ISSN: 2576-2842

Case Report

Journal of Gynecology & Reproductive Medicine

Effects of RTTN Gene Mutations and the Need for Complementary cDNA Analysis for Some Transcripts

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Submitted: 16 Jul 2020; **Accepted**: 20 Aug 2020; **Published**: 03 Sep 2020

Abstract

Introduction: *Prenatal WES analysis is currently required in prenatal diagnosis in case of multiple congenital anomalies and in families where some genetic diseases are reported. However, with development of prenatal WES, practitioners are sometimes facing a lot of challenge regarding interpretation of the genetic results.*

Method: *Prenatal WES analysis.*

Result: *Detection of two different RTTN gene transcripts in fetal WES. One of the transcripts showed RTTN homozygous gene mutation while the other transcript was normal.*

Discussion: *This result emphasizes difficulty of genetic counselling in case of absence of prenatal radiological findings or late findings. Conservative fetal follow up was advised because of absence of any positive radiological finding.*

Conclusion: *This article presents the multidisciplinary approach in prenatal and postnatal counselling for cases with quiry fetal genetic findings and illustrates the urgent need for development of transcriptome analysis for the fetus with WES findings of uncertain significance.*

Keywords: RTTN Gene, Rotatin Protein, Transcripts, Polymicrogyria, Microcephaly

Introduction

RTTN is a gene coding for Rotatin protein which has been localized at ciliary body and centriole of human fibroblasts. Its exact function in human is not well identified. However, it is thought to play important role in maintenance of normal structure of primary cilia and hence in left to right organ specification, axial rotation, development of notochord as well as early cellular division in mice. In human, RTTN gene coding for rotatin protein was physically mapped to chromosome 18q22.2 in 2002 [**1**]. It is made up of different transcripts with different exon length ranging from 48 to 54 exons. These transcripts have major differences between each other. In a given family only one transcript will be expressed in all the cells of different members of the same family. However, different isoforms of the same transcript can be observed in different cells of the same individual. These isoforms are considered a kind of minor modification of the same transcript to induce adaptation of the protein to cellular function according to the hosting tissue or organ [**2**].

Functionally, RTTN gene seems to play a role in left to right regulatory cascade of mice embryo with nodal, lefty and ptix2 genes. L-R regulatory cascade is essential for heart looping, axial rotation, neural tube and somite development in mice. Homozygous RTTN null mice embryos were smaller in size with failed axial rotation, randomized heart looping, delayed neurulation and they died early **[1].** In human, homozygous or compound heterozygote mutations causing premature stop codons in the transcript of this gene resulting in truncated protein production were reported to be associated with MSSP phenotype **[3]**.

At cellular and molecular level, Rotatin was recently proved to control different phases of cell cycle and cycle progression. It is an important regulator of centrosome, spindle formation and ciliogenesis. Rotatin protein has been localized to the basal body of centriole. It directly interacts with STIL (centriole assembly protein) to build full length centriole. Intact functional centrioles and hence centrosomes are important for cellular division, neuronal stem cell division, neuronal migration and apical-basal cell polarity. Its mutation leads to mitotic failure of progenitor neuronal cells due to

abnormal centrosome and multipolar spindle formation resulting in neuronal aneuploidy and apoptosis. Hence, microcephaly is thought to be a consequence of neuronal progenitor cell depletion. Primary microcephaly (MCPH) is characterized clinically by reduced brain size with or without decreased head circumference below three standard deviations at birth. By the same mechanism primordial dwarfism reported in some cases of RTTN gene mutation can be expected due to abnormal centrosomes and spindle formation **[4]**.

Rotatin protein is also essential for normal ciliogenesis, it has been localized to basal body and axoneme of cilia. Meanwhile intact primary ciliary function is essential for normal development and organization of human cortex. Cultured fibroblasts from RTTN mutated individuals have short cilia with structural abnormalities. This is associated with dysfunction of the cortex-organizing center that gives rise to Cajal-Retzius (CR) neurons. Cajal-Retzius (CR) cells are important regulator of brain development are responsible for radial migration of projection neurons through secretion of reelin protein. Also interaction between normal rotatin and neuronal myosin heavy chain results in neuronal migration. Rotatin malfunction results in cerebral malformation due to failure of proper neuronal migration resulting in lissencephaly and neuronal hetrotropia **[3]**.

To our knowledge, 28 cases have been reported with homozygous pathogenic mutations of RTTN gene to date. These cases presented with primary microcephaly, short stature, polymicrogyria with or without seizures (MPPS). Most of these symptoms appears in the late prenatal or even postnatal period **[1-4].**

Polymicrogyria (PMG) is a common brain malformation resulting from abnormal increased folding pattern of cerebral cortex as well as abnormal lamination and cortical organization. It is interesting to know that about 40 genes are described to be related to such a condition and they all fall in five different categories. These are the mTORopathies, tubulinopathies, alpha dystroglycanopathies, laminopathies, congenital glycosylation disorders as well as other groups of genes involved in early brain development such as RTTN gene [**5**]. Polymicrogyria associated with RTTN gene mutation was described to be variable, diffuse and asymmetric and is associated with primary microcephaly. Both abnormal neuronal migration and organization can result be associated with seizures. Many mutations in RTTN gene have been described to be pathogenic even intronic mutations indicating that heterogeneity is much more extensive than what is expected **[6]**.

Moreover, molecular analytic technical advances demonstrated that a same gene DNA template can give rise to different RNA transcripts. These transcripts differ from each other in transcription start and end sites, splice events or even transcribed exons giving rise to different proteins. A single gene often has more than one transcript. Non coding transcripts are known as transcripts of unknown functions (TUFs). On the other hand, coded transcript is known as a dominant transcript. This is the transcript which will be expressed in primary tissues. There is a single dominant transcript per gene in primary tissues. Slight structural and compositional variation of this dominant transcript can be observed in different tissues of the same individual which are called isoforms and it is considered as a kind of adaptation of the same transcript to the function of different cells **[2]**.

Keeping in mind this information, further evaluation of the effect of different transcripts is required for better identification of pathogenic variants. Studying cDNA is fundamental in order to determine the effect of variants of unknown significance and even for variants which are known to be pathogenic if the clinical findings are not present yet. This will be applicable for prenatal WES analysis which became a parental request for parents who had a previous child with undiagnosed handicap.

Case report

We report a young couple who are double first degree cousins. The couple decided to do prenatal WES analysis as they have family history of genetically undiagnosed non syndromic congenital progressive hearing impairment affecting their son and the nephew of the mother. The couple decided to go for natural pregnancy and to do prenatal diagnosis at 12 weeks of gestation by chorionic villous sampling for WES analysis.

Methods

Fetal karyotype was done after culture of trophoblasts using G banding technique with 550 bands resolution.

Fetal WES was performed with the Twist Human Core Exome Kit (Twist Biosciences) using an Illumina NextSeq 550 device. Identified variants were filtered using the software “Varvis” (Limbus Medical Technologies GmbH) for potentially pathogenic mutations.

The mutation was confirmed in the fetus and parents by Sanger sequencing of genetic DNA (Applied Biosystems Genetic Analyzer 3130xl device).

Results

Fetal karyotype obtained from cultured meschenymal cells of chorionic villous sample revealed normal male karyotype (46, XY) **(Figure 1).** WES analysis disclosed two RTTN transcripts, NM_173630.3 (49 exons) and NM_001318520.1 (48 exons). The first transcript, NM_173630.3 (49 exons) showed homozygote mutation of exon two of RTTN gene p.(Ala15Glyfs*16) resulting in a frame shift and premature stop codon at amino acid position 30.

However, the second transcript NM_001318520.1 (48 exons) differs from NM_173630.3 (49 exons) in exon two which is a non-coding, intronic region in NM_001318520.1. In such a case the mutation present in this site will not affect the transcribed protein.

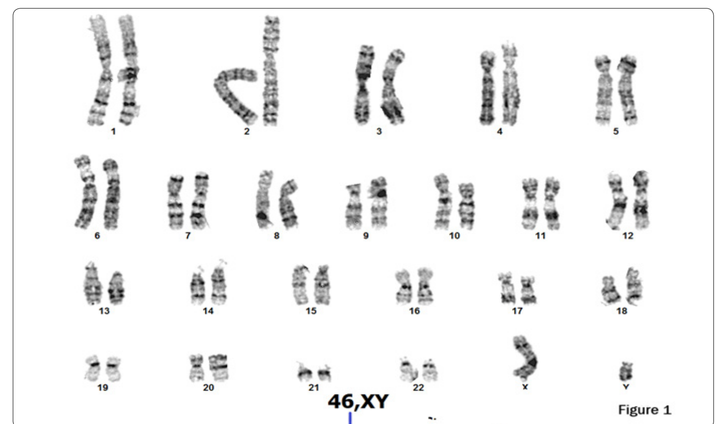

Figure 1: Chorionic villus sample analysis showing normal male karyotype (G-banding) at 12 weeks of gestation.

This means that if the first transcript (NM_173630.3) is the expressed one in the brain of the fetus, he will develop the clinical picture of RTTN gene mutation but if the second transcript (NM_001318520.1) is the expressed one, he will be healthy. The laboratory recommended cDNA for the fetus to be able to differentiate which transcript is translated in this family. Fetal cDNA analysis was not available in any laboratory, so it was not done. Analysis of the parents confirmed that each of them is heterozygote carrier of the first and second transcripts of RTTN gene.

It was concluded that the fetus might be affected by microcephaly, short stature, and polymicrogyria with or without seizure due to homozygous mutation in the RTTN gene. However, due to absence of radiological findings associated with RTTN gene mutation and due insufficient data regarding expression of different RTTN transcripts, this could not be confirmed. Hence, conservative close regular fetal follow up was recommended. Fetal brain MRI acquired at 32 weeks+ 5 days of gestational age **(Figure 2, 3 &4)** showed no evidence of acute hemorrhage or polymicrogyria.

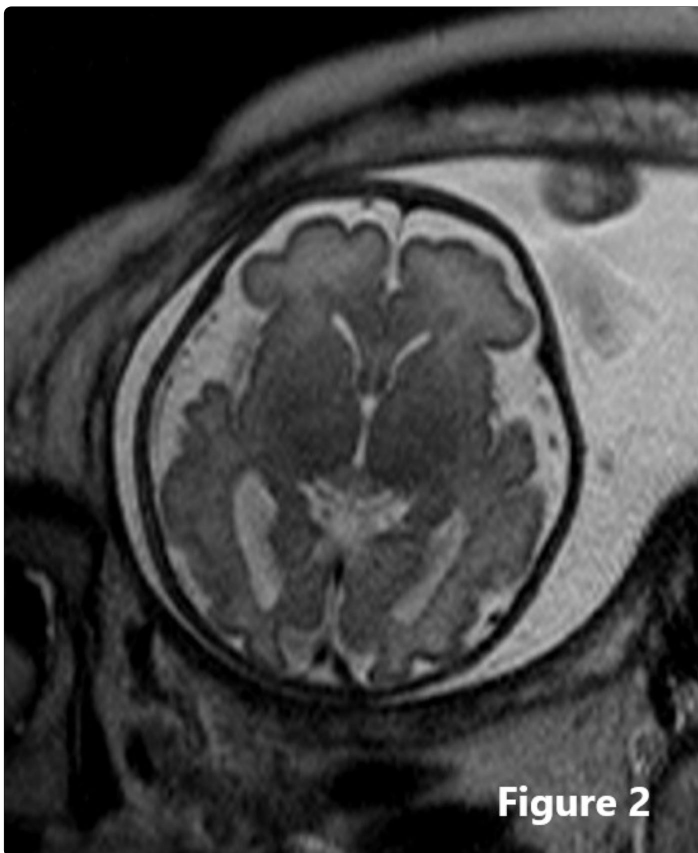

Figure 2: Fetal brain MRI acquired at 32 weeks and 5 days showing apparently normal axial section image.

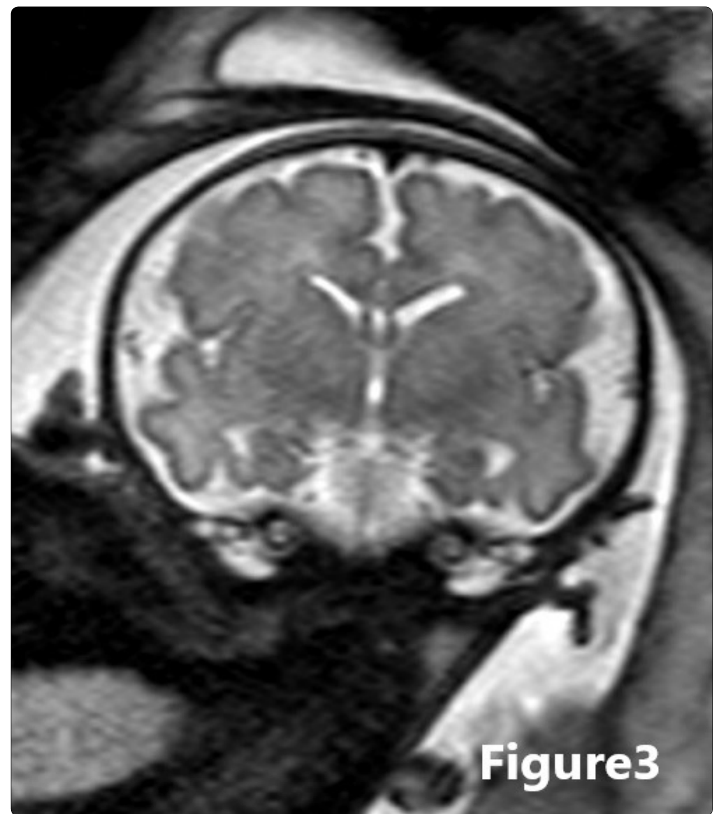

Figure 3: Fetal brain MRI acquired at 32 weeks and 5 days showing apparently normal coronal section image.

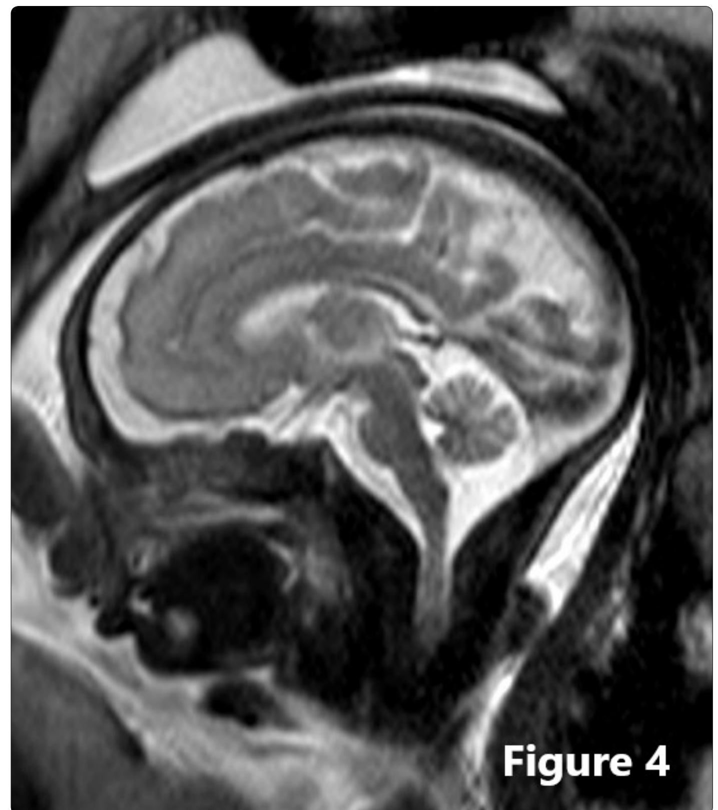

Figure 4: Fetal brain MRI acquired at 32 weeks and 5 days showing apparently normal sagittal section image.

Prenatal Management and Counselling

The parents were informed about the two possibilities for RTTN gene expression according to the expressed transcript. If the pathogenic transcript is the one expressed in this family, the fetus will be affected and if not, the fetus will be healthy. Unfortunately, microcephaly and polymicrogyria can be only detected late in pregnancy. Follow up of the fetus was done at 12 weeks, 16 weeks, 28 weeks, 32 weeks and 36 weeks. Fetal cerebral MRI was also performed at 32 weeks and 5 days. All the tests were normal.

Postnatal Management and Counselling

The baby was born at 35 weeks + 1 day because of placental calcification and decreased fetal perfusion. The fetus was delivered by NVD, his birthweight was 1.95 kg (10th centile) for a length of 44cm (~ 20th centile) and a head circumference measuring 31.2cm (~25th centile). APGAR score was 7 & 8 at 1 and 5 minutes respectively. He received nasal oxygen for one day in the neonatal day care unit.

After discharge, the infant was checked on monthly basis with serial anthropometric assessment for his weight, length and occiptofrontal circumference. At the age of 6 months, neurological assessment was done. His growth was following the normal growth charts. His weight was 8.3kg (50th centile), length 67.5 cm (~25th-50th centile) and occiptofrontal circumference was 44cm (~50th centile). He was alert, fixing and following in all directions. He turns appropriately to sounds. His developmental assessment was appropriate for his age for fine, gross motor as well as social and language skills using the Denver II developmental screening test[7]. Currently, he rolls over, sits without support for few seconds and bears weight on both legs. He fixes and follows, recognizes both parents, babbles with social smile and he reaches for toys with both hands. His neurological examination showed normal tone. His deep tendon reflexes were elicited all over the extremities. He has grossly intact tactile sensations. Bilateral fleshy overgrowth of both feet disproportionate to the skeletal development was noted.

Discussion

The clinical findings of pathogenic RTTN gene mutations manifested by MSSP (microcephaly, short stature, polymicrogyria with or without seizures) have been clearly defined and delineated by several authors [3-8]. None of these signs were detected prenatally in our case.

Microcephaly reported with RTTN gene mutations was either primary or secondary (81% and 30% of reported cases respectively). Both forms of microcephaly seem to be of different origins. Primary microcephaly is most likely to be due to inappropriate neuroprogenitor cellular division which can be expected with RTTN gene mutation. However, secondary microcephaly is more likely to be caused by late developmental, neurodegenerative issue or metabolic diseases **[9].** Primary microcephaly can be screened by fetal ultrasound and MRI. However, secondary microcephaly can be only detected postnatally. Moreover, primary microcephaly is usually detected late in the second trimester. It can't be ruled out by ultrasound only as fetal ultrasound is measuring head circumference and not brain circumference **[10]**. Fetal brain MRI is considered the most accurate modality for measuring the brain circumference in our reported case.

Polymicrogeria is one of the most common malformations of cortical development (MCD). However, it was reported only in about 30% of cases with RTTN mutation. It is characterized by excessive abnormal small and partly fused gyri separated by shallow sulci (8). It is a highly heterogeneous condition regarding the microscopic features, distribution, age of onset, and clinical manifestation. It is mainly classified according to the imaging pattern. It can be focal or diffuse, bilateral or unilateral, symmetric or asymmetric. It has many distributions but the most common one is the perisylvian PMG followed by frontal, fronto-parietal and other distributions. PMG reported with RTTN mutations has been described to be variable, diffuse and asymmetrical. PMG can be diagnosed by fetal ultrasound or MRI. In case of fetal ultrasound, It is best diagnosed by 27 weeks of gestation before development of secondary sulci which will make diagnosis difficult. However, late prenatal MRI has the same sensitivity of postnatal MRI. Common imaging pattern of PMG of the affected cortex (perisylvian in 60% of cases) will be closely packed microgyri with shallow sulci and irregular gray-white matter junction. It is also usually associated with mild ventricular dilatation and increased subarachnoid space at the site of the lesion. None of these findings were reported in our reported case prenatally either by ultrasound or MRI. Other reported MCD in RTTN mutations such as lissencephaly, pachygyria, heterotropia or cerebellar hypoplasia were not seen in fetal ultrasound or MRI of the reported case **[11, 12]**.

Polymicrogyria is usually associated with motor, intellectual disability and epilepsy. Epilepsy happens from 65 up to 78% of cases suggesting that polymicrogyria is an epileptogenic. Age of onset in large series varies from one month up to 37 years with a median age at 8 months. It can be generalized or focal **[11, 13]**.

Primordial dwarfism was reported in 35% of cases with RTTN gene mutations while postnatal short stature was reported in 43% of cases **[14]**. In the currently mentioned case, long bone was noticed to be at the 10th centile at 20 weeks. However, short long bones can be induced by a wide range of causes including familial factors, placental and environmental factors. It was not possible to predict if short long bone was related to mutation of one of the transcript of RTTN gene or it was related to familial or placental perfusion factors. On the other hand, our case during the fetal period did not show primordial dwarfism.

Prenatal features of all assessed cases such as microcephaly, primordial dwarfism and cortical brain development don't appear in all cases. 81% of cases presented with primary microcephaly, 35% with primordial dwarfism and around 30% with different forms cortical developmental malformation (CDM). Only postnatal moderate to severe developmental delay before the age of 2 years was noticed in all reported cases (100%) with speech delay being more affected (90%) **[8]**.

The development of prenatal WES enhanced our understanding regarding developmental anomalies. Before its development, only fetal karyotype, CGH microarray and targeted gene analysis were possible. Before the development of prenatal WES, prenatal counselling and management decision based only on phenotype anomalies in absence of detailed gene analysis was really challenging. This challenge was mainly due to broad range of differential diagnosis, prognosis and expectations as well as provided information by fetal ultrasound. Prenatal WES bridged the gap between abnormal fetal phenotype of unknown origin and fetal genotype. It also allowed phenotype/genotype correlations as well as discovery of new developmental genes. However, even prenatal WES is facing a lot of challenges which are mainly due to time factor required for analysis of big number of data, interpretation of variants of uncertain significance (VUS), incidental findings and determination of expressed transcript as in our case. A comprehensive fetal transcriptome is essential in such a case to detect the expressed gene and its effect **[15-17]**.

Physicians and prenatal counsellors are still facing a big issue with different gene expression for the following reasons. Each gene has multiple transcripts; hence it will not be able to know which one will be expressed in advance unless RNA analysis is performed, especially in case of incidental findings. Moreover, clinical findings might not manifest early in prenatal life like polymicrogyria and microcephaly which are mainly expressed by 27 weeks of gestation or even after birth. On the other hand, variable expressivity seen with the same pathogenic mutation results in subjective judgment on the effect of detected mutation especially when only one sign is present. Hence, other molecular diagnostic tests were required to be able to reassure the parents.

Generally speaking, it is not possible to differentiate between dominant (expressed) transcript and TUFs by traditional DNA analysis. This can be only detected by RNA analysis because RNA sequence mirrors the DNA template from which it was transcribed. RNA analysis techniques are known as transcriptomic technologies which underwent a lot of progress since 1990. Prenatal RNA analysis requires fresh fetal sample and can't be conducted on old sample as RNA is known to be much more fragile than DNA and because it is an expression analysis **[18]**. However, unfortunately, it is not applied in different laboratories for routine screening of positive WES results or for VUS. This was the case of our patient as no laboratory was able to do fetal RNA analysis. The laboratories which are performing transcriptome analysis were using it for research purposes and not for prenatal diagnostic situations.

Sometimes DNA analysis of other members of the family would be helpful if one of them is carrier of the homozygote gene anomaly but has normal phenotype so ruling out expression of the pathogenic transcript in this family. However, all the tested individuals from the family of this fetus were either heterozygote for the mutated transcripts or even non carrier of it. Finally, prenatal as well as postnatal follow up did not demonstrate any alerting sign of RTTN gene mutation which is a clinical proof that the transcribed transcript (dominant) of RTTN in this family is not the mutated one. However, prenatal transcriptomic would allow us to confirm it biologically and to reassure the family. Hence the importance of application of prenatal transcriptomics.

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