Research Article

Association of Combined Maternal-Fetal *TNF-α* Gene G308A Genotypes with Preterm Delivery: A Gene-Gene Interaction Study

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Preterm delivery (PTD) is a complicated perinatal adverse event. We were interested in association of G308A polymorphism in *tumor necrosis factor-α* (*TNF-α*) gene with PTD; so we conducted a genetic epidemiology study in Anqing City, Anhui Province, China. Case families and control families were all collected between July 1999 and June 2002. To control potential population stratification as we could, all eligible subjects were ethnic Han Chinese. 250 case families and 247 control families were included in data analysis. A hybrid design which combines case-parent triads and control parents was employed, to test maternal-fetal genotype (MFG) incompatibility. The method is based on a log-linear modeling approach. In summary, we found that when the mother's or child's genotype was G/A, there was a reduced risk of PTD; however when the mother's or child's genotype was genotype A/A, there was a relatively higher risk of PTD. Combined maternal-fetal genotype GA/GA showed the most reduced risk of PTD. Comparison of the LRTs showed that the model with maternal-fetal genotype effects fits significantly better than the model with only maternal and fetal genotype main effects (log-likelihood = -719.4, $P = .023$, significant at 0.05 level). That means that the combined maternal-fetal genotype incompatibility was significantly associated with PTD. The model with maternal-fetal genotype effects can be considered a gene-gene interaction model. We claim that both maternal effects and fetal effects should be considered together while investigating genetic factors of certain perinatal diseases.

1. Introduction

Preterm delivery (PTD) is one of the main causes of perinatal and neonatal death. It has been reported that PTD is associated with some severe complications, such as cerebral palsy, chronic respiratory illness, and blindness [1]. Even in some developed countries like the United States, more than 10% of newborns are preterm, and the PTD prevalence is still increasing [2–4]. Genetic factors may be important determinants of PTD because women who were born preterm are more likely to deliver preterm; approximately 20% of women who delivered preterm subsequently had another PTD with the same partner; to change partners reduces the risk of PTD by one third and twin studies of

pregnancy outcomes estimated the heritability of PTD as 17% to 36% [5].

Increasing clinical and laboratorial evidence suggests that amniochorionic-decidual infections may play an important role in PTD, by triggering a cascade of events that result in both spontaneous preterm labor (PTL) and preterm premature rupture of membranes (PPROMs). Researchers become interested in proinflammatory cytokines like tumor necrosis factor-α (*TNF-α*). *TNF-α* is a potent cytokine which has a wide range of proinflammatory activities [6]. Production of *TNF-α* gene is regulated partly at transcriptional level. An SNP from a normal guanine (G) allele to a variant adenine (A) allele at position 308 (G308A), which is located in the promotor region of *TNF-α*, is of particular interest.

Transfection studies indicated that *TNF-α* expression is higher in the presence of the −308A allele, compared with the −308G allele [7]. The G308A transition of *TNF-α* has been shown increasing both *TNF-α* concentration [8] and disease susceptibility in human subjects [9].

Large-scale studies of the association of the G308A polymorphism of *TNF-α* with PTD have been conducted only recently. However, no strong convincing evidence of association has been found. A systematic review has been reported, which reviewed studies investigating the association of the G308A polymorphism of *TNF-α* with PTD [10]. Those studies were reported between 1990 and 2005. Among the total seven studies involved in meta-analysis, only two reported positive results. A meta-analysis of the pooled dataset showed no statistically significant association. Trying to make progress, some researchers have tested population stratification as a potential confounder [11–13], and some have considered high-dimensional gene-gene interactions [14].

It is well known that pregnancy is a complicated course, depending on the balance between the mother and the fetus. Maternal-fetal incompatibility is thought to be one potential mechanism of adverse pregnancy outcomes. Therefore, while investigating certain perinatal diseases, it is recommended that one takes both maternal effects and fetal effects into consideration. Somewhat disappointingly, few studies have addressed association of G308A polymorphism of *TNF-α* with PTD in this way. In the current study, we used a hybrid design which combines case-parent triads and control parents in the data analysis [15], to explore the complicated effects of *TNF-α* G308A polymorphism on PTD. This hybrid design can bring the strengths of family-based designs and population-based designs together to test for maternal-fetal genotype (MFG) incompatibility, which can be considered a form of interaction between maternal genotypes and fetal genotypes.

2. Materials and Methods

2.1. Study Site and Population. Our study was conducted in Anqing City, Anhui Province, China. The city stretches about 80 km along the north bank of Yangtze River and includes eight counties. The total population of Anqing in 2000 was 6.8 million, with 20% of it living in urban areas. The birth rate was 15.1 per 1,000 people and the infant mortality rate was 3.8 per 1,000 live births.

Case families and control families were collected in Anqing Hospital between July 1999 and June 2002. Infants and their parents were all enrolled. Cases were defined as singleton, live, preterm infants (28 completed weeks or more but less than 37 completed weeks of gestation, regardless of birth weight); controls were defined as singleton, live, term infants (more than 37 completed weeks of gestation). Infants with birth defect were excluded. We matched cases and controls by maternal age (within 5 years) and delivery date (within 2 days). To control potential population stratification as we could, all enrolled subjects were ethnic Han Chinese. Besides, we enrolled only spontaneous PTD, to reduce heterogeneity within the case group to some extent.

2.2. Data Collection Procedures. All eligible mothers were approached by trained examiners soon after the delivery of their children. After informed consent (approved by the Ethics Committee of the Peking University Health Science Center) was obtained, a structured interview was conducted to obtain relevant information on demographic characteristics, cigarette smoking, alcohol consumption, as well as medical and reproductive history. Medical records of mothers and infants were reviewed to obtain clinical data including prenatal care, pregnancy complications, and birth outcomes (infant's gender, gestational age, and birth weight).

2.3. Blood Sample Collection and DNA Extraction. Cord blood samples (10 mL) from infants and peripheral blood samples (10 mL) from parents were collected. The blood samples were initially stored in a −20°C designated refrigerator in the *Labor and Delivery Ward* of the hospital and then transported to Peking University Health Science Center, via cold chain. DNA was extracted in our laboratory according to standard protocols [16].

2.4. Genotyping Methods. *TNF-α* is located in Chromosome 6p21.3, within the major histocompatibility gene complex [17]. We analyzed G308A polymorphism according to a reported method [18]. Primers used for PCR were 5′-GGGACACACAAGCATCAAGG-3′ and 5′-AATAGGTTT-TGAGGGCCATG-3′. After an initial denaturation step for 1 minute at 94°C, 40 cycles of amplification were performed as follows: 15 s at 94°C, 30 s at 60°C, and 15 s at 72°C followed by a final extension step for 7 minutes at 72°C. We used the Gene-Amp PCR System 9700 (Perkin-Elmer, Foster City, CA) for amplification. PCR products were digested with NcoI at 37°C. After ethidium bromide (EB) staining, diagnostic fragments were electrophoresed in 4% agarose gel and then visualized by UV transillumination. Genotyping accuracy was checked by duplicate genotyping within 10% random samples. Direct sequencing of PCR products was also performed to ensure genotyping results.

2.5. Statistical Methods. Our analysis employed the hybrid design reported by Weinberg and Umbach [15] as well as the ideas of testing maternal-fetal genotype (MFG) incompatibility reported by Sinsheimer and her colleagues [19]. For detailed information, please refer to the original papers.

The MFG test is based on a log-linear approach using only case-parent triads [20], while the hybrid design using both case-parents triads and control parents. Three key assumptions should be considered: Mendelian transmission of alleles, no population stratification, and mating symmetry. Mendelian transmission of the variant allele can be tested by *Transmission Disequilibrium Test (TDT)*, using control-parent triads. Because the MFG test employs only case-parent triads, mating symmetry cannot be tested [15, 21]. If one cannot trust the mating symmetry assumption, the appropriateness of using the MFG test is in doubt. The hybrid design provides ways of testing both the no population stratification and mating symmetry assumptions.

Luckily, even when one encounters mating asymmetry, a modification of the hybrid design using 9 ordered mating-types provides an alternative inference framework.

We first counted case-parent triads and control-parent triads based on six mating-types. After testing Mendelian transmission, we used the methods of testing no population stratification assumption and mating symmetry assumption proposed by the hybrid design.

Equation (1) is the standard alternative model presented by Weinberg and Umbach:

$$\ln[E(\text{count} \mid M, F, C, D)]$$
$$= \ln\left(\mu_j\right) + \beta_1 DI_{(C=1)} + \beta_2 DI_{(C=2)} \tag{1}$$
$$+ \alpha_1 DI_{(M=1)} + \alpha_2 DI_{(M=2)} + \gamma D + \ln(\text{Off}).$$

When $\beta_1 = \beta_2 = 0$, there are no child allelic effects in the model; when $\alpha_1 = \alpha_2 = 0$, there are no maternal allelic effects. Both of these restrictions hold under the null model. M, F, and C donate the number of copies (0, 1, or 2) of the variant allele carried by the mother, the father, and the affected child, respectively. D is an indicator variable that equals 1 for families with an affected offspring and 0 for control families. μ_j ($j = 1, \ldots, 6$) are proportional to the relative frequencies in the population for the mating-type categories. $\beta_1, \beta_2, \alpha_1$, and α_2 are relative risk parameters. "Off" is the probability multiplier (1, 1/2, or 1/4) for the particular cell (see Table 1 of [15]). $I_{(C=1)}$ denotes a dummy independent variable that equals 1 when $C = 1$ and 0 otherwise. It is similar for $I_{(C=2)}, I_{(M=1)}$, and $I_{(M=2)}$.

Augmenting (1) with an predictor $(M + F)D$ and testing for improvement in fit enables one to test whether there is bias due to population structure. Adding an interaction term between $I_{(M>F)}$ and mating-type ($I_{(M>F)}$ is defined as an indicator variable which is 1 when $M > F$ and 0 otherwise), one can test mating symmetry. Retaining any subset of (1) enables one to compare models with or without selected terms. We followed the ideas of the MFG test to test maternal-fetal genotype incompatibility by reparameterizing (1) with 6 maternal-fetal genotype relative risk combinations where the situation that both the mother and child have genotype G/G is considered the reference state. The *Likelihood Ratio Test (LRT)* is employed in model comparison. To ease understanding the five models we used in the current study, we present the models below symbolically.

Model I serves as a reference model and only includes 6 mother-father mating-type effects and disease effects:

$$\ln[E(\text{count} \mid M, F, C, D)] = \ln\left(\mu_j\right) + \gamma D + \ln(\text{Off}). \tag{I}$$

Model II has only fetal genotype effects, assuming no maternal genotype effects:

$$\ln[E(\text{count} \mid M, F, C, D)]$$
$$= \ln\left(\mu_j\right) + \beta_1 DI_{(C=1)} + \beta_2 DI_{(C=2)} + \gamma D + \ln(\text{Off}). \tag{II}$$

Model III has only maternal genotype effects, assuming no fetal genotype effects:

$$\ln[E(\text{count} \mid M, F, C, D)]$$
$$= \ln\left(\mu_j\right) + \alpha_1 DI_{(M=1)} + \alpha_2 DI_{(M=2)} + \gamma D + \ln(\text{Off}). \tag{III}$$

Model IV is (1).

Model V includes the combined maternal-fetal genotype effects instead of maternal genotype main effects and fetal genotype main effects:

$$\ln[(\text{count} \mid M, F, C, D)] =$$
$$\ln\left(\mu_j\right) + \delta_1 DI_{(M=2,C=2)} + \delta_2 DI_{(M=2,C=1)} + \delta_3 DI_{(M=1,C=2)}$$
$$+ \delta_4 DI_{(M=1,C=1)} + \delta_5 DI_{(M=1,C=0)} + \delta_6 DI_{(M=0,C=1)}$$
$$+ \gamma D + \ln(\text{Off}). \tag{V}$$

The hybrid design employs case-parent triads and control parents, but not controls. So the genotypes of controls in the later analysis are treated as missing. Under this circumstance, the expectation-maximization (EM) algorithm is used to estimate relative risk parameters. The calculation can be conducted in the LEM software (log-linear and event history analysis with missing data using the EM algorithm), which was developed by Vermunt [22]. To aid us in the somewhat complicated calculation of EM, Weinberg and Umbach have provided LEM scripts in their website [15].

3. Results

A total of 520 families, including 260 control families and 260 case families, participated in our study. The participation rate was 90% among the control group and 95% among the case group. We tested maternal ethnicity or other sociodemographic characteristics between participants and nonparticipants and found no significant differences. Four case families and five control families were excluded because we lacked their blood samples; six case families and eight control families were excluded because of genotyping failures. Reproducibility of genotyping was routinely greater than 99%. We didnot encounter any cases of Mendelian inconsistency. The final analysis included 250 case families and 247 control families.

Table 1 shows some general characteristics of mothers and children.

Table 2 shows the 6 mating-types and their corresponding counts, for control group and case group, respectively. In the test of Mendelian transmission of the variant allele, we found no statistical evidence of non-Mendelian transmission ($\chi^2 = 0.42, P = .522$).

Although we have genotypes of controls as well, to suit the hybrid design, we rearranged data in Table 2 to form 24 possible cells, just as described in [15, Table 1]. For simplicity of the context, we didnot provide another table here.

In the LRT testing whether there is bias due to population stratification, we could not reject the null hypothesis of no

TABLE 1: Some general characteristics of mothers and children.

| Variable | Control | Case | Preterm Delivery | | |
	Group	Group	OR	95%CI	P-value
Age, year					
19-	74	72	1	—	—	—
23-	99	105	1.09	0.72–1.67	.679
27-	74	73	1.01	0.64–1.60	.953
Education					
≤ elementary school	75	70	1	—	—	—
= middle school	133	145	1.18	0.79–1.75	.431
≥ middle school	39	35	0.95	0.54–1.66	.857
Occupation					
Farmer	110	111	1	—	—	—
worker	51	51	0.98	0.62–1.57	.935
Housewife	86	88	1.01	0.68–1.50	.955
Parity					
No	171	158	1	—	—	—
Yes	76	92	1.28	0.89–1.86	.187
Children gender					
Male	108	121	1	—	—	—
Female	139	129	0.82	0.58–1.17	.282

TABLE 2: Triad counts in control group and case group by genotype.

Mating-type[#]	Triad genotype (MFC)[*]	Control Group (N = 247)	Case Group (N = 250)
1	222	2	0
2	212	2	3
2	211	2	6
2	122	0	1
2	121	4	0
3	201	4	1
3	021	0	0
4	112	4	4
4	111	4	1
4	110	4	5
5	101	13	3
5	100	6	0
5	011	6	1
5	010	4	1
6	000	192	224

[#] Mating-type: decided by the number of copies of allele the parents carrying.
[*] Triad genotype (MFC): copies of the variant in mother, father, and child.
0: homozygous wild type; 1: heterozygous variant type; 2: homozygous variant type.

TABLE 3: LRTs for maternal and fetal genotype effects and their interaction.

| Model[#] | TNF-α G308A | |
	Log-likelihood[*]	P value[**]
I	−728.3	—
II	−724.8	.030[†]
III	−723.0	.005[†]
IV	−722.0	.013[†]
V	−719.4	.023[§]

[#]: Model I: with mating-type only; model II: with mating-type plus fetal genotype; model III: with mating-type plus maternal genotype; model IV: with mating-type plus fetal genotype and maternal genotype; model V: with mating-type plus combined maternal-fetal genotype.
[*]: The log-likelihoods were estimated using the LEM software.
[**]: LRTs, for a χ^2 distribution with df equal to the difference in the number of parameters being fitted.
[†]: Compared with Model I.
[§]: Compared with Model IV.

Table 3 shows the LRT results for the various models where model I serves as a reference. Comparing model II to model I, we can test for fetal genotype effects, assuming no maternal genotype effects. Comparing model III to model I, we can test for maternal genotype effects, assuming no fetal genotype effects. Comparing model IV to model I tests for both maternal genotype effects and fetal genotype effects simultaneously. Finally comparing model V to model IV tests for maternal-fetal genotype incompatibility and is a test of gene-gene interaction.

Table 4 shows the estimated relative risks of PTD, given maternal and fetal TNF-α G308A polymorphism. In summary, all the estimated relative risks of maternal genotype main effects and fetal genotype main effects were

population stratification at the 0.05 significance level (χ^2 = 3.13, P = .077). However in testing mating symmetry, we detected significant evidence of violation (χ^2 = 11.93, P = .008). Therefore, we conducted our analyses with nine ordered parental genotype categories instead of the usual six. That is, for mating-types 2, 3, and 5, we divided them into two ordered mating-types each.

TABLE 4: Associations of maternal and fetal *TNF-α* gene G308A genotypes with preterm delivery.

TNF-α G308A	Child		Mother	
Genotype*	RR	95%CI	RR	95%CI
GG†	1.0	—	1.0	—
GA	0.58	0.23–1.42	0.46	0.20–1.04
AA	0.93	0.28–3.04	1.23	0.34–4.46

*: GG: homozygous wild type; GA: heterozygous variant type; AA: homozygous variant type.
†: Reference category.

TABLE 5: Association of combined maternal-fetal *TNF-α* G308A genotypes with preterm delivery.

Genotypes*		Preterm delivery	
Child	Mother	RR	95%CI
GG	GG†	1.00	
GG	GA	0.36	0.14–0.96§
GA	GG	0.25	0.03–1.95
GA	GA	0.20	0.07–0.58§
GA	AA	1.12	0.41–3.10
AA	GA	0.82	0.30–2.24
AA	AA	0.59	0.15–2.25

*: Use LRTs, comparing the model with an interaction term for combined maternal-fetal genotypes against the model with maternal genotype and fetal genotype only (without interaction term).
†: Reference category.
§: Significant.

not significant (95%CI of RR includes 1). Nevertheless, mothers carrying 1 copy of the variant allele A had reduced risk, which was almost significant.

Table 5 presents estimated relative risks of PTD, given certain combined maternal-fetal genotypes. Combined maternal-fetal genotypes of GA/GG (RR = 0.36, 95%CI = 0.14–0.96) and GA/GA (RR = 0.20, 95%CI = 0.07–0.58) showed significant reduced risk of PTD.

4. Discussion

Because most reported studies investigating association of *TNF-α* G308A polymorphism with PTD didnot consider maternal effect and fetal effect together, we conducted the current study. We were interested in the combined maternal-fetal genotype effects on PTD. We used a hybrid design that combines case-parent triads and controls parents in the data analysis. Assumptions of Mendelian transmission of the variant allele and no population stratification were satisfied, but mating asymmetry was detected. So we conducted the following analysis based on models with nine ordered parental genotype categories instead of the usual six. Despite some loss of power, this hybrid design provides a still-valid analytic framework for inference, especially for maternal genetic effects [15].

Some of our results were insignificant, which may result from sparse data. Nevertheless, compared to heterozygote of variant allele A, homozygote of variant allele A showed

relatively higher risk of PTD (see Table 4). This is in accordance with some aspects of our current understanding of the underlying biological processes. The balance between pro- and anti-inflammatory cytokines is critical for implantation, placental development and pregnancy outcome. While the T helper 1 (Th1) cytokine is associated with inflammation, the T helper 2 (Th2) cytokine is associated with anti-inflammation. The predominant expression of Th2 cytokine is likely to be important to reduce aberrant inflammation and allograft rejection of the fetus [23].

Chorioamnionitis presents to most PTD [24]. It is known that chorioamnionitis with high-grade leukocyte infiltration usually indicates intrauterine infection [25, 26], accompanied by high concentrations of inflammatory mediators in amniotic fluid like proinflammatory cytokines (such as *TNF-α*) [27, 28]. The G308A transition in *TNF-α* promotor region can increase gene expression [29, 30]. When inflammation does happen, an elevated proinflammatory cytokines level may result in changes of Th1/Th2 cytokine profile at the fetal-maternal interface, so as to break down the status of the pregnant uterus as an immune-privileged organ and cause different adverse consequences [31]. Even if no infection exists, proinflammatory cytokines can transform the uterus from a quiescent to an active state. The cytokines stimulate uterine activity via production of uterine activation proteins (UAPs) [32]. An active status of the uterus increases the possibility of PTD. These may account for why the G308A transition increases the risk of PTD.

The effect of G308A transition is far more complicated. There is one result that needs to be mentioned: mothers and children whose genotypes are heterozygous A/G lead to reduced risk of PTD (see Table 4). Similar phenomenon can be seen in the result of testing maternal-fetal genotype incompatibility (see Table 5). Combined maternal-fetal genotype GA/GA showed the most reduced risk of PTD, and this result was significant. One possible explanation is there are some cell membrane-bound molecules expressed in tissues of the fetus (like cytotrophoblasts), such as Fas ligand (FasL, also called CD95L) and *TNF*-related apoptosis-inducing ligand (TRAIL) [33]. Proinflammatory cytokines like *TNF-α* can upregulate their expression, enhance their function, or downregulate counteracting elements against them, which favor the apoptosis of infiltrated inflammatory cells [34, 35]. Moderate extent of these processes might benefit pregnancy, which the genotype G/A might induce. But of course, on the other side of the coin, if these processes are too strong, an active status of the uterus ensues and inevitably increases the possibility of PTD, which the genotype A/A might induce.

Maternal genotype effects have long been involved in the study of perinatal diseases. In recent years, there have been emerging hypotheses and evidence that the fetal genome might be associated with pregnancy health, either by themselves or by interacting with maternal genes. The failure to recognize fetal contributions to pregnancy health may be at least partially responsible for the inability to consistently identify predisposing maternal genetic variants [36]. Since 1999, more than ten studies have investigated association of the G308A polymorphism with PTD, but only three of them

have involved both mothers and children in their studies. Even for these three studies, none considered the mother and the child together in their modeling. In our study, we used a modified version of the hybrid design of Weinberg and Umbach in an LRT framework to test. The model with combined maternal-fetal genotype effects is favored (log-likelihood $= -719.4$, $P = .023$) over the model with just maternal and fetal genotype main effects. It implies that fetal genotype effects might contribute to PTD by interacting with maternal genotype effects. This kind of combined maternal-fetal genotype effects can be viewed as a special maternal-fetal gene-gene interaction [36]. Pregnancy is a complicated biologic course, and there is interplay between the mother and the fetus. The mother needs to employ immune-privileged processes to maintain a maternal-fetal balance, while the fetus needs to survive immune rejection. Regarding the result presented in Table 5, one may find that risk of PTD could not be predicted just simply by number of copies carried by the mother and/or the child. It is far more complicated. Combination of maternal-fetal genotypes, that is maternal-fetal genotype incompatibility, deserves more of our attention as a potential source of risk.

There are limitations in our study. First, although we have enrolled a moderate sample size of triads, relative small numbers of informative triads might influence the precise of our results. Zero counts of cells may undermine the power of LEM. This limitation partly resulted from low frequency of the variant allele in our study population. Variant allele frequency varies remarkably among different races. Since there are few large sample size studies which can provide reliable information of *TNF-α* gene −308A allele frequency in Han Chinese, further studies are warranted to confirm our results. Another limitation is that there was mating asymmetry in our data. This limitation might be somehow relevant to the first one. Under mating asymmetry, the standard MFG test [19] cannot be applied to our study. However the hybrid design could be modified to include MFG incompatibility and saved our study by providing a still-valid analytic framework for inference. Finally, PTD is likely to be etiologically heterogeneous. Although we enrolled only spontaneous PTD, we couldn't specify detailed types of PTD due to practical reasons. This aspect should be improved in future. Since we were interested in *TNF-α* which is a proinflammatory cytokine, and chorioamnionitis presents to most PTD, the influence of heterogeneity within the case group may be mild. No population stratification may also lessen this limitation.

5. Conclusion

In this study, we used a hybrid design and ideas of the MFG test studying maternal-fetal genotype incompatibility, to explore association of *TNF-α* G308A polymorphism with PTD. For the first time, as far as we know, we have reported an association of combined maternal-fetal *TNF-α* gene G308A genotypes with PTD. This association was consistent and significant. Besides, G308A transition resulted in relatively higher risk of PTD for those who are

homozygous A/A, compared to those who are heterozygous A/G. Combined maternal-fetal genotype GA/GA showed the most reduced risk of PTD. But since there were zero counts in our data, the results should be interpreted with caution and our findings need to be confirmed by similar studies.

Understanding the interaction between maternal and fetal genes is important when studying genetic factors of perinatal diseases. Of special interest, the statistical methods we used here are robust to the potential confounding that can occur when investigating this interaction and use both family-based data and population-based data. There are few studies like ours, and so our study is of importance despite the previously mentioned limitations.

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