BASIC RESEARCH

Platelet membrane glycoprotein Iba gene –5T/C Kozak sequence polymorphism as an independent risk factor for the occurrence of coronary thrombosis

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Objective: To explore the potential of the GPIbα gene variable number tandem repeat (VNTR) and –5T/C Kozak polymorphisms to act as independent risk factors for myocardial infarction.

Methods: 256 patients aged 33–80 years (180 caucasian, 76 Indian Asian) were recruited at cardiac catheterisation for any diagnostic indication, and divided into two groups: group A, with confirmed previous myocardial infarction evident on ECG or ventriculogram (88 patients, 79 men, 9 women) and group B, with no evidence of myocardial infarction (168 patients, 101 men, 67 women).

Results: There was no significant difference in race, age, hypertension, smoking status, or family history between the infarct and non-infarct groups, though there was a significant difference in sex (89.8% male in group A, 60.1% male in group B, $p < 0.001$). Genotype analysis showed a strong association between the GPIbα Kozak homozygous TT genotype and the occurrence of myocardial infarction (group A: TT 85.2%, TC 12.5%, CC 2.3%; group B: TT 67.3%, TC 32.7%, $p = 0.001$). No significant association was found between myocardial infarction and the GPIbα VNTR, although analysis of the CC VNTR genotype against all other GPIbα VNTR genotypes showed a marginal association with myocardial infarction ($p = 0.059$). There was no association between the Kozak sequence polymorphism ($p = 0.797$) or GPIbα VNTR ($p = 0.714$) and the degree of vessel disease.

Conclusions: The homozygous TT Kozak genotype may be a significant factor in the outcome of coronary artery disease completed by myocardial infarction. Conversely, the Kozak C allele in the heterozygous state TC may confer some protection against myocardial infarction.

In the arterial circulation, platelets adhere to surface bound von Willebrand factor (vWF) through the platelet glycoprotein (GP) Ib/IX/V complex. This receptor is shear dependant and is “switched on” in the arterial microvasculature and environments such as those found in the coronary artery.^{1–4} GPIb/IX/V is composed of four subunits: GPIbα, GPIbβ, GPIX, and GPV, each being the product of a separate gene. GPIbα is disulfide linked to GPIbβ forming the heterodimer GPIb, which is non-covalently associated with GPIX and GPV.^{5–7} The binding site for vWF is located on the α subunit of GPIb.⁸

There is increasing evidence that predetermined genetic factors can influence haemostatic balance and consequently thrombotic risk.⁹ We hypothesised that two DNA polymorphisms in the GPIbα gene may be significant factors influencing the risk of myocardial infarction: firstly, the GPIbα –5T/C Kozak polymorphism which may alter the level of expression of GPIb/IX/V; and secondly, the GPIbα variable number tandem repeat (VNTR) which alters the structure of this essential platelet adhesion receptor.

Kozak sequences are DNA motifs that surround ATG initiator codons. The sequence context of ATG codons is important, in particular the positions –3 to –9.^{10–11} The GPIbα gene Kozak sequence polymorphism is the result of either a T (thymine) or a C (cytosine) at position –5 relative to the ATG start codon.¹² Recently, this Kozak sequence has been reported to affect the expression of the GPIb/IX/V complex on the platelet surface. The less common allele C is closer to the Kozak consensus sequence, which is said to be more efficient at translation, and there is evidence from in vitro studies that it is associated with increased expression of the GPIb/IX/V complex on the surface of CHO cells.¹³ The GPIbα VNTR consists of four variants ranging from 168 kDa to 153 kDa, designated A to D in order of decreasing molecular mass (D = 1, C = 2, B = 3, and A = 4

repeats).^{14–15} The A isoform is often found in Japanese Asians, but is rarely present in caucasian populations.¹⁶ Each additional repeat of 13 amino acids is predicted to add 32 Å to the distance that GPIbα extends from the platelet surface.⁶ It is therefore feasible that the length of GPIbα could affect the efficiency of platelet adhesion owing to differences in the distance between the vWF binding site and the vessel wall. Other polymorphisms in GPIbα include the platelet alloantigen HPA-2, coded by a Thr 145 Met dimorphism which shows strong linkage to the VNTR, and a TaqI polymorphism in the 3' untranslated region.^{17–18}

We designed this study to explore the potential of the Kozak and VNTR polymorphisms of GPIbα to act as independent risk factors for myocardial infarction. By comparing a group of patients who had experienced a myocardial infarct with a group who had not, rather than incorporating a traditional “normal control group,” we were able to assess two equivalent populations that were evenly matched for other known risk factors (table 1). Within any normal control group a degree of latent vessel disease would still be expected; therefore the infarct and non-infarct cohorts were both recruited from patients with known coronary angiographic data. The two study groups were well characterised, with detailed clinical information. This enabled us to dissect out the influence of each of the two polymorphisms on the occurrence of myocardial infarction and the degree of vessel disease.

Abbreviations: bp, base pair; GP, platelet glycoprotein; PCR, polymerase chain reaction; VNTR, variable number tandem repeat; vWF, von Willebrand factor

Table 1 Characteristics and prevalence of risk factors for ischaemic heart disease in the two subgroups with and without myocardial infarction

Characteristic	Infarct group (n=88)	Non-infarct group (n=168)	p Value OR (95% CI)
* ⁽¹⁰⁾ Age (years)			
Range	35 to 78	33 to 80	
Mean (SD)	59.1 (9.9)	57.0 (10.6)	0.117
Sex			
Male	79 (89.8)	101 (60.1)	<0.00001
Female	9 (10.2)	67 (39.9)	OR 5.82 (2.61 to 13.38)
Race			
Indian Asian	24 (27.3)	51 (30.5)	0.586
White	64 (72.7)	116 (69.5)	OR 1.17 (0.64 to 2.17)
Risk factors			
⁽¹²⁾ Family history	(yes) 51 (60.7) (no) 33 (39.3)	83 (51.9) 77 (48.1)	0.187 OR 1.43 (0.81 to 2.54)
⁽³⁾ Diabetes	(yes) 22 (25.30) (no) 65 (74.7)	28 (16.9) 138 (83.1)	0.110 OR 1.67 (0.85 to 3.28)
⁽⁵⁾ Hypertension	(yes) 30 (34.9) (no) 56 (65.1)	45 (27.3) 120 (72.7)	0.211 OR 1.43 (0.78 to 2.60)
⁽¹¹⁾ Cholesterol	(yes) 46 (52.9) (no) 41 (47.1)	70 (41.7) 98 (58.3)	0.088 OR 1.57 (0.90 to 2.74)
⁽⁷⁾ Smoker	(yes) 20 (23.8) (no) 64 (76.2)	36 (21.8) 129 (78.2)	0.72 OR 1.12 (0.57 to 2.18)
⁽¹⁶⁾ Ex-smoker	(yes) 49 (60.5) (no) 32 (39.5)	83 (52.2) 76 (47.8)	0.22 OR 1.40 (0.79 to 2.74)

Values are n (%) unless stated otherwise.
 *⁽ⁿ⁾ Indicates the number of missing values.
 CI, confidence interval; OR, odds ratio.

Table 2 The distribution and severity of vessel disease detected by coronary angiography in the infarct and non-infarct patient groups

Vessel disease	Infarct group (n=88)*	Non-infarct group (n=168)
0	2 (2.3)	79 (47.0)
X	4 (4.6)	14 (8.4)
1	10 (11.5)	17 (10.1)
2	24 (27.3)	23 (13.7)
3	46 (52.3)	35 (20.8)

Values are n (%).
 The difference in the degree of vessel disease between the infarct and non-infarct groups was highly significant ($p < 0.001$).
 *There were two subjects in the infarct group for whom the degree of vessel disease was unavailable.
 0, normal coronaries; X, irregularities on angiogram; 1, 2, and 3 = number of diseased vessels.

METHODS

Patients

Over a period of six months we recruited 256 consecutive patients (180 caucasian and 76 Indian Asian) aged between 33–80 years who were admitted for day case cardiac catheterisation at the Hammersmith Hospital. The study was approved by the research ethics committee and all patients gave their informed consent.

The patients were divided into two matched groups according to their clinical history. Group A consisted of 88 patients with definite previously diagnosed myocardial infarction (infarct group). Group B consisted of 168 patients with no previous myocardial infarction and with either normal or diseased coronary arteries (non-infarct group) (table 2). Group B patients were undergoing cardiac catheterisation for angina or reasons other than chest pain.

On presentation to the catheterisation laboratory, a history of myocardial infarction was established on the basis of documented evidence in the patients' hospital records or by ECG(Q

wave) or ventriculogram (area of akinesia). We also noted the presence or absence of the following risk factors for coronary atherosclerosis: hypercholesterolaemia (> 5.2 mmol/l or on cholesterol lowering drugs), hypertension (blood pressure $> 140/90$ mm Hg or on antihypertensive drug treatment), smoking (at presentation), diabetes mellitus, and a positive family history (first degree relatives) of ischaemic heart disease. The coronary arteriograms were interpreted by a single, independent observer. Each of the three main coronary vessels was considered to be diseased when the lumen was reduced to less than 50% of the normal diameter. Patients with irregularities on angiography formed a separate subgroup.

DNA genotyping

A 10 ml venous blood sample was taken into a tube containing EDTA as anticoagulant and stored at -70°C for subsequent DNA extraction. Genomic DNA was extracted from whole blood using standard techniques (BACC2 kit, Nucleon Biosciences, Lanarkshire, UK). The DNA sequence spanning the GPIIb VNTR polymorphism was amplified by polymerase chain reaction (PCR) of genomic DNA using Red Hot DNA polymerase (Advanced Biotechnologies, Surrey, UK) and the following primers: 5'-ATCCACTACTGAACCAACCC-3' nt 4199-4218 and 5'-GAGTGATACGGGTTTGTGG-3' nt 4451-4432 (GenBank Accession No AC22403). An initial DNA denaturation at 96°C for five minutes was followed by 32 cycles of 45 seconds denaturation at 96°C , one minute annealing at 55°C , and two minutes extension at 72°C . A 10 minute final extension at 72°C completed the reaction. The DNA fragments amplified were as follows: D allele, 253 bp; C allele, 292 bp; B allele, 331 bp; A allele, 370 bp.

The DNA sequence spanning the -5T/C Kozak polymorphism was amplified using the primers Kozak1 5'-GGGAGTAGGGAGGACAGGAG-3' and Kozak2 5'-AGTGTAAAGGCATCAGGGTTG-3'. Amplification conditions were as above but with an annealing temperature of 59°C and using a hot start technique. The 348 bp PCR product was digested with the restriction enzyme *Ava*II which recognises the T allele, resulting in two fragments of $129 + 219$ bp. Any apparently homozygous CC patients were checked with *Hae*III. In the presence of the C allele, *Hae*III generates two

Table 3 Genotype distribution and allele frequency of the GPIbA VNTR polymorphism in the Indian Asian and white patient subgroups

	Genotype							Allele				
	AC	BC	BD	CC	CD	DD	Total genotypes	A	B	C	D	Total alleles
% Distribution of total study group n	0.4 1	11.3 29	1.9 5	71.1 182	13.7 35	1.6 4	256	0.2 1	6.6 34	83.8 429	9.4 48	512
% Distribution, Asian n	1.3 1	11.8 9	1.3 1	56.6 43	25.0 19	4.0 3	76	0.7 1	6.6 10	75.6 115	17.1 26	152
% Distribution, white n	0 0	11.1 20	2.2 4	77.2 139	8.9 16	0.6 1	180	0 0	6.7 24	87.2 314	6.1 22	360

Table 4 Genotype distribution and allele frequency of the GPIb Kozak polymorphism in the study group

	Genotype			Total genotypes	Allele		Total alleles
	TT	TC	CC		T	C	
% Distribution n	73.4 188	25.8 66	0.8 2	256	86.3 442	13.7 70	512

fragments of 130 and 218 bp. A 10 μ l aliquot of the PCR products and the digests was analysed on 2.5% agarose gels (Seakem ME, FMC products, Maine, USA).

Statistical analysis

Association analysis was performed using the SPSS version 8.1 and EpiInfo 6.0 software. Sex, race, family history, diabetes mellitus, hypertension, cholesterol, and smoking are all categorical variables. Their individual effect on myocardial infarction was investigated using χ^2 or Fisher's exact test when necessary. The odds ratios (OR) are accompanied by Cornfield 95% confidence limits (CI). The effect of vessel disease on myocardial infarction was analysed using a Pearson χ^2 test. The effect of age on myocardial infarction was analysed as a single continuous variable using linear regression analysis of variance (ANOVA). The influence of the Kozak -5T/C and GPIb α VNTR polymorphisms on myocardial infarction and vessel disease was assessed using a Pearson χ^2 test. The effects of both genotype and allele frequency were examined individually. Probability values of $p < 0.05$ were considered significant.

RESULTS

The demographic and clinical characteristics of the patients in each group are listed in table 1. There were more men in the infarct group than in the non-infarct group (89.8% v 60.1%

respectively, $p < 0.001$). No significant difference was found between the two groups with regard to age (mean (SD): 59.06 (9.90) years in the infarct group v 56.96 (10.58) years in the non-infarct group, $p = 0.117$) or race (Asian, 27.3% v 30.5%, respectively, $p = 0.586$).

No significant differences were detected between the infarct and the non-infarct groups for smoking, hypertension, diabetes, and family history of ischaemic heart disease. However, there was a trend towards a higher plasma cholesterol in the infarct group (table 1). The difference in the number of diseased coronary arteries was highly significant ($\chi^2 = 18.951$, $p < 0.001$) with 55.4% of the non-infarct group having normal or slight irregularities compared with 6.9% of the infarct group. In the infarct group, 79.6% of the subjects had two or more main branches of the coronary arteries affected, compared with 34.5% in the non-infarct group (table 2).

Genotype frequency and haplotype analysis (tables 3 and 4) confirmed that both the Kozak polymorphism and GPIb α VNTR were in Hardy-Weinberg equilibrium in the study population. Analysis of the association of the Kozak polymorphism with myocardial infarction showed a very strong association with TT when analysing genotype frequency ($\chi^2 = 15.531$, $p < 0.001$), and an association with allele frequency ($\chi^2 = 6.02$, $p = 0.014$; odds ratio (OR) = 2.10; 95% CI, 1.11 to 4.02 (table 5)). Comparing the Kozak TT with the TC+CC genotypes also showed a very strong association with myocardial infarction ($\chi^2 = 9.56$, $p = 0.002$; OR = 2.81; 95% CI 1.37 to 5.82 (table 5)). There was no association between the GPIb α Kozak polymorphism ($\chi^2 = 3.092$, $p = 0.797$) or VNTR genotypes ($\chi^2 = 8.873$, $p = 0.714$) and the degree of vessel disease. In a multivariate analysis a marginal association was found between the Kozak polymorphism and cholesterol ($p = 0.046$; OR = 1.79; 95% CI, 1.04 to 3.09), but no association was seen with family history, diabetes, or hypertension. There was no difference in Kozak genotype distribution between the caucasian and Indian Asian subjects.

A significant variation in GPIb α VNTR genotype frequency was evident according to race, with 56.6% CC in Indian Asians

Table 5 Genotype distribution of the GPIbA VNTR and Kozak polymorphisms between the infarct and non-infarct patient groups

	VNTR						Kozak		
	AC	BC	BD	CC	CD	DD	TT*	TC	CC
Infarct group (n=88)	0 (0.0)	6 (6.8)	3 (3.4)	69 (78.4)	8 (9.1)	2 (2.3)	75 (85.2)	11 (12.5)	2 (2.3)
Non-infarct group (n=168)	1 (0.6)	23 (13.6)	2 (1.2)	113 (67.3)	27 (16.1)	2 (1.2)	113 (67.3)	55 (32.7)	0 (0.0)

Values are n (%).

No significant association was found between the VNTR genotypes and myocardial infarction ($p=0.60$). Considering the CC genotype v all other VNTR genotypes, there was a marginal association ($p=0.059$, OR 1.8, 95% CI 0.94 to 3.48).

*A significant association was found between the Kozak TT genotype and myocardial infarction ($p<0.001$, OR 2.10, 95% CI 1.11 to 4.02); the TT v TC+CC Kozak genotype also showed a significant association with myocardial infarction ($p=0.002$, OR 2.81, 95% CI 1.37 to 5.82).

CI, confidence interval; OR, odds ratio; VNTR, variable number tandem repeat.

v 77.2% CC in caucasians, respectively ($\chi^2 = 20.44$, $p = 0.001$; table 3). This drift reflected the increased incidence of the D allele in the Indian Asian population (25.0% CD) compared with the white population (8.9% CD) (table 3). However, this did not affect the analysis of the effect of genotype on the incidence of myocardial infarction, as both infarct and non-infarct groups were evenly matched with respect to the ratio of Indian Asian to caucasian subjects. The GPIIb VNTR showed no association with myocardial infarction when allele frequencies were compared ($\chi^2 = 1.89$, $p = 0.60$). When considering the VNTR CC genotype against all other VNTR genotypes, a marginal association was seen ($\chi^2 = 3.57$, $p = 0.059$; OR = 1.80; 95% CI 0.94 to 3.48) (table 5).

DISCUSSION

Coronary atherosclerosis with plaque disruption resulting in superimposed thrombosis is the main pathological mechanism in the acute coronary syndromes of unstable angina, myocardial infarction, and sudden death, with the amount and extent of associated thrombus formation affecting the final clinical outcome.¹⁹ The composition of thrombi found in arterial thrombosis is predominantly that of a platelet-rich core attached to an underlying atheromatous plaque in an epicardial segment of the corresponding coronary artery branch.²⁰ Blood passing through arteries that have been narrowed by atheroma will be subjected to higher local shear forces, resulting in an increased risk of thrombotic occlusion through the shear dependent vWF and GPIIb/IX/V interaction. It therefore appears that platelet adhesion to vascular subendothelium, and subsequent platelet aggregation, are critical elements in the mechanism of coronary thrombosis.²¹

The pathogenesis of thrombosis hinges on an imbalance in the haemostatic system, and polymorphisms of candidate coagulation genes have therefore been the targets of recent study.⁹ For example, the PLA2 allele of GPIIIa has been associated with myocardial infarction at a young age and has also been linked with sudden death from myocardial infarction in a series of middle aged men.^{22,23} Arterial thrombosis occurs predominantly in the older population and is affected by many environmental factors that may also act synergistically with pre-existing genetic influences. The GPIIb/IX/V receptor functions within a high shear environment, and altered binding to vWF caused by changes in structure or levels of surface expression may therefore be associated with the development of coronary thrombosis and subsequent myocardial infarction. The Kozak -5T/C and VNTR polymorphisms of the GPIIb gene may hypothetically modulate the efficiency of platelet adhesion in the arterial microvasculature. If this were the case, the genotype distribution may be expected to vary significantly between a group of patients with myocardial infarction and a matched group without myocardial infarction.

The results of our study show a significant difference in the genotype frequencies between the two study groups. In particular, the TT Kozak genotype was more common among patients with myocardial infarction and Kozak TC genotypes were more common in those without myocardial infarction (table 5). Based on the *in vitro* work it might be expected that the -5C/C genotype would confer the greatest risk.¹³ Paradoxically, the results of our study suggest that the presence of the -5T/T genotype in the Kozak sequence is significantly associated with the occurrence of myocardial infarction ($\chi^2 = 9.56$, $p = 0.002$; OR = 2.81, 95% CI 1.37 to 5.82). Furthermore, it could be argued that the presence of the Kozak C allele may confer a degree of protection against a coronary event when present in the heterozygous state (TC 12.5% in the infarct group v 32.7% in the non-infarct group). This may give the impression that the TT genotype is associated with increased risk. Clearly the Kozak -5T/C polymorphism has a potentially significant association with coronary thrombosis completed

by myocardial infarction, and this will need a larger prospective study for clarification.

In an elegant *in vitro* study with CHO cells,¹³ the effect of the T and C Kozak alleles on surface expression of GPIIb/IX/V was investigated; it was found that the Kozak C allele resulted in increased expression of the complex, which may correlate with increased platelet adhesion. However, studies in a static tissue culture system do not replicate the physiological environment that exists in the high shear forces of the arterial vasculature, on which the adhesive function of this complex depends. In these conditions, other regulatory sequences and transactivating factors may modulate *in vivo* gene expression.²⁴ It is therefore still credible that a C allele may confer protection, or alternatively that a T allele may act as a potential risk factor in the physiological situation.

Published data so far are inconsistent in their findings. In a series of patients with coronary heart disease or cerebrovascular accidents, GPIIb/IX/V expression was apparently not associated with the Kozak dimorphism and showed no association with arterial thrombosis.²⁵ In another recent study, no association was found between the Kozak sequence polymorphism and myocardial infarction.²⁶ In that study, however, there were significant differences in hypertension, cholesterol, and particularly smoking and diabetes between the normal control and patient groups. Although more patients were included than in our study, this was not a truly matched control group, and for neither patient nor control population were details of coronary vessel disease provided. During the submission process of the present paper, data were published from a series of non-fatal strokes and non-fatal myocardial infarcts in young women.²⁷ These suggested that the presence of a C allele apparently reduced the risk of myocardial infarction—in agreement with our results—but had no effect on cerebrovascular accidents. Our study was based on myocardial infarction survivors, and this may mask the true effect of genotype on risk. Interestingly, in our study, the only two patients with the CC genotype were in the infarct group. It could be argued that if patients dying suddenly from acute coronary syndromes could be included, then a different picture of genotype versus risk would emerge. The number of subjects in our study, although relatively small, was still adequate to provide useful data. A much larger prospective study will address this issue.

Although our study population consisted of patients undergoing an invasive diagnostic procedure, some having suspected coronary artery disease, the overall distribution of alleles and genotypes observed was consistent with previous reports, based on general population screening.^{13,28} Our study therefore provides some evidence that genetically determined differences in the structure of GPIIb may partly determine the risk of developing coronary thrombosis with consequent myocardial infarction. No relation was found between the severity of atheroma (stenosis) and the GPIIb VNTR or Kozak genotypes; thus the differences in risk associated with these polymorphisms appear to be more closely linked to thrombosis than to atheroma.

As the arterial lumen narrows in atheromatous coronary artery disease, the turbulent flow increases the shear forces. The presence of atheroma appears to be a necessary but not sufficient factor for the occurrence of coronary thrombosis. This study was designed to determine the frequency of genotypes in coronary artery disease completed by thrombosis. It was therefore quite appropriate that the whole study population, including the controls, be recruited from a population of patients likely to have atheromatous coronary disease rather than a population of healthy volunteers. No significant difference was found between the two study groups in the prevalence of established risk factors for coronary disease (table 1) and thus no further stratification of the sample was necessary.

The different GPIb α VNTR polymorphic variants determine the distance between the vWF binding domain and the platelet surface. Some of these could create a minor spatial advantage for platelet adhesion to exposed vascular subendothelium. It has been implied that a longer GPIb α polypeptide may lower the threshold of shear force required for platelet adhesion to immobilised vWF.¹⁵ A geometrically balanced arrangement of multiple complexes based on a hypothetical conformation of four copies of GPIb/IX and two copies of GPV has been proposed.²⁹ The adhesion efficiency of the complex may therefore have subtle differences, depending on whether it is assembled from GPIb α VNTR alleles of either equal or unequal length. In this study the CC VNTR genotype showed a marginal association with myocardial infarction when compared against all other VNTR genotypes ($\chi^2 = 3.57$, $p = 0.059$; OR = 1.80, 95% CI 0.94 to 3.48 (table 5)). However, in a previous study of Mediterranean caucasian patients ranging in age from 34–85 years, the C/B VNTR genotype was reported as showing an association with cerebrovascular accidents and coronary heart disease but not deep vein thrombosis.³⁰ All patients had individually matched controls with no documented history of heart disease, although no information was available about the degree of vessel disease. In a cohort of Japanese patients, the A allele showed an association with coronary heart disease.³¹ As reports to date are inconsistent, a definitive answer will require a much larger prospective study to clarify any potential association with coronary heart disease and myocardial infarction. The homozygous state also appears to be an important factor, as the VNTR CC genotype was more frequent (78.4% v 67.3%) and the CD genotype less frequent (9.1% v 16.1%) among the patients with myocardial infarction than among those without myocardial infarction (table 5). Why expression of a shorter GPIb α receptor component may be associated with myocardial infarction is open to speculation, and further in vitro and in vivo studies are required.

Conclusions

The preliminary findings of this study provide evidence that genetically determined variations in the structure and expression of the platelet GPIb α receptor could potentially influence the risk of developing coronary thrombosis and myocardial infarction. In particular, the hypothesis that the Kozak TC genotype may confer some protection against myocardial infarction, and the TT genotype may act as a risk factor, needs to be further explored in the context of a much larger study.

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