J Nepal Health Res Counc 2022 Apr-Jun;20(55):454-9

Knowledge and DdeI Based Confirmation of Sickle Cell Anemia Among the Tharu Community

Sajani Ghaju,[1] Karan Khadayat,[1,4] Raju Shrestha,[2] Salan Ghaju,[3] Trishna Manandhar,[1] Bishnu P. Marasini[1,5]

[1]Department of Biotechnology, National College, Tribhuvan University, Kathmandu, Nepal, [2]Department of Microbiology, National College, Tribhuvan University, Kathmandu, Nepal,[3]Department of Biomedical Sciences, Kent State University, Ohio, USA,[4]Central Department of Chemistry, Tribhuvan University, Kirtipur, Kathmandu, Nepal, [5]Nepal Health Research Council, Ramshah Path, Kathmandu, Nepal.

ABSTRACT

Background: Sickle cell anemia is an inherited blood disorder caused due to a point mutation at the sixth codon of the β-globin gene of both alleles. Sickle cell traits occur when the mutation is in one of the two alleles of the β-globin genes. This study was carried out in the Tharu community, which is an indigenous and minority group mostly residing in the Terai region of Nepal. They are also considered as the most vulnerable group for inheriting Sickle cell anemia.

Methods: Purposive sampling, which included 130 Tharu individuals of Kanchanpur district of Nepal, was considered for the study. The survey was conducted using a descriptive questionnaire that contained relevant information including the family history of Sickle cell anemia. This was followed by the analysis of blood samples to determine the prevalence of Sickle cell anemia and Sickle cell traits. Primer-mediated enzymatic amplification of target sequences in genomic DNA followed by restriction endonuclease assay with an enzyme DdeI was carried out for the confirmation.

Results: Among 130 individuals, only 55.4% had basic knowledge about Sickle cell anemia. After screening for sickle cell anemia from 60 participants, 27 (45%) of them were found to be in the heterozygous state (carrier, Hb AS) and 28 (46.7%) were in the homozygous (normal, AA Hb) state with 5 (8.3%) having the diseased hemoglobin (Hb SS) variant of Sickle cell anemia.

Conclusions: This study demonstrated a high prevalence of Sickle cell anemia and Sickle cell traits in the Tharu community. This study may be beneficial for concerned personnel policymakers to reduce sickle cell cases by improving genetic literacy among the Tharu community.

Keywords: Hemoglobin; sickle cell anemia (SCA); sickle cell traits (SCT)

INTRODUCTION

Sickle cell anemia (SCA) is caused due to the point mutation in the sixth codon of ß-globin gene by the substitution of glutamic acid with valine.[1] The estimated number of infants with SCA is 300,000 annually[2] and over 300 million people are affected with sickle cell trait (SCT) worldwide.[3] The high prevalence of SCA lies in the malaria-endemic Terai belt of Nepal where carriers are protected against death from malaria. SCT offers resistance to malaria, through the process of natural selection.[4,5] Individuals with SCA undergo inflammation and pain due to vaso-occlusion[6], and other clinical manifestations.[7] Supportive treatments include folic acid, analgesics, RBC transfusion; and hydroxycarbamide.[8-10] Curative treatment involves bone marrow transplantation.[11] Genetic counseling among couples could help in reducing the disease prevalence. The study may help to understand the knowledge about SCA and its prevalence in a small region of Nepal.

METHODS

This was a cross-sectional study carried out among 130 Tharu individuals, who were purposively selected based on clan history and geographical location of the malaria-endemic region. The sample size was not calculated as Kanchanpur is a district known for the high density of its Tharu community.[12] The duration of the survey was from January 2019 to June 2019. The ethical clearance for this study was provided by Nepal Health Research Council, Government of Nepal (Reg. no. 5/2018). Verbal

Correspondence: **Bishnu P. Marasini, Nepal Health Research Council, Ramshah Path, Kathmandu, Nepal.Email:bishnu.marasini@gmail.com;dr.bishnu@nhrc.gov.np, Phone:+9779847066906.**

informed consent and written consent were acquired from each participant who accepted to be enrolled in the study. Guardians' consent was taken for the children below 18 years.

All data were collected through face-to-face structured interviews. Venous blood samples (3 mL) were collected in Vacutainer tubes with EDTA (Ethylene Diamine Tetra Acetic Acid) from 60 participants based on questionnaire responses with resembled symptoms and family history of SCA/SCT, and the blood samples were preserved at -20 ℃ before transportation and application of DNA extraction were done within a week of collection in the Department of Biotechnology, National College, Kathmandu.

DNA was extracted from blood collected from participants by using Quick-gDNA™ Blood Miniprep kit (Zymo Research, USA) following the manufacturer's instructions. Primer for amplification of ß-globin gene was designed using Primer 3 software.[13] The ß-globin gene sequence was obtained from the NCBI database, NCBI Reference Sequence (NG_059281.1). The target DNA sequence was amplified by the polymerase chain reaction (PCR) using the primers SCA F (5'-GCAACCTCAAACAGACACCA -3'position at 32-52) and SCA R (5'-CCTCACCACCAACTTCATCC -3'position at 112-132) that primed amplification of 101-base-pair (bp) segment of a beta-globin gene.[14] The DNA clean and concentrator-5 kit (Zymo Research, USA) was used to purify impurities present in PCR products. The protocol was followed as described by manufacturer and electrophoresis of PCR product was performed with 3% agarose gel. Finally, the amplification of the PCR product was analyzed.

The amplified DNA was digested with a restriction endonuclease DdeI (Biolab, New England) according to the manufacturer's instructions. All restriction reactions were performed in a final volume of 10 µL: 1 µL buffer, 0.75 µL of restriction enzyme, 4 µL of DNA sample, and nuclease-free water to maintain the volume. The reaction tube was incubated in a water bath at 37ºC for 25 minutes. Then, gel electrophoresis was carried out using 5 % agarose gel in TAE (Tris – acetic acid – EDTA pH 8.3) with ethidium bromide for the confirmation of the sickle cell hemoglobin S (HbSS), hemoglobin T (HbAS), and normal hemoglobin (HbAA) genotypes. The electrophoresis was run at 100 volts for 45 minutes. A gel documentation system (Clever scientific, UK) was used for visualization of the electrophoretic mobility of the gel. The DNA sizes was determined using a 100 bp Ladder (Takara, USA).[15] Samples were analyzed based on a positive sample which was derived from a defined sickle cell anemic patient and was provided by Seti Zonal Hospital, Dhangadi.

Data was entered using MS-Excel 2010. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) version 19.0 software (IBM), and *P* values <0.05 were considered significant. The Chi-square test was used to analyze the qualitative variables.

RESULTS

A total of 130 participants were enrolled in the study. All the enrolled participants responded to the self-structured questionnaires developed at the Department of Biotechnology, National College, Kathmandu. Gender-wise distribution of the participants showed that the majority of them (69; 53.1%) were females and 61 (46.9%) were males. Almost half of the respondents, 65 (50%) were in the age group between 15-29 years whereas individuals between the age group 60-74 years had the least representation with only 6 (4.6%) respondents. The average age of the study participants was (28.7 ± 14.2 S.D.). The marital status of the study participants showed that both married and unmarried respondents were comparable with 62 participants (47.7%) each, whereas only 6 (4.6%) widows comprised the study population (Table 1).

Table 1. Socio-demographic characteristic of respondent.

Socio demographic characteristic	Total (n=130)
	Number (%)
Gender	
Male	61 (46.9)
Female	69 (53.1)
Age group	
1- 14	16 (12.3)
15 - 29	65 (50)
30 - 44	25 (19.2)
45- 59	18 (13.9)
60 - 74	6 (4.6)
Average age in year ± SD	28.7±14.2
Marital status	
Single	62 (47.7)
Married	62 (47.7)
Widow	6 (4.6)

Table 2. Association between knowledge of sickle cell anemia with socio- demographic characteristics of respondents.

Variables	Knowledge about sickle cell anemia		p value
	Having (n=72) Number (%)	Lacking (n=58) Number (%)	
Gender			
Male	30 (41.7)	31 (53.5)	0.253
Female	42 (58.3)	27 (46.5)	
Age group			
1- 14	2 (2.8)	14 (24.1)	
15 - 29	46 (63.9)	19 (32.8)	
30 - 44	15 (20.8)	10 (17.2)	0.001
45 - 59	6 (8.3)	12 (20.7)	
60 - 74	3 (4.2)	3 (5.2)	
Marital status			
Unmarried	34 (47.2)	26 (44.8)	
Married	35 (48.6)	29 (50)	0.959
Widow	3 (4.2)	3 (5.2)	

Out of 130 participants, the knowledge about sickle cell anemia (SCA) was present in 72 (55.4%) whereas 58 (44.6%) of the respondents lacked knowledge about the disease. Among 72 knowledgeable participants, 42(58.3%) were females and 30 (41.7%) were males. In contrast, 27 (46.5%) of the female respondents and 31(53.5%) of the male respondents lacked knowledge about sickle cell anemia (p = 0.253). Similarly, a significant association between knowledge of sickle cell anemia in the enrolled age groups was found (p = 0.001) (Table 2) where 46 (63.9%) of the individuals in the age group between 15-29 years had knowledge about the disease but in contrast, the same age group had 19 (32.8%) participants who lacked the knowledge about the disease. Furthermore, the response of married and unmarried individuals in relation to the knowledge about SCA comprised 35 (48.6%) and 34 (47.2%) respectively with only 3 (4.2%) widows being aware of the disease (Table 2). In addition, there was not much difference in the awareness of the SCA between married and unmarried individuals representing 29 (50%), 26 (44.8%) of the study population respectively with widows in minority 3 (5.2%) who were unfamiliar with the clinical condition (p = 0.959) (Table 2).

The Source of knowledge about sickle cell anemia among the participants was found to be mainly from medical workers and through an awareness program with a representation of 24 (33.3%) each, information from friends was 18 (25%), family 5 (7.0%) and the facts acquired from school was 1 (1.4%) (Table 3).

Table 3.Source of knowledge about sickle cell anemia among respondent.

Source of knowledge about sickle cell anemia	Total (n=72) Number (%)
Medical worker	24 (33.3)
Family	5 (7.0)
Friend	18 (25)
School	1 (1.4)
Awareness program	24 (33.3)

Table 4. Respondent with familial history of SCA.

Socio demographic characteristic	Total (n=60) Number (%)
Gender	
Male	38 (63.33)
Female	22 (36.67)
Age group	
1- 14	8 (13.3)
15 - 29	24 (40)
30 - 44	19 (31.7)
45 - 59	8 (13.3)
60 - 74	1 (1.7)
Average age in year ± SD	12±9.30

A total of 60 samples were collected from participants based on the family history of sickle cell anemia in addition to the information collected from questionnaires. The samples were processed and the results were analyzed (Table 4). A 101 bp fragment of the ß-globin gene was amplified using PCR. (Additional file, Table S1, and S2). Fig. 1 shows agarose gel electrophoresis of the amplified product. The amplified product was then digested with the help of DdeI restriction enzyme which showed three types of PCR bands. The normal sample has two bands of 37 bp and 67 bp. A single band of 101 bp was seen in patients with sickle cell anemia and samples containing sickle cell trait has PCR bands of lengths 101 bp, 67 bp, and 37 bp when compared to the 100 bp ladder (Additional file, Figure S1). Among 60 samples, 5 were found to have homozygous sickle cell disease (SS), 27 had sickle cell trait (AS), and the remaining were normal hemoglobin genotype (AA) (Figure 2).

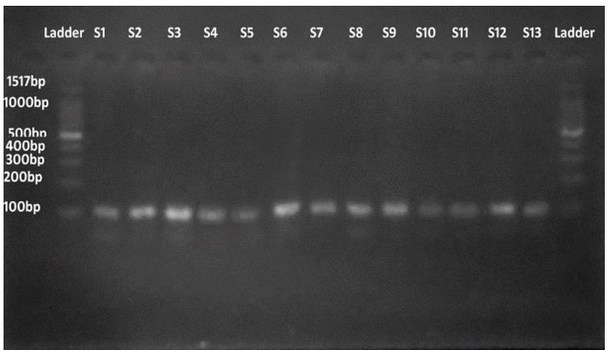

Figure 1. Amplified PCR product of ß globin gene in lane S1- S13.

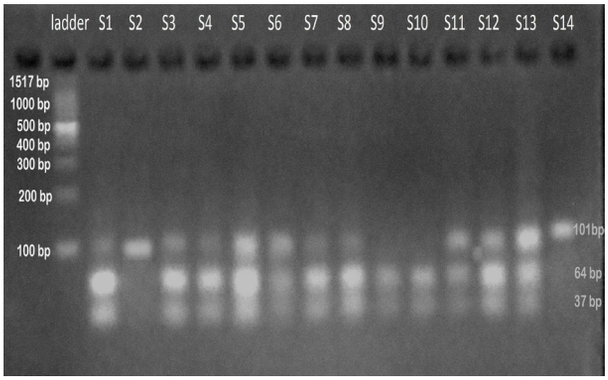

Figure 2. Restriction digestions of amplified products showing sickle cell mutation, heterozygous mutation, and normal ß globin gene. Lane 1: ladder; S1, S3, S4, S5, S6, S7, S8, S11, S12, S13: HbAS; S9, S10: HbAA; S14: HbSS; S2: positive control.

DISCUSSION

Almost half of the respondents had poor knowledge about sickle cell anemia. Therefore, health education programs and access to genetic counseling is needed at the primary level of health care to improve and increase the level of awareness. A study done in Manhattan and Nigeria has shown that there is a common misapprehension and poor understanding of sickle cell anemia even though a high prevalence of SCD is found among the respondents.[16,17] The findings in our study are consistent with the study done in Oakland, California conducted by Treadwell et al., in which over 68% of respondents had poor knowledge about the disease (32.8%).[18] In another study performed by Al-Suwaid et al., in Saudi Arabia it was found that the age group between 15-29 years constituted the highest number of people who lacked the knowledge about SCA.[19]

Medical personnel and awareness programs were a good source of information about SCA for the participants in our study. This was similar to a study conducted by Olakunle et al., in which the major source of information related to disease was chiefly from health professionals (36.5%).[17] A study done in Chicago, the United States by Acharya et al., showed that the most common sources of evidence related to clinical conditions were pediatricians (89%) and clinic staff (89%).[20] These findings recommend that extensive educational programs regarding diseases are mostly confined to hospitals, health posts, and communities.

The origin and demographic roots of sickle cell anemia are said to have begun in malarial endemic areas [21] with the Tharu being the popular indigenous ethnic group residing in these malaria-prone zones.[22] The study carried out among 100 suspected samples from the Tharu community of the southwestern region of Nepal revealed 5% SCA positive test, 38% SCT positive traits, and 36% normal cases [23] while in our study, we found 8.3% SCA positive test, 45% SCT positive traits, and 46.7% normal cases. From both studies, it is evident that there are high numbers of heterozygous cases among the Tharu community of Nepal. The study done among fertile aged women from western Nepal using the DdeI restriction enzyme reported 110, 54, and 56 bp in the heterozygous case.[24] Our study done among the Tharu community revealed an almost similar result with 101, 67, and 37 bp in the heterozygous case, 37 bp and 67 bp in normal samples, and 101 bp in the samples with gene mutations (Fig. 2). A study showed that a single point mutation eliminates new fragment of 376 bp in sickle cell anemia when compared to 201 bp and 175 bp fragments found in normal individuals and people with sickle cell traits respectively.[25] A similar study done by Monk et al showed DdeI digestion of amplified ß-globin sequencing revealed mutation by the formation of a PCR band of 381 bp and normal with 201 and 180 bp.[26] MstII restriction enzyme was used to detect sickle cell anemia producing PCR band of varying sizes of 54 bp and 56 bp in normal beta gene and 110 bp in mutated gene with a single fragment.[27]

Individuals with sickle cell trait (SCT) are generally healthy but bear a 50% chance of passing the trait on to their offspring.[28] Being asymptomatic, most SCT people have no clue about their condition, resulting in the potential of having a child with SCT or SCA.[29] Therefore, our study hopes to spread awareness about sickle cell anemia. A further large-scale study should be performed which would involve a more diverse population in order to extrapolate better generalizability of the target population and also for greater statistical reliability.

CONCLUSIONS

Based on the finding from our study, sickle cell trait is highly prevalent among the studied Tharu community ultimately increases the chance of sickle cell anemia in near future. Understanding knowledge about sickle cell inheritance is essential for its prevention. As certain population of the Tharu community is unknown about the disease therefore, formal genetic education about sickle cell anemia, trait condition, and access to genetic counseling, especially for people of the region is recommended.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Pauling L, Itano H, Singer S, Wells I. Sickle Cell Anemia, a Molecular Disease. Science, 1949;110(2865), 543-548. [Article]
2. Piel FB, Patil AP, Howes RE, Nyangiri OA, Gething PW, Dewi M, et al. Global epidemiology of sickle hemoglobin in neonates: a contemporary geostatistical model-based map and population estimates. Lancet. 2013;381(9861):142-51.[Article]
3. Tsaras G, Owusu-Ansah A, Boateng FO, Amoateng-AdjepongY. Complications associated with sickle cell trait: a brief narrative review. Am J Med. 2009;122(6):507-12. [Article]
4. Carlson J, Nash GB, Gabutti V, al-Yaman FA, Wahlgren M. Natural protection against severe *Plasmodium falciparum* malaria due to impaired rosette formation. Blood. 1994;84(11): 3909-3914.[Article]
5. Piel FB, Patil AP, Howes RE, Nyangiri OA, Gething PW, Williams TN, et al. Global distribution of the sickle cell gene and geographical confirmation of the malaria hypothesis. Nat Commun. 2010;1(1):1-7.[Article]
6. Ballas SK, Lusardi M. Hospital readmission for adult acute sickle cell painful episodes: frequency, etiology, and prognostic significance. Am J Hematol. 2005;79(1):17-25.[Article]
7. Ashley-Koch A, Yang Q, Olney RS. Sickle hemoglobin (Hb S) allele and sickle cell disease: a HuGE review. Am J Epidemiol. 2000;151(9):839-45.[Article]
8. Steinberg MH, Lu ZH, Barton FB, Terrin ML, Charache S, Dover GJ, Multicenter Study of Hydroxyurea. Fetal hemoglobin in sickle cell anemia: determinants of response to hydroxyurea. Blood. 1997;89(3):1078-88.[Article]
9. Adams RJ, Brambilla D. Optimizing Primary Stroke Prevention in Sickle Cell Anemia Trial I. Discontinuing prophylactic transfusions used to prevent stroke in sickle cell disease. N Engl J Med. 2005;353(26):2769-78. [Article]
10. Chou ST, Fasano RM. Management of patients with sickle cell disease using transfusion therapy: guidelines and complications. Hematol Oncol Clin North Am. 2016;30(3):591-608.[Article]
11. Hsieh MM, Fitzhugh CD, Weitzel RP, Link ME, Coles WA, Zhao X, et al. Nonmyeloablative HLA-matched sibling allogeneic hematopoietic stem cell transplantation for severe sickle cell phenotype. JAMA. 2014;312(1):48-56. [Article]
12. Marchand M, Gill C, Malhotra AK, Bell C, Busto E, McKeown MD, et al. The assessment and sustainable management of sickle cell disease in the indigenous tharu population of Nepal. Hemoglobin. 2017;41(4-6):278-82. [Article]
13. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3—new capabilities and interfaces. Nucleic Acids Res. 2012;40(15):e115- e115. [Article]
14. Williams DW, Wilson MJ, Lewis MA, Potts AJ. Identification of Candida species by PCR and restriction fragment length polymorphism analysis of intergenic spacer regions of ribosomal DNA J Clin Microbiol. 1995;33(9):2476-9.[PMC228446]
15. Kulozik AE, Lyons J, Kohne E, Bartram CR, Kleihauer E. Rapid and non-radioactive prenatal diagnosis of β thalassaemia and sickle cell disease: application of the polymerase chain reaction (PCR). Br J Haematol. 1988;70(4):455-8.[Article]
16. Siddiqui S, Schunk K, Batista M, Adames F, Ayala P, Stix B, et al. Awareness of sickle cell among people of reproductive age: Dominicans and African Americans in northern Manhattan. J Urban Health. 2012;89(1):53-8. [Article]
17. Olakunle OS, Kenneth E, Olakekan AW, Adenike OB. Knowledge and attitude of secondary school students in Jos, Nigeria on sickle cell disease. Pan Afr Med J. 2013;15(1).[Article]
18. Treadwell MJ, McClough L, Vichinsky E. Using qualitative and quantitative strategies to evaluate knowledge and perceptions about sickle cell disease and sickle cell trait. J Natl Med Assoc. 2006;98(5):704. PMCID: PMC2569269
19. Al-Suwaid HA, Darwish MA, Sabra AA. Knowledge and misconceptions about sickle cell anemia and glucose-6-phosphate dehydrogenase deficiency among adult sickle

cell anemia patients in al Qatif Area (eastern KSA). Int J Med Public Health. 2015;5(1) 86-92.[Article]

20. Acharya K, Lang CW, Ross LF. A pilot study to explore knowledge, attitudes, and beliefs about sickle cell trait and disease. J Natl Med Assoc. 2009;101(11):1163-72. [Article]

21. Lew VL, Bookchin RM. Ion transport pathology in the mechanism of sickle cell dehydration. Physiol Rev. 2005;85(1):179-200.[Article]

22. Terrenato L, Shrestha S, Dixit KA, Luzzatto L, Modiano G, Morpurgo G, et al. Decreased malaria morbidity in the Tharu people compared to sympatric populations in Nepal. Ann Trop Med Parasitol. 1988;82(1):1-1.[Article]

23. Gautam N, Gaire B, Manandhar T, Marasini BP, Parajuli N, Lekhak S, et al. Glucose 6 phosphate dehydrogenase deficiency and hemoglobinopathy in South Western Region Nepal: a boon or burden. BMC Res Notes 2019;*12*(1):1-6.[Article]

24. Tripathi GR. A Simplified and Cheapest Method for the Diagnosis of Sickle Cell using Whole Blood PCR and RFLP in Nepal. Tribhuvan University Journal. 2016;30(2);57–64.[Article]

25. Wilson JT, Milner PF, Summer ME, Nallaseth FS, Fadel HE, Reindollar RH, et al. Use of restriction endonucleases for mapping the allele for beta s-globin. Proc Natl Acad Sci U. S. A. 1982;79(11):3628-3631.[Article]

26. Monk M, Kenealy MR, Mohadjerani S. Detection of both the normal and mutant alleles in single cells of individuals heterozygous for the sickle cell mutation—prelude to preimplantation diagnosis. Prenat Diagn. 1993;13(1):45-53.[Article]

27. Ayatollahi M, Zakerinia M, Haghshenas M. Molecular analysis of Iranian families with sickle cell disease. J Trop Pediatr. 2005;51(3):136-40.[Article]

28. Gallo AM, Wilkie D, Suarez M, Labotka R, Molokie R, Thompson A, et al. Reproductive decisions in people with sickle cell disease or sickle cell trait. West J Nurs Res. 2010;32(8):1073-90.[Article]

29. Ojodu J, Hulihan MM, Pope SN, Grant AM. Incidence of sickle cell trait—United States, 2010. MMWR. Morb Mortal Wkly Rep. 2014;63(49):1155. PMCID: PMC4584538