GENETIC DISORDERS OF PITUITARY DEVELOPMENT IN PATIENTS WITH SHEEHAN'S SYNDROME

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Abstract

Introduction. Genetic disorders associated with the development of the pituitary gland and cranial bones may cause a genetic tendency toward Sheehan's syndrome (SS). Our aim in this study was to investigate expression disorders in the genes responsible for the development of the pituitary gland and cranial bones in patients with SS.

Materials and Methods. Forty-four patients who were previously diagnosed with SS and 43 healthy women were compared in terms of the mean expression values of genes including the prophet of PIT-1 (PROP1), HESX homeobox 1 (HESX1), POU class 1 homeobox 1 (POU1F1), LIM homeobox 3 (LHX3), LHX4, glioma-associated oncogene homolog 2 (GLI2), orthodenticle homeobox 2 (OTX2), SIX homeobox 3 (SIX3), SIX6, T-box transcription factor 19 (TBX19), transducin-like enhancer protein 1 (TLE1), TLE3, distal-less homeobox 2 (DLX2), DLX5, MSH homeobox 2 (MSX2), and paired box 3 (PAX3).

Results. The mean expression values of the HESX1, TLE1, TLE3, and MSX2 genes were significantly different in the SS group from the healthy control group, while the mean expression values of the remaining genes were similar.

Conclusion. The present study concludes that abnormal expressions of HESX1, TLE1, TLE3, and MSX2 genes may cause a genetic predisposition to the development of SS.

Key words: Sheehan's syndrome, etiopathogenesis, genetic, pituitary development.

INTRODUCTION

Sheehan's syndrome (SS) results from massive postpartum uterine hemorrhage which causes pituitary infarction, and thereby, hypopituitarism (1, 2). The two essential factors for the development of SS are the physiological enlargement of the pituitary gland during pregnancy and an arrest of the pituitary arterial blood flow due to severe hemorrhage, while a smaller sella turcica volume, hypercoagulation, and pituitary

autoimmunity may be contributing factors in proportion of patients (2-7).

Investigations regarding the molecular basis of pituitary organogenesis have shown that genetic disorders of pituitary development can be underlying mechanisms responsible for congenital hypopituitarism (8, 9). Importantly, most human diseases develop as a result of interactions among multiple genes and environmental factors (10). In that regard, it can be hypothesized that some types of late-onset hypopituitarism may develop in adults as a result of both genetic tendency and essential environmental factors. Massive postpartum hemorrhage may cause pituitary ischemic necrosis in women who also have particular genetic features, and thus a predisposition to SS. Therefore, we hypothesized that some genetic disorders involved in pituitary differentiation may make the development of SS easier.

Previous studies showed that, approximately half of the patients with SS, not all of them, have smaller mean sella turcica volume than healthy women (3, 11, 12). Therefore, smaller sella turcica volume is not an essential factor, but can be a contributing factor, for the development of SS. The characterization of patients with disorders in the genes responsible for the development of cranial bones may provide an opportunity to discover mechanisms responsible for smaller sellar volume.

The aim of this study was to investigate the expression profiles of some genes involved in the development of the pituitary gland and of the cranial bones. The determination of genetic disorders of pituitary development among patients with SS may be beneficial in identifying women prone to SS.

PATIENTS AND METHODS

Study Design

Forty-four patients who were previously

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diagnosed with SS and followed up by ‘Endocrinology Division of Erciyes University Medical School’ and 43 sex-matched healthy controls were enrolled into the study. Medical records, past medical histories and physical examinations of all participants were reviewed. The inclusion criteria for patients were having all of the following criteria for the diagnosis of SS: a) at least one pituitary hormone deficiency; b) severe hypotension or shock, for which blood transfusion or fluid replacement was necessary c) medical history of massive postpartum uterine hemorrhage; d) agalactia and amenorrhea after the index delivery; e) partial or complete empty sella on magnetic resonance imaging (MRI) (13). The exclusion criteria for patients with SS were having any disease other than SS, and receiving any medications other than thyroid hormone and glucocorticoid replacements. Healthy control group consisted of women who were hospital staff or their relatives. The control group did not have medical records, medical histories, or any physical findings of a disease and were not on any drug, while all of them had given at least one birth. The SS and control groups were compared in terms of mean gene expression levels after the local ethic committee’s approval according to declaration of Helsinki, and written informed consent was obtained from all participants.

Genes and Sample Collection

The genes that were investigated in terms of mean expression values were the prophet of PIT 1 (PROP1), HESX homeobox 1 (HESX1), POU class 1 homeobox 1 (POU1F1), LIM homeobox 3 (LHX3), LHX4, glioma-associated oncogene homolog 2 (GLI2), orthodenticle homeobox 2 (OTX2), SIX homeobox 3 (SIX3), SIX6, T-box transcription factor 19 (TBX19), transducin-like enhancer protein 1 (TLE1), TLE3, distal-less homeobox 2 (DLX2), DLX5, MSH homeobox 2 (MSX2), and paired box 3 (PAX3). For the genetic analyses, venous blood samples were collected from the participants in 2 mL ethylenediaminetetraacetic acid (EDTA)-containing tubes after overnight fasting. Peripheral blood mononuclear cells were stored at -80°C until the genetic expression analyses were performed. The genetic analyses were performed at the Erciyes University Genome and Stem Cell Center (GENKOK).

RNA extraction, cDNA synthesis and preamplification: Peripheral blood mononuclear cells were isolated from the collected blood samples by using standard methods (14). The total RNA of the blood mononuclear cells was extracted using a TRIzol-based

extraction method (Roche, Germany). The final RNA concentration was determined with NanoDrop 2000 (Thermo Scientific, USA). Two-hundred ng RNA was used for the cDNA synthesis with random hexamers using the SuperScript® III First-Strand Synthesis SuperMix (ABI Life Technologies, USA). The thermal cycler protocol was as follows: 10 min at 25°C , 120 min at 37°C , 5 min at 85°C , and 4°C hold. The cDNA was then preamplified using TaqMan® PreAmp Master Mix (Applied Biosystems, USA) with all 19 pairs of gene-specific primers, and 1.25 μL of each cDNA sample was combined with 2.5 μL of 2x TaqMan PreAmp Master Mix in the wells of a 96-well polymerase chain reaction (PCR) plate. The PCR cycle conditions were 95°C for 10 min, followed by 15 s at 95°C and 4 min at 60°C , repeated for 14 cycles. The samples were treated with Exonuclease I (Thermo Scientific, USA) to remove unconjugated primers, and then the PCR plate was moved to a thermal cycler (SensoQuest GmbH, Germany). All samples were diluted 1/10 in a Tris-EDTA (TE) buffer (pH 8) (Life Technologies, USA).

Gene Expression Profiling with High-throughput Quantitative PCR: An EvaGreen detection assay on a Biomark machine following standard Fluidigm protocols was used to determine the gene expression values. A quantitative RT-PCR was performed using Fluidigm 96x96 nano fluidic arrays targeting the 19 genes that we selected. Samples were prepared for loading on a 96X96 Dynamic Array™ IFC by preparing 2.25 μL of each preamplified cDNA sample with 2.5 μL 2x SsoFast™ EvaGreen® Supermix with 0.25 μL 20x DNA binding dye loading reagent (Fluidigm®) and low ROX (BioRad, USA). Five μL of each preamplified cDNA sample and 5 μL of each DeltaGene assay were mixed inside the chip using the Nanoflex IFC controller (Fluidigm, USA). Thermocycling parameters included an initial phase of 40 min at 70°C ; 30 s at 60°C ; 1 min at 95°C ; followed by 35 cycles consisting of 96°C for 5 s and 60°C for 20 s. After the completion of the run, a melting curve of the amplified products was determined using a ramp from 60°C to 95°C at $1^{\circ}\text{C}/3\text{ s}$. $\beta 2$ microglobulin (B2M), beta actin (ACTB), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as reference genes to normalize the gene expression data. The samples were run in duplicate and the Ct values were converted to relative expression values (negative delta Ct) by subtracting the mean of the three reference genes from the mean of each target gene. The mean expression values of the genes were represented as $2^{-\Delta\Delta\text{CT}}$ (15).

Table 1. Distributions of deficient pituitary hormones in patients with Sheehan's syndrome

Deficient hormones	Number and percentage of patients
FSH-LH + GH + TSH + ACTH + PRL	33 (75 %)
FSH-LH + GH + ACTH + PRL	3 (6.8 %)
FSH-LH + GH + ACTH + TSH	3 (6.8 %)
FSH-LH + GH + TSH	3 (6.8 %)
FSH-LH + GH + PRL	1 (2.3 %)
FSH-LH + GH	1 (2.3 %)
Total	44 (100 %)

Table 2. Comparison of mean expression values ($2^{-\Delta\Delta CT}$) of the genes between the groups

Gene	Control (n=43)	Patient (n=44)	p value	Adjusted p value	Odds ratio (95% CI)
<i>HESX1</i>	16.65 (4.74-40.06)	4.75 (2.72-8.65)	3.998×10^{-4}	6.660×10^{-4}	0.98 (0.95-0.99)
<i>TLE1</i>	0.45 (0.21-1.30)	0.19 (0.15-0.34)	7.982×10^{-4}	9.977×10^{-4}	0.98 (0.94-1.01)
<i>TLE3</i>	0.22 (0.12-0.65)	1.02 (0.67-1.31)	5.504×10^{-6}	2.752×10^{-5}	0.94 (0.82-1.07)
<i>MSX2</i>	11.19 (3.65-50.59)	2.22 (1.38-6.15)	2.198×10^{-5}	5.494×10^{-4}	0.96 (0.93-0.99)

Values are expressed either as mean $\pm$ SD or median (1st-3rd quartiles); CI: confidence interval.

Data Analysis

Statistical analyses were conducted by using the SPSS 15.0 software program. The descriptive statistics included mean and $\pm$ standard deviation (SD) values. Cq values of 999 and Cq values larger than 23 were removed from the data analysis. Histogram and q-q values were examined and Shapiro-Wilk's test was performed to assess data normality. Levene's test was used to assess variance homogeneity. To compare the differences between the groups, independent samples t test or Mann-Whitney U test was applied. Odds ratios were calculated with 95% confidence

intervals. After multiple adjustment using Benjamini-Hochberg procedure, adjusted p values less than 5% were considered statistically significant.

RESULTS

Examining the diagnostic criteria of the SS group revealed that all of the patients were recorded to have at least 2 pituitary hormone deficiencies. Panhypopituitarism was detected in 33 (75%) patients with SS, while partial hypopituitarism was detected in 11 (25%) patients (Table 1). All patients with SS had a medical history of massive postpartum uterine hemorrhage which had led to severe hypotension or shock; and agalactia and amenorrhea developed after their last delivery. Although all SS patients had a MRI report of partial or complete empty sella, magnetic resonance images of most of them were not available. Therefore sella turcica volumes of the patients could not be measured. Furthermore, craniofacial deformities or major optic or auditory problems were not found in the physical examinations or the past medical histories of the patients.

The mean ages of the women in the SS and control groups were 63.2 ± 12.5 and 60.3 ± 9.3 years, respectively. There was no significant difference in terms of the mean age between the two groups ($p > 0.05$). When the two groups were compared in terms of mean expression values of *HESX1*, *TLE1*, *TLE3*, and *MSX2* genes, a significant difference was found (Fig. 1 and Table 2). The expression values of the remaining genes, which included *PROP1*, *POU1F1*, *LHX3*, *LHX4*, *GLI2*, *OTX2*, *SIX3*, *SIX6*, *TBX19*, *DLX2*, *DLX5*, and *PAX3*, were similar when the groups were compared with each other.

Figure 1. Heat map reporting the expression values of *HESX1*, *TLE1*, *TLE3*, and *MSX2* genes in 44 Sheehan's syndrome patients and 43 healthy controls using Biomark Fluidigm. In the cluster figure, columns represent samples and rows represent genes (black, red and green correspond to unchanged, down-regulated, and up-regulated expressions, respectively).

DISCUSSION

Although it is not completely understood, the process of pituitary organogenesis has been shown to be under the control of coordinated expressions of multiple transcription factors and signaling molecules. This complex genetic process is regulated by the expressions of genes including bone morphogenetic protein 4 (BMP4), thyroid transcription factor 1 (TTF1), fibroblast growth factor 8 (FGF8), FGF10, wingless-type MMTV integration site family-member 5A (WNT5A), OTX2, GLI1, GLI2, LHX3, LHX4, paired-like homeodomain 1 (PITX1), PITX2, HESX1, insulin gene enhancer protein 1 (ISL1), PAX6, SIX3, SIX6, TLE1, TLE2, POU1F1, PROP1, GATA binding protein 2 (GATA2), steroidogenic factor 1 (SF1), and TBX19 (8, 9, 16, 17). The genetic disorders of those genes can be associated with isolated or combined hypopituitarism, septo-optic dysplasia, holoprosencephaly, and multisystemic syndromes (16-19). On the other hand, BMP2, sonic hedgehog (SHH), FGF8, fibroblast growth factor receptor 2 (FGFR2), GLI3, short stature homeobox 2 (SHOX2), twist basic helix-loop-helix transcription factor (TWIST), DLX2, DLX5, MSX2, and PAX3 genes regulate the development of cranial bones, eyes, and ears (20-23); and involved genetic disorders may cause craniofacial deformities, sensorineural hearing loss, and orbital deformities (22-26).

Genetic expression can simply be defined as the production process of functional proteins from their special genes (27). The expression levels of genes are under the control of multiple factors, such as other genes and various environmental factors (10). In other words, increases or decreases in the expression amount of a gene may be results of changes in expressions of other genes or environmental factors (28). In that regard, an environmental factor, for example, massive postpartum hemorrhage, along with a change in the expression of particular genes may facilitate ischemic pituitary necrosis.

In our patients with SS, the mean expression values of the HESX1, TLE1, TLE3, and MSX2 genes were significantly different from healthy subjects. The genetic disorders of HESX1, TLE1, and TLE3, which are closely interacting genes, were shown to cause pituitary developmental abnormalities, and thereby hypopituitarism (29, 30). Mutations in HESX1 gene were shown to cause either increased or decreased expression which involves defects in pituitary differentiation (9). Abnormal expressions of TLE1 and

TLE3 genes may disrupt differentiations of pituitary cells, since they augment the HESX2 and repress the PROP1 expression (9, 29). Therefore, the results of abnormal HESX1, TLE1, and TLE3 expressions raise the question of whether patients with SS had a genetic tendency towards ischemic pituitary necrosis previous to their index pregnancies. It seems that genetic disorders of pituitary organogenesis may be contributing factors in the development of SS, however further studies are required to advance our understanding of the roles of HESX1, TLE1, and TLE3 gene expressions.

A smaller sella turcica volume with pituitary enlargement in pregnant women may lead to a compression on the hypophyseal arteries against the walls of the sella turcica and the diaphragma sellae (11). As a small sella size was found in approximately half of the patients with SS, it can be a contributing factor in some of the patients with SS (13). The DLX2, DLX5, PAX3, and MSX2 genes are associated with the development of cranial bones (20-23), and might involve the development of sphenoid bone, which forms the sella turcica. However, the expression profiles of DLX2, DLX5, and PAX3, but not MSX2, were not abnormal in the SS group. Furthermore, no craniofacial deformities or major optic and auditory problems were found in the patients with SS. It is difficult to associate the decreased expression of the MSX2 gene in SS patients with a smaller sella size, because a correlation between expression values of MSX2 and sella turcica volumes was not performed in the present study.

Although it was found that SS is a very rare disorder in developed countries due to advanced obstetric care (31, 32), more recent studies have shown that it is not as rare as was thought, probably because of migrant women (33-35). On the other hand, it is still encountered frequently in underdeveloped and developing countries (36, 37). Therefore, it will be beneficial to identify genetic disorders that cause a tendency for the development of SS. The present study concludes that HESX1, TLE1, TLE3, and MSX2 are candidate genes for screening patients with SS in further studies. A limitation of the present study is investigating most, but not all, of the above-listed genes associated with the pituitary development. Understanding the molecular basis of the etiopathogenesis of SS may be useful to identify women, who have a particular genetic background, prior to the disease's development.

Conflict of interest

The authors declare that they have no conflict of interest concerning this article.

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