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Matrix Metalloproteinase-9 Genetic Polymorphisms and the Risk for Advanced Pelvic Organ Prolapse

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Abstract

Objective—Matrix metalloproteinase-9 (MMP-9) is a protease associated with degradation of collagen and elastin. Because increased MMP-9 activity in vaginal tissue has been associated with pelvic organ prolapse, we sought to comprehensively estimate *MMP-9* genetic variants and the risk for advanced prolapse.

Methods—This is a candidate gene association study of women with stage III-IV prolapse (cases, n=239) and women with stage 0-1 prolapse (controls, n=197). We attempted to oversample “extreme” phenotypes, including younger women with severe prolapse and older women without prolapse, in an attempt to concentrate the genetic effect. We utilized a linkage disequilibrium tagged approach to identify single nucleotide polymorphisms (SNPs) in *MMP-9* to evaluate in our study. In order to minimize potential confounding by race, our analysis focused on non-Hispanic white women. We performed multivariable logistic regression to estimate the association between *MMP-9* SNPs and case-control status, adjusting for age and vaginal parity.

Results—Women with advanced prolapse were slightly younger (64.8 ± 10.3 vs 69.0 ± 10.2 years, $p < 0.001$) and more likely to have had one or more vaginal deliveries (96.6% vs 82.2%, $p < 0.001$) when compared to controls. Eight SNPs were assessed, which represented 93% coverage of the *MMP-9* gene. Of these, two were associated with advanced prolapse: 1) rs3918253 (adjusted OR 0.64 [95% CI 0.41, 1.0], $p = 0.05$) and 2) rs3918256 (adjusted OR 0.64 [95% CI 0.41, 1.01], $p = 0.05$).

Conclusions—Matrix metalloproteinase-9 (*MMP-9*) is a biologically-plausible candidate gene for pelvic organ prolapse given our results.

INTRODUCTION

Pelvic organ prolapse is a major public health burden for women given its high prevalence, (1) economic cost of over \$1 billion annually,(2) and impairment in quality of life and body

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image.(3) Symptomatic prolapse can be managed surgically; however, the recurrence rate is as high as 30%.(4) Currently, the underlying pathophysiology of prolapse is not well understood, and as a result, effective long-term treatment options are limited. Given the tremendous public health effects of this disease, it is critical to identify risk factors for prolapse, which would enable physicians to risk-stratify patients.

While the etiology of pelvic organ prolapse is likely multifactorial, a growing body of evidence supports a genetic predisposition for prolapse. Epidemiologic data suggest that a family history of prolapse is a significant risk factor.(5, 6) Several genetic epidemiologic studies have also identified potential candidate genes associated with prolapse,(7-9) and a recent genome wide association study of familial cases of prolapse reported six loci associated with advanced disease.(10)

Matrix metalloproteinase-9 (*MMP-9*) is a possible candidate gene given its biologic plausibility and data from preliminary studies. *MMP-9* is a protease that has been associated with the degradation of both collagen and elastin in the extracellular matrix.(11) A recent study by Budatha et al. showed that the genesis of prolapse in a mouse model is related to a relationship between *MMP-9* and fibulin-5, a protein critical to the development of normal elastic fibers.(12) They found that 96% of mice with knockout of fibulin-5 developed prolapse due to an increase in *MMP-9* activity and impaired elastogenesis. In humans, several studies have shown that *MMP-9* activity is higher in the vaginal tissue of women with prolapse compared to those without prolapse.(12-14)

Limited data exist regarding the impact of genetic variants in the gene encoding the *MMP-9* protease and risk of pelvic organ prolapse in humans. Chen et al. evaluated three single nucleotide polymorphisms (SNP) in the *MMP-9* gene in 92 Taiwanese women with prolapse and 152 controls and found that the SNP rs17576 was associated with prolapse.(9) Given this promising finding in Asian women and the biologic role that *MMP-9* protease may play in the pathogenesis of prolapse, the *MMP-9* gene is certainly an important susceptibility gene to evaluate for prolapse. Thus, the objective of our study was to estimate *MMP-9* genetic polymorphisms and the risk for advanced prolapse in a case-control association study of non-Hispanic white women.

MATERIALS & METHODS

Study population

We recruited subjects from the Division of Urogynecology at a single academic institution from February 2009 to December 2010 after Duke University institutional review board approval. For this case control association genetic study, cases were defined as women with stage III-IV prolapse based on the Pelvic Organ Prolapse Quantification (POP-Q) examination, and controls were women with stage 0-I prolapse and no prior history of prolapse surgery.(15) We excluded pregnant women, women with a gynecologic malignancy, or those with connective tissue diseases, including rheumatoid arthritis, systemic lupus erythematosus, scleroderma, polymyositis, dermatomyositis or Marfan or Ehlers-Danlos syndromes. After obtaining informed consent from all subjects, we collected data regarding sociodemographics, past medical and obstetric history, and physical examination.

Because the objective was to estimate *MMP-9* as a susceptibility gene for advanced prolapse, we enhanced our ability to detect genetic differences between our two groups, by selecting more “extreme” cases and controls. This is a common approach in genetic epidemiologic studies. For example, although we recruited subjects of all ages with advanced prolapse, we preferentially enrolled younger subjects with more advanced disease

in order to select a “high risk” group with a presumably higher genetic load.(16, 17) We also preferentially recruited older women as controls. This is an important point because prolapse is more prevalent with increasing age. If younger controls are selected, it is possible that they are still at-risk for developing prolapse but they have not yet reached a sufficient age to manifest the disease. Despite purposeful selection of cases and controls of different ages, this does not limit the generalizability of the study, as inherent genetic susceptibility should not change based on age. Thus, this approach improves our ability to identify a susceptibility gene while not significantly impacting our external validity.

It is critical to carefully consider race and ethnicity in genetic epidemiologic studies as different racial/ethnic groups may have different frequencies of genetic variants, as well as different linkage disequilibrium structures. In order to avoid population stratification, or confounding by race, we focused on non-Hispanic white women in this study.(18) This decision helped to ensure that our cases and controls were derived from the same source population.(19) By evaluating a specific racial and ethnic group, we minimized the differences in the genetic background of our two groups. For evidence regarding the varying allele frequencies by race/ethnicity for *MMP9* SNPs, one useful resource is dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) which presents detailed information about SNPs, including allele frequencies by race/ethnicity.

Genotyping and SNP selection

After genomic DNA was extracted from frozen stored peripheral venous whole blood, we used a linkage disequilibrium (LD) tagged SNP approach in order to accomplish a more comprehensive evaluation of the *MMP-9* gene. A tagged SNP approach takes advantage of the fact that most SNPs are in linkage disequilibrium (i.e. genetically linked and thus correlated) with other SNPs.(20-22) Because of LD, there are groups of SNPs within each gene that are highly correlated with each other. Representative SNPs from each group (i.e. tagged SNPs) can be evaluated, thereby representing a detailed evaluation of the gene without having to genotype each SNP. Thus, a tagged SNP approach is a common methodology in candidate gene studies, as it is comprehensive as well as time- and cost-efficient. The Tagger subsection of Haploview 4.2 software (Broad Institute, Cambridge, MA)(23) was utilized to identify tagged SNPs in *MMP-9* as well as in the 5 kilobases (kb) upstream and downstream of the gene. In our search criteria, we used a “correlation” (r^2) of > 0.8 and SNPs with a minor allele frequency (MAF) of > 0.05 . We also included known and putative functional coding SNPs (i.e. SNPs that result in changes at the amino acid level and thus result in alterations in quantitative or qualitative protein function) with a MAF > 0.05 regardless of LD.

Genotyping was performed using the 7900 HT TaqMan™ system, which uses a standard PCR-based, dual fluorescence, allele discrimination assay with a dual laser scanner. Assays by Design and Assays on Demand were purchased through Applied Biosystems (Applied Biosystems, Foster City, CA). Quality control (QC) samples, composed of 12 reference controls, were included in each quadrant of the plate. All SNPs examined were successfully genotyped for 98% of the individuals in the study. Error rate estimates for SNPs meeting QC benchmarks were 0.2%.

Statistical analysis

In order to compare the sociodemographic data and physical examination findings for cases and controls, a chi-square test was used for categorical variables and a Student's t-test was used for continuous variables. A two-sided alpha of 0.05 was considered significant. We used a chi-square statistic to test for deviation from Hardy-Weinberg equilibrium (HWE) for

all SNPs. Genotype clusters and genotyping calling for SNPs that were significantly out of HWE were re-reviewed, and the genotyping accuracy was confirmed.

In order to test for association of single SNPs with advanced prolapse, we constructed logistic regression models and evaluated both unadjusted and adjusted models, using both dominant and additive models of genetic risk. The additive genetic model considers the risk allele to act in an additive fashion, i.e. two risk alleles confers a larger effect than one risk allele, and one risk allele confers risk over carrying no alleles. The dominant model tests for whether there is increased risk associated with carrying just one allele, with the assumption that carrying two alleles does not confer any additional risk over carrying one allele. In dominant analyses, we group heterozygotes with homozygotes of the minor allele, and compare these individuals to homozygote carriers of the major allele. A dominant model biologically suggests that carrying one minor (risk) allele is detrimental enough by itself to confer risk of disease. For example, the minor allele causes a quantitative or qualitative deficit in a protein that was vital and necessary for a normal phenotype. In these multivariate models, we adjusted for age and vaginal parity (dichotomous variable regarding any prior vaginal deliveries). For these regression models, we report the OR for the minor allele. Thus, an OR < 1.0 suggests a protective effect of the minor allele, and an OR > 1.0 suggests that the minor allele is the risk allele.

We utilized the QUANTO program (Gauderman and Morrison, <http://hydra.usc.edu/gxe>) for our power calculation, which was conducted for a non-familial case-control association.(24) We considered a range of allele frequencies of 0.05 – 0.5, varying effect sizes, and both dominant and additive models. While these calculations are based on model parameters that we cannot know with certainty, we can estimate a sense of the expected power for the proposed sample sizes. Given a sample size of 150 cases and 150 controls, we calculated >80% power to detect an effect size of approximately 2.0 and higher for most allele frequencies and genetic models at an $\alpha=0.05$. We felt that an effect size of 2.0 is a clinically meaningful difference to detect, and that it represented a balance between clinical/biological significance and feasibility. All statistical analyses were conducted with SAS version 9.2 (Cary, NC) unless otherwise noted.

RESULTS

Of a total of 436 non-Hispanic white women, 239 were cases with advanced prolapse and 197 were controls with no or minimal prolapse. Women with advanced prolapse were slightly younger (64.8 ± 10.3 vs 69.0 ± 10.2 years, $p<0.001$) and were more likely to have had 1 vaginal delivery (96.6% vs 82.2% , $p<0.001$) when compared to controls (Table 1). Our two cohorts were similar in Charlson comorbidity index scores,(25) smoking status, and body mass index (27.8 ± 5.1 vs 28.5 ± 6.3 kg/m², $p=0.18$). A majority of cases had stage III prolapse (94.6%) and 5.4% had stage IV prolapse. For controls, 57.4% had stage 0 prolapse and 42.6% had stage I prolapse.

We genotyped 8 SNPs based on our LD tagging selection. Figure 1 is a schematic of the *MMP-9* gene structure, which indicates exons, introns, sites of translation initiation and termination. The genotyped SNPs are marked on the schematic with descriptions of the different types of SNPs, whether nonsynonymous coding or intronic. All SNPs were in Hardy Weinberg equilibrium. At an r^2 of 0.80, the 8 SNPs evaluated provided 93% coverage of the variability within the gene including 5 kb upstream and downstream of *MMP-9*.

In this cohort of non-Hispanic white women, there were two SNPs associated with advanced prolapse in the dominant adjusted model: rs3918253, an intronic SNP (adjusted OR 0.64 [95% CI 0.41, 1.00], $p=0.05$) and rs3918256, an intronic SNP (adjusted OR 0.64 [95% CI

0.41, 1.01], $p=0.05$) (Table 2). Given $OR < 1.0$ for both of these SNPs, these results suggest that the minor allele is protective. It is also important to note that these two SNPs were in perfect linkage disequilibrium, or perfectly correlated, with each other ($r^2 = 1.0$) (Figure 1).

Regarding *MMP-9* SNP rs17576, which was associated with prolapse in Taiwanese women, this SNP was not associated with advanced prolapse in our cohort of non-Hispanic white women in either the dominant or additive adjusted models (OR 0.74 (95% CI 0.48, 1.14, $p=0.17$) and OR 0.81 (95% CI 0.59, 1.09, $p=0.17$), respectively) (Table 2). In this analysis, the remaining *MMP-9* SNPs were not significantly associated with advanced prolapse.

DISCUSSION

In this novel analysis of *MMP-9* genetic polymorphisms, we found two single nucleotide polymorphisms that were associated with advanced pelvic organ prolapse in a cohort of non-Hispanic white women. We utilized a candidate gene approach, selecting *MMP-9* as a candidate gene to evaluate given its potential biologic role in the pathogenesis of prolapse. (12-14) Importantly, we employed a linkage equilibrium tagged-SNP approach to comprehensively evaluate the gene. The significance of identifying SNPs associated with advanced prolapse is that these SNPs provide insight into the potential genetic location that is associated with disease susceptibility and could represent genetic biomarkers that could be used in risk stratification.

Limited genetic epidemiologic data exist regarding *MMP-9* and advanced prolapse, especially in a non-Hispanic white population. Chen et al evaluated *MMP-9* genetic variants in a case control association study of Taiwanese women.(9) They evaluated three SNPs, rs3918242, rs17576, and rs2250889 in 92 women with stage II prolapse and 152 controls with stage 0-I prolapse. They found that the G allele for SNP rs17576, a coding missense SNP, was the at-risk allele for prolapse. Although our two suggestive SNPs are in partial LD with rs17576, rs17576 did not show statistical significance in our study. In addition, there are conflicting reports about the directionality and functionality of this SNP in other disorders,(26, 27) which may be related to marked variation in the MAF depending on ethnicity.(28) Future studies will be necessary to assess whether our two SNPs are independently reporting on prolapse or whether they are serving as surrogates for rs17576.

The association of *MMP-9* genetic variants with advance prolapse in this genetic epidemiologic study provides additional support regarding the role of *MMP-9* in the pathogenesis of pelvic organ prolapse. A recent study by Budatha et al. evaluated the risk of prolapse in a mouse model based on the interaction between *MMP-9* and fibulin-5, a protein essential for the assembly of elastic fibers.(12) They found that when fibulin-5 and *MMP-9* were expressed at normal levels, prolapse does not occur. However, when fibulin-5 was absent which led to abnormal elastic fibers, *and* *MMP-9* activity was increased, 96% of mice developed prolapse. When fibulin-5 was absent *and* there was no increase in *MMP-9* activity, only 40% of mice developed prolapse. Their findings suggest the importance of both abnormal elastic fiber synthesis and increased *MMP-9* activity in the pathogenesis of prolapse.(29, 30) Several studies in humans have also supported the role of *MMP-9*. These studies have compared *MMP-9* activity in the vaginal tissue of women with prolapse compared to those without prolapse and have found increased *MMP-9* activity associated with disease.(13, 14, 31, 32) While the association of increased *MMP-9* activity and prolapse cannot confirm the causative role of *MMP-9*, as it is possible that elevated *MMP-9* levels may be an end result of prolapse, these preliminary data certainly supports further investigation into *MMP-9* as a potential candidate gene for prolapse.

The strengths of our study include that we performed a comprehensive evaluation of the genetic variation within the *MMP-9* gene by utilizing a tagged-SNP approach. By selecting tagged SNPs that are representative of the genetic variation within the gene, we were able to comprehensively and efficiently conduct this study. In addition, we attempted to concentrate the genetic effect by evaluating more extreme phenotypes. Specifically, our cases were those with advanced prolapse defined as stage III-IV based on the POP-Q examination and our controls were those with stage 0-I. We purposely excluded those women with stage II prolapse.

A potential limitation of our study was that we only evaluated non-Hispanic white women. However, as discussed previously, it is important to avoid confounding by race in genetic epidemiologic studies. Thus, we focused on one racial/ethnic group and are not able to comment on genetic risk factors in other racial and ethnic groups. Certainly, it will be important to evaluate all racial and ethnic groups in future, large scale genetic epidemiologic studies. In this study, we evaluated unrelated individuals. It is possible that there are different genetic risk factors for families with a genetic predisposition for advanced prolapse. Lastly, while we attempted to recruit more extreme phenotypes in this study, we may have not selected an extreme enough phenotype to detect a more significant association. Ideally, all cases would be young, premenopausal and nulliparous while all controls would be elderly postmenopausal women who have had numerous vaginal deliveries. In our study, the proportion of women who had vaginal deliveries was actually higher in our cases than our controls. These issues underscore the importance of careful phenotyping and support future investigations into the best method of phenotyping pelvic organ prolapse.

This study provides additional support for the role of *MMP-9* in the pathogenesis of pelvic organ prolapse. Hopefully, future translational and basic science research efforts will provide additional insight into the etiology of pelvic organ prolapse. We hope that by focusing on the genetic susceptibility for prolapse, we will ultimately be able to risk-stratify women. This has profound clinical implications, as the ability to identify women at risk and prior to development of disease could lead to implementation of prevention strategies and life-style modifications. These interventions could decrease the need for invasive surgery and prevent the reduced quality of life associated with severe, end-stage prolapse.

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Figure 1. *MMP-9* structure, genotyped single nucleotide polymorphisms (SNPs) and linkage disequilibrium between SNPs

The *MMP-9* gene structure is displayed diagrammatically with exons represented as black discs at the top of the diagram. Genotyped SNPs are noted with arrows and the different types of SNPs are listed in the figure legend. The bottom panel illustrates a linkage disequilibrium (LD) graphic with r^2 values (a measure of LD) in each box. A simplified method of describing LD is the degree of correlation between two SNPs. The closer the r^2 is to 1.0, the higher the correlation between two SNPs. In this figure, the r^2 value between 2 SNPs is determined by finding the box at the intersection of the rows for each SNP. For example, the r^2 value is 0.14 between the 1st SNP rs3918278 and the 3rd SNP rs2274755; thus, these 2 SNPs are not highly correlated. The r^2 value for the 3rd SNP rs2274755 and the 5th SNP rs2236416 is 0.98, so these 2 SNPs are highly correlated. The black squares marked "100" indicate perfect LD ($r^2=1.0$). An example of perfect LD is between the second SNP rs3918253 and the sixth SNP rs3918256, where the box at the intersection of these two SNPs is blank.

Table 1

Sociodemographic Characteristics and Prolapse Examination for Cases and Controls

Characteristic	Controls n=197	Cases n=239	P
Prolapse stage			
0	113 (57.4)	0 (0)	
I	84 (42.6)	0 (0)	
II	0 (0)	0 (0)	
III	0 (0)	226 (94.6)	
IV	0 (0)	13 (5.4)	
Age (years)			
Mean	69.0 ± 10.2	64.8 ± 10.3	<0.001
Median	68.5 (62.2, 76.3)	65.6 (57.8, 71.4)	<0.001
Vaginal parity	162 (82.2)	230 (96.6)	<0.001
Charlson comorbidity index	0.48 ± 0.90	0.42 ± 0.83	0.51
Current smoker	8 (4.1)	16 (6.8)	0.26
Body mass index (kg/m ²)	28.5 ± 6.3	27.8 ± 5.1	0.18

Data are mean ± standard deviation or n (%) or median (interquartile range) unless otherwise specified.

Logistic Regression Results for Both the Additive and Dominant Adjusted Models for *MMP-9* Single Nucleotide Polymorphisms, Adjusted for Age and Vaginal Parity

Table 2

SNP	Minor Allele (Frequency)	Dominant Adjusted Model		Additive Adjusted Model	
		OR (95% CI)	P	OR (95% CI)	P
RS3918253	C (0.44)	0.64 (0.41, 1.00)	0.05	0.78 (0.58, 1.05)	0.10
RS3918256	G (0.44)	0.64 (0.41, 1.01)	0.05	0.78 (0.58, 1.05)	0.10
RS3918278	A (0.02)	0.40 (0.14, 1.12)	0.08	0.40 (0.14, 1.12)	0.08
RS17576	G (0.36)	0.74 (0.48, 1.14)	0.17	0.81 (0.59, 1.09)	0.17
RS2274755	T (0.13)	0.78 (0.48, 1.26)	0.31	0.82 (0.53, 1.27)	0.38
RS17577	A (0.13)	0.78 (0.48, 1.26)	0.31	0.82 (0.53, 1.27)	0.38
RS2236416	G (0.14)	0.79 (0.49, 1.28)	0.34	0.83 (0.54, 1.29)	0.41
RS3787268	A (0.23)	0.82 (0.53, 1.25)	0.36	0.89 (0.64, 1.24)	0.48

SNP, single nucleotide polymorphisms; OR, odds ratio; CI, confidence interval.