ORIGINAL ARTICLE

LDLR and ApoB are Major Genetic Causes of Autosomal Dominant Hypercholesterolemia in a Taiwanese Population

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Background/Purpose: Autosomal dominant hypercholesterolemia (ADH) is an autosomal dominant inherited disease characterized by an increase in low-density lipoprotein cholesterol levels and premature coronary heart disease, which can be caused by mutations in genes encoding the low-density lipoprotein receptor (*LDLR*), apolipoprotein B (*APOB*) and proprotein convertase subtilisin/kexin type 9 (*PCSK9*). There is scant information with regard to the role played by each gene in the Taiwanese ADH population, especially the newly discovered *PCSK9* gene.

Methods: We used coupling heteroduplex analysis based on a denaturing high performance liquid chromatography system and DNA sequencing to screen for the *LDLR* gene, *APOB* gene and *PCSK9* gene in 87 ADH cases recruited from 30 unrelated Taiwanese families.

Results: We did not find any mutation-causing variant of the *PCSK9* gene in our cases and thus excluded *PCSK9* as the major culprit mutation in these families. On the other hand, we identified six previously reported *LDLR* gene mutations (C107Y, D69N, R385W, W462X, G170X, V408M), two novel *LDLR* gene mutations (FsG631 and splice junction mutation of intron 10), and one known mutation (R3500W) and one novel missense mutation (T3540M) in the *APOB* gene that were present in 55 members from 18 ADH families (60%). R3500W, rather than R3500Q, could be the principle mutation responsible for familial defective apolipoprotein B in Taiwanese.

Conclusion: The results of our study reveal a characteristic mutation pattern of ADH in Taiwan, mainly in the *LDLR* and *APOB* genes. However, *PCSK9* gene mutation may not be a major cause of ADH in our study population. [*J Formos Med Assoc* 2007;106(10):799–807]

Key Words: *APOB*, autosomal dominant hypercholesterolemia, *LDLR*, *PCSK9*, Taiwanese

Autosomal dominant hypercholesterolemia (ADH) is an inherited disorder of cholesterol metabolism characterized by a high concentration of plasma low-density lipoprotein cholesterol (LDL-C), deposition of cholesterol in tendons and skin, and increased risk of premature coronary heart disease (CHD). ADH is most commonly caused by mutations in the LDL receptor (*LDLR*) gene, which can lead to reduced hepatic clearance of LDL from the blood. The estimated prevalence of *LDLR* gene mutation is 1 in 500 in its heterozygous form.[1] To date, more than 800 mutations in

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the *LDLR* gene have been reported.[2] ADH can also be caused by certain mutations in the apolipoprotein B (*APOB*) gene, which encodes for the ligand for LDLR, named familial defective apolipoprotein B (FDB). The prevalence of FDB is 1 in 1000 in most populations.[3,4] Until recently, a third locus responsible for ADH (FH3) was identified at 1p34.1-p32 in several large ADH kindreds without mutations in the *LDLR* or *APOB* genes.[5,6] The proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene, localized to the third FH locus, has been proposed to be the third gene with pathogenic mutations accounting for ADH.[7,8] *PCSK9* encodes for neural-apoptosis-regulated convertase-1, a novel protein that may play a crucial role in cholesterol homeostasis, though the exact molecular mechanisms are still obscure.[9]

Although heterozygous ADH is presumed to be a common disorder resulting in atherosclerosis in Asians,[10] there are limited epidemiologic and genetic data with regard to Taiwanese ADH patients.[11] The role of the *PCSK9* gene in causing ADH in Taiwanese especially needs to be clarified so that a large-scale ADH screening program in Taiwan can be designed in the future. To confirm the role of the *PCSK9* gene in Taiwanese with ADH and to determine the molecular basis of ADH in Taiwan, we investigated the *LDLR*, *APOB* and *PCSK9* genes in Taiwanese ADH patients for mutations.

Methods

Subjects

Patients attending the Lipid Clinic at National Taiwan University Hospital, diagnosed as having ADH, were recruited into our study. The diagnostic criteria of ADH included: (1) fasting plasma total cholesterol and LDL-C levels above the 95th percentiles for adult Taiwanese after adjustments for age and gender,[12,13] and triglycerides < 220 mg/dL (2.5 mmol/L); (2) presence of tendon xanthomata/xantholesma/corneal arcus or premature CHD in the index case or a first degree relative, or a family history of hypercholesterolemia consistent with an autosomal dominant inheritance pattern. Patients with secondary causes of hypercholesterolemia, such as hypothyroidism, renal or hepatic disease, and those with clinically suspected familial combined hyperlipidemia were excluded. Family members of the index ADH case were invited to participate in the screening program and those who met the diagnostic criteria for ADH were also recruited.

Control subjects were healthy adult volunteers without known underlying dyslipidemia, cardiovascular disease, active inflammation or malignancy. We collected blood samples from 50 control subjects for genetic analysis (described later).

Lipid measurements

Blood samples from fasting patients without concurrent lipid-lowering therapy were obtained for measurements. The concentration of plasma total cholesterol, high-density lipoprotein cholesterol, and triglycerides were determined with commercially available kits (Boehringer Mannheim). LDL-C was estimated with the aid of the Friedewald formula.[14]

Molecular analysis

DNA preparation

Genomic DNA was isolated from EDTA whole blood with the Puregene DNA Isolation Kit (Gentra Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

Polymerase chain reaction

Polymerase chain reaction (PCR) amplification of the *LDLR* gene (including the promoter, 18 coding exons and flanking intron regions), *APOB* gene (exon 26 of the *APOB* gene containing codons 3473–3561, which harbors two known pathogenic mutation sites, R3500Q and R3500W, as well as four other known genetic variants of potential pathogenic importance[15–18]), and *PCSK9* gene (the 12 exons and flanking intron regions) were performed with the primers as shown in Table 1. Each PCR mixture, with a total volume of 25 mL, contained 50 ng of genomic DNA, 0.12 mM of each primer, 100 mM dNTPs, 0.5 units of

Table 1. Primers used for polymerase chain reaction of the *LDLR* gene, 26th exon of the *APOB* gene, and the *PCSK9* gene, and denaturing high performance liquid chromatography (DHPLC) temperatures

	Forward primer	Reverse primer	Annealing temperature (°C)	DHPLC temperature (°C)
LDLR				
Exon 1	5′tttccagctaggacacagca3′	5′ctcaccctgtggagacttgg3′	57	60
Exon 2	5′tgggttccttctttgtgtcc3′	5′tggcgagaccctgtctctat3′	56	58
Exon 3	5′tcttgaacccctgacctcac3′	5′caggaccccgtagagacaaa3′	57	62
Exon 4-1	5′gacttcacacggtgatggtg3′	5′ccccttggaacacgtaaaga3′	57	65
Exon 4-2	5′aagtgcatctctcggcagtt3′	5′ccagggacaggtgataggac3′	57	65
Exon 5	5′caggctggtcttgaactcct3′	5′agcagcaaggcacagagaat3′	55	62.5
Exon 6	5′ctcccaaagtgctgggatta3′	5′ttcccaaaaccctacagcac3′	57	62
Exon 7	5′cagctacttgggaggctgag3′	5′gttttccatgcaggtggaat3′	57	63.5
Exon 8	5′cttcgaaggtgtgggttttg3′	5′gcaagcccaagtcctaacag3′	57	62
Exon 9	5′gaggcactcttggttccatc3′	5′tctctgctgatgacggtgtc3′	57	64
Exon 10	5′ggtctgacctgtcccagaga3′	5′cttcctgctccctccattc3′	57	62
Exon 11	5′aagccacatttggagtttgg3′	5′aaaccttcagggagcagctt3′	57	62
Exon 12	5′ccaggtgcttttctgctagg3′	5′caaccagttttctgcgttca3′	57	61.5
Exon 13	5′cgagattgggccactgtact3′	5′tccacaaggaggtttcaagg3′	57	62
Exon 14	5′caagaggtaagggtgggtca3′	5′gagcagagagaggctcagga3′	57	64
Exon 15	5′cctcccaaggtcatttgaga3′	5′gtcagcaagggagtgaggac3′	57	64
Exon 16	5′tgtggcctctcacagacttg3′	5′ttccctgtccaggagaaaaa3′	57	63
Exon 17	5′tatggtacgatgcccgtgtt3′	5′cgcacagaagcattcaccta3′	57	61
Exon 18	5′cggtgggaagtgactgaatc3′	5′ggcaatgctttggtcttctc3′	57	62
Prom	5′cagctcttcaccggagaccc3′	5′acctgctgtgtcctagctgg3′	57	61.5
APOB				
Exon 26	5′tgtcaagggttcggttcttt3′	5′gggtggctttgcttgtatgt3′	56	58.5
PCSK9				
Exon 1	5′cttcagctcctgcacagtc3′	5′gaaactgaggcccgagag3′	57	62
Exon 2	5′aggggtgagataaagtacacct3′	5′aagcacagtccccagtgtat3′	56	60.5
Exon 3	5′gggacaggtttgatcaggta3′	5′tcagtggaggtgctgagtc3′	57	61
Exon 4	5′tgtgctctgtagtttgtgtgtg3′	5′atgctctggggtggcagt3′	57	59
Exon 5	5′ctgtactcctgggttgcac3′	5′cacagcattcttggttagga3′	57	64
Exon 6	5′ccatcactctgtgcctgtaa3′	5′ggaacgtgccacaagaag3′	57	62
Exon 7	5′aaggcctgagtctgcctct3′	5′ccatcaggcctacttcatct3′	55	60
Exon 8	5′gtgtatgtgtgtgcgtgtgt3′	5′agggagaagggagagactgt3′	57	63.5
Exon 9	5′cctcctctctcctaccatga3′	5′acagaagagctggagtctgg3′	57	61
Exon 10	5′atgagggtgcttgagttgat3′	5′gatcacacttgtgaggacca3′	57	62
Exon 11	5′agctcttgcctcagacctta3′	5′ggcacaaactgacacagaaa3′	56	63
Exon 12	5′gagggagaaatgaagtgtgg3′	5′agtcggaaccattttaaagc3′	57	62

AmpliTaq Gold™ enzyme (PE Applied Biosystems, Foster City, CA, USA), and 2.5 mL of GeneAmp 10X buffer II (10 mM Tris-HCl; pH, 8.3; 50 mM KCl), in 2 mM $MgCl_2$ as provided by the manufacturer. Amplification was performed in a multiblock system thermocycler (ThermoHybaid, Ashford, UK). PCR amplification was performed with an initial denaturation step at 95°C for 10 minutes, followed by 35 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 55–57°C for 60 seconds (specific annealing temperatures for each PCR product are listed in Table 1), extension at 72°C for 30 seconds, and then a final extension step at 72°C for 10 minutes.

Table 2. Clinical and biochemical characteristics of the autosomal dominant hypercholesterolemia cases*

	All ($N=87$)	Men ($N=46$)	Women ($N=41$)
Age (yr)	42.3 ± 14.3 (14–70)	40.4 ± 14.0 (14–56)	44.2 ± 15.0 (18–70)
Hypertension†	18 (21)	12 (26)	6 (15)
Total cholesterol (mg/dL)‡	316.0 ± 68.5 (268–500)	312.0 ± 79.3 (268–500)	320.8 ± 59.8 (278–475)
LDL-C (mg/dL)‡	220.6 ± 52.6 (172–400)	208.4 ± 17.6 (172–400)	231.4 ± 70.5 (174–389)
HDL-C (mg/dL)‡	48.3 ± 11.6 (29–73)	38.9 ± 5.6 (29–47)	55.6 ± 9.5 (42–73)
Triglyceride (mg/dL)‡	108.8 ± 52.2 (18–219)	118.2 ± 36.7 (77–186)	99.3 ± 65.1 (18–219)
CHD history	18 (20.7)	12 (26.1)	6 (14.6)
Presence of tendon xanthomata/corneal arcus/xantholesma	14 (16.1)	9 (19.5)	5 (12.2)

**Data are presented as mean ± standard deviation (range) or n (%); †none of the included subjects had diabetes mellitus; ‡to convert total cholesterol, LDL-C and HDL-C in mg/dL to mmol/L, divide by 38.7, and to convert triglyceride in mg/dL to mmol/L, divide by 88.6. LDL-C = low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol; CHD = coronary heart disease.*

Denaturing high performance liquid chromatography analysis

Mutation analysis was performed on a Transgenomic Wave Nucleic Acid Fragment Analysis System (Transgenomic Inc., San Jose, CA, USA). Denaturing high performance liquid chromatography (DHPLC) was carried out on an automated HPLC instrument equipped with a DNASep column (Transgenomic Inc.). DHPLC-grade acetonitrile (9017-03; JT Baker, Phillipsburg, NJ, USA) and triethylammonium acetate (TEAA; Transgenomic Inc., Crewe, UK) were used to constitute the mobile phase. The mobile phases comprised 0.05% acetonitrile in 0.1 M TEAA (eluent A) and 25% acetonitrile in 0.1 M TEAA (eluent B). For heteroduplex detection of crude PCR products, they were subjected to an additional 3-minute 95°C denaturing step followed by gradual reannealing from 95°C to 65°C over a period of 30 minutes prior to analysis, and were eluted at a flow rate of 0.9 mL/min. The start- and end-points of the gradient obtained by mixing eluents A and B and the temperature required for successful resolution of heteroduplex molecules, were deduced from the WAVEmaker system control software version 4.1.42 (Transgenomic Inc.). Eight microliters of PCR product were injected for analysis in each run. The DHPLC temperatures for each PCR product are listed in Table 1. Heterozygous profiles were identified by visual inspection of the chromatograms on the basis of the appearance of additional earlier eluting peaks. Corresponding homozygous profiles are shown as only one peak.

Direct sequencing analysis

The PCR products from index cases of ADH who showed abnormal DHPLC heteroduplex patterns compared with controls were sequenced. Amplicons were purified by solid-phase extraction and bidirectionally sequenced with the PE Applied Biosystems Taq DyeDeoxy terminator cycle sequencing kit according to the manufacturer's instructions. Sequencing reactions were separated on a PE Applied Biosystems 373A/3100 sequencer. Mutation was defined as base alterations that were not found in volunteer samples, while single nucleotide polymorphisms (SNPs) were defined as base alterations that were found in control samples (50 control blood samples were screened utilizing DHPLC for mutation/SNP confirmation).

Results

Clinical characteristics

Thirty unrelated ADH families, with a total of 87 members, were recruited. The demographic data, clinical features and lipid profiles of the subjects are shown in Table 2. Eighteen (20.7%) of the ADH cases had a history of CHD. Pretreatment plasma

Table 3. Mutations identified in the *LDLR*, *APOB* and *PCSK9* genes among the autosomal dominant hypercholesterolemia families

Family*	Exon	Nucleotide change	Amino acid change	Mutation class†	Reference
LDLR gene					
F_1, F_{12}	4	C→T571	G170X	Class 1	Chinese [19]
F_3, F_{13}	3	G→A268	D69N	Class 2B	UK [20]; Chinese [19]
F_4, F_{15}	9	G→A1285	V408M	Class 5	South African [21]
F_5	9	C→T1216	R385W	Class 5	Israel [22]
F_7	4	G→A383	C107Y	Class 3	French [23]
F_{10}	10	G→A1448	W462X	Class 2B/5	Chinese; Austrian [24]
F_{16}, F_{28}, F_{30}	13	delAT1954	FsG631	Class 5	Novel
F_{20}, F_{29}	Intron 10	G→C1586+5	Skipping of exon 10	Class 2B/5	Novel
APOB gene					
F_{23}, F_{25}, F_{27}	26	C→T10707	R3500W		Gaffney et al [3]
T_1	26	C→T10828	T3540M		Novel

**The numbering system for autosomal dominant hypercholesterolemia families consisted of a capitalized F or T, which denotes family, and a following digit, which indicates the number assigned to the autosomal dominant hypercholesterolemia family; †mutation class was assigned according to the functional domains described in the references.*

total cholesterol ranged from 268 to 500 mg/dL (6.9–12.9 mmol/L), and LDL-C ranged from 172 to 400 mg/dL (4.5–10.3 mmol/L).

LDLR gene variants

Using DHPLC analysis, we discovered a total of 14 nucleotide changes in the *LDLR* gene. Direct DNA sequencing of the amplicons confirmed eight mutations and six polymorphisms (Table 3[3,19–24] and Table 4[25–28]). The discovered *LDLR* gene mutations included four missense mutations (C107Y, D69N, R385W, V408M), two nonsense mutations (G170X, W462X), one novel deletion (c.1954_1955 del AT) leading to frameshift (FsG631), and one novel splice site mutation at intron 10 (c.1586+5G>C). Both of the newly-discovered *LDLR* gene mutations were shown to co-segregate well with the clinical ADH phenotype and were not found in control subjects. Among the identified *LDLR* gene polymorphisms, two have been reported previously (C>T at 1617 and C>T at 81) and two are novel (A>G at 1415 and C>T at 2558). The number of families carrying these polymorphisms is listed in Table 4.[25–28]

APOB gene variants

Four of our study families were discovered to carry mutations in the *APOB* gene. Three of them were heterozygous carriers of FDB causing missense mutation of R3500W, and the other one carried a novel missense mutation of T3540M. Missense mutation of R3500Q and the other four *APOB* genetic variants of pathogenic importance in hypercholesterolemia were not identified in our ADH families.[15–18] The T3540M mutation was found to co-segregate with the hypercholesterolemia trait in this family.

Table 4. Polymorphisms identified in the *LDLR* gene among 30 Taiwanese autosomal dominant hypercholesterolemia probands

Exon	Nucleotide change	Families carrying the SNP, *n*	Reference
2	C→T81	1	25
10	G→A1413	1	26
10	A→G1415	3	Novel
11	C→T1617	4	27
13	C→T1959	1	28
16	C→T2258	1	Novel

SNP = single nucleotide polymorphism.

PCSK9 gene variants

PCSK9 mutations were not detected in any of the 30 ADH probands. DHPLC and sequence analysis of the *PCSK9* gene revealed only three previously described polymorphisms (Table 5).[29,30]

Table 5. Polymorphisms identified in the *PCSK9* gene among 30 Taiwanese autosomal dominant hypercholesterolemia probands

Exon/ Intron	Nucleotide change	Families carrying the SNP, *n*	Reference
5	C→T658 –7	1	29
9	G→A1624	1	30
10	A→G1680 +64	1	29

SNP = single nucleotide polymorphism.

Discussion

We identified eight mutations in the *LDLR* gene and two mutations in the *APOB* gene among 55 members from 18 out of 30 ADH families in this study. None of our cases were found to harbor mutations in the *PCSK9* gene. All the mutations we identified were confirmed in at least two of the recruited ADH families. This is the first comprehensive mutation study of the three known genes causing ADH in Taiwanese.

Among the identified *LDLR* gene mutations, only three (D69N, W462X, G170X) have previously been reported in Chinese.[19,24] The novel deletion of AT at nucleotide 1954–1955 results in a frameshift from codon 631 of the EGF precursor homology region. This novel deletion was identified in three of our ADH families (F16, F28, F30), in which affected members all had markedly elevated plasma LDL-C levels. Another novel mutation was identified at the splice site c.1586+5G>C of the *LDLR* gene in families F20 and F29, which may result in an erroneous skipping of exon 10, since the G nucleotide, a 5′ splice donor, is essential for proper splicing.[31] The co-segregation of mutations and hypercholesterolemia in ADH probands and their family members implied that the newly identified genetic variants were presumably responsible for ADH. However, further expression studies are needed to confirm the functional role of the two novel *LDLR* gene mutations. Interestingly, these two recurrent novel mutations accounted for 17% (5 of 30) of the recruited ADH families, which may suggest the presence of characteristic common mutations due to a founder effect in the Taiwanese ADH population. It is necessary to screen a larger number of ADH subjects to confirm the hypothesis in the future.

Among the 30 recruited ADH families, three were found to have the R3500W mutation in the *APOB* gene. Interestingly, the prevalent R3500Q mutation in Caucasians was not identified in our ADH population. This finding is consistent with one previous study that evaluated the prevalence of FDB among hypercholesterolemic subjects in Taiwan,[32] in which the R3500W allele is far more prevalent than the R3500Q one (2.4% *vs.* 0.3%) among those with moderate hypercholesterolemia. In family T1, we identified a c.10828C>T nucleotide change in the *APOB* gene, which results in a substitution of threonine to methionine at codon 3540 (T3540M). This novel missense mutation is, to our knowledge, the seventh *APOB* genetic variant to be associated with hypercholesterolemia. However, even some well-known *APOB* mutations (such as R3531C) are not consistently found to be related to hypercholesterolemia.[33] The pathogenic role and clinical significance of the T3540M mutation require further investigation.

Unexpectedly, we failed to find mutation-causing *PCSK9* gene variants other than polymorphism in our study subjects. *PCSK9* gene mutations were found in 12.5% of the families without *LDLR*/*APOB* mutation in the original French series.[7] However, in a recent Danish study,[34] the *PCSK9* gene was excluded to be responsible for ADH in 20 *LDLR*/*APOB* mutation-negative families. The results suggest that the genetic variants of *PCSK9* may not play a significant role in causing ADH in certain ethnic groups. Or at the very least, the prevalence of ADH families carrying a *PCSK9* mutation could be very low among these populations.

Tendon xanthomata, corneal arcus or xantholesma was present in 16.1% (14 of 87) of our study subjects (Table 2). Several of these cases were found to have mutations in the *LDLR* gene (three had delAT1954, two had G→C 1586+5, four had R385W, W462X, V408M and C107Y).

None of these cases were associated with *APOB* gene mutation. Although cases with such pathognomonic physical findings were more often found to have a mutation in the *LDLR* gene (64.3%, 9 of 14), there were still five cases without an identifiable mutation in the *LDLR*, *APOB* or *PCSK9* genes.

We failed to identify mutations responsible for ADH among 35 of our cases (from 12 different families), making a mutation detection rate of 60%, while the mutation detection rate was 41% in another report on Taiwanese familial hypercholesterolemia patients (without taking the *PCSK9* gene into consideration).[11] The mutation detection rate in Taiwanese ADH patients was relatively low in comparison with previous studies.[19] One reason for the result is probably the technical problems. The sensitivity and specificity of DHPLC are approximately 95%, irrespective of sequence variations, except for some high-melting regions surrounded by lower-melting sequences. The optimal size of an amplicon varies between 150 bp and 700 bp, depending on GC content. Any deletion beyond that size will not be detected by DHPLC, and alternative methods such as long PCR or Southern blotting would be required to exclude large deletions or rearrangements. We could have missed some mutations like these in our analyses, if they had occurred in the *LDLR* and *PCSK9* genes.

Another reason for this result could be the existence of additional monogenic or polygenic causes responsible for the ADH phenotype due to ethnic differences. For example, despite an extensive search for mutations in the *LDLR* and *APOB* genes, the underlying genetic changes still could not be identified in a significant portion of ADH patients in the United Kingdom[20] and Malaysia.[35] The molecular basis of the ethnic differences will require further effort to determine.

A third reason could be due to the relatively less strict lipid criteria for ADH. Take the US MEDPED (Make Early Diagnosis to Prevent Early Death) program[36] cut points for ADH diagnosis as an example: adult patients older than 30 years need to have a serum cholesterol level of 290–340 mg/dL (7.5–9.3 mmol/L) to be diagnosed as an index case of ADH. However, the cut point for total cholesterol used in this study was 260 mg/dL (6.8 mmol/L) for Taiwanese of the same age. Although the lower absolute levels of cholesterol and LDL-C in Taiwanese could be due to a different diet and lifestyle from that in Western countries and would not affect the diagnostic power of the percentile criteria, we cannot exclude the possibility that some non-ADH probands would still be included due to frank hypercholesterolemia and an ambiguous family history.

In conclusion, the genetic background of Taiwanese ADH patients is highly heterogeneous, consisting of a variety of different mutations in the *LDLR* and *APOB* genes. However, there may be some common mutations responsible for a significant portion of the ADH population in Taiwan. The mutations of the *PCSK9* gene seem not to play a significant role in causing ADH in Taiwanese. These observations reflect the heterogeneous ethnic origins of Taiwanese and a characteristic mutation pattern that is different from that in other countries. A larger screening program is required to clarify the epidemiologic features of ADH in Taiwan. *In vitro* expression study is also needed to confirm the functional implications of the newly identified mutations in ADH patients.

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