Brief report

Association of klotho, bone morphogenic protein 6, and annexin A2 polymorphisms with sickle cell osteonecrosis

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In patients with sickle cell disease, clinical complications including osteonecrosis can vary in frequency and severity, presumably due to the effects of genes that modify the pathophysiology initiated by the sickle mutation. Here, we examined the association of single nucleotide polymorphisms (SNPs) in candidate genes (cytokines, inflammation, oxidant stress, bone metabolism) with osteonecrosis in patients with sickle cell disease. Genotype distributions were compared between cases and controls using multiple logistic regression techniques. An initial screen and follow-up studies showed that individual SNPs and haplotypes composed of several SNPs in bone morphogenic protein 6, annexin A2, and klotho were associated with sickle cell osteonecrosis. These genes are important in bone morphology, metabolism, and vascular disease. Our results may provide insight into the pathogenesis of osteonecrosis in sickle cell disease, help identify individuals who are at high risk for osteonecrosis, and thus allow earlier and more effective therapeutic intervention. (Blood. 2005;106:372-375)

Introduction

Sickle cell anemia results from homozygosity for the Glu6Val mutation in the hemoglobin beta chain gene *(HBB).* Osteonecrosis is a common sequela of sickle cell disease; studies suggest that by the age of 35 years, one half of all patients with sickle cell anemia have osteonecrosis.[1,2] We hypothesize that the presence or absence of osteonecrosis in sickle cell patients is influenced by genetic variability in genes other than *HBB* that are expressed in either bone or the vasculature. To test this hypothesis, we examined the potential association of osteonecrosis with single nucleotide polymorphisms (SNPs) in candidate genes of different functional classes, including those involved in vascular function, inflammation, oxidant stress, and endothelial cell biology.

Study design

Patients provided informed consent at the time of recruitment in 1987. For this study, samples were obtained from a public repository and were de-identified. This study was approved by the Boston University Institutional Review Board.

DNA samples, clinical information, and demographic information were obtained from the Cooperative Study of Sickle Cell Disease (CSSCD) and have been described elsewhere.[3,4] These studies were approved by the Boston University institutional review board. Cases are patients with sickle cell anemia, with or without concurrent α thalassemia with radiologically documented osteonecrosis of the hip and/or shoulder. Controls were patients older than 20 years who received a radiologic exam and did not have a diagnosis of osteonecrosis. We reasoned that any osteonecrosis "genotype" was less likely to be present in older, osteonecrosis-free patients.

Validated candidate gene SNPs with population frequency information and heterozygosity values of more than 0.1 were selected from public databases (http://www.ncbi.nlm.nih.gov/),[5] with follow-up studies also using the Celera SNP Reference Database.[6] When possible, SNPs in exons, promoters, and sequences important for RNA processing were chosen, however, none of the SNPs is known to affect protein function or gene expression levels. Genotyping was performed using the Sequenom Mass Array/Mass Spectrometer System (Sequenom, San Diego, CA) or the Applied Biosystem TaqMan probes (Applied Biosystems, Foster City, CA). Assay designs can be found in the supplemental data available at the *Blood* website; see the Supplemental Materials link at the top of the online article.

Approximately 3% of the DNA samples were genotyped twice for quality-control purposes. Hardy-Weinberg equilibrium (HWE) was assessed for each SNP among controls using an χ^2 test. SNPs that had more than 25% missing genotypes were not considered in the analysis. This resulted in 233 SNPs being tested for association.

Genotypic counts were compared between sickle cell anemia patients with osteonecrosis (cases) and without osteonecrosis (controls) using multiple logistic regression. In our initial screen, we considered an SNP to have an association with the phenotype when the *P* value was equal to or less than .01, or if this and other SNPs in the same gene were significant at the .05 level. If an SNP met these criteria, additional SNPs were typed to ascertain haplotypes that could be used to elucidate and better define the pattern of association in a particular gene. Because the large number of tests conducted could inflate the rate of falsely significant associations, the final selection of significant SNPs was carried out by controlling the false discovery rate (FDR) as described previously.[7] The selection of individual

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Submitted February 8, 2005; accepted March 10, 2005. Prepublished online as *Blood* First Edition Paper, March 22, 2005; DOI 10.1182/blood-2005-02-0548.

Supported by National Heart, Lung, and Blood Institute (NHLBI) grant HL R01 68970 (M.H.S.).

The online version of the article contains a data supplement.

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Table 1. SNPs associated with osteonecrosis

SNP no.	Gene	Genotype	AVN	Control	Reference genotype	AVN	Control	OR	95% CI	*P*
rs1019856*	*TGFBR2*	AA	55	36	AG	143	156	1.75	1.08-2.86	.023
rs934328†	*TGFBR2*	CC	75	60	T_	137	242	2.36	1.56-3.51	< .001
rs284157*†	*TGFBR3*	A_	209	344	GG	174	65	4.95	3.53-6.94	< .001
rs270393*†	*BMP6*	GG	63	39	C_	211	226	1.80	1.15-2.81	.009
rs267196†	*BMP6*	TT	253	226	AT	78	116	1.85	1.30-2.63	.001
rs267201	*BMP6*	CT	161	161	TT	92	142	1.60	1.13-2.28	.008
rs449853†	*BMP6*	TT	71	47	C_	308	338	1.68	1.12-2.52	.012
rs1225934†	*BMP6*	CC	308	265	AC	70	123	1.96	1.41-2.78	.001
rs212527†	*ECE1*	GG	75	22	A_	123	196	5.68	3.33-9.62	< .001
rs5369*†	*EDN1*	AG	48	41	GG	57	128	3.04	1.76-5.24	.001
hCV7464888†	*EDN1*	AG	49	61	AA	209	141	2.22	1.41-3.45	.001
rs979091*	*ERG*	CC	39	13	AA	9	12	3.85	1.32-11.10	.014
rs2836430	*ERG*	AA	373	353	AC	33	59	1.96	1.22-3.13	.005
rs7163836	*ANXA2*	GG	149	119	AA	44	70	1.82	1.15-2.86	.010
hCV11770326†	*ANXA2*	CC	227	204	G_	55	138	3.38	2.28-4.97	< .001
rs7170178†	*ANXA2*	AA	164	140	G	148	210	1.98	1.44-2.72	< .001
rs1033028†	*ANXA2*	T_	368	368	GG	14	36	2.43	1.28-4.57	.007
hCV26910500†	*ANXA2*	G_	156	122	AA	102	166	2.56	1.79-3.68	< .001
hCV1571628	*ANXA2*	TT	124	138	AT	111	179	1.45	1.03-2.08	.034
rs538874†	*STARD13*	G_	286	331	AA	37	51	1.79	1.12-2.85	.015
rs475303	*STARD13*	CT	132	93	CC	278	279	1.42	1.04-1.96	.029
rs648464†	*STARD13*	AG	193	126	GG	155	185	1.91	1.39-2.61	.001
rs480780*†	*KL*	AC	69	44	CC	6	5	2.97	1.83-4.84	.001
rs211235†	*KL*	C_	150	194	AA	9	46	3.97	1.81-8.69	.001
rs2149860†	*KL*	G_	290	231	AA	134	148	1.42	1.05-1.90	.021
rs685417†	*KL*	AG	199	133	GG	155	164	1.66	1.21-2.28	.002
rs516306†	*KL*	T_	411	365	CC	8	19	2.86	1.19-6.90	.019
rs565587†	*KL*	AG	142	113	AA	130	172	1.80	1.28-2.53	.001
rs211239†	*KL*	A_	388	314	GG	27	53	2.58	1.56-4.28	.001
rs211234†	*KL*	G_	402	333	AA	8	24	4.08	1.73-9.62	.001
rs2238166	*KL*	C_	408	360	TT	8	16	2.50	1.02-6.16	.046
rs499091†	*KL*	A_	368	335	GG	8	19	2.85	1.18-6.89	.020
rs576404†	*KL*	C_	411	372	AA	5	16	4.23	1.40-12.82	.010
hCV3118898†	*APRIN*	C_	396	333	AA	26	53	2.51	1.51-4.14	.001
hCV11710292	*APRIN*	AG	174	125	AA	225	232	1.45	1.08-1.95	.014

The selection of individual genotypes was based on 512 tests, so that the largest *P* value to accept a significant association with 20% FDR is .0016. The selection of pooled genotypes was based on 233 tests so that the largest *P* value to accept a significant association with 20% FDR was .0309. The *STARD13* and *APRIN* genes flank the *KL* gene.

*SNPs that were typed as part of the first, low-density SNP screen for the candidate genes.

†SNPs that are significant, with 20% FDR.

genotypes was based on 512 tests so that the largest *P* value to accept a significant association with 20% FDR was .001. The selection of pooled genotypes was based on 233 tests, so that the largest *P* value to accept a significant association with 20% FDR was .0309.

Pairwise linkage disequilibrium (LD) between SNPs was evaluated using the software Haploview (version 2.05, http://www.broad.mit.edu/mpg/haploview/download.php),[8] which implements a maximum likelihood method to infer phase for dual heterozygotes and expresses the magnitude of LD as D′. Haplotypes were inferred using Bayesian methods as implemented in the PHASE computer program (version 2.02; http://www.stat.washington.edu/stephens/software.html).[9,10] Haplotype association between cases and controls was assessed using PHASE.

Results and discussion

We studied 442 subjects with osteonecrosis and 455 controls. Males had a slightly higher proportion of avascular necrosis (AVN) when compared with females ($P = .02$); because of the study design, those with osteonecrosis, on average, were 6 years younger than the controls. Individuals with osteonecrosis had a higher prevalence of coincident α thalassemia ($P = .03$), and there was no difference in total hemoglobin or fetal hemoglobin (HbF) levels ($P > .28$). See the Supplemental Materials for detailed clinical information.

In the initial screen, 3 to 5 SNPs in 66 candidate genes involved in vascular function, inflammation, oxidant stress, and endothelial cell biology were genotyped, and significant associations were observed with 7 SNPs in 7 genes *(BMP6, TGFBR2, TGFBR3, EDN1, ERG, KL, ECE1).* Additional SNPs, equally distributed within the gene, were typed in all 7 genes, and a significant association with many SNPs in *KL* and *BMP6* (Table 1) was found. SNPs in *ANXA2* were also typed because of a previous finding of association between this gene and stroke among patients with sickle cell disease. In this study, we did not confirm these results in an independent population of sickle cell patients with AVN. Power calculations indicate that, for associated SNPs with an odds ratio of more than 2, examination of 100 additional cases and 200 additional controls would be sufficient to confirm our findings.

Of the 18 SNPs typed in *KL,* 10 were significantly associated with osteonecrosis (Table 1). Most of these SNPs were located in the 20-kilobase (kb) region representing the first half of the first *KL* intron and were in LD with each other. SNPs in *BMP6* (5/14) and *ANXA2* (6/13) were also associated with osteonecrosis (Table 1);

however, these SNPs were distributed throughout the intronic and 3′ untranslated regions of the gene. Similar to the finding in *KL,* there was a tendency for the disease-associated SNPs to be in LD with each other.

On the basis of visual inspection of the LD pattern in these 3 genes (see Supplemental Materials), 2 haplotype blocks were defined in *KL,* 1 block in *ANXA2,* and 1 block in *BMP6* (Table 2). For each of these LD blocks, haplotypes were estimated (PHASE) and for all 3 genes the distribution of haplotypes was significantly different among those with and without osteonecrosis *($P = .01$ for KL and BMP6; $P = .03$ for ANXA2).*

The 3 genes we identified are important in bone metabolism. *KL* encodes a glycosyl hydrolase that participates in a negative regulatory network of the vitamin D endocrine system and may be important for a wide variety of other cellular processes, including regulation of antioxidative defense, angiotensin converting enzyme activity, arteriosclerosis, and aging.[11-13] Bone morphogenic proteins (BMPs), including *BMP6,* are pleiotropic secreted proteins structurally related to transforming growth factor β (TGF-β) and activins. *BMP6* is involved in inflammatory processes[14] and is important for bone formation[15] and, in association with parathyroid hormone (PTH) and vitamin D, appears to be involved in inducing bone development by human bone marrow–derived mesenchymal stem cells.[16] *ANXA2* is a member of the calcium-dependent phospholipid-binding protein family and regulates cell growth and is involved in signal transduction pathways. It is involved in osteoblast mineralization; lipid rafts containing annexin 2 appear to be important for alkaline phosphatase activity in bone[17] and the neuronal response to hypoxia.[18]

Although we identified genes that may play a significant role in the pathogenesis of sickle cell osteonecrosis by altering protein function or gene expression, the mechanisms by which variants in these genes predispose sickle cell patients to vascular complications are unknown. We also have observed an association between *KL* and priapism[19] as well as between *BMP6/ANXA2* and stroke[20] in patients with sickle cell disease. This suggests that the vascular complications in sickle disease may have some common underlying molecular basis.

Understanding the genetic risk factors for the development of sickle cell osteonecrosis may provide new insight into the pathogenesis of this disease and eventually provide opportunities for its treatment, which now is limited.[21] For example, regulating the activity of the TGF-β pathway to modulate its effects on bone may be possible.[22-24] Ultimately, our results should help identify at an early age patients at high risk for osteonecrosis, and thus allow earlier and more effective therapeutic intervention.

Table 2. Haplotype analysis of SNP markers in *KL, BMP6,* and *ANXA2*

	Control, %	Case, %
KL block 1 haplotype*		
CACGAATTGT	34	41
CACGAGATAT	14	21
CACGGAATGT	12	8
AATAGGACAG	7	11
CACGAAATGT	7	5
CACGAATTAT	3	2
CACGAAATAT	2	1
CACGAGTTAT	2	1
CATAGAATGT	2	1
CACGGATTGT	2	1
Rare (< 1%)	16	7
KL block 2 haplotype*		
CCATTC	41	45
ACGCCC	27	32
ATGCCC	7	7
CCATCC	9	5
CCATTT	4	6
ACGCTC	7	3
BMP6 haplotype*		
CTTTCAC	16	12
CTTCCCC	13	13
CTTCCCT	10	10
CTTCTCT	8	9
TACCTCT	7	7
CTTTCCC	6	7
CTTCTCC	5	8
TACCTCC	6	6
CTCCCCC	4	4
TTCCTCT	3	4
CTTTCCT	3	3
TACTCCT	3	2
TACCCCC	2	2
TTCCTCC	1	2
TACCCCT	1	2
Rare (< 1%)	9	9
ANXA2 haplotype†		
GACACG	28	23
TACGCA	14	18
TACACG	14	14
TGCGCG	12	12
TGGGCG	11	9
TGGGGG	10	8
TACGCG	4	8
TGGGCA	2	2
TACGGG	1	3
TGCGCA	2	1
Rare (< 1%)	3	3

Haplotypes for *KL* block 1 are composed of SNPs rs576404, rs499091, rs2238166, rs211234, rs211239, rs565587, rs495764, rs516306, rs685417, and rs1334928. Haplotypes in *KL* block 2 are composed of SNPs rs656525, rs1888057, rs657049, rs497050, rs2249358, and rs560014. Haplotypes for *BMP6* are composed of SNPs rs267192, rs267196, rs267201, rs408505, rs449853, rs1225934, and rs267170. Haplotypes for *ANXA2* comprise SNPs rs1033028, rs7170178, hCV11770326, rs7163836, rs8037326, and hCV9036132.

*$P = .01$, global.

†$P = .03$, global.

Acknowledgments

We thank the investigators of the CSSCD who obtained blood samples for DNA-based studies and analyzed data from these studies for the original study publications cited in this paper.

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