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Variants in genes belonging to the Fibroblast Growth Factor family are associated with lower extremity amputation in non-Hispanic whites: findings from the Chronic Renal Insufficiency Cohort study

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Abstract

Diabetes is the major risk factor for non-traumatic lower extremity amputation (LEA). The role of genetic polymorphisms in predisposing diabetics to impaired wound healing leading to LEA has not been sufficiently explored. We investigated the association between a set of genes belonging to the angiogenesis/wound repair pathway with LEA in the Chronic Renal Insufficiency Cohort (CRIC), a study of adults with chronic kidney disease (CKD) that includes a subgroup with diabetes. This study was performed on 3772 CRIC participants who were genotyped on the ITMAT-Broad-CARe array (IBC) chip. A total of 1017 single-nucleotide polymorphisms (SNPs) in 22 genes belonging to the angiogenesis/would repair pathway were investigated. LEA was determined from patient self-report. The association between genetic variants and LEA status was examined using logistic regression and additive genetic models after stratifying the cohort by race/

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ethnicity and diabetic status. Unadjusted analyses as well as analyses adjusted for age, sex, estimated glomerular filtration rate (eGFR), body mass index (BMI), peripheral vascular disease (PVD), hemoglobin A1c (HgbA1c) and population stratification were performed. In non-Hispanic white participants with diabetes, **rs11938826** and **rs1960669**, both intronic SNPs in the gene basic fibroblast growth factor-2 (*FGF2*), were significantly associated with LEA in covariate-adjusted analysis (OR: 2.83 (95% CI: 1.73, 4.62); p-value: 0.000034; Bonferroni adjusted p-value: 0.0006) and (OR: 2.61 (95% CI: 1.48, 4.61); p-value: 0.00095; Bonferroni adjusted p-value: 0.02). In the same subgroup, **rs10883688**, an *FGF8* SNP of unknown functional effect, was also associated with LEA (OR: 1.72 (95% Confidence Interval: 1.14, 2.6); p-value: 0.00999; Bonferroni adjusted p-value: 0.04). No statistically significant associations were identified in the other ethnic groups. In conclusion, variant/s in *FGF2* and *FGF8* may predispose diabetics with CKD to LEA. Dysregulation of the *FGF2* gene represents an opportunity to understand further, and possibly intervene upon, mechanisms of wound healing in diabetics with CKD.

Keywords

diabetes; angiogenesis; wound repair

Introduction

In the US, more than 60,000 lower extremity amputations (LEAs) occur each year among people with diabetes with an incidence rate of about 4 per 1000 person years of diabetes(1, 2). It occurs 10 times more frequently in those with diabetes than those without the condition. LEA in people with diabetes is generally associated with yearly costs between $30,000 and $60,000 and a risk of mortality of about 20% per year(3). There are varying reasons that influence the decision to treat with LEA; however, about 90% of those with diabetes who have an LEA have a history of foot ulcers(4–7). About 34% of those who have an LEA will have a second more extensive amputation within 16 weeks of their initial amputation, indicating that those who are treated with an amputation may have a persistent inability to heal(8, 9). However, as with many other complications of diabetes, regardless of the severity or the duration of the disease, not everyone with diabetes will develop a foot ulcer or have an LEA. Several recent studies in the US and UK have shown that the rate of foot ulcer and amputation varies by geographic location, socioeconomic status, gender and race/ethnicity(10–13). As with type II diabetes, it is likely that defective wound healing that ultimately leads to non-healed foot ulcers and LEA is a complex process with both environmental and genetic risk factors.

Normal wound healing proceeds through an orderly sequence of steps that includes the removal of necrotic debris and infection, resolution of inflammation, repair of the connective tissue matrix, neovascularization and resurfacing(14). Chronic wounds are those that have failed to follow this sequence and do not achieve a sustained anatomic and/or functional result(14). Soon after tissue injury, as part of the repair process, devitalized tissue is removed and the repair phase begins as keratinocytes migrate and proliferate to the wound edge; while granulation tissue, which is primarily composed of fibroblasts and endothelial cells, begins to form. Granulation tissue contains excessive neovascular proliferation. This process

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includes the repair, restoration and regeneration of blood vessels; however, the origin of the cells needed for this process is not well understood. Neovascularization of wound granulation tissue occurs by angiogenesis and/or vasculogenesis and this process is at least in part regulated by the cytokines and their receptors that are assumed to be a part of the angiogenesis/wound repair pathway. It follows that variations in genes coding for these cytokines that result in functional changes in the protein could influence wound healing. However, the association between genetic variation and impaired wound healing in subjects with diabetes has been only minimally explored and not in a systematic manner(15). In a recently published report, we demonstrated an association between the Neuronal nitric oxide synthase associated protein (*NOS1AP*) gene and diabetic neuropathy(15). In this study, we further evaluate a set of genes specifically belonging to the angiogenesis/wound repair pathway for their association with LEA in participants of the CRIC study, a longitudinal multi-center prospective study of adults with CKD.

Materials and methods

Study population

The Chronic Renal Insufficiency Cohort study (CRIC) is a multicenter investigation undertaken to pursue the relationship between chronic renal insufficiency and cardiovascular disease(16, 17). The CRIC clinical research centers are located at the University of Pennsylvania, Johns Hopkins University/University of Maryland, Case Western Reserve University, University of Michigan, University of Illinois-Chicago, Tulane University Health Science Center, and Kaiser Permanente of Northern California/University of California at San Francisco. All participants are examined at their local CRIC site each year. Briefly, the yearly visit includes history, physical, and laboratory evaluations focused on factors that might contribute to or explain rate of progressive loss of kidney function in CKD and its relation to the progression of chronic renal insufficiency and atherosclerotic vascular disease. These evaluations include information on LEA, lower extremity peripheral vascular disease (PVD), diabetes, initial hemoglobin A1c (HgbA1c), gender, race/ethnicity, age, body mass index (BMI), estimated glomerular filtration rate (eGFR), and ankle brachial index. All participants enrolled in CRIC had decreased kidney function as defined by the Modification of Diet in Renal Disease (MDRD) equation(18). About half of the CRIC participants have diabetes. This study was conducted on 3772 participants in the CRIC study (through August 2011) who were genotyped on the ITMAT-Broad-CARe array (IBC) chip(19).

The study is in compliance with the Declaration of Helsinki. The study protocol was approved by individual institutional review boards at the participating centers. Written informed consent was obtained from all participants. Individuals who agreed to participate in the CRIC study were separately consented by CRIC investigators to participate in studies of genetic variation.

Genotyping

The IBC chip consists of more than 50,000 tag SNPs in genes that are hypothesized to be associated with cardiovascular-related phenotypes and is described in more detail

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elsewhere(19). Genotyping was performed using the Illumina iSelect (http://www.illumina.com).

Selection of genes and SNPs

SNPs in cytokine or associated receptor genes belonging to the wound healing/angiogenesis pathway were extracted from the IBC chip. There were a total of 1017 tagging SNPs in the following 22 genes belonging to the wound healing/angiogenesis pathway: *FGF2*, *FGF8*, *FLT1*, *EGF*, *HIF1A*, *HIF1AN, KDR, ANGPT1*, *ANGPT2*, *EGFR*, *PDGFB*, *PDGFC*, *PDGFD*, *PDGFRA*, *PDGFRB*, *TGFB2*, *TGFB3*, *TGFBR1*, *TGFBR2*, *TGFBR3*, *TIE1* and *VEGFA* (for full gene names, vide "Abbreviations: gene names" section).

Quality control

Quality control in a genetic association study is undertaken to identify and remove DNA samples and genetic markers that have the potential to introduce bias(20)). Samples were excluded (n = 226, 5.9%) based on the following criteria: 1) sample call rate < 0.97; 2) higher or lower than expected heterozygosity (inbreeding, |F| < 0.2); or 3) cryptic relatedness (PI_HAT identity-by-descent <0.2). The first two criteria ensure good sample quality whereas the third criterion is a safeguard against including related individuals. Next, principal components analysis, which is a popular method to categorize individuals according to ancestry, as described in Price et al (21) was performed to generate 4 categories of genetically-inferred race (non-Hispanic white, non-Hispanic African-American, Hispanic, and Asian/other). Because of the small sample size in the Asian/other group, we restricted our current analysis to non-Hispanic white, non-Hispanic African-American and Hispanic populations. SNP level quality control was then conducted within each race/ethnicity separately. SNPs were excluded if the SNP call rate was < 97% (to safeguard against including poorly characterized markers with too many missing genotypes), minor allele frequency (MAF) was < 3% (to ensure sufficient power), or Hardy-Weinberg equilibrium (HWE) p-value was < 0.001 (to safeguard against genotyping errors).

Statistical analysis

Demographic and clinical characteristics of the study population were compared between patients with or without an LEA using the Chi-square test for categorical variables and the independent samples t-test for continuous variables. The association between genetic variants and LEA status was examined using logistic regression under additive genetic models as implemented in the software PLINK(http://pngu.mgh.harvard.edu/purcell/plink/) (22). The inheritance pattern of LEA being unknown, additive genetic models, where the three genotypes AA, AB, and BB (A being the major allele and B being the minor allele) were coded as AA = 0, AB = 1, and BB =2, were used. All association analyses were conducted separately for each race/ethnicity subgroup. Further stratification was based on diabetic status. Only the subgroups with diabetes had sufficient sample sizes for meaningful analyses; therefore, analyses were restricted to these subgroups. Both unadjusted analyses as well as analyses adjusted for age, sex, eGFR, BMI, peripheral vascular disease, HgbA1c and the first three principal components (to correct for residual population stratification) were performed. Given that we had selected each gene based on *a priori* hypothesis, we corrected

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for the number of SNPs in each gene to account for multiple testing. Therefore, each gene had its own Bonferroni corrected p-value depending on the number of SNPs tested.

Results

At the time of analysis, our CRIC study sample consisted of 3546 participants of whom 210 had an LEA (5.9%). Overall, 1591 were female (44.9%), 1490 were non-Hispanic Black (42.0%), and 1726 had diabetes (48.6%). Selected demographic and clinical characteristics of the combined study population grouped according to LEA status are presented in Table 1. The proportion of participants who were males, non-Hispanic Blacks, had diabetes and PVD was significantly higher in the LEA group. Also, the mean eGFR value was lower while the mean BMI and HgbA1c values were significantly higher in those with LEA. We specifically present descriptive statistics for non-Hispanic white participants with diabetes as subsequent analyses identified this subgroup to be of particular interest (Table 2). In this subgroup, the prevalence of PVD was significantly higher among those with LEA and while not statistically significant (likely due to the small size of the LEA group), mean eGFR was lower and BMI and HgbA1c higher in the LEA group.

After SNP level quality control, there were 643 SNPs belonging to the angiogenesis/wound repair pathway left for analysis in the subgroup of non-Hispanic white participants with diabetes. The strongest association of LEA was with the fibroblast growth factor 2 (basic) (*FGF2*) gene, which codes for the protein basic fibroblast growth factor-2. **rs11938826**, an intronic SNP in *FGF2* that had a MAF of 15.9%, was significantly associated with LEA in covariate-adjusted analysis [Odds ratio (OR): 2.83 (95% Confidence Intervals: 1.73, 4.62)]; p-value: 0.000034; Bonferroni adjusted p-value: 0.0006). Another *FGF2* intronic SNP, **rs1960669** with a MAF of 12.8%, had a similar effect estimate [2.61 (1.48, 4.61)] and a p-value of 0.00095, which became 0.02 after Bonferroni adjustment. Another gene associated with LEA was fibroblast growth factor 8 (*FGF8*), which codes for the protein fibroblast growth factor 8. **rs10883688** in gene *FGF8*, functional significance unknown, had a MAF of 48% and an OR of 1.72 (1.14, 2.6); p-value = 0.01, Bonferroni adjusted p: 0.04. (Table 3 and supplement).

In other subgroup analyses (non-Hispanic Blacks with diabetes and Hispanics with diabetes), we found several other SNPs in *FGF2* as well as other genes that were nominally significant, but none withstood Bonferroni correction (Supplementary materials).

Discussion

We hypothesized that variation in genes that are part of the angiogenesis/tissue repair pathway would be associated with LEA, a measure of poor wound healing. To test this hypothesis, we used phenotype and genotype information from the CRIC study. We found that previously reported socio-demographic and clinical risk factors for LEA such as gender, race/ethnicity, eGFR, PVD and poor glycemic control were associated with LEA in the CRIC population(1, 7, 9, 23–26). Among non-Hispanic white participants with diabetes, the proportion of patients with PVD was significantly higher in the LEA group, while the other risk factors, though not statistically significant, demonstrated trends those were consistent

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with the literature. In this subgroup, two intronic SNPs in *FGF2*, **rs11938826 and rs1960669** were significantly associated with LEA in covariate-adjusted analysis. These findings are especially interesting given the structure and variation within the *FGF2* gene(27–29). A SNP **rs10883688** of unknown functional effect, in *FGF8*, was also found to be associated with LEA. It is important to note that the adjustment factors included PVD, which is well known to be the most potent predictor of amputation. We also adjusted for confounding due to residual population stratification, which can arise from further systematic differences in allele frequency between subpopulations within even a genetically defined single population (e.g. non-Hispanic whites), due to hidden and fine scale genetic sub-structure(20).

FGF2, first isolated in the 1970's, has been described in several species(30). Like other members of the class, it has been shown to be important in cell growth, differentiation, and death/survival. It has been specifically studied with respect to mediating cardiac reperfusion injury, wound repair, angiogenesis, neurogenesis, pulmonary fibrosis, pancreatic stromal proliferation, as well as other disease states(27, 28, 30, 31). While topical FGF2 is available in Japan for the treatment of chronic wounds (Fiblast Spray-trafermin launched in 2001), trials of topical FGF2 have not been successful in other countries(32, 33).

In humans, the gene fibroblast growth factor 2 (basic) (*FGF2*) is located on chromosome 4 (q26–q27) and encodes for five structurally different proteins (only three in mice) that are derived from alternative start sites(27, 28, 30, 31, 34). The five protein isoforms vary in size from 18–34 kDa, and are defined as low molecular weight (18kDa) and high molecular weight (21, 22.5, 24, and 34 kDa) isoforms(27, 31). The low molecular weight (LWM) isoform is predominantly cytoplasmic due to its ability to act as a ligand with cell surface receptors and the higher molecular weight (HWM) forms are primarily nuclear due to the presence of a nuclear localization sequence (27, 31, 34, 35).

Interestingly, in cardiac reperfusion injury LMW and HMW FGF2 isoforms appear to have opposing roles in that overexpression of HMW FGF2 results in greater tissue injury (this effect may be dose dependent) while the LMW isoform is cardioprotective[34]. Varied effects of HMW and LMW FGF2 have also been noted in neurogenesis where in general HMW FGF2 acts as a proliferative factor and LMW acts as a mitogenic factor, but even these effects can vary by cell type(29). We speculate that *FGF2* variants would influence the expression of specific isoforms, which might then uniquely influence wound healing.

FGF8, expressed widely during embryonic development, has a very limited expression pattern in the normal human adult tissue. Specifically, it is expressed only in steroid hormone target tissues such as the testes and the ovaries and in hormone regulated cancers of the breast, prostate and ovary(36). *FGF8* loss-of function variants have been associated with Kallman's Syndrome(37). Given the current state of knowledge, it is difficult to explain the role of *FGF8* variants in LEA. It is however, possible that *FGF8* has a wider expression pattern than currently thought. In fact, a study did report *FGF8* expression in normal hematological tissues and the investigators speculated on its potential role in hematopoiesis(38). However, this finding needs to be confirmed by others.

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It is to be noted that our findings in the subgroup of white participants with diabetes could not be replicated in either African-American or Hispanic subgroups. This is not unexpected, given that both rs11938826 and rs1960669 are tag SNPs located in the gene intron, while **rs10883688** is an intergenic SNP, situated between the genes *FGF8* and *FBXW4* (The ALlele FREquency Database, http://alfred.med.yale.edu/alfred/recordinfo.asp?condition=loci.locus_uid=%27LO046371V) that are presumably in linkage disequilibrium with the causative variant/s. Since the pattern of linkage disequilibrium differs among populations often due to race/ethnicity, it is possible that the marker SNPs that could be associated with the causative variants in African-American and Hispanic participants were not adequately represented in the IBC chip. Also, other genes outside of the FGF family that have established roles in the wound repair/angiogenesis pathway were not found to be significantly associated with LEA in our study. However, many variants in these genes did show nominally significant associations that could not withstand corrections for multiple testing (please see supplementary materials). Wound repair is a complex process with multiple genes and variants acting in concert, each presumably with a small effect. It is possible that in the current study, we were underpowered to detect many such true but small effects due to the modest number of outcomes after stratification by genetic ancestry.

Our study has a few limitations. First, we are unaware of an independent cohort that would be suitable for replication of our findings. Second, in all studies designed to evaluate multiple genetic factors there is always concern about false discovery. In our study we used an unbiased approach by *a priori* specifying genes of interest and then using a Bonferroni correction to adjust for multiple comparisons. The association that we ultimately discovered between the *FGF2* SNP rs11938826 and LEA had an adjusted odds ratio (OR) of nearly 3, while rs1960669 had an adjusted OR greater than 2.5. The multiplicity adjusted p-values and the high ORs are consistent with a greater likelihood that our findings are not false discoveries. However, confirmation of these finding in other studies is necessary. Third, it is known that LEA is more common in individuals with CKD and diabetes, the subgroup that makes up our parent cohort(26, 39, 40). However, our analyses allowed for the adjustment of effects of CKD based on eGFR. Finally, LEA is an imperfect marker for impaired wound healing in that the indications for LEA vary by healthcare provider and patient(13). While the decision to amputate is likely based on the failure of a wound to heal, it is also based on other important health, behavioral and social concerns. Therefore, there were likely other unmeasured risk factors that could have affected the likelihood of LEA. While unlikely to be true confounders, the inclusion of these variables in the model could have decreased variability and increased precision(41, 42). It is therefore important that future studies also evaluate other measures of impaired wound healing.

In conclusion, we present the first report of an association between genes known to be important in wound repair, *FGF2* and *FGF8*, with LEA, in a cohort of non-Hispanic white participants with diabetes and CKD. Both FGF2 and FGF8 proteins are created as four separately transcribed isoforms and thus may be especially sensitive to genetic variations. We suggest future studies be conducted to confirm our findings and to localize the likely causal genetic variants. In addition, future studies will need to demonstrate the effect that these variants have on the production and function of FGF2 and FGF8 proteins.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BMI	body mass index
CKD	chronic kidney disease
CRIC	Chronic Renal Insufficiency Cohort
eGFR	estimated glomerular filtration rate
HgbA1c	hemoglobin A1c
HWE	Hardy-Weinberg equilibrium
IBC	ITMAT-Broad-CARe array
LEA	lower extremity amputation
MAF	minor allele frequency
MDRD	Modification of Diet in Renal Disease
NOS1AP	Neuronal nitric oxide synthase associated protein
OR	odds ratio
PVD	peripheral vascular disease
SNP	single nucleotide polymorphism

Abbreviations (gene names)

ANGPT1	angiopoietin 1
ANGPT2	angiopoietin 2
EGFR	epidermal growth factor receptor

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FGF2	fibroblast growth factor 2 (basic)
FGF8	fibroblast growth factor 8
FLT1	fms-related tyrosine kinase 1
EGF	epidermal growth factor
HIF1A	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
HIF1AN	hypoxia inducible factor 1, alpha subunit inhibitor
KDR	kinase insert domain receptor
PDGFB	platelet-derived growth factor beta polypeptide
PDGFC	platelet derived growth factor C
PDGFD	platelet derived growth factor D
PDGFRA	platelet-derived growth factor receptor, alpha polypeptide
PDGFRB	platelet-derived growth factor receptor, beta polypeptide
TGFB2	transforming growth factor beta 2
TGFB3	transforming growth factor beta 3
TGFBR1	transforming growth factor, beta receptor 1
TGFBR2	transforming growth factor beta receptor II
TGFBR3	transforming growth factor beta receptor III
TIE1	tyrosine kinase with immunoglobulin-like and EGF-like domains 1
VEGFA	vascular endothelial growth factor A

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Table 1

Baseline demographic and clinical characteristics of CRIC Study participants who were genotyped on the IBC chip and passed quality control measures.

	No LEA (n = 3336)	LEA (n = 210)	p-value
Values presented as n (%)			
Females	1520 (45.6)	71 (33.8)	**0.0009**
Race/ethnicity			**<0.0001**
Non-Hispanic white	1483 (44.5)	67 (31.9)	
Non-Hispanic Black	1399 (41.9)	91 (43.3)	
Hispanic	323 (9.7)	50 (23.8)	
Other	131 (3.9)	2 (1.0)	
Diabetes	1540 (46.2)	186 (88.6)	**<0.0001**
PVD	177 (5.3)	64 (30.5)	**<0.0001**
Values presented as mean (standard deviation)			
Age (years)	58.2 (11.0)	58.5 (9.8)	0.65
eGFR (ml/min/1.73m^2)	43.3 (13.5)	38.5 (13.2)	**<0.0001**
BMI (kg/m^2)	32.0 (7.8)	33.6 (7.9)	**0.005**
HgbA1c	6.6 (1.5)	7.9 (1.9)	**<0.0001**

Bold = significant at the 0.05 alpha level

BMI = body mass index

CRIC = Chronic Renal Insufficiency Cohort

eGFR = estimated glomerular filtration rate

HgbA1c = hemoglobin A1c

IBC = ITMAT-Broad-CARe array

LEA = lower extremity amputation

PVD = peripheral vascular disease

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Table 2

Baseline demographic and clinical characteristics of non-Hispanic white participants with diabetes in the CRIC study who were genotyped on the IBC chip and passed quality control measures.

	No LEA (n = 571)	LEA (n = 56)	p-value
Values presented as n (%)			
Females	196 (34.3)	19 (33.9)	1.0
PVD	50 (8.8)	25 (44.6)	**<0.0001**
Values presented as mean (standard deviation)			
Age (years)	59.5 (10.0)	59.4 (8.4)	0.92
eGFR (ml/min/1.73m^2)	42.0 (12.3)	39.4 (10.8)	0.10
BMI (kg/m^2)	33.8 (8.2)	34.5 (8.5)	0.58
HgbA1c	7.4 (1.5)	7.7 (1.2)	0.18

Bold = significant at the 0.05 alpha level

BMI = body mass index

CRIC = Chronic Renal Insufficiency Cohort

eGFR = estimated glomerular filtration rate

HgbA1c = hemoglobin A1c

IBC = ITMAT-Broad-CARe array

LEA = lower extremity amputation

PVD = peripheral vascular disease

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Table 3

Association of variants in genes belonging to the angiogenesis/wound repair pathway with lower extremity amputation in non-Hispanic white participants with diabetes in the CRIC study. Only significant associations in *FGF2* and *FGF8* are shown.

SNP ID	Type	CHR	Gene	MAF	OR, unadjusted (95% CI) (n = 627)	p-value, unadjusted	OR, adjusted (95% CI) (n = 621)	p-value, adjusted	p-value, adjusted (Bonferroni corrected)*
rs11938826	intron	4	*FGF2*	0.16	2.42 (1.56, 3.77)	0.0000896	2.83 (1.73, 4.62)	0.000034	0.00063
rs1960669	intron	4	*FGF2*	0.13	2.15 (1.28, 3.60)	0.003686	2.61 (1.48, 4.61)	0.00095	0.01995
rs10883688	unknown	10	*FGF8*	0.48	1.67 (1.13, 2.47)	0.01057	1.72 (1.14, 2.60)	0.009992	0.03996

CHR = chromosome

CI = confidence intervals

FGF2 = fibroblast growth factor 2 (basic)

FGF8 = fibroblast growth factor 8

MAF = minor allele frequency

OR = odds ratio

SNP = single nucleotide polymorphism

* Bonferroni correction was based on 21 SNPs in *FGF2* and 4 SNPs in *FGF8*

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