Association between rs267196 and rs267201 of BMP6 gene and osteonecrosis among Sickle Cell Anemia patients

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Aims. The skeletal manifestations of sickle cell disease are the result of changes in bone and bone marrow caused by chronic tissue hypoxia that is exacerbated by episodic occlusion of the microcirculation by the abnormal sickle cells. Furthermore, the occurrence of osteonecrosis is under the control of some modifier gene. BMP6 (Bone morphogenetic protein) has been reported as associated with osteonecrosis in sickle cell anemia (SCA). Herein, we intend to study the impact of rs267196, rs267201, rs408505 and rs449853 of BMP6 gene in the occurrence of osteonecrosis among sickle cell patients in Tunisia.

Methods. Our study involved 100 SCA patients among whom 19 have osteonecrosis of the head of the femur. The latter polymorphisms of BMP6 gene were analyzed for all subjects by PCR/sequencing. To test for trait association with the candidate SNPs, genotype and allele frequencies between cases (osteonecrosis group) and controls (non-osteonecrosis group) were compared using Pearson's chi-square test with a significance threshold of $P < 0.05$ (compare 2, version 1.02).

Results. Our findings showed that the patients carried genotype TA of rs 267196 and genotype AG of rs267201 present a high risk factor for developing osteonecrosis $RR = 1.317$ and $RR = 1.3$ respectively. The results showed a significant association between the alleles A of rs 267196 and G of rs267201 and osteonecrosis $P = 0.0023$; $RR = 2.42$ and $P = 0.041$; $RR = 2.24$ respectively. Interestingly, SCA patients with the combined genotype TA/AG were found to be at higher risk of developing osteonecrosis ($P = 0.009$). As for rs408505 and rs449853 of BMP6 gene no significant association was found among SCA patients.

Key words: Sickle Cell Anemia, BMP6 polymorphisms, osteonecrosis

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INTRODUCTION

SCA is a single-gene mutation genetic disease caused by change of Glu6Val at the hemoglobin beta chain gene and characterized by high variable clinical complications¹. The skeletal manifestations of sickle cell disease are the result of changes in bone and bone marrow caused by the chronic tissue hypoxia that is exacerbated by episodic occlusion of the microcirculation by the abnormal sickle cells. Furthermore, the occurrence of osteonecrosis is under the control of some modifier gene. BMP6 (Bone morphogenetic protein) has been reported as associated with osteonecrosis in sickle cell anemia (SCA) (ref.²). Bone morphogenetic proteins (BMPs) are members of the TGF-beta superfamily of molecules. They are multi-functional cytokines involved in many aspects of tissue development and morphogenesis. BMP6 is involved in inflammatory processes and is important for bone formation 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 cells³. BMP6 promotes osteoblast differentiation from mesenchymal stem cells^{4,5} studied the role of BMP6

in cartilage homeostasis; they discovered an essential involvement of this gene in the repair/maintenance of human articular cartilage. In sickle cell osteonecrosis, the progressive degeneration of the bone eventually led to its collapse and destruction of articular cartilage, supporting a role of BMP6 in the pathogenesis of this complication. Indeed, Baldwin et al.⁶ have reported the role of rs267196, rs267201 and rs449853 in the occurrence of osteonecrosis in sickle cell disease.

Herein, we aimed to explore the implication of four known polymorphisms of BMP6: rs408505, rs449853, rs267201 and rs267196 in osteonecrosis among SCA patients.

MATERIAL AND METHODS

Material

100 sickle cell patients were involved in this study. Patients were selected on the basis of homozygosity for β^s -globin gene. Demographic, hematological and clinical data of subjects studied are summarized in Table 1.

METHODS

Clinical events

Data and clinical events were taken from the patient's history via search of the clinical registry with radiologically documented osteonecrosis. Patients chosen in this study have osteonecrosis of the head of the femur.

Laboratory methods

Diagnosis of sickle cell patient is performed using cation-exchange high performance liquid chromatography (HPLC) (D10 Biorad) and further confirmation by means of molecular diagnosis by restriction fragment length polymorphism (RFLP) using DdeI as previously described by Romana 2000⁷. Biochemical data were averaged for each patient in steady state (at least three values). We determined total and fetal hemoglobin (HbF) concentrations (D10, Biorad), and reticulocyte count and other hematologic parameters using (ABX pentra 60c+).

Genotyping of BMP6 polymorphisms

Genomic DNA was isolated from white blood cells of total blood using standard method (phenol/chloroform). A polymerase chain reaction (PCR) was performed using 4 couple of primers as described in Table 2. Polymerase chain reaction was performed in 25 µL reaction volumes containing 100 ng of genomic DNA, 0.2 mmol/L of each dNTP, 50 mmol/L KCl, 15 mmol/L Tris-HCl PH 8.0, 2.5 mmol/L MgCl₂, 0.5 U Amplitaq polymerase (Invitrogen life technologies, Carlsbad, CA, USA), and 10 pmol of each forward and reverse primers. The PCR cycling conditions included an initial denaturation of 10 min at 94 °C followed by 35 cycles of 94 °C for 60 s, annealing at 59 °C for 60 s, and extension at 72 °C for 1 min. The run was ended by a final extension at 72 °C for 10 min.

PCR products were then purified and doubly sequenced (forward and reverse) by ABI PRISM Big Dye Terminator on ready reaction kit (Applied Biosystems, Foster City, CA, USA) and an ABI 310 DNA sequencer (PEApplied Biosystems, Foster City, USA).

Statistical analysis

The demographic and hematologic data were normally distributed. The Hardy-Weinberg equilibrium was tested using the software package Arlequin (version 3.01). Genetic differences between cases and controls were evaluated applying tests to genotypic or allelic contingency tables (Compare 2, version 1.02). We used Fisher's exact test and chi-squared tests where appropriate.

RESULTS

The sample of patients was divided into two groups according to the presence or absence of osteonecrosis. Hematologic data such as hemoglobin and other parameters suggested similar hemolytic rates in these groups (Table 1). The two groups of patients stratified according to the occurrence of osteonecrosis were compared for age, sex ratio and hematological data including HbF. No significant association was found.

For each polymorphism the samples were found to be in Hardy-Weinberg equilibrium. As for *BMP6* polymorphisms, the distribution of genotypes and alleles was analyzed for all patients (Table 3). Indeed, for the polymorphisms rs408505 and rs449853 the repartition of genotypes and alleles between the two groups of patients showed no significant association. Further, the analysis of genetic profile of rs267196 showed that the major genotype in SCA patients without osteonecrosis is the normal TT (50.62%). Although it is the TA (63.16%) among SCA with osteonecrosis. As for rs267201, our results showed that the major genotype in SCA patients without osteonecrosis was the normal AA (53.09%) and AG (57.9%) in SCA patients with osteonecrosis. The distribution of genotypes between SCA patients according to the presence or absence of osteonecrosis revealed that patients carried genotype TA of rs267196 and genotype AG of rs267201 present a risk factor for developing osteonecrosis OR=1.317 and OR=1.3 respectively (Table 3). The analysis of allelic distribution showed the association of variant A of rs267196 and variant G of rs267201 with the

Table 1. Hematological, demographic and clinical data of studied population.

	SCA patients Without osteonecrosis N=81	SCA patients With osteonecrosis N=19	P
Age	30±5	30±5	1
Sex ratio	41/59	9/10	0.120
Hb (g/dl)	9.7	9.3	0.530
RBC (10 ¹² /L)	3.29±0.9	2.89±1.02	0.080
MCV(fl)	79.7±0.9	74.2±1.3	0.095
MCH(pg)	34.9±2.1	35.7±1.02	0.075
RDW(%)	4.83±0.5	5.29±1.02	0.120
HbA	0	0	1
HbS (%)	86±0.3	86.4±0.4	1
HbF(%)	11±0.1	10.6±0.3	0.900
HbA2	3±0.2	3±0.1	1

Hb: hemoglobin, RBC: red blood cell, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin and RDW: red blood distribution.

risk of developing osteonecrosis (Table 3). Interestingly, the stratification of genotypes found using logistic regression according to the presence or absence of osteonecrosis revealed the association of both genotypes TA of rs267196 and AG of rs267201 with osteonecrosis and thus appears to present a risk factor for the occurrence of osteonecrosis ($P=0.009$) (Table 4). The repartition of genetic profile according to hematological parameters showed no significant differences between the groups with risk genotype and whose with normal genotype (Table 5).

DISCUSSION

Osteonecrosis is a common sequela of sickle cell disease; studies suggest that some modifier genes are found to be associated with this complication². Genetic association studies, which attempt to link gene polymorphisms with selected complications, highlighted some variants

Table 2. Primer sequences.

rs	Primers
rs267196	F 1: 5'-GGCAGGCATGTTATCCTTGAGA-3'
	R 1: 5'-AGAAGCATTCTTGGGGTGTCTG-3'
rs267201	F 2: 5'-GTTGCCTTGCTTGGTTGCTCTA-3'
	R 2: 5'-ACATTGGGTCCCGATCTTGTTT-3'
rs408505	F 3: 5'-GGCTATTTTTGGAATTGTGCTGTC-3'
	R3: 5'-TGGTAAGAAATGGAGGCTGAAAAA-3'
rs449853	F 4: 5'-GGTTTTGATGAATGGGCTGAAG-3'
	R 4: 5'-TCTGACTCATGGGTCTGGGATT-3'

Table 3. Genotypic and allelic distribution of studied BMP6 polymorphisms.

	Osteonecrosis		<i>P</i>	RR CI95%
	Absence N=81	Presence N=19		
rs 408505				
C	0.519	0.553	1*	-
T	0.481	0.447	NS	-
CC	22	5	1*	-
CT	40	11	NS	-
TT	19	3	NS	-
rs 449853				
A	0.420	0.526	1*	-
G	0.580	0.474	NS	-
AA	18	5	1*	-
AG	32	10	NS	-
GG	31	4	NS	-
rs 267196				
T	0.685	0.474	1*	-
A	0.315	0.526	0.0023	2.42 (1.105-5.286)
TT	41	3	1*	-
TA	29	12	0.009	1.317 (1.07-1.63)
AA	11	4	NS	-
rs 267201				
A	0.691	0.5	1*	-
G	0.309	0.5	0.041	2.24 (1.021-4.335)
AA	43	4	1*	-
AG	26	11	0.02	1.3 (1.04-1.63)
GG	12	4	NS	-

P: index of significance.

NS: Non-Significant.

RR: Relative-Risk.

CI: confidence interval.

1*: reference group.

Table 4. Repartition of combined genotypes of rs267196/rs267201 according to presence or not of osteonecrosis.

Combined genotypes		Osteonecrosis		<i>P</i>	RR CI 95%
		Presence N=19	Absence N=81		
rs267196/rs267201	TT/AA	3	39	1*	-
	TA/AA	1	3	NS	-
	TT/GG	0	2	NS	-
	TA/AG	11	25	0.009	1.337 (1.06-1.69)
	TA/GG	0	2	NS	-
	AA/AG	0	2	NS	-
	AA/GG	4	8	NS	-

P: index of significance.

NS: Non-Significant.

RR: Relative-Risk.

1*: reference group.

Table 5. Repartition of genetic profile of rs267196/rs267201 according to hematological parameters.

Genotypes	Rs 267196			Rs 267201			rs267196/rs267201		
	TT*	TA	<i>P</i>	AA*	AG	<i>P</i>	TT/AA*	TA/AG	<i>P</i>
Hb (g/dL)	9.6	9.1	0.620	9.7	9.2	0.530	9.7	9.3	0.530
RBC (10 ¹² /L)	3.2±0.5	2.8±0.8	0.100	3.29±0.9	2.79±0.8	0.080	3.2±1.02	2.79±0.9	0.080
MCV(fl)	79.5±0.7	74.7±0.9	0.095	79.7±0.9	75±0.9	0.095	79.6±1.3	75.7±0.9	0.095
MCH(pg)	34.9±2.1	35.9±2.1	0.075	34.9±2.1	35±2	0.075	34.9±2.1	35.4±2.1	0.075
RDW(%)	4.83±0.5	5.23±0.5	0.120	4.83±0.5	5±0.2	0.120	4.83±0.5	5.16±0.7	0.120

P: index of significance.

*: reference group.

of candidate gene. We examined the association of single nucleotide polymorphisms (SNPs) in candidate gene of bone metabolism with osteonecrosis in patients with SCA namely *BMP6*. This gene is important in bone morphology. *BMP-6* was also shown to be associated with other vascular complications in SCD such as priapism⁸ and stroke⁹, suggesting a common underlying molecular basis for these vascular complications.

In the current study, 4 SNPs in *BMP6* were genotyped, and significant associations with osteonecrosis were observed with 2 SNPs namely: rs267196 and rs267201. As for rs408505 and rs449853 of *BMP6* gene, no significant association was found among SCA patients. Our findings show that the patients who carried genotype TA of rs267196 and genotype AG of rs267201 present a high risk factor for developing osteonecrosis RR=1.317 and RR=1.3 respectively. Our results showed a significant association between the alleles A of rs267196 and G of rs267201 and osteonecrosis *P*=0.0023; RR=2.42 and *P*=0.041; RR=2.24 respectively. Interestingly, SCA patients with the combined genotype TA/AG were found to be at higher risk of developing osteonecrosis (*P*=0.009). Only one study has identified the latter polymorphisms as associated with osteonecrosis⁶. Whereas Ulug et al.¹⁰ found no association between rs267196 and avascular necrosis among SCA patients. Two studies have described the implication of

BMP6 polymorphisms in patients with sickle cell disease including SS, Sβ⁺, Sβ⁰ and SC. By cons, in our study we focused on SS patients. The mechanisms by which rs267196 and rs267201 in *BMP6* gene predispose sickle cell patients to osteonecrosis complication are unknown.

Elucidating the genetic basis for the development of sickle cell osteonecrosis may provide new insight into the pathogenesis in SCA patients and hence provide treatment options, which are still limited. As for *BMP6*, regulating the activity of the TGF-β pathway to modulate its effects on bone may be possible¹¹. Ultimately, rs267196 and rs267201 of *BMP6* may be considered as a reliable biomarker for predicting at an early age, patients at high risk for osteonecrosis, and thus allow earlier and more effective therapeutic intervention.

ABBREVIATIONS

BMP6, bone morphogenetic protein6; CI, confidence interval; OR, odds ratio; *P*, index of significance; RR, relative risk; SCA, Sickle Cell Anemia.

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REFERENCES

- West MS, Wethers D, Smith J, Steinberg M. Laboratory profile of sickle cell disease: across-sectional analysis. The Cooperative Study of Sickle Cell Disease. *J Clin Epidemiol* 1992;45:893-909.
- Driss A, Asare KO, Hibbert JM, Gee BE, Adamkiewicz TV, Stiles JK. Cell Disease in the Post Genomic Era: A Monogenic Disease with a Polygenic Phenotype. *Genomics insights* 2009;2:23-48.
- Rosendahl A, Pardali E, Speletas M, Ten Dijke P, Heldin CH, Sideras P. Activation of bone morphogenetic protein/Smad signaling in bronchial epithelial cells during airway inflammation. *Am J Respir Cell Mol Biol* 2002;27:160-9.
- Lavery K, Swain P, Falb D, Alaoui-Ismaili MH. BMP-2/4 and BMP-6/7 differentially utilize cell surface receptors to induce osteoblastic differentiation of human bone marrow-derived mesenchymal stem cells. *J Biol Chem* 2008;283:20948-58.
- Bobacz K, Gruber R, Soleiman A, Erlacher L, Smolen JS, Graninger WB. Expression of bone morphogenetic protein 6 in healthy and osteoarthritic human articular chondrocytes and stimulation of matrix synthesis in vitro. *Arthritis Rheum* 2003;48(9):2501-08.
- Baldwin C, Nolan VG, Wyszynski DF, Ma QL, Sebastiani P, Embury SH, Bisbee A, Farrell J, Farrer L, Steinberg MH. Association of klotho, bone morphogenetic protein 6, and annexin A2 polymorphisms with sickle cell osteonecroses. *Blood journal* 2005;106:372-5.
- Romana M, Keclard L, Froger A, Lavocat E, Saint-Martin C, Berchel C, Mèrault EG. Diverse genetic mechanisms operate to generate atypical bS haplotypes in the population of Guadeloupe. *Hemoglobin* 2000;24:77-87.
- Nolan VG, Baldwin C, Ma Q, Wyszynski DF, Amirault Y, Farrell JJ, Bisbee A, Embury SH, Farrer LA, Steinberg MH. Association of single nucleotide polymorphisms in klotho with priapism in sickle cell anaemia. *Br J Haematol* 2005;128:266-72.
- Sharan K, Surrey S, Ballas S, Borowski M, Devoto M, Wang KF, Sandler E, Keller M. Association of T-786C eNOS gene polymorphism with increased susceptibility to acute chest syndrome in females with sickle cell disease. *Br J Haematol* 2004;124:240-3.
- Ulug P, Vasavda N, Awogbade M Juliette Cunningham, Stephan Menzel, Swee Lay Thein. Association of sickle avascular necrosis with bone morphogenetic protein 6. *Ann Hematol* 2009;88:803-5.
- Callahan JF, Burgess JL, Fornwald JA, Gaster LM, Harling JD, Harrington FP, Heer J, Kwon C, Lehr R, Mathur A, Olson BA, Weinstock J, Laping NJ. Identification of novel inhibitors of the transforming growth factor- β 1 (TGF- β 1) type 1 receptor (ALK5). *J Med Chem* 2002;45:999-1001.