BMC Biology

Research article

A single-nucleotide polymorphism in the human p27^{kip1} gene (-838C>A) affects basal promoter activity and the risk of myocardial infarction

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Published: 02 April 2004

BMC Biology 2004, **2**:5

Received: 19 December 2003
Accepted: 02 April 2004

This article is available from: http://www.biomedcentral.com/1741-7007/2/5

Abstract

Background: Excessive proliferation of vascular smooth muscle cells and leukocytes within the artery wall is a major event in the development of atherosclerosis. The growth suppressor p27^{kip1} associates with several cyclin-dependent kinase/cyclin complexes, thereby abrogating their capacity to induce progression through the cell cycle. Recent studies have implicated p27^{kip1} in the control of neointimal hyperplasia. For instance, p27^{kip1} ablation in apolipoprotein-E-null mice enhanced arterial cell proliferation and accelerated atherogenesis induced by dietary cholesterol. Therefore, p27^{kip1} is a candidate gene to modify the risk of developing atherosclerosis and associated ischaemic events (i.e., myocardial infarction and stroke).

Results: In this study we found three common single-nucleotide polymorphisms in the human p27^{kip1} gene (+326T>G [V109G], -79C>T, and -838C>A). The frequency of -838A carriers was significantly increased in myocardial infarction patients compared to healthy controls (odds ratio [OR] = 1.73, 95% confidence interval [95%CI] = 1.12–2.70). In addition, luciferase reporter constructs driven by the human p27^{kip1} gene promoter containing A at position -838 had decreased basal transcriptional activity when transiently transfected in Jurkat cells, compared with constructs bearing C in -838 (P = 0.04).

Conclusions: These data suggest that -838A is associated with reduced p27^{kip1} promoter activity and increased risk of myocardial infarction.

Background

The proliferation of leukocytes and vascular smooth muscle cells (VSMCs) within the artery wall is a hallmark of the atherosclerotic process [1]. The exposure of coronary

arteries to chemical and mechanical injury triggers an inflammatory response characterized by excessive arterial cell proliferation. Progression through the cell cycle depends on the sequential activation of several cyclin-dependent kinases (CDKs). Activation of CDKs requires their interaction with regulatory subunits named cyclins. In resting cells, cyclin–CDK complexes are inhibited by the reversible association with CDK inhibitory proteins (CKIs) of the Cip/Kip family ($p21^{cip1}$, $p27^{kip1}$, and $p57^{kip2}$) and Ink4 family ($p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$, $p19^{Ink4d}$) [2].

In recent years, accumulating evidence has implicated $p27^{kip1}$ as an important endogenous regulator of leukocyte and VSMC proliferation in various pathophysiological situations [3-7]. Remarkably, $p27^{kip1}$ expression and proliferation of VSMCs and leukocytes are inversely correlated in human atheroma, and a significantly lower level of $p27^{kip1}$ expression has been reported in primary atherosclerotic and restenotic tissue versus nondiseased arterial tissue [8-10]. We have previously shown that fat-fed mice deficient in both apolipoprotein E (apoE) and $p27^{kip1}$ display increased arterial cell proliferation and accelerated atherogenesis compared to apoE-null mice with an intact $p27^{kip1}$ gene [11]. We also found that reconstitution of sublethally irradiated apoE-null mice with $p27^{kip1}$-deficient bone marrow was sufficient to enhance arterial macrophage proliferation and atherosclerosis induced by fat feeding [12]. In view of these findings, the present study was designed to assess whether $p27^{kip1}$ is a candidate gene to modify the risk of developing atherosclerosis and consecutive myocardial infarction (MI) in humans.

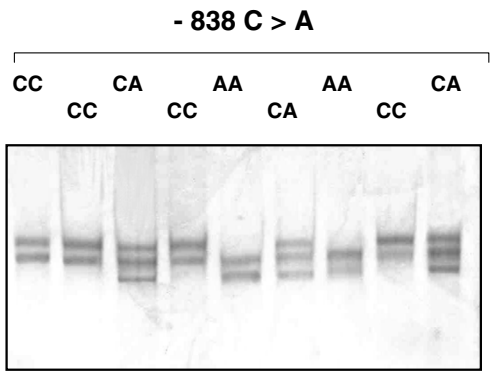

Figure 1
Single-strand conformation analysis of the -838C>A polymorphism within the human $p27^{kip1}$ gene. Note the distinct electrophoretic patterns for AA homozygotes, CC homozygotes, and CA heterozygotes.

Results

Identification of $p27^{kip1}$ polymorphisms and genotyping of MI patients and controls

The complete coding sequence as well as 1500 base pairs of the promoter region of the human $p27^{kip1}$ gene were analysed through single-strand conformation analysis (SSCA) in 50 MI patients and 50 controls. As shown in Fig. 1, we identified different electrophoretic patterns in $p27^{kip1}$ DNA fragments obtained with primers P3-F+P3-R3, P1-F+P1-R, and E1-F+E1-R (Table 1). After direct sequencing, we identified three single-nucleotide polymorphisms (SNPs) within the human $p27^{kip1}$ gene: +326T>G (V109G), -79C>T, and -838C>A (nucleotide positions numbered relative to the start of transcription according to Genbank accession number AB003688). +326T>G (V109G) and -79C>T have been previously described (rs2066827 and rs34330, respectively), and -838C>A is a novel polymorphism. +326T>G (V109G) is a missense polymorphism in exon 1 which has been previously analysed in the context of susceptibility to several types of cancer [13-15]. This polymorphism lies within the domain of $p27^{kip}$ that is required for interaction with $p38^{jab1}$, a protein–protein association that promotes $p27^{kip1}$ proteolysis [16]. The -79C>T is a polymorphism within a U-rich element in the 5' untranslated sequence of $p27^{kip1}$ which is necessary for the translation of the $p27^{kip1}$ mRNA [17].

To genotype these polymorphisms in MI patients and controls, we developed a protocol based on endonuclease digestion of DNA fragments obtained by amplification with the primers shown in Table 2. Table 3 summarizes the genotype and allele frequencies for the three polymorphisms in 180 MI patients and 250 controls. The frequencies for the -79C>T and +326T>G (V109G) polymorphisms did not differ between the two groups. For the -838C>A polymorphism, the A allele was significantly more frequent in cases compared to control subjects ($P = 0.002$; OR = 1.53; 95%CI = 1.15–2.03). Carriers of the A allele (AA+AC genotypes) would have an almost twofold risk of suffering an episode of MI compared to controls ($P = 0.009$; OR = 1.73; 95%CI = 1.12–2.70).

Functional effect of the -838C>A polymorphism in the promoter region of the human $p27^{kip1}$ gene

Having demonstrated that the -838A allele of the $p27^{kip1}$ gene is associated with MI, we sought to determine whether the -838C>A genetic variation has functional consequences. Because this SNP lies within the promoter region, we examined in transiently transfected Jurkat cells the activity of reporter constructs driven by the human $p27^{kip1}$ gene promoter containing either nucleotide A or C at position -838. We generated two different pGL3-$p27^{kip1}$ constructs for each A and C alleles (pGL3-$p27^{kip1}_A$ and pGL3-$p27^{kip1}_C$, respectively), and performed three

Table 1: Primers used to amplify the p27^{kip1} gene for single-strand conformation analysis (SSCA).

Amplified sequence	Primer sequence*	Annealing temperature	PCR product length (bp)
promoter	P5-F: GGAGGAAGGAAGGAGCTGTTGTA P5-R: AAGCCAAAGCGAACGTCTTTC	62°C	360
promoter	P4-F: ATGATAAGTGCCGCGTCTACTCCT P4-F: ACCAGAGGACCGCGAAGGT	62°C	310
promoter	P3-F: GGCCGAGCTGGGGGCAGCT P3-R: TTAACCTGGCCCCGGCGC	66°C	362
promoter	P2-F: TCGGGGAGGCGGCGCGCTCG P2-R: AGAGGGTGGCAAAGCCCGTC	62°C	360
promoter	P1-F: TGGGTTCGCGGGACCGCG P1-R: CTGCCTGGCGTCCATCCG	62°C	385
Exon 1	E1-F: TGCGAGTGTCTAACGGGAGC E1-R: TTACCGTCGGTTGCAGGTC	58°C	472
Exon 2	E2-F: TAAAGATTGTGTGTTCTTTTTAA E2-R: TATCGTGAGGTCTGAAGGCC	55°C	230

* F: forward; R, reverse.

Table 2: Primers used to genotype the single-nucleotide polymorphisms (SNPs).

SNP	Primer sequence*	Annealing temperature (Enzyme)	PCR product length after digestion (bp)
-838C>A	F: TCCAGGTCCCGGCTTCCCGGt** R: CCTGCTCTGGCTGGCCTCGGAG	65°C (*Taq*I)	177 (A allele) 153+24 (C allele)
-79C>T	F: TGATCAGCGGAGACTCGGCG R:CTGCCTGGCGTCCATCCG	58°C (*Hae*III)	275 (T allele) 140+135 (C allele)
+326T>G (V109G)	F: TGCGAGTGTCTAACGGGAGC R: TTACCGTCGGTTGCAGGTC	58°C (*Bgl*I)	472 (T allele) 316+156 (G allele)

* F: forward; R, reverse. ** A mismatch to create a *Taq*I site when -838C is present is shown in lower case.

independent transfections with each construct. To correct for differences in transfection efficiency, cells were cotransfected with a plasmid encoding for the green fluorescent protein (GFP) (see Methods). After normalizing luciferase activity by the internal GFP control, the average transcriptional activity of pGL3-p27$^{kip1}{}_{A}$ was 34% less than that of pGL3-p27$^{kip1}{}_{C}$ (P = 0.04) (Fig. 2).

Discussion

VSMC and macrophage proliferation within the artery wall is a major event in the development of the atherosclerotic lesion, a pathological hallmark of coronary artery disease. The CKI p27^{kip1} is a tumour suppressor that functions as a brake against excessive cell proliferation [2]. Several recent studies have suggested an important role for p27^{kip1} as a negative regulator of arterial cell proliferation and atheroma development. First, both whole body genetic ablation of p27^{kip1} and selective p27^{kip1} inactivation in haematopoietic precursors increase neointimal cell proliferation and accelerates atherosclerosis in fat-fed apoE-null mice [11,12]. Second, evidence has been presented suggesting that p27^{kip1} expression and arterial cell proliferation are inversely correlated within human neointimal lesions [8-10].

In addition to environmental risk factors, such as smoking, hypertension, and hypercholesterolaemia, genetic factors may also increase the risk of atherosclerosis and associated ischaemic events. For example, evidence exists indicating that polymorphisms in the components of the renin–angiotensin system, nitric oxide synthases, apolipoproteins, and several cytokines/chemokines and growth factors could modulate the risk of suffering an episode of MI. Genetic variations must fulfil two criteria to be considered candidate modifiers of cardiovascular risk: the corresponding gene encodes a protein involved in vascular physiology or in atheroma development, and the nucleotide change affects gene expression and/or function. In the present study, we have investigated whether genetic variations within the p27^{kip1} are associated with increased risk of MI. Among the three SNPs described here, only -838C>A showed differences in allele frequency

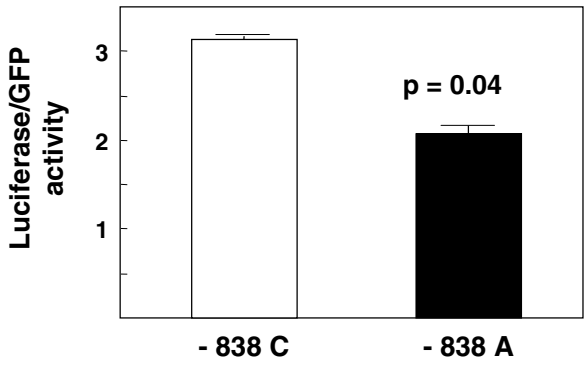

Figure 2
Transcriptional activity in Jurkat cells transfected with luciferase reporter gene constructs driven by the p27^{kip1} gene promoter containing the -838C or A allelic variants. Luciferase activity was normalized by the internal control green fluorescent protein. Data represent the means ± SD of six independent transfections (P = 0.04 in t-test).

Table 3: Genotype frequencies for the -838C>A, -79C>T, and V109G p27^{kip1} single-nucleotide polymorphisms (SNPs).

	-838C>A*		
	CC	**CA**	**AA**
Patients	47 (26%)	88 (49%)	45 (25%)
Controls	95 (38%)	115 (46%)	40 (16%)
	-79C>T		
	CC	**CT**	**TT**
Patients	99 (55%)	70 (39%)	11 (6%)
Controls	152 (61%)	82 (32%)	16 (7%)
	V109G		
	VV	**VG**	**GG**
Patients	100 (55%)	71 (40%)	9 (5%)
Controls	143 (57%)	98 (39%)	9 (4%)

*P = 0.009; OR = 1.73, 95%CI = 1.12–2.70 (AA+AC) vs CC. For each SNP, the table shows the total number of cases for each genotype and their relative frequencies among controls and patients with myocardial infarction.

between cases and controls. Carriers of the -838A allele (AA+AC genotypes) would have an almost twofold risk of suffering an episode of MI compared to controls (OR = 1.73; 95%CI = 1.12–2.70), and the risk increased to twofold for AA homozygous (OR = 2.27; 95%CI = 1.26–4.10; AA versus CC).

Because -838C>A lies within the promoter region of p27^{kip1}, we examined whether this SNP affects p27^{kip1} gene expression. Transient transfection experiments revealed a 34% reduction in promoter activity for -838A compared with the -838C allele. Thus, reduced p27^{kip1} expression in -838A carriers might facilitate neointimal hyperplasia and therefore accelerate atheroma progression and increase the risk of MI. Distinct transcription factor binding and/or activity may contribute to the difference in -838A and -838C p27^{kip1} promoter activity. Indeed, analysis of the promoter sequence containing the -838C>A SNP using the MatInspector v2.2 and TRANSFAC v7.3 software revealed potential binding sites for several transcription factors, including STAT and IK-2. However, additional studies are required to conclusively address whether specific transcription factors are involved in different transcriptional activation of -838A versus -838C. These studies should provide valuable insight not only for atherosclerosis but also for cancer, as p27^{kip1} expression appears to be altered in some human tumors [18,19].

The use of SSCA to identify p27^{kip1} SNPs may be a limitation of our study, because this technique apparently fails to identify approximately 20% of SNPs. Therefore, additional functionally relevant p27^{kip1} SNPs may exist. We also recognize that the number of cases analysed in our study is limited. This was mostly because we included only patients with angiographically diseased vessels, a strategy that was deemed important because p27^{kip1} has been involved in the control of neointimal thickening [8-12]. The possibility of false-positive results due to population stratification must be considered in association studies, but this is unlikely to occur in our study because we analysed individuals from a homogeneous white population. In addition, genotype frequencies were in the Hardy–Weinberg equilibrium in both groups, suggesting that the observed frequencies are representative of cases and controls.

Conclusions

Three polymorphisms of the human p27^{kip1} gene (-838C>A, -79C>T, and V109G) were analysed as candidates to modify the risk of suffering MI. We found a significantly higher frequency of the -838A carriers in MI patients compared to controls. Importantly, this polymorphism was associated with reduced basal p27^{kip1}gene promoter activity. We suggest that reduced

$p27^{kip1}$ expression in -838A carriers may facilitate the proliferative response associated with atherosclerosis, thus increasing the risk of MI in these individuals.

Methods

Subjects

A total of 180 patients who had suffered an episode of MI were included in the present study. All patients were male, no more than 55 years of age, and had at least one angiographically diseased vessel (a vessel was considered diseased when the angiography showed a narrowing of at least 70% in the lumen diameter). These patients were recruited for a search of genetic factors involved in MI [20].

The control group consisted of 250 healthy males younger than 55 years (blood bank donors and Hospital Universitario Central de Asturias staff). Although control subjects did not have a history of cardiovascular disease, they were not angiographically analysed to exclude the presence of diseased vessels. Patients and controls were white and were all from the same region (Asturias, northern Spain, 1 million total population) and gave their informed consent to participate in the study, which was approved by the Ethical Committee of the Hospital Universitario Central de Asturias.

Identification and genotyping of SNPs within the human $p27^{kip1}$ gene

Genomic DNA was obtained from each individual included in the study. In the search for $p27^{kip1}$ SNPs, we analysed 50 patients and 50 controls using SSCA. The entire $p27^{kip1}$ coding sequence and 1500 nucleotides of the promoter region were amplified in overlapping fragments of 300–450 base pairs by PCR (Table 1 shows the sequences of the PCR primers; the sequence of the $p27^{kip1}$ gene was obtained from http://ncbi.nlm.nih.gov, accession number NT009714). SSCA analysis was performed as previously described [21]. The DNA from samples showing atypical electrophoretic patterns was amplified, purified, and sequenced using an automated ABI310 system.

For genotyping of the three SNPs in patients and controls, genomic DNA was amplified by PCR to generate DNA fragments containing the SNP, and these fragments were digested with restriction enzymes that produced distinct patterns of digestion depending on the allele present (primers and restriction enzymes are listed in Table 2). The digested fragments were electrophoresed on 3% agarose gels, and the pattern corresponding to each individual was visualized after ethidium bromide staining of the gel.

Construction of $p27^{kip1}$ reporter vectors

DNA fragments from the 5' flanking region of the $p27^{kip1}$gene between nucleotides – 1100 to -27, containing either C or A at -838, were generated through PCR of two CC and AA homozygous individuals with forward (TATGATG*GTACC*AGACGTTCGCTTTGGCTTC) and reverse (GCACGA*AAGCTT*CTCTCGCACTCTCAAAAA) primers containing *Kpn*I and *Hin*dIII sites, respectively. The PCR products were cloned into the pGL3-Basic plasmid (Promega, Madison, WI, USA) to generate pGL3-$p27^{kip1}{}_C$ (-838C) and pGL3-$p27^{kip1}{}_A$ (-838A). For each genotype, two plasmids were generated and confirmed by direct sequencing.

Cell culture and luciferase reporter gene assays

Jurkat cells (American Type Culture Collection) were incubated at 37°C in a humidified 5% CO_2–95% O_2 atmosphere in RPMI medium supplemented with 100U/ml penicillin, 0.1 mg/ml streptomycin, 2 mmol/l L-glutamine, and 10% fetal calf serum. Transfections were performed with SuperFect Transfection Reagent following the manufacturer's protocol (Quiagen, Valencia, CA, USA). In a blinded manner, the promoter/luciferase reporter gene (2 μg) was cotransfected with 1 μg of pEGFP-N1 control vector. Three independent transfections were done for each of the four reporter genes (2 pGL3-$p27^{kip1}{}_C$ and 2 pGL3-$p27^{kip1}{}_A$, a total of six transfections for each SNP). For luciferase activity assay, cells were collected by centrifugation, washed twice with PBS, and lysed with 100 μl of cell lysis reagent (Promega). Insoluble protein was removed by a 10-minute centrifugation at 15,000 *g*, and the supernatants were immediately assayed for luciferase activity and green fluorescence. Luciferase assays were performed using a Victor 4120 multilabel counter (Perkin Elmer, Boston, MA, USA) and the Promega luciferase assay kit. Cell extract (50 μl) and 100 μl of luciferin mixture were incubated for 5 s and light output was monitored for 5 s. For each sample, arbitrary light units from luciferase were normalized versus the measure of green fluorescence.

Statistical analysis

Allele and genotype frequencies in patients and controls were compared using a chi-square test. This test was also used to determine if the observed genotype frequencies in cases and controls differed from those expected under the Hardy–Weinberg equilibrium. Multivariate analysis was used to compare the genotype and allele frequencies between the groups according to the presence/absence of classical risk factors. Odds ratios (OR) with 95% confidence intervals (CI) were obtained to calculate the relative risk of MI associated with the genotypes. The results of transfections were analysed by two-tailed unpaired *t*-test. All statistical analyses were performed with the SPSS statistical package (v.11.0).

Authors' contributions

JRR and AB recruited the patients and controls and performed all the clinical analysis. PG, EC, and VÁlvarez performed the genetic analysis (search for $p27^{kip1}$ polymorphisms and genotyping) and the statistical analysis. AD-J, VAndrés, and PG designed and performed the analysis of $p27^{kip1}$-promoter polymorphisms with reporter vectors. All the authors read and approved the final version of the manuscript.

Acknowledgements

PG was the recipient of a fellowship from the Fundación para el Fomento en Asturias de la Investigación Científica Aplicada y Tecnológica (FICYT, BP 01-082). AD-J received salary support from Fondo Social Europeo (CSIC-Programa I3P). We thank MJ Andrés-Manzano for preparing the figures. This work was supported by grants from the Spanish Fondo de Investigaciones Sanitarias to EC (FIS 03/05) and from the Spanish Ministry of Science and Technology and Fondo Europeo de Desarrollo Regional to VAndrés (SAF2002-1443).

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