Evolution ROP18 gene expression of Toxoplasma gondii in pregnant and aborted women

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Abstract
Toxoplasma gondii is an opportunistic protozoan infecting almost one-third of the world's population. T. gondii rhoptry protein 18 (TgROP18) is a key virulence factor determining the parasite's acute virulence and is secreted into host cells during infection. This study assessed the evolution of ROP18 gene expression in 192 cases that were categorized into 156 pregnant women, and 36 women with abortion. Out of the 156 pregnant women, 23 cases were positive for real time-PCR and out of all 36 aborted women, 16 of them were positive for real time-PCR investigation for toxoplasma gondii. The result showed the level of ROP-18 expression was higher significantly in pregnant when compared to pregnancy groups, 17.15 versus 7.11, respectively (p = 0.019). These finding also suggest high expression of ROP 16 gene in placenta than blood tissue.
Keyword: Toxoplasma gondii, ROP18, RT-PCR, Pregnant

1. Introduction

Toxoplasma gondii is a protozoan parasite, classified under the phylum Apicomplexa, that contains obligatory intracellular pathogens. In general, infection of immunocompetent individuals is either asymptomatic or causes mild flu-like symptoms (Schneider et al., 2013). In order to establish an infection, T. gondii manipulates the host cells via altering the cellular metabolism (Ma et al., 2019), dysregulating the gene expression (He et al., 2016), for example, certain proteins in rhoptries are important determinants of virulence of parasite (Shwab et al., 2016; Mammari et al., 2019). ROP18 is a Ser/Thr kinase related to the ROP2 subfamily, secreted by the rhoptries into the PV and host cytosol. Its action as effector molecule is anticipated to modulate host factors; it inhibits host cell apoptosis by blocking the release of cytochrome-c, upregulating the ratio of Bcl-2/Bax, and inducing p53 degradation (Wu et al., 2016; Yang et al., 2017b; Xia et al., 2018). The catalytic core of the ROP18 virulence complex regulates gene expression and apoptosis. ROP18 directly phosphorylates and subsequently degrades activating transcription factor 6ß (ATF6ß) (Yamamoto etal., 2011). Additionally, ROP18 can phosphorylate RTN1-C, an ER protein expressed in the central nervous system (CNS), leading to the induction of ER stress-mediated apoptosis in neural cells (An et al., 2018).

2. Materials and Methods

Study subjects and case definition

The following study was designed in order to diagnose parasitic virulence factor ROP16 in pregnant and aborted women by using molecular diagnosis Real time PCR method. Specimens were collected aseptically via venous blood sampling of 192 pregnant and aborted women; 100 mg Human placenta tissue samples were transported to a sterile 1.5ml micro centrifuge tube and then stored in -80C for genomic DNA and RNA extracted.

Genomic DNA and RNA Extraction

Genomic DNA was extracted from blood and placenta tissue samples by using (gSYNCTM DNA Extraction Kit / Geneaid Biotech Ltd. Taiwan) and total RNA were extracted from specimens by using (TRIzol® reagent kit) and done according to company instructions. Real Time PCR was performed for detection of T.gondii from blood samples and placenta tissue by using the specific primers and TaqMan probe specific for B1 gene in Toxoplasma gondii this technique was carried out according to method described by (Lin et al., 2000). qPCR master mix was prepared by using (RealMOD™ Probe HP 5X qPCR Mix Kit iNtRON /Korea) and this master mix done according to company instructions. The reactions were done with an AB Step One real-time PCR system (Applied Biosystems) in a final volume of 20µl. the reaction mixture contained 10µl of qReal Master Mix (Amplicon, Denmark), 1 µl of each primer (B1 forward, B1reverse and B1probe primer), PCR water (2µl) and 5µl extracted DNA. The RT-PCR primer that used in gene expression of T.gondii virulence factors genes and housekeeping GAPDH gene were designed in this study by using NCBI Genbank database and perimer3 plus. (Scientific Researcher provided all these primers. Co. Ltd. Iraq) are showed in Table 1.

doi.org/10.31838/hiv23.03.230 Received: 27.11.22, Revised: 23.12.22, Accepted: 12.01.23.

Table 1: Nucleotide sequences of real time PCR primer/probe sets			
Primer	Sequence (5'-3')		Product size
B1gene primer	F	TCCCCTCTGCTGGCGAAAAGT	94bp
	R	AGCGTTCGTGGTCAACTATCGATTG	
B1gene probe	FAM- TCTGTGCAACTTTGGTGTATTCGCAG-TAMRA		
T.gondii rop18 gene	F	TTCGTGAAGCTTGGCCAATG	117bp
	R	TCCAGCAATGAAACGTCTCG	
Human GAPDH gene	F	AATTCCATGGCACCGTCAAG	104bp
	R	ATCGCCCCACTTGATTTTGG	

The extracted RNA were treated with DNase I enzyme to remove the trace amounts of genomic DNA from the eluted total RNA by using samples (DNase I enzyme kit) and done according to method described by Promega company. After that, the mixture was incubated at 37C° for 30 minutes. Then, 1µl stop reaction was added and incubated at 65C° for 10 minutes for inactivation of DNase enzyme action. DNase-I treated RNA samples were also used in cDNA using M-MLV Reverse Transcriptase kit and done according to company instructions, Than RNA and primer was denatured for 10 min at 65 °C, after that immediately cool on ice, After that, these qPCR master mix component that mentioned above placed in qPCR plate strip tubes and mixed by Exispin vortex, centrifuge for 3 minutes, then placed in Miniopticon Real-Time PCR system. After that, the qPCR plate was loaded and the following thermocycler protocol.

3. Result

The present study enrolled 192 cases that were categorized into 156 pregnant women and 36 women with abortion. Out of the 156 pregnant women, 23 cases and out of all 36 aborted women, 16 of them were positive for real time-PCR investigation for *T.gondii*. Comparison of results of RT-PCR for *toxoplasma gondii* in blood and placenta between abortion group and pregnancy group is shown in table 1

Table 1: Comparison of results of RT-PCR for toxoplasma gondii in blood and placenta between abortion group and pregnancy group			
RT-PCR	Abortion n = 36	Pregnancy n = 156	P
Blood RT-PCR toxoplasma gondii			
Positive, n (%)	9 (25.0 %)	23 (14.7 %)	0.137 C NS
Negative, n (%)	27 (75.0 %)	133 (85.3 %)	
Placenta RT-PCR toxoplasma gondii			
Positive, n (%)	7 (19.4 %)		
Negative, n (%)	29 (80.6 %)		
n: number of cases; **C**: chi-square test; **NS**: not significant; *: significant at $p \leq 0.05$			

The level of ROP-18 expression was lower significantly in abortion group when compared to pregnancy group, 32 versus 14.83, respectively (p = 0.019).

Table2: Comparison of median blood ROP-18 expression in fold change between abortion group and pregnancy group				
Cases	Abortion Group	Pregnancy Group	P value Mann Whitney U test	interpretation
ROP 18 fold change (blood)				
Median	7.11	17.15	0.019	Significant

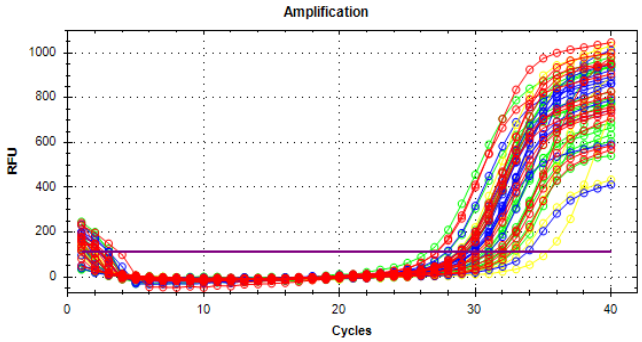

Fig 1: Real Time PCR amplification plot for rop18 gene in T.gondii positive samples. Green plots (Aborted woman group samples), yellow plots (Patient placenta group samples), Blue plots (Patient Blood group samples), and the Red plots (Patient pregnant group samples).

Comparison of median ROP-18 fold change expression between placenta and blood in abortion group is shown in table 3 The level of ROP-18 was lower significantly in blood in comparison with placenta, 21.41 versus 7.11, respectively (p = 0.002).

Table 3: Comparison of median p53, ROP-16 and ROP-18 fold change expression between placenta and blood in abortion group

Fold change expression	Placenta	Blood	*P* value *Wilcoxon test*	Interpretation
ROP 18				
Median	21.41	7.11	0.002	Significant

4. Discussions

In this study, we demonstrated T. gondii virulence factor ROP18 in aborted and pregnant women.it members of the subgroup known as ROP2-like genes, as shown by comparison of their kinase domains. Additionally Fentress et al., (2010) and Etheridge et al., (2014) revealed that despite this divergence, ROP18 target a common pathway in the host by phosphorylating members of the Immunity-related GTPases (IRG) family. Consistent with their overlapping functions, single deletion of either gene had only a modest effect on virulence, characterized by a delayed time to death, but a nonetheless lethal outcome even with low inoculate. A study by Mahdi and AL-sakee (2021) conducted to evaluated seroprevalence toxoplasmosis in aborted and non-aborted women and investigate the expression of rhoptry protein 18 genes in women in Erbil. The results among 50 samples by polymerase chain reaction, confirmed the expression of these genes in toxoplasmosis condition ROP18 genes were positive 6 (12 %) of abortive women, versus 1 (2 %) for in non-abortive women. there was ROP18 show no significant at (p=0.229) differ with us.

Present study indicated that the level of ROP-18 was lower significantly in blood in comparison with placenta, 21.41 versus 7.11, respectively (p = 0.002). High expression of ROP18 in placenta is closely associated with high toxoplasma infection state, while low expression is associated with a virulence in type III strains in placenta more than blood this was in accordance with Saeij et al., (2006), Taylor et al., (2006) and Hakim et al., (2017).

However, the causative mechanism of ROP18 as a key factor in Toxoplasma infections remains a mystery. According to our knowledge, all studies mentioned the same fact, in other diseases but not in abortion or pregnancy. ROP18-induced host cell apoptosis has been previously reported. During pregnancy, fetal development is directly related to the proliferation, differentiation, and apoptosis of trophoblast cells (Wang et al., 2018). Increased trophoblast cell apoptosis could be damaging to fetal health and even cause adverse pregnancy outcomes (Chen et al., 2012; Li et al. 2012). The above finding contributes novel knowledge to current understanding in regards to Toxoplasma gondii-induced apoptosis, and may help to illustrate the underlying mechanism of Toxoplasma gondii-induced pregnancy failure.

Li et al. (2020) study presents the first RNA-Seq-based analysis of the transcriptomic responses of HEK239T cells to ROP18 expression. Identified 22,460 host genes, and the expression of 750 genes was significantly altered by ROP18, including 467 upregulated genes and 283 downregulated genes. Data revealed several potential new roles of ROP18 in the transcriptional regulation of host cells. Further investigations of the effects of a catalytic inactive mutant of ROP18 on the host cell transcriptome and using different cell lines (e.g. neurons and immune cells) will deepen our understanding of T. gondii interactions with the host cell processes (Li et al., 2021). In addition, using methods such as siRNA and gene editing to alter ROP18 protein expression can improve the evaluation of the effects of ROP18 protein with the concomitant entry of live parasites (Li et al., 2020). Regarding ROP18 Dincel and Atmaca (2016) study suggested that Toxoplasma-mediated apoptosis might play a pivotal role in neurodegeneration and neuropathology in the process of thrombo embolism in abortion and pregnant women with infections. Based on the results obtained An et al., (2018) was found that ROP18 was strongly bound to RTN1-C via its N-terminal 20 amino acids, A better understanding of the interaction between RTN1-C and ROP18 might offer insights into the mechanism of neural tropism of Toxoplasma infection related to other infections. Our data were in agreement with outcomes.

Conclusion

Our data revealed several potential new roles of ROP18 in the transcriptional regulation of host cells. Further investigations of the effects of a catalytic inactive mutant of ROP18 on the host cell transcriptome and using different cell lines (e.g. neurons and immune cells) will deepen our understanding of T. gondii interactions with the host cell processes. Also, using methods such as siRNA and gene editing to alter ROP18 protein expression can improve the evaluation of the effects of ROP18 protein with the concomitant entry of live parasites.

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