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Research article

Zika Virus Congenital Syndrome and *MTOR* gene variants: insights from a family of dizygotic twins

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ABSTRACT

Congenital Zika virus syndrome (CZS) is associated with damage to neural progenitor cells by ZIKA virus infection. There are no accurate statistics on the percentage of pregnant mothers who have had babies affected by the syndrome. Few cases of discordant twins have been described in the literature and, therefore, we hypothesize that the genetic background of the progeny and/or mother may play a role in the fate of the syndrome. We performed a complete exome sequencing in a set of dizygotic individuals and their parents. After that, we selected discordant variants on the MTOR gene between the affected and unaffected twin and we observed a mutation (rs2295079), placed in a region restricted to proximal 5′-UTR, as a strong possible causal variant. In addition, in most brain tissues (including fetal brain) evaluated for expression quantitative trait loci (eQTL), this locus is strongly correlated with post-translational modifications of histones (promoter and enhancer marks) and hypersensitivity to DNAse I (open chromatin mark). Taken together, our data suggest that changes in the MTOR gene may be related to CZS. Additional functional studies should be carried out to prove how and why a MTOR mutation can predispose the fetus to the syndrome.

1. Introduction

Cases of ZIKV infections have decreased worldwide, but the virus that has the ability to cause birth defects in fetuses and babies, as exemplified by the microcephaly epidemic in Brazil, still represents a threat to public health, mainly because of the large number of susceptible people that reside in Aedes-infested regions, which makes their resurgence likely [1, 2].

It is evident then that there is still a critical need to mobilize support and improve the capacity in low- and middle-income countries to respond to future ZIKV epidemics and their possible consequences, such as the Zika Virus Congenital Syndrome [2].

The syndrome named Congenital Zika Syndrome (ZCS) [3] characterized by severe abnormalities including brain abnormalities and ocular changes are among the most commonly observed features of the disease [4, 5] and the degree of severity of these changes was closely related to the gestational period in which exposure to ZIKV occurs [6].

However, it appears that the host genetics can play a role on the fate of the intrauterine infection since a recent study conducted by Caires-Junior et al. (2018) has described a pairs of discordant twins for ZCS and it was shown that the neural progenitor cells of affected individuals have approximately 60 genes with differences in expression, the most significant of which was *DDIT4L*, an mTOR signaling inhibitor [7]. In addition, a significant difference in gene expression of mTOR and Wnt pathway regulators was also observed after *in vitro* ZIKV infection. In such study, cells from affected individuals had significantly higher ZIKV replication and reduced cell growth. In another study, Figueiredo and collaborators (2019) demonstrated that

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ZIKV also replicates in the brain tissue of adult humans and mice [8]. ZIKV targets memory-related brain regions preferentially, inhibiting long-term hippocampal potentiation and inducing memory impairment in adult mice. Up-regulation of TNF and microgliosis are induced by ZIKV infection [8]. Such results suggest that ZIKV leads to synapse dysfunction and subsequent impairment of memory through the aberrant activation of TNF, microglia and proteins of the complement system C1q and C3. In fact, the action of the microglia phagocyting pre-synaptic terminals of the hippocampus during acute infection seems to be exacerbated by TNF, or C1q/C3 signaling, preventing synapse and thus compromising memory in ZIKV-infected mice [8]. The signaling pathways related to the neurogenesis and cellular differentiation in neurons are known to be activated mainly during embryogenesis, persisting until prenatal development and one of these pathways is the PI3K-Akt-mTOR [9, 10, 11]. PI3K-Akt-mTOR is an essential pathway in the cellular differentiation of neural stem cells in neurons, as well as in the migration, maturation and regulation of the autophagy process [12]. Some studies have demonstrated that, in relation to mTOR signaling, there is an important role on antigen-induced TNF expression, which also activates microglia, which can lead to several neurological outcomes [11, 12]. These studies also demonstrate that mTOR pathway activity is regulated in response to intracellular and extracellular signals, including TNF which along with other growth factors, regulate mTORC1 activity. Another study that evaluated the role of autophagy in neurodegeneration provided evidence that TNF inhibits autophagy flow and leads to a change in the microglia M1 phenotype by activating AKT/mTOR signaling, generating neuroinflammation and autophagy dysregulation. M1 or M2 polarization generates different functions of the microglia, where the microglia in the classic activation state, so called M1, induces the iNOS and NF-κB pathways and produces various proinflammatory cytokines such as TNF, IL-1 β and IL-6 and the M2 microglia subset which is considered an alternative activation profile [13, 14].

Considering the above, in our study we evaluated a couple and their dizygotic twins. Both twins were exposed and infected with ZIKV during pregnancy, but only one was born with ZCS. We performed a whole exome sequencing and prioritized the *MTOR* as target gene, considering it is the most consistent pathway described so far related to the infection outcome [10, 13, 15].

2. Methods

2.1. Case report

In early 2017, a family with a discordant dizygotic twins for microcephaly entered a public health department follow-up program. At the end of April 2015, the 39-year-old mother, two months pregnant, was clinically diagnosed with a "mild dengue-like disease". Four days after the onset of her clinical manifestations, the 49-year-old husband had similar symptoms. They were seen by a primary care physician who made the clinical diagnosis of a "mild dengue-like disease" - at that time, ZIKV had not yet been reported in Brazil.

During pregnancy, she underwent routine serological tests for Hepatitis B, Hepatitis C, Syphilis, Human Immunodeficiency Virus, Human T Leukemia Virus, Anti-Cytomegalovirus IgG/IgM, Anti-Toxoplasmosis IgG/IgM.

The twins were born in October 2015 at 35 weeks. The male child 1 (C3) weighed 2,065 g and the female child 2 (C4) weighed 1,335 g (low birth weight below the 3rd percentile). At birth, the cranial circumference of C1 was 31.5 cm and C4 was 28.5 cm. They underwent neonatal cranial ultrasound (NCU). NCU for 23 showed normal results, while for C4, NCU revealed corpus callosum agenesis. C4 computed tomography showed thinning of the frontal, parietal, occipital and temporal cortex; cerebral calcifications in the left cerebral hemisphere and in the basal ganglia and dilation of the lateral ventricle. All of these findings resemble ZCS.

On July 20, 2017, blood samples from the entire family were collected. Informed consent was obtained from adults and parental permission was obtained for children. The Couto Maia Hospital Ethics Committee (45483115.9.000.0046) approved the study. Viral RNA was extracted from serum and molecular diagnosis was performed by RT-qPCR as described by Lanciotti et al. (2007). Molecular tests were performed for Zika and Chikungunya viruses and serological survey using enzyme-linked immunosorbent assay (ELISA) Anti-Zika Virus ELISA IgG and IgM kit (Euroimmun, Medizinische Labordiagnostika AG, Lübek, Germany) [16].

2.2. DNA extraction and whole exome sequencing

The peripheral blood DNA was extracted from the individuals according to the Flexigene® Blood Kit (Qiagen) protocol. DNA was then subjected to whole exome sequencing using the Nextera ChIP [San Diego, California] via the Illumina HiSeq 2000 platform, Illumina, San Diego, CA [17].

2.3. Variation calling and annotation

Raw sequence files were prepared using the Genome Analysis Tool Kit [GATK] for each of the sequenced samples. Each fastq file was aligned against the human GRCh38/hg38 reference genome. PCR duplicates were removed using Picard [http://picard.sourceforge.net/], reads around known and detected indels were realigned, and base quality was recalibrated using GATK. In order to call variants from the processed BAM files, a variant calling pipeline from GATK was applied. All generated VCF files were analyzed using VarAFT software (http://varaft.eu).

2.4. Filtering data

VarAFT was used to search for mutations in the genes of the mTOR pathway associated with the CZS. VarAFT software uses a series of filters that can be applied to ensure the rapid exclusion of common variants present in public databases (1000 Genomes Project, Exome Aggregation Consortium [ExAC], 6500 Exome Sequencing Project [ESP]) and non-deleterious variants (Desvignes et al., 2018). Assuming that the CZS is not rare, no MAF thresholds were applied. The prediction of amino acid substitution on the biological function of the protein was evaluated using PolyPhen2 [18].

2.5. Expression analysis using GTEx browser

Gene expression analysis was conducted using the GTEx expression portal (www.gtexportal.org), created by the National Institutes of Health Common Fund (Genotype-Tissue Expression Project).

The GTEx (Genotype Tissue Expression) eQTL Browser was used to identify SNPs that are correlated with modulation of gene expression in different brain tissues. Cis-eQTL was evaluated using FastQTL [19, 20] to identify SNPs that are correlated with modulation of *MTOR* gene expression in different brain tissues. Nominal p-values were generated for each variant-gene pair by testing the alternative hypothesis that the slope of a linear regression model between genotype and expression deviates from 0.

2.6. Linkage disequilibrium analysis and sequence annotations

Linkage disequilibrium values between the evaluated SNP were determined by r^2 (Reference population: CEU, 1000 Genomes Project), using the Haploview program, version 4.

Comparative genomic data and regulatory features were obtained from the Ensembl (http://www.ensembl.org) genome browser. SNP positions were cross-referenced with sequence annotations, including

Table 1. Molecular and serological assays for ZKV infection.

Family member	Serum* (Ct)	Virus isolation (Ct)		Zika serology	
		Vero cells	C6/36	IgM	IgG
C1	35.28 (±0.46)	35.87	36.88	-	+
C2	32.25 (±0.19)	35.38	36.18	-	+
C3	37.46 (±0.76)	35.82	36.65	-	-
C4	35.92 (±0.54)	36.74	36.52	-	+

Numbers represent the Ct (cycle threshold) of RT-qPCR assays.
* Mean of three assays; standard deviation of the mean are between parentheses. **C1**. Mother; **C2.** Father; **C3**. Unaffected twin; **C4**. Affected twin.

Table 2. Obstetric Ultrasound results during twin pregnancy.

Date	Fetus	BPD*	FL†	EFW§	CCL#
April 30	C3	14	ND	ND	46
	C4	13	ND	ND	45
July 08	C3	51	33	370	ND
	C4	46	31	303	ND
Sept. 09	C3	72	57	1,458	ND
	C4	56	47	862	ND
Oct. 10	C3	83	62	2,188	ND
	C4	64	56	1,423	ND

ND = No data, C3. For the Unaffected twin; C4. For the Affected twin.
* Biparietal diameter (mm).
† Femur length (mm).
§ Estimated fetal weight (grams).
# Craniocaudal length (mm).

genomic evolutionary rate profiling–constrained elements (GERP) [21], chromatin segmentation state and enrichment for marks of open chromatin (only brain tissues were considered; Roadmap Epigenomics Consortium, 2015) [22]. These last two types of information were obtained from the ENCODE project.

3. Results

3.1. Clinical data

Cycle thresholds (Ct) showed that children mother and father had viremia detected in the serum, suggesting a viral persistence (Campo et al., 2020). These results were confirmed in ZIKV isolated from cultured cells (Table 1). C3 was negative for anti-ZIKV IgG and IgM and C4 positive only for anti-ZIKV IgG. The ZIKV rapid test for mother and father was positive for anti-ZIKV IgG and negative for anti-ZIKV IgM (Table 1). Until the baby was born, it was she performed several obstetrical ultrasonographies. Table 2 presents the ultrasound results, where underdevelopment of fetus C4 can be observed.

3.2. Exome sequencing and filtering strategy

After applying filters for prioritization of candidate genes related to the mTOR pathway, we observed, considering the four exomes analyzed (parents and both twins), twenty-two variants found in the MTOR locus. Fifteen of them presented discordant genotypes between the affected and unaffected twins (Table 3).

3.3. Expression analysis using GTEx browser

Next, the GTEx (Genotype Tissue Expression) eQTL Browser was used to identify SNPs that are correlated with modulation of gene expression. Only eleven SNPs showed significant correlation with *MTOR* gene expression in two or more different brain tissues and were selected for further analyses. Those eleven variants are in high linkage disequilibrium ($r^2 \geq 73$; Table 4 and Figure 1).

3.4. In silico functional analyses

In order to identify the causal variant in this LD block we performed several *in silico* functional analyses. SNP positions were cross-referenced with sequence annotations, including genomic evolutionary rate profiling–constrained elements (GERP)[12], chromatin segmentation state and enrichment for marks of open chromatin (only brain tissues were considered; Roadmap Epigenomics Consortium, 2015) [23]. These results strongly indicate the variant rs2295079 as a strong factor involved in ZCS because it is placed in a *MTOR* proximal 5′-UTR constrained region (Table 5). Moreover, in most of the brain tissues evaluated (including fetal brain) this locus is strongly correlated with post-translational modifications of histones (promoter and enhancer marks) and DNAse I hypersensitivity (mark of open chromatin).

4. Discussion

In this study, we evaluated the exome of a pair of twins exposed to ZIKV infection during pregnancy, where only one of the babies was born with congenital ZIKV syndrome (ZCS). Following a recent publication reporting discordant twins and the ZCS [7], we hypothesized that there may be a genetic component of susceptibility to the virus, which is not random, and that the mTOR pathway may be playing a significant role since it seems that the mTOR pathway, which so far is the best-described pathway involved in the outcome of the infection [10, 15, 24]. We identified a mutation (rs2295079) as a strong candidate for the causal variant since it is placed in a region restricted to the proximal region (5′-UTR) of the *MTOR* gene. Moreover, in most of the brain tissues evaluated (including fetal brain) this locus is strongly correlated with post-translational modifications of histones (promoter and enhancer marks) and DNAse I hypersensitivity (mark of open chromatin).

The *MTOR* gene codes for the mTOR protein. This protein interacts with other molecules to form two distinct protein groups, called the 1 mTOR complex (mTORC1) and the mTOR 2 complex (mTORC2). Both complexes transmit downstream signals linked to several biological routes that direct cell function. Signaling through mTORC1 and mTORC2

Table 3. SNPs showing discordant genotypes between samples 23 and 24 which were selected for additional analysis.

Individual	Coordinate	SNP	REF	ALT	GENO
C1	11145001	rs1057079	C	T	CT
C2					CT
C3					**CT**
C4					**TT**
C1	11228701	rs1064261	G	A	GA
C2					GA
C3					**GA**
C4					**AA**
C1	11121270	rs11121691	C	T	CT
C2	11121270	rs11121691	C	T	CT
C3	11121270	rs11121691	C	T	**CT**
C4	11121270	rs11121691	C	T	**TT**
C1	11243421	rs11121707	G	A	GG
C2	11243421	rs11121707	G	A	GA
C3	11243421	rs11121707	G	A	**GG**
C4	11243421	rs11121707	G	A	**GA**
C1	11122204	rs113055615	G	GT,A	GTGT
C2	11122204	rs113055615	G	GT,A	GT/A
C3	11122204	rs113055615	G	GT,A	**GTGT**
C4	11122204	rs113055615	G	GT,A	**GT/A**
C1	11241657	rs1135172	A	G	AG
C2	11241657	rs1135172	A	G	AG
C3	11241657	rs1135172	A	G	**AG**
C4	11241657	rs1135172	A	G	**GG**
C1	11257263	rs2092642	C	T	CT
C2	11257263	rs2092642	C	T	CT
C3	11257263	rs2092642	C	T	**CT**
C4	11257263	rs2092642	C	T	**CC**
C1	11130589	rs2275527	G	A	GA
C2	11130589	rs2275527	G	A	GA
C3	11130589	rs2275527	G	A	**GA**
C4	11130589	rs2275527	G	A	**AA**
C1	11262508	rs2295079	C	G	CG
C2	11262508	rs2295079	C	G	CG
C3	11262508	rs2295079	C	G	**CG**
C4	11262508	rs2295079	C	G	**GG**
C1	11228561	rs4845985	G	A	GA
C2	11228561	rs4845985	G	A	GA
C3	11228561	rs4845985	G	A	**GA**
C4	11228561	rs4845985	G	A	**GG**
C1	11228576	rs4845986	G	C	GC
C2	11228576	rs4845986	G	C	GC
C3	11228576	rs4845986	G	C	**GC**
C4	11228576	rs4845986	G	C	**GG**
C1	11259530	rs4845988	A	G	AG
C2	11259530	rs4845988	A	G	AG
C3	11259530	rs4845988	A	G	**AG**
C4	11259530	rs4845988	A	G	**AA**
C1	11234417	rs718206	T	A	TA
C2	11234417	rs718206	T	A	TA
C3	11234417	rs718206	T	A	**TA**
C4	11234417	rs718206	T	A	**TT**
C1	11204516	rs72856909	G	A	GG
C2	11204516	rs72856909	G	A	GA
C3	11204516	rs72856909	G	A	**GG**
C4	11204516	rs72856909	G	A	**GA**

(*continued on next page*)

Table 3 (*continued*)

Individual	Coordinate	SNP	REF	ALT	GENO
C1	11237705	rs7524202	T	C	TC
C2	11237705	rs7524202	T	C	TC
C3	11237705	rs7524202	T	C	**TC**
C4	11237705	rs7524202	T	C	**TT**

C1. For the Mother; C2. For the Father; C3. For the Unaffected twin; C4. For the Affected twin; SNP: single nucleotide polymorphism; REF: allele reference; ALT: allele altered; GENO: genotype.

Table 4. GTEx-Gene expression level of *MTOR* according to SNP alleles in different brain tissues.

SNP ID	REF	ALT	*Caudate (basal ganglia)	Cortex	*Putamen (basal ganglia)	*eQTL hits
rs1057079	C	T	7,0e-6		1,4e-6	2
rs1064261	G	A	5,5e-6	3,1e-5	1.4e-7	3
rs11121691	C	T	2,5e-5		6,0e-6	2
rs11121707	G	A				0
rs113055615	G	GT,A				0
rs1135172	A	G	2,1e-5		9.6e-8	2
rs2092642	C	T	1,8e-5		1.4e-7	2
rs2295079	C	G	3,8e-6		2.4e-7	2
rs4845985	G	A	1,0e-5		8.2e-8	2
rs4845986	G	C	1,0e-5		8.2e-8	2
rs4845988	A	G	1,8e-6		1.4e-7	2
rs718206	T	A	1,5e-5	3,3e-5	1.2e-7	3
rs72856909	G	A				0
rs7524202	T	C	7,2e-6	3,1e-5	1.7e-7	3
rs7525957	C	T			3.1e-7	1

* eQTL p-values are shown.

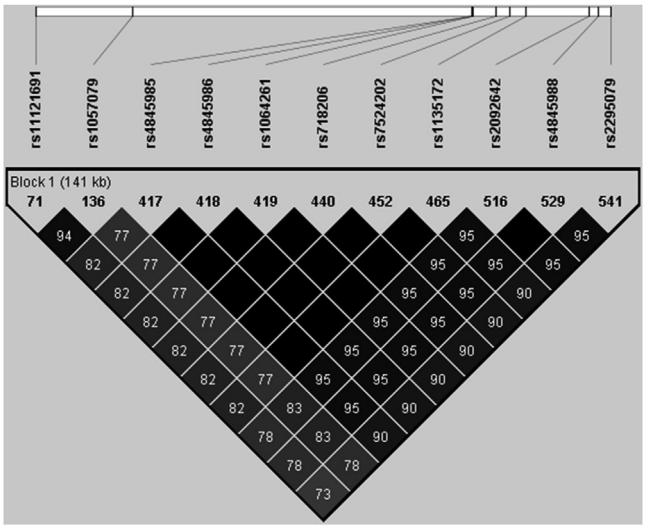

Figure 1. Linkage disequilibrium (r^2) analysis of discordant *MTOR* SNPs genotypes showing 2 or more eQTL hits in Brain tissues (GTEx). Ref population: CEU (1000 genomes project).

regulates the production of protein, which influences cell growth, division and survival, including brain growth and, development [25].

It is well established in the literature that homozygous mutations in the *MTOR* gene cause a neurological disorder called Smith-Kingsmore syndrome. Individuals with this condition usually have a larger head (macrocephaly), intellectual disability and, seizures. Affected individuals may also have unusual facial features, a behavioral condition called Attention Deficit Hyperactivity Disorder or Autism Spectrum Disorder, which affects communication and social interaction [26]. Recent studies have shown that activating mutations in the PI3K-Akt-mTOR pathway may lead to brain overdevelopment syndromes, including malformations involving macrocephaly [27] and inhibition of mTOR in the developing brain may cause microcephaly in mice [7]. However, neurological complications affecting the central nervous system in adults have also been reported in patients infected with ZIKV, such as acute myelitis, encephalomyelitis, encephalitis, meningoencephalitis and sensory polyneuropathy. Although their mechanisms are still unknown, recent work has demonstrated neutralization of necrosis. Tumor factor signaling can be blocked to activate the microglial, leading to neurological dysfunctions [8]. It is now known that in the central nervous system (CNS) TNF plays a critical role as an inflammatory mediator and in the generation of microgliosis and synapse/memory deficits [11].

Table 5. Functional Analysis: Epigenomics: Regulatory chromatin states from DNAse and histone ChIP-Seq (Roadmap Epigenomics Consortium, 2015) - Only Brain tissues were considered. Comparative genomics: GERP constrained elements (Davydov, 2010).

SNPs	rs2295079
GERP constrained elements	yes
Promoter histone marks	Hippocampus Middle/Substantia Nigra/Anterior Caudate/Cingulate Gyrus/Inferior Temporal Lobe/Angular Gyrus/Dorsolateral_Prefrontal_Cortex/Germinal Matrix/Fetal brain female/Fetal Brain male
Enhancer histone marks	Hippocampus Middle/Substantia Nigra/Anterior Caudate/Cingulate Gyrus/Inferior Temporal Lobe/Angular Gyrus/Dorsolateral_Prefrontal_Cortex/Germinal Matrix/Fetal brain female/Fetal Brain male
DNase I hypersensitive site	Fetal brain female/Fetal Brain male
Funcional annotation	5′-UTR

Signaling pathways related to neurogenesis and cellular differentiation of neural progenitor cells in neurons are activated primarily during embryogenesis and persist until prenatal development. One of these signaling pathways is PI3K-Akt-mTOR, which is essential in the cellular differentiation of neural stem cells in neurons, as well as in the migration, maturation of these cells and regulation of the autophagy process [9, 10, 12]. The present work demonstrates once again, according to previous studies, the mTOR signaling pathway as possibly playing an important role in the outcome of Zika infection. This signaling pathway plays an important role in the mechanism of application of antigen-induced TNF expression that also activates resident brain defense cells called microglia, and as a result of this activation, microglia attacks and destroys synapses (connections between neurons). which can lead to various outcomes with neurological disorders [12]. We observe here a modulation of *MTOR* gene expression in brain regions according to SNP alleles, reinforcing the idea that this variant would lead to an increase in neurogenesis. However, according to Liang (2016), the PI3K-Akt-mTOR pathway is modulated after exposure of two ZIKV nonstructural proteins (NS4A and NS4B), leading to impairment of neurogenesis and autophagy [10]. Therefore, we hypothesized that individuals with altered levels of the *MTOR* gene expression (rs2295079) may also have a greater chance of interaction with the viral proteins NS4A and NS5B, significantly altering the signaling pathway and leading to the suppression of neurogenesis.

In the future, we intend to investigate this mutation in additional children affected by the ZCS to evaluate the expression of the mTOR protein in such individuals. Our report demonstrates for the first time that the mutation a in *MTOR* could be involved in the manifestation of ZCS, expanding our understanding of the underlying pathology of the ZIKV disease, which has to date caused a significant public health impact on Brazilians.

Declarations

Author contribution statement

Luciana Reboredo de O. da Silva, Ryan dos Santos Costa, Valdirene Leão Carneiro, Camila A Figueiredo: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Pablo Oliveira, Nicholas Rafaels, Monica Campbell, Tonya Brunetti, Kristy Crooks, Michelle Daya, Kathleen Barnes: Analyzed and interpreted the data; Wrote the paper.

Silvia Sardi, Gubio Soares, Antônio Carlos Bandeira: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Maria Glória Teixeira: Performed the experiments; Wrote the paper.

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Data availability statement

The data that has been used is confidential.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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