The Kozak sequence polymorphism of platelet glycoprotein Ibα and risk of nonfatal myocardial infarction and nonfatal stroke in young women

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Several platelet membrane glycoprotein polymorphisms have been identified as potential risk factors for cardiovascular disease. Recently a nucleotide −5T/C dimorphism in the translation initiation site (Kozak sequence) of the platelet glycoprotein Ibα (*GPIbα*) gene was associated with increased platelet surface levels of the GPIb-IX-V receptor complex. The role of this GPIbα Kozak sequence polymorphism in the occurrence of arterial thrombotic disease is unknown. We performed genotype analysis of the Kozak sequence polymorphism of GPIbα in a population-based study of 18- to 44-year-old women with nonfatal myocardial infarction (MI) (n = 78), nonfatal stroke (n = 106), and 384 demographically similar female control subjects. Analysis of −5T/C genotypes revealed that at least one copy of the C allele was present in 14.1% of MI cases, 23.6% of stroke cases, and 23.7% of controls. The age-adjusted odds ratio for MI in women carrying at least one copy of the C allele was 0.53 (95% confidence interval [CI] 0.27-1.05). The age-adjusted odds ratio for stroke in women carrying at least one copy of the C allele was 0.99 (95% CI 0.59-1.65). Analyses stratified by stroke type (ischemic, hemorrhagic) yielded similar results. In conclusion, young women carrying the C allele of the Kozak sequence polymorphism of GPIbα are not at increased risk of MI or stroke. Paradoxically, the C allele may even be associated with a reduced risk of MI in this population. This finding requires further study. (Blood. 2001;97: 875-879)

Introduction

The glycoprotein (GP) Ib-IX-V complex is the major platelet surface receptor for von Willebrand factor (vWF) GPIb-IX-V plays a key role in the adhesion of platelets to injured vascular subendothelium and mediates shear-induced platelet activation [1,2] Two polymorphisms in the *GPIbα* gene affect the structure of the protein The first is a size polymorphism resulting from a variable number of tandem repeats (VNTR) of a 13-amino acid sequence with designated alleles A, B, C, and D The second is a Thr/Met dimorphism at position 145 located in the vWF binding domain of GPIbα, which defines the human platelet alloantigen system HPA-2 The HPA-2 and VNTR polymorphisms are in strong linkage disequilibrium [3-5]

A third polymorphism of the *GPIbα* gene that has been described more recently involves a −5T/C dimorphism in the translation initiation sequence (Kozak sequence) [6,7] From previous studies of the translation initiation site, the nucleotides preceding the AUG initiator codons are thought to be important in translation efficiency in eukaryotic cells [8] The original consensus Kozak sequence contains nucleotide C at position −5, and therefore the switch to T at the −5 position of *GPIbα* may theoretically decrease translation efficiency of the receptor Afshar-Khargan et al[6] reported an association between the C allele of the Kozak dimorphism and increased platelet GPIb-IX-V receptor levels both in vitro and in vivo

Because GPIb-IX-V receptor density may influence platelet adhesion to exposed vascular subendothelium after atherosclerotic plaque rupture, and thus affect risk of thrombosis, we assessed the relationship between the C allele of the Kozak sequence dimorphism of *GPIbα* and risk of nonfatal MI and stroke among young women in a population-based case-control study To assess potential gene-environment interactions, we also performed analyses stratified by other known cardiovascular risk factors Finally, because of the possible association between the HPA-2 and VNTR polymorphisms and risk of atherothrombotic disease,[9-11] we also considered the potential confounding effects of these other *GPIbα* polymorphisms in our analysis

Patients, materials, and methods

Subjects

Approval for these studies was obtained from the University of Washington's institutional review board Informed consent was provided according to the Declaration of Helsinki We used data and specimens from a population-based case control study among women ages 18 to 44 years living in 3 contiguous counties of western Washington state between July 1, 1991, and February 28, 1995 [12-14] Potential MI and stroke cases were initially identified by chart review of discharge diagnoses from all 34 hospitals and Emergency Medical Service incident reports in the study

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region Eligible MI cases were women who had a probable MI diagnosed using criteria of the Cardiovascular Health Study (CHS)[15] Stroke cases were defined as women with new neurologic deficits lasting more than 24 hours or resulting in death in less than 24 hours and were classified by the study neurologist as ischemic, hemorrhagic, venous, or "other" (including arterial dissections) based on brain imaging studies and lumbar puncture results Two hundred eight eligible MI patients and 249 stroke patients were identified, and 161 of MI patients and 198 of stroke patients were living at the time recruitment was initiated One hundred twelve MI and 149 stroke patients were recruited and interviewed Six hundred eighty-four age-matched female control subjects without history of cardiovascular disease (CVD) were identified from the same geographic area by random digit telephone dialing Five hundred twenty six control subjects were recruited and interviewed for the study (Table 1)

Data collection

Case patients and control subjects were interviewed in person regarding histories of cardiovascular risk factors, including hypertension, diabetes mellitus, hypercholesterolemia, cigarette smoking, height and weight, menstrual history, contraceptive practices, history of MI in first-degree relatives, and demographic characteristics All questions elicited information from the period before the MI or stroke in each case patient or an equivalent date for control subjects A venous blood sample was collected from 79 MI cases, 106 stroke cases, and 391 control subjects into EDTA treated vacuum tubes Aliquots of plasma and buffy coat were frozen at −70°C Samples were sent to Leiden, The Netherlands, where DNA was extracted as described by Miller et al[16] Analyzable DNA was available for 78 MI cases, 106 stroke cases, and 384 controls

Laboratory analysis

Platelet genotyping for the *GPIbα* Kozak polymorphism was performed by polymerase chain reaction (PCR) amplification of genomic DNA, followed by restriction enzyme digestion, according to the method of Kaski et al[7] Each reaction contained 100 ng DNA, 4 pmol of each primer, 200 μM dNTP, 0 4 units of Taq polymerase, 2 μL 10× reaction buffer, and 16 μL water in a total volume of 20 μL For the amplification, the samples were heated to 93°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 1 minute, ending with a final extension step of 72°C for 5 minutes The amplification products were digested in a mixture of 5 units of the restriction enzyme *Hae*III, 1 μL 10× buffer, 3 5 μL water, and 5 μL PCR product, which was incubated for 2 hours at 37°C Samples were analyzed by electrophoresis on a 2% agarose gel and stained with ethidium bromide Thirty-eight samples were randomly selected for duplicate testing using the methods previously described with consistent results In addition, 5 samples of each genotype (T/T, T/C, and C/C) were selected for direct nucleotide sequencing with confirmation of accuracy on all 15 samples

Platelet genotyping for the HPA-2 (Thr/Met^{145})[17] and VNTR[4] polymorphisms of *GPIbα* were performed as previously described

Variable definitions and data analysis

Current smokers were defined as subjects who reported smoking currently and regularly (at least 5 cigarettes per week for at least 6 consecutive months), and all others were classified as nonsmokers Obesity was defined as a body mass index (BMI) 27 3 kg/m^2 or greater Hyperhomocysteinemia was defined as a serum homocysteine level greater than 12 6 μM A woman was classified as hypertensive, hypercholesterolemic, or diabetic if she reported ever receiving the diagnosis by a physician Women were classified as premenopausal if they (1) reported still having menstrual periods, (2) were currently pregnant or nursing, or (3) had undergone hysterectomy but had at least one remaining functioning ovary Family history of early onset MI was defined as having at least one first degree relative who had an acute MI before the age of 55

The association of the C allele of the *GPIbα* Kozak sequence polymorphism with MI and stroke was examined by calculation of the age adjusted odds ratio (OR) and 95% confidence interval (CI) using unconditional logistic regression The extent to which the association of the C allele with risk of MI or stroke was modified by other cardiovascular risk factors (smoking, hypertension, diabetes mellitus, hypercholesterolemia, obesity, and homocysteinemia) was assessed through analyses stratified by these other risk factors To test for the presence of interaction between the Kozak C allele and these other cardiovascular risk factors, multiplicative terms were introduced into the logistic regression model, and the *P* value was computed for the likelihood ratio test comparing the model containing the interaction term with the model lacking the interaction term To control for the potential confounding effects of the *GPIbα* HPA 2 and VNTR polymorphisms on the risk of MI and stroke due to linkage disequilibrium with the Kozak sequence C allele, we also performed separate logistic regression models that included either (1) an indicator variable encoding the presence (one copy or more) of the less common HPA-2b (Met^{145}) allele or (2) 3 indicator variables encoding the presence (one copy or more) of the VNTR B, VNTR-C, and VNTR D alleles All data were analyzed using the SAS package (Cary, NC)

Table 1 Recruitment of study cases and control subjects

	Myocardial infarction	Stroke	Controls
Eligible cases by chart review	208	249	
Living at time recruitment initiated	161	198	684
Recruited and interviewed	112	149	526
Blood samples collected	79	106	391
Analyzable DNA available for genotyping	78	106	384

Results

The characteristics of the MI patients, stroke patients, and control subjects are summarized in Table 2 The 78 women with acute MI had a median age of 39 7 years (range 23 to 44) and the 106 women with stroke a median age of 36 6 years (range 18 to 44) Most of the study patients and control subjects were white There was a higher proportion of African Americans among the hemorrhagic stroke and MI cases than in the control group The frequency of cardiovascular risk factors such as smoking, obesity, hypercholesterolemia, hypertension, diabetes mellitus, and hyperhomocysteinemia was higher in the MI patients than control subjects For both ischemic and hemorrhagic subtypes, the frequency of smoking, hypertension, and hyperhomocysteinemia was higher in the stroke patients than control subjects In contrast, higher frequencies of obesity and diabetes were confined to the ischemic stroke cases

The distribution of the *GPIbα*-5C/T genotypes among the study subjects is shown in Table 3 Among the control subjects, the *GPIbα*-5C/T genotype distribution was in Hardy-Weinberg equilibrium MI patients were less likely to have at least one copy of the C allele compared with the control subjects (14 1% vs 23 7%), although the frequency of the −5C/C genotype was similar in both groups (1 3% vs 1 6%) The age-adjusted odds ratio for women carrying at least one copy of the C allele (0 53, 95% CI = 0 27-1 05) was virtually identical to the unadjusted OR (0 53, 95% CI = 0 27-1 04) When adjusted additionally for obesity, homocysteine level, and diagnosis of hypertension, diabetes mellitus, or hypercholesterolemia, the OR was 0 46 (95% CI = 0 22-0 96) With the analysis restricted to the white population, the OR for the age-adjusted model was 0 58 (95% CI = 0 28-1 20)

When stratified according to the presence or absence of known cardiovascular risk factors, the presence of the C allele was not associated with increased risk of MI even in women with known cardiovascular risk factors (Table 4) In women with obesity, the age-adjusted OR was 0 81 (95% CI 0 33-1 94), with diabetes,

Table 2 Characteristics of MI patients, stroke patients, and control subjects

	Myocardial infarction (n = 78)	Ischemic stroke (n = 41)	Hemorrhagic stroke (n = 54)	Controls (n = 384)
Age (y)				
Median	39 7	37 6	36 0	37 8
Mean	41	39 0	37 0	39 0
Range	23-44	21-44	18-44	19-44
Race (%)				
White	87	88	78	90
African American	6	2	9	2
Other	7	10	13	8
Current smokers (%)	69 2	31 7	44 4	20 8
Obesity* (%)	56 4	48 7	25 9	26 5
Current oral contraceptive use (%)	3 8	10 0	13 0	10 9
Premenopausal (%)	89 7	92 7	88 8	95 6
Diagnosis of†				
Hypercholesterolemia (%)	41 8	9 8	16 7	15 7
Hypertension (%)	34 2	31 7	27 8	9 7
Diabetes (%)	15 2	19 5	3 7	2 9
Hyperhomocysteinemia‡ (%)	51 3	35	39 6	24 2

MI indicates myocardial infarction
*Defined as BMI ≥ 27 3 kg/m²
†Ever received the diagnosis based on subject interview
‡Defined as plasma homocysteine ≥ 12 6 mg/dL

hypercholesterolemia, or hypertension was 0 83 (95%CI 0 35-1 99), and with hyperhomocysteinemia was 0 92 (95% CI 0 34-2 48) By contrast, in certain subgroups of women without traditional cardiovascular risk factors, the risk of MI associated with the C allele was particularly low The age-adjusted OR in women without obesity was 0 20 (95% CI 0 05 0 87), without diabetes, hypercholesterolemia, or hypertension was 0 22 (95% CI 0 05 0 96), and without hyperhomocysteinemia was 0 36 (0 12-1 05) However, none of these interactions between traditional cardiovascular risk factors and the Kozak −5C allele were statistically significant (Table 4)

Examination of the *GPIbα* genotypes in cases and controls revealed strong linkage disequilibrium between the Kozak −5C allele and the *GPIbα* HPA-2a/VNTR C haplotypes For example, analysis of the 551 subjects (1002 haplotypes) who are genetically informative for the HPA-2 and −5C/T dimorphisms (ie, homozygous for one or both dimorphisms) revealed that all 120 of the haplotypes that contain the −5C allele also contain the HPA 2a allele In contrast, all 83 HPA-2b haplotypes were associated with the −5T allele In the remaining 899 haplotypes, the −5T allele was associated with HPA-2a Because the *GPIbα* HPA-2 and VNTR polymorphisms have been associated with risk of atherothrombotic disease in other population studies,[9 11] we reanalyzed the risk of MI associated with the Kozak sequence polymorphism in our subjects, while separately controlling for the effects of the HPA-2 and VNTR polymorphisms The OR for MI associated with the presence of the Kozak C allele was 0 53 (95% CI 0 27-1 04) after adjusting for HPA-2, and 0 50 (95% CI 0 25-0 99) after adjusting for VNTR Furthermore, there was no evidence of effect modification of the risk of MI associated with the Kozak C allele when the subjects were stratified according to presence or absence of the HPA-2b allele (Table 4)

Stroke patients overall were as likely as control subjects to have at least one copy of the C allele (23 6% vs 23 7%) The age-adjusted OR associated with ischemic stroke for women who possessed at least one copy of the C allele was 0 99 (95% CI = 0 59-1 65) When analyzed according to stroke subtype, there was no association between carrying at least one copy of the C allele and risk of hemorrhagic or ischemic events (Table 3) Additional adjustment for the HPA-2 and VNTR polymorphisms did not appreciably change the results (data not shown) The 3 stroke cases who possessed the C/C genotype all had thromboembolic events (2 had ischemic strokes and one had a cerebral venous thrombosis) The frequency of the C/C genotype in the ischemic stroke cases (4 9%) was higher compared with controls (1 6%), but the difference was not statistically significant The age-adjusted OR associated with ischemic stroke for women who possessed 2 copies of the C allele was 3 21, but the confidence interval is wide (0 62-16 54), reflecting the small numbers of subjects

Discussion

The nucleotide −5C Kozak sequence variant of *GPIbα* was recently associated with increased translational efficiency and increased platelet surface density of the GPIb IX-V receptor on

Table 3 Kozak genotype frequencies for MI cases, stroke cases, and control subjects

	TT (%)	TC (%)	CC (%)	Total	OR*	(95% CI)*
Control subjects	293 (76 3)	85 (22 1)	6 (1 6)	384		
MI cases	67 (85 9)	10 (12 8)	1 (1 3)	78	0 53	(0 27-1 05)
Total stroke cases	81 (76 4)	22 (20 8)	3 (2 8)	106	0 99	(0 59 1 65)
Ischemic stroke	31 (75 6)	8 (19 5)	2 (4 9)	41	0 89	(0 44-1 82)
Hemorrhagic stroke	41 (75 9)	13 (24 1)	0	54	1 01	(0 51 1 99)

MI indicates myocardial infarction OR odds ratio CI confidence interval
*OR and 95% CI calculated for presence of C allele (homozygous or heterozygous) compared with TT genotype and adjusted for age

Table 4 Risk of MI according to presence of Kozak C allele analysis stratified by cardiovascular risk factors

Risk factor	MI cases (n = 78)		Controls (n = 384)		OR*	(95% CI)*	*P* value for interaction
	T/C or C/C	T/T	T/C or C/C	T/T			
Overall	11	67	91	293	0 53	(0 27-1 05)	
Current smoking							
No	4	20	74	231	0 62	(0 21 1 88)	0 88
Yes	7	47	17	62	0 55	(0 21-1 46)	
Obesity							
BMI < 27 3 kg/m^2	2	32	66	213	0 20	(0 05-0 87)	0 09
BMI ≥ 27 3 kg/m^2	9	35	25	77	0 81	(0 33 1 94)	
Hypertension diabetes, or hypercholesterolemia							
No	2	28	70	218	0 22	(0 05-0 96)	0 10
Yes	9	39	21	75	0 83	(0 35 1 99)	
Early family history of MI in first degree relative							
No	7	43	75	249	0 55	(0 24 1 26)	0 88
Yes	4	22	14	39	0 43	(0 11-1 62)	
Plasma homocysteine							
<12 6 mg/dL	4	34	70	216	0 36	(0 12-1 05)	0 21
≥12 6 mg/dL	7	33	19	74	0 92	(0 34-2 48)	
Factor V or prothrombin mutations							
No	10	56	88	272	0 55	(0 27-1 13)	0 93
Yes	1	11	3	18	0 61	(0 05 6 88)	
HPA 2b allele							
No	57	10	242	79	0 54	(0 26-1 10)	0 87
Yes	1	10	12	51	0 45	(0 05-3 89)	

MI indicates myocardial infarction, OR odds ratio, CI confidence interval, BMI body mass index
*OR and 95% CI calculated for presence of C allele (homozygous or heterozygous) compared with TT genotype and adjusted for age

platelets as well as transfected cells expressing the complex [6] These findings support the possibility of the Kozak sequence C allele as a candidate genetic susceptibility marker for atherothrombotic disease However, the role of the *GPIbα* Kozak sequence polymorphism in the pathophysiology of arterial thrombotic disease has not been well characterized We have assessed the *GPIbα* Kozak polymorphism in young women, a study sample that we have previously used to demonstrate the importance of other genetic prothrombotic factors in atherothrombotic disease and their potential interaction with other cardiovascular risk factors [12 13] We found no increased risk of MI or stroke in young women who carry one or more copies of the C allele of the Kozak sequence of *GPIbα*

MI and stroke are unusual events in young women, thus our study population is unique and well suited for analyzing potential prothrombotic risk factors The incidence of cardiovascular risk factors such as smoking and obesity are quite high in our population, allowing us to analyze potential interactions between these known risk factors and new genetic factors We have previously shown that factor V Leiden and prothrombin 20210 G to A each increase the risk of MI in young women and interact synergistically with other cardiovascular risk factors such as smoking [12,13] It is therefore important to note that we find no association between the *GPIbα*-5C allele and increased risk of MI in the same study population even in subgroups of women with other cardiovascular risk factors

Our negative findings with respect to the C allele of the *GPIbα* Kozak sequence and increased risk of atherothrombotic events in young women are consistent with several recent reports involving predominantly middle-aged to older men In a case-control study of 539 patients with nonfatal MI, Croft et al[18] found no association with carriership of the C allele either overall (OR 1 03) or in subgroups of patients younger than 55 years old or defined by other cardiovascular risk factors In a smaller hospital-based case-control study, Corral et al[19] observed no association between Kozak genotype and acute coronary or cerebrovascular disease Similarly, a preliminary report indicated no association with unstable angina [20] In contrast to these findings, another preliminary report noted an increased frequency of the C variant of the *GPIbα* Kozak sequence in men younger than 55 and women younger than 65 years of age with a family history of early onset MI, but only when individuals with a history of diabetes mellitus, hypertension, and hypercholesterolemia were excluded [21]

In addition to the absence of an association with increased risk of atherothrombotic disease in young women, our data suggest that the C allele may even be associated with reduced risk of MI in this population However, because of the relatively small numbers of subjects and multiple subgroup comparisons, these findings should be interpreted cautiously This trend toward an inverse association between the Kozak C allele and the risk of MI in young women clearly requires confirmation in larger studies

The mechanism for the possible inverse relationship of the C allele of the *GPIbα* Kozak sequence is not readily apparent One may speculate that the "high expression" C allele is associated not only with higher levels of the receptor on the platelet surface,[6] but also with increased circulating levels of the soluble extracellular portion of *GPIbα* glycocalicin Alternatively, the C allele may have functional consequences for *GPIbα* on another cell type (eg, endothelial cells) that may influence the development of atherothrombotic disease via a causal pathway distinct from increased platelet adhesion Finally, because we analyzed only nonfatal MI cases, it is theoretically possible that the apparent inverse association results from selection bias due to a strong effect of the Kozak C allele on case fatality (discussed below)

As previously reported in the Spanish population,[5] our results indicate strong linkage disequilibrium between the *GPIbα* Kozak C allele and the *GPIbα* HPA-2a/VNTR-C haplotype The less common HPA-2b/VNTR-B haplotype, which appears to be linked to the Kozak T allele, has been associated with increased risk of MI and acute cerebral ischemia in some case-control studies[9 11] but not in others [19 22 24] Thus, it is possible that the apparent inverse

association between the Kozak −5C allele and risk of MI could result from its association with the "low risk" HPA-2a/VNTR-C haplotype In our study population of young women, there was a trend toward an increased risk of ischemic stroke associated with HPA-2b,[25] but no evidence of an association between HPA-2 and risk of MI (manuscript submitted) Furthermore, adjustment for HPA-2 and VNTR did not significantly alter the estimates of effect for the *GPIbα* Kozak C allele in our current analysis Although Afshar-Kharghan et al[6] described a dose-dependent increase in GPIb-V-IX receptor levels associated with the C allele among healthy platelet donors, Corral et al[19] were unable to confirm any difference in receptor levels between T/T and T/C individuals in a larger number of subjects Thus, another possible explanation for a possible inverse association with MI is that the Kozak polymorphism may be linked to a nearby gene that is important in atherosclerosis or thrombosis

Our study has several limitations, including a small number of subjects and multiple subgroup comparisons, which can potentially lead to spurious associations arising by chance The small sample size also lacks the statistical power to detect some associations, including the effect of the C/C genotype Lastly, the cases from whom blood specimens for DNA analysis were obtained included only women who survived at least 3 months after their MI or stroke This resulted in exclusion of approximately 20% of the cases initially identified by chart review Thus, our study design can only assess the relationship between genotype and nonfatal events, and an association may have been missed if the C allele is strongly associated with a high case fatality rate immediately after the acute event However, the case-control study design is the only practicable approach, because MI and stroke among women under the age of 45 are very rare Furthermore, it is highly improbable that early mortality is strongly influenced by *GPIbα* Kozak genotype However, this issue can be resolved empirically only by genotype analysis of blood or tissue from fatal cases

In conclusion, our results suggest that young women carrying the C allele of the Kozak sequence polymorphism of *GPIbα* are not at increased risk of MI or stroke Paradoxically, the C allele may even be associated with a reduced risk of MI in this population These findings require confirmation in studies that involve larger numbers of subjects and fatal events, as well as further elucidation of the phenotypic consequences of the *GPIbα* Kozak sequence variant

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