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Phenotyping and Genotyping of HNA: Prevalence, Risk of Alloimmunization, and HNA Incompatibilities in Indians

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Keywords

Human neutrophil antigen alloimmunization · Human neutrophil alloantigens · Indians · Neutropenia · Transfusion-related acute lung injury

Abstract

Background: Antibodies to human neutrophil alloantigens (HNA) are involved in the pathophysiology of several clinical conditions including transfusion-related acute lung injury (TRALI), alloimmune and autoimmune neutropenia, and febrile nonhemolytic transfusion reactions leading to neutropenia. The cognate antigens are polymorphic structures expressed on several glycoproteins on the neutrophils, i.e., antigens HNA-1a, -1b, -1c, and -1d on Fc-γ-receptor IIIb; HNA-2 on CD177; HNA-3a and -3b on choline transporter-like protein 2; HNA-4a and -4b on CD11b/αM subunit of the αMβ2-integrin (CD11b/CD18, Mac-1, CR3); and HNA-5a and -5b on αL-subunit (CD11a) of the αLβ2 integrin (CD11a/CD18), leukocyte function associated molecule (LFA)-1. Currently, there is a lacuna of diagnostic methods for detection of HNA in India. This study aimed to determine the HNA frequencies in Indians, estimate the risk of alloimmunization, and prepare typed neutrophil panels, which can be used to detect HNA antibodies in neutropenia cases. ***Material and Methods:*** EDTA blood samples were collected from random 1,054 blood donors. HNA-2 was phenotyped on fresh EDTA samples using FITC labelled monoclonal anti-CD177 by flowcytometry. HNA-1 (*FCGR3B*) genotyping was carried out by DNA sequencing and PCR-RFLP. Antigens of HNA-3 (*SLC44A2*) and HNA-5 (*ITGAL*) were genotyped by PCR-RFLP using *TaqaI* and *Bsp1286I* restriction enzymes, respectively, while HNA-4 (*ITGAM*) was genotyped by PCR-SSP. ***Results:*** Allele frequencies of *FCGR3B*01*, *FCGR3B*02*, and *FCGR3B*03* were found to be 0.433, 0.444, and 0.087, respectively. FCGR3B*01+*02+*03− was the most common genotype (33.78%). Ten individuals showed deficiency of FCGR3B individuals, while 23 showed hyperexpression, i.e., *FCGR3B*01+*02+*03+*. *FCGR3B*04* and **05* occurred with a frequency of 0.002 and 0.024. HNA-2 was found to be a high frequency antigen occurring in 98.8% population. Four percent individuals showed atypical expression of CD177 on their neutrophils. Allele frequencies of *SLC44A2*01* and *SLC44A2*02* were 0.812 and 0.188, respectively, and that of *ITGAM*01*, *ITGAM*02*, *ITGAL*01*, and *ITGAL*02* were 0.9546, 0.0454, 0.2372, and 0.7628, respectively. ***Conclusion:*** This is the first study in India to report the frequencies of HNA among blood donors. Typed neutrophil panels identified in the present study will enable us to investigate suspected cases of immune neutropenia in future.

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Introduction

Antibodies to human neutrophil antigens (HNA) are implicated in various clinical conditions such as neonatal alloimmune neutropenia (NAN), transfusion-related acute lung injury (TRALI), febrile transfusion reactions, immune neutropenia post stem cell transplantation, autoimmune neutropenia (AIN), and drug-induced neutropenias [1]. The cognate antigens are expressed on glycoproteins located on the neutrophil membrane. The first granulocyte antigen HNA-1a (formerly known as NA1) was discovered by Lalezari et al. [2–9] during their investigation of a neonate with transient NAN and since then, many other antigens have been identified from sera of patients with autoantibodies or alloantibodies directed against these granulocyte antigens. The Granulocyte Immunobiology Working Party of the International Society of Blood Transfusion (ISBT) has now recognized nine polymorphic antigens (HNA-1a, -1b, -1c, -1d, -2, -3a, -3b, -4a, -4b, and 5a) assigned into five HNA systems and encoded by fourteen different alleles [10].

Due to the lack of commercial antisera and short life span of granulocytes, serological assays such as granulocyte immunofluorescence test (GIFT) and granulocyte agglutination test (GAT) have limited applicability. However, the molecular basis of these antigens has been elucidated thus enabling use of various DNA-based methods to successfully genotype HNA. HNA can be differentiated by single nucleotide polymorphisms (SNP) in the genes encoding them, i.e., *FCGR3B* (HNA-1a, -1b, -1c, and -1d), *SLC44A2* (HNA-3a and -3b), *ITGAM* (HNA-4a and -4b), and *ITGAL* (HNA-5a and -5b).

In contrast, HNA-2 is an isoantigen and has differential expression on neutrophils. mRNA splicing defects along with DNA polymorphism *CD177*787A>T* in association with c.1254G>A, c.955delG, c.1291G>A SNPs have been identified in several HNA-2-deficient individuals in different ethnic groups [11–14]. HNA-2 expression has been more commonly studied by flow-cytometry using monoclonal antibodies.

Due to the increasingly role of the antibodies to human neutrophil antigens in the pathophysiology of various immune neutropenias, it is necessary to know their incidence in a population as they may cause differential risk of HNA alloimmunisation. Various populations worldwide have reported HNA frequencies [15–44]. There are no comprehensive data on the distribution of HNA among Indian blood donors. Hence, the aim of the present study was to detect HNA in blood donors and thereby determine their frequencies and estimate the risk of alloimmunization in Indian population. These data will help us to prepare typed neutrophil panels which can be used for detecting HNA associated antibodies and thereby provide diagnosis for clinical conditions where these antigens and antibodies play an important role.

Material and Methods

This study was carried out between 2016 and 2020 at the Department of Transfusion Medicine, ICMR-National Institute of Immunohematology (NIIH), Mumbai, India, after ethical approval (NIIH/IEC/02-2016) from the Institutional Ethics Committee. Five millilitres EDTA blood sample was collected from randomly selected 1,054 blood donors from cosmopolitan city of Mumbai (belonging to different ethnic groups) attending blood donation camps organized by Department of Transfusion Medicine, K. E. M. Hospital, Parel, Mumbai, after informed consent had been taken. They were between the age group of 18–55 years and approximately 11% were females. They had no preexisting medical conditions.

Phenotyping HNA-2

On freshly collected sample, HNA-2 was phenotyped using commercially available anti-CD177 monoclonal antibody using an in-house standardized flowcytometry-based method. Briefly, polymorphonuclear cells (PMNs) from 100 µL of EDTA blood sample were stained with FITC anti-CD177 antibody [MEM-166] (Abcam, Cambridge, UK; Catalogue No. ab26013). Post incubation, the red blood cells were lysed with FACS lysing solution (Becton, Dickinson and Company, New Jersey, USA). The supernatant containing the lysed RBCs was discarded and the residual WBCs (pellet) were washed twice with phosphate buffered saline (PBS) and subsequently resuspended in 200 µL of PBS. HNA-2 expression on neutrophils was determined by analysing about 1×10^4 cells/mL on a flow cytometer (BD FACS Calibur; Becton-Dickinson) using Cell Quest Pro software. Individuals showing less than 5% of reactive neutrophil population with low mean fluorescence intensity (MFI) were considered as HNA-2 negative.

Genotyping Antigens of HNA-1, -3, -4, and -5 Systems

From the remaining EDTA sample, DNA extraction was carried out using standard phenol-chloroform method [45]. PCR-based assays were then used to genotype antigens of HNA-1, -3, -4, and -5. All primers were diluted to 10 pmoles. before use. The primers and PCR cycling conditions are listed in online supplementary Table S1 (for all online suppl. material, see www.karger.com/doi/10.1159/000525654).

HNA-1 Genotyping

Six polymorphic sites in the exon 3 of the *FCGR3B* gene are responsible for formation of antigens of HNA-1 system (online suppl. Fig. S1). These were analysed by direct DNA sequencing as described by He et al. [34]. To further differentiate between presence of both or any of the two alleles (*FCGR3B*02* and *FCGR3B*03*), PCR-RFLP with *SfaNI* restriction enzyme (Thermo Fisher Scientific, USA) was carried out as recommended by Steffensen et al. [46]. HNA-1c homozygous showed presence of two fragments (126 bp and 93 bp) when electrophoresed on 2% agarose gel.

HNA-3 Genotyping

HNA-3 genotyping (*SLC44A2*01:* 455G [3a] and *SLC44A2*02:* 455A [3b]; rs2288094) was carried out by PCR-restriction fragment length polymorphism (RFLP) as recommended by Lopes et al. [47] as presence of an additional nonsynonymous SNP c.451C>T (rs147820753) in the *SLC44A2* gene can affect the PCR-SSP primer binding and subsequent assay results. HNA-3 specific fragment (271 bp) was digested with enzyme *Taqα1* (New England Biolabs, Beverly, MA, USA), for 60 min at 65°C. Presence of two bands 171 bp and 100 bp post digestion indicated presence of *SLC44A2*01*, while presence of only undigested 271 bp band indicated presence of *SLC44A2*02*.

HNA-4 Genotyping

HNA-4 (*ITGAM*01*: 230G [4a] and *ITGAM*02*: 230A [4b]; rs1143679) genotyping was carried out by PCR using sequence specific primers (SSP) [48]. Human growth hormone (HGH) control was co-amplified as internal control. Presence of 249 bp allele-specific band along with 434 bp internal control indicated presence of the antigen.

HNA-5 Genotyping

HNA-5 genotyping (*ITGAL*01*: 2372G [5a] and *ITGAL*02*: 2372C [5b]; rs2230433) was performed using a previously described PCR-RFLP technique [42]. The PCR product post amplification was digested with *Bsp1286I* (Fermentas, California, USA) at 37°C. Presence of two bands, i.e., 136-bp and 65-bp fragments, were indicative of presence of *ITGAL*01* in homozygous state.

DNA control samples with known HNA-1a, -1b, -3a, -3b, -4a, -4b, -5a, and -5b alleles were kindly provided by Dr. Hitoshi Okazaki, Department of Blood Transfusion, The University of Tokyo Hospital, Japan [23]. Additionally, some of our anonymized samples were sent to Dr. Marie Audrain, Laboratoire d'Immunologie, CHU Nantes, France, who genotyped them. The results were concordant with our HNA genotyping [49].

Statistical Analysis

Hardy-Weinberg equilibrium was validated by calculating expected numbers of subjects for each genotype and its agreement with the observed genotypes was determined using the χ^2 test. The level of statistical significance was set at $p < 0.05$. The probability of HNA incompatibilities after random transfusions was estimated using the formula: $a^2 (1 - a^2) + b^2 (1 - b^2) = 2ab (1 - ab)$, where "a" and "b" are the HNA allele frequencies. The potential risk of alloimmunisation against the HNA systems was calculated using the – formula: risk of alloimmunization for "a" phenotype = bb (ab + aa) and for "b" phenotype = aa (ab + bb), where "aa," "ab," and "bb" are the genotype frequencies [50].

Results

In the present study, HNA typing was carried out using flow cytometry and DNA-based assays.

HNA-2 Phenotyping

In 1,004 healthy Indian blood donors (88.15% males and 11.85% females; age: mean ± SD: 33.28 ± 9.54), HNA-2 showed biphenotypical expression (one reactive and one nonreactive cell population). The frequency of HNA-2 null phenotype was found to be 1.2%. As CD177 was variably expressed, the donors were classified into four categories based on their % of CD177 reactive neutrophils subpopulations as shown in online supplementary Table S2. 46.7% individuals showed an increased expression of CD177 on the neutrophils while, 18.43% showed low expression. In 80.38% ($n = 806$) donors, ≥40% of neutrophil subpopulation was reactive. Forty one donors (4.08%) presented an atypical expression of the HNA-2, i.e., along with a nonreactive subpopulation, two reactive cell populations were observed (online suppl. Fig. S2). The distribution of HNA-2 expression showed no significant differences among age groups ($p < 0.05$) at 95% confidence interval (−2.386 to 4.366). Mean HNA-2 expression was slightly higher in women (59.16 ± 17.80) as compared to that in men (56.69 ± 19.86).

HNA-1 Genotyping

On genotyping 906 donors for *FCGR3B* gene using DNA sequencing (online suppl. Fig. S3), the allele frequencies of *FCGR3B*01*, *FCGR3B*02*, and *FCGR3B*03* were found to be 0.4327, 0.4441, and 0.0866, respectively. *FCGR3B*01+*02+*03* the most common genotype (36.56%) of which, 92.97% genotypes were encoded by the common allele, *FCGR3B*02*, and remaining by *FCGR3B*05* (Table 1). *FCGR3B*01−*02+*03−* homozygous was the second most common genotype (23.07%). Ten individuals were found to be *FCGR3B* deficient, i.e., (*FCGR3B*01−*02−*03−* or HNA-1 null). Fifty-three donors showed polymorphisms corresponding to all the three alleles namely, *FCGR3B*01, *02*, and **03* alleles. On carrying out PCR-RFLP analysis for further differentiating between presence of both *FCGR3B*02* and *FCGR3B*03* or only *FCGR3B*03* along with *FCGR3B*01*, 23 donors showed presence of 3 bands and thus carried both *FCGR3B*02 and *03* along with *FCGR3B*01* allele, *FCGR3B*01+*02+*03+*, i.e., hyperexpression of FCGR3B receptor. Remaining donors carried only *FCGR3B*03* along with *FCGR3B*01*, i.e., *FCGR3B*01+*02−*03+* (showed presence of two bands post digestion with *SfaNI*). Variant alleles *FCGR3B*04* encoding HNA-1a and *FCGR3B*05* encoding HNA-1b were found to have an allele frequency of 0.0016 and 0.024 respectively. One individual with very rare *FCGR3B*04/FCGR3B*05* genotype was also identified. Apart from these, two additional substitutions 230T>G and 230T>A (old nomenclature) were also observed in 54 donor samples; of which majority (75.93%) of them displayed 230T>G polymorphism.

HNA-3 Genotyping

Allele frequencies of *SLC44A2*01* and *SLC44A2*02* were found to be 0.812 and 0.188 respectively among 1,040 tested blood donors by PCR-RFLP (online suppl. Fig. S4). HNA-3a homozygous was the most common phenotype occurring with a frequency of 0.6596 (Table 1, Table 2). Genotype frequencies suggest that about 3.6% of Indian population is at the risk of producing anti-HNA-3a antibodies.

HNA-4 and HNA-5 Genotyping

Out of the 1,046 tested donors (using PCR-SSP (online suppl. Fig. S5), the allelic frequencies of *ITGAM*01* and *ITGAM*02* were found to be 0.9546 and 0.0454 respectively. HNA-4a homozygous was the most common genotype with 91% frequency (Table 1). Similarly, the frequencies of *ITGAL*01* and *ITGAL*02* were found to be,

Table 1. Percentage distribution of genotype frequencies of HNA in Indian blood donors

HNA system	Genotype	Phenotype	Frequency	HNA system	Genotype	Phenotype	Frequency
HNA-1	FCGR3B*01+, *02–, *03–	HNA-1a/1a	0.2208	HNA-3	SLC44A2*01+, *02–	HNA-3a/3a	0.6596
	FCGR3B*01+, *02+, *03–	HNA-1a/1b	0.3377		SLC44A2*01+, *02+	HNA-3a/3b	0.3048
	FCGR3B*01–, *02+, *03–	HNA-1b/1b	0.2307		SLC44A2*01–, *02+	HNA-3b/3b	0.0356
	FCGR3B*01–, *02–, *03+	HNA-1c/1c	0.0166				
	FCGR3B*01–, *02+, *03+	HNA-1b/1c	0.0728	HNA-4	ITGAM*01+, *02–	HNA-4a/4a	0.9120
	FCGR3B*01+, *02–, *03+	HNA-1a/1c	0.0442		ITGAM*01+, *02+	HNA-4a/4b	0.0851
	FCGR3B*01+, *05+, *03–	HNA-1a/1b	0.0276		ITGAM*01–, *02+	HNA-4b/4b	0.0029
	FCGR3B*01–, *02–, *03–	HNA-1null	0.0110				
	FCGR3B*01–, *05+, *03–	HNA-1b/1b	0.0099	HNA-5	ITGAL*01+, *02–	HNA-5a/5a	0.0626
	FCGR3B*04+, *05+, *03–	HNA-1a/1b	0.0011		ITGAL*01+, *02+	HNA-5a/5b	0.3491
	FCGR3B*04+, *02+, *03–	HNA-1a/1b	0.0022		ITGAL*01–, *02+	HNA-5b/5b	0.5882
	FCGR3B*01+, *02+, *03+	HNA-1a/1b/1c	0.0254				

0.2372 and 0.7628 respectively by using PCR-RFLP (online suppl. Fig. S6). HNA-5b homozygous was the most common genotype (approximately 59%) in 1,054 tested donors (Table 1).

HNA genotypes were consistent with the Hardy-Weinberg equilibrium. After random transfusions, 31.5%, 25.9%, 8%, and 29.6% of the HNA-1, -3, -4, and -5 incompatibilities, respectively, are expected. The risk of alloimmunization against HNA-1a, -1b, -3a, -3b, -4b, -5a, and -5b was found to be 0.129, 0.126, 0.035, 0.225, 0.08, 0.242, and 0.059, respectively.

Discussion

Investigators have observed that HNA and associated antibodies are involved in the pathophysiology of various clinical conditions leading to the decrease in the neutrophil count, i.e., neutropenia [51–57]. Hence, it is necessary to know the prevalence of HNA in a population to better understand the risk of HNA alloimmunization they may cause due introduction of foreign antigen via blood transfusion or feto-maternal route and thereby the specificities of HNA antibodies produced. In India, there are no comprehensive data on the distribution of HNA among donors. Also, as currently there are no HNA diagnostics available, cases of immune neutropenia where these antigens and antibodies are involved remain unresolved.

In the present study, HNA-2 was phenotyped using a flow cytometry-based method using anti-human CD177 monoclonal antibody CD177 clone MEM-166. HNA-2 was found to be a high frequency antigen occurring in 98.8% of Indians and presenting in most cases as a biphenotypical expression profile by flow cytometric analysis. This frequency was similar to those in Thais, Chinese, Taiwanese, Brazilians, Japanese, Americans, and Africans but significantly lower than that Eastern Japanese, Koreans, and French populations (Fig. 1) [15–24]. These findings suggest that risk of alloimmunization against HNA-2 is very low. Hence, chances of producing anti-HNA-2 antibodies and subsequently causing clinical disorders, especially NAN or TRALI, may also be less.

No significant association was found between CD177 expression and donors' age. Similar results have been observed in a Thai study, who also did not find any association with donors age [24]. However, in Japanese women, as age decreased, HNA-2 expression also decreased though it remained constant in men [19]. Regarding the effect of sex on HNA-2 expression, the CD177 expression was slightly decreased in males as compared to females as compared to males suggesting that oestrogen levels may affect HNA-2 expression on neutrophils. Significantly higher association has been found in females expressing CD177 among Thais, Japanese, and Brazilian populations [19, 20, 24, 58].

Atypical expression of HNA-2, i.e., 2 reactive cell populations along with a nonreactive cell population has been reported by Moritz et al. [58], in 8 out of 135 individuals which had strong correlation with the presence of three polymorphism viz. A134T, G156A, and G1333A in *CD177* gene. Matsuo et al. [19] also found 19 (8.5%) individuals showing a nonreactive population with 2 distinct reactive cell populations. Caruccio et al. [59] also found that 6 out of 52 tested blood donors had two CD177 reactive cell population along with one HNA-2 null population, this type of expression was not seen in pregnant women (n = 44). In the present study, 41 donors showed atypical expression of CD177 on their neutrophils. Though the exact role of the HNA-2 atypical expression on the neutrophil function is not known, it is unclear whether it confers any selective advantage. Molecular analysis of mRNA among these donors may throw some light if any polymorphisms are detected which may be responsible for causing atypical expression of HNA-2.

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Table 2. Comparison of HNA alleles in different populations worldwide

Population	*n*	HNA-1			HNA-3		HNA-4		HNA-5	
		1a	1b	1c	3a	3b	4a	4b	5a	5b
Indians	1,054	0.433 (n = 906)[§, ‡]	0.444 (n = 906)[§, ‡]	0.086 (n = 906)[§, ‡]	0.812 (n = 1,040)[‡]	0.188 (n = 1,040)[‡]	0.955 (n = 1,046)	0.045 (n = 1,046)	0.237 (n = 1,054)[‡]	0.763 (n = 1,054)[‡]
Germans [26]	364	0.360*	0.631*	0.019*	0.801*	0.199*	0.889*	0.111*	0.665*	0.335*
Syrians [27]	100	0.375	0.58	0.04	0.742	0.258	0.86	0.14	0.66	0.34
Southern Thais [28]	427	0.619[†]	0.365[†]	0.012[†]	0.808[†]	0.192[†]	0.973[†]	0.027[†]	0.656[†]	0.344[†]
North-eastern Thais [29]	400	0.696	0.301	0.0	0.785	0.215	0.972	0.028	0.676	0.324
Burmese [29]	216	0.605	0.395	0.031	0.747	0.253	0.971	0.029	0.559	0.441
Karen [29]	249	0.725	0.275	0.0	0.845	0.155	0.956	0.044	0.693	0.307
Hongkong, Chinese [30]	300	0.678	0.315	0.0	0.71	0.29	0.995	0.005	0.852	0.148
Total Malays [32]	192	0.706	0.294	0.037	0.758	0.242	0.977	0.023	0.708[‡]	0.292[‡]
Central Thais [33]	300	0.47	0.53	0.005	0.49	0.51	0.97	0.03	0.79[‡]	0.21[‡]
Zhejiang, Chinese [34]	400	0.613[§]	0.387[§]	0[§]	0.654[§]	0.346[§]	1.0[§]	0.0[§]	0.896[§]	0.104[§]
Caucasians [35]	140	0.318**	0.668**[a]	0.014**	0.768**	0.232**	0.882**	0·118**	0.736**	0·264**
Japanese [36]	570	0.623 (n = 523)[b]	0.377 (n = 523)[b]	0.0 (n = 523)[b]	0.654 (n = 570)	0.346 (n = 570)	1 (n = 570)	0.0 (n = 570)	0.84 (n = 508)	0.16 (n = 508)
Danish [37]	200	0.365 (n = 200)[c]	0.635 (n = 200)[c]	0.030 (n = 200)[c]	0.814 (n = 366)[c]	0.186 (n = 366)[c]	0.881 (n = 210)[c]	0.119 (n = 210)[c]	0.724 (n = 210)[c]	0.276 (n = 210)[c]
Zambian [37]	200	0.398 (n = 200)[c]	0.341 (n = 200)[c]	0.252 (n = 200)[c]	0.974 (n = 193)[c]	0.026 (n = 193)[c]	0.892 (n = 181)[c]	0.108 (n = 181)[c]	0.5 (n = 189)[c]	0.5 (n = 189)[c]
Guangzhou Han, Chinese [22]	493	0.667	0.333	0	0.738 (n = 195)	0.262 (n = 195)	0.996	0.004	0.854	0.146
Chinese [38]	400	0.613	0.387	0	0.654	0.346	1	0	0.896	0.104
Turkish [39]	118	0.42	0.564	0.03	0.737	0.263	0.881	0.119	0.754	0.246
German [39]	119	0.391	0.601	0.025	0.744	0.256	0.908	0.092	0.731	0.269
Brazilians [40, 42]	123	0.65 (n = 100)[d]	0.83 (n = 100)[d]	0.0 (n = 100)[d]	0.95 (n = 100)[d]	0.05 (n = 100)[d]	0.822 (n = 121)[d]	0.178 (n = 121)[d]	0.711 (n = 123)[‡]	0.289 (n = 123)[‡]
African American [43, 44]	99	0.31	0.69	0.225 (n = 204)	NT	NT	NT	NT	NT	NT
Asian Indians [43, 44]	92	0.30	0.70	0.159 (n = 88)	NT	NT	NT	NT	NT	NT
Caucasians [43, 44]	90	0.37	0.63	0.045 (n = 222)	NT	NT	NT	NT	NT	NT
Hispanic [43, 44]	99	0.53	0.47	0.078 (n = 90)	NT	NT	NT	NT	NT	NT
Native American [43, 44]	98	0.55	0.45	0.011 (n = 94)	NT	NT	NT	NT	NT	NT

For all the test methods used by different study groups, except for PCR-SSP, any other method used has been indicated using symbols in superscript: [†]Multiplex PCR, [‡]PCR-RFLP, [§]DNA sequencing, *Multiplex PCR followed by Fragment Analysis, **Luminex allele-specific primer extension (ASPE) assay; [a]Additionally PCR-SSP was used to resolve the presence of the HNA-1b allele in samples assigned as HNA-1a/-1b/-1c or HNA-1b/-1c by the Luminex allele-specific primer extension (ASPE) assay, [b]PCR-rSSOP, [c]TaqMan-based real time PCR (Q-PCR), [d]Flow cytometry.

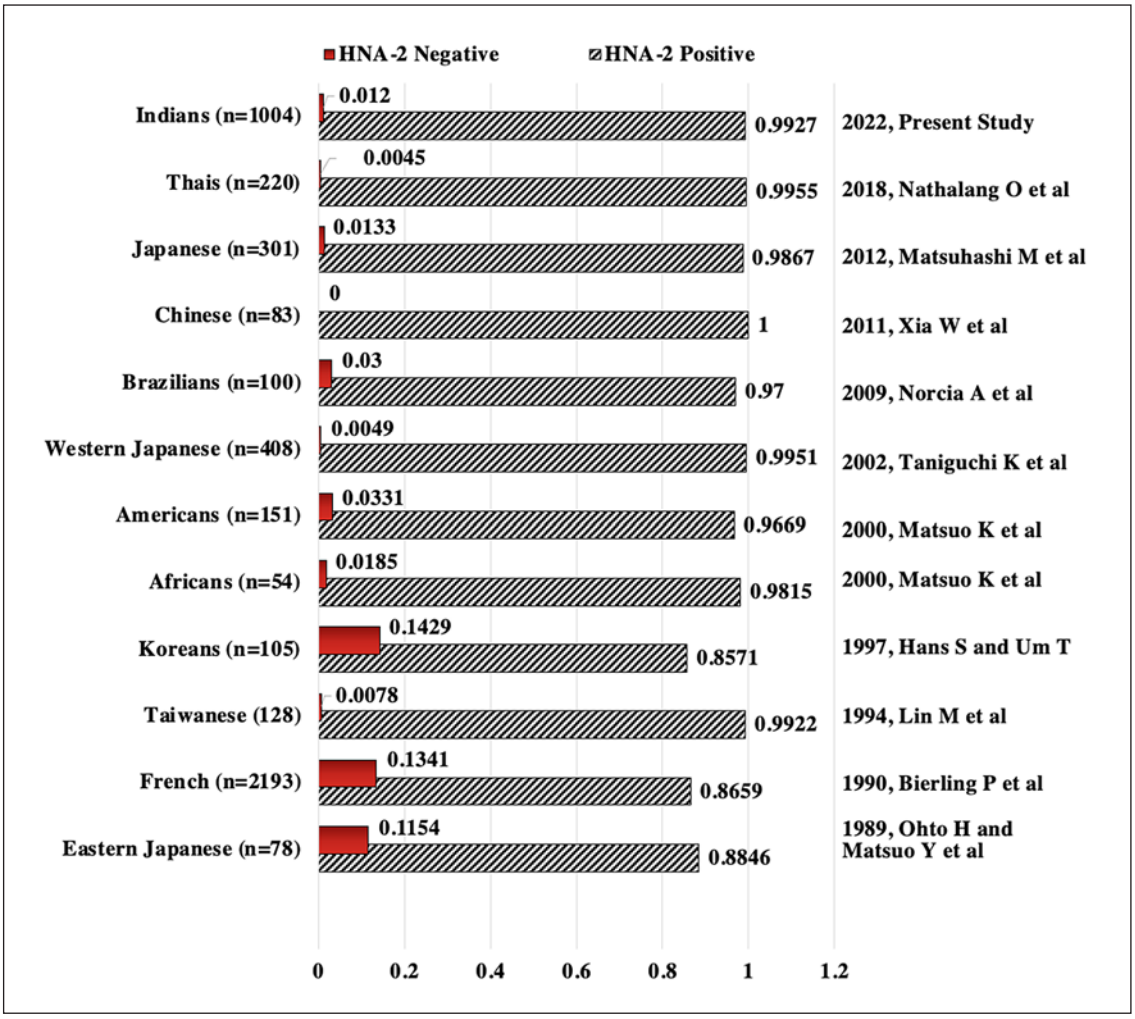

Fig. 1. Comparison of HNA-2 frequencies (determined by flowcytometry) among different populations studied worldwide.

FCGR3B alleles (HNA-1) were identified by direct DNA sequencing. *FCGR3B*01+02*+*03–* was the most common genotype as seen in other populations. Allele frequency of *FCGR3B*01* was comparable to Central Thais, Turkish, Germans but higher than Caucasians, Zambian and lower than Chinese and Japanese [22, 26, 30, 33, 35–37, 39]. Among all Asians, it was the lowest (Table 2). Frequency of *FCGR3B*02* was comparable to Chinese and Thais but was higher than Japanese and lower than Caucasians, Germans, Brazilians, and Turkish [22, 26, 30, 33, 35–37, 39, 42]. Frequency of *FCGR3B*03* (8.76%) was highest as compared to all other populations [22, 25–44] except Zambians. This may be due to the ethnic differences among both population groups and/or sample size variation. Higher frequency of HNA-1c suggests that the alloimmunization for HNA-1c may be greater in our population as compared to other populations. Hence, HNA-1c positive cells should be included in typed neutrophil antibody detection panels along with HNA-1a and 1b. In a recent study by Simtong et al. [60], it has been shown that individuals harbouring the HNA-1c allele, have higher affinity towards the human IgG, leading to enhanced neutrophil activation and thereby, clearance of *P. falciparum* via intracellular ROS. This corroborates to the fact that HNA-1c may be found higher in malaria endemic regions as compared to nonendemic regions. In the present study, higher frequency of HNA-1c may also be due to the selective pressure of malarial infection as it is endemic in India.

Hyperexpression of FCGR3B was observed in 2.5% individuals resulting from gene duplication events as reported among other populations [35, 37, 61]. Contrastingly, deficiency of FCGR3B or HNA-null genotype was found in approximately (1: 105) Indian donors. This frequency is significantly higher as compared to that among Chinese (Han) from Guangzhou (1 in 493) and from Southern (1 in 214) and Northern Thais (1 in 400) [22, 28–30].

This is the first study to report the frequencies of *FCGR3B*04* and *FCGR3B*05* in Indian donors. All *FCGR3B*04* positive individuals carried only two alleles and were negative for *FCGR3B*03*. This finding excludes that both alleles are located on the same chromosome in these individuals which has been earlier described for Caucasians. HNA-1 genotyping results indicates that instead of PCR-SSP, other medium to high throughput genotyping methods must be adopted which can detect these alleles in Indians. The presence of additional 230T>G and 230T>A (old nomenclature) substitutions found in the present study could be explained by a mechanism of somatic permutation [62].

HNA-3 genotyping was carried out using PCR-RFLP. Though it is slightly more expensive and time consuming than PCR-SSP, it was used to overcome shortcomings of PCR-SSP primer binding and further amplification if c.451C>T SNP was present. *SLC44A2*01+*02–* was the most common genotype identified among Indians as observed in other populations. The frequency of *SLC44A2*01* (0.81) found in Indians was higher than that seen among Central Thais, Chinese from Zheijiang and Guangzhou, Burmese, and Japanese, while it was comparable to that seen among Caucasians, Germans, and Turkish (Table 2) [22, 25, 29, 33–36, 38, 39]. Among all the Asian groups it was the highest. However, it was lower than seen among African Americans and Brazilians [40, 63]. Low frequency of *SLC44A*02* homozygous genotypes (3.6%) suggests that these individuals are at a lower risk of alloimmunization against HNA-3a as compared to Chinese from Zhejiang Han (13.2%), Japanese (11%), Chinese Dongs (7%), and Thais (6%) but they are at a higher risk as compared to the African Americans (0.04%) and English Caucasoid (2.9%) [33–36, 63, 64].

The allele frequency of *ITGAM*01* (95.5%) found in the present study is higher than that found among the Germans, Caucasians, and Turkish, but it is slightly lower than that seen among the other Asian groups such as Japanese, Thais, and Chinese (Table 2). The genotype *ITGAM*01+*02–* (HNA-4a homozygous) was found to be the most common genotype in all the previous studies [22, 25–42]. This suggests that antibodies to HNA-4a may also be involved in Indian patients with NAN and AIN as reported previously in other studies [40]. The frequency of HNA-5a i.e., *ITGAL*01* (23.7%) found in Indians is significantly lower ($p < 0.05$) as compared to that seen among all other populations worldwide (range: 50–89.6%) (Table 2) [22, 25–42]. These data confirm that the chances of alloimmunization and immune neutropenia due to anti-HNA-5a in the Indian population will occur more frequently as compared to any other population worldwide.

In the present study, HNA incompatibilities and risk of alloimmunisation against the HNA antigens in Indian population has been determined. As compared to other populations, quite similar rates of alloimmunization against HNA-1 and -3 antigens were seen among the Indians [22, 25–42]. However, HNA-4 alloimmunization risk was lower than Caucasian and African populations ($p < 0.05$) but similar to Asian populations. Though the estimated mismatch probability of HNA-5 is similar to other populations, the risk of alloimmunization against HNA-5a antigen in Indian population is significantly higher ($p < 0.05$) as compared to that seen among all other populations worldwide [18–44].

The data on HNA frequencies and their risk of alloimmunization have helped us understand the antigens which may be more commonly involved in causing immune neutropenias in Indian patients. Based on this, we prepared a 2-cell and a 3-cell indigenous panel which can now be used for detection of granulocyte reacting antibodies and thereby help us to investigate immune neutropenia cases.

Conclusion

This is the first study to report the frequencies of HNA among Indian blood donors. Varying distribution of HNA in our population as compared to other studies suggests the need to prepare population specific typed neutrophil panels for identifying HNA antibody specificities prevalent in Indian patients. Assays established for HNA detection assays in this study can now be utilized as diagnostic tools for evaluating any clinical scenario of immune mediated neutropenia where these antigens and their associated antibodies are involved.

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Statement of Ethics

This study was approved by the Institutional Ethics Committee, ICMR-NIIH with approval no: NIIH/IEC/02-2016.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Harita Gogri, Swati Kulkarni, and Ajit Gorakshakar designed the research study. Jayashree Sharma overlooked recruitment of study subjects. Harita Gogri and Meghana Parihar carried out the experiments. Harita Gogri analysed the data and wrote the manuscript. Ajit Gorakshakar, Swati Kulkarni, and Manisha Madkaikar critically reviewed the manuscript.

Data Availability Statement

All data generated or analysed during this study are included in this article and its online supplementary material. Further enquiries can be directed to the corresponding author.

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