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Asian flushing, *ADH1B*, aldehyde dehydrogenase 2 genotypic status among the unique ethnic population of the Himalayan state of Sikkim, India

Yazum Bhutia[1], Sanjiba Dutta[2], Mingma Lhamu Sherpa[3]

[1]PhD Scholar, [3]Professor and Head, Department of Biochemistry, [2]Professor and Head, Department of Psychiatry, Sikkim Manipal Institute of Medical Sciences, Sikkim Manipal University, Gangtok, Sikkim, India

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ABSTRACT

Background: Alcohol induced flushing a phenotypic instrument variable also known as "Asian flush" was first reported by Wolff (1972), commonly seen among East Asians. This phenotype is indicative of inactive aldehyde dehydrogenase 2 (*ALDH2*) and high activity of alcohol dehydrogenase (*ADH1B*). **Aims and Objectives:** This study aimed to examine the sensitivity and specificity of the simple flushing questionnaire for identifying inactive *ALDH2* and to examine the flushing, *ADH1B* and *ALDH2* status across the three unique ethnic groups in Sikkim, India. **Materials and Methods:** Two hundred and fifty consenting adults (age, ≥18 years) visiting the referral hospitals in East Sikkim were enrolled. Flushing questionnaire was assessed among the 201 alcohol users, who were categorized as "non-flusher," "current flusher," and "former flusher." *ALDH2* and *ADH1B* genotyping were done on the all 250 subjects by polymerase chain reaction - restriction fragment length polymorphism blinded from flushing response status. **Results:** In the study, 201 were alcohol users and 49 were non-alcohol users. The sensitivity and specificity of the flushing questionnaire against inactive *ALDH2* genotypes were 84.6% and 93%, and the positive predictive value was 45.8%. 19.5% Bhutia/Sherpa, 8.3% Nepalese and 11.1% Lepcha reported current flushing. Only two Bhutia reported former flushing while remaining were "non-flushers." *ALDH2* (6%) and *ADH1B* (4.4%) genotypes, respectively, were seen with allele frequency of 0.06 for *ALDH2*1/2*2*, and 0.004 for *ADH1B*2/*2*, 0.044 for *ADH1B*1/*2. ALDH2*2/*2* were not observed in this study. **Conclusion:** Alcohol induced flushing, *ALDH2* and *ADH1B* genotype is reported across the ethnicity, among Sikkimese people, and this flushing is the first report from India.

Key words: *ADH1B*; Aldehyde dehydrogenase 2; Alcohol induced flushing; Sensitivity; Specificity, Sikkim

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INTRODUCTION

Liver metabolizes 92–95% of alcohol, by alcohol dehydrogenase (*ADH1B)* and aldehyde dehydrogenase 2 (*ALDH2)*. Functional polymorphism of these genes is responsible for variability of alcohol metabolism between individuals.[1,2] The polymorphic alleles *ADH1B*2, ALDH2*2* are common among East Asians reported to provide protection from excessive alcohol consumption. This aversion to alcohol is due to unpleasant alcohol-related reactions like flushing response attributed to acetaldehyde accumulation due to the effect of high *ADH1B* and or lowered *ALDH2* activity, the major enzymes responsible for alcohol metabolism. Although this unpleasant response provides protection from excessive alcohol consumption, those that continue to drink, develop tolerance, and these individuals are also reported to be at higher risk for esophageal cancer and other alcohol-related diseases.[3-8]

Address for Correspondence:
Dr. Mingma Lhamu Sherpa, Professor and Head, Department of Biochemistry, Sikkim Manipal Institute of Medical Sciences, Sikkim Manipal University, Gangtok - 737 102, Sikkim, India. **Mobile:** +91-8967583004. **E-mail:** mingmals@yahoo.com

Wolff first reported racial differences in facial flushing after consuming small amounts of alcohol.[9] The alcohol induced flushing questionnaire consisting of two simple questions have demonstrated to be 90% sensitive and specific for detecting inactive *ALDH2*.[10] This flushing questionnaire indicates adverse reactions related to toxic effects of alcohol consumption. Many studies reported that *ADH1B* and *ALDH2* variants are associated with alcohol flushing in East Asian population.[10-13] There are no reported studies on alcohol flushing from India, neighboring countries Nepal and Bhutan, and only few inconsistent studies have examined *ADH1B* and *ALDH2* genes in the Indian subcontinent. This study was taken up with the view that Sikkimese people might share Mongolian, East Asian origin, and the investigators had come across people who reported unpleasant flushing reaction to even small doses of alcohol in the community; therefore, the study aimed to know the *ADH1B*, *ALDH2*, alcohol induced flushing status across the ethnic group of Sikkim, and to examine the reliability of the flushing questionnaire in this population. This report also intends to inform the clinicians, the role of flushing response as a proxy for *ALDH2* enzyme activity due to polymorphic genes for identifying candidates for screening the patients who are at the increased risk for alcohol-related diseases and cancers.

Aims and objectives

This study aimed to examine the sensitivity and specificity of the simple flushing questionnaire for identifying inactive *ALDH2* and to examine the flushing, *ADH1B* and *ALDH2* status across the three unique ethnic groups in Sikkim, India.

MATERIALS AND METHODS

This cross-sectional study was duly approved by the Institutional Ethics Committee (IEC/255/14–56), Sikkim Manipal Institute of Medical Sciences (SMIMS) and was conducted in the department of Biochemistry, SMIMS.

Inclusion criteria

Adults of 18 years and above visiting the referral hospitals, East Sikkim, from 2017 to 2021 were enrolled as participants.

Exclusion criteria

Any individual (age, <18 years), diagnosed with neurodegenerative disorders (dementia and mental retardation), psychosis, and cognitive impairment were excluded from the study.

The sample size of the study was calculated based on the reported 6.6% of *ADH1B*2* allele frequency in the Kachari population of Assam, at 95% confidence interval and a desired level of precision at 0.033% the proportion "P" was estimated to be 0.066 and a sample size of 236 participants was calculated.[14,15] An informed consent and an intake form were collected from all the participants. A total of 250 were enrolled in the study, and 201 participants were alcohol users who had consumed alcohol at least once in their lifetime and the rest (n=49) were non-alcohol users. Alcohol users were further classified as "ex-drinkers" who had consumed alcohol at least once in their lifetime but not during the past 12 months and "current drinkers" as those who had consumed alcohol at least once during the past 12 months.

The alcohol induced flushing questionnaire by Yokoyama et al., 2003 was administered to the 201 alcohol users. Both current and ex-drinkers completed the flushing questionnaire with responses categorized as "non-flusher," "current flusher," and "former flusher." For the sensitivity and specificity of "flushing questionnaire," the current and "former flusher" were basketed into one group as "flushers" and were measured using *ALDH2* expression as a gold standard (Table 1). Both *ADH1B* and *ALDH2* genotyping were done for all 250 subjects. This *ADH1B* and *ALDH2* genotyping was done without the information on flushing response from the questionnaire.

DNA from whole blood was extracted and quantification of DNA was done by nano-drop spectrophotometer.[16] *ADH1B* and *ALDH2* genes were amplified using standard polymerase chain reaction (PCR) reaction.[17] The PCR product of size ~108 bp was observed. The amplified product was subjected to restriction fragment length polymorphism (RFLP) wherein the PCR product of *ADH1B* was digested with *NmuCI* restriction enzyme, and *ALDH2* with *EcoRI*. The digested products run through 3% agarose gel electrophoresis for confirmation of the product.

All the data were entered using Microsoft excel sheet and exported to Statistical Package for the Social Sciences

Table 1: Simple flushing questionnaire to detect inactive *ALDH2*[10]

Q1	Do you have a tendency to develop facial flushing immediately after drinking a glass of beer (180 mL) or any drink containing alcohol?	Yes, No or Unknown
Q2	Do you have a tendency to develop facial flushing immediately after drinking a glass of beer (180 mL) or any drink containing alcohol in the first one or 2 years after you started drinking?	
Responses	Current flushers: Q1=Yes Former flushers: Q1≠Yes, But Q2=Yes Never flushers: Q1=Q2=No or Unknown	Inactive *ALDH2* Active *ALDH2*

ALDH2: Aldehyde dehydrogenase 2

version 20 for statistical analysis. Descriptive statistics for proportions and averages were used to report the demographic profile, alcohol use, *ADH1B* and *ALDH2* genotyping and flushing response. Fisher's exact test was used to study the gene distribution and association with alcohol induced flushing among the ethnic groups as the expected value in a cell was <5. *P*=0.05 was considered statistically significant where applicable.

RESULTS

The 250 participants enrolled in this study were with a mean age of 42.5 (±14.2) years, and 64.4% of the participants were females. Majority (81.6%) of the participants were married, 16.8% were single and only one divorced and three widowed. Most of the individuals were living in nuclear families and residing in urban areas. Among the participants 68.4% were Hindus, 27.6% were Buddhists and 4% were Christians. Of the total 250 participants, 201 (80.4%) were alcohol users, out of which 154 (76.4%) were current users, and 47 (23.3%) were ex-users (Table 2).

The genotype profile of *ADH1B* and *ALDH2* genotype revealed that majority of the participants were homozygous (*2*1/2*1*) for *ADH1B* (95%) and *ALDH2 (*94%). Only 4.4% (11) were heterozygous for *ADH1B*1/*2*. One (0.4%) of the participants were homozygous for *ADH1B*2/*2*. Fifteen (6%) were heterozygous for *ALDH2*1/2*2*. None of the participants were homozygous for *ALDH2*2/*2*. The allele frequency of *ADH1B* and *ALDH2* were within the Hardy-Weinberg equilibrium (Table 3).

Table 2: Demographic profile of the study participants

Variables	Frequency (n=250) (%)
Age (in years) (Mean, SD)	42.5±14.2
Gender	
Male	89 (35.6)
Female	161 (64.4)
Ethnicity	
Bhutia/Sherpa	49 (19.6)
Lepcha	11 (4.4)
Nepalese	190 (76)
Marital status	
Married	204 (81.6)
Single	42 (16.8)
Divorced/separated	1 (0.4)
Widowed	3 (1.2)
Family status	
Joint	53 (21.7)
Nuclear	197 (78.8)
Residence	
Urban	134 (53.6)
Rural	116 (46.4)
Religion	
Hindu	171 (68.4)
Buddhist	69 (27.6)
Christian	10 (4)
Alcohol use	
Yes	201 (80.4)
No	49 (19.6)
Alcohol use in past 12 months (n=201)	
Yes	154 (76.4)
No	47 (23.3)

The *ALDH2* distribution among the three ethnic groups of Sikkim-Nepalese, Bhutia/Sherpa, Lepcha were as follows. Out of 250, 182 Nepalese, 42 Bhutia/Sherpa, and 11 Lepcha were homozygous for *ALDH2 (2*1/2*1)*. The heterozygous *ALDH2 (2*1/2*2)* were seen in eight Nepalese and seven Bhutia/Sherpa; however, none were seen in Lepchas. The *ALDH2 (2*2/*2)* allele of *ALDH2* was not found in any of the participants. Similarly, the *ADH1B (2*1/2*1)* distribution among the three ethnic groups of Sikkim was similar. The homozygous expression for *ADH1B (2*2/*2*) was seen in only one Nepalese. None of the Bhutia/Sherpa and Lepchas were homozygous for *ADH1B (2*2/2*2)*, but two Bhutia/Sherpa and one Lepcha expressed heterozygous *ADH1B (2*1/2*2)* (Table 4).

For *ADH1B* polymorphism, the RFLP was 95 bp for the *GG (2*1/2*1)* genotype, and 60 bp for the *AA (2*2/2*2)* genotype; heterozygotes exhibited both fragments (95+60) for *AG (2*1/2*2)*.

ALDH2 polymorphism (RFLP) showed 86 bp for *GG (2*1/2*1)* genotype, and 108 bp for *AA (2*2/2*2)* genotype, which was not found among the participants, while both fragments were seen for *GA (2*1/2*2)* genotype (Figure 1).

The flushing questionnaire was administered to 201 alcohol users only, and 24 reported flushing, wherein they were categorized into "flushers" and "non-flushers" as described in the methodology. Sensitivity and specificity of flushing questionnaire using gene expression of *ALDH2* as exposure and flushing as an outcome were done for this study population. This study showed that 46% (11 of 24) of the "flushers" had inactive *ALDH2*, whereas only 1.1% (2 of 177) of "non-flushers" had inactive *ALDH2*. The sensitivity and specificity of the flushing questionnaire against inactive *ALDH2* genotypes of the study were 84.6% and 93%, and the positive predictive value was 45.8% and negative predictive value was 98.8%. For *ADH1B* gene, 0.6% (1 of 177) of "non-flushers" and 37.5% (9 of 24) of "flushers" had active polymorphic allele (*ADH1B1/*2*).

The ethnic profiling of alcohol users and alcohol induced flushing revealed that 144 (71.6%) Nepalese, 48 (23.8%) Bhutia/Sherpa, 9 (4.4%) Lepcha were alcohol users. Furthermore, 22 (8.8%) of the alcohol users were "current

Table 3: *ADH1B* and *ALDH2* distribution of the study participants

Gene	Genotype	No. of sample/ genotype	Genotype frequency (%)	Allele frequency*
ALDH2	*2*2/2*2	0	0	*1(p)=0.97
	*2*1/2*2	15	0.06 (6)	*2(q)=0.03
	*2*1/2*1	235	0.94 (94)	
ADH1B	*2*2/2*2	1	0.004 (0.4)	*1(p)=0.97
	*2*1/2*2	11	0.044 (4.4)	*2(q)=0.02
	*2*1/2*1	238	0.95 (95)	

*Was within Hardy Weinberg equilibrium. *ALDH2*: Aldehyde dehydrogenase 2

Table 4: Distribution of *ADH1B* and *ALDH2* among the three major ethnic groups in Sikkim

Gene	Genotype	Ethnicity (n=250)			Fisher's exact	P-value
		Lepcha	Bhutia/Sherpa	Nepalese		
ALDH2	*2*1/2*2	0	7	8	6.066	*0.04
	*2*1/2*1	11	42	182		
ADH1B	*2*2/2*2	0	0	1	3.192	0.65
	*2*1/2*2	1	2	8		
	*2*1/2*1	10	47	181		

*P=0.05 was considered to be statistically significant. *ALDH2*: Aldehyde dehydrogenase 2

flushers" and only 2 (0.8%) were "former flushers." The ethnic profile of "current flushers" were only 9.2% (13/144) of Nepalese, whereas 17.3% (8/46) Bhutia/Sherpa, and 11.1% (1/9) Lepcha. Only two Bhutia/Sherpa were "former flushers" while remaining were "non-flushers" (Figure 2).

The *ADH1B* and *ALDH2* genotypic variation with alcohol induced flushing was found to be statistically significant in Bhutia/Sherpa (P=0.05) and Nepalese (P<0.001) but not statistically significant in Lepcha. Since only one individual reported homozygous allele *ADH1B*2/*2, it was included in the heterozygous polymorphic group. The *ALDH2* variant was not found in Lepcha (Table 5).

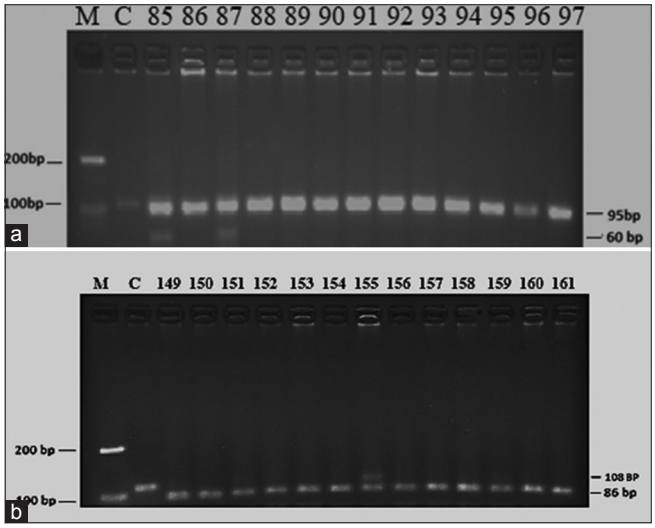

Figure 1: Restriction fragment length polymorphism (a) for *ADH1B*, Lane M- DNA ladder, C - control, 164 - AG, 159 - AA, other lane shows GG. (b) for aldehyde dehydrogenase 2, lane 155 - GA, other lane - GG

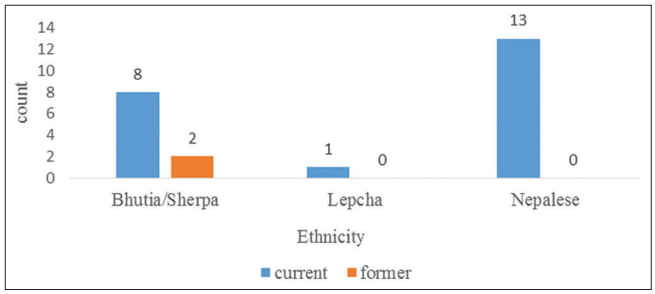

Figure 2: Alcohol flushing status across the three ethnic group of Sikkim

DISCUSSION

The complete metabolism of alcohol by the two major enzymes is encoded by the gene *ADH1B* and *ALDH2*. Polymorphic variants like *ADH1B*2, ADH1C*1, ALDH2*2* are expressed more commonly among East Asians and are more likely to have a protective role against excessive alcohol consumption. This protection is due to the increased blood level of acetaldehyde and its unpleasant toxic effects such as flushing, headache, tachycardia, nausea, vomiting, and hypotension.[10,13,17] Till date, there is no study on alcohol induced flushing response from the Indian population and moreover reports on these genes are few and inconsistent. Study involving 28 Indian tribal population in Southern India found complete absence of *ADH1B*2* allele and another study from North Indian Population reported the presence of polymorphic *ALDH2*2* and *ADH2*2* allele.[18,19] Allelic variation of *ADH1B* and *ALDH2* genes studied in a Native American Indian population with respect to alcohol dependence and two related intermediate phenotypes, flushing and binge drinking also reported absence of protective

Table 5: Association of *ADH1B* and *ALDH2* genotype with flushing status among three ethnic groups of Sikkim

Ethnicity/flushing status	*ADH1B*			*ALDH2*		
	*2*1/2*1*	*2*1/2*2 2*2/2*2*	P-value[a]	*2*1/2*1*	*2*1/2*2*	P-value[a]
Nepalese, n=144 (%)						
Non-flushers	130 (99.2)	1 (0.8)	<0.001**	131 (100)	0 (0)	<0.001**
Flushers	7 (53.8)	6 (46.1)		7 (53.8)	6 (46.2)	
Bhutia/Sherpa, n=48 (%)						
Non-flushers	38 (100)	0 (0)	0.04*	36 (94.7)	2 (5.3)	0.003*
Flushers	8 (80)	2 (20)		5 (50)	5 (50)	
Lepcha, n=9 (%)						
Non-flushers	8	0	0.11	8	0	b
Flushers	0	1		1	0	

[a]Fisher's exact test, [b]constant, *P=0.05, **P<0.001 was considered to be statistically significant. *ALDH2*: Aldehyde dehydrogenase 2

alleles *ADH1B*2* and *ALDH2*2* with a high prevalence of alcohol dependence among the subjects.[20] This study was undertaken with the view that the Sikkimese people might share Mongolian, East Asian, origin and might show a flushing response. This study observed the polymorphic *ADH1B* and *ALDH2* gene (Table 3), which was similar to study conducted by Goedde et al., reporting 9.9% of *ADH1B*2* allele frequency from the Indian population, whereas another study from India (on Kachari population, Assam) reported 6.6%.[14,21] A study conducted among aadibasi/janajati in Nepal reported high prevalence of *ADH1B*1/*1* genotype similar to this study.[22] Nepalese, Bhutia/Sherpa, amd Lepcha are unique ethnicities residing in the sub Himalayan region of Sikkim state, Darjeeling District of West Bengal, India and in Nepal. Moreover, the polymorphic allele for both genes *ADH1B* and *ALDH2*, the alcohol use report and flushing response, was proportionately higher among Bhutia/Sherpa (23.9%) when compared to Nepalese (8.3%) (Table 4 and Figure 2). The study also reported statistically significant relation between the both gene variant with alcohol induced flushing among Nepalese and Bhutia/Sherpa (Table 5). The sensitivity and specificity of the alcohol flushing questionnaire to identify inactive *ALDH2* used for the study was found to be high, and this may be considered as a candidate tool for screening for adverse phenotypic effect of alcohol consumption. Furthermore this tool may be used as a tool to guide the intervention and prediction of the risk for chronic toxic effects of alcohol including, chronic liver disease and cancer.

Limitations of the study

Recall bias was part of the design since the data collected on flushing response were self-reported but to mitigate that, the participants were categorized into former and current flushers. The study cannot draw any causal inference due to study design and sampling technique used.

CONCLUSION

The alcohol induced flushing, prevalence of *ADH1B* and *ALDH2* gene variants is reported across the ethnicity, and among Sikkimese people is the first report from not only Sikkim but India. Although a larger community-based study will need to be undertaken to establish the validity of the screening tool in the community at large, this simple flushing questionnaire tested in our study with high sensitivity and specificity makes it possible to propose alternative for the *ALDH2* genotyping which is laboratory intensive and expensive.

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Authors' Contributions:
YB- Concepts, design, literature search, data acquisition and analysis, statistical analysis, manuscript preparation, and editing and review**; SD-** Study concept and design, literature search, data acquisition, and manuscript editing and review**; MLS-** Concepts, design, definition of intellectual content, literature search, data acquisition and analysis, statistical analysis, manuscript preparation, and editing and review.

Work attributed to:
Department of Biochemistry, Sikkim Manipal Institute of Medical Sciences, Sikkim Manipal University, Gangtok, Sikkim, India.

Orcid ID:
Dr. Yazum Bhutia - https://orcid.org/0000-0002-2712-0462
Dr. Sanjiba Dutta - https://orcid.org/0000-0002-9957-2409
Dr. Mingma Lhamu Sherpa - https://orcid.org/0000-0002-5905-6127

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