LETTER TO JMG

Association of single nucleotide polymorphisms in the oxidised LDL receptor 1 (OLR1) gene in patients with acute myocardial infarction

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Acute myocardial infarction (AMI) is a significant cause of mortality and morbidity. Substantial data support a plausible role for oxidised LDL (oxLDL) in the aetiology of this disease.[1 2] The human OLR1 (or LOX 1) gene encodes the endothelium derived lectin-like oxidised low density lipoprotein (oxLDL) receptor, which is involved in the binding, internalisation, and proteolytic degradation of oxLDL, suggesting that it may play a significant role in atherogenesis.[3] OLR1 is considered a good candidate for atherosclerosis and AMI since it is induced in vitro by inflammatory cytokines and in vivo by pro-atherogenic conditions like hypertension, hyperlipidaemia, and diabetes mellitus.[4] Recently, upregulation of OLR1 has been shown in ischaemia reperfusion injury in the rat.[5] OLR1 acts as a mediator of ''endothelial dysfunction'' favouring superoxide generation, inhibiting nitric oxide production, and enhancing endothelial adhesiveness for monocytes.[6–8] It is noteworthy that the versatile activities of OLR1 also include the ability to bind not only oxLDL, but also aged red blood cells, apoptotic cells, and activated platelets.[4] With this background, we sought to validate the hypothesis of OLR1 involvement in atherosclerosis and AMI by defining OLR1 genetic variation by an association study of intragenic SNPs.

Key points

- OLR1 is a cell surface endocytosis receptor for atherogenic oxidised LDL (oxLDL), which is strongly expressed in endothelial cells. Recent studies suggest that it may be involved in the development of atherosclerosis and its complications.
- We genotyped 253 individuals for OLR1 gene polymorphisms: 150 individuals with acute myocardial infarction (AMI) and 103 healthy controls.
- We identified six novel single nucleotide polymorphisms (SNPs) within intron 4, intron 5, and the 3′UTR. The six polymorphisms were in complete linkage disequilibrium.
- Carriers of the T allele (CT/TT) at the SNP located in the 3′ UTR (C>T, 188 bp from the stop codon) exhibited a significant association with AMI ($p<0.0001$) with an odds ratio (OR) of 3.74.

METHODS

Study subjects

The study included 150 individuals with AMI who were referred to the Centre of Atherosclerosis at the Medical School of the Tor Vergata University of Rome. All cases were clinically evaluated and all underwent coronary angiography and left ventriculography. The diagnosis of AMI was based on typical electrocardiographic changes and increased serum activities of at least two enzymes, such as creatine kinase, aspartate aminotransferase, and lactate dehydrogenase. The diagnosis was confirmed by the presence of wall motion abnormality on left ventriculography and attendant stenosis (>50%) in any of the major coronary arteries or in the left main coronary artery on coronary angiography. One hundred and three control subjects were recruited from persons found to have at least one conventional risk factor for coronary artery disease (CAD), such as cigarette smoking (10 cigarettes daily), hypertension (systolic blood pressure of 140 mm Hg or diastolic blood pressure 90 mm Hg), diabetes mellitus (blood glucose level 126 mg per decilitre (6.93 mmol per litre)), hypercholesterolaemia (total serum cholesterol level 220 mg per decilitre (5.72 mmol per litre)). These subjects had no evidence of active myocardial ischaemia, but underwent left ventriculography and coronary angiography as part of valvular disease. All these control subjects were found to have normal coronary arteries. The mean age of the AMI patients was 64.4 (5.8 years) (mean (SD)) with a mean age at onset of symptoms 60.4 (4.5) years. The mean age of the control subjects was 63.4 (6.2) years. The study was approved by the Tor Vergata University Ethics Committee.

SNP discovery and genotyping

All six exons and intron/exon boundaries of OLR1 were screened using denaturing high performance liquid chromatography (DHPLC, Transgenomic, Crewe, UK) in 50 AMI patients and 50 controls according to the conditions reported in table 1. Identified SNPs were characterised by sequencing on a CEQ2000 automated sequencer (Beckman Coulter Inc., USA). All samples were genotyped for the OLR1/3′UTR and the K167N polymorphisms by allelic discrimination assays. We designed primers and MGB probes (OLR1/ 3′UTR forward primer: 5′-GCCTGGCACCTTTATGTCAAC-3′; OLR1/3′UTR reverse primer: 5′-CTTGGGACAAGCTAGGTGAAATAATA-3′; 3′UTR/C allele MGB probe : 5′-FAM-TTTTTGATTCTAGCTACCTG-3′; 3′UTR/T allele MGB probe: 5′-VIC-ATTTTTGATTCTAGCTATCTG-3′; K167N forward primer: 5′-GCAACTTGGCATCCAAAGACA-3′; K167N reverse primer: 5′-CCTATTTTCCTCGGGCTCATT-3′; K167N/G allele 5′-FAM-TTCTCTTGGCTCTTTT-3′; K167N/C allele 5′-VIC-CTTCTCTTGGCTGTTTT-3′) using Primer Express 2.0 software (Applied Biosystems, Foster City, CA). PCR amplification was carried out in a reaction

Abbreviations: AMI, acute myocardial infarction; CAD, coronary artery disease; OLR1, oxidised LDL receptor 1; OR, odds ratio; oxLDL, oxidised LDL; SNP, single nucleotide polymorphism; TNF, tumour necrosis factor

Table 1 Primers, PCR, and DHPLC conditions used for the analysis of the OLR1 gene

OLR1	Fragment (bp)	Primers (5′-3′)	PCR conditions	DHPLC conditions
Exon 1	238	F: acttctgcagaagctcac R: ctaatcccattcttcggtg	94°C 15 s, 54°C 30 s, 72°C 30 s over 35 cycles	57°C
Exon 2	230	F: ctccaaggaatggtttgctt R: taacctcatttccaacacccc	94°C 15 s, 60°C 30 s, 72°C 30 s over 35 cycles	59°C
Exon 3	355	F: cctggagggatttttatttgg R: tgattggacagaaagcttcca	94°C 15 s, 60°C 30 s, 72°C 30 s over 35 cycles	59°C
Exon 4	333	F: atgcacgtgagagactaaggg R: tggctctcaaacaagaattcc	94°C 15 s, 62°C 30 s, 72°C 30 s over 35 cycles	58°C
Exon 5	263	F: cagtcaaggggatgtcaaaga R: gaggcatcaaaaagaatggg	94°C 15 s, 60°C 30 s, 72°C 30 s over 35 cycles	59°C
Exon 6	400	F: tgaaggctctggaagaaaagg R: tgtgaggggaaggtgataatg	94°C 15 s, 59°C 30 s, 72°C 30 s over 35 cycles	58°C
3′UTR	403	F: tgaatttgaaggctctggaag R: tttttgctctctgcctgcact	94°C 15 s, 60°C 30 s, 72°C 30 s over 35 cycles	58°C

volume of 25 µl containing 900 nM of each primer, 200 nM of each probe, 12.5 µl of TaqMan universal Master Mix (Applied Biosystems) and 50 ng of genomic DNA. PCR was performed on an ABI prism 7000 sequence detection system (Applied Biosystems) using the following conditions: after enzyme activation for 10 min at 95°C, 40 two step cycles were performed; 15 s denaturation at 95°C followed by 1 min annealing/elongation at 60°C for both OLR1/3′UTR and K167N polymorphisms. The allelic specific fluorescence was measured on the ABI prism 7000 sequence detection system using the ABI Prism 7000 SDS software. The genotype calls were attributed automatically.

Statistical analysis

Differences in genotype and allele proportion between patients and controls and differences between observed and expected genotype frequencies (assuming Hardy-Weinberg equilibrium) were evaluated by Pearson's χ^2 test of independence. Linear association was assessed using the Mantel-Haenszel test. Analysis of risk factors was also conducted using the χ^2 test and discriminant analysis. All these statistical analyses (and the OR determination) were performed using SPSS (Statistical package for social sciences, SPSS Inc., Chicago, IL). Haplotype frequencies were maximum-likelihood estimates (MENDEL software, Department of Biostatistics, University of Michigan, Ann Arbor, MI). The disequilibrium coefficient (D) was calculated as:
(product of coupling types) ÷ (product of repulsion types).

RESULTS

To identify SNPs in the OLR1 gene, we screened all exons and intron/exon boundaries in 50 patients and 50 controls using DHPLC. The samples that showed an anomalous elution profile were sequenced to characterise the allelic variants. We identified seven SNPs in the OLR1 gene using this method. These SNPs were then genotyped in the sample using allelic discrimination assays. Allele and genotype frequencies of the seven studied polymorphisms are reported in table 2 for AMI patients and control subjects. All genotype distributions were compatible with Hardy-Weinberg equilibrium. Among the seven polymorphisms, six of them (located within introns 4, 5 and 3′UTR) comprised a complete linkage disequilibrium block behaving as a single SNP. This suggests the existence of a linkage disequilibrium block which includes at least intron 4, exon 5, intron 5 and 3′UTR. The OLR1/3′UTR polymorphism (C>T, 188 bp from the stop codon) showed the most significant association when AMI patients and controls were compared ($p<0.0001$) (table 2). Genotypes with the T allele (CT or TT) at this SNP were found in 91.3% of the patients compared with 73.8% of the controls, ($p = 0.0003$) which resulted in an odds ratio (OR) of 3.74 (95% confidence limits: 1.73 and 8.18). The SNP located in exon 4, which produces a conservative amino acid change (K>N at codon 167) demonstrated a weak association. We also performed haplotype analysis considering the OLR1/3′UTR and the K167N polymorphisms but the association found with AMI was not higher than that observed considering OLR1/3′UTR

Table 2 Description of studied OLR1 SNPs and distribution of their genotypic and allelic frequencies in controls and AMI patients*

			Genotypic frequencies			Total no	χ^2	df	p	p^	*Allelic frequencies*		χ^2	df	p
Location	**Substitution**		***GG***	***GC***	***CC***						***G***	***C***			
Exon 4	K167N (501G>C)	**Controls**	0.825	0.165	0.010	103					0.908	0.092			
		Patients	0,913	0,087	0,000	150	5.16	2	0.07	0.03	0.957	0.043	4.139	1	0.04
			CC	***CT***	***TT***						***C***	***T***			
3′UTR	*188 C>T	**Controls**	0.262	0.573	0.165	103					0.548	0.452			
		Patients	0.087	0.546	0.367	150	20.69	2	<0.0001	0	0.360	0.640	16.89	1	0.0004
Intron 4	IVS4+27 G>C														
Intron 4	IVS4−73 C>T														
Intron 4	IVS4−14 A>G		genotypic and allelic frequencies are identical to the 3′UTR *188 C>T												
Intron 5	IVS5−70 A>G														
Intron 5	IVS5−27 G>T														

*Deposited in HGVbase (http://hgvbase.cgb.ki.se/)
p^ refers to Mantel-Haenszel test of linearity;

Table 3 Percentage distribution of risk factors in controls and in AMI patients and comparisons of each risk factor with SNPs

Risk factor		Controls	Patients	p	Comparison with K167N	Comparison with *188 C>T
Hypertension	Absent	41.4%	40.7%	ns	ns	ns
	Present	58.6%	59.3%			
Diabetes	Absent	94.3%	75.9%	0.001	ns	ns
	Present	5.7%	24.1%			
Hyperlipidaemia	Absent	65.7%	38.4%	0	ns	ns
	Present	34.3%	61.6%			
Cigarette smoking	Absent	64.3%	28.3%	0	ns	ns
	Present	35.7%	71.7%			
Familiarity	Absent	82.9%	62.4%	0.002	ns	ns
	Present	17.1%	37.6%			
Sex	Male	56%	72%			
	Female	44%	28%	0.01	ns	ns

ns, not significant.

polymorphism alone. Since this analysis does not add further information to the association, these data are not included. To explore the interaction of OLR1 SNPs on different risk factors, we performed the χ^2 test which revealed a complete independence of each single risk factor (table 3) from SNPs detected. When all risk factors (K167N, OLR1/3'UTR polymorphism and CAD risk factors) were considered by discriminant analysis (direct method), a significant separation between patients and controls subjects was observed ($p<0.0001$). The two variables that mainly contributed to the discrimination of the two groups were cigarette smoking and OLR1/3'UTR polymorphism with a correlation of 0.54 and 0.47, respectively. Significance of contribution of a variable to separation of the two groups was also evaluated by the change in Rao's V produced by entering the variable into the discriminant function. The results confirmed that cigarette smoking and OLR1/3'UTR polymorphism showed the highest significance levels.

DISCUSSION

The identification of susceptibility genes is of major biomedical interest. Genetic association studies and linkage analysis have identified several genetic loci involved in atherosclerosis and its major life threatening complication, the acute coronary syndrome.[9–11] The present study provides support for a significant novel genetic component to the development of AMI. There is accumulating evidence that acute coronary syndromes relate to the activation of chronic inflammation in the stable atherosclerotic plaque resulting in abrupt rupture. The vulnerable plaque has been shown to contain large amounts of oxLDL, over expression of its newly described lectin-like receptor OLR1, and a large number of monocytes/macrophages. Recent studies suggest that OLR1 expression is also upregulated in the myocardium in acute myocardial ischaemia.[5] OLR1 specifically interacts with oxLDL, but not the native LDL, suggesting that OLR1 expression may be involved in atherogenesis. The expression of OLR1 is upregulated by various pro-atherosclerotic stimulants, including angiotensin II, TNF-α, PMA, and lysophosphatidytilcholine.[12] These observations suggest that OLR1 may be expressed locally and play important roles in inflammatory responses in vivo and rupture of the atherosclerotic plaque resulting in acute coronary syndrome.[13] Further evidence that OLR1 is directly involved in the atherogenesis comes from inbred strains of LDLr−/− mice and Olr1 knockout mice (JL Mehta et al, unpublished data) in which the development of atherosclerosis is attenuated. Interestingly, Welch et al[9] identified and mapped a susceptibility to atherosclerosis locus in mice (Athsq2) in a syntenic region with the human chromosome 12p13–12, which contains the OLR1 gene. The present study was designed to verify whether OLR1 SNPs influence the AMI susceptibility in the Italian population. We observed that the OLR1/3'UTR SNP genotype and allele frequencies were differed significantly between the control and the AMI groups. Our data showed that subjects with the T/T or C/T genotype at OLR1/3'UTR polymorphism are at higher risk of developing acute AMI (OR, 3.74). The K167N polymorphism, despite its weak association, causes an amino acid substitution at codon 167 (Lys>Asn) which is located within the C-type lectin domain of the protein. Replacement of this Lys residue causes reduced binding and internalisation of oxLDL.[14] Therefore, we can speculate that this SNP could exert a protective effect. However, recently, Tatsuguchi et al[15], has reported a positive association of the K167N polymorphism with the risk of myocardial infarction in a sample of Japanese patients. This discrepancy may be attributed to a different genetic background in the population studied, or to the different selection of the patients and healthy controls. During the preparation of the present manuscript, Chen et al[16] reported the association of three OLR1 SNPs with the risk of CAD in white women. The three OLR1 polymorphisms studied by these authors correspond to our SNPs. These authors reported an odds ratio of 1.67 ($p = 0.025$) and an absence of interaction between the OLR1 and APOE genes. Notably, they observed that the OLR1/3'UTR polymorphism may affect the binding of a putative transcription factor, as deduced by the electrophoretic mobility shift assay. This study further supports the function of OLR1 as a risk related gene for heart disease.

Many studies have examined the relations between polymorphisms and coronary artery disease or myocardial infarction. The results of most of these studies, however, remain controversial, with no consensus in their implications, mainly because of the limited size of the study populations, the ethnic diversity of polymorphisms, and complicating environmental factors. In this context, OLR1 seems to have all the features required (large sample sizes, small p values, SNPs with putative biological effects, independent replication of studies) to be of clinical relevance. The association reported in the present study confirms the previously reported association of the OLR1/3'UTR SNP and so it provides credence to the hypothesis that the OLR1 gene product is involved in the pathogenesis of AMI.

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