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The *ITGAV rs3738919-C* allele is associated with rheumatoid arthritis in the European Caucasian population: a family-based study

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ABSTRACT

The integrin α v β 3, whose α v subunit is encoded by the *ITGAV* gene, plays a key role in angiogenesis. Hyper-angiogenesis is involved in rheumatoid arthritis (RA) and *ITGAV* is located in 2q31, one of the suggested RA susceptibility loci. Our aim was to test the *ITGAV* gene for association and linkage to RA in a family-based study from the European Caucasian population.

Two single nucleotide polymorphisms were genotyped by PCR-restriction fragment length polymorphism in 100 French Caucasian RA Trio families (one RA patient and both parents), 100 other French families and 265 European families being available for replication. The genetic analyses for association and linkage were performed using the comparison of allelic frequencies (AFBAC), the transmission disequilibrium test (TDT), and the genotype relative risk (GRR).

We observed a significant RA association for the *C* allele of rs3738919 in the first sample (AFBAC RA index cases 66.5% vs. controls 56.7%, $P = 0.04$). The second sample showed the same trend and the third sample showed again a significant RA association. When all sets were combined the association was confirmed (AFBAC RA index cases 64.6% vs. controls 58.1%, $P = 0.005$). The rs3738919-*C* allele was also linked to RA (TDT 56.5% of transmission vs. 50%,

$P = 0.009$) and the *C* containing genotype was more frequent in RA index cases than in controls (RA index cases 372 vs. controls 339, $P = 0.002$, odds ratio [OR] = 1.94; 95% confidence interval [CI]: 1.3 to 2.9).

The *rs3738919-C* allele of *ITGAV* is associated with RA in the European Caucasian population, suggesting *ITGAV* as a new minor RA susceptibility gene.

Introduction

Rheumatoid arthritis (RA) is the most common human systemic autoimmune disease (0.8% prevalence in the European Caucasian population) affecting preferentially women [1]. It is characterized by a chronic inflammation of the synovial tissues leading to the formation of the rheumatoid pannus, which erodes adjacent cartilage and bone, causing subsequent joint destruction. One hallmark of the pannus is hyper-angiogenesis [2].

Previous studies have indicated that the risk of developing the disease in siblings of affected individuals is 2–17 times higher than in the general population, suggesting the importance of genetic factors [1]. Two RA genes have been established so far and confirmed using familial material, *HLA-DRB1* and *PTPN22* [3, 4], but they account only for a part of the RA genetic component. The dense genome scan realized in our laboratory suggested 19 non-*HLA* regions in the French Caucasian population [5] and one of these, 2q31, contains the *ITGAV* gene (alias *CD51*, *alphav*), which encodes the alphav subunit of the integrin family. This family is composed of at least 24 heterodimeric combinations of 18 alpha and 9 beta subunits. These transmembranous receptors are expressed at the surface of numerous cells (endothelial cells, macrophages, monocytes, osteoclasts, platelets) and recognize the RGD sequence (Arg-Gly-Asp) of many ligands (such as vitronectin, fibronectin, osteopontin, sialoprotein,

thrombospondin, fibrinogen, von Willebrand factor, tenascin, agrin, matrix metalloproteinases, and prothrombin) [6]. The integrins are involved in several functions including adhesion of activated endothelial cells with the extra cellular matrix, proliferation, migration and differentiation signals of vascular cells [6].

The alphavbeta3 integrin is well documented to play a key role in angiogenesis, and the *ITGAV* knock out animal model is lethal in utero for 80% with presence of large vascular anomalies [7, 8].

Angiogenesis also plays a key role in RA where the synovial membrane becomes hyperplastic and destroys the cartilage. We can observe an excess of blood cells (macrophages, T lymphocytes) in the synovial membrane and fluid, and some alphavbeta3 ligands (i.e. fibrinogen or osteopontin) are abundant in the RA synovial fluid [7]. Moreover, some pro-angiogenic mediators (i.e. vascular endothelial growth factor) are over expressed in RA synovial and serum [9, 10].

In addition, several alphavbeta3 antagonists and angiogenesis inhibitors were successfully tested on RA animal models [11, 12, 13, 14]. Thus, alphavbeta3 could become a new therapeutic target in RA and some clinical studies have already started [15].

Our aim was to use RA familial material to test two intronic *ITGAV* single nucleotide polymorphisms (SNP) for RA association and linkage in the European Caucasian population.

Materials and methods

All subjects provided informed consent, and the ethics committee of Hôpital Bicêtre (Kremlin-Bicêtre, Assistance Publique-Hôpitaux de Paris, France), approved the study. RA families were recruited through a national media campaign followed by selection of individuals who fulfilled the 1987 American College of Rheumatology criteria for RA according to the physicians in charge of the patients [16]. A rheumatologist university fellow reviewed all clinical data.

Sample 1 (Table 1)

The sample 1 was constituted with the DNA of 100 French Caucasian unrelated Trio families (one patient and both parents) with the four grand-parents of French Caucasian origin. Among the 100 RA patients, 87 were women; their mean age at disease onset was 32. In all, 81 were rheumatoid factor (RF) positive, 78 carried at least one *HLA-DRB1* 'shared epitope' (SE) susceptibility

alleles (DRB1*0101, 0102, 0401, 0404, 0405, 0408, 1001) [17] and 90 presented erosion.

Sample 2 (Table 1)

The sample 2 was made with the DNA of 100 French Caucasian unrelated Trio families of same characteristics as in sample 1. Among the 100 RA patients, 90 were women; their mean age at disease onset was 31 years. In all, 76 were RF positive, 80 carried at least one *HLA-DRB1* SE and 79 had an erosive disease.

Sample 3 (Table 1)

The sample 3 contained the DNA of 265 European Caucasian unrelated Trio families of same characteristics than sample 1, excepted for the shorter mean disease duration and the ethnic origin (Caucasian families from France, Italy, Portugal, Spain, Belgium, and Netherlands).

Genotyping

DNA was isolated and purified from whole blood according to standard protocols [18]. Two intronic SNPs were selected in the 5' and 3' ends of the gene with a minor allele frequency > 25% for European population databases.

Moreover the presence of a restriction site for one of the allele was required (SNP1: rs3768777, SNP2: rs3738919, [19, 20]). Genotyping was performed by the polymerase chain reaction followed by restriction fragment length polymorphism (PCR-RFLP) method [21]. The designed primers were: sense 5'AAGTTGCCAACGTTCCGCGTTGCA3' and anti-sense 5'GTAGTAGAAGATGGTCCTATCCACG3', sense 5'ATTTCAGGTGGAAGTCTTTTGGGA3' and anti-sense 5'TCACAATTCAGATTTTGGCCACTGG3' for SNP1 and 2 respectively. PCR amplification of SNP1 and SNP2 was performed on each sample in a 25 µl reaction volume consisting of 10U PCR buffer (Perkin Elmer, Boston, MA, USA), 1.25 mM of each dNTP, 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), 3 mM MgCl₂, 0.0125 nM of the two primers and 50 ng of genomic DNA, diluted to the final volume with H₂O on Eppendorf thermocycler using a hot start procedure. The PCR program was carried out using a first denaturation cycle of 94°C for 10 minutes followed by 37 cycles of denaturation at 94°C for 40 s, with annealing temperature at 67°C for 30 s followed by an elongation step at 72°C for 1 min. One final cycle of the extension was performed at 72°C for 2 min.

For the SNP1, a 341-bp amplified fragment was digested with *Nla*III, generating two fragments when the restriction site was present (A allele). For the SNP2, the resulting 501-bp fragment was digested with *Alu*I, generating 3 fragments for

the C allele (126-bp, 161-bp, 214-bp), and 2 for the A allele (permanent restriction site allowing to validate the restriction protocol, 161-bp, 340-bp). Genotypes were assessed blindly by 2 independent investigators (LJ and CP). CEPH controls (1347-02 and 884-15) and 40 patients choose at random were genotyped for quality control. All genotype data will be available [22].

Power calculation

Using the European population minor allele frequency of 29% and 35% for SNP1 and SNP2 respectively, a sample size of 100 patients and 100 controls, and the arc sinus transformation method precedently described by Garnier *et al.* [23], we had a 80% power to detect an association ($P < 0.05$) if the difference in allelic frequencies between patients and controls was at least of 11% for SNP1 and 12.2% for SNP2.

Statistical analysis

Prior to association tests, we checked the Hardy–Weinberg equilibrium in “virtual controls” (constituted by parental un-transmitted alleles to RA index cases).

Association and linkage between each polymorphism and RA was examined by three different methods: the affected family-based controls (AFBAC) was used

to compare transmitted and untransmitted allelic frequencies across all families, the transmission disequilibrium test (TDT) to detect linkage through preferential transmission of one allele to the affected subjects, and the genotype relative risk (GRR) to compare the genotypic distribution in patients and controls [24, 25, 26]. Significance of p-value was assessed at 5% and led to replication tests in the upper described sample 2 and, in case of relevant results, in the larger sample 3.

Results

Hardy–Weinberg equilibrium

The Hardy-Weinberg equilibrium in the virtual controls was respected for SNP1 and SNP2 in the sample 1 and in the replication samples (data not shown).

Test for association and linkage in the sample 1 (Table 2, 3)

We observed neither significant association nor linkage between SNP1 and RA in the sample 1.

For SNP2, we observed a significant association for the *C* allele and a strong trend for a RA linkage (AFBAC RA index cases 66.5% vs. controls 56.7%, $P = 0.04$, TDT 59.7% of transmission vs. 50%, $P = 0.06$). The GRR test showed a

significant increase of the *C/C* genotype and an excess of *C* containing genotypes in patients.

The linkage disequilibrium (LD) test showed a weak LD between SNP 1 and SNP 2 ($D' = 0.33$) thus considered independent. The results of the haplotypic TDT analysis showed a significant under transmission of the SNP1/SNP2 *GA* haplotype (21 vs. 37, $P = 0.03$), and a trend for an over transmission of the two haplotypes containing the *C* allele of SNP2 (data not shown).

When stratifying the sample for the families with index presenting at least one *PTPN22-620W* allele or the *HLA-DRB1* allele shared epitope status, no correlation with the *ITGAV* genotypes could be observed (data not shown).

Test for association and linkage in the sample 2 (Table 4, 5)

The significant association observed for SNP2 in the sample 1 led to a replication test in a second set of 100 French Caucasian Trio families (sample 2) on the hypothesis of an association of the *C* allele.

In this sample, we observed a trend for association and linkage of the *C* allele with RA (AFBAC RA index cases 63.1% vs. controls 59.6%, $P = 0.4$, TDT 52.6% of transmission, $P = 0.6$). The GRR test showed a trend for an excess of

the *C* containing genotype in RA index cases compared to controls (90 RA index cases *vs.* 79 controls, $P = 0.09$) but not for the *C/C* genotype.

The combination of the 2 samples, authorized by the absence of significant clinical difference between them, showed a marginally significant association of the *C* allele (AFBAC RA index cases 64.8% *vs.* controls 58.2%, $P = 0.05$, TDT 56.1% of transmission, $P = 0.09$) and a significant excess of the *C* containing genotype in RA index cases compared to controls (173 RA index cases *vs.* 157 controls, $P = 0.02$).

Test for association and linkage in the sample 3 (Table 6, 7)

The trend for association of the *C* allele observed in the sample 2 was in the same direction than the significant association observed in the sample 1, without reaching statistical significance, notably due to a lack of power (the power to detect a significant association in the sample 2, based on the allelic frequencies in the sample 1, with $P < 0.05$, was only 51%). Thus, a larger replication test (265 families, sample 3) was conducted on the hypothesis of an association of the *C* allele and of the *C* containing genotype.

We observed a significant RA association and linkage for the *C* allele (AFBAC RA index cases 64.4% *vs.* controls 57.8%, $P = 0.03$, TDT 57% of transmission

vs. 50%, $P = 0.04$), which was supported by a significant increase of the *C* containing genotype in patients (199 RA index cases vs. 182 controls, $P = 0.02$).

Test for association and linkage in the combined sample 1 + 2 + 3 (Table 8, 9)

The combination of the three samples, authorized by the absence of significant clinical difference between them, confirmed association and linkage for the *C* allele (AFBAC 64.6% vs. 58.1%, $P = 0.005$, TDT 56.5% of transmission, $P = 0.009$). The GRR showed an excess of the *C* containing genotype in patients (372 RA index cases vs. 339 controls, $P = 0.002$, OR = 1.94; 95% CI: 1.3 to 2.9).

Discussion

We studied the *ITGAV* gene, a good RA candidate gene for its function implicated in angiogenesis, and its chromosomal location (in one of the 19 non-*HLA* suggested loci of our dense genome scan) [5]. We observed a significant RA association for the *C* allele of rs3738919 in a first sample of French Caucasian families, a same trend in the replication sample 2, and again a significant association in the replication sample 3 (European Caucasian families). Finally, significant RA association and linkage were observed when all sets were combined.

The association and linkage evidences provided by this study remain nevertheless statistically modest, suggesting at most a minor RA susceptibility marker. Further studies in independent samples will be needed to definitively establish association and linkage of the *ITGAV* *rs3738919-C* allele to RA. For the observed allelic frequencies of 64.6% in patients vs. 58.1% in controls, a sample size of 350 families would be required to obtain, with 80% power ($P < 0.05$), an independent replication of the association evidence reported here. Once this association would have been replicated, resequencing will be necessary to identify exonic and promoter SNP to refine the associated haplotype.

In the same way, the chromosome 2 linkage suggestion observed in the genome scan of our laboratory could not be totally explained by the findings of the *ITGAV* linkage hence with the overtransmission observed in the TDT, the allele sharing expected for the Affected Sib-Pairs (ASP) siblings would be about 53% and would necessitate thousands of sibling pairs to be revealed. Other RA genes in this chromosomal location and/or epistatic effects could be expected to be stronger RA factors which remain to be discovered.

Because this association is modest, no genetic testing would be clinically indicated. Instead, the clinical relevance of the finding is likely to come through

the better understanding of RA pathophysiology and may lead to new therapeutic targets.

Contrary to the result of the GRR test in the sample 1 which suggested a recessive effect of the *ITGAV rs3738919-C* allele, the result of the larger combined sample is more in favour of a dominant effect of this marker, and this could be explained by the relative small size of the first sample.

Finally, regarding the key function of angiogenesis in others diseases, and in particular in cancers, it would be interesting to test the *ITGAV rs3738919-C* allele in these phenotypes.

Conclusion

Our study showed a significant association and linkage for the *rs3738919-C* allele of the *ITGAV* gene with RA in the European Caucasian population, suggesting *ITGAV* as a new minor RA susceptibility gene in this population.

List of abbreviations

RA = rheumatoid arthritis, AFBAC = affected family-based controls, TDT = transmission disequilibrium test, GRR = genotype relative risk, PCR =

polymerase chain reaction, RFLP = restriction fragment length polymorphism,
SNP = single nucleotide polymorphism.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LJ, CP, EG and SG carried out the molecular genetic studies. LJ, CP, SB, SG, PD, LM, HM, VHT, BP, EPT and FC performed acquisition and analysis of the data. LM, SL, IL, PQ, PH, PM, AB, RW, PB, HA, CV, MF, DP-S, SB, JD, TRR, PVR, LvdP, AL-V, TB, and ECRAF contributed to the recruitment of families and to the acquisition of clinical data. All authors read and approved the final manuscript.

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Table 1. Characteristics of rheumatoid arthritis (RA) index cases from the investigated samples

n: number of cases, NA: non available

	<u>Sample 1 (<i>n</i> = 100)</u>	<u>S.2 (<i>n</i> = 100)</u>	<u>S.3 (<i>n</i> = 265)</u>
Females (%)	87	90	86
Mean age (\pm standard deviation) disease onset (years)	32 (\pm 10)	31 (\pm 6)	30 (\pm 9)
Mean (\pm standard deviation) disease duration (years)	18 (\pm 7)	16 (\pm 8)	8 (\pm 7)
RA patients with bone erosions (%)	90	79	72
RA patients seropositive for rheumatoid factor (%)	81	76	73
RA patients carrying at least one <i>HLA-DRB1</i> shared epitope allele (%)	78	80	NA

Table 2. AFBAC and TDT analysis for the SNP1 and SNP2 in the sample 1 of RA Trio families

n: number of heterozygote parents

<u>Alleles</u>	<u>AFBAC</u>		<i>P</i>	<u>TDT</u>		
	<u>RA cases</u>	<u>Controls</u>		<u>% of Trans</u>	<i>n</i>	<i>P</i>
SNP1 A	0.360	0.320	0.39	54.4	90	0.39
<i>G</i>	0.640	0.680				
SNP2 C	0.665	0.567	0.04	59.7	92	0.06
<i>A</i>	0.335	0.433				

Table 3. GRR analysis for the SNP1 and SNP2 in the sample 1 of RA Trio families

<u>Genotypes</u>	<u>RA cases</u>	<u>Controls</u>	<u>P</u>
SNP1 A/A	16	7	0.1 (Global)
A/G	40	50	
G/G	44	43	
SNP2 C/C	45	32	0.03 (C/C vs. C/A + A/A)
C/A	38	46	0.36 (C/C + C/A vs. A/A)
A/A	13	18	

Table 4. AFBAC and TDT analysis for SNP2 in the sample 2 of RA Trio families

n: number of heterozygote parents

<u>Allele</u>	<u>AFBAC</u>			<u>TDT</u>		
	<u>RA cases</u>	<u>Controls</u>	<u>P</u>	<u>% of Trans</u>	<u>n</u>	<u>P</u>
SNP2 C	0.631	0.596	0.4	52.6	95	0.6

Table 5. GRR analysis for SNP2 in the sample 2 of RA Trio families

<u>Genotypes</u>	<u>RA cases</u>	<u>Controls</u>	<u>P</u>
SNP2 C/C	33	39	0.4 (C/C vs. C/A + A/A)
C/A	57	40	0.09 (C/C + C/A vs. A/A)
A/A	8	19	

Table 6. AFBAC and TDT analysis for SNP2 in the sample 3 of RA Trio families

n: number of heterozygote parents

<u>Allele</u>	<u>AFBAC</u>			<u>TDT</u>		
	<u>RA cases</u>	<u>Controls</u>	<u>P</u>	<u>% of Trans</u>	<u>n</u>	<u>P</u>
SNP2 C	0.644	0.578	0.03	57	200	0.04

Table 7. GRR analysis for SNP2 in the sample 3 of RA Trio families

<u>Genotypes</u>	<u>RA cases</u>	<u>Controls</u>	<u>P</u>
SNP2 C/C	88	76	0.2 (C/C vs. C/A + A/A)
C/A	111	106	0.02 (C/C + C/A vs. A/A)
A/A	22	39	

Table 8. AFBAC and TDT analysis for SNP2 in the combined sample 1 + 2 + 3

n: number of heterozygote parents

<u>Allele</u>	<u>AFBAC</u>			<u>TDT</u>		
	<u>RA cases</u>	<u>Controls</u>	<u>P</u>	<u>% of Trans</u>	<u>n</u>	<u>P</u>
SNP2 C	0.646	0.581	0.005	56.5	387	0.009

Table 9. GRR analysis for SNP2 in the combined sample 1 + 2 + 3

<u>Genotypes</u>	<u>RA cases</u>	<u>Controls</u>	<u>P</u>
SNP2 C/C	166	148	0.1 (C/C vs. C/A + A/A)
C/A	206	191	0.002 (C/C + C/A vs. A/A)
A/A	43	76	