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Muscle-Specific Creatine Kinase Gene Polymorphisms in Korean Elite Athletes

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Abstract. In view of the importance of muscle-specific creatine kinase (CKMM) gene as a genetic factor for athletic performance, we investigate the relationship between elite athletic performance and two restriction fragment length polymorphisms (*Nco*I and *Taq*I RFLPs) in the CKMM gene. Genomic DNA was extracted from white blood cells of 98 unrelated male Korean elite athletes and 64 sedentary controls, respectively. Two genetic polymorphisms in the CKMM gene were detected by the polymerase chain reaction and the digestion with restriction endonucleases, *Nco*I and *Taq*I, respectively. There were no significant associations between two genetic polymorphisms in the CKMM gene and elite athletic performance or clinical parameters in our subjects. Therefore, these findings suggest that two genetic polymorphisms in the CKMM gene may not be useful as genetic markers to predict the athletic performance in male Koreans.

Keywords: Endurance, Genotype, Muscle-specific creatine kinase.

INTRODUCTION

ATP regeneration capacity is an important element of athletic performance, and DNA sequence variations in genes encoding enzymes involved in this function may be related to athletic performance.

Creatine kinase is a key enzyme in energy metabolism that catalyzes the following reaction: phosphocreatine + MgADP + $H^+ \leftrightarrow$ MgATP + creatine. This enzyme is abundantly present in skeletal muscle, heart, and brain, and several isozymes are reported (Wallimann *et al.,* 1992). In addition, recent studies indicate its presence in the intestine, uterus, and sperm (Koretsky, 1995). There are two distinct CK mitochondrial forms (ubiquitous and sarcomeric) and two cytosolic subunits (brain and muscle) that are encoded by at least four different CK genes (Wallimann *et al.,* 1992).

The cytosolic CKMM gene has been mapped to the q13.2-q13.3 region of chromosome 19 (Nigro *et al.,* 1987). This gene extends over 17.5 kilobase pairs, and contains 8 exons and 7 introns (Trask *et al.,* 1988). The CKMM gene is a good candidate in athletic performance for several reasons. Skeletal muscles of athletes involved in athletic performance are characterized by a high proportion of type I (slow twitch) fibers as well as by high activity levels of marker enzymes of the aerobic oxidative metabolism (Holloszy and Coyle, 1984). There are several reports that genetic factors explain some of the inter-individual differences observed in these characteristics (Bouchard *et al.,* 1986; Simoneau and Bouchard, 1995). Considering that CKMM activity level is two-fold higher in type II (fast-twitch) than in type I fibers (Yamashita and Yoshioka, 1991), a low CKMM activity level is often another typical feature of the skeletal muscle of endurance athletes. Experiments with knock-out mouse suggest that low CKMM activity resulted in an improved endurance performance during repeated muscular contractions (van Deursen *et al.,* 1993). In addition, other study using isoelectric focusing suggest that a CKMM protein charge variant was weakly associated with the ability to perform during a 90 min endurance test (Bouchard *et al.,* 1989).

Two restriction fragment length polymorphisms (RFLPs) of CKMM gene have already been identified, either with the *Nco*I or the *Taq*I restriction endonucleases (Lavedan

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et al., 1990; Gennarelli *et al.*, 1991), and used as genetic markers to perform the association study. Until now, there are some reports regarding the role of CKMM gene in athletic performance. Rivera *et al.* (1997a) proposed that there was the significant association between the *Nco*I RFLP in this gene and the VO_{2max} response to endurance training in biologically unrelated sedentary adult Caucasians, but their another study suggested that *Nco*I and *Taq*I RFLPs of this gene was not significantly associated with the elite endurance athlete status (Rivera *et al.*, 1997b).

It could be, however, excluded the possibility that two genetic markers of CKMM gene influence the athletic performance in other ethnic group. Therefore, the present study tested the hypothesis of association between two genetic polymorphisms (*Nco*I and *Taq*I RFLPs) of CKMM gene and athletic performance in ethnically homogeneous Korean population.

MATERIALS AND METHODS

Study Subjects

98 unrelated male elite athletes and 64 sedentary controls were randomly chosen from the students of department of physical education, Hanyang University, Seoul, Korea and from outpatients of department of clinical pathology, Seoul Hygiene Hospital, Seoul, Korea. Elite athletes belonged to the following sporting disciplines: 15 basketball players, 22 soccer players, 28 baseball players, 10 gymnastics players, 10 volleyball players, 4 >5,000 m middle-distant runners, 6 judo players and 3 marathon players.

Determination of Clinical Parameters

Blood samples were obtained in EDTA tubes from individuals who had been fasting for 12~16 hr. Systolic and diastolic blood pressures was measured by mercury sphigmomanometer. The mean arterial pressure (MAP) is calculaed by DBP-1/3 (SBP-DBP) (mmHg). The body mass index (BMI) value was calculated by the body weight (kg) devided by the square of the height (m^2). The VO_2max index was determined by using incremental exercise tests on cycle ergometers or motor-driven treadmills (Strømme *et al.*, 1977) when the athletes were at their peak or from previous laboratory assessments of their training status. Concentrations of serum total cholesterol (TC), triglyceride (TG) and glucose were measured by enzymatic colorimetry methods with commercial kit (Boehringer Mannheim, Germany) and chemistry analyzer. Serum HDL-cholesterol level was determined by measuring cholesterol in the supernatant after precipitation of the serum with $MgCl_2$ and dextran sulfate, with a Gilford Impact 400E automated analyzer with reagents and calibrators from Boehringer Mannheim. Serum LDL-cholesterol level was calculated by using the formular of Friedewald *et al.* (1972). Serum LDH and creatine phosphokinase activities were measured by ultraviolet assay.

PCR Amplification

Blood samples were collected in EDTA-containing tubes, and centrifuged at 1,500×g for 10 min. Total genomic DNA was isolated from buffy coat by the method of Sambrook *et al.* (1989) with slight modification. The polymerase chain reaction (PCR) was performed in a DNA thermal cycler (Perkin Elmer Cetus GeneAmp 9700, Norwalk, CT, USA). Primers for the *Nco*I and *Taq*I RFLPs in the CKMM gene were as follows:

sense 5'-GTGCGGTGGACACAGCTGCCG-3'

anti-sense 5'-CAGCTTGGTCAAAGACATTGAGG-3'

(Gennarell *et al.*, 1991).

Briefly, total 50 μl of the reaction mixture contained 200~400 ng of genomic DNA, 100 ng of each primer, 200 μl of each dNTP, and buffers recommended by the manufacturer. Amplification was performed according to the following temperature condition: one cycle at 94°C for 5 min, 30 cycles at 95°C for 30 sec, at 60°C for

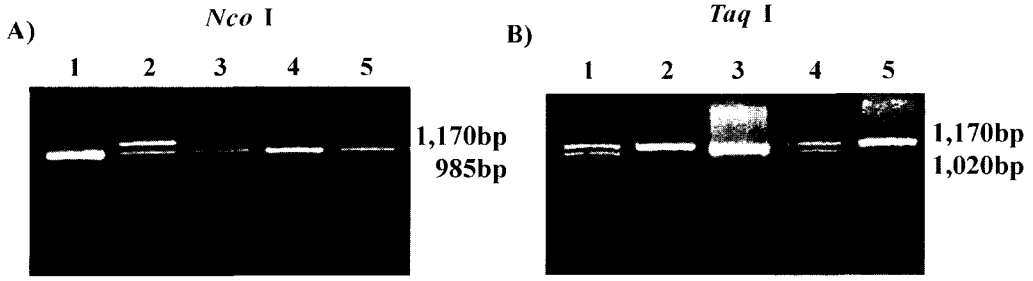

Fig. 1. Two RFLP patterns of muscle-specific creatine kinase gene. A) *Nco*I RFLP patterns. Lane 1 and 3~5, N2N2 genotypes; lane 2, N1N2 genotype. B) *Taq*I RFLP patterns. Lanes 1 and 4, T1T2 genotypes; lane 2 and 5, T1T1 genotypes; lane 3, T2T2 genotype.

30 sec and at 72°C for 45 sec with a final polymerization at 72°C for 5 min.

RFLP Analysis

After each amplification, 10 ul of the PCR product was digested in separate tube with 10 U of restriction enzymes, *Nco*I and *Taq*I, respectively. The digested fragments were separated by 2% agarose gel electrophoresis. The alleles without *Nco*I or *Taq*I restriction sites were designated with N1 or T1 alleles (all 1,170 bp), respectively, whereas the alleles with the polymorphic *Nco*I or *Taq*I restriction sites were designated as N2 (985 + 185 bp) or T2 (1,020 + 150 bp) alleles, respectively (Fig. 1).

Statistical Anaysis

The gene counting method estimated allele frequency. The heterozygosity and polymorphism information content (PIC) values were estimated by the method of Bostein *et al.* (1980). The significance of differences in allele frequencies between populations was estimated by χ^2-test, while the significance of differences in haplotype frequencies assessed in a 2 X N contingency table using Monte-Carlo simulation (Sham and Curtis, 1995). One-way ANOVA test was performed to compare the mean levels of clinical parameters among different genotypes.

The evidence for linkage disequilibrium between the two RFLP sites in the CKMM gene was estimated, we reported two-locus disequilibrium statistic of Δ (Hill and Robertson, 1968) and D' (Lewontin, 1964). Although the D' measure has the advantage of being less sensitive than Δ to the frequencies of the associated alleles (Hedrick, 1987), Δ has been reported in many previous studies, and we therefore reported both measure to facilitate comparisons. The measures are calculated as follows. If p is the frequency of the rarer allele at the first locus, q is the frequency of the rarer allele at the second locus, and h is the frequency of the haplotype with the rarer allele at both loci. Then, $D = (h - pq)/D_{max}$. D_{max} is the smaller of pq or $(1 - p)(1 - q)$ for $(h - pq) < 0$, or D_{max} is the smaller of $p(1 - q)$ or $(1 - p)q$ for $(h - pq) > 0$. To calculate the value of Δ, $\Delta = (h - pq)/\sqrt{p(1 - p)q(1 - q)}$. The significance of the observed disequilibrium is assessed with $n\,\Delta^2$ (where n is the number of haplotypes observed), which is asymptotically distributed as a χ^2 with 1 df.

Significance levels were corrected for multiple comparisons as follows: $\acute{\alpha} = 1 - (1 - \alpha)1/k$, where α is the risk of type I error chosen as significance level. Thus, corrected statistical significance was accepted at the P = 0.0016 level ($\alpha = 0.05$, k = 33). All statistical analyses were performed using the computer program of SPSSWIN (version 11.0).

RESULTS

Genotype Distribution

In the present study, we attempted to clarify the gene frequencies of two genetic polymorphisms in the CKMM gene from subjects of Korean origin. Table 1 displays the gene frequencies and the values of heterozygosity and PIC for *Nco*I RFLP of the CKMM gene in Korean normal controls and pooled elite athletes, respectively. The genotype and allele frequencies of *Nco*I RFLP were not significantly different between both groups, respectively. The frequencies of N1N1, N1N2 and N2N2 genotypes were 0, 31 and 69% in normal controls, and 5, 15 and 80% in elite athletes, respectively. N1N1 homozygote was only detected in elite athletes. The heterozygosity and PIC values of *Nco*I RFLP represented the values of 0.2637 and 0.2289 for normol controls, and 0.2226 and 0.1978 for elite athletes, respectively. Table 2 represents the allele distribution of the *Nco*I RFLP among various athletic groups. There was no significant difference in allele frequency among sporting disciplines studied.

Table 3 shows gene frequencies and the values of

Table 1. Genotype and allele frequencies of the *Nco*I RFLP in the muscle-specific creatine kinase gene between Korean male controls and Korean male elite athletes

	Genotype No. (%)			Allele No. (%)		H^1	PIC^2
	N1N1	N1N2	N2N2	N1	N2		
Controls	0(0)	20(31)	44(69)	20(16)	108(84)	0.2637	0.2289
Athletes[4]	5(5)	15(15)	78(80)	25(13)	171(87)	0.2226	0.1978
Chi-square		8.4250		3.7080			
Probability		0.0150		0.0540			
Odds ratio (CI)[3]			0.79(0.42-1.49)				

[1]Heterozygosity, [2]Polymorphism Information Content, [3]95% Confidence Interval.
Frequency is given as a percentage in parenthesis.
[4]The observed genotype distribution was significantly deviated from Hardy-Weinberg equilibrium (P<0.05).

Table 2. Distribution of *Nco*I RFLP of muscle-specific creatine kinase gene in Korean male controls and Korean male elite athletic groups

Subjects	Muscle-specific creatine kinase gene				
	Genotypes			Alleles	
	N1N1	N1N2	N2N2	N1	N2
Controls (n = 64)	0(0)	20(31)	44(69)	20(16)	108(84)
Athletes (n = 98)	5(5)	15(15)	78(80)	25(13)	171(87)
Basketball (n = 15)	0(0)	1(7)	14(93)	1(3)	29(97)
Soccer (n = 22)	1(4)	3(14)	18(82)	5(11)	39(89)
Baseball (n = 28)	2(7)	3(11)	23(82)	7(13)	49(87)
Gymnastics (n = 10)	0(0)	2(20)	8(80)	2(10)	18(90)
Volleyball (n = 10)	2(20)	3(30)	5(50)	7(35)	13(65)
Runner (n = 4)[1]	0(0)	1(25)	3(75)	1(13)	7(87)
Judo (n = 6)	0(0)	1(17)	5(83)	1(8)	11(92)
Marathon (n = 3)	0(0)	1(33)	2(67)	1(17)	5(87)
Total (n = 162)	5(3)	35(22)	122(75)	45(14)	279(86)

[1]>5000 m distance runner.

Table 4. Distribution of *Taq*I RFLP of muscle-specific creatine kinase gene in Korean male controls and Korean male elite athletic groups

Subjects	Muscle-specific creatine kinase gene				
	Genotypes			Alleles	
	T1T1	T1T2	T2T2	T1	T2
Controls (n = 54)	37(68)	15(28)	2(4)	89(82)	19(18)
Athletes (n = 97)	71(73)	21(22)	5(5)	163(84)	31(16)
Basketball (n = 14)	11(79)	3(21)	0(0)	25(89)	3(11)
Soccer (n = 22)	19(86)	2(9)	1(5)	40(91)	4(9)
Baseball (n = 29)	20(69)	6(21)	3(10)	46(79)	12(21)
Gymnastics (n = 10)	6(60)	4(40)	0(0)	16(80)	4(20)
Volleyball (n = 9)	5(56)	3(33)	1(11)	13(72)	5(28)
Runner (n = 4)[1]	3(75)	1(25)	0(0)	7(87)	1(13)
Judo (n = 6)	5(83)	1(17)	0(0)	11(92)	1(8)
Marathon (n = 3)	2(67)	1(33)	0(0)	5(83)	1(17)
Total (n = 151)	108(71)	36(24)	7(5)	252(83)	50(17)

[1]>5000 m distance runner.

heterozygosity and PIC for *Taq*I RFLP of the CKMM gene in Korean normal controls and pooled elite athletes, respectively. Likewise *Nco*I RFLP, the genotype and allele frequencies of *Taq* I RFLP were not also significantly different between both groups, respectively. The frequencies of T1T1, T1T2 and T2T2 genotypes were 68, 28 and 4% in normal controls, and 73, 22 and 5% in elite athletes, respectively. The heterozygosity and PIC values of *Taq*I RFLP represented the values of 0.2900 and 0.2479 for normol controls, and 0.2685 and 0.2325 for elite athletes, respectively. According to the heterozygosity and PIC values, *Taq*I RFLP showed a relatively high degree of polymorphism in the both groups compared with *Nco*I RFLP. Table 4 represents the allele distribution of the *Taq*I RFLP among various athletic groups. Likewise *Nco*I RFLP, there was no also significant difference in allele frequency among sporting disciplines studied.

Association with Clinical Parameters

Table 5 displays the comparison of clinical parameters across *Nco*I RFLP in elite athletes. There were no significant differences in clinical parameters across the genotypes. Table 6 shows the comparison of clinical parameters across *Taq*I RFLP in elite athletes. Likewise *Nco*I RFLP, *Taq*I RFLP was not significantly associated with any clinical parameters.

Haplotype Analysis

The haplotype frequencies and the linkage disequilibrium statistics reflecting the extent or significance of pair-wise nonrandom associations between the two RFLP sites in the CKMM gene are displyed in Table 7. There was no significant difference in haplotype frequency between normal controls and elite athletes (Monte-Carlo simulation, T_3 = 0.4105, df = 1, P = 0.5217, simulation number = 10,000). However, the significant pair-wise linkage disequilibrium between *Nco*I RFLP and *Taq*I RFLP in the CKMM gene was observed in the both groups by χ^2-test (P < 0.0016).

DISCUSSION

Athletic performance is a very complex and heteroge-

Table 3. Genotype and allele frequencies of the *Taq*I RFLP in the muscle-specific creatine kinase gene between Korean male controls and Korean male elite athletes

	Genotype No. (%)			Allele No. (%)		H^1	PIC^2
	T1T1	T1T2	T2T2	T1	T2		
Controls	37(68)	15(28)	2(4)	89(82)	19(18)	0.2900	0.2479
Athletes	71(73)	21(22)	5(5)	163(84)	31(16)	0.2685	0.2325
Chi-square		0.8100		0.1310			
Probability		0.6670		0.7180			
Odds ratio(CI)[3]			1.12(0.60-2.10)				

[1]Heterozygosity, [2]Polymorphism Information Content, [3]95% Confidence Interval.
Frequency is given as a percentage in parenthesis.

Table 5. The comparison of the anthropometric data and intermediate phenotypes according to *Ncol* RFLP of the muscle-specific creatine kinase gene in Korean male elite athletes

Variables	Genotypes		
	N1N1(No.)[11]	N1N2(No.)	N2N2(No.)
BMI (kg/m^2)[1]	22.7±2.0(5)	22.5±2.0(8)	23.1±1.8(65)
VO_2max (ml/kg/min)	55.5±1.6(5)	55.9±1.6(8)	55.7±1.5(65)
SBP (mmHg)[2]	116.2±3.9(5)	117.5±8.1(10)	119.5±8.1(66)
DBP (mmHg)[3]	68.2±8.5(5)	72.0±6.4(10)	73.1±6.6(66)
MAP (mmHg)[4]	84.2±5.9(5)	87.3±6.5(10)	88.6±6.0(66)
Tg (mg/dl)[5]	108.4±43.9(5)	113.1±126.0(15)	103.2±64.9(78)
TC (mg/dl)[6]	185.6±21.3(5)	165.4±22.5(15)	175.6±44.7(78)
LDL-chol (mg/dl)[7]	98.9±18.0(5)	84.2±25.9(15)	96.3±46.1(78)
HDL-chol (mg/dl)[8]	70.0±18.8(5)	57.1±10.0(15)	57.2±11.8(78)
CPK (IU/l)[9]	281.6±143.8(5)	347.9±236.7(14)	640.7±1011.7(78)
LDH (IU/l)[10]	479.2±21.3(5)	448.5±115.7(15)	457.8±103.3(78)
Glucose (mg/dl)	55.8±19.3(5)	54.8±11.9(15)	55.6±15.7(78)

[1]Body Mass Index, [2]Systolic blood pressure, [3]Diastolic blood pressure, [4]Mean arterial pressure, [5]Triglyceride, [6]Total cholesterol, [7]LDL-cholesterol, [8]HDL-cholesterol, [9]Creatine phosphokinase, [10]Lactate dehydrogenase and [11]Number. Value are mean±SD (standard deviation).

Table 6. The comparison of the anthropometric data and intermediate phenotypes according to *TaqI* RFLP of the muscle-specific creatine kinase gene in Korean male elite athletes

Variables	Genotypes		
	T1T1(No.)[11]	T1T2(No.)	T2T2(No.)
BMI (kg/m^2)[1]	23.2±2.0(58)	22.8±1.8(14)	23.0±1.8(5)
VO_2max (ml/kg/min)	55.6±1.6(58)	55.8±1.3(14)	55.2±1.4(5)
SBP (mmHg)[2]	120.0±8.3(59)	116.6±7.2(16)	117.0±4.7(5)
DBP (mmHg)[3]	73.3±6.3(59)	71.2±7.3(16)	69.8±9.6(5)
MAP (mmHg)[4]	88.8±5.8(59)	86.4±6.8(16)	85.6±7.1(5)
Tg (mg/dl)[5]	101.2±66.5(71)	115.6±108.2(21)	124.6±29.6(5)
TC (mg/dl)[6]	173.2±45.9(71)	171.7±24.3(21)	196.2±15.3(5)
LDL-chol (mg/dl)[7]	93.8±47.6(71)	92.0±27.7(21)	105.9±20.3(5)
HDL-chol (mg/dl)[8]	57.6±12.1(71)	55.5±8.8(21)	65.6±18.5(5)
CPK (IU/l)[9]	542.6±763.4(71)	577.9±1097.3(20)	228.2±134.4(5)
LDH (IU/l)[10]	452.3±97.6(71)	456.2±116.9(21)	480.2±22.2(5)
Glucose (mg/dl)	55.5±16.3(71)	54.9±14.0(21)	49.8±16.7(5)

[1]Body Mass Index, [2]Systolic blood pressure, [3]Diastolic blood pressure, [4]Mean arterial pressure, [5]Triglyceride, [6]Total cholesterol, [7]LDL-cholesterol, [8]HDL-cholesterol, [9]Creatine phosphokinase, [10]Lactate dehydrogenase and [11]Number. Value are mean±SD (standard deviation).

Table 7. Haplotype frequencies and linkage disequilibrium statistics (D, D') between pairs of two RFLPs in the muscle-specific creatine kinase gene

Haplotype		Controls	Athletes
Ncol	*TaqI*		
N1	T1	0.030382	0.010842
N1	T2	0.120562	0.108950
N2	T1	0.790373	0.827700
N2	T2	0.058684	0.052509
Total chromosomes		106	192
D		0.681234	0.749829
Δ'		0.775060	0.891989
χ^2		49.192455	107.9508
P		2.3204×10^{-12}	2.7553×10^{-25}

There were no significant differences in haplotype frequencies between normotensive and essential hypertensive individuals (Monte-Carlo simulation, T_3 = 0.4105, df = 1, P = 0.5217, simulation number = 10,000).

neous phenotype, and it is very unlikely that only a few major genes are responsible for its genetic component (Rankinen *et al.*, 2001). It is more likely that several genetic loci, each with a small but significant contribution, will be responsible for this genetic component. A limited number of candidate genes have been tested so far in this context (Gayagay *et al.*, 1998; Hagberg *et al.*, 2001).

The results of the present study do not support the hypothesis that two genetic polymorphisms in the CKMM gene have an influence on athletic performance in male subjects of Korean origin. Therefore, these genetic variations are not likely to be important determinants of athletic performance.

In the case of *Ncol* RFLP, considerable caution is needed in interpreting the marginal statistical significance (P = 0.015) between genotype distribution of this

Table 8. Comparison of the allele frequencies of the muscle-specific creatine kinase gene in other ethnic groups and Korean population

RFLP site	Allele	Caucasian[1]	Caucasian[2]	Caucasian[3]	Caucasian[4]	Korean[5]
Ncol						
	N1	0.33	0.33	0.30	0.27	0.16
	N2	0.67	0.67	0.70	0.73	0.84
Taql						
	T1	0.78	0.27		0.26	0.82
	T2	0.23	0.73		0.74	0.18

[1]Perryman *et al.*, 1988; [2]Gennarelli *et al.*, 1991; [3]Rivera *et al.*, 1997a; [4]Rivera *et al.*, 1997b;[5]*This study.*

polymorphism and athletic performance observed in present study. In this study, N1N1 genotype was detected in only elite athletic group, but the frequency of N1N2 heterozygote was rather higher in normal control group than that in athletic group, showing the excess of N1 allele in normal contol group. In other words, gene dosage effect of N1 allele for athletic performance was not detected in our subjects. Accordingly, we could only set a limited value on this marginal association, and finally judged the statistical significance (P = 0.0016) considering the possibility of type I error by multiple comparison. Supposedly, chance effect may explain the presence of N1N1 homozygote in our athletic subjects.

Pair-wise haplotype analysis detected the significant linkage disequilibrium between *Ncol* and *Taql* RFLP pairs in our subjects. This may be explained by the physical proximity between two polymorphic sites in the chromosome. Actually, The *Ncol* and *Taql* polymorphisms are about 479 bp and 1119 bp downstream of the polyadenylation site, respectively. The presence of significant linkage disequilibrium between two polymorphic sites decreases the information content for linkage analysis, whereas it did not require the large sample size to perform the association study. Thus, association study may be more suitable than linkage analysis to discover the causative gene.

The allele distribution of *Ncol* RFLP in the CKMM gene was different between Koreans and Caucasians studied (Table 8). N1 allele frequency in Koreans (0.16) was very lower than those in Caucasians (0.27~0.33), but relatively similar among Caucasians. These data may reflect differences in the genetic backgrounds of the populations, and indicate that this RFLP is not a variant but a polymorphism whose distribution exhibits clear diversity between different racial groups.

In contrast, the allele distribution of *Taql* RFLP was very diverse among populations studied. T1 allele frequency in Koreans (0.82) was higher those in Caucasians studied (0.27~0.78), and indicated marked heterogeneity in the allele distribution among Caucasians. The reason for this phenomenon may be, at least in part, explained by ethnic heterogeneity of Caucasians. The genetic heterogeneity in Caucasian populations is considered to be relatively larger than that in Asian populations (Ogihara *et al.*, 2000). Even if the larger genetic heterogeneity is an advantage for the survival from the natural selection, it should be a disadvantage for the positional cloning of the gene for polygenic trait. Thus, this genetic study using Korean population must play an important role in the identification of genetic factors specific to Korean population but also in revealing common genetic factors in humans regardless of race.

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