University of Sistan and Baluchestan

Journal of Epigenetics

Online ISSN: 2676 - 4350

Homepage: https://jep.usb.ac.ir

Iranian Genetics Society

Analysis of Expression Profiles of GLI3A, LATS2 and MOB1A genes in Patients with Congenital hypothyroidism

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ARTICLE INFO

Article type:
Research Article

Article history:
Received: 20 June 2021
Revised: 19 March 2022
Accepted: 10 April 2022

Keywords:
Gene Expression,
Transcriptional activator GLI3,
MOB kinase activator 1A,
Large tumor suppressor kinase 2,
Hypothyroidism,
Congenital,
Real-Time Polymerase Chain Reaction.

A B S T R A C T

In recent years, congenital hypothyroidism (CH) has been reported as the most prevalent endocrine disorder and most common cause of preventable mental retardation in many parts of the world, especially in Asia. Delays in early diagnosis and treatment of CH can lead to growth retardation, as well as neurological and psychological disorders. Many genetic defects along with their molecular mechanisms based on pathophysiology have not been identified in many babies with congenital hypothyroidism. This study aimed to evaluate the expression of GLI3A and LATS2, MOB1A genes in patients with congenital hypothyroidism. In this study, the expression pattern of GLI3A, LATS2, and MOB1A genes after RNA extraction and converted to cDNA from the blood of 20 patients and 20 control samples were examined by the Real-time quantitative PCR (qRT-PCR). The obtained data were analyzed using Spss software and Mann-Whitney statistical method. Statistical analysis of findings has shown significant differences in the expression of GLI3A gene in two groups of patients and control. However, in the study on the expression of two genes LATS2 and MOB1A, there was no significant difference, so it can be assumed that expression of GLI3A gene may play a role in risk of congenital hypothyroidism while changes in expression of LATS2 and MOB1A genes are not involved in this disease. Results of the present study demonstrated that GLI3A gene expression could be a genetic risk factor for congenital hypothyroidism. In the present study, LATS2 and MOB1A gene expression did not show significant risk for susceptibility CH disease.

Introduction

Thyroid hormones play an important functions in creating central nervous system, with the deficiency of thyroid hormones at birth being known as congenital hypothyroidism (CH) (Bajracharya et al., 2019). This disease is the most common endocrine disorder and one of the major causes of preventable mental retardation (Kwak et al., 2018). Congenital hypothyroidism affects almost 1 in 2000 to 1 in 4000 babies. Clinical manifestations at birth are often mild or indistinguishable (Rastogi et al., 2010). Girls are almost twice more likely to be affected than boys. Thyroid hormones in early life are essential for development of central nervous system (CNS) as they are involved dendritic and axonal growth,

DOI: 10.22111/jep.2022.38960.1029

Publisher: University of Sistan and Baluchestan

How to Cite: Eisazaei, S., Nakhaee-Moghadam, M., Kordi Tamandani, D. (2022). Analysis of Expression Profiles of GLI3A, LATS2 and MOB1A genes in Patients with Congenital hypothyroidism. Journal of Epigenetics, 3(1), 21-26. DOI: 10.22111/jep.2022.38960.1029

neuronal immigration, neurogenesis, and synaptogenesis, with regulation of neurotransmitters (Pardo et al., 2017). CH being often sporadic and only 2% of cases of CH (thyroid dysgenesis) being inherited (Park et al., 2005). Congenital hypothyroidism is etiologically divided into either primary, secondary, or environmental types. Transient CH involves temporary deficiency of thyroid hormone that develops at birth and returns to normal after birth. Most cases of permanent congenital hypothyroidism (80-85%) are caused by defect in the formation of thyroid gland during embryogenesis (thyroid dysgenesis). Fewer cases of congenital hypothyroidism are associated with defects in thyroid hormone synthesis (10-15%) (dyshormonogenesis) (LaFRANCHI et al., 1999). Deficiencies of thyroid morphogenesis are classified as athyreosis (lack of thyroid tissue) (~ 35–40%), ectopy (~30–45%), and hemiagenesis (hypoplasia of thyroid) (~5%) (Bona et al., 2015). Genes associated with thyroid dysgenesis include PAX8, NKX2-1, FOXE1, NKX2, HHE5, TSH while the genes associated with dyshormonogenesis are DUOX2, DUOX2A, TPO, TG, SLC26A4 (PDS), SLC5A5 (NIS) and IYD (DHEAL1) (Wassner et al., 2018). As familial TD is rare, it is considered as sporadic disease which is mainly caused by non-genetic causes such as environmental factors or accidental events during embryogenesis. Molecular mechanisms that lead to TD are largely unknown (Szinnai et al., 2014).

Dyshormonogenesis is usually associated with pattern of recessive-inheritance and is more common in close relatives (Nakajima et al., 2006). Secondary or central congenital hypothyroidism is usually caused by defects in TSH production, with this defect being typically part of disorder that causes congenital hypopituitarism (Lania et al., 2008). Environmental hypothyroidism may be due to resistance of target tissues to thyroid hormone function and in most cases, it is associated with mutation in the gene coding subunit B of thyroid hormone receptor (THRβ) (Torres-Manzo et al., 2014).

Epigenetic Risk factors involved in permanent and transient CH include family history of CH, birth in geographical areas with very high prevalence of disease, premature birth, high maternal age, gestational diabetes, twin pregnancy, ethnicity, retinopathy of premature infants, birth season, puberty, birth weight, CH, and kinship degree (Waller DK et al, 2000; Hashemipour et al., 2019). Neonatal screening (NS) with CH is one of the most important achievements in preventive medicine (Clause et al., 2013). Experimental screening programs for newborns with congenital hypothyroidism in North America began in 1972 and were performed in Iran in 2006 (Fisher et al., 1979; Delange et al., 1997). However, neonatal screening for hypothyroidism has not been performed in many third-world countries and only about one-third of world's newborn has been screened. Thus, it is important for doctors to be able to diagnose and treat the disorder early (Dorreh et al., 2014).

LATS2 and MOB1A genes are the main components of Hippo signaling pathway and play important roles in processing the proliferation and apoptosis, i.e. in determining appropriate number and size of cells. Further, and increases or decreases in the expression of these two genes have been reported in many human abnormalities and cancers (Hansen et al., 2015). In addition, GLI3A gene acts as one of main mediators of Hedgehog signaling pathway and plays a vital role in growth and development of primary embryo, organ growth and birth defects, as well as many abnormalities (Rimkus et al., 2016). Numerous studies have shown interrelationship between Hippo and Hedgehog signaling pathways (Ou et al., 2017; Ehmer et al., 2016). Due to high importance of these genes in cell growth and development, it can be assumed that changes in the expression of GLI3A, LATS2, and MOB1A genes may play a role in the development of congenital hypothyroidism. In this study, this hypothesis was evaluated experimentally for the first time in world.

Materials and methods

Twenty blood samples with congenital hypothyroidism and twenty normal control samples were collected from the Pasteur Laboratory in Zahedan, Iran, between 2018 and 2019. Conscious consent of all participants in this study has been obtained and this study has been approved by the local ethics committee of

Zahedan University of Medical Sciences. Demographic information of statistical population are given in Table1.

Table1: Demographic data on cases and controls.

		Controls N=20		Cases N=20	
Gender		Girl	Boy	Girl	Boy
Number		10	10	9	11
Age grouping	=<1	4	4	7	8
	1-9	6	5	2	1
	9-17	0	1	0	2
Mean ± SD		4/20 ± 2/85		2/45 ± 3/98	
Minimum age		29 days	4 days	22 days	31 days
Maximum age		7 years	11 years	3 years	17 years

RNA extraction and Real-time quantitative PCR (qRT-PCR)

Total RNA from whole frozen EDTA blood was prepared to analyze gene expression using Total RNA Extraction Kit tests (Cat. No. A101231 .Pars tous biotechnology, Iran) according to the manufacturer‘s protocol. Subsequently, first-strand cDNA was synthesized from 1–10 µg of total RNA using Easy cDNA Reverse Transcription kit 50 tests (Cat. No. A101161.Sinaclon, Tehran, Iran) according to the supplier’s protocol. RNA 18s was used as an internal standard. Real-time quantitative PCR (qRT-PCR) was performed in reactions of 20 µl volume reaction mixture containing 10 µL of 2× Sybergreen, 2 µL of cDNA, 1 µL of each forward and reverse primer, and 6 µL of DEPC water. An AB15700 sequence detection system (Applied Biosystems) was used to estimate the involved cDNA using Real-time quantitative PCR (qRT-PCR). Reactions were run with the following thermal cycling parameters: initial denaturation at 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 30 s (18s rRNA:60 °C, GLI3A: 59.4 °C, LATS2: 58.1 °C, MOB1A: 59.2 °C), and 72 °C for 40 s, followed by 1 cycle of final extension at 72 °C for 10 min; finally, the melting curve was obtained over the range 60–95 °C. The sequences of the primers used for expression analysis are listed in Table 2. The PCR products were tested on 2% agarose gels by electrophoresis and visualized with ethidium bromide staining. The mRNA expression levels of GLI3A, MOB1A and LATS2 were normalized to 18s rRNA and was given as $2^{-\Delta\Delta c}$.

Table2: Real-time Primer sequences and annealing temperatures

Genes	Sequence	Product size(bp)	Annealing temp (°C)
GLI3A-F	5′-ACTTCCGCCTTATCTAGTAGCC-3′	188 bp	59.4 °C
GLI3A-R	5′-CCACGGGTTGCTGAGATCAT-3′		
LATS2-F	5′-ACTTTTCCTGCCACGACTTATTC-3′	٧٧ bp	58.1 °C
LATS2-R	5′-GATGGCTGTTTTAACCCCTCA-3′		
MOB1A-F	5′-CAGCAGCCGCTCTTCTAAAAC -3′	134 bp	59.2 °C
MOB1A-R	5′-CCTCAGGCAACATAACAGCTTG-3′		
18S RNA-F	5′-GTAACCCGTTGAACCCCATT-3′	112 bp	60 °C
18S RNA-R	5′-CCATCCAATCGGTAGTAGCG-3′		

Statistical analysis

Data were analyzed using the T-test, with SPSS Version 20.0 and the association between GLI3A, MOB1A and LATS2 gene expression and risk of congenital hypothyroidism was examined. Mann–Whitney test was used for comparing expression data between cases and normal samples. The level of statistical significance was set at $P \leq 0.05$.

Results

The statistical results showed a significant differences between GLI3A gene expression in congenital hypothyroidism patients (N= 20, mean ± SD: 0/409 ± 0/784) and healthy controls (N= 20, mean ± SD: 0/091 ± 0/219, $p < 0.023$). However, LATS2 and MOB1A showed no significant difference between the studied groups with respect to the gene expression level (Table 3).

Table 3: Comparison of relative gene expression for GLI3A, LATS2, and MOB1A between patients and healthy controls.

Genes		N	Mean ± S.D.	*p*-value[a]
GLI3A	Cases	20	0/409 ± 0/784	0/023
	Controls	20	0/091 ± 0/219	
LATS2	Cases	20	0/815 ± 2/204	0/234
	Controls	20	0/117 ± 0/232	
MOB1A	Cases	20	2/292 ± 6/133	0/725
	Controls	20	0/550 ± 0/562	

[a] Mann–Whitney test.
N: number, SD: standard deviation.

Discussion

In the present study, we examined the expression of GLI3A, LATS2 and MOB1A genes at risk of congenital hypothyroidism. The results showed that the expression GLI3A of gene in the risk of congenital hypothyroidism was statistically significant and the LATS2 and MOB1A genes did not show a statistically significant difference. Many studies have shown the importance of the Hh signaling pathway in development. Proper homeostasis and cell function are maintained by convergent integration of different signaling pathways, enabling the cell to respond to intracellular and extracellular changes (Furth and Aylon 2017). Many diseases, including familial and sporadic cancers as well as abnormalities at birth, appear to be associated with the abnormal function of Hh signaling pathway. The Hh signaling pathway is the main regulator of cell differentiation, proliferation and tissue polarity. Zinc finger transcription factors from GLI family play an important role in mediating and interpreting Hh signals. Explaining how do GLI proteins work enables us to increase our knowledge of cell proliferation, differentiation, or survival in response to Hedgehog (Hh) signals, as well as the design of logical therapies for diseases associated with Hh signals (HSDs) (i Altaba 1999). Recently, targeting GLI transcription factors has become a major focus of treatment protocols (Sabol et al. 2018). Shh is essential for biosynthesis of thyroid hormone as well as thyroid-binding to UBB (Ultimobranchial Body) during thyroid development. Accordingly, a homozygous mutation in the mouse Shh causes both thyroid hypoplasia and impaired thyroid binding to UBB (Nilsson and Fagman 2017). Shh, as a novel regulator of thyroid development, was introduced in the research by Fagman et al in 2004 (Fagman et al. 2004). The study by Wang et al. in 2013 showed that the signaling pathway hedgehog is essential for cerebellar neuronal precursor's (CGNPs) proliferation (Wang et al. 2014). Furthermore, regulation of the Wnt gene by GLI3 in telencephalon was first reported in the study by Grove et al. in 1998 (Grove et al. 1998). GLIS3 is another component of the hedgehog signaling pathway that plays an important role in thyroid hormone biosynthesis. The lack of this gene in humans and mice leads to several defects, including neonatal diabetes and congenital hypothyroidism (Jetten 2018). A study by Szczepanek-Parulska et al. In 2020 was the first to show a possible role for the GLI3 gene in thyroid growth (Szczepanek-Parulska et al,. 2020). The Association of a defect in this gene with CH was demonstrated by FU et al. in 2018 by studying 592 Chinese people with congenital hypothyroidism (Fu et al. 2018). Therefore, the result of this study is in line with the research done so far. Organ size adjustment is a very coordinated process that involves complex mechanisms in response to physiological symptoms. The hippo signaling pathway plays an important role in cell growth, apoptosis, development, and migration. It is also important for regenerating stem cells and maintaining genomic stability. Among Hippo pathway proteins, the MOB1 family is involved in cell proliferation and apoptosis, thus controlling the number of cells and right size of organs (Pinosa et al. 2013). MOB1A is one of the major members of the Hippo signaling pathway and is mutant or inactivated in many human cancers. Double-mutant in MOB1A / B gene in mice increases the risk of cancer and fetal death. The two components MOB1A and MOB1B act as tumor suppressors by regulating downstream elements of the Hippo-signaling pathway (Nishio et al. 2012). A study by Rong et all showed overexpression of MOB1A in most colorectal cancers (Zhang et al.1997). According to Lee, LATS2 which is else member of Hippo signaling harmonizes the cell cycle, proliferation, and cell death (Nishio et al. 2012). Recently, LATS1 and

LATS2 kinases have received much attention in research. Mutations in the LATS gene are almost rare. The regulation of LATS2 genes varies at the transcriptional level (Furth et al. 2017). The potential role of LATS2 in many cancers has been reported in recent years. expression of LATS2 results in cessation of G2/M by inhibition of Cdc2 kinase activity, inhibition of G1/ S transfer by regulation of cyclin E/Cdk2 kinase activity, and induction of apoptosis by regulation of apoptotic inhibitors such as Bcl2 and Bcl-xL in the downstream pathway (Lee et al. 2010). A variety of tumors, including soft tissue sarcoma, leukemia, breast, prostate, lung, and esophageal cancers are caused by loss of function LATS1 or LATS2 (Tian et al. 2013). LATS2 prevents the proliferation, migration and invasion of glioma cells by inactivating YAP. (Shi et al. 2019). The findings of Si-Hyong et al Showed that tumor growth and invasive status are greatly increased by low expression of the LATS2 gene in NSCLC (Jang et al. 2019). Overexpression of the LATS2 gene is involved in the development of AML (Gholami et al. 2014). Research on the effect of LATS2 and MOB1A gene expression on the risk of thyroid disease has not been reported so far and often studies done on the role of these two genes in the development of most cancers. Therefore, this is the first study worldwide to examine express on these genes in congenital hypothyroidism. Based on studies that showed the very important role of these genes in growth and development, we decided to investigate the expression of these genes in congenital hypothyroidism. However, to confirm the results of the present study, a large-scale study with a high statistical population is needed.

Conclusion

Results of the present study demonstrated that GLI3A gene expression could be a genetic risk factor for congenital hypothyroidism. In the present study, LATS2 and MOB1A gene expression did not show a significant risk for susceptibility to CH disease.

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