5* rs1799987 A alleles and *CCL2* rs1024611 G/G genotype was observed in JE cases. *TNFA* rs1800629 A and *CCR5* rs1799987 A alleles were associated with susceptibility while combination lacking *TNFA* rs1800629 A, *CCR5* rs333 Δ32, and rs1799987 A alleles and *CCL2* rs1024611 G/G genotype was associated with protection to JE.

1. Introduction

Acute encephalitis syndrome (AES) due to Japanese Encephalitis virus (JEV) is one of the most commonly diagnosed types of encephalitis. Japanese Encephalitis (JE) cases dominated the number of AES cases upto 2010 in the Gorakhpur and Basti division, of eastern Uttar Pradesh, North India. After the introduction of JE vaccine, though the incidence of JE cases have declined, still they account for about 10.0% of the AES cases (Murhekar et al., 2018; Mittal et al., 2018). JEV is a single stranded RNA virus belonging to family of Flaviviridae and is transmitted by the bite of female *Culex* mosquitoes. Majority of the infections are asymptomatic and mild, 0.1 to 1% of JEV infections develop in to encephalitis. About 45% of the JE cases experience permanent neurological sequelae and approximately an average of 18% of JE cases experience fatal outcome (Turtle and Solomon, 2018).

JEV induced inflammation has been identified as one of the major contributor to disease severity. Pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, and interleukin (IL)-1β, and chemokines such as CXCL-8 and monocyte chemoattractant protein (MCP)-1, also called as CCL-2 produced during JEV infection facilitates the neuroinvasion of the virus as well as inflammatory monocytes (Lannes et al., 2017). An insufficient anti-inflammatory cytokine response indicated by reduced IL-10 production in mice brain has also been reported to increase tissue pathology and viral load contributing to encephalitis development (Saxena et al., 2008). C−C chemokine receptor 5 (CCR5) has been reported to play important role in the immune response to West Nile virus (WNV), another flavivirus known to cause encephalitis (Lim et al., 2008). A recent study has reported that CCR5 ablation exacerbated progression to JE (Kim et al., 2016). Recent studies have shown that damaged neurons release danger associated molecular patterns (DAMP) that are associated with neuroinflammatory effects. High mobility box protein (HMGB)-1 is one such DAMP reported to mediate neuroinflammatory effects in tick borne viral encephalitis and neuroborreliosis (Moniuszko-Malinowska et al., 2018).

The expression of these inflammatory mediators and chemokine receptors are influenced by single nucleotide polymorphisms (SNPs) in the genes coding for them. SNPs in the promoter and intron regions of *TNFA, IL1B, CCL2* and *CXCL8* have been shown to be associated with altered levels of cytokines and chemokines (Umapathy et al., 2018; Xuan et al., 2005; Chowdhury and Khan, 2017; Hull et al., 2000). SNPs in the promoter region and a 32-base pair (bp) deletion mutation in the coding region of *CCR5*gene have been reported to affect CCR5 expression (Samson et al., 1996). Hence, it is plausible that SNPs in the genes coding for inflammatory mediators might be associated with development of JE in JEV infected children. In the present study, the association of SNPs in the *TNFA, IL1B, CCL2, HMGB1, IL10* and *CCR5* with JE

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Table 1
Percent allele frequencies of cytokine, chemokine and chemokine receptor gene polymorphisms in healthy controls and Japanese Encephalitis cases.

SNPs	Alleles	Japanese Encephalitis cases[a]	Healthy controls[b]	Odds ratio with 95% confidence intervals	*P* value
IL1B rs16944	T	228 (63.3)[c]	457 (64.2)	–	
	C	132 (36.7)	255 (35.8)	1.04 (0.79–1.35)	0.783
IL1B rs1143627	C	226 (62.8)	459 (64.1)	–	
	T	134 (37.2)	257 (35.9)	1.06 (0.81–1.38)	0.669
TNFA rs1799964	T	225 (61.8)	444 (61.8)	–	
	C	139 (38.2)	274 (38.2)	1.00 (0.77–1.30)	0.992
TNFA rs1800629	G	340 (93.4)	693 (96.8)	–	
	A	24 (6.7)	23 (3.2)	**2.12 (1.17–3.85)**	**0.013**
IL10 rs1800896	A	238 (65.0)	453 (63.1)	–	
	G	128 (35.0)	265 (36.9)	0.92 (0.71–1.19)	0.532
IL10 rs1800872	C	209 (57.7)	391 (54.2)	–	
	A	153 (42.3)	331 (45.8)	0.86 (0.67–1.12)	0.264
CCL2 rs1024611	A	234 (64.3)	472 (66.9)	–	
	G	130 (35.7)	234 (33.1)	1.12 (0.86–1.46)	0.401
CXCL8 rs4073	T	237 (65.1)	485 (67.2)	–	
	A	127 (34.9)	237 (32.8)	1.10 (0.84–1.43)	0.496
CCR5 rs1799987	G	243 (66.8)	513 (71.8)	–	
	A	121 (33.2)	201 (28.2)	1.27 (0.97–1.67)	0.086
CCR5 rs333	Wt	360 (98.4)	717 (99.3)	–	
	Δ32	6 (1.6)	5 (0.7)	1.66 (0.46–5.72)	0.418
HMGB1 rs3742305	G	327 (90.8)	638 (89.9)	–	
	C	33 (9.2)	72 (10.1)	0.89 (0.57–0.1.37)	0.62

Bold values represent significant values.

[a] For cases 2n = 360 for *IL1B* rs16944, *IL1B* rs1143627 and *HMGB1* rs3742305; 362 for *IL10* rs1800872; 364 for *TNFA* rs1799964, *TNFA* rs1800629, *CCL2* rs1024611, *CXCL8* rs4073, and *CCR5* rs1799987; 366 for *IL10* rs1800896 and *CCR5* rs333.

[b] For healthy controls, 2n = 706 for *CCL2* rs1024611; 708 for *CCR5* rs1799987; 710 for *HMGB1* rs3742305; 712 for *IL1B* rs16944; 716 for *IL1B* rs1143627 and *TNFA* rs1800629; 718 for *TNFA* rs1799964 and *IL10* rs1800896; 722 for *IL10* rs1800872, *CXCL8* rs4073, and *CCR5* rs333.

[c] Numbers represent allelic counts and numbers within parentheses indicate percentages.

was investigated in children from Gorakhpur and Basti division of Uttar Pradesh, a region endemic for JE.

2. Materials and methods

2.1. Study subjects and blood samples

ICMR-Regional Research Centre, Gorakhpur (formerly National Institute of Virology Field Unit, Gorakhpur) is providing diagnosis to the acute encephalitis cases admitted to BRD Medical college Hospital at Gorakhpur, Uttar Pradesh. The line list of the all AES cases is available with NIV Gorakhpur Unit. AES cases with history of laboratory confirmation for anti JEV specific IgM antibodies during the acute phase of the disease were contacted for participation in the study. AES cases were defined as those having acute onset of fever with an altered mental status or new onset of seizures but excluding febrile seizures. Altered mental status includes the following symptoms: confusion, disorientation, coma or inability to talk (Solomon et al., 2008). The case definition is according to the 2006 guidelines of national vector Borne Disease Control Program of India, which is the apex agency for surveillance of AES cases (NVBDCP, 2006). Prospective AES cases with positivity to JEV specific IgM antibodies were also included in the study. Two controls per case from the same geographical region without any known history of AES were included in the study. The samples were collected between July 2017 and December 2018. A written informed consent was obtained from the study subjects or their legal guardians. Two milliliters of blood samples were obtained in an EDTA vacutainer and brought to the laboratory. DNA was isolated using a commercial DNA isolation kit according to the protocol of the manufacturer (QIAmp DNA blood midi kit, USA). The DNA samples were shipped in dry ice to ICMR-National Institute of Virology, Pune for genotyping.

2.2. Genotyping of single nucleotide polymorphisms in the TNFA, IL1B, IL10, CCR5. CCL2, CXCL8 and HMGB1 genes and 32 base pair deletion mutation in the CCR5 gene

Genotyping of *TNFA* (rs1800629), *IL10* (rs1800896 & rs1800872), *CXCL8* (rs4073), and *CCR5* (rs1799987 &rs333) gene polymorphisms were performed using a polymerase chain reaction (PCR) with sequence specific primers (SSP) as described earlier (Warzocha et al., 1998; Kingkeow et al., 2011; van de Sande et al., 2007; Petrek et al., 2000; Easterbrook et al., 1999). *IL1B* polymorphisms (rs16944 & rs1143627), *TNFA* (rs1799964) *CCL2* (rs1024611) and *HMGB1* (rs3742305) were genotyped using a PCR-restriction fragment length polymorphism (RFLP) based methods as described earlier (Ma et al., 2012; Karray et al., 2011; Takada et al., 2002; Deng et al., 2013).

2.3. Statistical analysis

Allele and genotypic counts were enumerated using direct counting method. Allele frequencies of different SNPs were compared between cases and controls using two by two table method (mid p exact test) and the strength of association was expressed in terms of odds ratio (OR) with 95% confidence intervals (CI). Whether the genotype frequencies for each SNPs in control group deviated from Hardy-Weinberg equilibrium was assessed using χ^2 test. Logistic regression analysis, adjusting for age and gender, was performed to find out the association between genotypes and disease status under different genetic models: codominant, dominant, recessive, over dominant and log-additive. Akaike information criterion (AIC) and Bayesian information criterion (BIC) were used to infer the appropriate model. The strength of association was summarized in terms of *P* values and odds ratio (OR) with 95% confidence intervals (CI). A *P*-value of $< .05$ was considered significant. SNPstats and OpenEpi online softwares were used for statistical analysis (Solé et al., 2006; Dean et al., 2013).

3. Results

3.1. Demographic profile of study subjects andallele frequencies of cytokine, chemokine and chemokine receptor gene polymorphisms in JE cases and healthy controls

A total of 183 JE cases (mean age ± standard deviation 7.56 ± 3.59) and 361 healthy controls (mean age ± standard deviation 9.70 ± 3.56) without any history of encephalitis were included in the study. Male to female ratio is 1.3 for cases and 0.93 for controls.

Allele frequencies of different SNPs in the genes coding for various cytokine, chemokine and chemokine receptor were provided in Table 1. A significantly higher frequency of *TNFA* rs1800629 'A' allele was observed in JE cases compared to healthy controls (OR with 95% CI 2.12 (1.17–3.85), $P = .013$). A higher frequency of *CCR5*rs1799987 'A' allele was observed in JE cases as compared to healthy controls. However, the difference was not significant ($P = .086$). Allele frequencies of other studied SNPs were not different between JE cases and healthy controls.

3.2. Genotype frequencies of SNPs in the genes coding for pro-inflammatory cytokines (IL1B and TNFA), HMGB1gene coding for pro-inflammatory mediator and gene coding for anti-inflammatory cytokine (IL10) in JE cases and healthy controls

Genotype frequencies of *IL1B, TNFA, HMGB1* and *IL10* SNPs were presented in Table 2. Genotype frequencies for all SNPs conformed to hardy-Weinberg equilibrium in healthy controls ($P > .05$) except for *TNFA* rs1799964 ($P = .04$). A significantly higher frequency of *TNFA* rs1800629 'G/A' genotype was observed in JE cases in comparison to healthy controls (OR with 95% CI 1.97 (1.04–3.97), $P = .037$). The genotypes frequencies of other SNPs were not different between cases and controls ($P > .05$).

3.3. Genotype frequencies of SNPs in the genes coding for chemokines (CXCL8 and CCL2) and chemokine receptor gene (CCR5) in JE cases and healthy controls

Genotypes frequencies of SNPs in the *CXCL8* (rs4073), *CCL2* (rs1024611) and *CCR5* (rs1799987) and a32 base pair mutation in the *CCR5* gene (rs333) were given in Table 3. A higher frequency of *CCL2* rs1024611 'G/G' genotype was noticed in JE cases in relation to healthy controls though not significant ($P > .05$). Only heterozygous genotype of CCR rs333 was observed in the study subjects. The frequency of CCR5 rs333 wild type/Δ32 was 3.3% in JE cases and 1.4% in healthy controls. The frequency of CCR5 rs1799987 'A' allele containing genotypes (G/A and A/A) were higher among JE cases with respect to healthy controls and was significantly associated with JE in a log-additive mode (OR with 95% CI 1.38 (1.01–1.87), $P = .04$).

3.4. Predicted haplotype frequencies of ILB, TNFA, IL10 and CCR5 gene polymorphisms in JE cases and healthy controls

The haplotype frequencies of *ILB*, *TNFA*, *IL10* and *CCR5* gene polymorphisms were tabulated in Table 4. The *TNFA* haplotype (rs1799964-rs1800629) T-A and CCR5 haplotype (rs1799987-rs333) A-Wt were observed at a higher frequency in JE cases as compared to controls (For *TNFA*, OR with 95% CI 1.97 (1.03–3.76) $P = .042$; For *CCR5,*OR with 95% CI 1.37 (1.01–1.87) $P = .046$). Haplotype frequencies of SNPs in other genes were not significantly different between cases and healthy controls ($P > .05$).

Table 2
Percent genotype frequencies of pro-inflammatory cytokines, pro-inflammatory mediator and anti-inflammatory cytokine gene polymorphisms in healthy controls and Japanese Encephalitis cases.

SNPs and allele	HWE *P* value in controls	Japanese Encephalitis cases[a]	Healthy controls[b]	Odds ratio with 95% confidence intervals	*P* value
IL1B rs16944					
T/T		69 (38.3%)[c]	141 (39.6%)	1.00	
T/C	0.21	90 (50%)	175 (49.2%)	1.21 (0.81–1.80)	0.62
C/C		21 (11.7%)	40 (11.2%)	1.23 (0.66–2.31)	
IL1B rs1143627					
C/C		69 (38.3%)	145 (40.5%)	1.00	
C/T	0.73	88 (48.9%)	169 (47.2%)	1.25 (0.84–1.88)	0.48
T/T		23 (12.8%)	44 (12.3%)	1.30 (0.71–2.39)	
TNFA rs1799964					
T/T		69 (37.9%)	128 (35.6%)	1.00	
T/C	0.04	87 (47.8%)	188 (52.4%)	0.79 (0.53–1.19)	0.47
C/C		26 (14.3%)	43 (12%)	1.00 (0.55–1.81)	
TNFA rs1800629					
G/G	1.00	158 (86.8%)	335 (93.6%)	1.00	
G/A		24 (13.2%)	23 (6.4%)	**1.97 (1.04–3.71)**	**0.037**
IL10 rs1800896					
A/A		84 (45.9%)	147 (41%)	1.00	
A/G	0.37	70 (38.2%)	159 (44.3%)	0.70 (0.47–1.05)	0.20
G/G		29 (15.8%)	53 (14.8%)	0.95 (0.55–1.65)	
IL10 rs1800872					
C/C		61 (33.7%)	101 (28%)	1.00	
C/A	0.34	87 (48.1%)	189 (52.4%)	0.75 (0.49–1.14)	0.39
A/A		33 (18.2%)	71 (19.7%)	0.78 (0.45–1.34)	
HMGB1 rs3742305					
G/G		147 (81.7%)	286 (80.6%)	1.00	
G/C	1.00	33 (18.3%)	66 (18.6%)	0.84 (0.52–1.37)	0.24
C/C		0	3 (0.8%)	0	

Bold values represent significant values.

[a] For cases $n = 180$ for *IL1B* rs16944, *IL1B* rs1143627 and *HMGB1* rs3742305; 181 for *IL10* rs1800872; 182 for *TNFA* rs1799964 and *TNFA* rs1800629; 183 for *IL10* rs1800896.

[b] For healthy controls, $n = 355$ for *HMGB1* rs3742305; 356 for *IL1B* rs16944; 358 for *IL1B* rs1143627 and *TNFA* rs1800629; 359 for *TNFA* rs1799964 and *IL10* rs1800896; 361 for *IL10* rs1800872, and *CXCL8* rs4073.

[c] Numbers represent genotypic counts and numbers within parentheses indicate percentages.

Table 3
Percent genotype frequencies of chemokine and chemokine receptor gene polymorphisms in healthy controls and Japanese Encephalitis cases.

SNPs and allele	HWEP value in controls	Japanese Encephalitis cases[a]	Healthy controls[b]	Odds ratio with 95% confidence intervals	*P* value
CXCL8 rs4073					
T/T		87 (47.8%)[c]	169 (46.8%)	1.00	
T/A	0.18	63 (34.6%)	147 (40.7%)	0.94 (0.62–1.42)	0.33
A/A		32 (17.6%)	45 (12.5%)	1.43 (0.83–2.48)	
CCL2 rs1024611					
A/A		82 (45%)	156 (44.2%)	1.00	
A/G	0.72	70 (38.5%)	160 (45.3%)	0.83 (0.56–1.25)	0.19
G/G		30 (16.5%)	37 (10.5%)	1.43 (0.80–2.54)	
CCR5 rs1799987					
G/G		76 (41.8%)	177 (49.6%)	1.00	
G/A	0.067	91 (50%)	159 (44.5%)	1.33 (0.90–1.97)	0.12
A/A		15 (8.2%)	21 (5.9%)	2.02 (0.95–4.27)	
CCR5 rs333					
Wt/Wt	1.00	177 (96.7%)	356 (98.6%)	1.00	
Wt/Δ32		6 (3.3%)	5 (1.4%)	2.04 (0.60–6.92)	0.25

Bold values represent significant values.
[a] For cases $n = 182$ for *CCL2* rs1024611, *CXCL8* rs4073, and *CCR5* rs1799987; 183 for and *CCR5* rs333.
[b] For healthy controls, $n = 353$ for *CCL2* rs1024611; 354 for *CCR5* rs1799987; 361 for *CXCL8* rs4073, and *CCR5* rs333.
[c] Numbers represent genotypic counts and numbers within parentheses indicate percentages.

3.5. Percentage frequencies of combinations of genetic risk factors in JE cases and healthy controls

Based on the present study results and earlier studies, certain alleles and genotypes were identified as risk factors and both cases and controls were classified in to those with presence of at least one risk factor or complete absence of all the risk factors and compared. The risk factors include *TNFA* rs1800629 'A' allele, *CCR5* rs333 Δ32 allele, *CCR5* rs1799987 'A' allele and *CCL2* rs1024611 'G/G' genotype. The results revealed significantly lower frequency of complete absence of all the risk factors in cases with JE as compared to healthy controls (OR with 95% CI 0.56 (0.38–0.83), $P = .0037$) (Table 5).

4. Discussion

Studies on the genetics of susceptibility to viral encephalitis have mainly focused on WNV and tick-borne encephalitis virus (TBEV) induced encephalitis while for JE, only three studies exist (Lim et al., 2008; Mickienė et al., 2014; Barkhash et al., 2018; Ellwanger and Chies, 2019). A study from Chandigarh, India with 66 JE cases and 60 healthy controls reported the association of *TNFA* rs1800629 A allele with JE while another study from Assam, India with 87 JE cases and 94 healthy controls reported the association of *CCL2* rs1024611 G, *CCL5 rs*2280788 G and *CCR2* rs1799864 A alleles with JE (Pujhari et al., 2012; Chowdhury and Khan, 2017). A study from Lucknow, North India involving 103 JE cases and 103 healthy controls reported the association of *TLR3* rs3775291 T allele with JE (Biyani et al., 2015). The present study is from a region in which seasonal outbreaks of AES cases occurs annually with high case fatality rate and JE accounts for about 10% of AES cases (Murhekar et al., 2018; Mittal et al., 2018). In the present study, SNPs in the genes coding for pro-inflammatory cytokines (*TNFA* and *IL1B*), anti-inflammatory cytokine (IL10) pro-inflammatory mediator (HMGB1), chemokines (*CCL2* and *CXCL8*) and chemokine receptor (*CCR5*) were investigated for their association with JE involving 183 JE cases and 361 healthy controls without any history of encephalitis from a region endemic for JE and known for high mortality rates in children due to AES.

The results revealed the association of *TNFA* rs1800629 A allele and

Table 4
Percent predicted haplotype frequencies of *IL1B*, *TNFA*, *IL10* and *CCR5* gene polymorphismsin healthy controls and Japanese Encephalitis cases.

Genes and haplotypes	Japanese Encephalitis cases[a]	Healthy Controls[b]	Odds ratio with 95% confidence intervals	*P* value
IL1B rs16944-rs1143627				
T-C	60.8	60.8	1.00	–
C-T	35.1	32.7	1.17 (0.88–1.57)	0.28
C-C	1.7	3.5	0.58 (0.23–1.46)	0.25
T-T	2.4	3.0	0.97 (0.44–2.12)	0.94
TNFA rs1799964-rs1800629				
T-G	55.2	58.6	1.00	–
C-G	38.2	38.2	1.00 (0.75–1.33)	1.00
T-A	6.6	3.2	**1.97 (1.03–3.76)**	**0.042**
IL10 rs1800896-rs1800872				
A-A	37.8	39.2	1.00	–
G-C	30.7	30.3	1.04 (0.75–1.42)	0.83
A-C	27.2	23.8	1.16 (0.81–1.65)	0.42
G-A	4.2	6.6	0.59 (0.28–1.27)	0.18
CCR5 rs1799987-rs333				
G-Wt	66.4	71.8	1.00	–
A-Wt	32.0	27.4	**1.37 (1.01–1.87)**	**0.046**
Rare haplotypes	1.7	0.7	2.33 (0.68–8.03)	0.18
G-Δ32	0.4	0		
A-Δ32	1.3	0.7		

Bold values represent significant values.
[a] For cases $n = 180$ for *IL1B*; 181 for *IL10*; 182 for *TNFA*; 182 for *CCR5*.
[b] For healthy controls, $n = 356$ for *IL1B*; 358 for *TNFA*; 359 for *IL10* rs1800896; 354 for *CCR5*.

Table 5
Percentage frequencies of combinations of genetic risk factors in JE cases and healthy controls.

Combination of risk factors	Japanese Encephalitis cases (n = 180)	Healthy controls (n = 348)	Odds ratio with 95% confidence intervals	P value
Subjects bearing *CCR5* Δ32 allele and/or *CCR5* rs1799987 A allele and/or *CCL2* rs1024611 G/G genotype and/or *TNFA* rs1800629 'A' allele	130 (72.2%)[a]	207 (59.5%)	1.00	–
Subjects lacking all of these: *CCR5* Δ32 allele, *CCR5* rs1799987 A allele, *CCL2* rs1024611 G/G genotype and *TNFA* rs1800629 'A' allele	50 (27.8)	141 (40.5%)	**0.56 (0.38–0.83)**	**0.0037**

Bold values represent significant values.
[a] Numbers represent combined genotypic counts and numbers within parentheses indicate percentages.

CCR5 rs1799987 A allele in log-additive mode with JE. The association of *TNFA* rs1800629 A allele was further corroborated by the observation of increased frequency of *TNFA* haplotypes containing rs1800629 A allele in JE cases. The study results also confirm the earlier report from Chandigarh, India (Pujhari et al., 2012). TNF-α has been demonstrated to affect the expression and cellular distribution of tight junction proteins and cause damage to the BBB in JEV infection (Wang et al., 2018). *TNFA* rs1800629 A allele has been reported to be associated with increased transcription as compared to G allele (Kroeger et al., 1997). Elevated levels of the TNF-α has been reported in patients with rs1800629 A allele (Umapathy et al., 2018). It is possible that in JEV infected patients, *TNFA* rs1800629 A might lead to higher TNF-α resulting in subsequent development of encephalitis. In the present study, no association between *TNFA* rs1799964 was observed. It is possible that the effect of rs1799964 on TNF-α level might be lower as compared to rs1800629 and may not have a role to play in JE pathogenesis.

Association of *CCR5* rs1799987 A allele with JE in log-additive mode is reported for the first time. However, no association between *CCR5* rs333Δ32 allele and susceptibility to JE was observed. Though the frequency of Δ32 allele was higher in JE cases, the frequency is too low and requires a larger sample size to detect an association with JE. CCR5 deficiency has been reported to be associated with susceptibility to WNV and TBEV induced encephalitis (Lim et al., 2008; Ellwanger and Chies, 2019; Kindberg et al., 2008). *CCR5* rs1799987 A allele has been reported to be associated with higher expression of CCR5 (McDermott et al., 1998). However, studies have reported that CCR5 expression is regulated by multiple promoter alleles and the resulting haplotypes have different phenotypic effects in different populations (Gonzalez et al., 1999). It is possible that the *CCR5* rs1799987 A in combination with other promoter alleles might alter the expression of CCR5 and affect susceptibility to JE. However, further studies investigating the other promoter alleles and effect of different haplotypes on CCR5 expression are needed.

In the present study, SNPs in the *HMGB1*, *CXCL8*, *CCL2* and *IL10* were not associated with JE though a trend towards increased frequency of *CCL2* rs1024611 'G/G' genotype was observed in JE cases. In similar way, a study from Assam, India has reported the association of *CCL2* rs1024611 G allele (associated with higher CCL2 levels) with susceptibility to JE [11]. The observation of no association between *HMGB1, CXCL8* and *IL10* SNPs with JE suggests that the effect of HMGB1, CXCL8 and IL10 on JE pathogenesis might be minor.

Based on the present study as well as the earlier studies conducted in flavivirus encephalitis, certain genotypes or alleles were identified as risk factor for JE. The risk factors identified include *TNFA* rs1800629 'A' allele, *CCR5* rs333 Δ32 allele, *CCR5* rs1799987 'A' allele and *CCL2* rs1024611 'G/G' genotype. When the study subjects were classified into those with atleast one risk factor or with complete absence of all the risk factors and compared between cases and controls, complete absence of the all the risk factors was associated with protection to JE. This strengthens the findings of our study as well as the earlier studies and suggests a role for TNF-α, CCR5 and CCL2 in the pathogenesis of JE. Since the study region is endemic for AES irrespective of etiologies, more studies are needed to find out whether the risk factors identified in the present study is also contributing to the AES with other etiologies.

One limitation of the study is that the status of exposure to JEV among healthy controls is not known. However, JE is endemic in the study region and the ratio of clinical JE to asymptomatic infections had been reported to be 1:250 suggesting that large number of asymptomatic/mild JE infections might occur in regions endemic to JE (WHO, 2015). To ensure that healthy controls had the possibility of equal exposure to the virus as JE patients, the healthy controls included in the study belonged to the same villages where cases reside and were living nearby to the cases.

To conclude, the present study reports the association of *TNFA*

rs1800629 'A' and *CCR5* rs1799987 'A' alleles with susceptibility to JE. Moreover, complete absence of combination of *TNFA* rs1800629 'A' allele, *CCR5* rs333 Δ32 allele, *CCR5* rs1799987 'A' allele and *CCL2* rs1024611 'G/G' genotype might be associated with protection to JE.

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Author's contributions

HD and KA conceived the concept and designed the study, HD, KA, MM, NS, RB, AG, UC, DC, VB performed the work. KA did the statistical analyses. KA and HD wrote the manuscript. All the authors have approved the manuscript.

Declaration of competing interests

None declared.

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