Abstract

Autism spectrum disorder (ASD) is characterized by a range of social and behavioral challenges. Neuronal characteristics of ASD include altered synaptic transmission and neuronal excitability. Microexons have been found to be misregulated, resulting more often in microexon exclusion, in neuronal genes of autistic individuals. These autism-related genes have been postulated to be regulated by the neural splicing regulator nSR100. However, in three cell lines, four different genes exhibited differing patterns of alternative splicing, indicating that these genes are not regulated by the same regulatory factor.

Introduction

Autism spectrum disorder (ASD) refers to a range of conditions characterized by challenges with social interaction, communication, behavior, and sensory sensitivities. The symptoms associated with ASD can result in mild social impairment to severe disability. Approximately 1 in 68 children has been identified to have some form of ASD (Christensen. 2012.). Neuronal characteristics of autism have also been identified, including altered synaptic transmission and neuronal excitability. *De novo* missense mutations, *de novo* likely gene-disrupting mutations, and copy number variants have been found to be contributing factors to some ASD diagnoses (Iossifov et al. 2014.; Krumm et al. 2015.). Numerous other factors exist as potential causal contributors to autism, which encompasses a wide variety symptoms, likely with heterogenous causes.

Microexons, exons between 3 and 27 nucleotides long, have been found to be frequently misregulated in individuals with ASD. 30% of microexons have been found to be misregulated in the brains of individuals with ASD, compared to 5% of longer exons; 90% of these misregulated microexons have also been found to display neural-differential regulation (Irimia et al. 2014). Exclusion of microexons is more likely to occur in the brains of individuals with ASD than in the brains of individuals unaffected by ASD. The neural splicing factor nSR100 facilitates the inclusion of most neural microexons (Irimia et al. 2014). Neuronal activation, characteristic of autistic brains, decreases nSR100 levels and therefore increases microexon exclusion. Altered synaptic transmission and neuronal excitability has been shown to occur in nSR100 deficient mice. nSR100 deficient mice also exhibited altered social behavior and autistic-like behaviors (Quesnel-Vallières et al. 2016).

If autism-related genes containing microexons are regulated by nSR100, the same pattern of alternative splice variants would be expected to occur. In order to determine whether the same patterns were exhibited in the same cell line by multiple genes, four genes were analyzed in three cell lines.

Methods

RNA Purification: RNA was purified from the three cell lines Hep G2, A549, and THP-1 using the QIAGEN RNeasy© Plus Mini Kit protocol for purification of total RNA from animal cells. Concentration of RNA was determined using a spectrophotometer, and the purified RNA samples were aliquoted to make 5 ng/µL stocks for each cell line.

Primer Design: Primers were designed for four genes. Primers were created in exons adjacent to the location of the microexon, except in one case when one primer was created in an exon not adjacent to the microexon to ensure the target piece was large enough.

Gene	Direction	5' → 3' Code	Target Product Length Without Microexon	Target Product Length with Microexon
ABI1	Forward	GAAAGTGGCACGAAGAGAG	185 nucleotides	200 nucleotides
	Reverse	CAGTTCTTGCAGGCTGG		
AGRN	Forward	TGGAGTTCCGCTACGACCTG	175 nucleotides	187 nucleotides
	Reverse	TTGAGGACGGTGTGCGGAAC		
ITSN1	Forward	CACTTACCATTTCTGCACAGG	316 nucleotides	331 nucleotides
	Reverse	GAAGAGGTTACTGCCAAAGG		
RAPGEF6	Forward	GCACTGTAGGCATAGTCTG	252 nucleotides	267 nucleotides
	Reverse	GAATATGTGTCGGATGCAC		

Table 1. Primers used for RT-PCR designed around microexons.

Reverse Transcriptase PCR: RT-PCR was run in duplicate for each of the genes in each of the cell lines. The final volume for each reaction was 10 µL. Each reaction contained 2.0 µL of 5x buffer, 0.5 µL of forward primer, 0.5 µL of reverse primer, 0.4 µL of dNTP mix, 0.4 µL of enzyme mix, and 2.2 µL of dH_2O. 4.0 µL of RNA was added for a total concentration of 20 ng of RNA in each mix and a total volume of 10 µL for each reaction. The RT-PCR cycle conditions had two holds prior to the start of cycling at 50 °C and 95 °C for 30 minutes and 25 minutes respectively. For RT-PCR cycling, melting occurred at 95 °C, annealing occurred at 58 °C, and elongation occurred at 72 °C for 30 seconds for each stage for a total of 40 cycles.

RT-PCR Product Visualization: RT-PCR products were run on a 4% agarose electrophoresis gel to visualize bands.

DNA Purification: Remaining RT-PCR product was purified using the QIAGEN DNeasy protocol.

Sequence Analysis and Alignment: Purified RT-PCR product was sent to GeneWiz for sequencing for ABI1, AGRN, and ITSN1. ABI1 showed two bands, so these sequences were also aligned. The sequence of both bands was identical until the location of the microexon, where two peaks occurred. The sequence of the lower peaks was determined visually. These two sequences were aligned using Clustal Omega using the NCBI database as a reference.

Results

AGRN and ITSN1 each exhibited only one band, which sequencing showed to be the splice variant lacking the microexon in all three cell lines. The microexon that was lacking from the sequence of AGRN was a 12 nucleotide microexon. A 15 nucleotide microexon was not observed in the ITSN1 sequence. ABI1 showed two bands of similar intensities in cell lines THP1 and A549. However, in the cell line HepG2 the splice variant lacking the microexon showed a more intense band than the variant that contains the microexon. ABI1 sequences were confirmed to be the expected sequences by sequencing through GeneWiz. Analysis of RAPGEF6 indicated the presence of two bands in the cell lines HepG2 and A549, but only showed one apparent band in the cell line THP1. The RAPGEF6 alternative splice variant containing the microexon exhibited a more intense band in the three cell lines than the splice variant lacking the microexon, which was light in the HepG2 and A549 cell lines, and not visible in the THP1 cell line (Figure 1).

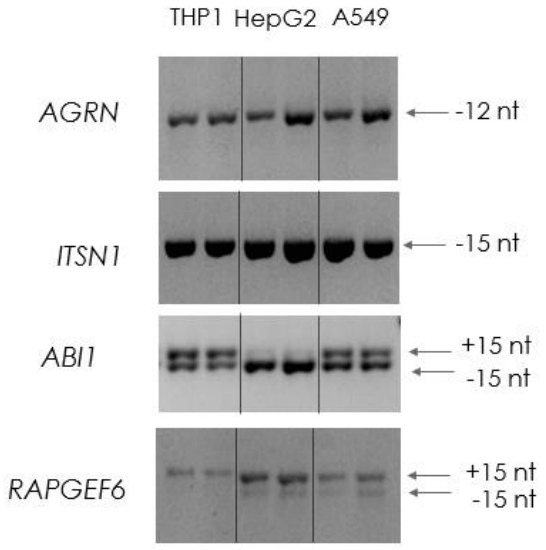

Figure 1. RT-PCR results visualized on a 4% agarose gel for electrophoresis. Primers were used for the genes AGRN, ITSN1, ABI1, and RAPGEF6 in the cell lines THP1, Hep G2, and A549.

Alignment of the two sequences for ABI1 showed both of the expected sequences, one of which had the 15 nucleotide microexon and the other of which was missing the 15 nucleotide microexon (Figure 2). The microexon was in the expected location with the expected sequence. Additionally, analysis of intronic sequences indicated that no unexpected nucleotides occur at the intronic splice sites of ABI1 (Figure 3).

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CLUSTAL O(1.2.4) multiple sequence alignment

-15 nt      AATAAGAATACATCAAGAACTCACAAAATAATAGCACCTGCGAATATGGAGCGCCCTGTA
+15 nt      AATAAGAATACATCAAGAACTCACAAAATAATAGCACCTGCGAATATGGAGCGCCCTGTA
            ************************************************************

-15 nt      AGGTATATTCGGAAACCTATCGATTACACAGTTCTGGATGATGTGGGCCATGGTGTCAAG
+15 nt      AGGTATATTCGGAAACCTATCGATTACACAGTTCTGGATGATGTGGGCCATGGTGTCAAG
            ************************************************************

-15 nt      --------------CATGGAAATAACCCGCCTGCAAGAACTGCTATAGTGTCACCTAAA
+15 nt      TGGCTAAAAGCCAAGCATGGAAATAACCCGCCTGCAAGAACTGCTATAGTGTCACCTAAA
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Figure 2. Alignment of the DNA sequences obtained from GeneWiz via Clustal Omega. The upper sequence is the sequence lacking the 15 nucleotide microexon. The lower strand is the sequence containing the 15 nucleotide microexon. The microexon is highlighted by the red square.

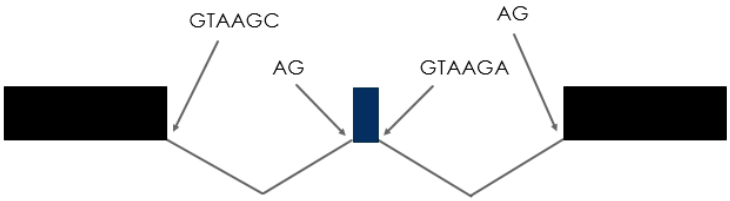

Figure 3. ABI1 showed expected sequences in intronic splice site regions.

Discussion

If the degree of inclusion of microexons depends on the same splicing regulator, it would be expected that different genes in the same cell line would show the same splicing pattern. Therefore, the genes ABI1, AGRN, ITSN1, and RAPGEF6 would be expected to exhibit the same patterns of alternative splicing within in the same cell line of the three observed: THP1, Hep G2, and A549. We observed the same pattern in AGRN and ITSN1 in all cell lines, where there was only one variant and that variant was that in which the microexon had been excluded. ABI1 showed a unique pattern, where all three cell lines

exhibited two bands (Figure 1). The two bands were of approximately the same intensity in the THP1 and A549 cell lines, but in the HepG2 cell line the lower band, which does not contain a microexon, was more intense than the microexon-containing band, indicating a different splicing regulator than that of AGRN and ITSN1. Analysis of RAPGEF6 showed a unique pattern because in all three cell lines the band of the splice variant containing the microexon was more intense than the band lacking the microexon. Furthermore, THP1 exhibited a novel pattern where the splice variant containing the microexon was the only visible band (Figure 1). This indicates RAPGEF6 is regulated by a different splicing regulator than the other three genes. Because different splicing patterns were observed among the microexon-containing genes ABI1, ITSN1, AGRN, and RAPGEF6 within specific cells lines, autism-related genes appear to be differentially regulated and may not be dependent on nSR100.

The sequencing results indicated the expected sequences for the three genes that were sent out for sequencing: ABI1, AGRN, and ITSN1. Analysis of ABI1 showed that the intronic sequences were those that would be expected at the 5' and 3' splice sites. Further analysis of these genes and their regulators could allow scientists to elucidate the mechanisms by which microexons tend to be excluded in the brains of individuals with ASD.

Additional next steps would include identifying which neuronal microexon-containing genes are regulated by known splicing regulators. When groups of genes regulated by the same splicing regulator are identified, the effects of different compounds on differential splicing could be tested. This is a significant area of research because 1 in 68 children have been diagnosed with some form of ASD. If exclusion of microexons could be decreased by treatment with different compounds that could be taken as supplements or consumed in the diet, ASD symptoms may be decreased, potentially increasing quality of life for people with ASD and their families.

Citations

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