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ORIGINAL

Study of the expression rates of *RORA* and *COX5b* genes amongst MS patients compared with healthy individuals as an emerging diagnostic biomarker

Estudio de los índices de expresión de los genes RORA y COX5b entre los pacientes con EM en comparación con los individuos sanos como biomarcador diagnóstico emergente

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Abstract

Background: Multiple Sclerosis is a degenerative disease of the central nervous system accompanied with both environmental and genetic backgrounds. The present survey was performed to assess the expression rates of *COX5B* and *RORA* genes in MS and healthy individuals.

Methods: Sixty blood samples were taken from MS (n= 30) patients and healthy (n= 30) individuals. RNA was extracted from blood samples and used for cDNA synthesis. DNA samples were subjected to real-time PCR to assess the *COX5B* and *RORA* genes expression by specific primers.

Results: MS patients had the higher history of smoking and alcohol. Statistically significant differences were obtained for the history of alcohol and smoking between MS and healthy population (P <0.05). There were no statistical differences between MS patients and healthy individuals for age, sex, BMI, and weight. *COX5B* gene expression was significantly decreased in MS patients compared to healthy individuals (P = 0.0433). *RORA* gene expression was increased in MS patients compared to the control group (P = 0.481).

Conclusion: *COX5B* gene expression analysis can be used as an emerging biomarker for MS diagnosis in individuals. It seems that RORA gene expression should be studied more to find its specific role in MS pathogenicity.

Keywords: Multiple sclerosis, *RORA, COX5B*, Gene expression, Real-Time PCR.

Resumen

Antecedentes: La esclerosis múltiple es una enfermedad degenerativa del sistema nervioso central acompañada de antecedentes tanto ambientales como genéticos. El presente estudio se realizó para evaluar los índices de expresión de los genes *COX5B* y *RORA* en la EM y en individuos sanos.

Métodos: Se tomaron sesenta muestras de sangre de pacientes con EM (n= 30) y de individuos sanos (n= 30). Se extrajo el ARN de las muestras de sangre y se utilizó para la síntesis de ADNc. Las muestras de ADN se sometieron a PCR en tiempo real para evaluar la expresión de los genes *COX5B* y *RORA* mediante cebadores específicos.

Resultados: Los pacientes con EM tenían mayores antecedentes de tabaquismo y alcohol. Se obtuvieron diferencias estadísticamente significativas para los antecedentes de alcohol y tabaquismo entre la población con EM y la población sana (*P* <0,05). No hubo diferencias estadísticas entre los pacientes con EM y los individuos sanos para la edad, el sexo, el IMC y el peso. La expresión del gen *COX5B* disminuyó significativamente en los pacientes con EM en comparación con los individuos sanos (*P* = 0,0433). La expresión del gen *RORA* aumentó en los pacientes con EM en comparación con el grupo de control (*P* = 0,481).

Conclusión: El análisis de la expresión del gen *COX5B* puede utilizarse como un biomarcador emergente para el diagnóstico de la EM en individuos. Parece que la expresión del gen *RORA* debería estudiarse más para encontrar su papel específico en la patogenicidad de la EM.

Palabras clave: Esclerosis múltiple, *RORA, COX5B*, expresión génica, PCR en tiempo real.

Introduction

Despite the high advances of the medical sciences[1-5], some diseases remain threat for human health[6-10]. Multiple sclerosis (MS) is a neurodegenerative, demyelinating disease of the nervous system that respects few demographic boundaries. It has an autoimmune basis, which leads to widespread nervous system tissue lesions and dysfunction, resulting in communication breakdown between neurons[11]. As MS research has progressed, it has become clearer that environmental and genetic factors underlie the etiology of MS. The cooperation of these two factors in the etiology raises the question of whether one is more important than the other in posing risk, and whether co-morbidities elevate risk[12].

Several genetic markers are involved in the pathogenesis of MS. *COX* is composed of 13 subunits, of which the three largest are encoded by the mtDNA and form the catalytic core of the enzyme. The remaining ten, evolutionary younger, nuclear-encoded subunits are involved in assembly and regulation of the enzyme. *COX* can be physiologically modulated and the enzyme represents one of the key regulatory sites of energy metabolism. *COX* gene has also some functions on the immunological reaction in the human body and its expression can decrease or increase the exacerbation of different diseases[13]. The retinoid acid-related orphan receptor (ROR) subfamily of orphan nuclear receptors consists of three members, α, β, and γ, that regulate multiple cellular processes including cell growth, differentiation, and apoptosis[14]. The *RORA* gene is expressed in several tissues and it regulates inflammatory responses, neuronal cell development, bone metabolism, and arteriosclerosis and also it is involved in the differentiation of Th17 cells[15].

Rendering the high importance of the MS and role of genetic markers in its pathogenesis, the present survey was done to assess the role of *COX5B* and *RORA* genes expression in MS patients and compared it with healthy individuals.

Materials and methods

Study designs

This survey was designed to assess the expression rates of *RORA* and *COX5b* genes in blood samples taken from MS patients and healthy individuals. For this purpose, 35 MS patients and 35 healthy individuals were examined.

Inclusion and exclusion criteria

Thirty MS patients of both sexes and all ages who were confirmed by the neurologist were included in the study. For a control group, 30 healthy individuals with no personal or family history of autoimmune diseases were also included. Written informed consent was obtained from each individual.

Samples

Peripheral blood samples (5 ml) were obtained from the cubital vein and collected in cell preparation tubes containing an anticoagulant Ethylene diamine tetraacetic acid (EDTA). Peripheral blood mononuclear cells were isolated by EDTA density centrifugation

RNA extraction and cDNA synthesis

Total RNA was extracted from the blood samples using a RNeasy kit ((Qiagen, Gaithersburg, MD, USA), according to the manufacturer's instructions. The RNA samples were incubated with RNAse-free DNAse I at 37°C for 15 min. RNA samples were purified with a RNeasy kit. Extracted RNA were immediately stored at -80°C. Total RNA concentration was assessed by ultraviolet absorbance at 260 nm (1 absorbance unit at 260 nm = 40 ng/µl RNA). Total RNA was run on 1% agarose gels to check size and integrity. The quality of RNA was confirmed by the detection of 18S and 28S bands after 1% agarose gel electrophoresis.

Total extracted RNA was used to generate cDNA with a Reverse Transcriptase cDNA synthesis kit (Roche, Germany) with oligo deoxythymine (dT) primers, according to the manufacturer's protocol. Synthetic cDNA samples were stored at -20°C. The quality and quantity of extracted DNA were assessed according to previous studies[16-22].

Real-Time PCR analysis

The real-time PCR technique was used to assess the expression rates of *RORA* and *COX5b* genes amongst the DNA samples of MS patients and healthy individuals. **Table I** shows the primer sequences used in this regard. The Biosystems TaqMan R, Universal PCR Master Mix was used to perform the real-time PCR, using the real-time PCR thermal cycler (Corbett Research, Rotor Gene 6000). The GAPDH and β-actin were applied as house-keeping genes for *COX5b* and *RORA* genes, respectively. The data were evaluated by the Δ Δ CT method, which measures the expression level of the target genes normalized to a reference gene and relative to the expression of the genes in the calibrator samples.

Statistical analysis

Statistically significant differences were calculated by the Student's t-test, using the SPSS 21.0 and Excel. Finally a P-value <0.05 was accepted as the level of significance[25-28].

Results

Study principles

The present survey was performed to assess the expression rates of COX5b and RORA genes amongst the MS patients and healthy individuals as an emerging biomarkers of MS.

Table I: Primer sequences used in this study.

Targeted genes	Primer sequence (5'-3')	References
COX5B	F: CCCAAAGGGAGCTTCAGG R: CGACGCTGGTATTGTCCTCT	23
GAPDH	F: CCACTCCTCCACCTTTGAC R: ACCCTGTTGCTGTAGCCA	
RORA	F: GTCAGCAGCTTCTACCTGGAC R: GTGTTGTTCTGAGAGTGAAAGGCACG	24
β-actin	F: TCTACAATGAGCTGCGTGTGG R: GGAACCGCTCATTGCCAATG	

Table II: Demographic charters of the examined MS patients and healthy individuals.

Demographic characters	Individuals (60 people)		P value
	MS (30 cases)	Healthy (30 cases)	
Mean age (SD)	61.32	60.72	0.87
Sex (M/F)	18/12	14/16	0.52
Mean weight (SD)	70.02 (8.14)	68.33 (9.69)	0.63
Mean BMI (SD)	24.6 (2.7)	25.1 (3.1)	0.89
History of alcohol (%)	19 (63.33)	12 (40)	0.042
History of smoking (%)	22 (73.33)	10 (33.33)	0.028

Figure 1: The expression rate of *COX5B* gene amongst the MS patients and healthy individuals.

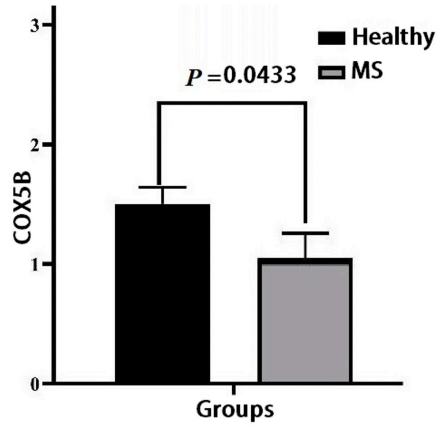
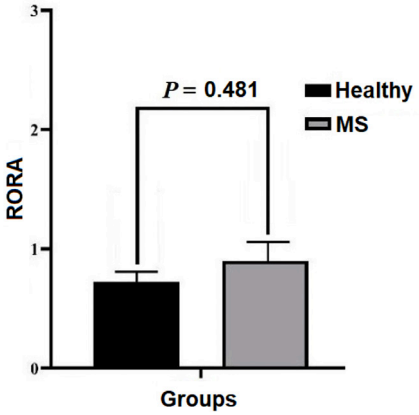


Figure 2: The expression rate of RORA gene amongst the MS patients and healthy individuals.



Demographic charters

Table II shows the demographic charters of the examined MS patients and healthy individuals. As shown, the mean age of the studied MS and healthy population was 61.32 and 60.72 years with a male to female ratio of 18/12 and 14/16, respectively. Statistically significant differences were obtained for the history of alcohol and smoking between MS and healthy population (P <0.05). MS patients had the higher history of smoking and alcohol.

COX5B gene expression

Figure 1 shows the expression rate of *COX5B* gene amongst the MS patients and healthy individuals. Findings showed that *COX5B* gene expression was significantly decreased in MS patients compared to healthy individuals (P = 0.0433).

RORA gene expression

Figure 2 shows the expression rate of *RORA* gene amongst the MS patients and healthy individuals. Findings showed that *RORA* gene expression was increased in MS patients compared to the control group, but it was not significant (P = 0.481).

Discussion

Infectious[29-33] and autoimmune diseases[34] have been considered as the most important causes of morbidity and mortality in the last century. In the present study, two important genetic markers have been determined for MS. Findings revealed that alcohol and smoking histories may affect the occurrence of MS among individuals. This claim was also approved by Hedström et al. (2021) (Sweden)[35], Ivashynka et al. (2019) (Italy)[36], and D'hooghe et al. (2012) (Belgium)[37]. Our findings also showed that sex and age didn't have any relation to the occurrence of MS, which was similar to previous surveys[38, 39]. As *COX5B* gene expression was significantly decreased in MS patients compared to healthy individual, it can introduce as a good marker for the MS diagnosis and further evaluation. Similarly, Safavizadeh et al. (2012)[40]

reported that *COX5B* gene expression is significantly reduced in MS patients compared to control (P <0.05), whereas there was no significant difference in the *COX2* gene expression. Role of the *COX5B* gene in MS has also been determined previously[41].

In despite of the increase in the *RORA* gene expression in MS group compared to the healthy individuals, difference was not statistically significant. Thus, it seems that further researches should perform to obtain the exact role of *RORA* gene in MS occurrence and pathogenicity. *RORA* is proposed to promote Th17 cells differentiation that play a crucial role in many inflammatory diseases, including MS. The gene is also involved in regulation of inflammatory responses and neuronal cell development. In a survey conducted by Eftekharian et al. (2016)[42], the relationship between *RORA* rs11639084 and rs4774388 gene polymorphisms on the individual susceptibility of MS was examined. Their findings revealed that both variants showed significant differences in allele and genotype distributions between the studied groups. Genotypes were risk associated in additive (*P*-value of 0.0003 and odds ratio equal to 1.7 (95% CI: 1.27-2.26)), dominant (*P*-value of <0.0001 and odds ratio equal to 0.55 (95% CI: 0.41-0.73)) and recessive (*P*-value of 0.04 and odds ratio equal to 0.33 (95% CI: (0.12-0.96)) models for rs11639084. However, the rs4774388 genotypes were risk associated in recessive model with a P-value of 0.036 and an odds ratio of 0.62 (95% CI: (0.4-0.97)). Down-regulation of *RORA* gene expression in the blood of MS patients was also reported by Sayad et al (2018)[43].

Conclusion

The present survey is the first report on the assessment of both *COX5B* and *RORA* gene expression in MS patients. As only significant difference was found for the expression rate of *COX5B* gene, its analysis can be effective for diagnosis of MS in individuals. However, several works should perform to obtain the main activity of *RORA* gene in MS pathogenicity.

Interests conflict

The authors declare no conflict of interest.

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