Evolutionary Triangulation to Refine Genetic Association Studies of Spontaneous Preterm Birth

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Abstract

Objective The objective of this study was to apply evolutionary triangulation, a novel technique exploiting evolutionary differentiation among three populations with variable disease prevalence, to spontaneous preterm birth (PTB) genetic association studies.

Study Design Single nucleotide polymorphism (SNP) allele frequency data were obtained from HapMap for CEU, GIH/MEX, and YRI/ASW populations. Evolutionary triangulation SNPs, then genes, were selected according to the overlaps of genetic population differences (CEU = outlier). Evolutionary triangulation genes were then compared with three PTB gene lists: (1) top maternal and fetal genes from a large genome-wide association study of PTB, (2) 640 genes from the database for PTB, and (3) 118 genes from a recent systematic review. Empirical *p*-values were calculated to determine whether evolutionary triangulation enriched for putative PTB associating genes compared with randomly selected sample genes.

Results Evolutionary triangulation identified 5/17 maternal genes and 8/16 fetal genes from PTB gene list 1. From list 2, 79/640 were identified by CEU_GIH_YRI evolutionary triangulation, and 57/640 were identified by CEU_ASW_MEX evolutionary triangulation. Finally, 20/118 genes were identified by evolutionary triangulation from gene list 3. For all analyses, $p < 0.001$ except CEU_ASW_MEX analysis of list 3 where $p = 0.002$.

Conclusion Genes identified in prior PTB association studies confirmed by evolutionary triangulation should be prioritized for further genetic prematurity research.

Keywords
- preterm birth
- evolutionary triangulation
- genetic
- susceptibility
- racial disparity

Preterm birth (PTB) remains a major public health problem worldwide,[1] and is the leading cause of neonatal morbidity and mortality of nonanomalous infants in the United States.[2,3] Approximately two-thirds of PTB are spontaneous, and though there appears to be a genetic component to spontaneous PTB susceptibility, genetic association studies have traditionally yielded inconsistent results and have been difficult to replicate. Spontaneous PTB is known to vary by population, and significant differences are seen in PTB rates across the world. Though sociodemographic factors can account for some of the differences in spontaneous PTB rates, it cannot account for all differences. Variations in rates of prematurity by population are also seen within countries, including the United States, where disparities are pronounced. Non-Hispanic black women have a rate of spontaneous PTB that is 49% higher than non-Hispanic white women.[3,4] Hispanic women also have an elevated rate of spontaneous PTB compared with non-Hispanic white women.

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Genetic variation (e.g., allele frequencies at specific single nucleotide polymorphisms [SNPs]) is known to be population specific. All populations have unique genetic variation inherent to each group. For example, in general, non-Hispanic black populations are known to have more variation and many more low-frequency variation compared with other populations. Across the genome, though some regions are highly conserved with minimal variations between individual and between populations, at other sites, minor allele frequencies have the potential to vary between individuals and between populations.

Evolutionary triangulation is a novel genetic filtering and prioritization method that capitalizes on the differences in allele frequencies between populations to refine results from genetic association studies.[5] The technique identifies alleles that differ among populations with different disease prevalence, such as spontaneous PTB. It has previously successfully identified genes implicated in lactose intolerance, Smith–Lemli–Opitz, and albinism.[5]

We hypothesized that genes identified by evolutionary triangulation could refine results from previous spontaneous PTB gene association studies.

Materials and Methods

First, we generated our evolutionary triangulation gene lists. We obtained baseline SNP allele frequency data for populations selected to represent non-Hispanic black, non-Hispanic white, and Hispanic women using data from the International Haplotype Map Project HapMap[6,7] (►**Table 1**). Each population was chosen based on population differences in rates of PTB. HapMap is an international consortium with publically available SNP frequencies, genotypes, and haplotypes by population, and can be applied to estimate genetic ancestry.[7–9] Next, to assess population differences, we calculated Wright's fixation index (F_{ST}),[10,11] a metric assessing population genetic differences by pairwise allele comparisons between groups, according to the Cockerham and Weir's formula.[12] We first compared alleles between the first two populations, and then compared the allelic differences between populations 1 and 3. Finally, we compared differences between populations 2 and 3. A list of evolutionary triangulation SNPs was then generated according to the overlaps of low F_{ST} between the populations with similarly high rates of spontaneous PTB (YRI, GIH, MEX, and ASW) and high F_{ST} with CEU as the outlier population with lower rates of spontaneous PTB. We used several thresholds of high and low F_{ST}, that is, several degrees of differentiation. Each evolutionary triangulation SNP was then used to identify nearby genes as evolutionary triangulation genes, as recent literature suggests that variants in the regulatory regions or near transcription start sites of genes may impact function and possibly disease. For that reason, genes located within 100 kb of each evolutionary triangulation SNP were compiled to comprise the final evolutionary triangulation gene list. This methodology is summarized in ►**Fig. 1**. We generated two separate evolutionary triangulation SNP lists, each containing a representative non-Hispanic white, Hispanic, and non-Hispanic black populations: (1) CEU_GIH_YRI and (2) CEU_MEX_ASW.

Next, we compared the evolutionary triangulation gene lists generated earlier to three separate gene lists. Spontaneous PTB gene list 1 comprised the top 20 maternal and fetal SNPs from a multicenter genome-wide association study of 1,025 spontaneous PTB cases delivering less than 34 weeks' gestation and 1,015 matched term controls with spontaneous labor; these top 20 SNPs were located in 32 different genes.[13] Spontaneous PTB gene list 2 consisted of 640 genes on the online database for PTB "dbPTB" Web site (www.ptbdb.cs.brown.edu).[14,15] This Web site, hosted by Brown University, provides a web-based aggregation site of published genes previously associated with spontaneous PTB in one or more studies.[14,15] Our third spontaneous PTB gene list comprised the 118 candidate genes identified from a recent systematic review of 92 genetic studies of spontaneous PTB published from 2007 to 2015.[16]

Finally, we applied principles of evolutionary triangulation to each gene list. Genes found in both our evolutionary triangulation gene list and the spontaneous PTB gene lists were considered top candidate genes, and were examined for evidence of expression in the myometrium or placenta. We also analyzed genes identified by evolutionary triangulation using the STRING database (http://string-db.org)[17] to assess functional relationships, interactions between genes, and to classify genes into ontology groups as applicable.

Permutation testing was performed to determine whether evolutionary triangulation significantly enriched in the lists of genes associated with spontaneous PTB. Specifically, this was performed by calculating empirical *p*-values, comparing the ability of evolutionary triangulation to detect putative spontaneous PTB-associated genes from a list of randomly sample genes. We first generated a null (random) list of genes by sampling from the list of genes in the entire genome 10,000 times. For each resample, we

Table 1 Selected populations used to generate evolutionary triangulation gene lists

Population	Source from HapMap	Representative population
CEU	Utah residents with European ancestry	Non-Hispanic white
GIH	Gujarati Indians (Houston, TX)	Hispanic
MEX	Mexican ancestry (Los Angeles, CA)	Hispanic
YRI	Yoruba (Ibadan, Nigeria)	Non-Hispanic black
ASW	African ancestry in southwestern United States	Non-Hispanic black

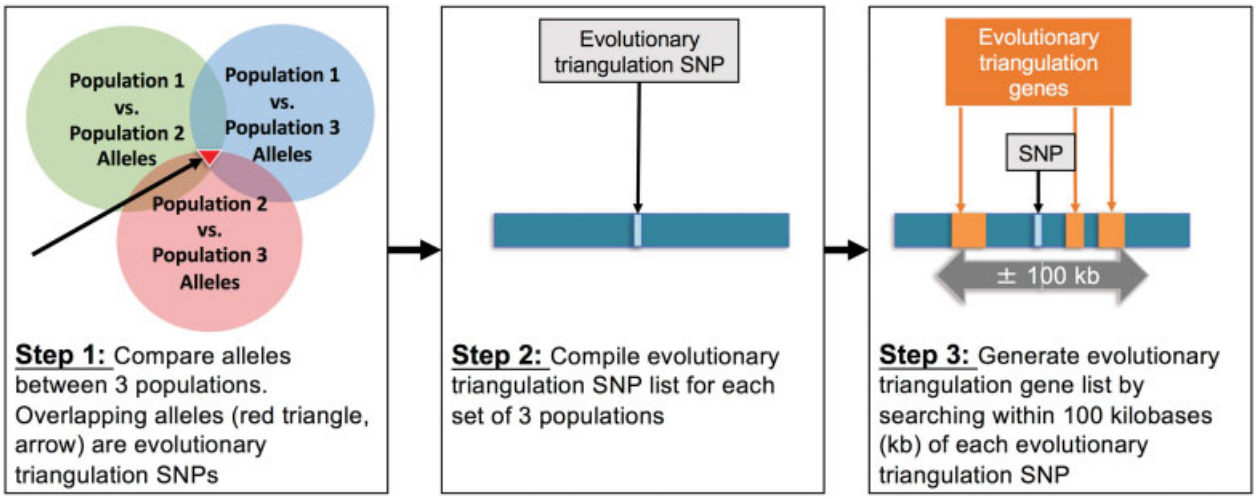

Fig. 1 Summary of evolutionary triangulation methodology. SNP, single nucleotide polymorphism.

randomly selected the number of genes corresponding to each of the three gene lists (32, 640, and 118, respectively). We then counted the number of genes from each resampling that appeared in each of the three lists. From these counts, we generated three null distribution lists of spontaneous PTB genes. Empirical *p*-values were calculated by dividing the number of counts that exceed evolutionary triangulation detected PTB genes in CEU_GIH_YRI and CEU_MEX_ASW separately and CEU_GIH_YRI and CEU_MEX_ASW combined.

Results

We examined both maternal and fetal SNPs distributed in 32 different genes from the large genome-wide association study of early spontaneous PTB (PTB gene list 1). Multiple top genes in the genome-wide association study were confirmed by evolutionary triangulation filtering methods, using both evolutionary triangulation population gene lists. In total, 13 of the 32 genes were identified using evolutionary triangulation. The enrichment for spontaneous PTB genes from list 1 was significant (empiric $p < 0.0001$ for CEU_GIH_YRI; $p < 0.0001$ for CEU_MEX_ASW; $p < 0.0001$ for combined CEU_GIH_YRI and CEU_MEX_ASW). These results included three maternal genes expressed in the myometrium [myopalladin [*MYPN*], ethanolamine kinase 1 [*ETNK1*], contactin 5 [*CNTN5*]) as well as six fetal genes expressed in the placenta (ribonuclease T2 [*RNASET2*], SMAD family member 9 [*SMAD9*], ras responsive element-binding protein 1 [*RREB1*], sortilin-related receptor 1 [SORL1], potassium voltage-gated channel subfamily H member 7 [*KCNH7*], and nucleolar protein 10 [*NOL10*]), ►**Table 2**. These nine significant genes were not overrepresented in any gene ontology (GO) groups and no functional enrichment was appreciated.

We then evaluated PTB gene list 2, the 640 genes listed in the online dbPTB. In total, evolutionary triangulation identified 123 unique genes from the online dbPTB; 19% overall. This number represented significant enrichment as compared with random sampling of the genome (empiric $p < 0.0001$ for CEU_GIH_YRI; $p < 0.0001$ for CEU_MEX_ASW; $p < 0.0001$ for combined CEU_GIH_YRI and CEU_MEX_ASW). Overall, the CEU_GIH_YRI evolutionary triangulation gene list identified a higher proportion of genes from the online dbPTB (77/640, 12.0%) compared with the CEU_ASW_MEX list (55/640, 8.6%). These 123 genes were analyzed by STRING for evidence of biologic pathway enrichment. Notably, 44 genes were implicated in response to stress pathways (GO:0006950), 25 genes in immune response (GO:006955), 20 in innate immunity (GO:0045087), and 17 in positive regulation of the immune system process pathways (GO:0002684). Eleven genes from the online dbPTB were identified by both the CEU_GIH_YRI and the CEU_ASW_MEX list; their names and previous associations with PTB and other pregnancy complications are shown in ►**Table 3**. From these top 11 genes identified in both evolutionary triangulation lists, functional enrichments were found within receptor signaling protein (GO:0005057; kinase insert domain receptor [*KDR*], anaplastic lymphoma receptor tyrosine kinase [*ALK*], interleukin 1 receptor-associated kinase 1 [*IRAK1*]) and nuclear factor-κB–inducing kinase activity (GO:0004704; *ALK*, *IRAK1*) pathways.

Finally, we found 20/118 candidate genes from spontaneous PTB gene list 3 by evolutionary triangulation (►**Table 4**). These associations for spontaneous PTB list 3 were also significant (empiric $p < 0.001$ for CEU_GIH_YRI; $p = 0.002$ for CEU_MEX_ASW; $p < 0.001$ for combined CEU_GIH_YRI and CEU_MEX_ASW). Of these, 10 (interleukin 1 receptor type 2 [*IL1R2*], nitric oxide synthase 2 [*NOS2*], FMS-related tyrosine kinase 1 [*FLT1*], interleukin-6 [*IL6*], *KDR*, colony-stimulating factor 2 [*CSF2*], Sp3 transcription factor [*SP3*], *IRAK1*, toll-like receptor 10 [*TLR10*], protein kinase c α (*PRKCA*]) are genes previously implicated in innate immunity (GO:0002376) or the immune response (GO:0006955). In addition, eight genes (angiotensinogen [*AGT*], *FLT1*, nitric oxide synthase 3 [*NOS3*], *PRKCA*, *IL6*, *KDR*, collagen type IV α 3 chain [*COL4A3*], and collagen type IV α 2 chain [*COL4A2*]) are involved in the regulation of angiogenesis (GO:00045765).

Table 2 Genes from PTB gene list 1 confirmed by evolutionary triangulation

Gene	Chr.	Gene description	Originally published findings		Evolutionary triangulation list	
			Location	*p*-Value	CEU, GIH, YRI	CEU, ASW, MEX
SHROOM3	4	Shroom family member 3	Maternal	$5.6e^{-6}$	Yes	No
LOC100128865	4	Methyltransferase like 5	Maternal	$2.7e^{-5}$	Yes	Yes
MYPN	10	Myopalladin	Maternal	$3.3e^{-5}$	Yes	No
ETNK1	12	Ethanolamine kinase 1	Maternal	$3.7e^{-5}$	Yes	No
CNTN5	11	Contactin 5	Maternal	$4.1e^{-5}$	Yes	No
LOC100128365	6	Interferon-stimulated exonuclease	Fetal	$2.7e^{-12}$	Yes	No
RNASET2	6	Ribonuclease T2	Fetal	$1.4e^{-10}$	No	Yes
L3MBTL3	6	Lethal(3) malignant brain tumor-like protein 3	Fetal	$8.3e^{-7}$	Yes	No
SMAD9	13	SMAD family member 9	Fetal	$1.1e^{-6}$	No	Yes
RREB1	6	Ras responsive element-binding protein 1	Fetal	$2.3e^{-6}$	Yes	No
SORL1	11	Sortilin-related receptor 1	Fetal	$2.8e^{-6}$	Yes	No
KCNH7	2	Potassium voltage gated channel subfamily H member 7	Fetal	$6.2e^{-6}$	No	Yes
NOL10	2	Nucleolar protein 10	Fetal	$6.4e^{-6}$	Yes	No

Abbreviations: Chr, chromosome; PTB, preterm birth.

Table 3 Genes from PTB list 2 (dbPTB) identified by both CEU_GIH_YRI and CEU_ASW_MEX evolutionary triangulation lists

Gene	Chr.	Gene description	Prior association(s) with PTB and other adverse pregnancy outcomes
ALK	2	Anaplastic lymphoma receptor tyrosine kinase	Peripheral blood gene expression in early pregnancy associated with PTB[25]
CNTLN	9	Centlein, centrosomal protein	Peripheral blood gene expression in early pregnancy associated with PTB[25]
IRAK1	X	Interleukin 1 receptor-associated kinase 1	Increased in response to intrauterine inflammation[26]
KCNK2	1	Potassium channel, subfamily K, member 2	Maintenance of uterine quiescence[27]
KDR	4	Kinase insert domain receptor	Some genotypes associated with higher PTB risk among overweight or obese Caucasian women[28]; reduced expression in gestational hypertension, small for gestational age, and PTB[29]
KLHL2	4	Kelch-like 2	Not applicable
NAA10	X	N(α)-acetyltransferase 10, NatA catalytic subunit	Not applicable
STMN3	20	Stathmin-like 3	Decidualization in murine uterus in early pregnancy[30]; peripheral blood gene expression in early pregnancy associated with PTB[25]
TMEFF2	2	Transmembrane protein with EGF-like and two follistatin-like domains 2	Expression in uterine leiomyomas[31]
WDR90	16	WD repeat domain 90	Peripheral blood gene expression in early pregnancy associated with PTB[25]
XPNPEP1	10	X-prolyl aminopeptidase (aminopeptidase P) 1, soluble	Peripheral blood gene expression in early pregnancy associated with PTB[25]

Abbreviations: Chr, chromosome; dbPTB, database for PTB; EGF, epidermal growth factor; PTB, preterm birth.

Table 4 Genes from preterm birth list 3 identified by either CEU_GIH_YRI or CEU_ASW_MEX evolutionary triangulation lists

Gene	Chr.	Gene description	Other prior association(s) with preterm birth and adverse pregnancy outcomes
AGT	1	Angiotensinogen	Polymorphisms associated with placental hemorrhage[32]
COL4A2	13	Collagen type IV	Increased expression at decidual interface associated with preeclampsia[33]
COL4A3	2	Collagen type IV	Neonatal respiratory distress syndrome[34]
COL4A4	2	Collagen type IV	Not applicable
CSF2	5	Colony-stimulating factor 2	Not applicable
CYP1A1	15	Cytochrome P4501A1	Genotype associated with preterm birth among women exposed to passive cigarette smoke[35]
NOS3	7	Endothelial nitric oxide synthases	Not applicable
FLT1	13	FMS-like tyrosine kinase 1	Genotype associated with spontaneous preterm birth $<$ 34 wk in African American women[36]
FSHR	2	Follicle-stimulating hormone receptor	Multiple haplotypes, polymorphisms associated with preterm birth[24,37]
GSTM1	1	Glutathione S-transferase mu 1	Genotype associated with preterm birth[38] particularly in the setting of high air pollution[39]
NOS2	17	Inducible nitric oxide synthases	Fetal genotype associated with prematurity[40]
IL1R2	2	Interleukin 1 receptor 2	Genotypes modify risk for preterm birth[41]
IL6	7	Interleukin 6	Cytokine levels,[42] genotype[43] associated with preterm birth, and cervical insufficiency in multiple studies
IRAK1	X	Interleukin 1 receptor-associated kinase 1	Increases in response to uterine inflammation[26,44]
KDR	4	Kinase insert domain receptor	Differential methylation and expression in preeclampsia[45]
PTGDR	14	Prostanoid DP receptor	Genotype associated with postcoital associated preterm birth[46]
PRKCA	17	Protein kinase C α	Genotype associated with preterm birth in African American women[36]
KCNN3	1	Small conductance calcium-activated potassium channel 3	Maternal[47] and fetal[48] genotype associated with preterm birth
SP3	2	Specificity protein 3	Overexpressed in maternal blood in early pregnancy among women destined to deliver preterm[25]
TLR10	4	Toll-like receptor 10	Fetal membrane response to inflammation[49]

Abbreviation: Chr, chromosome.

Comment

We found that applying evolutionary triangulation analysis to spontaneous PTB provided independent support for multiple genes previously associated with disease in separate genome-wide association and candidate gene studies. Therefore, evolutionary triangulation presents an alternative filtering metric for genetic analyses based on evolutionary history. Multiple identified genes have biologic plausibility for spontaneous PTB and have been previously associated with spontaneous PTB in at least one study, as shown in ►**Tables 3** and **4**. These genes fall into a variety of broad functional categories and classifications theorized to contribute to a predisposition to spontaneous PTB.

Though multiple genes and pathways were identified, it is notable that both spontaneous PTB gene lists 2 and 3 contained multiple genes within key immune response regulatory pathways. Specifically, the immune response gene ontology framework (GO:0006955) was represented by significant results in both lists 2 and 3. Exposure to (and maternal and fetal response to) inflammation and infection have long been implicated in the pathogenesis of spontaneous PTB.[18] IL6 and other cytokine gene genotypes,[19,20] antimicrobial immunity,[21] and the composition of the vaginal microbiome[22] are known to vary by race and ethnicity[23]; these factors are hypothesized to contribute to some of the racial disparity in spontaneous PTB. These data provide additional support, at the evolutionary level, for the theory that inflammatory pathways contribute to spontaneous PTB.

Other researchers have used evolutionary approaches to examine the genetic contribution to spontaneous PTB. In 2011, Plunkett et al used a phylogenetic approach to identify candidate genes along the human and human–chimpanzee ancestor lineages.[24] Eight out of the top 10 SNP differences were found within the follicle-stimulating hormone receptor (*FSHR*) gene.[24] Notably, the *FSHR* gene was on list 3 and was confirmed by our evolutionary triangulation methodology. The *FSHR* gene was not in the dbPTB and therefore not evaluated as a part of list 2.

Our study should be interpreted with several limitations in mind. Evolutionary triangulation and this analysis is limited by the unknown degree of similarity between the selected populations and the non-Hispanic white, Hispanic, and non-Hispanic black populations used to generate the spontaneous PTB gene lists we examined. HapMap populations, by design, are generally less diverse in comparison to a random sampling of pregnant women at risk for PTB in the United States. Because of the uncertainty regarding which HapMap populations might best represent the admixed Hispanic and non-Hispanic black populations within the United States, we generated two separate evolutionary triangulation gene lists (CEU_GIH_YRI and CEU_ASW_MEX). It is possible that other combinations of HapMap populations may more appropriately estimate the populations studied. The small number of genes analyzed from list 1 likely limited our ability to find statistically significant functional enrichment within this list. However, our focus on gene lists derived from large, multicenter studies or aggregated genetic data minimizes the likelihood that isolated differences local ancestry would limit our results or generalizability. In addition, the literature curation is current for the dbPTB Web site only through July 2013. Recent findings published over the past 4 years could therefore not be included in a systematic manner as they are unavailable online.[14,15] For that reason, we incorporated the spontaneous PTB gene list by Sheikh et al,[16] to provide more a contemporaneous gene list.

This study also had several strengths. Application of a novel genetic filtering technique provides additional "verification" of genes or single nucleotide polymorphisms that were marginally significant in previous prematurity studies, and provide a much-needed focus for future investigations given the complex nature of this phenotype. Further, these results present additional proof of concept and suggest that the filtering methodology of evolutionary triangulation may be applied to other disorders of pregnancy that disproportionately affect different populations of women (e.g., preeclampsia or gestational diabetes).

In conclusion, the application of evolutionary triangulation analysis—a novel filtering metric based on evolutionary history—to spontaneous PTB provided independent support for multiple genes previously associated with disease in genome-wide association and candidate gene studies. Identification of genes from prior spontaneous PTB genetic association studies through evolutionary triangulation filtering highlights a prioritized list of genes for future investigations of prematurity.

Condensation

Evolutionary triangulation, a novel bioinformatics approach, provides independent support for multiple genes previously associated with PTB and presents an alternate filtering metric for genetic analyses using evolutionary history.

Note

This study was presented in part at the Society for Maternal Fetal Medicine's 37th Annual Meeting 2017 (Las Vegas, NV), as an oral concurrent presentation (1/26/17), final abstract ID #11.

Conflict of Interest

None.

Acknowledgments

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