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Investigation of Genomic Imprinting in the X-linked Transketolase-like Protein 1 (TKTL1) in Human and the Implications for Autism

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Contents

Abstract

Autism and Autism Spectrum Disorders effect up to 2% of the population, with males being 2-3 times more likely to develop a disorder compared to females. Current data suggests that a combination of genetic and environmental factors lead to the development of these disorders. One of the candidates for involvement in the onset of the disease is oxidative stress since levels are affected by both internal and external factors. Increased oxidative stress has been linked to Autism and Autism Spectrum Disorders. *Transketolase-like 1* (*TKTL1*) is believed to be involved in the non-oxidative phase of the pentose phosphate pathway (PPP) which produces ribose. This pathway is separate from the oxidative phase of the PPP which produces ribose along with NADPH. NADPH is essential for the production of antioxidants and the maintenance of reactive oxygen species at levels low enough to prevent oxidative stress. Altered expression of *TKTL1* could be correlated with an increased production of ribose from the non-oxidative phase causing negative feedback to decrease production of ribose and NADPH from the oxidative phase and increase risk for oxidative stress. This investigation analyzed genomic and cDNA sequencing data as well as RNA expression data from control and autistic sample groups (pedigree lymphoblast cell lines and human brain samples) and from Turner's Syndrome lymphoblast cell lines (X_m and X_p) in order to determine the imprinting status of the *TKTL1* gene and to compare the expression levels between the autistic and control groups. The RNA expression results from this analysis indicate that there is no statistically significant difference in *TKTL1* when comparing the autistic group with the control group but there is significant variation between individuals of both groups. The sequencing results demonstrate that *TKTL1* is potentially imprinted in a sub-region specific manner within the brain in the control group but that this imprinting is not present in the autistic samples.

Background

The pentose phosphate pathway is involved in glucose metabolism and primarily produces NADPH, ribose, or both. There are two phases to this pathway: oxidative and non-oxidative. The oxidative phase is irreversible and converts glucose 6-phosphate to ribose 5-phosphate which can be used in the synthesis of nucleotides or production of coenzymes. During the production of ribose in the oxidative phase, NADP+ is reduced to NADPH, an essential cofactor in many cellular reactions. The non-oxidative phase of the pentose phosphate pathway is reversible but produces ribose only, it does not produce NADPH [1]. This is an important distinction to make because of the role NADPH plays in the cell. NADPH is essential for the synthesis of fatty acids and steroids within the liver as well in the synthesis of deoxyribonucleotides for DNA. In this context though, its most important function is that it facilitates the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) [1]. GSH is an antioxidant and reacts with reactive oxidative species to prevent them from damaging the cellular environment. In this case, GSH reacts with peroxides to produce water. NADPH itself is also involved in reactions which eradicate reactive oxygen species (ROS) [2].

Maintaining appropriate antioxidant levels is essential to avoiding oxidative stress within the cell. If reactive oxygen species are not reduced in a controlled manner they can react with reduced transition metals in the cell to produce a very reactive compound called a hydroxyl radical. These reactive species interact with cellular components including proteins, lipids, and nucleic acids and eventually lead to apoptosis or necrosis [2]. Levels of ROS that are too high to be counteracted or controlled by a cell's antioxidant capabilities lead to this damage, the process is termed oxidative stress [2]. GSH is the most important antioxidant for the reaction with and the elimination of environmental toxins; it is therefore essential in avoiding oxidative stress [2].

While all organs can be damaged by the effects of oxidative stress, the brain is one of the more susceptible because it has larger concentrations of potential reactants for ROS as well as high energy needs compared to other tissues. Studies have shown that oxidative stress in the brain during development can be especially damaging and could be a contributing factor in certain developmental diseases [2].

In recent research, Chauhan et al has shown that there is a potential link between increased oxidative stress and autism. Specifically, this research showed higher levels of lipid peroxidation in individuals with autism compared to a control group. This is a sign that ROS have interacted with lipids in the cell. The group also found lower levels of total glutathione (both reduced and oxidized) as well as a decreased ratio of reduced glutathione to oxidized glutathione in autistic versus control subjects. Since reduced glutathione is an important antioxidant, this indicated a higher susceptibility to reactive species in the autistic individuals. This research also found differences in the metabolism of certain metals and increased levels of the free radical, nitric oxide (NO), and the pro-oxidant, xanthine oxidase (XO), in the subjects with autism compared to the control group [2]. Based on these results, Chauhan et al stated that increased levels of oxidative stress could cause membrane lipid abnormalities, mitochondrial dysfunction, excitotoxicity, inflammation, and immune dysregulation in autism [2]. These impacts may play a part in the behavioral abnormalities, sleep disorder, and gastrointestinal disturbances that can be found in individuals with autism [2].

Autism and autistic spectrum disorders (ASD) are terms that refer to a group of neuro developmental disorders with two key characteristics. In order to be considered for the diagnosis of autism, an individual must have social and communicative deficits as well as limited and repeated interests and behavior [3]. Autism and associated disorders cause a wide range of

symptoms and are associated with several concomitant conditions and with increased risk of certain medical problems. Current estimates suggests that the worldwide prevalence of autism and other autism spectrum disorders could be up to 2%, with 2 - 3 times more males affected compared with females. A large percentage of those diagnosed with autism struggle with the associated difficulties for the rest of their lives [3]. Studies suggest that there is an environmental role in the development of autism, but twin studies show that there is a relatively high degree of heritability. It is likely that the interaction between the two that leads to the disorder [3]. This causal theory is supported by the idea that oxidative stress may contribute to the development of autism. Since the production of reactive oxygenated species and antioxidants can be influenced by both environmental and genetic components, both factors influence the balance essential for protection against oxidative stress.

Transketolase-like 1 (*TKTL1*) is a gene on the q arm of the X chromosome that produces the Tranksketolase-like 1 protein, a protein involved in the non-oxidative phase of the pentose phosphate pathway. TKTL1 is believed to behave similar to the transketolase enzyme. Transketolase facilitates the reversible reaction of 5 Fructose-6-Phosphate and Glyceraldehyde-3-Phosphate to produce Erythrose-4-Phosphate and Xylulose-5-Phosphate (which can then be converted to ribose) and the reversible reaction between Glyceraldehyde-3-Phosphate and Sedoheptulose-7-Phosphate which produces Ribose-5-Phosphate directly as well as Xylulose-5-Phosphate [1]. *TKTL1* is an important gene to consider when investigating the link between autism and oxidative stress because of its role in this pathway. It is possible that altered levels of *TKTL1* expression result in increased production of ribose through the non-oxidative phase of the PPP. This potentially causes negative feedback that reduces the production of NADPH through the oxidative phase and increases the risk for oxidative stress. An aspect crucial for

understanding the impacts of *TKTL1* and its expression in a cell is the fact that it is an imprinted gene.

Genomic imprinting is a term used to describe a type of epigenetic gene regulation, where epigenetic refers to the phenomenon of regulation stemming from differences not in the actual sequence of the DNA but rather in non-DNA elements. In genomic imprinting, these differences result in differential expression of certain genes in a parent-or-origin-dependent manner [4]. Skuse et al were the first to find evidence for a parent-of-origin effect involving the human X chromosome. In their research they looked at 80 females with Turner Syndrome (females with only one fully intact X chromosome, often referred to as X0). 55 of these individuals had only a maternal X chromosome (referred to as $45,X_m$) and 25 had only a paternal X chromosome (referred to as $45,X_p$). Skuse et al compared these two groups to each other as well as to normal males (who have a Y chromosome and a maternal X chromosome) and normal females (who have both a paternal and maternal X chromosome) [5].

The first part of the Skuse et al research relied on surveys of parents and teachers. From this, the research group found that 40% of $45,X_m$ individuals had been labeled as having special education needs compared to 16% of $45,X_p$ individuals and only 2% of the general population [5]. They also found that 72.4% of $45,X_m$ individuals were affected by social difficulties compared to 28.6% of $45,X_p$ individuals [5]. Continuing this study, $45,X_m$ individuals showed poorer performance on social cognitive, verbal intelligence, and behavioral inhibition tasks compared with $45,X_p$ individuals. In the social cognitive and behavioral inhibition tasks normal females outperformed normal males as well, with the normal females having similar mean scores to the $45,X_p$ females in the behavioral inhibition tasks [5]. Skuse et al also noted that 3 of the Turner Syndrome females were diagnosed with autism and all three of them had only the

maternal X chromosome [5]. All of this data supports the hypothesis that there is imprinting on the X chromosome that is able to influence cognition. In order to narrow down the potential location of the imprinted locus, the Skuse et al group also looked at 8 females with two X chromosomes but for whom there was a p arm deletion on the paternal X chromosome. This deletion led to a preferential inactivation of the X_p chromosome compared with the X_m chromosome. They found that these individuals performed comparably to the 45,X_p Turner individuals on their tasks [5]. From this information, Skuse et al determined that imprinted locus influencing these differences was not deleted (and therefore is located somewhere on the q arm or on the p arm closer to the centromere) and also that the locus was not subject to X inactivation [5]. The data from this experiment suggests that it is possible that an X-linked imprinted gene may contribute to social cognitive development and that this type of imprinting could explain the increased prevalence of autism in males compared with females.

The purpose of this research was to look at expression of *TKTL1* in human brain for both control and autistic samples as well as in human lymphoblast Turner's Syndrome X_m and X_p cell lines. Differential expression of *TKTL1* among these samples may provide further insight into the cause and development of autism or the potential risk factors for the developmental disorders. Further knowledge in this area may help to propel development of diagnostic tools as well as prevention or intervention strategies to help reduce the impacts of the disorder.

Materials and Methods

Samples

The human brain samples were provided by Dr. Abha Chauhan of the Institute for Brain Research, Staten Island, NY. They were collected from the occipital and parietal lobes of both autistic and control subjects. The samples and the associated information is listed in Figure 1.

UMB#	Sample Type	Age (years)	Sex	PMI (hours)
4670	Control	4.6	M	17
1185	Control	4.7	M	17
1500	Control	6.9	M	18
4898	Control	7.7	M	12
1708	Control	8.1	F	20
1706	Control	8.6	F	20
1407	Control	9.1	F	20
4722	Control	14.5	M	16
1846	Control	20.6	F	9
4645	Control	39.2	M	12
4671	Autistic	4.5	F	13
1349	Autistic	5.6	M	39
4849	Autistic	7.5	M	20
1174	Autistic	7.8	F	14
4231	Autistic	8.8	M	12
797	Autistic	9.3	M	13
1182	Autistic	10	F	24
4899	Autistic	14.3	M	20
1638	Autistic	20.8	F	50
5027	Autistic	38	M	26

Figure 1. Human Brain Samples
PMI stands for post mortem interval.

The first set of cell lines were Turner Syndrome cells ($45X_0$) one line contained only a maternal X chromosome; the other, only a paternal X chromosome. The second set was a pedigree that consisted of sibling pairs of autistic and normal cell lines, the samples are listed in Figure 2.

Lymphoblast Cell Lines	Serial Number	Sex	Autism Status	Age	Info.	Pedigree
16	03C23216	M	**Autistic**	9		1
18	03C23218	M	Control			1
15	03C23215	M	**Autistic**	12		1
23	03C23223	F	**Autistic**	21		1
28	03C23228	M	Control			1
12	03C23212	F	Control			1
75	06C53575	F	Control			2
77	06C53577	M	**Autistic**	12		2
74	06C53574	M	**Autistic**	10		2
76	06C53576	M	**Autistic**	8		2
72	06C53572	M	**Autistic**	7		2
82	06C53582	F	Control			2
83	06C53583	F	**Autistic**	3		2
69	03C15169	M	Control			3
68	03C15168	F	**Autistic**	7		3
67	03C15167	M	**Autistic**	6		3
22	03C16522	F	Control			3
26	03C16526	M	Control			3
23	03C16523	F	**Autistic**	10		3
25	03C16525	F	Control			3
4	01C05904	F	**Autistic**	4		4
3	01C05903	M	**Autistic**	8		4
2	01C05902	M	**Autistic**	12		4
5	01C05905	F	**Autistic**	2		4
42	01C06842	M	Control	8	twin	5
43	01C06843	F	Control			5
44	01C06844	F	**Autistic**	8	twin	5
45	01C06845	F	**Autistic**	9		5
48	01C06848	F	Control			5
34	01C07334	M	Control			6
35	01C07335	F	**Autistic**	30		6
33	01C07333	M	**Autistic**	36		6
36	01C07336	M	Control			6
37	01C07337	F	Control			6

Figure 2. Pedigree Lymphoblast Cell Lines
Related groups are blocked together, blank spaces indicate information not provided. Samples are referred to by the number listed in the first column.

RNA Extraction and cDNA Synthesis

RNA was extracted from both sets of cell culture lines and human brain samples. RNA extraction was performed using the QIAGEN RNeasy Kit. cDNA was synthesized from some of the collected RNA using the qScript cDNA Super mix from Quanta. The cDNA was used in sequencing analysis and the remainder of the RNA was using in RNA expression analysis.

DNA Extraction

DNA was extracted from the pedigree cell lines as well as from the human brain samples using Phenochloroform Isoamyl (PCI) Extraction.

Sequencing and Sequencing Analysis

Both the DNA extracted from samples and the cDNA prepared from extracted RNA was sequenced and then analyzed. The sequencing was performed using the BigDye 3.1 protocol, single nucleotide polymorphism (SNP) analysis utilized Finch TV software. SNPs appear as a double peak in sequencing results indicating the presence of two different nucleotides at the same position in the sequence, also known as heterogeneity in the genome.

RNA Expression Analysis

Analysis of the expression of RNA across different samples, including the Turner Syndrome cell lines, the pedigree cell lines, and the human brain samples, was performed using reverse transcriptase quantitative polymerase chain reactions (qRT-PCRs). The results from the qRT-PCRs were normalized to expression of the β-Actin gene. Fold change was calculated using the ΔΔCt method and data was analyzed using BioRad CFX Manager and Microsoft Excel.

Results

Sequencing and Sequencing Analysis

Sequencing was run on all Pedigree Lymphoblast Cell Line samples as well as all of the human brain samples. In sequencing of the genomic DNA, the majority of samples came back as clean sequences. Many of these sequences showed no SNPs; however, samples 67, 1174, 1407, 1846 and 4671 did have evidence of diagnostic SNPs. Sample 1407 had evidence of two different SNPs, one of which is the same as the SNP that appears in samples 67, 1174, 1846 and 4671. In this analysis, a double peak in the sequence is indicative of a SNP at that point in the gene. Some of the sequences containing these SNPs are shown in Figure 3. Others listed, though not shown, have the same SNP. The SNP in Figure 3A, B, and D is the SNP that was used in analysis of cDNA to determine if imprinting was present.

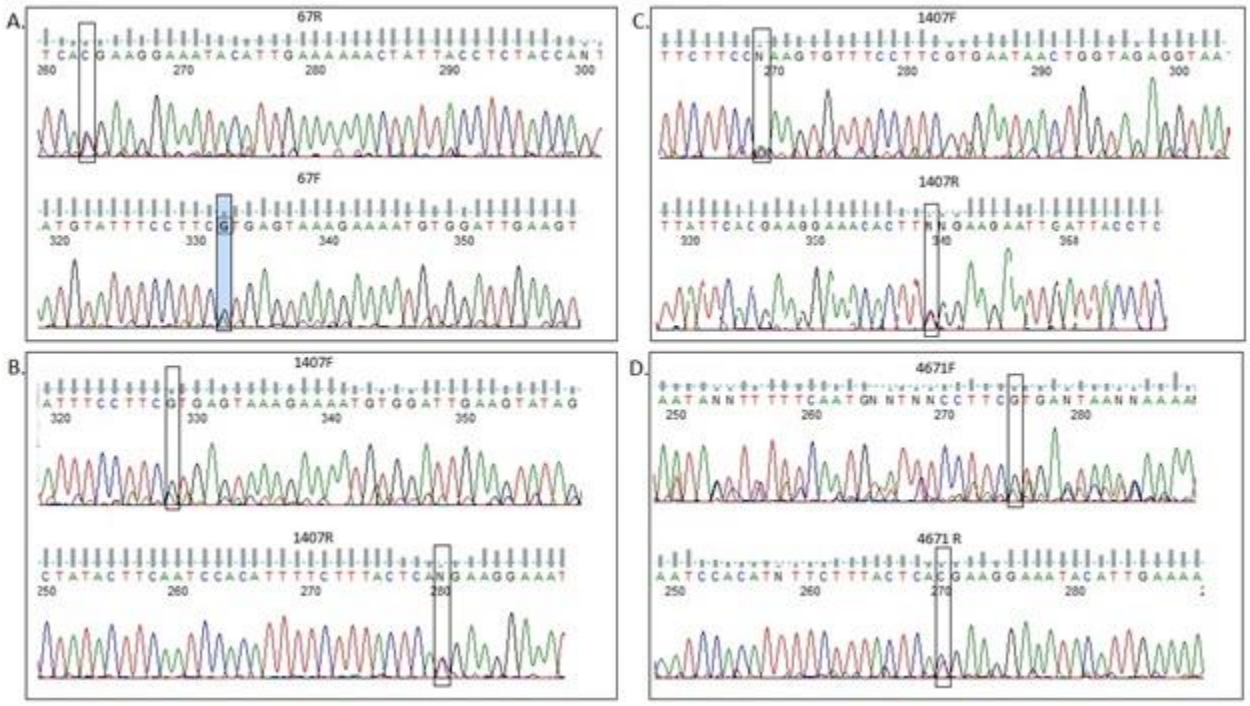

Figure 3. SNP Analysis for genomic DNA
A, B, and D show the same SNP in three different samples: 67, 1407, and 4671. The same SNP was also found in samples 1174 and 1846. C shows a second SNP found in sample 1407. Nucleotide positions with SNPs are indicated by the outlined sections.

In the analysis of cDNA sequences, the presence of a SNP is indicative of biallelic expression while a single peak at a position where a SNP exists in the genomic DNA is indicative of monoallelic expression. In the sequencing of the cDNA from the samples with diagnostic SNPs present, we found evidence of monoallelic expression in the parietal samples from control sample 1407, and evidence for biallelic expression in the occipital samples from 1407. The same pattern of SNPs was found in control sample 1846 as well, once again indicating parietal monoallelic expression and occipital biallelic expression. The sequencing results for both of these samples is shown in Figure 4.

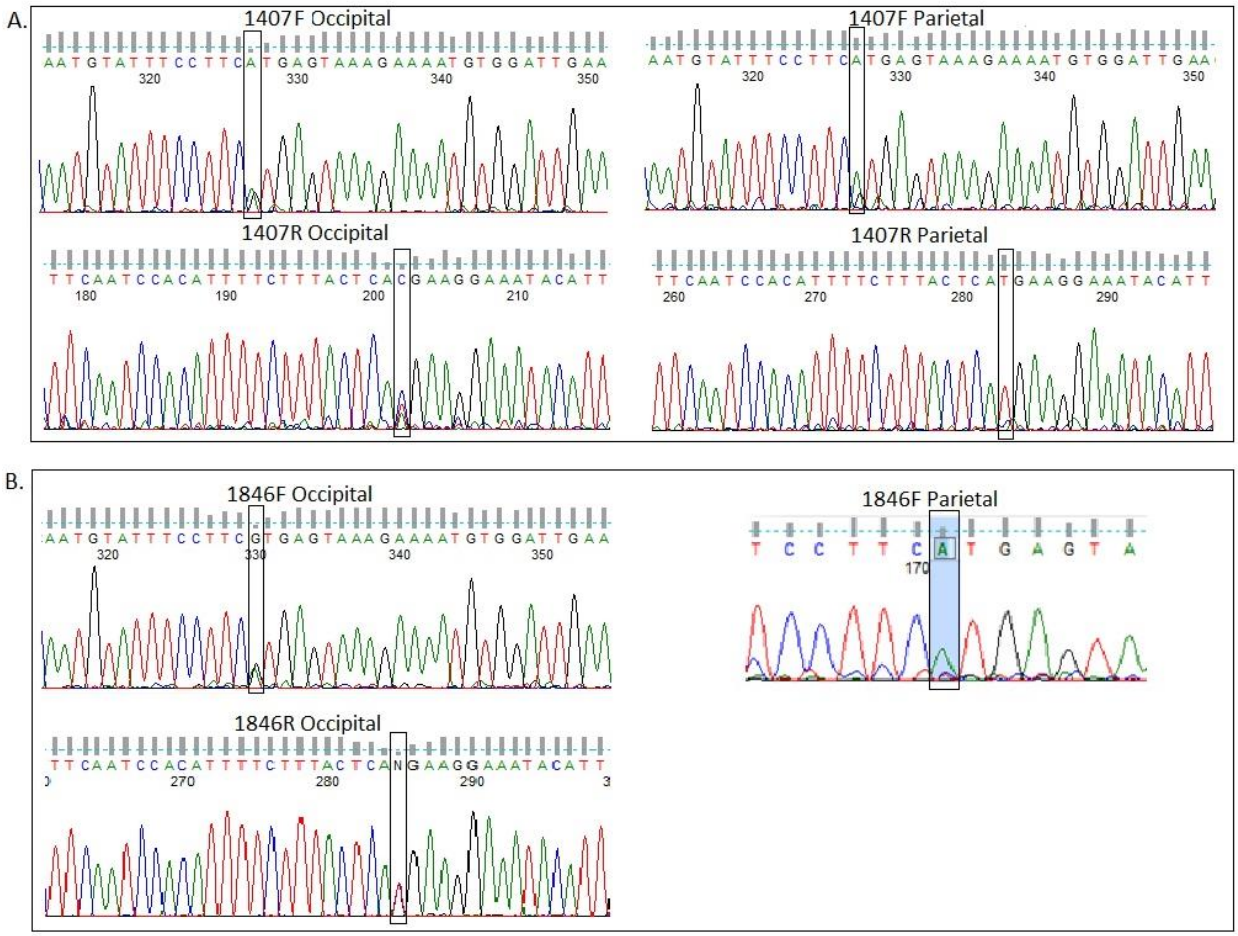

Figure 4. SNP Analysis for Control Samples cDNA
Figure 4A shows sample 1407 occipital and parietal and demonstrates monoallelic expression in the parietal and biallelic expression in the occipital. Figure 4B shows the same trend in sample 1846.

Analysis of the same SNP in the cDNA of the autistic samples revealed a different result. Neither of the autistic samples with diagnostic SNPs, 1174 and 4671, showed monoallelic expression in the parietal. Both of these samples had evidence of biallelic expression of *TKTL1* in occipital tissue and parietal tissue. The sequencing data for these autistic samples is shown in Figure 5.

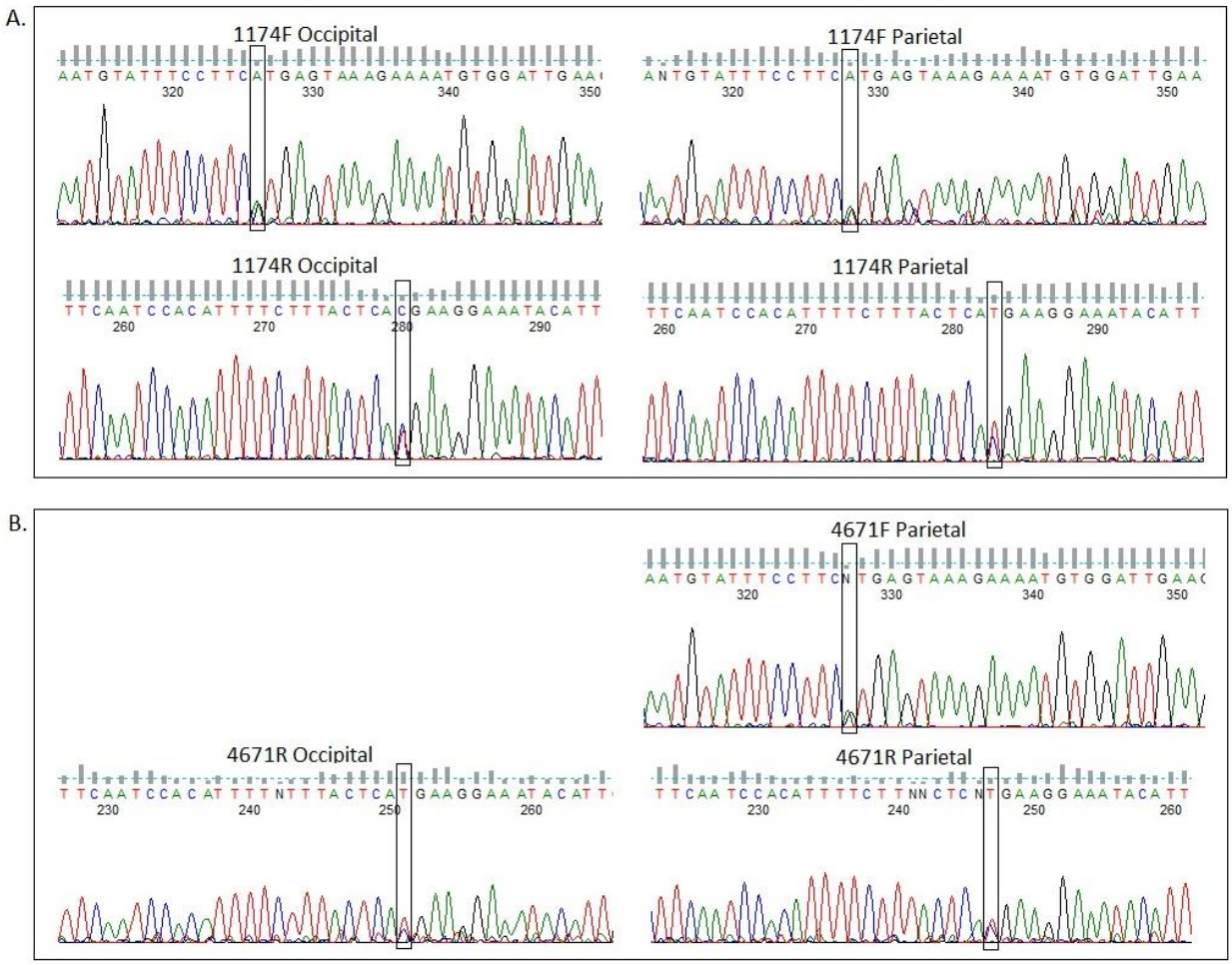

Figure 5. SNP Analysis for Autistic Samples cDNA
Figure 4A shows sample 1174 and demonstrates biallelic expression in both the occipital and parietal tissue. Figure 4B shows the same pattern in sample 4671.

RNA Expression Analysis

The first expression analysis compared expression of several genes between the two Turner's Syndrome cell lines using a qRT-PCR. This analysis revealed no significant difference in *TKTL1* expression between the X_m and X_p samples. Graphical representation of the results of this analysis is shown in Figure 6.

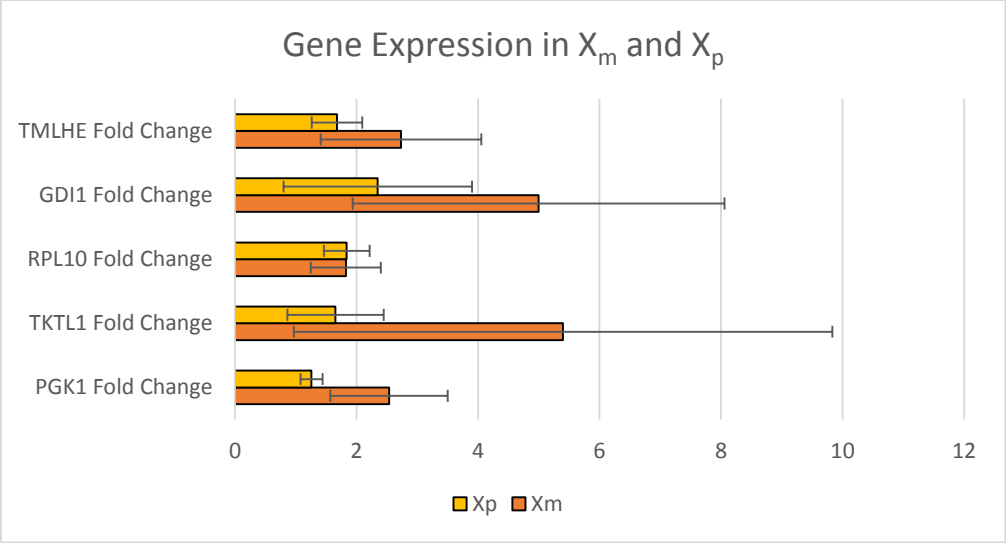

Figure 6. Gene Expression in Turner's Syndrome Cell Lines
The gene of interest in this figure is *TKTL1*. This analysis of RNA expression shows no significant difference in expression between the two cell lines.

The second RNA expression analysis investigated potential differences in *TKTL1* expression between several of the pedigree lymphoblast cell lines. This analysis showed significant variability between individual samples both within and between the autistic and control groups but the analysis did not show significant differences between the autistic and control groups due to the high individual variation. Graphical representation of this analysis is shown in Figure 7.

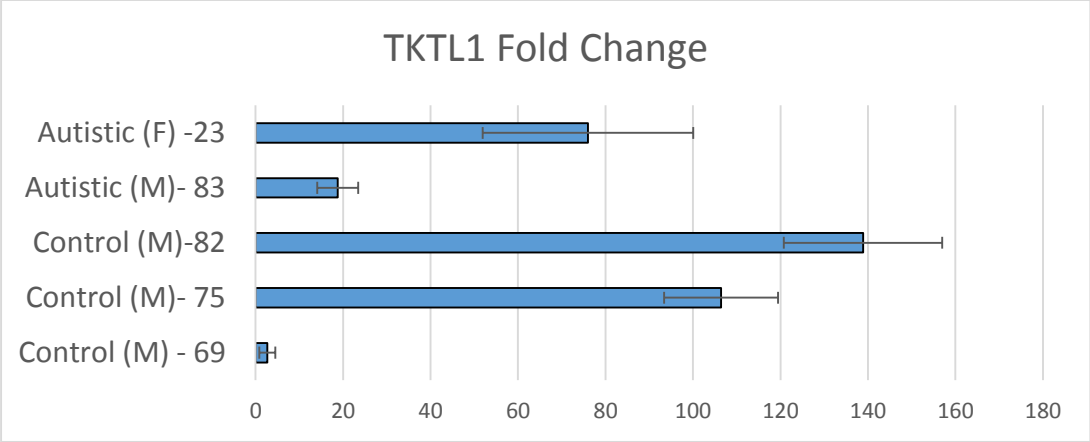

Figure 7. *TKTL1* Expression in Pedigree Lymphoblast Cell Lines
This analysis shows variation between individual samples but no significant difference between the autistic and control groups. Individuals 23 and 69 are a sibling set, as are individuals 83, 82, and 75.

The third RNA expression analysis utilized the human brain samples. The samples were divided into four categories: female parietal, female occipital, male parietal, and male occipital. Analysis looked for differences in expression within these four groups. Two different primer sets were used; labeled as T3 and T1, they capture different portions of the *TKTL1* gene. The sequence elucidated by the T1 primer is located in the 5' untranslated region of the gene, the sequence amplified by the T3 primer is located in the 3' untranslated region of the gene. In general the T3 primer was a more efficient amplifier than the T1 primer. All four cohorts showed significant individual variation between samples both within and between the autistic and control sample groups. However, due to this high individual variation there is no statistically significant difference between the autistic group as a whole and the control group as a whole. Graphical representations of these analyses are shown in Figures 8, 9, 10, 11, 12, and 13.

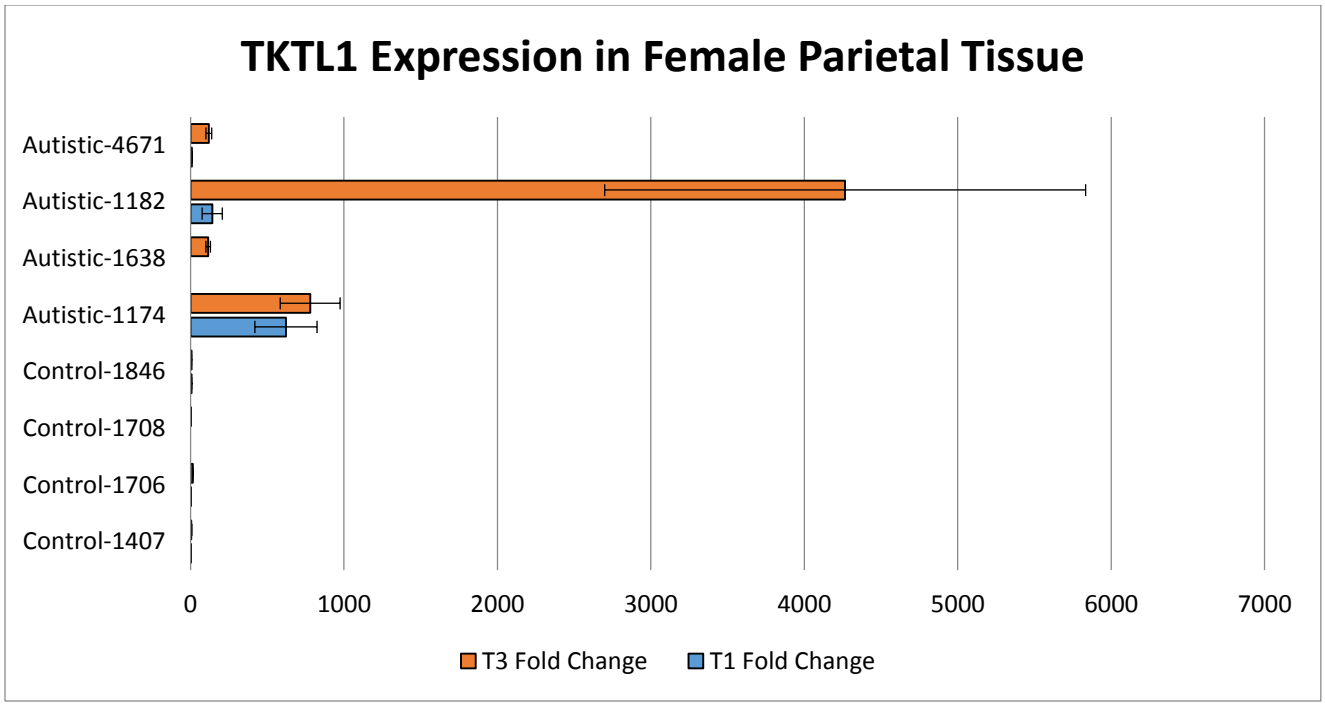

Figure 8. *TKTL1* Expression in Female Parietal Brain Samples
This graph includes all female parietal brain samples, this analysis shows significant individual variation but no significant difference between the autistic and control groups.

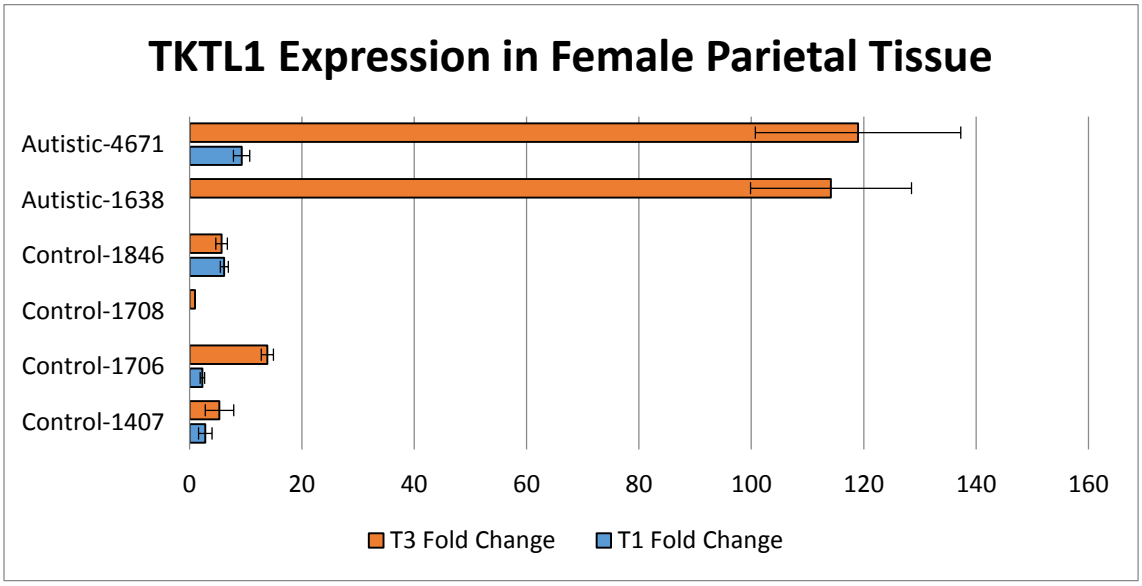

Figure 9. *TKTL1* Expression in Female Parietal Brain Samples without Samples 1174 and 1182
This is the same graph as shown in Figure 7 but samples 1174 and 1182 have been removed for increased clarity of those samples with lower levels of expression.

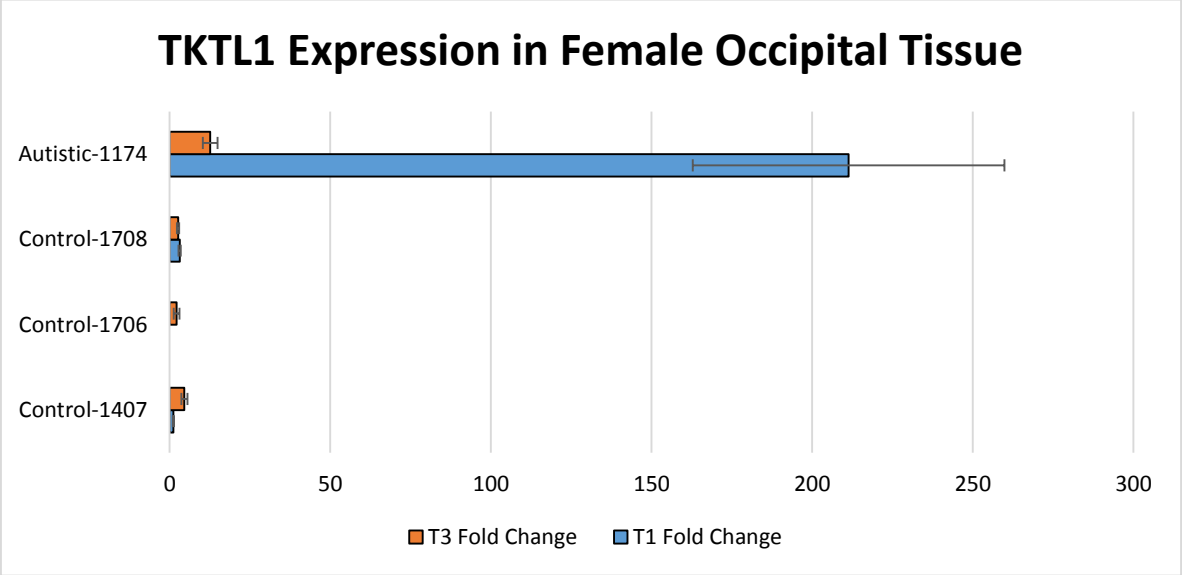

Figure 10. *TKTL1* Expression in Female Occipital Brain Samples
This analysis shows individual variation similar to that found in the parietal samples and shows no significant differences between the autistic and control groups.

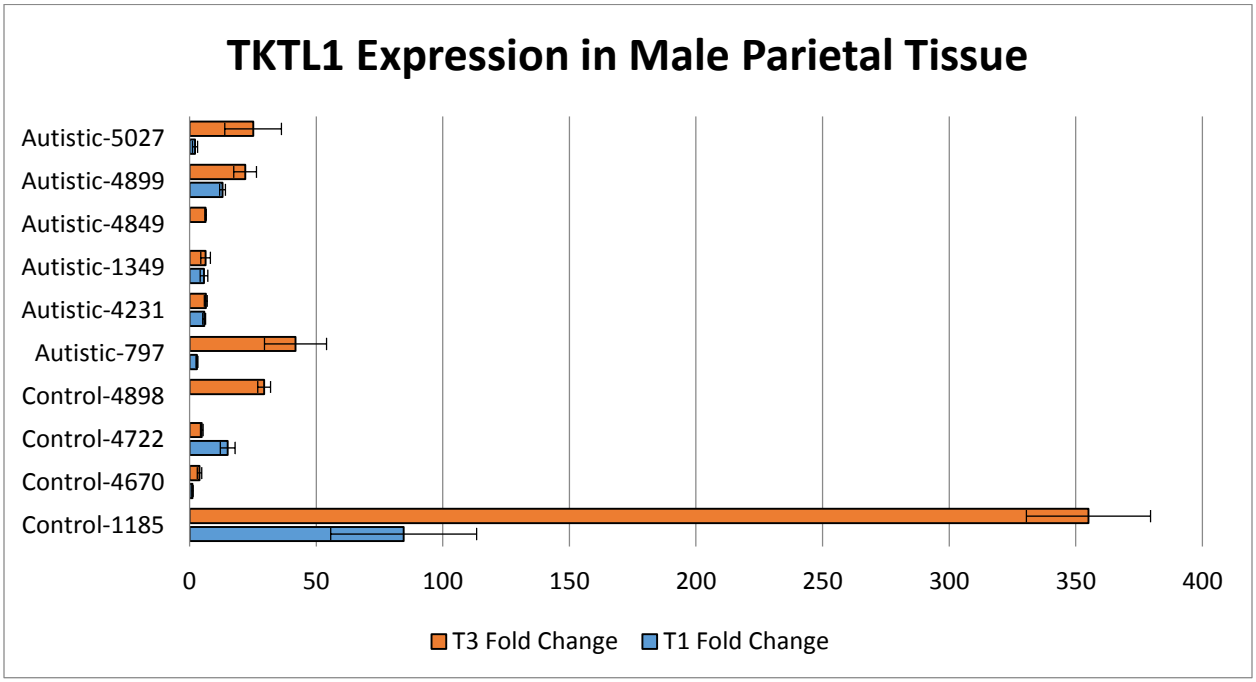

Figure 11. *TKTL1* Expression in Male Parietal Brain Samples
This analysis showed significant individual variation but no significant difference in expression between the control and autistic groups.

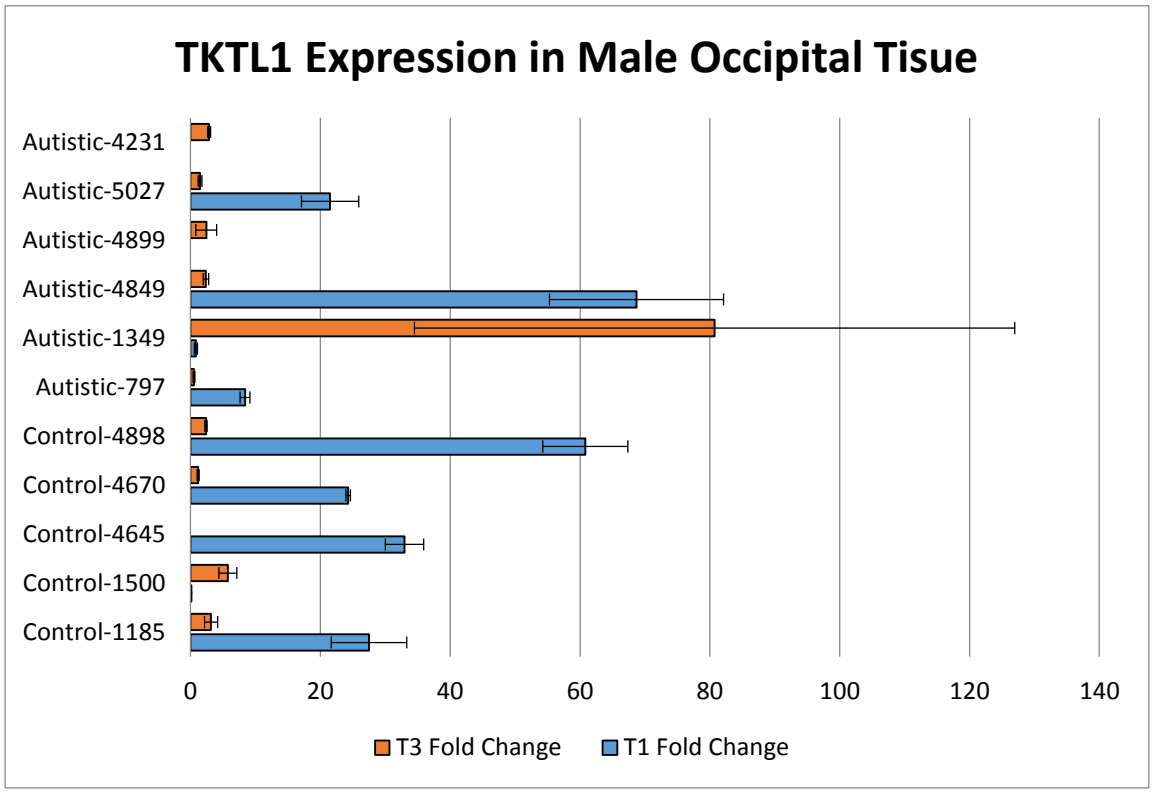

Figure 13. *TKTL1* Expression in Male Occipital Brain Samples
This analysis showed significant individual variation but no significant difference in expression between the control and autistic groups.

Discussion

Sequencing Analysis

Sequencing analysis showed a SNP in the genomic DNA for control sample 1407 and that same SNP in the occipital cDNA for the same sample. However, there was no SNP in the parietal cDNA. This suggests that *TKTL1* shows monoallelic expression in the parietal lobe but biallelic expression in the occipital lobe of the brain. Control sample, 1846 also showed a SNP in the genomic DNA but not in the parietal cDNA, indicating monoallelic expression in the parietal lobe for that sample as well. The occipital tissue from sample 1846 was also sequenced and it

showed biallelic expression. Although n=2 is not a large sample, all of the control samples with diagnostic SNPs show this monoallelic expression in the parietal paired with biallelic expression in the occipital. This supports the idea that there is sub-region specific expression of *TKTL1* in the human brain. Overall, the sequencing data shows evidence for sub-region specific imprinting in brain tissue in control samples.

The Autistic samples, 1174 and 4671, also have the same diagnostic SNP that was seen in samples 1407 and 1846. Sequencing data from 4671 shows biallelic expression in both the occipital and the parietal, signifying an absence of imprinting compared to the control samples. Sample 1174 also showed biallelic expression in both sub-regions and therefore, no evidence of imprinting. It is possible that this misregulated imprinting may explain higher *TKTL1* expression levels seen in these two autistic samples in the RNA expression analysis (Figure 8 and Figure 9). Samples 1182 and 1638 also had high levels of *TKTL1* expression but they did not have this same diagnostic SNP so it was not possible to tell whether there was sub-region specific imprinting or not in those samples.

The absence of imprinting in the autistic samples, 1174 and 4671, correlates with earlier findings by the Michael O'Neill Lab. Sequencing with different primers for these samples demonstrated that they both had a guanine to thymine point mutation (G>T transversion) in the 5' UTR region of *TKTL1*. This point mutation is found in less than 1% of the general population and it is therefore highly unlikely that it would be found in both of these samples by random chance. Because it is a G to T mutation, it has the potential to disrupt methylation and therefore imprinting as well. A disruption like this may explain the discrepancies between the control and autistic samples in terms of monoallelic vs biallelic expression of *TKTL1* in parietal tissue.

RNA Expression Analysis

RNA expression analysis did not show any statistically significant differences in expression between the X_m and X_p lymphoblast Turner's Syndrome cell lines. As a result, there is not enough information from that data to determine whether there is *TKTL1* imprinting. It is possible that lack of differential expression here may be the result of a loss of imprinting due to the alterations in the process of establishing a cell line. Other genes analyzed in that qRT-PCR showed expression levels different than those expected with the X_m cell line showing higher expression of all genes tested when compared to the X_p cell line. It is also important to note that these were lymphoblast cell lines and therefore, may not show the same imprinting that the sequencing data suggests is present in the parietal brain tissue of the control samples. These result do not contradict the sequencing evidence of imprinting in parietal tissue.

Expression analysis did not reveal any differences in average *TKTL1* expression between the autistic and control sample groups. There was significant individual variation between samples but both the Autistic and the Control groups had samples that were significantly different from the others within the group leading to large standard deviation values for the data when averaged together. The large standard deviations prevents the average group values from being significant, but there are some clear differences between the autistic and control groups. Specifically, as depicted in Figure 8 there is a significant difference in *TKTL1* expression between individual autistic female parietal tissue samples and individual control female parietal tissue samples. The sequencing data shows imprinting in the parietal tissue of control females but not autistic samples, further supporting the differences between these two groups.

Limitations

Some potential complications and limitations exist with the use of this data, particularly the use of the cell lines (both the pedigree and the Turner's Syndrome lines) as well as the human brain samples. As indicated above, it is debated whether or not gene regulation or expression data from cell lines can be trusted due to the genetic alterations made in order to produce a sustainable as a cell line. As of now, a majority agree that the information can be trusted but there is no definitive answer as to whether the process of establishing a cell line alters the expression of *TKTL1* in particular. The human brain sample sets were provided with limited associated information. Since our analysis of these samples was largely through RNA expression analysis, it is possible that not all relevant information was received. There is also the possibility of RNA degradation occurring in these samples since the times that the samples were collected varied from 9 to 50 hours post mortem. Overall, the numbers for each sample set were quite small, limiting the level of confidence that the results yielded can provide.

Future Directions

Future work for this investigation into *TKTL1* will include further sequencing analysis of samples as well as continued RNA expression analyses. There are a few genomic DNA samples did not come through clear enough to identify any diagnostic SNPs. Sequencing these samples will be tried again in order to attempt to increase the sample number and increase the support for the sub-region specific monoallelic expression that has been found in sequenced control samples. More sequencing will be performed with autistic samples as well to try and determine if that pattern of expression is conserved within those samples as well. Results of the sequencing analysis will be compared to the RNA expression analysis to determine whether there is a

correlation between increased expression of *TKTL1* and potential loss of imprinting. The possible acquisition of more brain samples would allow expansion of both sequencing and RNA expression analysis efforts. Additional brain samples, leading to a larger sample size, would potentially provide increased confidence in the existing findings.

In addition to the RNA expression data, future work will include investigation of methylation in the human brain samples. Using bisulfate sequencing, methylation patterns in control samples will be compared to those in autistic samples. Specifically of interest is the methylation pattern in the region where the G to T point mutation has been identified in the female autistic samples. A change in methylation in the samples that show this mutation will provide potential evidence for the cause of a misregulation in imprinting which may lead to the increased *TKTL1* expression that was seen in the autistic female parietal samples.

Further confirmation of RNA expression data will be sought using Precise technology by Cellular Research Inc., this technology creates molecular indexes by labeling specific RNA molecules in a way that allows you to determine relative abundance of lowly expressed genes. This would eliminate any potential PCR bias that may exist in the RNA expression data from qRT-PCR. The resulting product from this technique will need to be analyzed using the Illumina MiSeq sequencer and will give valuable additional information which may confirm the *TKTL1* expression data found through this research project.

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