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Original Article

Investigations of Microtubule-associated Protein 2 Gene Expression in Spinal Muscular Atrophy

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ABSTRACT

Aim: Spinal muscular atrophy (SMA) is a devastating genetic disease in childhood andff is caused by the absence of functional survival motor neuron (SMN) protein, which leads to impairments of the cytoskeleton, especially in neurons. Dysregulation of actin dynamics have been linked to SMA patho mechanisms, however involvement of altered microtubule dynamics is largely unknown. In this study, we investigated differentially expressed microtubule-related genes using *in vitro* and *in vivo* SMA model systems.

Materials and Methods: By focusing on microtubule-related genes, we re-analyzed publically available gene expression arrays, which were previously performed with induced pluripotent stem cell-derived motor neurons of SMA patients and the spinal cords of SMA mice. We found altered expressions of microtubule-associated protein 2 (MAP2), which was validated by real time reverse-transcription polymerase chain reaction using the SMN knock-down NSC34 cell line and the severe SMA mouse model.

Results: We showed that the expression of *MAP2* gene was significantly upregulated in both expression arrays. Upregulation was also detected in the brain and spinal cord tissues of severe SMA mice at different developmental stages.

Conclusion: Our findings suggest that microtubule regulatory proteins may be altered in SMN depleted cells and further research is needed to elucidate the contribution of dysregulated microtubule dynamics towards SMA.

Keywords: Spinal muscular atrophy, exon-array, microtubule-associated protein 2

Introduction

Spinal muscular atrophy (SMA) is an inherited neurodegenerative/neuromuscular disease and the leading genetic cause of infant mortality. The incidence of SMA is reported as 1 in 11.000 live births, however, due to a high rate of consanguinity, it is estimated to be higher in Turkey (1). SMA is characterized by the loss of alpha motor neurons in the spinal cord and progressive muscle atrophy. Since patients have different clinical phenotypes, SMA is grouped into V Types (0-IV) according to the age of disease onset and achieved motor functions (2,3). Type 0 refers to the most severe and the Type IV refers to the mildest form of SMA. Mutations or conversions of the *Survival of motor neuron 1 (SMN1)* gene are responsible for SMA and regardless of disease severity, homozygous deletion of exon 7 and 8 or exon 7 only is the most frequent mutation in patients (4-6). It has been shown that the absence of ubiquitously expressed, functional SMN protein leads to defects in both axon and dendrite growth, axonal transport and neuromuscular junction

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maturation in model systems and also patient samples (7-11). Dysregulation of F-actin dynamics have been linked to these defects due to alterations in either actin-regulatory proteins such as profilin, plastin 3, coronin 1C or Rho-kinase (ROCK) signaling pathways in SMN depleted cells (6,7,12,13). Although significant alterations in some microtubule-related proteins (stathmin and tau) have been shown, the contribution of altered microtubule dynamics to SMA patho mechanisms is largely unknown (14,15).

Re-analysis of publicly available gene expression data is a powerful and cost-efficient technique to better understand disease mechanisms. This technique has been used to explore the molecular mechanisms of various diseases, such as different cancers (16,17), osteoarthritis (18) and degenerative diseases (19). Furthermore, meta-analysis and the comparison of gene expression profiles of different species enables researchers to discover conserved molecular mechanisms (20).

Therefore, in this study, we re-analyzed human and mouse microarray gene expression data and specifically focused on genes regulating microtubule structure and function. We found that the expression of MAP2 was significantly altered in both induced pluripotent stem cell (iPSC) derived motor neurons of SMA patient and the spinal cords of SMA mice when compared to controls. MAP2 is primarily expressed in neurons and localizes to cell bodies and dendrites in mature neurons. The MAP2 protein binds to microtubules and regulates their stability. It also binds to F-actin and bundle filaments *in vitro* (21). Therefore, we focused on the *MAP2* gene and analyzed its expression in both the SMN knock-down motor neuron like NSC34 cell line and the Taiwanese SMA mouse model (22).

Materials and Methods

Human and Mouse Dataset Retrieval

The Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/, (23,24) database was searched for Human Exon arrays of motor neuron samples obtained from an SMA patient and a control. A record GSE27205 (25), which was on the Affymetrix Human Exon 1.0 ST platform (HuEx-1_0-st), was identified and CEL files of iPSC-derived motor neurons from an SMA patient (n=3, n is different clones from an SMA patient; GSM672172, GSM672173 and GSM672174) and a heterozygous father (n=3, n is different clones from an SMA patient's father; GSM672178, GSM672179 and GSM672180) were selected and extracted from the GEO database. This article does not contain any studies with human participants performed by the authors. Informed consent wasn't obtained. Mouse microarray data GSE19674, which was on an Affymetrix Mouse Genome 430A 2.0 Array platform (Mouse430A_2), including CEL files of spinal cords of homozygous knock-out SMA mice (n=4, $SMN2^{+/+}$; SMN $\Delta7^{+/+}$; $mSmn^{-/-}$; FVB.Cg-Tg (SMN2*delta7) 4299Ahmb Tg (SMN2) 89Ahmb $Smn1^{tm1Msd}$) and heterozygous SMN knock-out mice (n=4, $SMN2^{+/+}$; $Smn\Delta7^{+/+}$; $mSmn^{+/-}$; FVB.Cg-Tg (SMN2*delta7) 4299Ahmb Tg (SMN2) 89Ahmb $Smn1^{tm1Msd}$) (26). CEL files for SMA mice spinal cord (GSM491297, GSM491298, GSM491299 and GSM491300) and heterozygous SMN knock-out mice spinal cord (GSM491293, GSM491294, GSM491295 and GSM491296) were obtained from the GEO database.

Human and Mouse Gene Expression Analysis

Affymetrix Human Exon 1.0 ST array CEL files of an SMA patient and heterozygous father, and Affymetrix Mouse Genome 430A 2.0 Array CEL files of an SMA and heterozygous SMN knock-out mouse were analyzed via the Transcriptome Analysis Console 4.0.1.36 (TAC, https://www.thermofisher.com). For the human exon array analysis, Gene Level-Core, robust multichip average (RMA)-sketch workflow was applied to create probe level summarization files. For Mouse430A_2 arrays, the RMA algorithm (27,28) was used to normalize CEL files. The annotation files HuEx-1_0-st-v2.na36.hg19.transcript.csv for HuEx-1_0-st array and Mouse430A_2.na36.annot.csv for Mouse430A_2 array were used to annotate human and mouse arrays, respectively. Probe sets with ANOVA (eBayes) p-value <0.05 and fold change <-1.5 or fold change >1.5 were considered as differentially expressed for both human and mouse datasets.

Venn Diagram Analysis

The significantly altered genes, which are involved in microtubule structure and regulation of its dynamics, of human and mouse datasets were compared using Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/) (29).

Cell Culture and siRNA Transfections

Motor neuron-like murine NSC34 cells were grown in Dulbecco's modified Eagle medium (DMEM, 4.5 g/D-glucose), containing 5% fetal calf serum and 1% penicillin/streptomycin at 37 °C, 5% CO_2. The cells were transfected with siRNA against murine SMN (5'-CAGAAGUAAAGCACACAGCAA-3') or scrambled control siRNA (5'-GCGCAAAUAAACCGAAAGACA-3') by using OptiMeM (Thermo Scientific) and Lipofectamine 2000 (Invitrogen) in differentiation medium, containing 1% FSC for 72 hours. The SMN knock down efficiency of NSC34 cells was about 80%, which was routinely tested by Western blot.

RNA Isolation and Real Time RT-PCR

Total RNA was isolated from NSC34 cells by the RNeasy mini kit (Qiagen) using the manufacturer's protocol. Spinal

cord (p1, p5 and p8) and brain (p8) RNA samples of severe the SMA Taiwanese mice model (FVB.Cg-Tg (SMN2) 2Hung SMN1^{tm1Hung}/J (22) (Jackson Laboratory) and heterozygous control littermates, which were previously isolated and stored at -80 °C, according to German animal welfare regulations (breeding approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES, reference number 15/1774 LAVES). The numbers of mice used from different developmental stages are provided in the figure legends. cDNA synthesis was performed as previously reported (30). Briefly, 2.5µg of RNA was incubated with random hexamer primers (3µg/µl, Invitrogen) at 70 °C for 2 min, then M-MLV-transcriptase (200U/µl, Invitrogen), RNase-Inhibitor (40U/µl), DTT (0.1M, Invitrogen) and dNTP (10mM) was added. The reaction mix was incubated at 42 °C for 90 min and then 70 °C for 15 min for transcriptase deactivation. Real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed using 5µl diluted cDNAs (1:200), 7µl SYBR green (Applied Biosystems, PowerSYBR green mix), 2µl MAP2 primers (1.75µM, forward: 5'TCTAAAGAACATCCGTCACAGG3', reverse: 5'GGTGAGCATTGTCAAGTGAGC 3') or PPIA primers (1.75µM, forward: 5'TGCACTGCCAAGACTGAATG 3', reverse: 5'CCATGGCTTCCACAATGTTC 3') as the housekeeping gene. The reaction was performed in triplicate and StepOnePlus Real-time PCR System (Applied Biosystems) was used with the following conditions; 95 °C for 10 min (initial denaturation), 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. A comparative threshold cycle method (2-$^{\Delta\Delta CT}$) was used for the quantitation of the results.

Statistical Analysis

Statistical analyses were performed using Graphpad prism version 8 (La Jolla California USA). Mann-Whitney U test was used and a result of $p<0.05$ was considered as statistically significant.

Results

After the re-analysis of human and mouse array CEL files, we identified differentially expressed genes of SMA samples when compared to their related control samples in both human and mouse datasets (ANOVA (eBayes) p-value <0.05 and fold change <-1.5 or fold change >1.5). In order to find expressional alterations of microtubule-related genes in SMA patients and SMA mice, we analyzed the expression of several transcripts, which are involved in both microtubule structure and the regulation of its dynamics. Differentially expressed gene lists of human and mouse datasets are given in Table I and II, respectively.

Among the microtubule-related genes that we analyzed in this study, 5 genes were differentially expressed in both the SMA patient and mouse model, while MAP2, MAP7 and TUBB4A showed similar gene expression alteration patterns (Figure 1A). The *MAP2* gene was the only upregulated target, which drew our attention since the altered protein level of MAP2 was reported in a mouse model of amyotrophic lateral sclerosis (ALS), which is another motor neuron disease (31). Additionally, it has also been reported that either the protein level or the post-translational modifications of TAU, which is another microtubule-associated protein from the same protein family, was altered in ALS and SMA models, respectively (14,21,31). Therefore, we subsequently focused on the *MAP2* gene. To validate exon array results, we first used motor neuron-like NSC34 cells, which are murine neuroblastoma and spinal cord hybrid cell line as an *in vitro* model (32). We knocked down SMN by siRNA in the NSC34 cells and detected an upregulation in *MAP2* gene expression by real time RT-PCR in SMN-depleted cells compared to scrambled controls (Figure 1B). Since the increase in gene expression was close to the significance level, we decided to analyze *MAP2* gene expression in the severe SMA Taiwanese mouse model (22). First, we analyzed *MAP2* gene expression in the total brain and spinal cord of late symptomatic p8 mice. We found a significant upregulation in both tissues of SMA mice compared to control littermates (Figure 1C). Considering that spinal cord motor neurons are primarily affected by SMN loss, we further analyzed *MAP2* gene expression in the spinal cords of both pre-symptomatic (p1) and early symptomatic (p5) mice. A significant increase was detected in p5 but not p1 SMA mice (Figure 1C).

Discussion

SMA is a devastating genetic disease of childhood. Despite the recent therapeutic achievements with antisense oligonucleotide and successful clinical trials with gene therapy and small molecules, elucidating the functions of SMN protein and understanding the patho mechanisms of SMA is still needed. Re-analysis of public gene expression data is a promising tool to understand disease mechanisms. In this study, we re-analyzed raw data of previously published human exon-array and mouse microarray data that we obtained from the GEO database (23,24) and specifically focused on genes regulating microtubule structure and/or function, since there is little knowledge about microtubule dynamics in SMA. Previously, an altered organization of microtubules in the presynaptic terminals of the axons innervating transverse abdominis of SMA mice have been reported in this commonly affected muscle (33). Additionally, stathmin, a microtubule depolymerizing protein, is upregulated in both SMN-depleted NSC34 cells and SMA mice and has

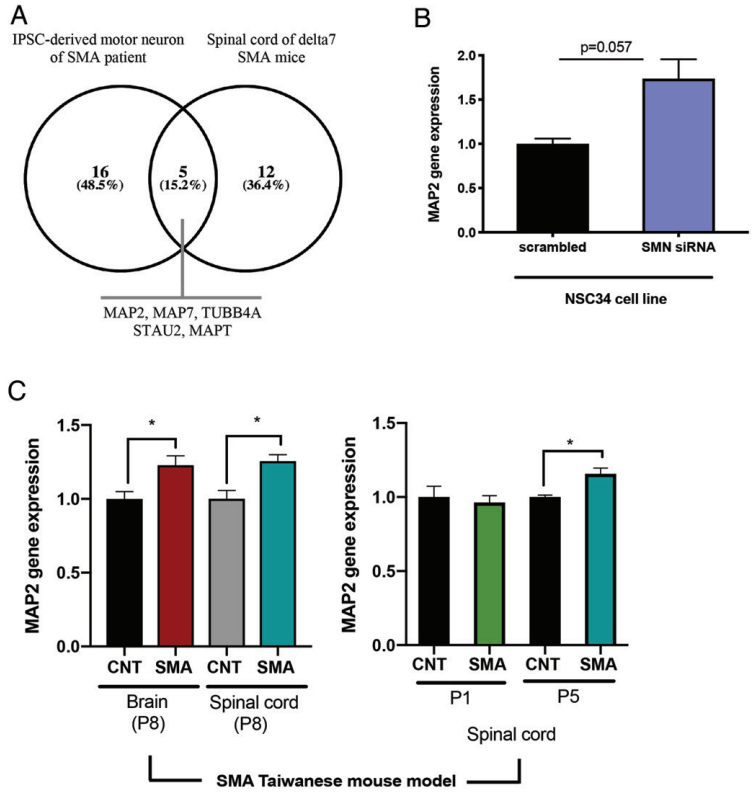

Figure 1. Expression analysis of genes regulating microtubule structure and its dynamics, A) Venn diagram of significantly altered genes in induced pluripotent stem cell-derived motor neurons of SMA patient and delta7 SMA mice using HuEx-1_0-st and Mouse430A_2 datasets, respectively. Fold changes of MAP2 transcript level in B) SMN knock down NSC34 cell line, n=4 biological replicates, C) spinal cord and brain tissues of p1, p5 and p8 severe SMA Taiwanese mouse model and heterozygous control littermates, for p1; n=5 (control) and n=4 (SMA) mice, for p5; n=6 (control) and n=5 (SMA) mice, for p8; n=5 (control) and n=5 (SMA) mice. PPIA gene was used for normalization. Mann-Whitney U, *p<0.05, Data are presented as means with standard error of mean.

MAP: Microtubule-associated protein, SMN: Survival of motor neuron, SMA: Spinal muscular atrophy

Table I. Expressional alterations of microtubule-related genes in iPSC-derived motor neurons of SMA patient

ID	Fold change	p-value	FDR p-value	Gene symbol	Description
*3591459	2.24	0.0426	0.1914	MAP1A; KRTAP6-1	microtubule associated protein 1A; keratin associated protein 6-1
3860229	1.71	0.001	0.0441	CLIP3	CAP-GLY domain containing linker protein 3
2790823	1.61	0.021	0.1322	MAP9	microtubule-associated protein 9
*3784208	1.59	0.0151	0.1103	DTNA; MAPRE2	dystrobrevin, alpha; microtubule-associated protein, RP/EB family, member 2
2525533	1.56	0.006	0.0718	MAP2	microtubule associated protein 2
3740126	-1.5	0.0472	0.2023	YWHAE; PAFAH1B1	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon; platelet-activating factor acetylhydrolase 1b, regulatory subunit 1 (45kDa)
3526151	-1.56	0.0258	0.1464	TUBGCP3	tubulin, gamma complex associated protein 3
4002081	-1.62	0.0034	0.0575	MAP7D2	MAP7 domain containing 2
2654394	-1.68	0.0467	0.2014	FXR1	fragile X mental retardation, autosomal homolog 1
3721926	-1.73	0.0046	0.0642	TUBG1	tubulin, gamma 1
3140640	-1.75	0.0081	0.0823	STAU2	staufen double-stranded RNA binding protein 2

Table I. Continued

ID	Fold change	p-value	FDR p-value	Gene symbol	Description
*3723687	-1.87	0.0026	0.0524	MAPT; MAPT-IT1	microtubule associated protein tau; MAPT intronic transcript 1
2901913	-1.88	0.0022	0.0504	TUBB	tubulin, beta class I
3764933	-2.03	0.0193	0.1264	TUBD1	tubulin, delta 1
*4050485	-2.05	0.0116	0.0968	GRIN1; TUBB4B	glutamate receptor, ionotropic, N-methyl D-aspartate 1; tubulin, beta 4B class IVb
3847959	-2.24	0.0012	0.0443	TUBB4A	tubulin, beta 4A class IVa
2600068	-2.27	0.0026	0.0524	TUBA4A	tubulin, alpha 4a
2878662	-2.51	0.0045	0.064	DIAPH1	diaphanous-related formin 1
3515965	-2.73	0.0071	0.0777	DIAPH3	diaphanous-related formin 3
2975741	-2.81	0.0002	0.0436	MAP7	microtubule-associated protein 7
*3453732	-16.34	0.0447	0.1965	TUBA1B; LMBR1L; TUBA1A	tubulin, alpha 1b; limb development membrane protein 1-like; tubulin, alpha 1a

*is used where the probe group is annotated to different gene symbols

been linked to defective microtubule polymerization (15). Hyperphosphorylation of TAU protein has been reported in the spinal cord of both SMA mice and patient samples (15). We observed an opposite gene expression profile of microtubule associated protein TAU (MAPT) between human and mouse gene expression results. According to our analysis, this target showed downregulation in human iPSC-derived motoneurons but it was upregulated in the spinal cords of SMA mice, which might be related to the presence of glial cells in the spinal cord (Table I and Table II). We focused on genes which have similar expression pattern in both arrays such as MAP2, MAP7 and TUBB4A. Among them, the *MAP2* gene was the only upregulated one in both SMA patient and SMA delta 7 mice. It has been known that MAP2 plays a role on neuronal growth and degeneration (34). Considering its function in regulating microtubule stability in neurons and previous reports on gene expression alterations in ALS, we analyzed *MAP2* gene expression for both *in vitro* and *in vivo* SMA model systems. Our experimental results were consistent with mouse microarray results, which showed a significant upregulation of MAP2 in the spinal cords of

Table II. Expressional alterations of microtubule-related genes in spinal cord of SMA delta 7 mice

ID	Fold Change	p-value	FDR p-value	Gene Symbol	Description
1417885_at	2.95	5.86E-06	0.0002	Mapt	microtubule-associated protein tau
*1449682_s_at	2.87	4.93E-08	5.60E-05	Tubb2a-ps2; Tubb2b	tubulin, beta 2a, pseudogene 2; tubulin, beta 2B class IIB
1424718_at	2.67	4.80E-06	0.0002	Mapt	microtubule-associated protein tau
1421327_at	2.42	0.0002	0.0019	Map2	microtubule-associated protein 2
1424719_a_at	2.41	1.13E-05	0.0003	Mapt	microtubule-associated protein tau
1450397_at	2.07	0.0039	0.0166	Map1b	microtubule-associated protein 1B
1421328_at	2.04	6.44E-05	0.0009	Map2	microtubule-associated protein 2
1425534_at	1.95	4.88E-05	0.0008	Stau2	staufen (RNA binding protein) homolog 2 (Drosophila)
1452679_at	1.88	7.85E-06	0.0003	Tubb2b	tubulin, beta 2B class IIB
1418066_at	1.79	1.01E-05	0.0003	Cfl2	cofilin 2, muscle
1428819_at	1.77	7.75E-05	0.001	Mapre1	microtubule-associated protein, RP/EB family, member 1

Table II. Continued

ID	Fold Change	p-value	FDR p-value	Gene Symbol	Description
1415978_at	1.71	0.0001	0.0016	Tubb3	tubulin, beta 3 class III
1425533_a_at	1.67	0.0002	0.0019	Stau2	staufen (RNA binding protein) homolog 2 (Drosophila)
1416256_a_at	1.59	1.44E-05	0.0004	Tubb5	tubulin, beta 5 class I
1435347_at	1.59	8.30E-05	0.0011	Stau1	staufen (RNA binding protein) homolog 1 (Drosophila)
1422765_at	1.53	0.0029	0.0133	Mapre1	microtubule-associated protein, RP/EB family, member 1
1424040_at	-1.56	0.0045	0.0186	Map7d1	MAP7 domain containing 1
1450407_a_at	-1.64	0.0012	0.0071	Anp32a	acidic (leucine-rich) nuclear phosphoprotein 32 family, member A
1418868_at	-1.67	0.0028	0.0128	En2	engrailed 2
1426518_at	-1.74	2.01E-05	0.0004	Tubgcp5	tubulin, gamma complex associated protein 5
1429894_a_at	-1.95	8.66E-05	0.0011	Map7	microtubule-associated protein 7
1423221_at	-2.08	0.0009	0.0056	Tubb4a	tubulin, beta 4A class IVA
1421836_at	-2.79	0.0001	0.0014	Map7	microtubule-associated protein 7
1421835_at	-3.21	0.0001	0.0013	Map7	microtubule-associated protein 7
1460219_at	-4.39	0.0002	0.002	Mag	myelin-associated glycoprotein

*is used where the probe group is annotated to different gene symbols

delta 7 SMA mice. We analyzed *MAP2* gene expression in the brain and spinal cord tissues of the SMA Taiwanese mouse model and detected a significant increase in *MAP2* gene expression in both tissues in the late symptomatic stage, which suggests a global differential expression of the *MAP2* gene in the central nervous system. Results obtained from earlier developmental stages of SMA mice showed that MAP2 upregulation in the spinal cord occurs during the onset of disease symptoms. MAP2 induction may be a compensatory mechanism in an impaired cytoskeletal environment to maintain both microtubule stability and actin-microtubule crosslink during disease progression. Detailed studies on MAP2 expression in both neuronal and surrounding non-neuronal cells will help to reveal any functional consequences of this alteration.

Conclusion

Our findings may indicate an altered expression of the *MAP2* gene during disease progression. Although our work is limited due to a lack of protein studies, our preliminary results indicate that microtubule regulatory proteins may be altered in SMN depleted cells. Further studies will be valuable in understanding the involvement of both MAP2 and other microtubule-related proteins to SMA patho mechanisms.

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Ethics

Ethics Committee Approval: This article does not contain any studies with human participants performed by the authors.

Informed Consent: Informed consent wasn't obtained.

Peer-review: External and internal peer-reviewed.

Authorship Contributions

Concept: G.B., H.E.Y., Design: G.B., C.S., Data Collection or Processing: G.B., C.S., N.H., Analysis or Interpretation: G.B., C.S., N.H., P.C., H.E.Y., Literature Search: G.B., C.S., Writing: G.B., C.S., N.H., P.C., H.E.Y.

Conflict of Interest: The authors have no conflicts of interest to declare.

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