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Whole Genome Sequencing Incidentally Identified Intrauterine Cytomegalovirus Infection in a Fetus with Fetal Growth Restriction: A Case Study

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Abstract

Cytomegalovirus (CMV) intrauterine infection is a risk factor underlying Fetal Growth Restriction (FGR). Maternal secondary infection is of lower vertical transmission rate compared to primary infection during pregnancy. Whole Genome Sequencing (WGS) detects nearly all types of genomic variants and is increasingly applied to prenatal diagnosis. In the present study, we describe a growth-restricted fetus, of which CMV intrauterine infection was initially not suspected due to prior maternal immunity. WGS of DNA extracted from amniotic-fluid cells incidentally detected the presence of CMV. Our experience highlights incorporating pathogen analysis in WGS is of clinical value.

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Abbreviations

BPD: Biparietal Diameter; CMA: Chromosomal Microarray Analysis; CMV: *Cytomegalovirus*; CNV: Copy Number Variant; EFW: Estimated Fetal Weight; FGR: Fetal Growth Restriction; HC: Head Circumference; HSV: Herpes Simplex Virus-1/2; HIV: Human Immunodeficiency Virus; Indel: Small Insertion/Deletion; MCA-PSV: Middle Cerebral Arterial Peak Systolic Velocity; mNGS: Metagenomic Next Generation Sequencing; NGS: Next Generation Sequencing; OFD: Occipitofrontal Diameter; PVB19: Human Parvovirus B19; RV: Rubella Virus; SNV: Single-Nucleotide Variant; WES: Whole Exome Sequencing; WGS: Whole Genome Sequencing; ZIKV: Zika Virus

Introduction

FGR is a common pregnancy complication and associated with many adverse perinatal outcomes. A fetus with an EFW less than the 10th percentile for gestational age would be diagnosed as FGR [1]. Fetal malnutrition due to disrupted uterine-placental perfusion [2] is the major etiology, other factors include genetic abnormalities, substance use, teratogen exposure, and infectious disease. CMV is a double-stranded DNA virus and belongs to the *Herpesviridae* family. CMV has been recognized as the leading infectious cause of newborn malformation, with a prevalence of congenital infection 0.2% to 2.0% worldwide [3]. Vertical transmission of CMV may occur in maternal primary infection with the fetal transmission risk approximately 30% to 40% [4]. Correspondingly, fetal transmission rate in nonprimary infection is 0.15% to 2% [5,6] due to maternal prior immunity. Moderate-to-severe fetal sequelae are associated with intrauterine CMV infection while worse manifestations are observed in primary maternal infection [7]. Clinically, fetal CMV infection is diagnosed by detecting CMV nucleic acid in amniotic fluid after 20 to 21 weeks of gestation and at least 6 weeks from the time of maternal infection [8]. With the progress of Next Generation Sequencing (NGS), Whole Exome Sequencing (WES) [9,10] and WGS [11] have been increasingly applied to screen genomic variants including Single-Nucleotide Variants (SNVs), small Insertions and Deletions (Indels), and large Copy Number Variations (CNVs). Compare to WES, WGS requires no enrichment/capture

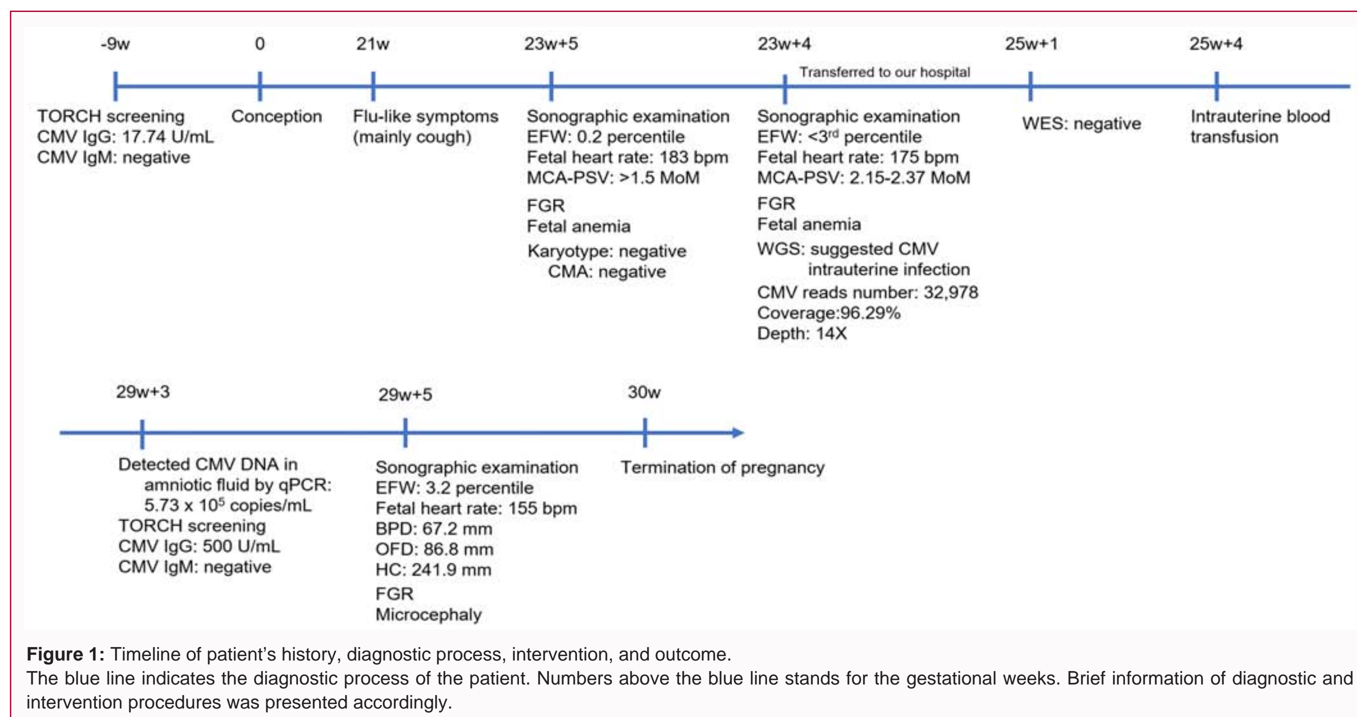

Figure 1: Timeline of patient's history, diagnostic process, intervention, and outcome.
The blue line indicates the diagnostic process of the patient. Numbers above the blue line stands for the gestational weeks. Brief information of diagnostic and intervention procedures was presented accordingly.

of target regions when constructing the library. Therefore, WGS has increased potential to identify some microorganisms within the clinical sample which might have clinical significance [12]. However, the value of integrating pathogen analysis in clinical WGS in prenatal diagnosis remains poorly defined.

Case Presentation

The patient (a 30-year-old-woman) received voluntary TORCH screening 9 weeks before pregnancy and diagnosed as CMV IgG positive (17.74 U/mL) and IgM negative (Figure 1). The patient conceived naturally and was normal during routine pregnancy examination. At 21 weeks gestation, the patient developed cough and flu-like symptoms. Ultrasonographic examination was offered at 23 weeks (Figure 2), FGR was determined with an Estimated Fetal Weight (EFW) equivalent to 0.2 percentile for gestational age according to the Hadlock formula [13]. Additionally, fetal tachycardia (183 bpm) and increased Middle Cerebral Arterial Peak Systolic Velocity (MCA-PSV) (>1.5 MoM) were determined. Fetal anemia was suspected. Four days later, the patient was transferred to Fetal Medicine Department of our hospital. Similar diagnoses were made, and the fetus was determined as severe FGR (EFW less than 3rd percentile for gestational age), tachycardia (175 bpm), and increased MCA-PSV (2.15-2.37 MoM). Due to fetal anemia, intrauterine blood transfusion was provided at 25 weeks gestation with an initial fetal hemoglobin of 4.8 g/dl, and hematocrit of 17.2%. The couples were not consanguineous marriage and denied any genetic disorder within three generations.

To investigate the genetic cause of fetal abnormalities, karyotype and CMA were offered and got no definitive findings. Trios WES and trios WGS were further offered. The sequencing depth of WGS was 59.66X and the ratio for targeted regions with ≥ 1X coverage was above 99% and that with ≥ 10X coverage was above 98% (Table 1). After bioinformatic filtering, we identified a total of 421 variations, including 3 nonsense, 176 missense, 9 indels, and 233 other types of variations. However, no causal variants responsible for the clinical presentations were identified. Interestingly, when interpreting the sequencing variants, we noticed some long insertions. We observed that reads were partially mapped to the reference human genome at the insertion sites. We speculated these reads were generated from other organisms, due to sequence similarity, these reads were interpreted as human reads and partially mapped to the human genome. Thus, the remaining unmapped fragments were classified as insertions by our bioinformatic pipeline. To test our assumption, we extracted these reads and blasted against nt/nr database and determined these reads showed 100% similarity to CMV reference genome (Figure 3, 4A). We then discarded all human reads and aligned the unmapped reads (32,978 reads in total) to CMV reference genome using BWA method. We determined the unmapped reads covered 96.29% of CMV genome with a 14X average coverage. The above results suggested the presence of CMV within the sample. We confirmed the presence of CMV using qPCR and determined the viral load was 5.73×10^5 copies/mL (Figure 4B). We further provided TORCH screening to the patient and determined CMV IgG positive (500 U/mL) and IgM negative, which indicates nonprimary CMV infection. An additional sonographic examination was offered at 29 weeks. FGR continued in the fetus with EFW at 3.2 percentile for gestational age. Fetal heart rate was 155 bpm and MCA-PSV was normal. However, the fetus developed severe microcephaly with reduced biparietal diameter (BPD=67.2 mm), occipitofrontal diameter (OFD=86.8 mm), and head circumference (HC=241.9 mm). Given the severed fetal manifestations and poor estimated prognosis, the patient decided to terminate the pregnancy at 30 weeks.

Discussion

We report the identification of intrauterine CMV infection by WGS in a fetus with FGR at 23 weeks of gestation. Given the maternal immunity against CMV before pregnancy (IgG+/IgM- was determined in preconception serological screening) and a considerable low fetal transmission risk (0.5% to 2%) of CMV nonprimary infection, intrauterine CMV infection was not suspected

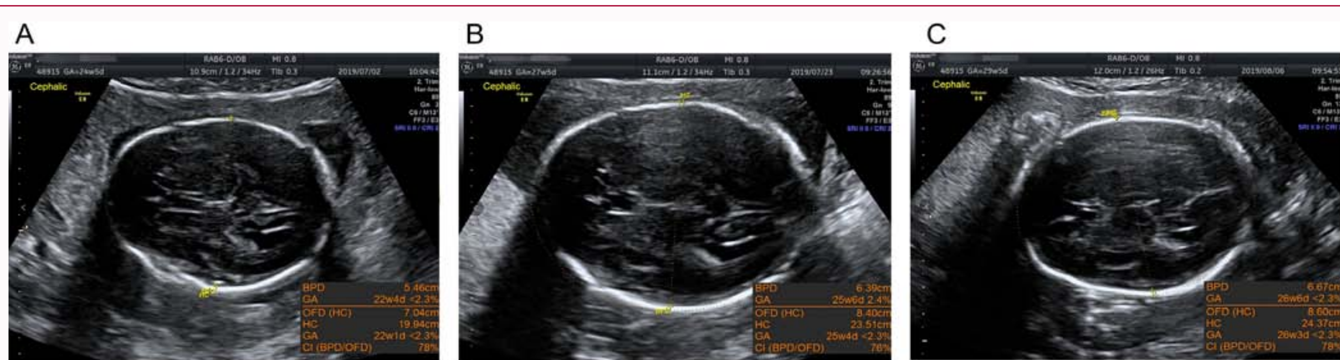

Figure 2: Sonographic examinations of the fetus.
The patient received sonographic examination at 22w+4 (A), 25w+6 (B), 26w+6 (C). Pictures show the growth-restricted fetus and growth indexes including BPD (biparietal diameter), OFD (occipitofrontal diameter), HC (head circumference), and GA (gestation age).

A Long insertions in the variation list

```
Ins 1 Chr9 : 137591840_137591841insCTGACCACCTTTGTCAAGCACATCGACGCCGCGGTTTTTAAGACGGTACGCGATTGCGTCTTCGACATCGC
Ins 2 Chr10: 131761188_131761189insTGACACGTCCTTCCTCATCATCTTCGACCGTTTCTGTCGTTTTTACGTGGTGATTGTCAAGTCGCATCTGGACC
Ins 3 Chr18: 47753942_47753943insATACAACAGCGACAGTTCGGTGTACATAGCGTATAATATATATATAGGTCACATACAGACTTATGAGCTTTTTACCAAA
Ins 4 Chr19: 38966089_38966090insTGCGACGTTCCAGAGCAAGGCTGCCTGAGCTCCGCTGGTTTATAAGAAGACTCCACCGAGACGCTCACCCGTTCACTCG
```

B Insertions were mapped to cytomegalovirus genome

```
Ins 1 Human betaherpesvirus 5 strain SYD-SCT1, complete genome (71/71, 100% indentities)
  Query  1       CTGACCACCTTTGTCAAGCACATCGACGCCGCGGTTTTTAAGACGGTACGCGATTGCGTCTTCGACATCGC  71
  Sbjct  63749   CTGACCACCTTTGTCAAGCACATCGACGCCGCGGTTTTTAAGACGGTACGCGATTGCGTCTTCGACATCGC  63819
Ins 2 Human betaherpesvirus 5 strain SYD-SCT1, complete genome (74/74, 100% indentities)
  Query  1       TGACACGTCCTTCCTCATCATCTTCGACCGTTTCTGTCGTTTTTACGTGGTGATTGTCAAGTCGCATCTGGACC 74
  Sbjct  219075  TGACACGTCCTTCCTCATCATCTTCGACCGTTTCTGTCGTTTTTACGTGGTGATTGTCAAGTCGCATCTGGACC 219002
Ins 3 Human betaherpesvirus 5 strain SYD-SCT1, complete genome (79/79, 100% indentities)
  Query  1       ATACAACAGCGACAGTTCGGTGTACATAGCGTATAATATATATATAGGTCACATACAGACTTATGAGCTTTTTACCAAA  79
  Sbjct  97035   ATACAACAGCGACAGTTCGGTGTACATAGCGTATAATATATATATAGGTCACATACAGACTTATGAGCTTTTTACCAAA  96957
Ins 4 Human betaherpesvirus 5 strain GLA-SOT2, complete genome (79/79, 100% indentities)
  Query  1       TGCGACGTTCCAGAGCAAGGCTGCCTGAGCTCCGCTGGTTTATAAGAAGACTCCACCGAGACGCTCACCCGTTCACTCG  79
  Sbjct  208431  TGCGACGTTCCAGAGCAAGGCTGCCTGAGCTCCGCTGGTTTATAAGAAGACTCCACCGAGACGCTCACCCGTTCACTCG  208355
```

C Cytomegalovirus sequences were called as long insertions due to its sequence similarity to human genome

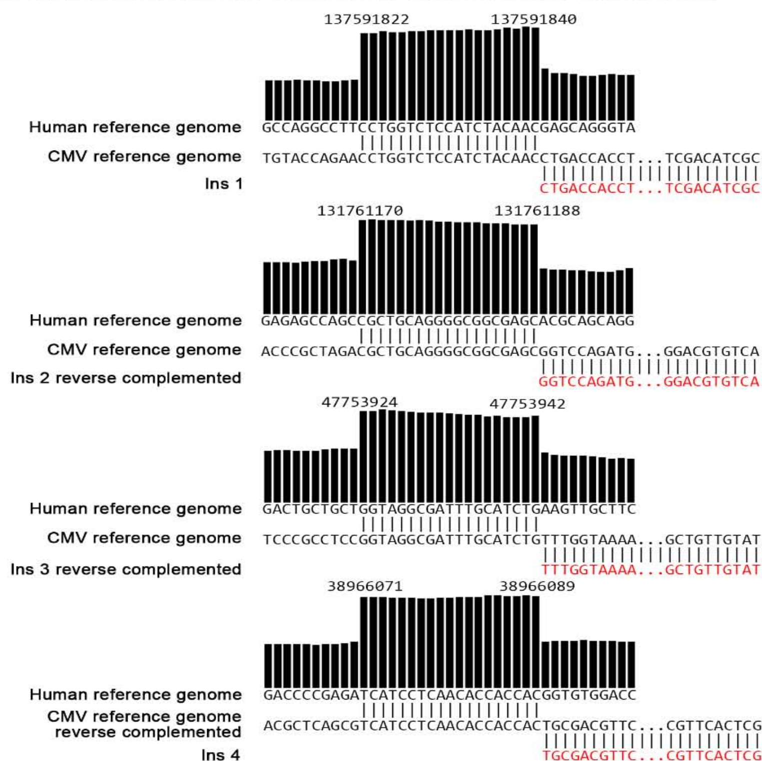

Figure 3: Identification of CMV sequence in WGS data.
A: Long insertions in the variants list of the growth-restricted fetus; B: Insertion sequences were blasted using nt/nr database. The insertions were mapped to cytomegalovirus genome with 100% identities; C: IGV plot of the insertion sites. Black bars indicate sequencing depth.

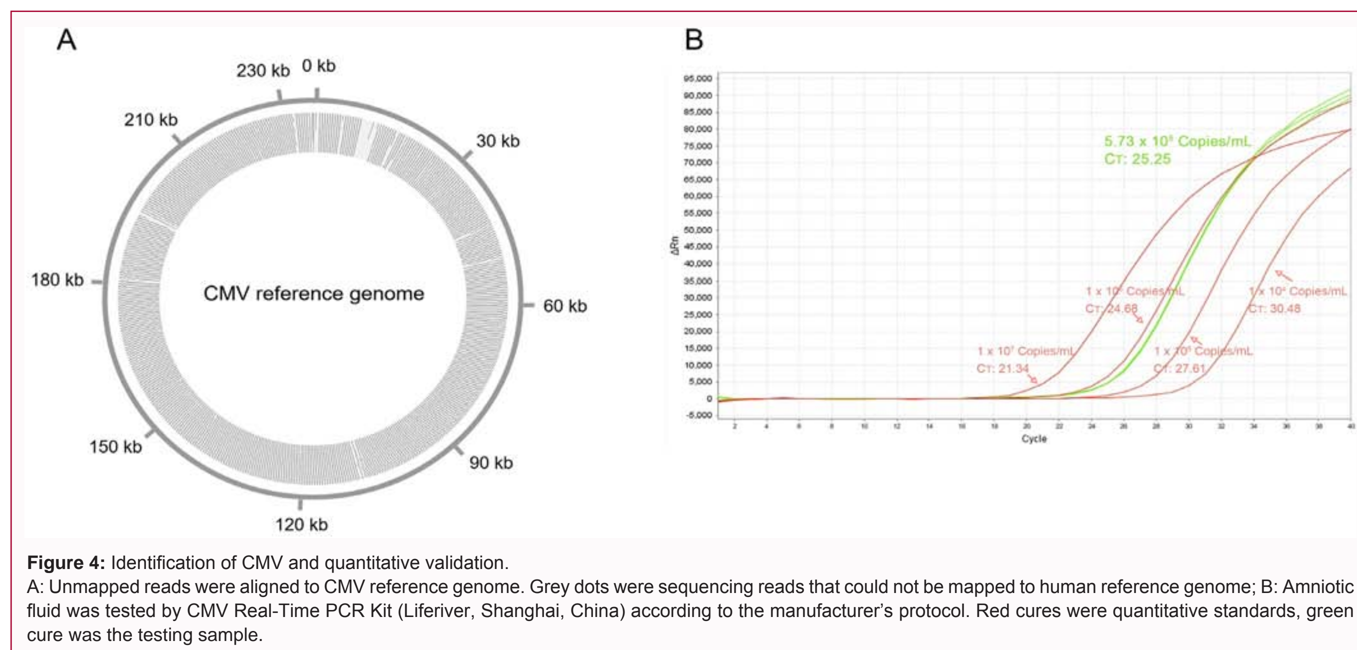

Figure 4: Identification of CMV and quantitative validation.
A: Unmapped reads were aligned to CMV reference genome. Grey dots were sequencing reads that could not be mapped to human reference genome; B: Amniotic fluid was tested by CMV Real-Time PCR Kit (Liferiver, Shanghai, China) according to the manufacturer's protocol. Red cures were quantitative standards, green cure was the testing sample.

Table 1: WGS sequencing data of the fetus.

Raw data (G)	175.83
Depth	59.66
coverage ≥ 1x (%)	99.27
coverage ≥ 4x (%)	99.13
coverage ≥ 10x (%)	98.91
coverage ≥ 20x (%)	98.26
GC rate (%)	41.4
Q20 rate (%)	97.1
Q30 rate (%)	89.64

and diagnostic measurements were not provided in the first place. Due to the non-genetic nature of the pathogenesis, karyotype, CMA, and WES failed to yield a diagnosis. WGS, which detects nearly all forms of genetic variations, was provided. However, no chromosomal numerical disorders, pathological CNVs, SNVs, or InDels were determined. Interestingly, we accidentally detected the genetic materials of CMV. Furthermore, the possibility of intrauterine CMV infection was reconsidered and diagnostic tests were offered. Our experience shows WGS is an effective tool in diagnosing the etiologies of FGR. Ideally, when FGR was detected, the possibility of fetal CMV infection would be considered and diagnosed with viral nucleic acid tests. This could have been the most efficient and economical practice to achieve a diagnosis in the present case. In this case, the clinical management was complicated by the maternal prior immunity to CMV. By applying WGS, we determine the limited fetal growth potential was caused by CMV intrauterine infection. Given the time-sensitive nature of prenatal diagnosis, this case demonstrates the potential of WGS as a first-line comprehensive diagnostic tool in prenatal diagnosis. Our experience also indicates nonprimary CMV infection should be considered in a growth-restricted fetus even preconception maternal immunity was established.

Apart from CMV and other TORCH agents whose clinical manifestation exhibits in a similar fashion with screening measurements routinely provided, there are other pathogens challenging reproductive health. These pathogens include Human Immunodeficiency Virus (HIV), influenza virus, vaccinia, Epstein-Barr virus, malaria, listeria, and Zika Virus (ZIKV). Generally, the clinical presentations of these pathogens are distinctive from TORCH and their diagnostic measurements are highly diversified. In perinatal settings, rather than inferring each agent individually, which is labor-intensive and time-consuming, there warrants a synergistic approach to simultaneously detect all candidate pathogens. Metagenomic Next Generation Sequencing (mNGS) provides an unprecedented opportunity to identify the potential cause of an ongoing infection in a comprehensive manner [14]. However, the application of mNGS in prenatal samples is limited. In the present study, we identified intrauterine CMV DNA by WGS, in which the sampling processing, library preparation, and sequencing steps are similar to mNGS. Our experience demonstrates the incorporation of pathogen analyses in WGS data interpretation is of substantial clinical value. However, it should be noted that pathogens such as RNA viruses and fungi/bacteria with a thick cell wall are not detectable by the current analytic process in our study. In future studies, fine adjusting of the experimental protocols such as the nucleic acid extraction and RNA sequencing process may be required.

In conclusion, our experience suggested nonprimary CMV infection should be considered in FGR fetuses even prior immunity was established. By incorporation of pathogen analysis, WGS is promising to serve as a first-line diagnostic tool in the prenatal setting.

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