行政院國家科學委員會補助專題研究計畫成果報告

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計畫類別：x **個別型計畫**　　□**整合型計畫**
計畫編號：NSC89－2320－B－039－005－
執行期間：1999 **年**　8 **月**　1 **日至** 2000 **年**　7 **月**　31 **日**

計畫主持人：鄔哲源
共同主持人：蔡輔仁 李正淳

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- □**國際合作研究計畫國外研究報告書一份**

執行單位：中國醫藥學院附設醫院醫學研究部

中　華　民　國 90 **年**　2　**月**　27　　**日**

行政院國家科學委員會專題研究計畫成果報告

威爾森氏症之分子研究

Molecular Analysis of Wilson Disease

計畫編號：NSC 89-2320-B-039-005-

執行期限：1999 年 8 月 1 日至 2000 年 7 月 31 日

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一、 Chinese Abstract

威爾森氏症是種有關銅代謝有關的的自體隱性遺傳疾病。據估計每三萬五千至十萬名新生兒就有一人罹患此症。患者主要症狀為肝臟硬化伴隨神精方面之症狀。威爾森氏症主要導因於以銅為輔胺之腺核甘三磷酸酵素（ATP7B)之突變造成。

之前一年的研究發現，以銅為輔胺之腺核甘三磷酸酵素突變研究發現約百分之七十的威爾森氏症突變染色體總計共十二個突變，其中六個突變是過去所未發表的。研究結果顯示國人威爾森氏症之突變非常多元性，本計劃即在延續之前的研究成果冀望對威爾森氏症做更深一層的研究。研究要點如下：

1. **結合單股多形性分析以及直接核酸定序分析，找出其餘未被偵測及新收集威爾森氏症的突變。**
2. **利用 ATP7B 點附近已清楚定性之微衛星標誌做單套性分析，結合已知之突變，分析威爾森氏症之家庭，藉以釐清各突變所對應之單套型，寄望發現單套型與突變種類之關聯性，以利日後之突變檢測。**
3. **功能研究，在上個計劃我們雖有提出。藉用功能研究以確定檢測之核酸改變為突變而非多型性。但鑑於所通過之人力及經費配置，勢難達成，因此本計劃乃延續上一計劃之功能研究，釐清突變原因。**
4. **基因型與表現型關聯性之研究，鑑於威爾森氏症突變之逐漸被釐清，我們將做基因型與臨床症狀之關聯性研究。**

關鍵詞：威爾森氏症、突變、以銅為輔胺之腺核甘三磷酸酵素（ATP7B）、單股多型性分析、直接核酸定序分析、單套型分析、微衛星標誌、功能分析。

二、English Abstract

In the previous year, we successfully identified more than 70% WND mutant alleles. The result was published (Human Mutation, 12: 370-376, 1998). We identified 12 mutations, of which six were not previously reported. While we are concentrating on identifying more WND mutations, we found the approved budget is limited for further characterization of Wilson disease. We think our finding justify more funding for this research. Our proposed study for the next year include the following:

I. Mutation detection of those unidentified WND alleles and those newly collected Wilson

disease patients.

II. Haplotype analysis of WND chromosomes to establish the correlationship between specific mutations and their respective haplotypess.

III. Functional characterization of those identified WND mutation. Phenotype and genotype correlation study.

Keywords：Wilson disease (WND), mutation analysis, single strand conformation polymorphism (SSCP), direct DNA sequencing, Haplotype analysis, microsatellite markers, Functional characterization, transient transfection

三、Background and Specific aims

I. Background

Wilson disease (WD) is inherited in an autosomal recessive model with a disease prevalence of about one in 35,000 and one in 100,000 live births (Danks, 1995). WD is a disorder of copper transport that is characterized by a reduced incorporation of copper into ceruloplasmin by decreased biliary excretion. This leads to copper accumulation in the liver and, consequently, to progressive liver damage. The clinical features of WD are attributable to the toxic accumulation of copper in the liver and other tissues, such as kidney, brain and cornea.

Wilson disease gene (ATP7B), consisting of 21 exons, codes for a putative copper transporting P-type ATPase, a closely related Menkes disease gene (ATP7A) (Bull et al. 1993; Chelly et al. 1993; Mercer et al. 1993; Vulpe et al. 1993). Earlier genetic linkage studies showed that the WD locus segregates with the red cell enzyme esterase D (Frydman et al. 1985) on chromosome 13. Subsequent linkage analysis refined the disease locus to a genomic region flanked proximally by the DNA marker, D13S31, and distally by D13S59 (Bowcock et al. 1987; Stewart et al. 1993). Haplotype analysis using three dinucleotide-repeat markers, D13S314, D13S301, and D13S316, has been a useful indicator of specific mutations (Thomas et al. 1995; Nanji et al. 1997).

II. Specific aims

The specific aims of this proposed study were listed as following:

1. To identify most of the WND alleles in Chinese:
2. To find out if there is correlation between mutations and their respective haplotypes in WND gene.
3. To shed light on the WND protein function through the functional study.
4. To study the genotype-phenotype correlation of WND disease

四、Results and Discussion

I. Results

Mutation analysis of ATP7B gene

Besides twelve mutations previously reported by Tsai et al (1998), we identified another four missense mutations (A874V, V1216M, E1173K, and D1279G) in Taiwanese WND

chromosomes. While A874V was recently reported in Japanese and Korean (Yamaguchi et al 1998; Kim et al 1998), V1216M and E1173K were identified in Mediterranean (Loudianos et al 1998, 1999). D1279G is novel mutations with nonconservative amino acid substitution. Glutamic acid 1173 is located near the ATP loop region and replacement with basic lysine residue might affect the binding of ATP. Aspartic acid 1279 is located between the hinge region and the 7th transmembrane domain of ATPase and replacement with glycine residue might affect the folding of the polypeptide.

Haplotype association of WND chromosomes

Allele size definition and allele distribution for STR markers of D13S314, D13S301, and D13S316 among normal and WND chromosomes were listed in Table 1. A total of 11, 14, and 10 alleles were identified for each marker, respectively. Table 2 listed all the identified WND chromosomes' haplotypes with their respective mutations. Among those identified WND mutations, we observed the haplotype-mutation association among most mutations. G943D mutation, which was reported only in Taiwanese WND patients (Tsai et al 1998), was found exclusively associated with 11-1.5-5.5 haplotype ((n=7). R778L, the most frequently found WND mutation among Taiwanese, was associated with either 8-4-4 (n=4) or 8-4-5.5 (n=4). Mutation P992L was associated with either 8.5-6.5-2 (n=2) or 8.5-6.5-5.5 (n=3). R778Q, the other mutation besides R778L found in the amino acid position 778, was exclusively associated with 7.5-0.5-5.5 (n=3). Q1142H, reported only in Taiwanese (Tsai et al 1998), was found associated with either 7-4-2 (n=3) or 7-4-5.5 (n=1). 11-0.5-5.5, the most frequent found haplotype among WND chromosomes, was associated with mutations IVS4-1G→C (n=3), A874V, 2304insC and other unidentified WND alleles.

Haplotype and Pedigree analysis in the WND families

Results of haplotype and pedigree analysis for eight WND families were shown in Fig. 1. Although some of the mutations were not identified yet, we were able to deduce those WND chromosomes' haplotypes. In the process of pedigree analysis of WND families, we found the haplotype data was useful in assessing status of either carrier or pre-symptomatic patients, whose sibling was diagnosed with Wilson disease and had yet to develop clinical symptoms. As shown in family F (Fig. 1), the *propositus* was newly diagnosed with Wilson disease. The haplotype data indicated she was homozygous with haplotype of 11-1.5-5.5, which was associated with mutation G943D. Direct sequencing analysis of G943D mutation confirmed that she was homozygous of G943D mutation (data not shown). In family G, the *propositus* was shown to carry 11-0.5-5.5 haplotype. Direct sequencing showed that she inherited G943D mutation from his father (data not shown). His sister, at the time of haplotype analysis, had not shown symptoms of Wilson disease. Although the other mutation had not been identified, haplotype data indicated that his sister shared the same haplotype like him and was subsequently diagnosed as pre-symptomatic WND patient (Fig. 1). In family H, the *propositus* was newly recruited Wilson disease patient. He was shown to carry haplotypes of 11-0.5-5.5 and 7-4-2. To identify WND mutations, those two haplotype-associated mutations were screened first. In a very short time, we identified that he was compound heterozygous for

Q1142H and IVS4-1G>C mutations.

II. DISCUSSION

Up to date, sixteen different mutations had been identified in Taiwanese. Since each non-identified WND allele was checked for the known mutations with the same haplotype, we could estimate that there are probably 11 more unidentified mutations existing. Due to the high heterogeneity of Wilson disease mutation spectrum in Taiwanese, haplotype analysis was suggested to be the initial step before full-scale mutation analysis was performed in newly recruited WND patients. The haplotype data of WND chromosomes was compared to previous study performed by Chuang et al (1996). In their study, only R778L and R778Q were identified and were reported to be associated with haplotypes 8-4-4 and 8-1-6, respectively. While R778L associated haplotypes were the same in both studies, R778Q associated haplotypes (7.5-0.5-5.5 in ours and 8-1-6 in Chuang's) had minor discrepancy. It was probably due to nonconsensual allele definition by different laboratories. We also noticed that haplotypes 11-2-6 and 11-1-6 were mostly frequent in Chuang's study. Those two haplotypes would correspond to the haplotypes 11-0.5-5.5 and 11-1.5-5.5, respectively, in this study.

Some WND mutations, e.g. R778L, P992L, A874V, 2304insC, and N1270S were identified in both Taiwanese and Japanese (Chuang et al 1996; Nanji et al 1997; Tsai et al 1998; Yamaguchi et al 1998). It is interesting to know if those common mutations also shared the same haplotypes between the two ethnic populations. However, we found this task is difficult even among Japanese WND chromosomes themselves. For example, while R778L was found to be associated with haplotypes: 5-5-6, 7-5-4. 7-5-5, 7-5-5.5, and 7-5-7 in one study of Japanese WND patients (Nanji et al 1997), the same mutation was found to be associated with haplotype of either 8.5-6-5.5 or 8.5-6-7 in the other (Yamaguchi et al 1998). The discrepancy probably derived from either different amplification primers used or different allele definition used by different laboratories. Recently, R778L and A874V were identified in Korean WND chromosomes with R778L being the most frequent WND allele (37.5%) among Korean (Kim et al 1998). The WND chromosomes identified among those three different ethnic populations in Northeast Asia area implied that Taiwanese, Japanese, and Korean might originate from the same ancestor. Haplotype study of those shared mutations among those three populations should be able to prove this hypothesis. Consensual allele definition, however, is necessary for haplotype comparison among different ethnic populations. It is also the reason why we listed our allele-size definition in Table 1. On the other hand, the presence of other population specific mutations among those three ethnic peoples suggests that there still is heterogeneity present in WND chromosomes within each population (Nanji et al 1997; Tsai et al 1998; Yamaguchi et al 1998; Kim et al 1998).

Because non-identified WND mutations still account for about 20-30 % in Taiwanese WND patients, it is still difficult to make direct correlation between haplotypes and their respective WND mutations. We believe that, with more WND mutations being identified, the haplotype-mutation association will become more significant and it will also assist mutation identification for newly diagnosed WND patients.

五、Self-evaluation

During the past grant period (one year span), we identified four more mutations (see Results and Discussions Section) in Taiwanese Wilson disease patients. We also successfully established the haplotyopes for most identified WND mutations. The acquired data will be helpful for future mutation study. Because of the limited time span for this grant, we were not able to do the functional study to study the mutation effect of ATPase polypeptide. Our results had been accepted for publication in “Jounal of Human Genetics” (J Hum Genet. 2000; 45:275-9).

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七、 Tables and Figures

Table 1. Allele-size definition and distribution of STR markers in Taiwanese WND families

Marker	Band size (bp)	Allele	No. of Chromosomes	
			Normal	WND
D13S314[a]	154	5.5	1	0
	153	6	7	1
	151	7	32	9
	150	7.5	3	3
	149	8	10	10
	148	8.5	6	5
	147	9	1	0
	143	10.5	6	1
	141	11	46	25
	138	11.5	3	0
	136	12.5	1	0
D13S301[b]	158	-2	1	0
	157	-1.5	3	1
	155	-0.5	3	2
	153	0.5	25	13
	152	1	2	0
	151	1.5	10	11
	150	2	2	0
	149	2.5	4	0
	148	3	11	2
	146	4	44	18
	144	5	8	1
	142	6	1	0
	141	6.5	0	6
	131	11.5	2	0
D13S316[a]	152	2	20	7
	150	3	3	2
	149	3.5	1	0
	148	4	4	5
	147	4.5	3	0
	145	5.5	77	37
	144	6	2	0
	143	6.5	1	3
	142	7	2	0
	138	9	3	0

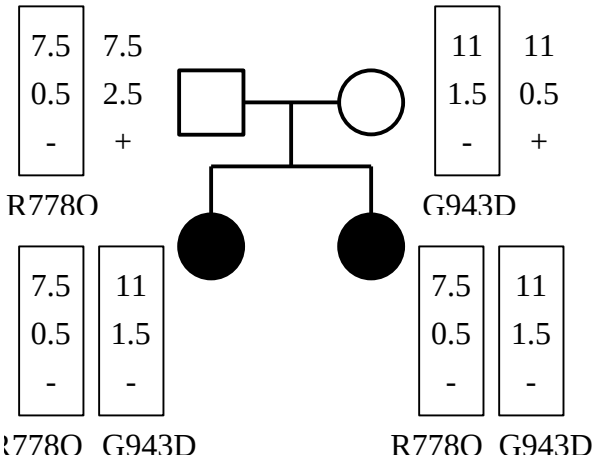
[a] Amplification primers for D13S314 and D13S316 previously described (Thomas et al 1994).
[b] Amplification primers used for D13S301 previously described (Petrukhin et al 1993).

Table 2. Haplotypes and Mutations in Patients with Wilson Disease in Taiwan

Haplotype	Mutation[a]	WND chromosome	Normal chromosome
6-(-1.5)-5.5	n.i.	1	0
7-4-2	Q1142H	3	1
7-4-3	n.i.	1	1
7-4-5.5	C490X	1	12
	Q1142H	1	
	W1153C	1	
7-5-5.5	n.i.	1	2
7-6.5-2	N1270S	1	0
7.5-0.5-5.5	R778Q	3	1
8-4-4	R778L	4	3
	IVS17-2A→T	1	
8-4-5.5	R778L	4	2
8.5-6.5-2	P992L	2	0
8.5-6.5-5.5	P992L	3	0
10.5-3-5.5	n.i.	1	1
11-(-0.5)-3	E1173K	1	0
11-(-0.5)-5.5	n.i.	1	1
11-0.5-5.5	A874V	1	11
	IVS4-1G→C	3	
	2304insC	1	
	n.i.	4	
11-0.5-6.5	n.i.	1	0
11-1.5-2	n.i.	1	0
11-1.5-5.5	G943D	7	5
	n.i.	2	
11-1.5-6.5	D1279G	1	0
11-3-6.5	n.i.	1	0
11-4-5.5	523insA	1	7
	V1216M	1	

[a] n.i.=mutation not identified

Figure 1. Haplotypes, identified mutations, and pedigrees of WND families. Numbers indicate alleles of each STR markers and are defined in Table 1. Blackened boxes indicated the affected WND probands. The haplotype was arranged from the top to bottom in the order of D13S314-D13S301-ATP7B-D13S316. Normal chromosomes and WND chromosomes are labeled as "+" and "-", respectively. Haplotypes carrying WND mutations are boxed. N.I. represents WND mutations not yet identified. (see next page for Fig. 1)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7.5 2.5 +
11 1.5 -
11 0.5 +
R778Q
G943D
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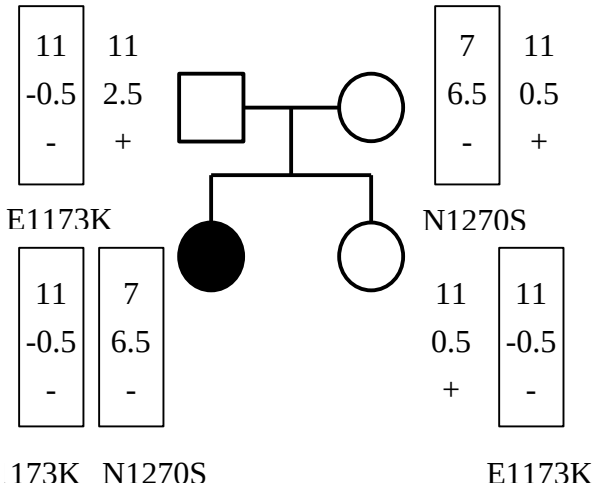
7.5 0.5 -
11 1.5 -
R778Q G943D
R778Q G943D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7 6.5 -
11 0.5 +
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N1270S
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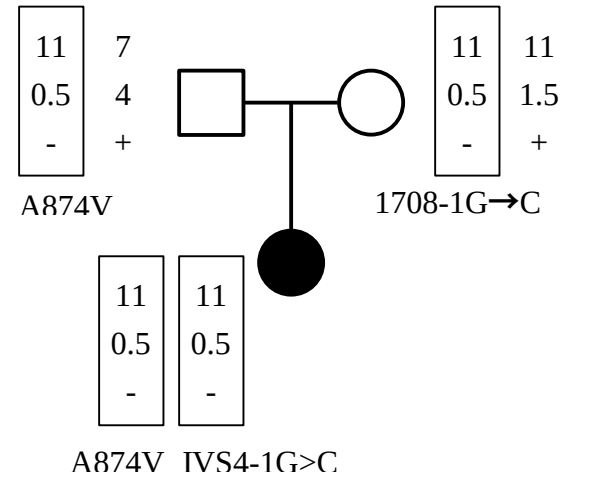
11 -0.5 -
173K N1270S
E1173K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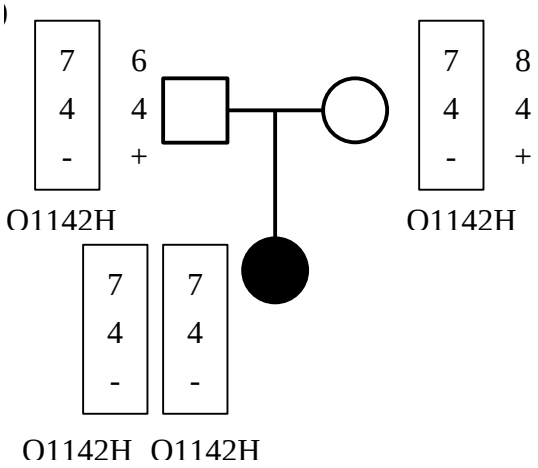
A874V IVS4-1G>C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Q1142H
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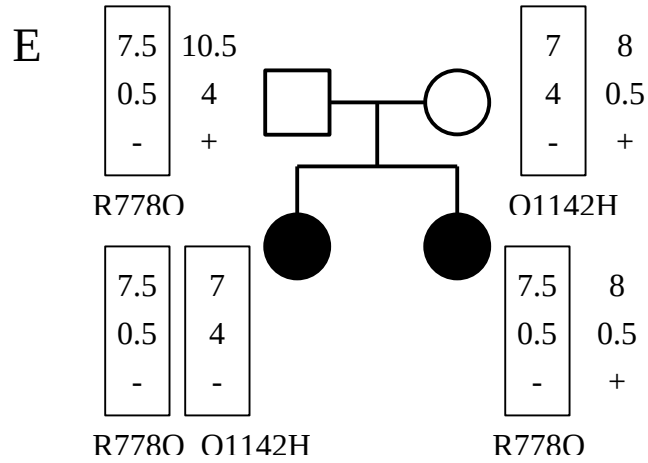
Q1142H Q1142H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7 4 -
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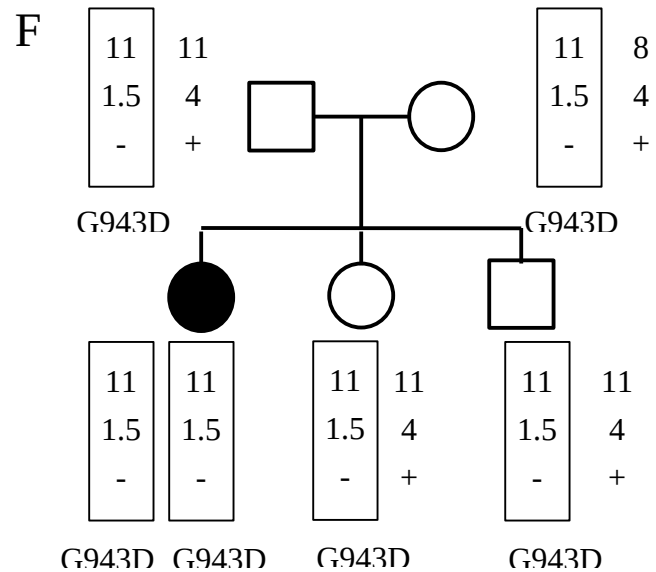
R778Q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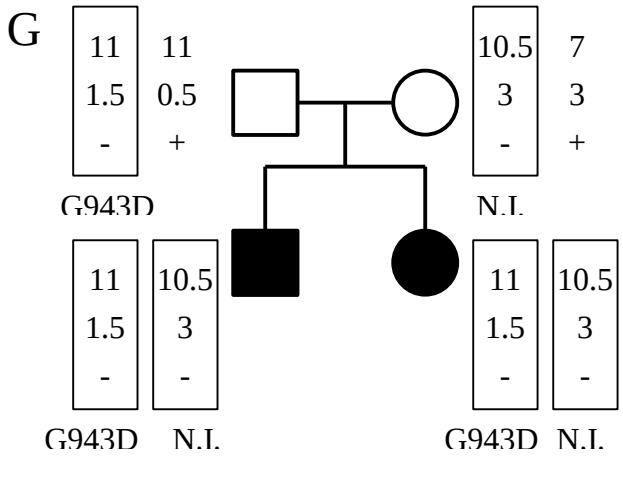
G943D G943D G943D G943D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7 3 +
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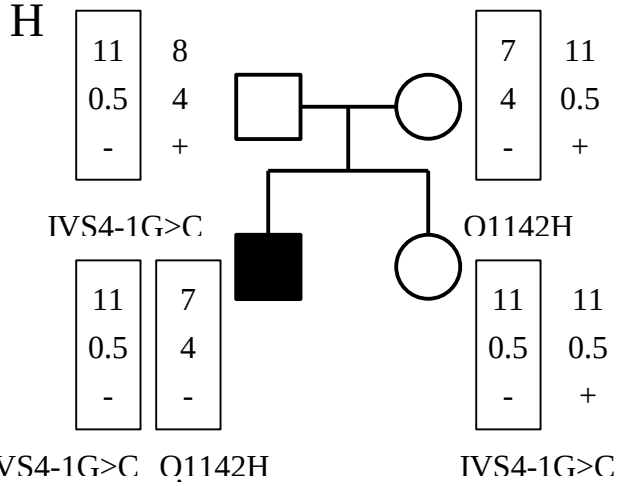
10.5 3 -
G943D N.I.
G943D N.I.


H
11 0.5 -
8 4 +
7 4 -
11 0.5 +
IVS4-1G>C
Q1142H
11 0.5 -
7 4 -
11 0.5 -
11 0.5 +
IVS4-1G>C Q1142H
IVS4-1G>C