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ORIGINAL INVESTIGATION

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Genetic susceptibility to pre-eclampsia and chromosome 7q36

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Abstract Pre-eclampsia is the most common serious medical disorder of human pregnancy. The human endothelial cell nitric oxide synthase (eNOS) gene is a candidate for pre-eclampsia/eclampsia (PE/E) susceptibility. A linkage study was performed on Australian PE/E families using 25 microsatellite markers from chromosome 7, one of which (eNOS-CA) resides within the eNOS gene. No significant linkage was found for the eNOS-CA marker using either parametric or non-parametric analysis. However, D7S1805 from the eNOS gene region on 7q36, gave a suggestion of linkage using parametric analysis (maximum LOD score =2.143 at θ=0.14) and non-parametric APM analysis ($T_{1/sqrt(p)}$=3.53; *P*=0.002). Further, an association study was performed on unrelated PE/E cases and controls from both Chinese and Australian populations to test for a relationship between the eNOS gene and PE/E. No association was found between the eNOS-CA marker and PE/E in either population. However, there was a significant difference in the allelic distribution of eNOS-CA between the two ethnic groups. The linkage results support the possibility that a susceptibility locus for pre-eclampsia resides in the 7q36 region, however, there is no definitive evidence to support the notion that the eNOS gene itself is responsible for susceptibility to pre-eclampsia.

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Introduction

Pre-eclampsia (PE) is the most common serious disease of human pregnancy. It has a world-wide incidence of 1–5% (Cooper et al. 1993). Occurring in the mid-to-late stages of gestation, PE is traditionally diagnosed by the three clinical signs: pregnancy-induced hypertension, proteinuria and oedema. Severe clinical deterioration is marked by the development of multi-system organ dysfunction and/or a convulsive condition called eclampsia (E). Severe pre-eclampsia and eclampsia carry a high morbidity and mortality for both mother and baby. The only effective treatment of PE/E is delivery of both fetus and placenta, irrespective of gestational length.

The aetiology of PE/E remains unknown. Epidemiological studies indicate that PE/E has a strong familial component (Chesley et al. 1968; Chesley and Cooper 1986; Cooper et al. 1993; Arngrimsson et al. 1995), and is primarily a placental disorder (Redman 1991; de Groot and Taylor 1993; Roberts and Redman 1993). Human endothelial nitric oxide synthase (eNOS) is widely distributed in placental tissue (Myatt et al. 1991; Buttery et al. 1994) and produces nitric oxide (NO), a potent vasodilator and inhibitor of platelet aggregation. Reduction of placental NO synthase activity has been demonstrated in pre-eclamptic placentae (Morris et al. 1995; Brennecke et al.

1997) implicating the eNOS gene in the pathophysiology of PE/E. A subsequent linkage study using PE/E families from Scotland and Iceland reported evidence for a PE/E susceptibility locus in the eNOS gene region on chromosome 7q36 (Arngrimsson et al. 1997). This, however, was not confirmed in a repetition study using the same markers in families from Amsterdam and the United Kingdom (Lewis et al. 1999). We now report our findings from a chromosome 7 linkage study with particular emphasis on the eNOS gene region, using multiple Australian PE/E affected families. In addition, we present the results of a case control association study in Chinese and Australian populations performed to investigate the relationship between the eNOS gene and PE/E.

Subjects and methods

PE/E families and definition

This study incorporated a total of 26 Australian PE/E families (Wilton et al. 1990; Harrison et al. 1997; Lade et al. 1999), which included 14 cases of eclampsia, 57 of severe pre-eclampsia and 28 of mild pre-eclampsia. For the association study, unrelated individuals from China (72 cases of severe pre-eclampsia and 48 normal pregnancy controls) and from Australia (46 cases of severe pre-eclampsia and 80 controls) were genotyped (Guo et al. 1997).

The criteria of the Australasian Society for the Study of Hypertension in Pregnancy (Brown et al. 1993) were used for clinical diagnosis of PE/E. Women were considered to have severe pre-eclampsia if they had: (1) a rise from baseline systolic blood pressure of at least 25 mm Hg and/or a rise from baseline diastolic pressure of at least 15 mm Hg; or (2) the presence of a systolic pressure of at least 140 mm Hg, and/or a diastolic pressure of at least 90 mm Hg. These levels had to occur on at least two occasions 6 h or more apart. The level of proteinuria had to be greater than 0.3 g/l in a 24 h specimen, or at least "2+" dipstick proteinuria on a random urine collection. Women who met these criteria and experienced convulsions or unconsciousness in the perinatal period were classified as having had eclampsia. Women with the pattern of hypertension outlined above but with no proteinuria were classified as mild pre-eclamptics. At the clinical level, PE covers a spectrum of severity from mild to severe to eclampsia itself, with the progression in severity in any given case being dependant upon individual susceptibility, pregnancy stage and the availability and standard of clinical care. Because PE/E is typically a disease of first pregnancies, only those women with the above features in their first, but not subsequent, pregnancies were included in this study. Ethics approval for this study was obtained from all relevant institutions and informed written consents were obtained from family members.

DNA genotyping

Eighteen chromosome 7 microsatellite markers from the Human Linkage Mapping Set Version I (LMS I, PE Applied Biosystems), six microsatellite markers in the 7q35–36 region and one microsatellite marker within intron 13 of the eNOS gene, were genotyped (Fig. 1).

The eNOS-CA repeat was amplified in a 10 μl reaction containing 30 ng genomic DNA, 2 pmol of each primer (Nadaud et al. 1994) with one fluorescently labelled, 200 μM of each dNTP, 3 mM $MgCl_2$ and 0.5 U Ampli *Taq* Gold DNA polymerase (PE Applied Biosystems). PCR was carried out on a GeneAmp PCR System 9600 (PE Applied Biosystems) with initial denaturation at 94°C for 10 min and two step cycles of 94°C for 1 min, 60°C for 1 min for 35 cycles followed by a final 10 min incubation at 72°C. D7S1824, D7S498, D7S1805 and D7S1826 were amplified and genotyped using the γ[^{32}P]dATP labelling method (Wilton et al. 1995). All other microsatellite markers were amplified using the standard LMS I protocol (PE Applied Biosystems) on a FST-960 microplate thermal sequencer (Corbett Research, Sydney, Australia) and PCR products were sized and genotyped using an ABI 377 DNA sequencer with GeneScan (Version 2.1) and Genotyper (Version 2.0) software (PE Applied Biosystems).

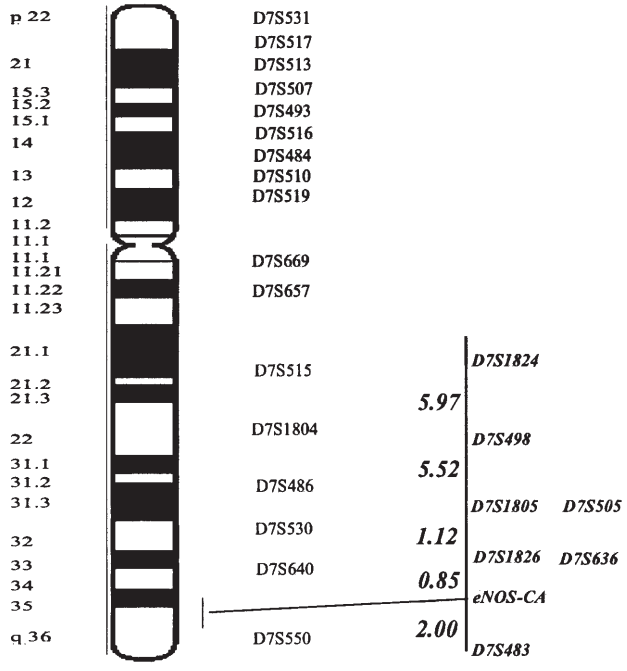

Fig. 1 Schematic diagram of chromosome 7 showing the microsatellite markers typed in this study. The relative map positions are as given at http://www.marshmed.org/genetics/ and http://www.CHLC.org/ABI/ABIRefMaps.html. The eNOS-CA marker was positioned as previously described (Nadaud et al. 1994; Arngrimsson et al. 1997). The distance between markers in the eNOS gene region is shown in Haldane cM. The eNOS-CA, D7S1805, D7S505 and D7S483 markers were typed on 26 PE/E families; D7S1824, D7S1804 and all LMSI markers were typed on 21 PE/E families; D7S498 and D7S1826 markers were typed on 15 PE/E families. The variations in the number of families for the various marker sets are explained by the ongoing recruitment of families during the course of this study

Computational analysis

All computational linkage analysis was carried out using two maternal disease state classifications. The two classifications are as follows. Firstly, MPE where mild pre-eclampsia, severe pre-eclampsia and eclampsia are treated as affected. Secondly, SPE where severe pre-eclampsia and eclampsia only were treated as affected, while mild pre-eclampsia was treated as unaffected, therefore accounting for the suggestion that mild pre-eclampsia may be inherited independently to severe pre-eclampsia and eclampsia (Cooper and Liston 1979). The paternal affection status was classified as "unknown".

Allele frequencies for all chromosome 7 markers (Fig. 1) were calculated using unrelated Australian individuals from pedigrees.

Pedigree power calculations

The SLINK program (Ott 1989; Weeks et al. 1990) was used to calculate the power of 26 multi-case Australian PE/E families, based on the seven inheritance models outlined in Table 1.

Table 1 Inheritance models used for parametric analyses

Model	Mode of inheritance	Gene frequency	Penetrance		Reference
			A_1 A_2	A_2 A_2	
1	Autosomal recessive	0.20	0.00	1.00	Cooper and Liston 1979; Chesley and Cooper 1986
2	Autosomal recessive	0.25	0.00	0.90	Wilton et al. 1995
3	Autosomal recessive Incomplete penetrance	0.39	0.00	0.41	Liston and Kilpatrick 1991
4	Partial dominance	0.10	0.21	1.00	Arngrimsson et al. 1995
5	Arbitrary autosomal dominance	0.02	0.90	0.90	Arngrimsson et al. 1997
6	Affecteds-only analysis	0.02	0.01	0.01	Arngrimsson et al. 1997
7	Autosomal dominant low penetrance	0.10	0.30	1.00	Arngrimsson et al. 1995

Parametric linkage analysis

Linkage analysis was carried out for all chromosome 7 markers (Fig. 1) using the ANALYZE package (ftp://linkage.cpmc.columbia.edu/software/analyze). Two-point parametric analysis was carried out using seven inheritance models (Table 1), six of which were used by Arngrimsson et al. (1997). Heterogeneity was tested using the HOMOG program (Ott 1991) in the ANALYZE package. Multi-point linkage analysis was carried out for microsatellite markers in the eNOS gene region using the LINKMAP program from the LINKAGE package (Lathrop et al. 1984). The eNOS-CA marker was positioned relative to the other seven markers in the eNOS gene region, according to published data (Nadaud et al. 1994; Arngrimsson et al. 1997). As different pedigrees may yield different estimates of genetic distance, both a published map and a map generated from our pedigrees were used for multi-point linkage analysis.

Non-parametric analysis

The affected pedigree member (APM) method (Weeks and Lange 1988) was used for non-parametric linkage analysis of eNOS-CA and D7S1805. In addition, multilocus APM (Weeks and Lange 1992) was used for the joint analysis of five microsatellite markers in the eNOS gene region, (D7S1805, eNOS, D7S505, D7S636 and D7S483) only for SPE. The exact *P* value for each *T* statistic was estimated by carrying out 10,000 simulations. Non-parametric linkage analysis transmission/disequilibrium test (TDT) was also carried out for all chromosome 7 markers (Fig. 1) using the TDTLIKE program (Terwilliger 1995) in the ANALYZE package.

Association study

Chi-squared analysis was used for the association study to examine the allele distributions of the eNOS-CA marker between PE-case and control groups in Chinese and Australian populations.

Results

Pedigree power calculations

Power calculations carried out on the 26 affected Australian PE/E families demonstrated that the power for detecting linkage under recessive inheritance models is greater than that for dominant models with low penetrance, as previously described (Harrison et al. 1997). Using a simulated marker with a heterozygosity of 0.78, an average maximum LOD score of 4.21 (Z_{max}=9.94) was observed at a recombination fraction of 0.05 under model 2 (Table 1), with 75% of replicates establishing linkage at the critical value of 3.0. For MPE under the same model, a marker with the same properties gave an average maximum LOD score of 4.74, with 80% of replicates establishing linkage at the critical value of 3.0.

Two-point LOD score analysis

Two-point LOD scores for a range of recombination fractions were calculated for each family and for all families combined. There was no suggestion of linkage based on two-point LOD score analysis for either the SPE or MPE classifications for any of the 18 LMS I chromosome 7 markers under the seven inheritance models tested (Table 1).

In addition, two-point LOD score analysis was also carried out for eight microsatellite markers in the eNOS gene region. D7S1805 gave the highest LOD score with a Z_{max} of 2.143 at θ=0.14 for SPE under model 1 and a Z_{max} of 2.024 at θ=0.12 for the SPE model 2. The eNOS-CA marker gave a Z_{max} of 1.45 at θ=0.20 for SPE under model 1 and a Z_{max} of 1.38 at θ=0.18 for the SPE under model 2. For SPE, four markers (D7S1824, D7S505, D7S1826, D7S483) gave inconclusive LOD scores <1, while D7S498 and D7S636 gave a Z_{max} of 1.28 and 1.25, respectively, under model 1. Two-point LOD scores for SPE under model 2 are shown for all markers typed in the eNOS gene region (Table 2). Two-point LOD scores for the MPE classification did not reach 1.00 under any of the models tested for the eight markers in the eNOS gene region.

Heterogeneity was shown for both D7S1805 and eNOS-CA under various models for SPE. Of the seven inheritance models tested, D7S1805 indicated heterogeneity for linkage to SPE (α=0.51 to 0.88) under models 1, 2, 3 and 6, while the eNOS-CA marker showed heterogeneity (α=0.28 to 0.88) under all models except model 4 (Table 3). Under heterogeneity D7S1805 gave a Z_{max} of 2.54 at θ=0.00 under model 1 and a Z_{max} of 2.30 at θ=0.00 under model 2. While the eNOS-CA marker gave a Z_{max} of 1.83 at θ=0.00 under model 1 and a Z_{max} of 1.58 at θ=0.00 under model 2. Polylocus LOD scores were calculated for families in which a given marker is untyped or uninfor-

Table 2 Two-point LOD score analyses for markers in the eNOS gene region under the severe pre-eclampsia autosomal recessive inheritance model (gene frequency =0.25; penetrance =0.90) (Z_{max} maximum LOD score, θ_{max} theta value at the maximum LOD score, $H\ Z_{max}$ maximum LOD score with heterogeneity, α the portion of linked families)

Locus	Haldane cM	Z_{max}	θ_{max}	$H\ Z_{max}$	θ_{max}	α
D7S1824	0.0000	0.588	0.20	0.588	0.20	1.00
D7S498	0.05973	1.141	0.10	1.172	0.00	0.61
D7S1805	0.11497	2.024	0.12	2.300	0.00	0.55
D7S505	0.11597	0.851	0.16	0.851	0.16	1.00
D7S1826	0.12710	0.549	0.20	0.581	0.08	0.49
D7S636	0.12810	1.170	0.14	1.202	0.06	0.69
eNOS-CA	0.13616	1.381	0.18	1.582	0.00	0.45
D7S483	0.15657	0.102	0.38	0.102	0.38	1.00

mative by using information from the next nearest marker. Under heterogeneity D7S1805 gave a polylocus Z_{max} of 2.64 under model 1 and 2.39 under model 2, while eNOS-CA give a polylocus Z_{max} of 1.93 under model 1 and 1.68 under model 2.

Multi-point LOD score analysis

Multi-point LOD score analysis was performed for the eight markers in the eNOS gene region to locate the putative PE locus position for SPE under model 2, as it is unlikely that the putative gene has 100% penetrance as in model 1 given the lack of concordance between monozygous twins (Cooper et al. 1993). The marker map used for multi-point LOD score analysis (Fig. 1) was derived from the Marshfield marker map and the position of eNOS was derived from published data (Arngrimsson et al.1997; Nadaud et al. 1994). Using this marker map, the maximum LOD score for the putative PE locus was 2.02 located in the interval between D7S498 and D7S1805 (Fig. 2). Using the marker map generated from our pedigree data, the maximum LOD score for the putative PE locus was 2.10, again between D7S498 and D7S1805, with an interval of 14 cM. A maximum LOD score of 2.87 was given by four-point analysis from the D7S1824, D7S498 and D7S1805 marker group using the Marshfield map, with a similar maximum LOD score of 2.78 for the same marker group using the map generated from our data. Four-point analysis for other marker groups did not give LOD scores greater than 2.

Non-parametric linkage analysis

The APM was used to measure the deviation from the expected levels of allele sharing in affected individuals. The APM results for this study gave no evidence for linkage between the eNOS-CA locus and PE/E. However, suggestive linkage for D7S1805 with the putative PE locus for SPE was found under the allele frequency weighting 1/sqrt (p), T=3.53, P=0.002 (Table 4). Multilocus APM analysis using the SPE classification for five markers (D7S1805, eNOS-CA, D7S505, D7S636 and D7S483) also indicated evidence of allelic distortion from independent segregation [1/sqrt (p) T=2.60, P=0.004]. TDT gave no significant evidence of allelic association for markers within the eNOS gene region.

An association study for eNOS-CA and PE/E

The eNOS-CA marker was highly polymorphic in the Chinese and Australian populations studied, with allele sizes ranging from 140 to 186 bp in the Chinese (Fig. 3A) and from 150 to 190 bp in the Australian populations (Fig. 3B). There were no significant differences between allele frequencies of the eNOS-CA repeat in the Chinese PE group and controls (χ^2=20.32, df=18, P=0.27). Also no significant differences were found between the allele frequencies of the eNOS-CA marker in the Australian PE group and controls (χ^2=22.87, df=18, P=0.20). However, there were significant differences between the eNOS-CA allele frequencies in different ethnic groups (Chinese to Australian, χ^2=38.67, df=20, P=0.0073) (Fig. 3C).

Table 3 Two-point LOD score analyses for D7S1805 and eNOS-CA for severe pre-eclampsia using various inheritance models (see Table 1) (Z_{max} maximum LOD score, θ_{max} theta value at the maximum LOD score, $H\ Z_{max}$ maximum LOD score with heterogeneity, α the portion of linked families)

Locus	LOD scores	Inheritance models						
		1	2	3	4	5	6	7
D7S1805	Z_{max}	2.143	2.024	1.299	1.568	0.019	0.280	1.175
	θ_{max}	0.14	0.12	0.02	0.00	0.44	0.22	0.00
	$H\ Z_{max}$	2.543	2.300	1.308	1.568	0.019	0.347	1.175
	θ_{max}	0.00	0.00	0.00	0.00	0.44	0.12	0.00
	α	0.51	0.55	0.88	1.00	0.99	0.56	0.98
eNOS-CA	Z_{max}	1.453	1.381	0.771	0.606	0.210	0.00	0.488
	θ_{max}	0.20	0.18	0.10	0.14	0.32	99.0	0.14
	$H\ Z_{max}$	1.828	1.582	0.783	0.606	0.278	0.043	0.490
	θ_{max}	0.00	0.00	0.00	0.14	0.20	0.20	0.12
	α	0.42	0.45	0.67	1.00	0.41	0.28	0.88

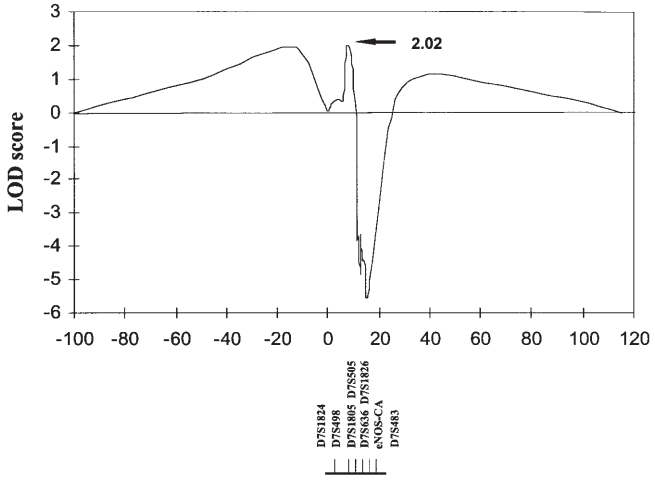

Fig. 2 Multipoint LOD scores analysis for eight microsatellite markers in the eNOS-CA gene region. A LOD score of 2.02 was obtained between D7S498 and D7S1805 for severe pre-eclampsia under model 2 (autosomal recessive with gene frequency =0.25, penetrance =0.90)

Table 4 Affected pedigree member (APM) method results for eNOS-CA and D7S1805 (*SPE* severe pre-eclampsia, *MPE* mild pre-eclampsia, *T* T-statistic, *P P*-value)

Weight function	eNOS-CA				D7S1805			
	SPE		MPE		SPE		MPE	
	T	P	T	P	T	P	T	P
f (p) = 1	0.57	0.27	–0.21	0.58	1.09	0.14	0.88	0.17
f (p) = 1/ sqrt (p)	–0.12	0.54	–0.35	0.65	3.53	0.002	2.74	0.008
f (p) = 1/p	–0.55	0.69	–0.52	0.67	4.95	0.002	3.61	0.007

Discussion

The genetic basis of PE/E is complex. It is unclear whether maternal gene(s), fetal gene(s) or maternal-fetal gene interaction is involved in susceptibility to PE/E. Further, the contribution of paternal gene(s) is unknown as it can only be inferred through their daughters' phenotype. A number of candidate gene studies based on the maternal genotype have been carried out, however no causative gene(s) for PE/E have been confirmed (Ward et al. 1993; Arngrimsson et al. 1993; Wilton et al. 1990;Wilton et al. 1995; Morgan et al. 1995). The evidence for a PE susceptibility locus in the eNOS gene region was reported based on a significant two-point linkage for the marker D7S505 (Z_{max}=4.03) and suggestive non-parametric linkage for eNOS-CA in 50 affected families, including 106 cases of severe pre-eclampsia and 42 cases of mild pre-eclampsia (Arngrimsson et al. 1997). In contrast, linkage was excluded for eNOS-CA under all models, and also excluded for two flanking markers, D7S505 and D7S483, under most models using 104 sibships and 21 PE families (Lewis et al. 1999).

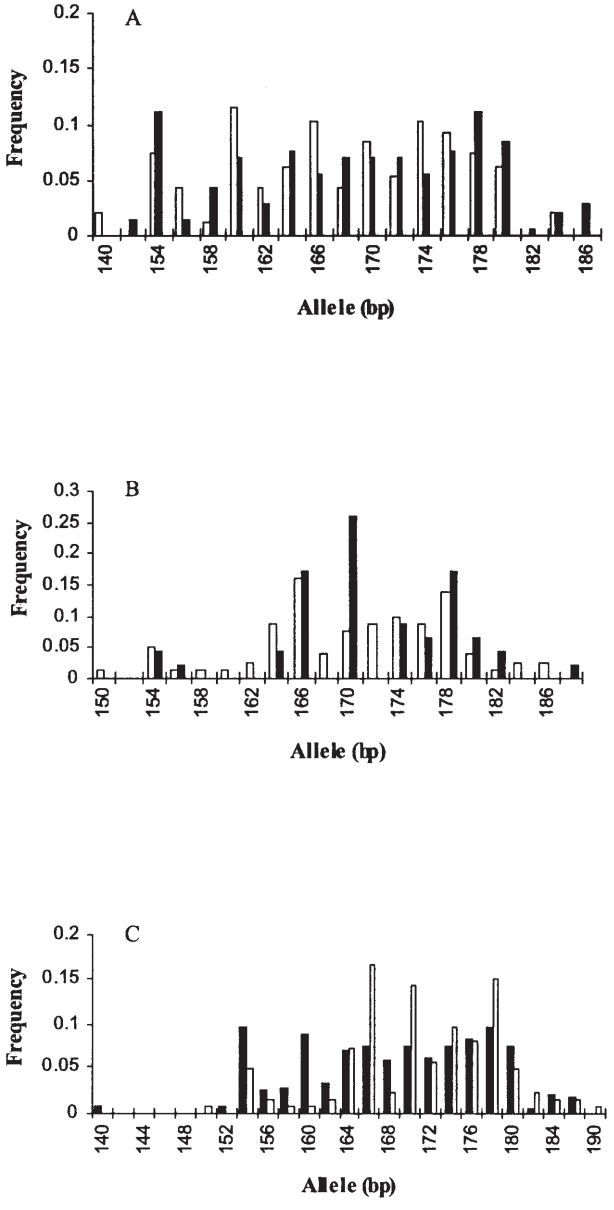

Fig. 3 Association study results illustrate allele distributions for the eNOS-CA repeat between pre-eclampsia cases and controls. **A** Between pre-eclampsia (*black bars*) and controls (*white bars*) in the Chinese population. **B** Between pre-eclampsia (*black bars*) and controls (*white bars*) in the Australian population. **C** Allele distribution of the eNOS-CA repeat between Chinese (*black bars*) and Australian (*white bars*) populations

In this study, no significant linkage was found for the marker D7S505 under any model tested, as reported in a previous study (Lade et al. 1999). However, a strong suggestion of linkage was given for D7S1805, which is located in approximately the same position as D7S505 (Fig. 1) by non-parametric analysis, using the APM method for SPE. Furthermore, a suggestive linkage was found for D7S1805 under SPE using parametric analysis, according to the guidelines for complex disease (Lander and Kruglyak 1995). The eNOS-CA marker gave positive LOD scores for SPE, but did not reach suggestive linkage,

nor did it demonstrate distortion in allele sharing under non-parametric analysis. In the Icelandic/Scottish study (Arngrimsson et al. 1997), eNOS-CA gave a Z_{max} of 1.95 at θ=0.04 for SPE and a Z_{max} of 2.44 at θ=0.06 for MPE by two-point analysis. In addition, suggestive linkage disequilibrium was given for eNOS-CA allele 14 under SPE by TDT (P=0.007), with 16 cases and 2 controls, only when Scottish and Icelandic families were combined. The results from our study, considering previous studies (Arngrimsson et al. 1997; Lade et al. 1999; Lewis et al. 1999), might be interpreted as follows. Firstly, the eNOS gene may not be involved in PE/E but a neighbouring gene linked to the D7S1805 marker within the eNOS gene region may be responsible for PE, as suggested by multipoint analysis (Fig. 2). Secondly, the eNOS gene may be a susceptibility gene for PE/E. However, the ability to identify the involvement of the eNOS gene may be affected by heterogeneity, or differences in marker information between populations, and particularly by differences in the diagnostic criteria used by our study in comparison to those used by Arngrimsson and co-workers (1997).

Association studies are useful to determine whether a candidate locus is causative of a disease in different populations. This method relies on either typing the disease-causing site (mutation) directly, or on there being linkage disequilibrium between the marker typed and the disease-causing site. No differences were found in the allele distribution of eNOS-CA between case group and control group in either the Chinese or Australian populations. However, there was a significant difference in allele distributions between the two ethnic groups. A comparison between Japanese and French Caucasians showed similar trends (Makayama et al. 1995). Caution is needed when undertaking an association study using markers that have significant differences in allele frequency in different populations, as it can lead to either false positive or negative results. Although no association was found between the eNOS-CA marker and PE in the case-control study, the possibility of functional variations of the eNOS gene being causative of PE cannot be ruled out, because the marker typed was a microsatellite. If there were strong linkage disequilibrium in the region, the disease association due to a nearby functional site would only be evident. For example, one variation (G894 T) in exon 7 of the eNOS gene was recently found to be associated with essential hypertension (Miyamoto et al. 1998).

It is particularly challenging to carry out linkage analysis for complex diseases, such as PE/E. The results from this study support the location of a putative PE/E susceptibility locus on 7q36. Fine-mapping studies with a higher density of markers within the 7q36 region, and association studies of functional variations within the eNOS gene in different ethnic groups may increase further understanding of the relationship between PE/E susceptibility and the 7q 36 eNOS gene region.

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