BIOMEDICAL AND ENVIRONMENTAL SCIENCES **18**, 58-64 (2005)

Relationship Between Polymorphism of Methylenetetrahydrofolate Dehydrogenase and Congenital Heart Defect[1]

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Objective To investigate the relationship between G1958A gene polymorphism of methylenetetrahydrofolate dehydrogenase (MTHFD) and occurrence of congenital heart disease (CHD) in North China. **Methods** One hundred and ninety-two CHD patients and their parents were included in this study as case group in Liaoning Province by birth defect registration cards, and 124 healthy subjects (age and gender matched) and their parents were simultaneously selected from the same geographic area as control. Their gene polymorphism of MTHFD G1958A locus was examined with PCR-RFLP, and serum folic acid and homocysteine (Hcy) levels were tested with radio-immunoassay and fluorescence polarization immunoassay (FPIA). **Results** There existed gene polymorphism at MTHFD G1958A locus in healthy subjects living in North China. The percentages of GG, GA, and AA genotype were 57.98%, 35.57%, and 6.45% respectively, and the A allele frequency was 24.23%, which was significantly different from Western population. No difference was observed when comparing genotype distribution and allele frequency between the case and control groups, so was the result from the comparison between genders. The A allele frequency of arterial septal defect patients' mothers (10.87%) was significantly lower than that of controls (28.15%) (P=0.014), with OR=0.31 (95% CI: 0.09-0.84), and no difference in the other subgroups. The percentage of at least one parent carrying A allele in arterial septal defect subgroup (43.48%) was significantly lower than that in controls (69.64%) (P=0.017), with OR=0.34 (95% CI: 0.12-0.92). The analysis of genetic transmission indicated that there was no transmission disequillibrium in CHD nuclear families. Their serum folic acid level was significantly higher than that of controls (P=0.000), and Hcy level of the former was higher than that of the latter with no statistical significance (P>0.05). Serum Hcy and folic acid levels of mothers with gene mutation were lower than those of mothers with no mutation. **Conclusion** No significant difference of genotype distribution and allele frequency existed between CHD patients and healthy population. MTHFD G1958A mutation in parents (particularly in mother) can decrease the risk of arterial septal defect in offspring. The possible mechanism of protection might be mutation, which can increase MTHFD enzyme activity, folic acid metabolism and homocysteine remethylation, and decrease Hcy level.

Key words: Methylenetetrahydrofolate dehydrogenase; Gene polymorphism; Congenital heart disease; Homocysteine; Folic acid

INTRODUCTION

Congenital heart defect (CHD), a structural malformation of embryo or fetus heart or vessel in uterus, is a group of birth defects affecting heart function factually or potentially. Its incidence rate was reported to be 10% and 0.5%-1.0% in abortion fetus and live infants, respectively, even up to 1.15%[1] and had a tendency to increase yearly[2], making up a significant proportion of today's birth defects. Although etiology and mechanism of CHD remain poorly understood, it has been well recognized that CHD is multifaceted in their etiology, having both genetic and environmental factors contributing to its development. Genetic and epidemiological studies showed that the genetic factor played an important role in CHD genetic susceptibility[3].

In recent years, the relationship between homocysteine (Hcy) and birth defects has become been a focus of studies. A large number of basic studies and epidemiological investigations showed that hyperhomocysteinemia was an important risk factor of birth defects, especially of neural tube defects (NTDs) which were closely related to heart in development[4-6]. Animal experiments indicated that Hcy could lead to the occurrence of CHD in chicken

[1]This work was supported by the Major State Basic Research Development Program of People's Republic of China (G1999055904) and the Danone's Diet and Nutrition Research and Education Grant (DIC2002-08).

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0895-3988/2005
CN 11-2816/Q

and rat fetus, implying that Hcy was a risk factor impairing or disturbing cardiovascular development in early developmental stage[7-9]. Genetic and epidemiological studies showed that Hcy metabolic key enzyme, methylenetetrahydro- folate reductase (MTHFR) gene mutation was a risk factor of CHD in infants[10], and MTHFR 677C→T mutation could decrease MTHFR enzyme activity and increase Hcy level[11]. All the above studies gave a clue that hyperhomocysteinemia might be related to the occurrence of CHD[12], while folic acid was closely related to Hcy metabolism. Hcy is a sulfur-containing amino acid derived from the metabolic conversion of methionine and one of its metabolic pathways is remethylation to methionine in which N-5-methyl-tetrahydrofolate (the dominating formation of folic acid *in vivo*) provides methyl. Methylenetetrahydro-folate dehydrogenase (MTHFD) can possess three enzymatic properties and catalyze three sequential reactions in the interconversion of one-carbon derivatives of tetrahydrofolate (FH_4) which are substrates for methionine, thymidilate, and *de novo* purine synthesis[13]. MTHFD gene defects might lead to deficiency of enzyme activity that obstructs folic acid metabolism and disturbs Hcy remethylation, resulting in hyperhomocysteinemia and occurrence of CHD. MTHFD 1958G→A mutation could act as a risk factor in human NTDs[14,15], while there was no report linking this mutation to CHD. This study is aimed at genetic and epidemiological study by molecular biology and analyzing the relationship of 1958G→A mutation in MTHFD gene and CHD.

MATERIALS AND METHODS

Subjects

This study was conducted in Liaoning Province. One hundred and ninety-two patients with CHD (0-31 years old, 93 males and 99 females) and their parents were included in this study as case group by birth defect registration cards. All CHD patients were diagnosed by specialized doctors. Of these patients, 17 (8.85%) had arterial septal defects, 82 (42.71%) had ventricular septal defects, 28 (14.58%) had patent arterial ducts, 19 (9.90%) had tetralogy of Fallot, 31 (16.15%) had other types of CHD (pulmonary artery stenosis, Ebstein's anomaly and so on), and 15 (7.81%) had combined types of CHD (there existed ⩾2 types of CHD simultaneously).

One hundred and twenty-four healthy subjects (0-31 years old, 71 males and 53 females) without personal and family birth defect history and their parents were simultaneously selected from the same geographic area of Liaoning Province as controls, with age and gender matched.

Sample Collection

Three to five mL fasting venous blood was collected for all subjects, and then the serum and blood clotting were separated. Genome DNA was extracted from blood clotting by salting-out method. Genotypes for MTHFD were determined with restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR).

PCR Reaction

Presence of the G1958A (R653Q) substitution in genomic DNA was investigated by PCR amplification using a pair of primers (referred to literature[14]): 5′-CAC TCC AGT GTT TGT CCA TG-3′ (cDNA# 1940-1959) and 5′-GCA TCT TGA GAG CCC TGA C-3′ (cDNA#2172-2154). The length of amplification fragment was 331 bp. PCR was performed in a total volume of 20 μL containing DNA template 35-70 ng, $MgCl_2$ 2.0 mmol/L, primers 0.5 μmol/L, dNTPs 0.25 mmol/L, and Taq DNA polymerase 2.0 U. Reaction was conducted as the initial denaturation at 94℃ for 2 min followed by 35 cycles consisting of denaturing at 94℃ for 30 s, annealing at 57℃ for 30 s, and extension at 72℃ for 45 s. A final extension was lasting for 7 min at 72℃ to end the procedure.

Restriction Digestion

Msp Ⅰ restriction endonuclease could recognize 5′-CCGG-3′ sequence. There were three CCGG sequences in the amplified product and it could be digested into four fragments (197 bp, 70 bp, 56 bp, and 8 bp). The G → A change abolished a Msp Ⅰ restriction site, therefore, the product would be digested into three fragments (267 bp, 56 bp, and 8 bp). Ten μL amplification fragment was digested by 6 U Msp Ⅰ restriction endonuclease for 4 h at 37℃ and subsequent electrophoresis was performed on a 12% polyacrylamide gel.

Serum Folic Aacid and Hcy Levels Assay

Radio-immunoassay and fluorescence polarization immunoassay (FPIA) were used to measure serum folic acid and Hcy level.

Statistical Analysis

Statistical analysis was performed using SPSS10.0 and Epi info2002 software. Genotype distribution and allele frequency were compared between the case and control groups by using chi-square test, and folic acid and Hcy level were

compared with *t*-test or rank sum test. The odds ratio (OR) was calculated by means of Epi info. *P* value less than 0.05 was considered statistically significant.

RESULTS

Comparison of Genotype Distribution and Allele Frequencies at MTHFD G1958A Locus Between the Case Group and the Control Group

The numbers of father, mother and children with genotype detected in case group were 172, 177, and 179 respectively, and those in control group were 116, 119, and 122 respectively. There were 170 families with genotypes of father, mother and child detected in case and 115 families in control. MTHFD G1958A genotype frequencies of subjects in case and control groups do not deviate from Hardy-Weinberg equilibrium.

In healthy subjects living in North China, there were three genotypes at MTHFD G1958A locus and no difference between genders (*P*=0.131) was observed. The A allele frequency was 21.24% and 27.49% in males and females, respectively, without significant difference (*P*=0.051) (Table 1).

There also existed gene polymorphism at this locus in CHD patients, and GG, GA and AA genotype percentage was 60.34%, 35.75%, and 3.91% respectively, with no difference between genders (*P*=0.401). Genotype distribution in CHD patients was not significantly different from that in control (*P*=0.818). The A allele frequency of CHD patients (21.79%) was slightly higher than that of control (21.31%), but there was no significant difference (*P*=0.889) (Table 1).

The comparison of genotype distribution and allele frequencies showed no significant difference between parents of CHD patients and the control, and no gender difference in either group (*P*>0.05). But A allele frequencies of father and mother in the control were 23.28% and 28.15% respectively, higher than those of counterparts in the case group (Table 1).

Comparison of MTHFD G1958A Genotype Distribution and Allele Frequency Between Different Types of CHD and the Control

Regarding one type of CHD with or without other types of CHD as a subgroup, the comparison of MTHFD G1958A genotype distribution and allele frequencies between different types of CHD patients and controls showed no statistical significance (Table 2).

The comparison of parents' genotype distribution between different subgroups and controls is listed in Table 3. *P*-value resulted from comparison between genotype distribution of mothers of arterial septal defect patients and that of counterparts in the control was nearly 0.05, and the A allele frequency of patients' mothers (10.87%) was significantly lower than that of the control (28.15%) (*P*=0.014), with *OR*=0.31 (95%CI: 0.09-0.84).

Comparison of Parents' Genotype Combination Between the Case and Control Groups

No significant difference in genotype combination between parents of the case and control groups was observed. Comparing genotype combination status of subgroups with that of the control, we found no significant difference except patent arterial duct subgroup, and *P*-value from comparison between arterial septal defect subgroup and the control was nearly 0.05. Further comparison between percentage of at least one parent carrying A allele and that of both parents carrying genotype G showed that this percentage in arterial septal defect subgroup was significantly lower than that in the control (43.48% vs 69.64%, *P*=0.017), and OR was 0.34 (95% CI: 0.12-0.92) (Table 4).

TABLE 1

Comparison of Genotype Distribution and Allele Frequency Between the Case and Control Groups at MTHFD G1958A Locus

Group		*n*	Genotype% (*n*)			*P*[a]	Allele Frequency		OR (95% CI)	*P*[a]
			GG	GA	AA		G	A		
Case Group	Father	172	65.70(113)	30.23(52)	4.07(7)	0.505	80.81	19.19	0.78(0.51-1.20)	0.236
	Mother	177	54.24(96)	41.24(73)	4.52(8)	0.391	74.86	25.14	0.86(0.58-1.26)	0.415
	Son	85	57.64(49)	36.47(31)	5.88(5)	0.284	75.88	24.12	1.46(0.81-2.65)	0.181
	Daughter	94	62.76(59)	35.11(33)	2.13(2)	0.128	80.32	19.68	0.70(0.38-1.28)	0.215
Control Group	Father	116	59.48(69)	34.47(40)	6.03(7)		76.72	23.28		
	Mother	119	52.10(62)	39.50(47)	8.40(10)		71.85	28.15		
	Son	70	65.71(46)	32.86(23)	1.43(1)		82.14	17.86		
	Daughter	52	57.69(30)	32.69(17)	9.62(5)		74.04	25.96		

Note.[a] Compared with control group by Chi-square test.

TABLE 2

Comparison of Allele Frequency and Genotype Distribution Between Different Types of CHD Patients and the Control Group at MTHFD G 1958A Locus

Group	*n*	Genotype % (*n*)			P^a	Allele Frequency		OR (95% CI)	P^a
		GG	GA	AA		G	A		
ASD	22	77.27(17)	22.73(5)	0.00(0)	0.311	88.64	11.36	0.47(0.14-1.29)	0.128
VSD	88	59.09(52)	36.36(32)	4.55(4)	0.864	77.27	22.73	1.09(0.66-1.78)	0.729
PAD	34	58.82(20)	38.24(13)	2.94(1)	0.773	77.94	22.06	1.04(0.52-2.09)	0.894
TF	22	72.73(16)	18.18(4)	9.09(2)	0.332	81.82	18.18	0.82(0.33-1.98)	0.638
Others	32	56.25(18)	43.75(14)	0.00(0)	0.273	78.13	21.87	1.03(0.50-2.11)	0.922
Control	122	62.29(76)	32.79(40)	4.92(6)		78.69	21.31		

Note. ASD-arterial septal defect, VSD-ventricular septal defct, PAD-patent arterial duct, TF-tetralogy of fallot. [a]Compared with control group by chi-square test. Regarding one type of CHD with or without other types of CHD as a subgroup.

TABLE 3

Comparison of Allele Frequency and Genotype Distribution in Parents Between Different Types of CHD and the Control at MTHFD G1958A Locus

Group		*n*	Genotype % (*n*)			P^*	Allele Frequency		OR (95% CI)	P^a
			GG	GA	AA		G	A		
ASD	Father	24	62.50(15)	25.00(6)	12.50(3)	0.419	75.00	25.00	1.10(0.50-2.38)	0.798
	Mother	23	78.26(18)	21.74(5)	0.00(0)	0.051	89.13	10.87	0.31(0.09-0.84)	0.014
VSD	Father	84	60.72(51)	34.52(29)	4.76(4)	0.925	77.98	22.02	0.93(0.56-1.54)	0.768
	Mother	83	49.40(41)	45.78(38)	4.82(4)	0.488	72.29	27.71	0.98(0.61-1.56)	0.923
PAD	Father	34	70.59(24)	26.47(9)	2.94(1)	0.470	83.82	16.18	0.64(0.29-1.36)	0.212
	Mother	34	61.76(21)	38.24(13)	0.00(0)	0.190	80.88	19.12	0.60(0.29-1.23)	0.135
TF	Father	19	63.16(12)	36.84(7)	0.00(0)	0.546	81.58	18.42	0.74(0.26-1.85)	0.508
	Mother	22	59.09(13)	31.82(7)	9.09(2)	0.792	75.00	25.00	0.85(0.38-1.87)	0.668
Control	Father	116	59.48(69)	34.48(40)	6.04(7)		76.72	23.28		
	Mother	119	52.10(62)	39.50(47)	8.40(10)		71.85	28.15		

Note. ASD-arterial septal defect, VSD-ventricular septal defct, PAD-patent arterial duct, TF-tetralogy of fallot. [a]Compared with the control group by chi-square test. Regarding one type of CHD with or without other types of CHD as a subgroup.

TABLE 4

Comparison of Genotype Combination Between CHD Parents and the Control

Group	*n*	% (*n*)			P^a	% (*n*)		OR (95% CI)	P^a
		GG+GG	GG+GA (or AA)	GA+GA (or AA)		Non-mutation	Mutation		
ASD	23	56.52(13)	30.43(7)	13.04(3)	0.055	56.52(13)	43.48(10)	0.34(0.12-0.92)	0.017
VSD	73	30.14(22)	46.57(34)	23.29(17)	0.737	30.14(22)	69.86(51)	1.01(0.68-1.49)	0.975
PAD	32	34.38(11)	65.62(21)	0.00(0)	0.028	34.38(11)	65.62(21)	0.83(0.34-2.08)	0.666
TF	19	36.84(7)	47.37(9)	15.79(3)	0.845	36.84(7)	63.16(12)	0.75(0.25-2.32)	0.574
Total	147	36.05(53)	48.30(71)	15.65(23)	0.588	36.05(53)	63.95(94)	0.77(0.44-1.35)	0.337
Control	112	30.36(34)	50.89(57)	18.75(21)		30.36(34)	69.64(78)		

Note. ASD-arterial septal defect, VSD-ventricular septal defect, PAD-patent arterial duct, TF-tetralogy of fallot. [a]Compared with the control group by chi-square test. Regarding one type of CHD with or without other types of CHD as a subgroup.

Analysis of Allele Transmission Disequillibrium in CHD Nuclear Families

Transmission disequillibrium of alleles was analyzed by transmitted disequilibrium test (TDT) (Table 5) and haplotype-based haplotype relative risk (HHRR) (Table 6) calculation. In 1:1 matched case-control design, that is, in TDT, the alleles of CHD patients were thought as "case" and the non-inherited alleles of parents as "intra-control". The data showed there was no transmission disequillibrium in CHD nuclear families, suggesting that G allele was not transmitted from parents to fetus more proportionally than A allele. Our data could not provide any evidence that MTHFD G1958A was related to CHD risk.

Comparison of Serum Folic Acid and Hcy Levels Between the Case and Control Groups

We measured the levels of folic acid and Hcy in part of subjects. Data given in Table 7 showed that serum folic acid level in the case group was significantly higher than that in the control, and serum Hcy level of CHD patients and mother in the case group was higher than that in the control, with no significant difference. Serum Hcy level of patients' mothers was higher than 14 μmol/L which was considered as the Hcy threshold value of healthy Chinese[16]. Serum Hcy level of mothers with gene mutation was lower than that of mother with no mutation (P=0.830), being (11.015±5.930) μmol/L and (13.202±15.033) μmol/L respectively. So was the result of comparison of serum folic acid level between these two groups (P=0.738), the former was (23.138±18.778) nmol/L and the latter was (24.406 ±26.733) nmol/L (not shown in Table 7).

TABLE 5

Analysis of TDT in CHD Nuclear Families

Allele Without Transmission From Parents	Allele Number of CHD Patients		Total
	G	A	
G	179	55	234
A	54	12	66
Total	233	67	300

Note. TDTd(χ^2)=0.009, P>0.05.

TABLE 6

Analysis of HHRR in CHD Nuclear Families

Transmission of Allele	Allele Number		Total
	G	A	
Transmitted From Parents	233	67	300
Not Transmitted From Parents	234	66	300

Note. HHRR ((χ^2)=0.01, P=0.922, OR=1.02 (95% CI: 0.68-1.53).

TABLE 7

Comparison of Serum Folic Acid and Hcy Levels Between the Case and Control Groups

Group		Serum Folic Acid (nmol/L)			Serum Hcy (μmol/L)		
		n	$\bar{x}$±s	P-value[a]	n	$\bar{x}$±s	P-value*
Case Group	Father	30	30.484±19.043	0.000			
	Mother	30	40.373±19.437	0.000	30	14.164±15.390	0.606
	Child	30	31.949±23.535	0.000	25	8.336±3.707	0.875
Control Group	Father	41	12.429±11.567				
	Mother	41	13.710±10.566		29	9.120±2.031	
	Child	41	19.720±23.887		23	8.194±2.464	

Note. [a]compared with the control by t-test or Rank Sum Test.

DISCUSSION

The human MTHFD gene, encoding a single protein with three specific enzymatic activities (methylenetetrahydrofolate-dehydrogenase, methenyltetrahydrofolate-cyclohydrolase, formyltetrahydrofolate synthetase), important in folate metabolism, has been considered as a candidate for human NTD. Two mutation loci of MTHFD gene on 14q24, G878A (R293H) and G1958A (R653Q), were reported. In healthy Dutch population, the percentage of GG, GA and AA genotype was 18.8%, 51.3% and 29.9% respectively, while the G allele frequency was 44% and A allele frequency was 56%[14]. In healthy Turkey population below 18 years old, the percentage of GG, GA,and AA genotype was 25.00%, 51.47% and 23.53% respectively, while the A allele frequency was 49.26%[17]. From the above data, we could know that

in western population, frequency of heterozygote (GA) was the highest among genotypes, and its mutation frequency was about 50%, while in healthy population living in North China, the highest was wild type (GG), and its mutation frequency was lower than 25%. All these data showed the presence of ethnic difference in gene polymorphism at MTHFD G1958A locus. Mutation frequency of Chinese at this locus was significantly lower than that of western population (P=0.000).

Analysis of relationship between MTHFD G1958A mutation and CHD showed no significant difference in genotype distribution and allele frequencies between the case and control groups, A allele frequency was 21.79% in CHD patients, OR 1.03 (95% CI: 0.68-1.56). By dividing CHD patients into subgroups according to their CHD type and comparing them with the control, we could not find any difference in genotype distribution and allele frequencies, with an OR of 0.47-1.09, so was the result of comparison in parents. But A allele frequency of mothers of arterial septal defect patients (10.87%) was significantly lower than that of the control (28.15%), with an OR of 0.31 (95% CI: 0.09-0.84), indicating that mothers carrying A allele could decrease arterial septal defect risk of offspring. Further analysis of parents' genotype combination was carried out in our study, and no difference was observed between the subgroups and the control except for patent arterial duct subgroup, but the percentage of at least one parent carrying A allele in arterial septal defect subgroup (43.48%) was signifyc-antly lower than that in the control (69.64%), with an OR of 0.34 (95% CI: 0.12-0.92). Similarly, this result could illuminate that gene mutation of parents might act as a protective factor and decrease the risk of arterial septal defect to offsprings.

A nuclear family consists of a child and his parents. In this kind of research design the alleles of offspring were thought as "case", and the non-inherited alleles of parent as "intra-control", to seek for the genetic biomarkers related to diseases or allele being transmission disequillibrium to adjoining locus. By the design the selection bias of genetic race difference could be overcome without looking for the same background control[18]. In our study, transmission disequillibrium was analyzed with case-parental control study in nuclear families, and results showed there was no existing transmission disequillibrium in CHD nuclear families, suggesting that G allele was not transmitted from parents to fetus more proportio-nally than A allele. The OR of A allele was 1.02 (95% CI: 0.68-1.53). As mentioned above, parents' (particularly mother) carrying mutation A allele could decrease arterial septal defect risk to offspring. Whether there existed transmission disequillibrium at this locus in arterial septal defect nuclear family could not be analyzed, because arterial septal defect patients were not available in adequate amounts. Further larger-sized study might be required.

FH_4 is a coenzyme of interconversion of one- carbon enzyme. Both Hcy metabolism-related enzymes and folate-metabolism enzymes are associated with the increased Hcy level, and MTHFD belongs to the latter. Serum folic acid level in the case group was significantly higher than that in the control, which disagreed to the existing result[6]. No significant difference of Hcy level between the case and control groups was observed, and the mean level in the case group was slightly higher than that in the control. Further analysis of Hcy status in mothers with gene mutation and those with no mutation showed that the mean Hcy level of the former was lower than that of the latter, so was the result of folic acid. G1958A substitution in MTHFD gene could cause the replacement of the arginine residue at position 653 by a glutamine in the deduced protein[14]. Because the amino acid coded by codon 653 was located within the enzyme activity area, the gene locus mutation might increase MTHFD enzyme activity by changing the secondary structure of protein, which caused the decrease of Hcy level. In mothers of CHD (particularly arterial septal defect) patients, their A allele frequency was lower than that of the control, and the transformation of folic acid to FH_4 decreased because of MTHFD enzyme activity was low, while 5-FH_4 acted as the principal methyl-group donor in the remethylation of homocysteine to methionine. Therefore, the reason why serum folic acid level of the case group was higher than that of the control was not that folic acid intake in the control was low, but that the decreased MTHFD enzyme activity in mothers of patients resulted in folic acid accumulation, leading to traffic of Hcy remethylation and increased Hcy level. Subsequently, the development of embryo or fetus heart was disturbed because of the high-risk environment in uterus. Assay of FH_4 level and MTHFD enzyme activity should be taken to determine whether this hypothesis was true.

In conclusion, gene polymorphism at MTHFD G1958A locus exists in healthy population living in North China, and their genotype distribution and allele frequencies differ from western population. No evidence could be given in our study that MTHFD G1958A mutation in parents is related to the occurrence of CHD in offspring, but our data could provide evidence that this mutation could decrease the risk of arterial septal defect in offspring. The possible mechanism of protection might be mutation which could increase MTHFD enzyme activity and

FH_4 level, homocysteine remethylation, and at the same time decrease Hcy level.

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(*Received July* 10, 2003 *Accepted November* 26, 2004)