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Molecular & Cellular Biomechanics 2025, 22(2), 1250.
https://doi.org/10.62617/mcb1250

Article

The molecular characteristics of congenital muscular torticollis patients living in Qinghai-Tibetan Plateau

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CITATION

Zhang T, Bao C, Wang Y. The molecular characteristics of congenital muscular torticollis patients living in Qinghai-Tibetan Plateau. Molecular & Cellular Biomechanics. 2025; 22(2): 1250. https://doi.org/10.62617/mcb1250

ARTICLE INFO

Received: 24 December 2024
Accepted: 7 February 2025
Available online: 11 February 2025

Abstract: Background: Congenital Muscular Torticollis (CMT) is defined as interstitial fibrosis and contracture of one side of the sternocleidomastoid muscle (SCM), typically resulting 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 China's largest and highest region, the Qinghai-Tibetan Plateau (QTP) is recognized as one of the world's critical biodiversity hotspots. **Methods:** The blood and SCM bio-samples from 20 patients and their parents at Qinghai Women and Children's Hospital were collected. The clinical properties of these individuals, including gender, ethnic group, and age at initial diagnosis, were analyzed. Whole exon sequencing was then performed on the blood and SCM bio-samples to characterize the molecular properties of CMT patients living in the Qinghai-Tibetan Plateau (QTP) region. **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 across different chromosome and lots of them were located in chromosome 1 and 2. The variation of C→T and G→A were the most common alteration types, and patients have no significant differences in various ethnic groups. The comparison of molecular variations in family members suggested the genetic variations for CMT, which could provide targets in treatment of CMT. **Conclusions:** The findings indicated that glucose and lipid metabolism, as well as antigen processing, might be involved in the development of CMT. This could increase the efficacy of diagnosis and prognosis, ultimately leading to the development of optimal targeted therapeutics for CMP patients living in QPT.

Keywords: Congenital Muscular Torticollis; Qinghai-Tibet Plateau; whole exon sequencing; genetic variations; cancer targeted therapeutics

1. 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 an ipsilateral (homolateral) tilt of the head and contralateral rotation of the face and chin. CMT usually occurs in the neonatal period or after birth [1]. Previous studies suggest that CMT occurs in 1 out of 250 newborns, which ranks as the third most common congenital orthopedic anomaly and usually following congenital hip dysplasia and calcaneovalgus feet [2]. The patients with CMT usually got head tilts to one side, 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 X-ray and

Ultrasound examination were the most common strategies to be the diagnostic procedures for CMT. Treatment options for CMT may include gentle stretching exercise program to relieve the tension and length of SCM, infant stimulation to help the infant learn to move and stretch the affected 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 can not explain why newborns 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 studies on the risk factors for Italy CMT patients with over 13 years old suggested that the presence of headache or vomiting were strongly correlated with an underlying urgent condition of CMT [8].

The worldwide incidence rate of CMT varies between 0.3% and 1.9% [9]. The incidence of CMT displays obvious regional differences across China, with the northwest and northeast regions being the most highly affected areas due to healthcare-related factors [10]. Qinghai province is located in the Qinghai-Tibet Plateau (QTP), which has a complex geological history. It is a common understanding that the central plateau uplifted first and formed the 'proto-QTP' as early as 40 million years ago (Mya), followed by outward extensions in early Miocene epoch [9–12]. The agricultural and pastoral areas of Qinghai province are vast, characterized by a challenging natural environment and relatively weak sanitary conditions and health awareness. There are many ethnic minorities in Qinghai province, which had very different living habits and health awareness, leading to more challenge in treatment of CMT. Therefore, we attempted to investigate 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, to better understand the molecular mechanism of CMT in plateau region 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. The initial diagnosis age, the gender, the residency, the position of illness, and the ethnic group were collected to characterize the clinical features of these patients. We found that the 20 CMT patients were between 1 to 13 years old, most of them were male, and all were living at plateau regions with an average altitude of 2200 m. Then the whole exome sequencing (WES) was conducted on blood and SCM tissue bio-samples, and their molecular characteristics was analzyed. As a result, the majority of single nucleotide polymorphisms (SNPs) were located on chromosomes 1 and 2, with C→T and G→A being the most common alteration types. Importantly, there were no significant differences in SNP profiles among the different ethnic groups. Most notably, the comparison of molecular variations between the patients and their parents indicated

that the patients shared over 70% of their genome variations with their parents, suggesting that genetic factors are likely involved in the development of CMT. The shared variations between blood and SCM tissues that represent the somatic mutations resulting from the development of disease hints the potential therapeutic targets for CMT. By analyzing the mutations shared by blood and SCM tissues, the glucose and lipid metabolism, as well as antigen processing were suggested to be involved in the development of CMT in QTP populations. This could increase the efficacy of diagnosis and prognosis, ultimately leading to the development of optimal targeted therapeutics.

2. Methods

2.1. Bio-sample specimens

The patients and their parents were received the ethics approval from ethics committee office at Qinghai Women and Children's Hospital, which includes the aims of this project, which information will be collected and how long will it be kept, how their private will be protected, and how to contact the ethics committee office once they have any concerns about this recruitment, etc. After agreeing and signing the ethics approval, 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. The WES was performed on blood and SCM tissues by a sequencing company, Frasergen, to capture the exonic DNA fragments and perform the sequencing. The sequencing fastaq files were used for further investigation. All methods were conducted in accordance with the relevant guidelines and regulations for human specimen research.

2.2. Whole exome sequencing and mutation calling

The WES was implemented via Illumina HiSeq 2000 instrument. 2×150 base paired-end reads were generated and the intial fastaq files were obtained for further analysis. Burrows–Wheeler Aligner (BWA) was introduced to align sequences to the human genome assembly (hg38) [13], and the BAM files were used to alignment and assemble. SAMtools [14], Bedtools [15], and Picard (https://broadinstitute.github.io/picard/) was applied to initially aligned sequenced sequences to human Hg38 data and pre-processed the aligned data, including remove duplicated reads, locally realign reads around potential small indels, and recalibrate base quality scores. Then SNPs was called via the Genome Analysis ToolKit (GATK) [16] and the duplicated and low-quality SNPs were removed. The detected SNPs were then annotated through an annotation software, ANNOVAR [17]. To filter out common population-level SNPs, the final set of SNPs was identified by removing any that were present in the dbSNP population variation database [18]. Those SNPs or variations were statistics according to their locations along chromosome and the variation type, such as A→T, C→G, etc, and the statistical significance was tested via wilcoxon rank sum test.

2.3. Prediction of disease driver gene

MaxMIF [19] that was reported to outperform the existing state-of-the-art methods, including MUFFINN [20], MuttSig2 [21], MutSigCV [22] on pan-cancer datasets sourced from The Cancer Genome Atlas (TCGA), was introduced to distinguish the disease driver genes from the passenger genes. The genome mutation data and human protein-protein interactions extracted from HumanNet [23] were integrated into MaxMIF model by a maximal mutational impact function. In particular, the mutation matrix M with row as gene, column as samples, and binary value indicating whether the gene was mutant or not at corresponding sample was obtained by summarizing the whole exon sequencing data. The MaxMIF score for gene a was determined as follows:

$$S_{\mathrm{MaxMIF}}(a) = \max_{j \in N_a} \mathrm{MIF}(a, j),$$

where N_a was the neighbouring gene set of gene a in PPI network, $\mathrm{MIF}(a, j) = \frac{S(a)S(j)}{r_{a,j}^2}$, $\mathrm{r_{a,j}}$ is the weight of interaction between gene a and gene j in PPI network, $S(x) = \frac{\sum M(x,)}{G}$, $M(x,)$ is the x row sums of mutation matrix M,G is the number of total samples. The genes with top 30 MaxMIF scores were defined as the disease driver genes.

3. Results

3.1. Clinical characteristics

The total of 53 blood and SCM tissues from 20 CMT patients and their parents were collected, and 43 of them sourced from 18 patients were successfully sequenced for characterize the molecular mechanisms of CMT patients living in QTP (Appendix **Table A1**). The clinical information, including the initial diagnosis age, the gender, the residency, the position of illness, and the ethnic group, were anazlyzed to summarize the clinical characteristics of CMT patients living in QTP.

Most of patients were male, and they were taken 55% (11/20) (**Figure 1A**). As for the position of CMT, there were 12 patients out of 20 ones found in the right side (**Figure 1B**), All 20 patients were minor ethnic group with hui and zang accounting for the majority, and both two ethnic group have seven patients (**Figure 1C**). Besides of zang and hui patients, there were five sala patients and one menggu patient. Zang and hui are the two largest ethnic minorities in Qinghai, they are account for about 25% and 15% of the entire population in Qinghai, respectively. Zang people usually live at Hainan, yusu, guoluo and huangnan district with altitude beyond 3500 m, and hui people usually live at haidong and haibei district with relative low altitude (altitude between 2000 and 3000 m). Therefore, our CMT patients were almost zang and hui people, and whether there were some genomic differences among these ethnic groups were discussed later.

Among all 20 patients, 17 patients had SCM Fibrosis, and 18 patients had CMT symptoms when they were born (**Figure 1D**). The initial diagnosis age was varied from one to 13 years old, and most of patients were around one to three years old or night to 11 years old (**Figure 1E**), and they were living in the different region in

Qinghai province (**Figure 1F**). While haidong district had the lowest average altitude at around 1800 m, other districts such as yushu, guoluo, and huangnan had average altitudes exceeding 4000 m.

In summary, the Congenital Muscular Torticollis (CMT) patients in this study lived at an average elevation of approximately 3000 m above sea level. Most of them exhibited CMT symptoms from birth, with the condition tending to manifest on the right side. The CMT patients were either diagnosed at a young age (1–3 years old) or between 9–11 years old.

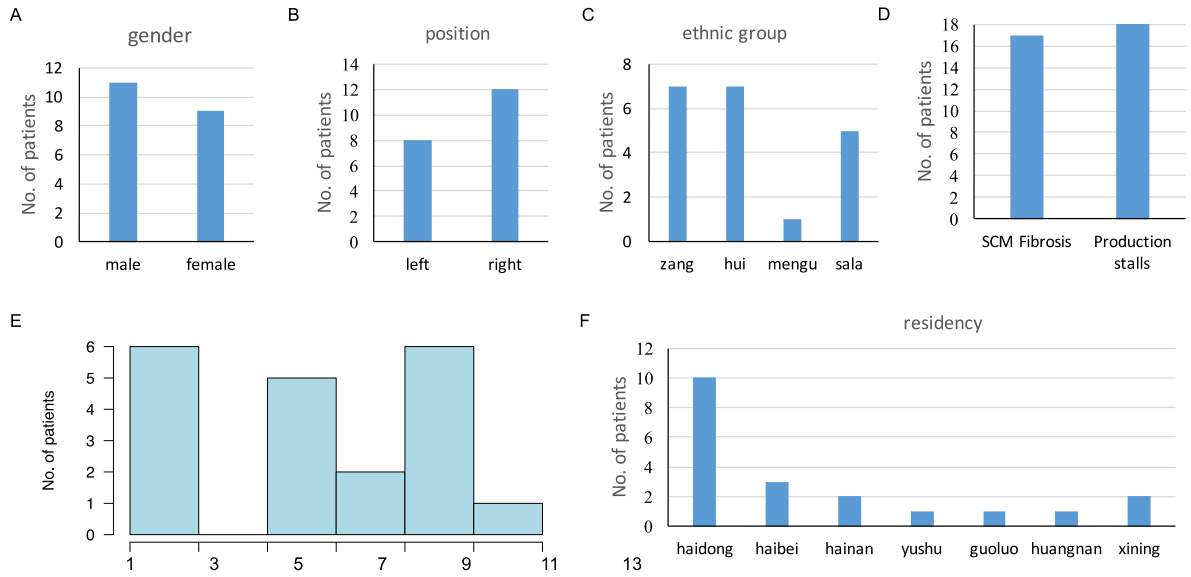


Figure 1. Clinical characteristics for 20 CMT patients living in plateau region. **(A)** The clinical information includes the gender; **(B)** illness position; **(C)** ethnic group; **(D)** status of SCM fibrosis and production stalls; **(E)** the initial diagnosis age and **(F)** residency.

3.2. Molecular characteristics

To clarify the molecular characteristics of CMT patients living in QTP, the genome data was generated by WES data. We firstly check the distributions of SNP along the 22 autosome and two heterosome. As shown in **Figure 1A**, the most of variations was located in chromosome 2, and decreased along the autosome (**Figure 2A**). DAVID functional and KEGG pathway enrichment analysis were conducted on mutant genes located in Chromosome 2 (a total of 226 genes) to identify the molecular functions and biological processes involved in CMT progression. The enriched terms in molecular function of protein binding, ATP binding, and guanyl-nucleotide exchange factor activity (Appendix **Figure A1A**), cellular component of cytosol, cytoplasm, and receptor complex (Appendix **Figure A1B**), biological process of activation of cysteine-type endopeptidase activity involved in apoptotic process, endocytosis, and phosphorylation (Appendix **Figure A1C**), and KEGG pathway of proteoglycans in cancer, regulation of actin cytoskeleton, and arrhythmogenic right ventricular cardiomyopathy were revealed, indicating the important roles of molecular binding, apoptotic process, and pathways linked to complex disease, such as cancer and cardio vascular disease in CMT progression.

The most of variations were located in chromosome 1 and 2. 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 showed the C→T and G→A were accounting for the vast majority (**Figure 2B**).

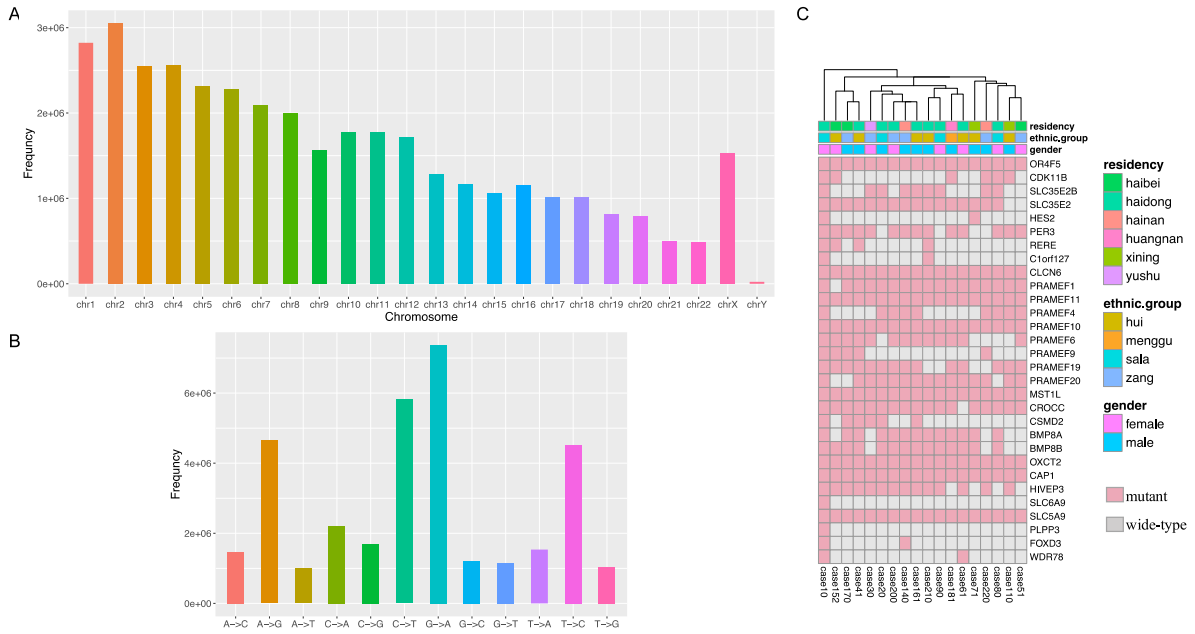


Figure 2. The molecular characteristics for 18 CMT patients with their bio-samples been successfully sequenced. **(A)** the distribution of genome variations along chromosome; **(B)** the distribution of variation type; **(C)** the mutation profile for top 30 disease driver genes. The patients' gender,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

The we checked that whether there some significant differences in molecular characteristics among various ethnic groups, gender, or residency in terms of disease driver genes. To distinguish the potential driver genes from passage genes, MaxMIF was introduced [19]. The first 30 genes with highest MaxMIF scores were selected as disease driver genes. The mutations in CMT patients with different ethnic groups, gender, and residency were displayed in **Figure 2C**. As you can see, there is no significant differences among either diverse ethnic groups or gender or residency in terms of disease driven genes. Particularly, there was no significant differences in number of mutations for top 30 disease driver genes happened in male and female patients (**Figure 3A**), in hui and zang patients (**Figure 3B**), and in patients living in district with altitude lower or higher than 3000 m (**Figure 3C**). The wilcoxon rank sum test was performed here and *p*-value less than 0.05 was accepted as the significance.

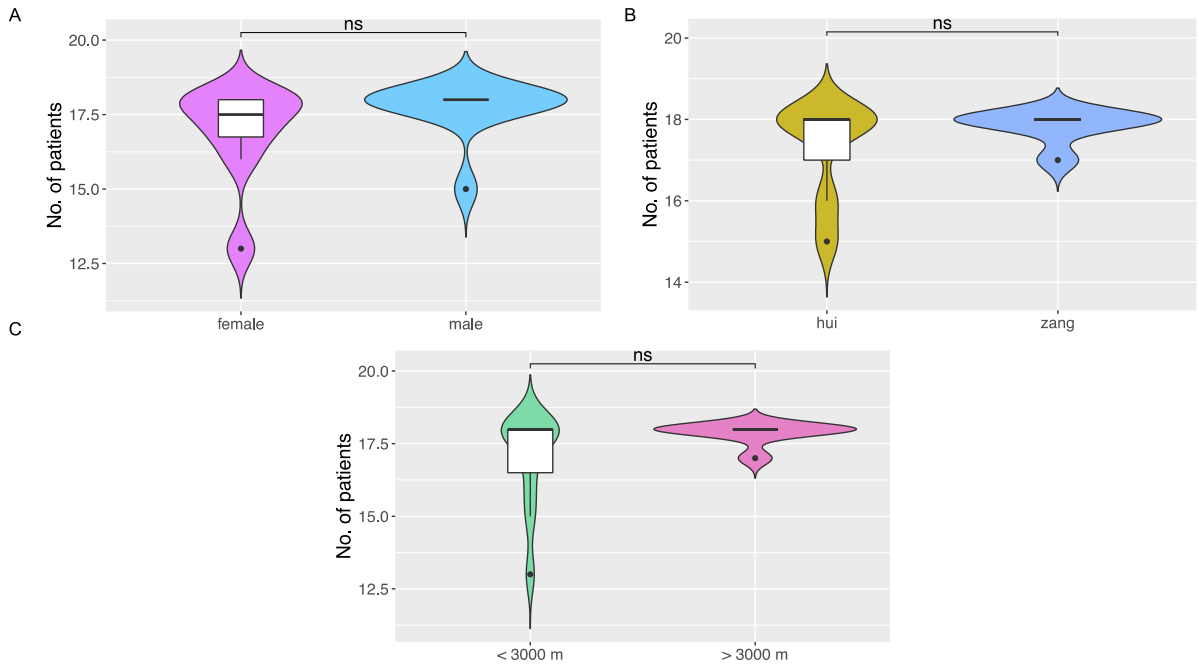
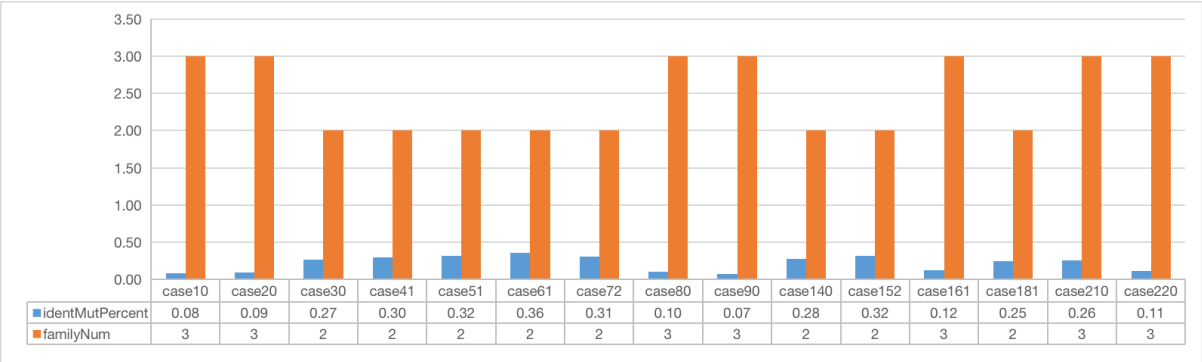


Figure 3. The differences in mutation profiles of top 30 disease driver genes among various groups of patients. **(A–C)** the boxplots show the differences in mutation profiles of top 30 disease driver genes between male and female patients, hui and zang patients, and patients living in place with altitude higher than 3000 m or not.

To identify the relationships of genomic features between patients and their family, we compared the SNPs obtained from patients and those from their parents. There were 15 patients with their parents' genome data available. To avoid the genetic factor, the variations for patients' parents were called by removing common human variations from Genome 10,000 and variations from both children' and parents' blood. Then the SNPs from parents were compared with those from children. From **Figure 4**, we can see that patients have about 8%~30% identity SNPs that were differ with their parents (**Figure 3**). That is, there were over 70% common variations between patients and their parents, which imply that CMT might be caused by genetic disorder.



	case10	case20	case30	case41	case51	case61	case72	case80	case90	case140	case152	case161	case181	case210	case220
identMutPercent	0.08	0.09	0.27	0.30	0.32	0.36	0.31	0.10	0.07	0.28	0.32	0.12	0.25	0.26	0.11
familyNum	3	3	2	2	2	2	2	3	3	2	2	3	2	3	3

Figure 4. The distribution of genome variations obtained from patients and their parents.

Note: identMutPercent (blue bar): the percentage of genome variations only obtained from patients; familyNum (orange bar): the number of family members for corresponding patient.

The somatic mutations represent the variations resulting from the development of disease. Therefore, to identify the potential therapeutic targets for CMT, we put our focus on three patients with their SCM tissue also be sequenced, and summarized the

shared mutations between blood and SCM tissue from same patient. **Figure 5** showed that, tissue and blood shared over 70% common variations, and the common variations shared by three patients' blood and SCM tissue represented the somatic mutations resulting from the disease progression that might offer promise targets for treatment of CMT. Among 281 common varied genes, 145 genes varied in all other patients. The DAVID enrichment analysis indicated that they were enriched in chromosome 19 with *p*-value of 0.016 and 13 genes (FCGBP, MUC16, CIB3, PSG11, DMKN, ADAMTS10, WDR87, FUT5, SDHAF1, PLIN4, POLR2E, HOMER3, ZNF772) were involved. HOMER3 (homer scaffold protein 3) was participate in FoxO signaling pathway and Glutamatergic synapse, FUT5 (fucosyltransferase 5) was particicapte in glycosphingolipid biosynthesis, while PLIN4 (perilipin 4) was involved in PPAR signalling pathway, suggesting the important role of glucose and lipid metabolism in CMT, which was also suggested by previous work [24]. The 2599 CMT patients in Shenzheng were collected from 2004 to 2020 to summarize the treatment options for CMT and suggested that physiotherapy combined with the injection of glucocorticoid drugs for CMT treatment [24].

DAVID functional and pathway enrichment analysis on 145 common varied genes (**Figure 5F**) showed the enriched items in biological process of angiogenesis with *p*-value of 0.049 and 4 genes (NRP1, PLXND1, FN1, RAPGEF3), in molecular function of Heparin-binding with *p*-value of 0.014 and 4 genes (NRP1, AOC1, SAA1, FN1), in KEGG signal pathway of antigen processing and presentation with *p*-value of 0.078 and 3 genes (KLRC2, KLRC3, HLA-B). All these results indicate the important role of glucose and lipid metabolism in CMT progression, and the angiogenesis and antigen processing might be also crucial in progressing of CMT in QTP.

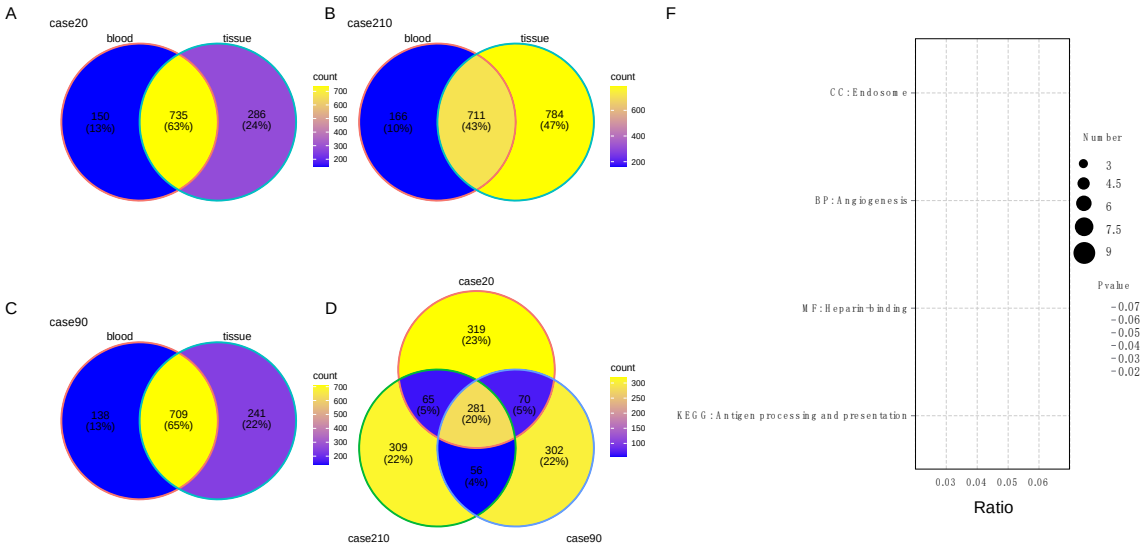


Figure 5. Potential molecular functions and biological processes in CMT that were indicated by shared variations between blood and CMT tissues. **(A–C)** Venn diagrams show genome variations shared by blood and SCM bio-samples for same patient; **(D)** the genome variations shared by all three patients with both blood and tissue been successfully sequenced; **(F)** the bubble plot shows the enriched GO terms and KEGG pathways for 145 common varied genes.

In total, the unique clinical and molecular characteristics for plateau CMT patients were detected, providing some therapeutic targets for them.

4. 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 [25]. Qinghai province is in the northeast QTP with average altitude of 4000 m. Han Chinese and lots of minor ethnic groups people, including hui, zang (Tibetan people), zhi, sala, and so on, live here. The unique area usually leads to the diverse dietary and living habit. For instance, climatic conditions are harsh and people here had relative low-quality healthcare, comparing with the east of China. Here, to better understand the molecular mechanism of CMT patients living in QTP, and predict the most promising targets to enhance the treatment effects 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. We attempted to discuss the clinical and molecular characteristics for those patients. The clinical data showed that 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. The molecular characteristics of CMT patients living in QTP were analyzed by the genomic features that were obtained based on the WES data. As a result, most of SNPs were located in chromosome 1 and 2, and the variation of C→G, T→A were the major types. By comparing the patients' variations with their parents', only 8%~30% unique variations were found, which means that CMT might be hereditary disorders. The treatment outcomes for these patients could potentially be improved by enhancing the quality of healthcare available and intervening at an earlier stage. The comparison of variations in blood with that obtained in SCM tissue suggested the potential treatment targets, and the enrichment analysis on these variations indicate genes in chromosome 19, glucose and lipid metabolism, and antigen processing might be involved in the development of CMT in QTP populations.

There are around five million people living around Qinghai province, and based on the recent statistic reports, only about two million people live around the capital of Qinghai province, Xining. Collecting samples and conducting follow-up tracing presented significant challenges in this region. It took us one and half years to collect these 20 patients. In future, we will collect much more patients and their parents' information, including the medication strategy and the followed-up treatment reports, to further show the unique characteristics of plateau patients and discuss their special treatment strategy. In particular, we will set some recruitment rules to select patients with balance ages, ethnic group and residency, collect their family and medicine history, operation information, etc., and build up the follow-up process to identify the best treatment strategy. Meanwhile, besides WES, the RNA-seq data will be also included to further investigate the molecular characteristics from both genome and transcriptome view of points.

There were a lot of environmental influences, such as dietary habits, healthcare access, or exposure to environmental toxins, might has impact on the progression of

CMT. In future work, we will attempt to collect more environmental influences and dietary habits to investigate the risk factors for CMT. The study suggests therapeutic targets for CMT based on shared variations between blood and SCM tissue. The further validation of these targets in larger cohorts will be performed. In particular, we will collect the molecular features from large cohorts based on literature and database search, and validate the potential targets suggested in this work. Either common or differences will highlight the molecular mechanism of CMT in QTP populations.

The comparison the molecular characteristics of CMT patients in Qinghai with those from other regions (such as low altitude or region with high-quality healthcare) will help identify region-specific genetic factors and treatment responses, and enhancing the understanding of CMT's etiology. There was literature research that collected therapeutic strategies from 2599 CMT patients in Shenzheng from 2004 to 2020. This research summarized the treatment options for CMT and suggested that physiotherapy combined with the injection of glucocorticoid drugs for CMT treatment. However, they did not collect molecular data based on high-throughput sequencing. In future, we will perform deep literature search and collect the molecular data for CMT patients in other region to implement the comparison and identify region-specific genetic factors.

5. Conclusions

Here, 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 to better understand the molecular characteristics of CMT patients living in QTP, and predict the most promise therapeutic targets for them. Several unique molecular characteristics for those CMT patients were found, including a higher prevalence of SNPs located on chromosome 1 and 2, with alteration types of C to T and G to A being the majority. Additionally, there was a lack of shared disease driver genes among the diverse ethnic groups. Moreover, 8%~30% unique variations were identified when comparing patients' variations with their parents', which suggest that there might some hereditary disorders involved in CMT development. The comparison of variations in blood with that obtained in SCM tissue hint that the glucose and lipid metabolism, as well as antigen processing might be a potential target to improve the treatment of CMT patients living in QTP.

Author contributions: Conceived the idea and supervised the study, TZ and YCW; designed and implemented the prediction model and performed data analysis, YCW; analyzed the results and drafted the manuscript, TZ and YCW; edited the manuscript, CYB. All authors have read and agreed to the published version of the manuscript.

Funding: 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.

Acknowledgments: We thank editors and reviewers for his contribution to this research.

Ethical approval: The study was conducted in accordance with the Declaration of Helsinki. The studies involving human participants were reviewed and approved by Qinghai Children and Women's Hospital ethics committee with the protocol code of 20201020. The patients/participants provided their written informed consent to participate in this study. All methods were performed in accordance with the relevant guidelines and regulations for research using human specimens.

Availability of data and materials: The datasets used and/or analysed during the current study available at Sequence Read Archive (SRA) with the BioProject ID of PRJNA948480 (http://www.ncbi.nlm.nih.gov/bioproject/948480).

Conflict of interest: The authors declare no conflict of interest.

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Appendix

Table A1. The details of sample collection.

No. of collected bio-samples/No of patients	No. of bio-samples with WES data/No of patients	No. of patients with their tissue bio-samples also be sequenced	No. of patients with their family member bio-samples also be sequenced
53/22	43/18	3	15

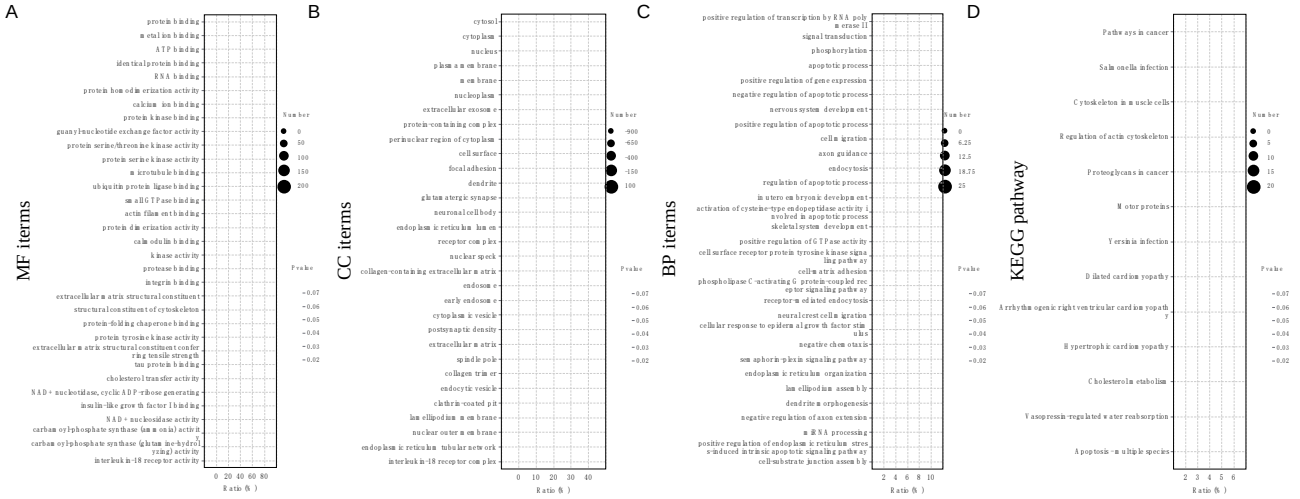

Figure A1. The functional and KEGG pathway enrichment analysis on mutant genes located in chromosome 2. A-D, the bubble plots show the enriched MF, BP, CC, and KEGG pathways for mutant genes located in chromosome 2.