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Association of Scn1a Gene with Neonatal Hypoxic-Ischemic Encephalopathy

Mavis Adusei-fosu[1], Hui Xu[1], Ying Li[1], Hongbin Qiu[2] and Ning Xie[3]

[1]Department of Biochemistry and Molecular Biology, Jiamusi University, School of Basic Medical Sciences, Jiamusi, Heilongjiang Province, China.

[2]Department of Molecular Epidemiology, Jiamusi University, School of Public Health, Jiamusi, Heilongjiang Province, China

[3]Department of Chinese Medicine Basic Theories, Heilongjiang University of Chinese Medicine, Harbin, Heilongjiang Province, China

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ABSTRACT

The study was to evaluate the association of SCN1A gene with hypoxic ischemic encephalopathy (HIE) in Heilongjiang province, Jiamusi city. Blood samples were taken from patients and genomic DNA extracted. Polymerase chain reaction (PCR) was used to amplify exons 10, 13 and 15 of SCN1A gene, and BstNI restriction enzyme used in digesting PRC products. Restriction fragment length polymorphism (RFLP) technique was used to detect gene polymorphism within SNP locus of SCN1A gene: exons 10, 13, and 15 among case and control groups. After screening of 210 blood samples from both case and control groups using PCR-RFLP technique, there was a significant difference between case and control groups' SCN1A gene exon 13, locus A221G but none for exon 10 and 15. There was a significant difference between case and control groups' SCN1A gene exon 13, locus A221G. AA genotype on comparison with AG and GG genotypes was found to be the highest risk factor for developing HIE. This study establishes an important foundation for further study on cause, prevention and treatment of neonatal HIE based on the gene mentioned above and other related genes.

Introduction

Hypoxic ischemic encephalopathy (HIE) in neonates is a condition that occurs when the entire brain is deprived of an adequate oxygen supply (asphyxia), this could be as a result of a number of reasons among which are; acute maternal hypotension, blood containing less oxygen due to poorly functioning lungs, trauma, among others. It can lead to cognitive disturbances and decreased motor control [1,2,3]. (Ferriero, 2004, Perlman, 2004 and Grow and Barks, 2002), skin may also appear bluish (cyanosis), and heart rate increases, severe cases may result in fainting, long term loss of consciousness, coma, seizures, cessation of brain stem reflexes, and brain death.

Although HIE treatment focuses on helping the child adapt to symptoms that result from the brain injury, asphyxia typically causes permanent damage, which sometimes continues to progress even after the asphyxia has been relieved. There is therefore the need to prevent the occurrence of HIE in neonates in order for children to have a normal life. So far, the best way HIE has been prevented is by eliminating asphyxia during pregnancy and delivery.

SCN1A gene encodes the large α-subunit of the neuronal voltage-gated sodium channel Nav1.1. Vertebrate sodium channel is a voltage-gated ion channel essential for the generation and propagation of action potentials, mainly in nerve and muscle. Mutations in this gene have been associated with several epilepsy, convulsion and migraine disorders.

A study carried out by Tang *et.at.* [4] suggests that SCN1A IVS5N+5G>A polymorphism is a risk factor of EFS and epilepsy, especially in Caucasian. Findings by Saitoh *et. al.* [5] provides further evidence that SCN1A mutations are predisposing factors for the onset of various types of acute encephalopathy. Also a study in China identified high percentage of SCN1A mutations in their Chinese cohort of Dravet syndrome patients but none in the rest of the patients [6]. Lin WD. *et. al.* [7]confirms the high sensitivity of SCN1A for the DS phenotype in a study carried out in Taiwanese DS patients, a suggestion of the possibility of mutations in SCN1A being involved in the cause of HIE.

6%-10% of children born in China yearly suffer from HIE, of which 20%-50% die, and about 300,000 of them suffer various degrees of disabilities, as many as 25% suffer permanent brain damage, epilepsy, mental retardation, visual and hearing impairments, paralysis and other serious complications which cause negative impact on families and the community, there are no effective HIE treatments at present [8]. The persistence of HIE however, has given the need for new preventive measures to be taken in order to if not eliminate, reduce it drastically. Scientists have hence found the need to study the aetiology of cerebral palsy, hence the need for HIE study at the molecular level, which has resulted in the awareness of SCN1A mutation as a possible cause of HIE.

This study was aimed at evaluating the association of SCN1A gene (exons: 10, 13 and 15) with hypoxic-ischemic encephalopathy in a number of neonates in Heilongjiang province in China.

Materials and Methods

Research objects

Blood samples were collected between the period of January 2012 to June 2013 in the Jiamusi University First Affiliated Hospital, Jiamusi Central Hospital, and Jiamusi Maternity and Child Health Care Clinic. 105 Blood samples taken from HIE patients were studied as the case group, whereas samples taken from patients without nervous system related diseases from Jiamusi University First Affiliated Hospital

Tele:

E-mail addresses: xuhui19782003@163.com

were studied as control group.

Experimental methods

200µl of each patient's blood sample was prepared for extraction of genomic DNA by following the protocol of the DNA extraction kit (TIANGEN, China). Exons 10 (480bp), 13 (374bp) and 15 (458bp) of SCN1A gene were amplified by PCR using the following primers:

1. Exon 10 (480bp), annealing temperature of 55°C
   Forward primer: 5'-GCCATGCAAATACTTCAGCCC-3'
   Reverse primer: 5'-CACAACAGTGGTTGATTCAGTTG-3'
2. Exon 13 (374bp), annealing temperature of 52°C
   Forward primer: 5'-GTATACCTTTTGGTGGTTCT-3'
   Reverse primer: 5'-TGGTTGAAAGACTGCTATAC-3'
3. Exon 15 (458bp), annealing temperature of 50°C
   Forward primer: 5'-ACCATTTCTAGGTAAAGCTC-3'
   Reverse primer: 5'-TGCATATCTTAAGTGGGTAC-3'

PCR products were then digested with BstNI restriction enzyme (BioLabs) and results detected by agarose gel electrophoresis.

Statistical analysis

Statistical analysis of the data was performed with statistical analysis software (SAS 9.13). The χ^2 test was used to verify whether the selected population was a genetic equilibrium group. The χ^2 test and Fisher exact test were used to compare the genotype distribution of case and control groups. The calculation of odd ratios (ORS) at 95% confidence intervals (CIS) was calculated by unconditional logistic regression analysis.

Results

Each of the case and control groups had a total of 105 samples, with ratio of men to women being equal in each group. There were 68 cases of males and 37 cases of females, accounting for 64.8% and 35.2% respectively. Refer to figures 1 to 7 for Electrophoresis results of extracted genomic DNA, PCR and genotyping results of exons 10, 13 and 15.

3.1. Electrophoresis result for genomic DNA of 10 samples (Fig. 1).

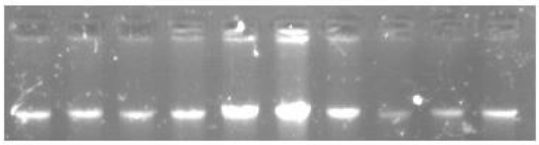

Figure 1. Electrophoresis results for genomic DNA

3.2. PCR-RFLP analysis results for exon 10 of SCN1A gene, SNP locus A150G (Fig. 2 & 3)

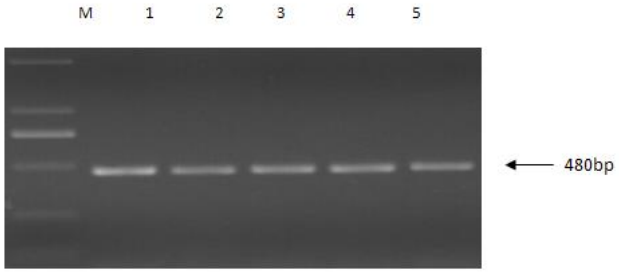

Figure 2. PCR results for exon 10 of SCN1A

1-2 for cases; 3-5 for controls; M for DL2000 Marker

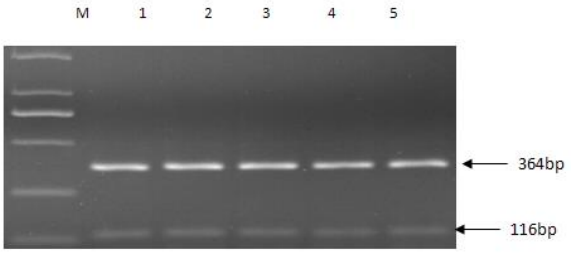

Figure 3. RFLP results for exon 10 of SCN1A

1-5 for restricted products; M for DL2000 maker

3.3. PCR-RFLP analysis results for exon 13 of SCN1A gene, SNP locus A150G (Fig. 4 & 5)

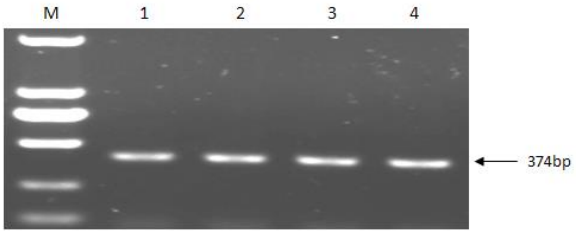

Figure 4. PCR results for exon 13 of SCN1A.

1-2 for cases; 3-4 for controls; M for DL2000 Marker

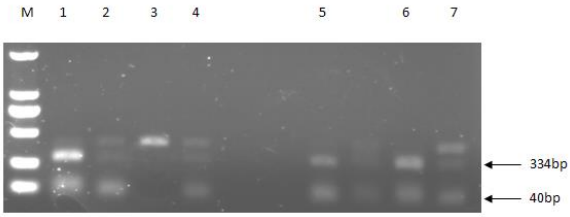

Figure 5. RFLP results for exon 13 of SCN1A

1, 5, 6 for homozygote AA; 2, 4, 7 for heterozygote AG; 3 for homozygote GG; M for DL2000 maker

3.4. PCR-RFLP analysis results for exon 15 of SCN1A gene, SNP locus C84T (Fig. 6 & 7).

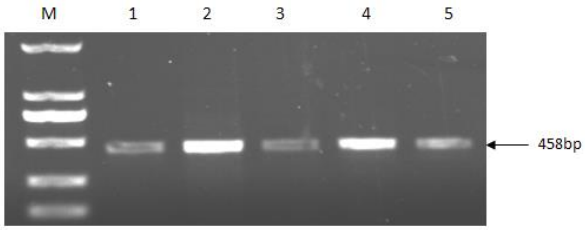

Figure 6. PCR results for exon 15 of SCN1A

1-2 for cases; 3-5 for controls; M represents DL2000 Marker

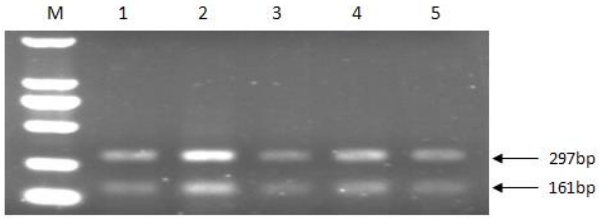

Figure 7. RFLP results for exon 15 of SCN1A

1-5 represents restricted product; M represents DL2000 maker

3.5. A221G allele and genotype frequencies distribution among case and control groups

Distribution of SCN1A genotype in case group and control group: there was no significant difference when observed and expected numbers of the different genotypes were compared using χ^2 test. The genotype distributions of case and control groups were corresponding with Hardy Weinberg equilibrium.

3.5.1. There was a significant difference in the genotype distribution of A221G locus in the case and control groups (P<0.05) (Table 1).

Table 1. A221G genotypic frequency distribution of case and control groups

Genotype	Control（N=10）	Case（N=10）	χ^2	P
AA（%）	53(50.5)	70(66.7)		
AG（%）	46(43.8)	32(30.5)		
GG（%）	6(5.7)	3(2.8)	3.721	0.036

3.5.2. There was a significant difference in the Allele frequency distribution of A221G locus in the case and control groups (P<0.05) (Table 2).

Table 2. A221G allele frequency distribution of case and control group

Genotype	Control（N=120）	Case（N=120）	χ^2	P
A	152（72.4%）	172（81.9%）		
G	58（27.6%）	38（18.1%）	5.262	0.026

3.5.3. Relationship between SCN1A gene and HIE: AA genotype proved to be the highest risk factor when all three genotypes were compared, at a P value of 0.036, OR = 0.383 at CI of 95% (Table 3).

Table 3. The relationship between A221G genotype and HIE

Geno type	Control（N=120）	Case（N=120）	P	OR(95%CI)
AA（%）	53(50.5)	70(66.7)		
AG（%）	46(43.8)	32(30.5)		
GG（%）	6(5.7)	3(2.8)	0.036	0.383 (0.201-0.962)

Discussion and Conclusion

With the initial completion of the human genome project, a large number of nucleotide information was deciphered. The study of genomic diversity was the first step towards the study of gene function, as well as understanding phenotypic differences between individuals and populations. The objective of the continuous genome research according to a hand book by Genomic Home Reference [9] include: Develop and apply genome-based strategies for the early detection, diagnosis, and treatment of disease, and find variations in the DNA sequence among people and determine their significance. The most common type of genetic variation is known as a single nucleotide polymorphism or SNP (pronounced “snip”). SNP types include deletion, insertion, missense, nonsense, etc. These small differences may help predict a person’s risk of particular diseases and response to certain medications. Each SNP represents a difference in a single DNA building block, called a nucleotide. SNPs occur normally throughout a person’s DNA. They occur once in every 300 nucleotides on average, which means there are roughly 10 million SNPs in the human genome. Most commonly, these variations are found in the DNA between genes and can act as biological markers [10], helping scientists locate genes that are associated with disease. Researchers have found SNPs to be relevant to drug therapy [11] and a risk for developing particular diseases such as Sickle–cell anaemia, β Thalassemia and Cystic fibrosis [12, 13, 14]. A genetic marker may be associated with a disease when the frequency of the genetic marker in patients are significantly more than that of non-patients, through the comparative analysis of the two haploid type and the study of linkage disequilibrium, any unknown disease causing gene may be identified within the genome. A considerable portion of the incidence of SNPs in different ethnic groups causes significant differences in the population. SNPs alone may cause mild or no changes to channel protein functions; they usually don't cause diseases directly but can change the individual's susceptibility to disease. SNPs may influence the pathogenic mutations; however the same gene mutation within different polymorphisms may behave differently under pathogenicity. As the commonest and widely distributed form of polymorphism and a source of genetic information, SNPs in addition to acting as genetic markers have the following advantages: used in disease linkage analysis, correlation analysis, and play an increasingly important role in susceptibility of individuals and populations to environmental factors and drug. With the completion of the Human Genome Project, there is growing emphasis on SNPs, which fully reflect genetic differences between individuals and groups, helps to design and use drugs based on specific individual SNPs, and helps to achieve a higher degree of individual disease screening, treatment and prevention.

Hardy Weinberg law is the most important population genetic theory which explains how reproductive gene frequency and genotype frequency groups are influenced. Hardy Weinberg equilibrium can be divided into three parts: (1) in an infinitely large randomly mating population, free from mutation migration, and natural selection; (2) The frequencies of the alleles do not change over time and (3) The sum of the genotypic frequencies can be predicted from allele frequencies. p^2 represents AA genotype, 2pq represents Aa genotype and q^2 represents aa genotype. The frequency of allele A (the dominant allele) is p and that of allele a (the recessive allele) is q. Genotype frequency must equal 1, that is: $p^2+2pq+q^2=1$. In short, the law states that: allele and genotype frequencies in a population will remain constant from generation to generation in the absence of other evolutionary influences. In this experiment, genotype distribution within cases and controls conform to Hardy Weinberg equilibrium, both case and control groups were drawn from a large population with presence of random mating between individuals, no inbreeding, migration and other interfering factors on population characteristics, a representative sample group.

With advances in genomics and molecular epidemiology, the role of genetic polymorphism in the occurrence and development of disease susceptibility in population has become of great interest to researchers. SCN1A gene mutation is one of the major causes of infant severe myoclonic epilepsy (SMEI) syndrome, can lead to seizures and severe mental retardation in children [15]. In-depth study of epilepsy, found that some of the symptoms of epilepsy are directly related to perinatal hypoxic-ischemic encephalopathy, suggesting SCN1A gene abnormalities may be related to the occurrence of HIE, while ischemia and hypoxia in different parts caused varying degrees of brain damage caused by the occurrence of epilepsy[16]. Various mutations in SCN1A gene (Nav1.1) cause voltage triggered disorder in the excitatory membrane, signal transmission repression, leading to reduced sodium ion channel function. In vitro experiments on rats have verified that SCN1A regulates the mechanism of ischemic brain injury expression [17]. In a study, Meta-analysis revealed a genome-wide significant association for mesial temporal lobe epilepsy with hippocampal sclerosis with febrile seizures at the sodium channel gene cluster on chromosome 2q24.3 [rs7587026] [18], within an intron of the SCN1A gene. Moreover, mutations were found to be present in

the SCN1A gene in generalized epilepsy with febrile seizures plus (GEFS +) patients after investigations [19, 20]. Wallace et. al. carried out SCN1A single strand conformation analysis on 53 cases of unrelated GEFS+ propositus, and found 3 abnormal SCN1A mutation sites[21].

PCR-RFLP method was used to analyze 105 cases, and the cases were compared with 105 controls, amplification of exons 10, 13, and 15 of the SCN1A gene was done by PCR, RFLP technique was then used to determine the relation between the SCN1A gene and HIE. Through the Research on relationship between SCN1A gene polymorphism and HIE, HIE mechanism from the molecular level can be expounded for provision of new approaches for prevention and treatment of HIE.

In this study, extracted genomic DNA from peripheral blood was amplified with SNP locus-specific primers, after which PCR products were digested with BstNI restriction enzyme. Experimental results show the following genotypic frequencies for SCN1A gene (A221G) polymorphism in case group: 6.7% AA genotype, 30.5% AG genotype, and 2.8% GG genotype; among control group there were 50.5% AA genotype , 43.8% AG genotype, and 5.7% GG genotype. There was significant difference between the genotype distribution of case and control groups by χ^2 test ($P<0.05$). It was estimated that AA genotype proved to be at the highest risk when AA, AG, and GG genotypes were compared, (adjust OR=0.383 at 95% confidence interval (CI) = 0.201-0.962, P=0.036). This suggests that the polymorphism in A221G locus of SCN1A gene is associated with HIE susceptibility. Based on this study, there was no detection of abnormalities in the A150G and C84T loci of SCN1A gene, and hence can be said to have no association with HIE.

The study of link of HIE with other SNP sites will continue, as well as the influence of other SNPs on heredity of HIE susceptibility. Occurrence of HIE is not solely due to mutations but also environmental factors. The results of this study may be due to sample size being small and not representative enough, as well as different genetic backgrounds of the studied population. There is hence the need for further research on a larger sample, conducting genetic screening for functional SNPs and their relationship with disease aetiology, mechanism and susceptibility, and lastly comparing of the difference between the gene polymorphism of different ethnic groups.

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