Journal of Clinical and Medical Images

Research Article

ISSN: 2640-9615 | Volume 8

The Molecular Characteristics of Congenital Muscular Torticollis Patients Living in Qinghai-Tibetan Plateau

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Received: 26 Sep 2024
Accepted: 15 Oct 2024
Published: 21 Oct 2024
J Short Name: JCMI

Keywords:

Congenital Muscular Torticollis; Qinghai-Tibet Plateau; Whole exon sequencing; Genetic variations; Cancer targeted therapeutics.

Citation:

Yongcui Wang, The Molecular Characteristics of Congenital Muscular Torticollis Patients Living in Qinghai-Tibetan Plateau. J Clin Med Img. 2024; V8(4): 1-6

1. Abstract

1.1. Background

Congenital Muscular Torticollis (CMT) defined as the interstitial fibrosis and contracture of one side of the sternocleidomastoid muscle (SCM) usually results in the head and neck deviating to the affected side, the lower jaw turning to the opposite side, and limitation of the rotation of the head and neck. As the largest and highest region in China, Qinghai-Tibetan Plateau (QTP) is one of the important global biodiversity hotspots.

1.2. Methods

The blood and SCM bio-samples from 20 patients and their parents from Qinghai Women and Children's Hospital. The clinical properties, including gender, ethnic group, initial diagnosis age, ect, were collected, and the whole exon sequencing was performed on blood and SCM bop-samples.

1.3. Results

The female to male ratio was 9:11 for these 20 patients, the age varied from 1 to 13 years old, 17 of them showed SCM fibrosis, and 18 of them were found CMT symptoms when they were born. The number of single nucleotide polymorphisms (SNP) varied a lot for different chromosome, the C->T and G->A were the most common alteration types, and patients have no significant differences in various ethnic groups in terms of cancer driver genes. The comparison of molecular variations in family members suggested the genetic variations for CMT, which could provide promise biomarkers in treatment of CMT in QTP.

1.4. Conclusions

The findings offer a great opportunity to further understanding the divergent mechanism of CMT in QTP, increase the efficacy for diagnosis and prognosis, finally lead the optimal targeted therapeutics.

2. Introduction

Congenital Muscular Torticollis (CMT) is one of the most common diseases in infants in the world with the incidence rate of CMT varied between 0.3% and 1.9%. It defined as a contracture or fibrosis of the Sternocleidomastoid muscle (SCM), on one side, leading to a homolateral inclination and contralateral rotation of the face and chin. Congenital torticollis usually manifests in the neonatal period or after birth [1]. The previous studies indicate a ratio of 1 per 250 new borns being the third congenital orthopaedic anomaly, more frequently following congenital hip dysplasia and the calcaneovalgus feet [2]. Most common symptoms include the infant's head tilts to one side, the infant's chin turns toward the opposite side of the head, and firm, small, one to two centimeter mass in the middle of the SCM [3]. The diagnostic procedures for

congenital muscular torticollis may include X-ray and Ultrasound examination. Treatment may include gentle stretching exercise program (to help relieve the tension and lengthen the SCM), infant stimulation (to help the infant learn to move and stretch the muscle), and surgery (to correct the shortened muscle) [4]. The cause of CMT remains unclear, and scholars have proposed a variety of theories based on different findings, such as birth trauma theory. The birth injury theory is based on the fact that one-third of the children with CMT have breech or dystocia, so it is speculated that SCM is squeezed by labor or forceps assisted delivery during delivery, resulting in CMT [5]. However, it cannot explain why new borns with section or vaginal delivery also have CMT, and pathological examination of postoperative specimens showed no signs or evidence of bleeding [6]. Some research reported that family inheritance of genes in two patients with partial trisomy on chromosome 13q, and the gene for CMT was suggested to be located on chromosome 13 [7]. It has also been suggested that CMT has a familial predisposition [7], and CMT might be hereditary disorders.

The worldwide incidence rate of congenital torticollis varies between 0.3% and 1.9 % [8]. The incidence in different regions of China also displays obvious differences, among which northwest China and northeast China are highly affected by the healthcare condition. Qinghai province is located in the Qinghai-Tibet Plateau (QTP). QTP has a complex geological history, and it is a common understanding that the central plateau uplifted first and formed the `proto-QTP' as early as 40 Mya, followed by outward extensions in early Miocene [9-12]. The agricultural and pastoral areas in Qinghai are vast, with difficult natural environment, relatively weak sanitary conditions and awareness. There are many ethnic minorities, leading to more challenge in treatment of CMT. Therefore, we analyzed the clinical and molecular characteristics of CMT patients living in Qinghai Province, in order to better understand its pathogenesis, so as to better treatment.

Here, for better understanding the molecular mechanism of CMT in plateau and offer the promise therapeutic strategies specially designed for patients living in QTP, we collected blood and SCM tissue bio-sample from 20 CMT patients and their parents at Qinghai Women and Children's Hospital, and discussed their unique molecular characteristics. We found that, these patients were among 1 to 13 years old, most of them were male, and all of them were living at place with average altitude of 2200. By sequenced 43 blood and CMT tissue bio-samples, we found that, most of single nucleotide polymorphisms (SNP) are in chromosome 1 and 2, with C->T and G->A as the most common alteration types, and has no significant differences among different ethnic group. Most importantly, the comparison of molecular variation among patients and their parents indicated that patients share over 70% genome variations with their parents, meaning the genetic disorder might be involved in CMT.

3. Methods

3.1. Bio-Sample Specimens

After receiving informed consent, about 2cm of diseased tissue was taken from patients undergoing surgery at Qinghai Women and Children's Hospital. Then they were fixed by 10% formaldehyde immediately after leaving the body, and stored in liquid nitrogen. The venous blood bio-samples for patients and their parents were also collected. This work was performed in compliance with all relevant ethical regulations for research using human specimens. The fresh frozen tissues and blood bio-samples were delivered to sequencing company, Frasergen (Table S1), to capture the exonic DNA fragments and perform the whole exome sequencing. All methods were performed in accordance with the relevant guidelines and regulations for research using human specimens.

Whole Exome sequencing and Mutation calling. The Illumina HiSeq 2000 instrument was applied for whole exome sequencing (WES), which generated 2×150 base paired-end reads. FASTQ files were aligned to the human genome assembly (hg38) via Burrows–Wheeler Aligner (BWA) [13]. Before further analysis, using SAMtools [14], Bedtools [15] and Picard (https://broadinstitute.github.io/picard/), the initially aligned BAM files were pre-processed to remove duplicated reads, locally realigned reads around potential small indels, and recalibrate base quality scores. The SNP was detected through the Genome Analysis ToolKit (GATK) [16] and annotated via ANNOVAR [17]. The duplicated and low-quality SNPs were removed before annotation. The final SNPs were called by removing common SNPs from dpSNP [18].

3.3. Identification of Cancer Driver Gene

To distinguish the cancer driver genes from the passenger genes, the Max MIF [19], which was reported to outperform the existing state-of-the-art methods, such as MUFFINN [20], MuttSig2 [21], MutSigCV [22], on TCGA pan-cancer datasets, was introduced. MaxMIF integrated the genome mutation data and molecular interaction data by a maximal mutational impact function. The protein-protein interaction (PPI) network from HumanNet v2 [23] was introduced to represent the molecular interaction.

4. Results

4.1. Clinical Characteristics

The total of 53 blood and SCM tissue bio-samples from 20 CMT patients and their patents were collected, 43 of them were sequenced for 18 patients (Table S1). The clinical information, including the initial diagnosis age, the gender, the residency, the position of illness, and the ethnic group, were shown in Figure 1. Most of patients were male (11/20) (Figure 1A) and the illness position were in the right place (Figure 1B), all these patients were minor ethnic group with hui and zang accounting for the majority (Figure 1C). The initial diagnosis age was from 1 to 13 years old, and most

of patients are around 1 to 3 years old or 9 to 11 years old (Figure 1E), and they were living in the different district in Qinghai province with average altitude higher than 2200 miters (Figure 1F). Among all 20 patients, 17 patients had SCM Fibrosis, and 18 patients had CMT symptoms when they were born (Figure 1D).

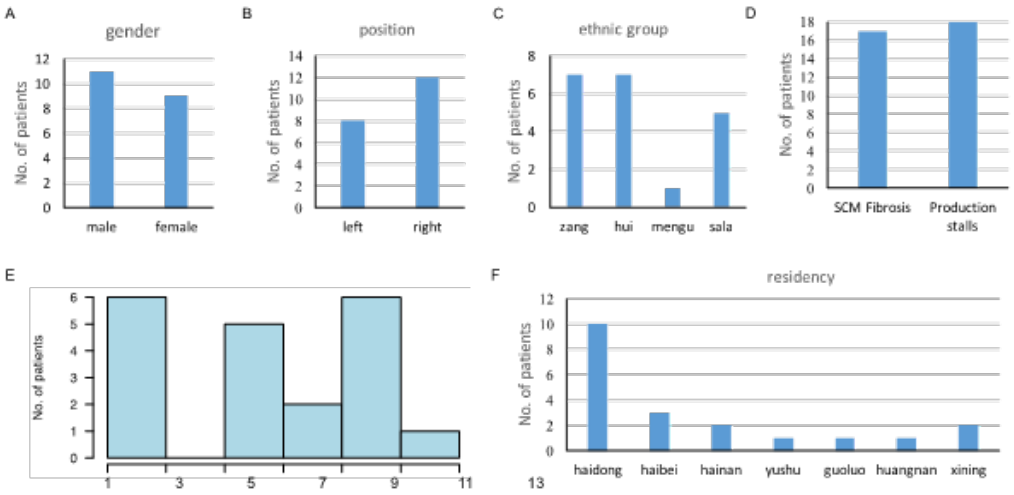

Figure 1: Clinical characteristics for 20 CMT patients in QTP. The clinical information includes the gender (subfigure A), illness position (subfigure B), ethnic group (subfigure C), status of SCM fibrosis and production stalls (subfigure D), the initial diagnosis age (subfigure E), and residency (subfigure F).

4.2. Molecular Characteristics

To clarify the molecular characteristics of CMT patients living in QTP, we performed the WES analysis. The SNP distribution along the chromosome showed that the number of variations was the largest in chromosome 1, and decreased along the autosome (Figure 2A). This was quite different form the reports for western patients, in which the most SNPs were found in chromosome 13 [7]. The distribution of SNP alteration type shows the C->T and G->A were accounting for the vast majority (Figure 2B).

To distinguish the potential driver genes from passage genes, MaxMIF was introduced [19]. The first 30 genes with highest MaxMIF scores were shown in Figure 2C. As you can see, there is no significant differences among diverse ethnic groups in terms of driven gene. The further comparison of 15 patients' variations indicates that, patients has about 8%~30% identity variations (Figure 3), and over 70% variations came from their parents, meaning CMT might be caused by genetic disorder.

To identify the potential biomarkers for CMT, we put our focus on three patients with their SCM tissue also be sequenced. Figure 4 showed that, tissue and blood shared over 70% common variations, and the common variations shared by three patients' blood and SCM tissue might offer promise biomarkers for treatment CMT. Among 281 common varied genes, 145 genes varied in all other 15 patients, the DAVID enrichment analysis indicated that they enriched in chromosome 19 with p-value of 0.016 and including 13 genes (FCGBP, MUC16, CIB3, PSG11, DMKN, ADAMTS10, WDR87, FUT5, SDHAF1, PLIN4, POLR2E, HOMER3, ZNF772), suggesting the chromosome 19 might be involved in CMT patients living in QTP. DAVID functional and pathway enrichment analysis showed that 145 common varied genes were enriched in biological process of angiogenesis with p-value of 0.049 and including 4 genes (NRP1, PLXND1, FN1, RAPGEF3), in molecular function of Heparin-binding with p-value of 0.014 and including 4 genes (NRP1, AOC1, SAA1, FN1), in KEGG signal pathway of antigen processing and presentation with p-value of 0.078 and including 3 genes (KLRC2, KLRC3, HLA-B). All these results indicate the biological function and pathways might be involved in progressing of CMT in QTP. In sum, the unique clinical and molecular characteristics for plateau CMT patients were detected, suggesting some therapeutic targets for them.

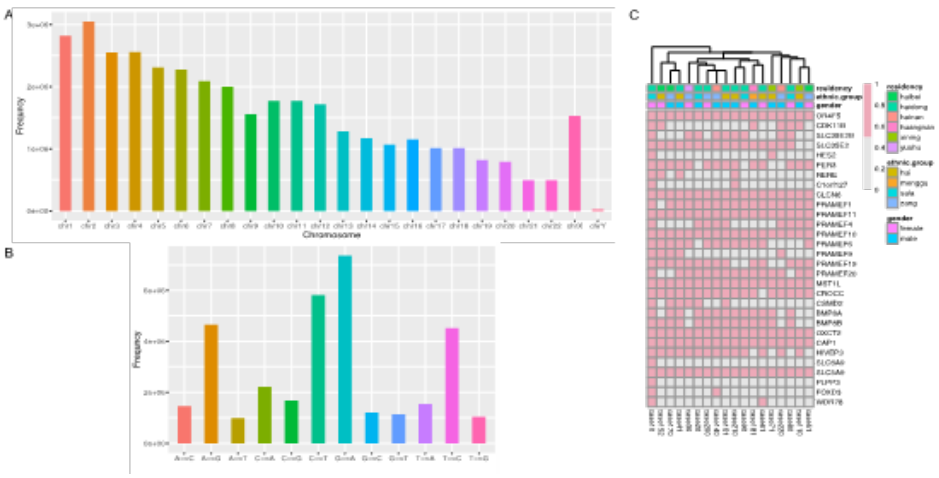

Figure 2: **The molecular characteristics for 18 CMT patients with their bio-samples been successfully sequenced**. A, the distribution of genome variations along chromosome. B, the distribution of variation type. C, the mutation profile for top 30 cancer drive genes. The patients' gender, ethnic group, and residency information were shown with distinct colours.

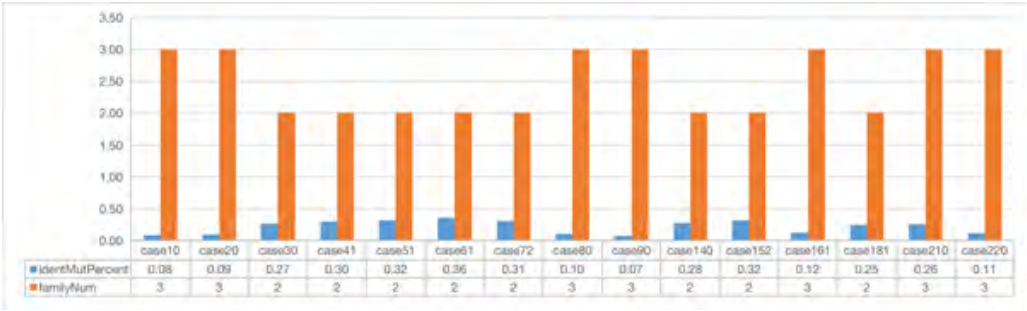

Figure 3: The distribution of genome variations obtained from patients and their parents. Note: identMutPercent: the percentage of genome variations only obtained from patients; familyNum: the number of family members for corresponding patient.

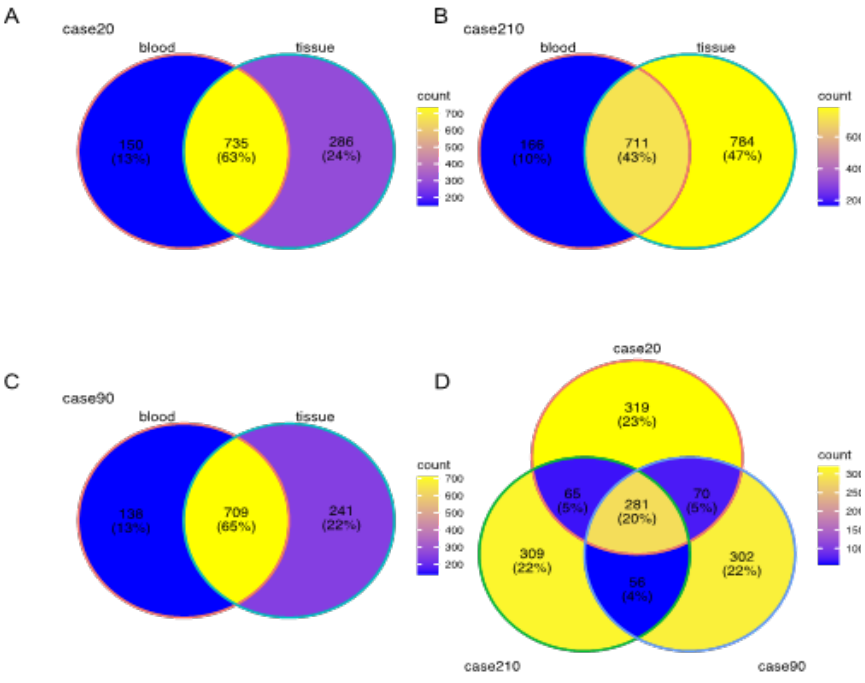

Figure 4: Potential biomarkers. A-C, Vene diagrams show genome variations shared by blood and SCM bio-samples for same patient. D, the genome variations shared by all three patients with both blood and tissue been successfully sequenced.

5. Discussion

Torticollis in children is the third most common pediatric orthopaedic diagnosis in childhood. Patients usual present with a stiff and tilted neck, and require a thorough and systematic work-up, including a complete physical and neurologic examination and cervical spine radiographs [24]. Qinghai province is in northeast QTP with average altitude of 4000 meter. Han Chinese and lots of minor ethnic groups people (such as hui, zang (Tibetan people), zhi, sala, and so on) live here. Here, to better understand the molecular mechanism of CMT patients living in QTP, and predict the most promise therapeutic targets for these patients, we collected the blood and SCM tissue bio-samples from 20 CMT patients and their parents at Qinghai Children and Women's Hospital, and discussed the clinical and molecular characteristics for those patients. The most of CMT patients were male (11/20), and most of them were right torticollis (12/20). Among 20 patients, 17 had their SCM fibrosis, and 18 were found torticollis when they were born. By comparing the patients' variations with their parents', only 8%~30% unique variations were found. The comparison of variations in blood with that obtained in SCM tissue showed that two types of bio-samples shared about 70% genome variations, the enrichment analysis on the common variations indicate the chromosome 19 might be involved in the development of CMT in QTP. There are around 5,000,000 people living in Qinghai province, and based on the recent statistic reports, only around 2,000,000 people live in around the capital of Qing Hai province, Xining. The sample collection and followed up tracing are quite challenge here. It took us one and half years to collect these 20 patients. In future, we will collect much more patients and their parents' information with followed up treatment reports to further show the unique characteristics of plateau patients and discuss their special treatment strategy.

6. Conclusions

Here, to better understand the molecular mechanism of CMT patients living in QTP, and predict the most promise therapeutic targets for these patients, the WES was performed on blood and SCM tissue bio-samples from 20 CMT patients and their parents at Qinghai Children and Women's Hospital. Several unique molecular characteristics for those CMT patients were found, including having more SNPs located in chromosome 1 with C->T and G->A as the most common alteration types, barely sharing the cancer driver genes among diverse ethnic group, and the chromosome 19 might be involved in the development of CMT in QTP, et al.

7. Funding Information

This work was supported by a grant from Qinghai Children and Women's Hospital Research Program (No. 2020-FEYN-02), Yunnan Fundamental Research Projects (202401AS070077), and Introducing talents start-up fund of Kunming Institute of Botany, Chinese Academy of Sciences.

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