

PHOSPHATIDYLINOSITOL 3-KINASE P85 REGULATORY SUBUNIT GENE AND SPINAL MUSCULAR ATROPHY DISEASE

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Abstract. Spinal muscular atrophy (SMA) is a frequent neuromuscular disorder caused by motoneuronal apoptosis, as a result of SMN (Survival Motor Neuron) protein deficiency. Although the SMA determining gene was identified, the molecular mechanism of the disease is not clearly understood, due to the heterogeneity of clinical manifestations. Trying to complete the molecular describing SMA picture, by identifying potential modulators factors, we investigated the relationship between phosphatidylinositol 3-kinase p85 regulatory subunit gene (*PIK3R1*) and SMA pathology. As IGF signaling pathway has been reported to play an important role in motoneurons survival and PIK3 is a key element of this cascade signaling, we focused on the relationship between *PIK3R1* gene *Met326Ile* polymorphism and SMA type I, the most severe form of the disease. A total of 80 subjects (40 SMA type I patients and 40 unrelated healthy controls) were included in the study. The statistical analyzes performed consequently to the genotyping by mismatch PCR-RFLP method, revealed that *Met326Ile* polymorphism is not associated with SMA type I disease: $OR_{Met/Met} = 0.398$ with a $p = 0.072$ meanwhile $OR_{Met} = 0.495$, $p = 0.063$. However, the Cochrane – Armitage test indicated that there is a statistically association trend between the analyzed polymorphism and SMA type I pathology: $OR_{Met} = 0.438$, $p = 0.032$. We concluded that additional researches with an increased subjects number and replicates studies in other populations will clarify the investigated relationship and it may contribute to the SMA molecular mechanism understanding.

Keywords: spinal muscular atrophy, *PIK3R1* gene, polymorphism

INTRODUCTION

Spinal muscular atrophy (SMA) is the second most common lethal autosomal recessive disorder in Caucasians, after cystic fibrosis [17, 12]. The disease is characterized by the progressive degeneration of the alpha motoneurons which leads to muscle atrophy, paralysis and even premature death [6]. From genetically point of view, SMA is caused by mutations in *SMN1* (Survival Motor Neuron) gene, localized in 5q12.2-13.3 region. Approximately 95 -98% [9, 18] of the patients have homozygous deletion in *SMN1* exon 7, meanwhile 2-5% are heterozygous compounds, bearing deletion of exon 7 in one chromosome and subtle mutations in the other. Despite the fact that other genes as *SMN2* [4, 20] and *PLS3* [15] are reported to be modulators of the disease severity, the heterogeneity of clinical features cannot be explained. The action of other genes on the SMA severity seems to be the most plausible hypothesis.

In order to identify some potential modulators, we focused our research on establishing the involvement of phosphatidylinositol 3-kinase p85 regulatory subunit gene (*PIK3R1*) in SMA type I, the most severe form of the disease. The PIK3 is a key enzyme in the IGF signaling pathway, as it mediates the effects of different tyrosine kinase receptors, including IGF-1R. The involving of this signaling pathway in the motoneuronal survival and the trophic action of IGF has been reported previously [2, 14]. From the single nucleotide polymorphisms (SNP) described in *PIK3R1* gene, we focused on *Met326Ile* genetic variant (rs3730089) which have been reported as a nonsynonymous (coding) SNP associated with a decreased activity of PIK3 [3]. In this case-control study we proposed to investigate the relationship between this polymorphism and SMA type I disease.

MATERIALS AND METHODS

In our study we included 40 unrelated SMA type I patients (24 boys and 16 girls) who referred to "Al. Obregia" Clinical Psychiatry Hospital. The patients (with ages between 1 and 6 months) were diagnosed according with International SMA Consortium criteria. Proximal hypotonia, neurogenic electromyography and normal values for creatininkinase serum level were recorded when clinical and paraclinical investigations were performed for these patients. Molecular analysis confirmed the clinical diagnosis, as homozygous deletion of *SMN1* exon 7 was identified in all DNA patients probes. Also, 98% of our SMA subjects were found with the homozygous deletion of *SMN1* exon 8. To evaluate the involvement of the *Met326Ile* polymorphism in the SMA pathology we recruited also a sex-matched lot of 40 unrelated subjects without personal or heredo-colateral neuromuscular or neurodegenerative disorders. The subjects were enrolled in the study after the informed consent was obtained, according to the Declaration of Helsinki. Taking into account the fact that all the patients are minor age, the consent was given by the parents or their legal representatives.

Genomic DNA was isolated from blood samples stored on EDTA anticoagulant. For *Met326Ile* polymorphism genotyping, mismatch PCR-RFLP method was used. Oligonucleotide primers were designed to amplify a 242 pb product: Fwd-5'CCAACAACGGTATGAATAACCATAT3' and Rev-5'ATCCAGCACCCACAGTTCTC3'. The forward primer was designed with one base mismatch in order to create a restriction site for *NdeI* enzyme. The PCR was performed in a 10 µl reaction and was carried out in the following conditions: 94°C – 1 min for 1 cycle, 94°C – 30 sec, 58°C - 30 sec, 72°C - 30 sec

repeated for 35 cycles, 72°C - 2 min, 4°C - 1 min. Amplicons were verified in 2% agarose gel, restricted with *NdeI* enzyme for 3 hours at 37°C and separated in a 8% PAGE. Additionally, the Single Strands Conformation Polymorphism (SSCP) method was used in order to identify new genetic variations.

The genotypes were counted by direct numbering and the Hardy-Weinberg condition respecting was verified using the chi-square test (χ^2). Also, two by two contingency tables [19] and Cochran-Armitage test for trend [21] were used in order to establish the relationship between the *Met326Ile* polymorphism and

SMA type I disease. A value of $p < 0.05$ was considered statistically significant.

RESULTS

The genotypes distribution of the *Met326Ile* polymorphism in the SMA type I lot fit the Hardy-Weinberg equilibrium condition, as well as in the control lot. The χ^2 value obtained was 3.83 with a p of 0.05 for the SMA group and 1.79 with a p value of 0.17 for the healthy subjects' lot. The frequency of the genotypes distribution is shown in Table 1.

Table 1. The distribution of *Met326Ile* polymorphism genotypes in analyzed groups.

<i>Met 326 Ile</i> genotypes	SMA type I cases N (%)	Controls N (%)	OR [95% CI]	χ^2 (p value)
<i>Met/Met</i>	17 (0.425)	26 (0.65)	0.398 [0.1613<OR<0.9817]	4.073 (0.0435)
<i>Met/Ile</i>	22 (0.55)	14 (0.35)		
<i>Ile/Ile</i>	1(0.025)	0 (0)		

Note: OR (odd-ratio), N% (frequency of genotypes)

As it can be observed in the table, the genotype distribution seems to be significantly different between the two groups, as p value obtained is < 0.05 . Taking into account the small number (for cases lot) and even the absence (for controls lot) of the subjects with *Ile/Ile* genotype, the Yates correction was applied and a $\chi^2 = 3.218$ with a $p = 0.072$ was obtained, suggesting that the results do not accomplish the significance statistic level. Comparing only *Met/Met* and *Met/Ile* genotypes we find that *Met/Met* genotype is not associated with the disease, as resulted values are: OR = 0.416, 95%CI = [0.168<OR<1.0306], $p = 0.056$. Analyzing the frequency of alleles distribution, we also observed that for allele *Met*, the p value is not at a statistically significance level: OR = 0.495, 95% CI = [0.234<OR<1.047], $p = 0.063$. However, when we applied the Cochran-Armitage test, we noticed that there is a significant association trend between allele *Met* and type I of SMA disease: OR = 0.438, $p = 0.032$. No significant differences were observed between genotypes or alleles distribution when we compared the two lots according with the sex of the subjects. Also, neither within the groups, there were no differences in distribution on sexes.

In order to identify other possible variations in this genome sequence, SSCP analyzes were performed. The *PIK3R1* gene amplicons (2 μ l)

of denaturing buffer and incubated 5 minutes at 95°C. The samples were placed immediately on ice and loaded in a 8% non-denaturing PAGE. After electrophoretic running for 3 hours, at a constant voltage of 800V and 4°C, the gel was silver stained in order to observe the single strands bands.

As it can be observed in the Fig. 1, we didn't identify a different migration pattern of ssDNA, so we concluded that there are no genetic variations for SMA type I patients, at least in this region of *PIK3R1* gene.

DISCUSSIONS

The SMA is a neurodegenerative disorder whose main pathologic characteristic is the severe proximal muscle hypotonia. IGF signaling pathway has been shown previously to be involved in neuronal survival as well in muscle atrophy [13]. One of the most important element of this signaling cascade is PIK3, a heterodimeric enzyme composed of a regulatory subunit (p85) and a catalytic subunit (p110 α or p110 β) [1]. In the p85 α subunit gene *PIK3R1* (5q13) a coding polymorphism in codon 326 lead to the replacement of methionine with isoleucine [8]. This aminoacid change takes place in the NH2 terminal SH2 domain, an important region for binding of receptor tyrosine kinases [7]. Many studies assessed the relationship between this polymorphism and different pathologies. A special attention was accorded for researches about the involvement of *Met326Ile* polymorphism in prostate [16] or colon cancer risk [10]. If no significant differences were observed between prostate cancer patients and control, the study about the association with colon cancer risk revealed a twofold increased risk for *Ile/Ile* variant. Also, association between *Ile/Ile* genotype and diabetes and hypertension has been proved previously [5]. In this literature screening performed in order to identify reported relationships between the *Met326Ile* polymorphism and different pathologies, an association between this SNP and Alzheimer disease [11] was identified.



Figure 1. SSCP migration pattern (silver nitrate staining of 8% PAGE gel). Lanes 5 and 15 - healthy subjects; lane 11 - DNA weight marker (*BIORON GmbH*); lanes 1-4, 6-10, 12-14, 16-20 - SMA type I patients

Comparing our results with those reported by other populations, we could notice that the procentual distribution of genotypes for the healthy control subjects lots are similar in all groups (about 67-75% for *Met/Met* genotype and 1.8 – 2% for *Ile/Ile* genotype). Taking into account that, about our knowledge, this is the first study regarding the involvement of *PIK3R1* gene *Met326Ile* polymorphism in SMA disease, no other data were available concerning this polymorphism genotypes distribution in a SMA group. Our results obtained when Cochran-Armitage test for trend was applied lead to the conclusion that *Met326Ile* polymorphism should be take into account in the future studies focused on possible modifiers of the SMA pathology. Because our study has as limiting factor the small number of subjects, we considered that additional studies and replicates researches will help us to clarify the relationship between *PIK3R1* gene and SMA type I pathology.

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