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PCR in Disease Diagnosis of WND

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1. Introduction

Polymerase chain reaction (PCR) invented by Karry Mullis in 1983 is a cornerstone of molecular biology techniques. Polymerase chain reaction (PCR) is used to generate millions of copies of a DNA sequence in a short interval. PCR is commonly used for a variety of applications including diagnosis of genetic disease, cloning, QTLR analysis, forensic analysis, diagnosis of infectious diseases. Polymerase chain reaction becomes indispensable technique in medical sciences these days for the diagnosis of infectious diseases. Molecular genetic testing has made possible to identify the mutations in first degree relatives of index case even in the absence of clinical and biochemical presentations of symptoms. PCR is a basic step in molecular genetic testing.

Wilson disease is a genetic disorder of copper metabolism with hepatic or neuropsychiatric presentations [1]. The copper-transporting P-type ATPase, *ATP7B* gene was identified in 1993 (ATP7B; OMIM 606882) [2] and found responsible for WND. Copper is a nutritional trace element and play indispensable role in variety of biological reactions [3, 4]. The homeostasis of copper is maintained by liver. It regulates excretion of copper into bile and into secretary pathway. Ceruloplasmin protein is abundant in blood and plays functional role in copper transport. Copper excretes from hepatocytes as ceruloplasmin or through bile [5, 6]. The human Cu-ATPases regulate the intracellular copper homeostasis. The copper is transported from cytosol to secretory pathway using energy released by the hydrolysis of ATP and supply the copper for various copper-dependent biological process and enzymes. The biosynthesis of copper-dependent ferroxidase ceruloplasmin is dependent on *ATP7B*. In addition, Cu-ATPases play part in export of excess copper out of the cell [7, 8, 9].

Liver is involved in the copper homeostasis and remove excess copper via bile [10] through the activity of *ATP7B* transporter. Therefore defect in *ATP7B* gene results in disturbance of copper homeostasis and caused WND [2, 11] due the copper accumulation in liver, brain and cornea.

ATP7B is present in the Golgi & trans-Golgi Network (TGN), travel to the vesicular compartment. There, *ATP7B* delivers copper to ceruloplasmin in the cell which is copper binding protein [12, 13]. *ATP7B* gene contains six copper binding domains, transduction domain, cation and phosphorylation domain, nucleotide-binding domain and eight hydrophobic transmembrane sequences [7]. The Wilson disease gene *ATP7B* was localized

at chromosome 13 on q14.3 band and cloned in 1993. *ATP7B* gene comprised of 21 exons which encodes 1465 amino acid residues [2, 14, 15].

The WND diagnosis is complex due to variable disease onset and clinical symptoms. Clinical symptoms of the disease include hepatic, neurologic and psychiatric disturbances [16]. WND patients with hepatic manifestation may present asymptomatic to mild hepatomegaly, cirrhosis, acute hepatitis and jaundice. Haemolysis can be present in acute liver failure [17, 18, 19].

Neurological symptoms mostly appeared in second decade of life due to the toxic copper accumulation in brain that damages the nerve cells. This leads to hypokinetic speech, tremor, dystonia and later dysphagia, mutism and Parkinsonism. Hepatic and neurological presentations in combination have been observed in 50% WND patients [20, 21].

Psychiatric symptoms have been seen in the early stages of WND including incompatible behavior, irritability, depression and cognitive impairment [22, 23].

Therefore, molecular testing of WND [21, 24] has served as a very useful approach for presymptomatic disease diagnosis in the absence of clinical symptoms. In present study, we investigated presymptomatic WND in siblings of two index case of Wilson disease.

2. Material and methods

The informed consent was obtained from all subjects and study was approved from ethnic committee of the institution.

2.1 Subjects

Two families with WND patients were enrolled for present study. The age of patients was between 7-11 years and of siblings was between 2-5 years. The children of family 1 were born to non-consanguineous parents. History of patient belongs to family I was described earlier [25]. The children of family II were born to consanguineous parents. Past family history was negative for presence of WND. However, liver disease was reported in family II. Patients were enrolled for mutational analysis based on clinical diagnosis. Siblings were screened for presymptomatic WND. The patients of family II were 11 & 8 years old. The laboratory investigations of respective patients were described in table 1 & 2. The siblings of index patients were included without clinical data. Patients of this family were presented with hepatic manifestation. The 11 year old boy was also patient of hepatitis C.

2.2 Mutational analysis

DNA was extracted from peripheral blood of all subjects by standard phenol/chloroform extraction method [26]. The quality plus quantity of DNA was checked through gel electrophoresis and spectrophotometer. PCR was done in 30µl volume containing 200ng genomic DNA, 1X *Taq* buffer, 200µM dNTPs mixture, 2.5 pmol of both primers, 1unit of *Taq* polymerase. The PCR conditions were optimized for 11 exons of *ATP7B* gene. The PCR products were purified through PCR purification kit (Genomed GmbH Inc). For sequencing, 0.1-0.5ng of PCR product was used as template and sequencing PCR was performed with quickstart DTCS kit (Beckman Coulter). The sequencing PCR program was comprised of 30

cycles: 96◦C for 20 seconds, 20 seconds at respective annealing temperature and 60◦C for 4 min. Salt precipitation method was used to remove unincorporated dye terminators as described by manufacturer. The sequencing was performed with forward and reverse primers on CEQ8000 Genetic Analyzer (Beckman Coulter). The sequences were compared for the detection of mutation through BioEdit Sequence Alignment Editor ver 7.0.9.0.

3. Results and discussion

Wilson disease is a recessive autosomal disorder caused by increased accumulation of copper in liver, brain, cornea [5]. The prevalence of WND is around 1:30,000 with carrier frequency 1:90 [27] while 4% carrier frequency is also reported [28]. Clinical presentation of disease is variable and mostly appears between ages 5 to 35 with rare case of onset in 2 to 72 years [29, 30]. The diagnosis of WND is based on presence of KF ring, low plasma ceruloplasmin, elevated urinary copper and liver copper concentration [1, 24]. WND is caused due to defect in *ATP7B* gene, which is copper transporter. The worldwide data revealed the population specific pattern of *ATP7B* gene mutations. The most common mutation found in Europe, American and Greece population was H1069Q in exon 14. About 50–80% of WND patients from these countries carry at least one allele with this mutation with an allele frequency ranging between 30 and 70% [28, 31, 32]. Mutational analysis of *ATP7B* gene has been extensively carried out in Chinese population and showed a high prevalence of WND. Mutations have been detected in all exons except 21. Most mutations were found in exons 8, 12, 13 and 16 accounts for 74.0% of the reported WND alleles. The most frequent WND mutations were p.Arg778Leu and p.Pro992Leu, which account for 50.43% of all the reported WND alleles in Chinese population [33, 34]. The R778L mutation was also frequent in Korean and Taiwan population with an allele frequency of 20-35% & 55.4% [35, 36, 37]. In addition to R778L mutation at exon 8, hotspot for *ATP7B* mutation in exon 12 were also detected in Taiwan WND patients [38] where 9.62% of all mutations occurred.

The spectrum of *ATP7B* gene mutations in our population is yet to be studied. In present study, the siblings of index patients of WND were screened for mutations in *ATP7B* gene. We enrolled two families for genetic testing and novel mutation was described previously in one patient [25]. The past history of patients revealed the sudden onset of disease while no WND patient was reported earlier in these families. Based on current family history, siblings were screened for presympotomatic WND. The exon 15 and 19 were amplified at 55°C. The exons 16, 17, & 21 were optimized at 64°C. Same annealing temperatures were used for sequencing PCR. Phenotypic data of subjects of family 1 was found normal. No laboratory investigations were performed at the time of genetic testing. The family members of patient were screened without relying on clinical data. The patients and siblings were born to non-consanguineous parents. The parents were also found normal in genetic testing.

The family-II was also presented with same history. The family did not have any past WND history. The two child of respective family were declared WND patient. The phenotypic presentations of sibling and parents were found normal. However, occurrence of liver diseases (details not available) was reported in three generations. The parents were not reported any type of liver disease. The Hb level of sibling was reported below normal range when he was 3years old. We encountered problem in collecting blood sample from family-II. Therefore we reamplified each exon through PCR with same product to get product in

sufficient quantity. This step reduced the chance for loss of sample. Because subjects were belonged to remote area and access to them was not feasible.

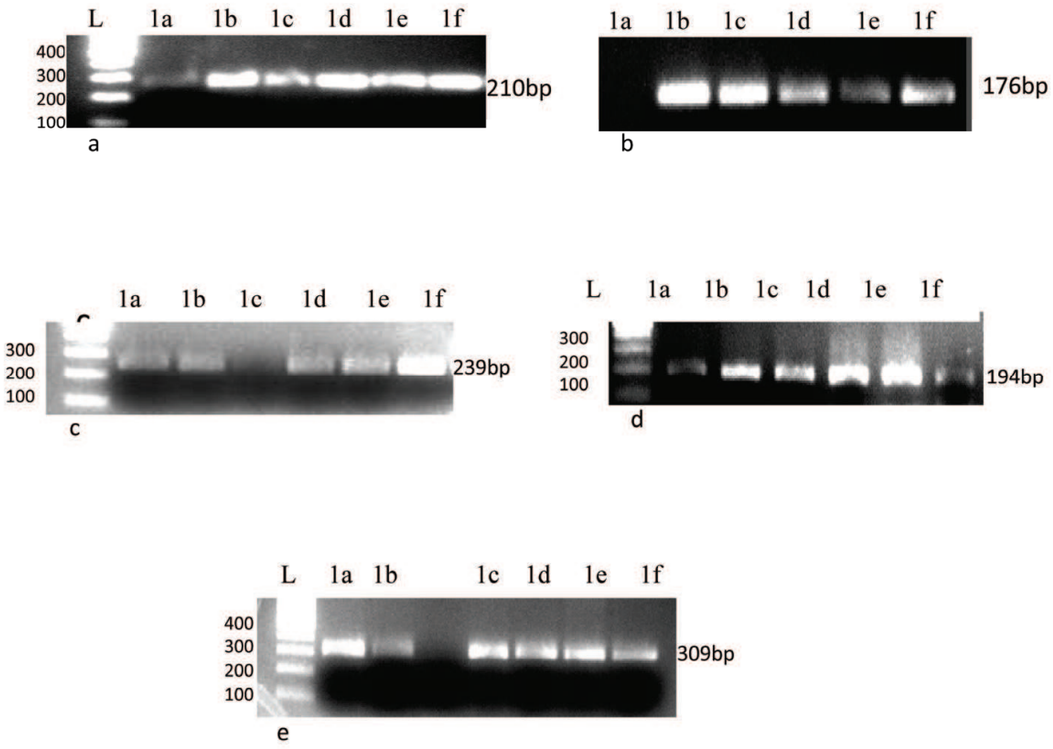


Fig. 1. PCR amplification of samples of family 1
a) Exon-15, b)Exon-16, c)Exon-17, d)Exon-19, e)Exon-21

Patients	**CP**	**U-Cu**	**ALT**
Normal	**<20mg/dl**	**>100mg/24h**	**7-45 U/L**
8a	6.8 mg/dl	1796mg/24h	40 U/L
8f	20 mg/dl	1000mg/24h	200 U/L

Table 1. Biochemical analysis of WND patients of family II

Patients	**Hb**	**Total Bilirubin**	**AST**	**ALP**	**Serum Albumin**
Normal	**13-17 g/dl**	**0.3-1.2 mg/dl**	**7-45U/L**	**98-279 U/L**	**3.5-5.5 g/dl**
8a	8.9 g/dl	1.8 mg/dl	1400 U/L	569 U/L	2.3 g/dl
8f	5 g/dl	30 mg/dl	450 U/L	350 U/L	2.2 g/dl

Table 2. Clinical features of WND patients of family II

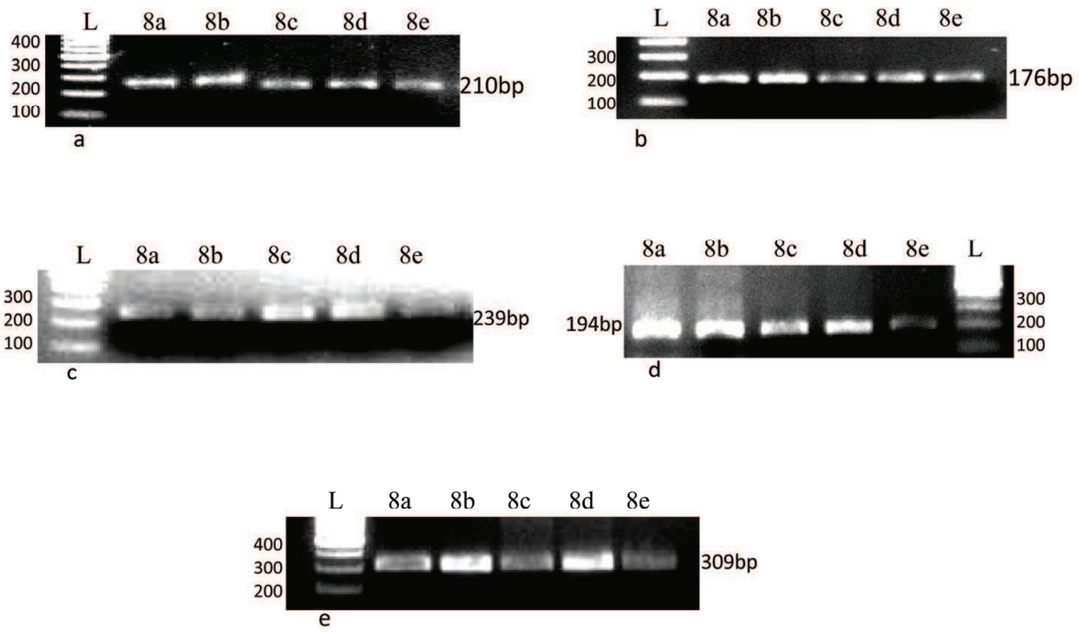


Fig. 2. PCR amplification of samples of family II
a) Exon-15, b)Exon-16, c)Exon-17, d)Exon-19, e)Exon-21

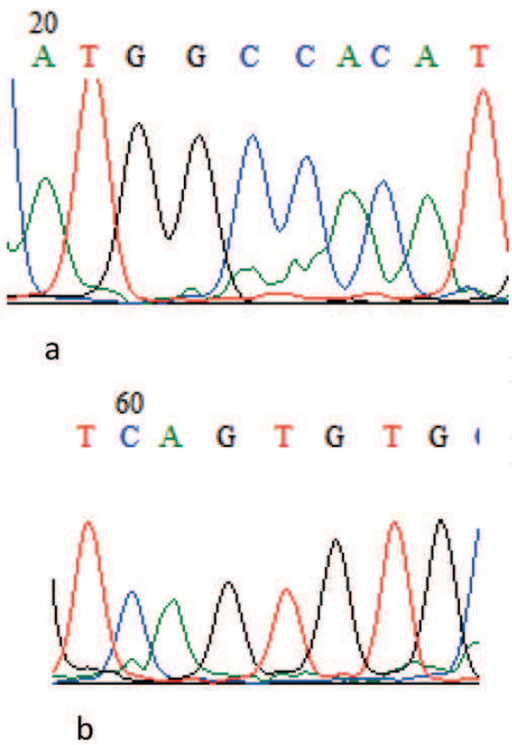


Fig. 3. Sequencing of *ATP7B* gene
a) Family 1, b) Family II

The phenotypic presentations of sibling were found normal. Fig 1 & Fig 2 shows the PCR amplification of respective exons. In mutational analysis, subjects of both families were detected negative for any mutation on exons 15, 16, 17, 19 & 21 (Fig 3).

The prospective of our study was the screening of carrier in respective families and inclusion of genetic testing for earlier diagnosis of WND. PCR and gene sequencing are the basic steps of genetic testing. We have not found prevalent mutation in our Wilson disease patients. Screening the siblings is a grueling task when common mutation was not identified. Genotyping of *ATP7B* gene in families of WND patients was first time carried out in our country. Genetic testing is useful tool for the screening of presymptomatic WND case or carriers in the family of index patient. It could help in genetic counseling of respective family based on molecular diagnosis. We have also found that optimization of parameters for PCR and sequencing is indispensible for effective screening. The result's integrity dependent on quantity and quality of template and is a paramount factors in PCR. Presymptomatic diagnosis through genetic analysis could help to stop progression & prevention of the disease and timely treatment. The Taqman allelic discrimination reported a valid technique for efficient screening of common mutation in index patient and sibs [39]. Single nucleotide polymorphism (SNP) have also been studied in combination with the prevalent mutations for WND diagnosis and evaluated as a comprehensive strategy for the detection presymptomatic or carrier sibs of WND patients [40].

4. Conclusion

This case has provided a base to establish a system for genetic testing for the earlier diagnosis of disease and detection of heterozygote carrier. PCR based genetic testing using different approaches like multiplex PCR, SNP markers and Taqman assay will turn into a cost effective screening. These results further more have provided a platform for haplotype analysis. Presymptomatic diagnosed patient can undergo regular treatment to prevent disease progression and onset.

5. References

[1] Roberts EA, Schilsky ML. Diagnosis and treatment of Wilson disease: an update. Hepat 2008;47(6): 2089-111.

[2] Bull PC, Thomas GR, Rommens JM, Frobes JR, Cox DW. The Wilson disease gene is a putative copper transporting p-type ATPase similar to the Menkes gene. Nat Gen 1993;5: 327-337

[3] Prohaska JR. Role of copper transporters in copper homeostasis. Amer J Clin Nutr 2008; 88(3):826S-829S

[4] Lallioti V, Muruais G, Tsuchiya Y, Pulido D, Sandoval IV. Molecular mechanisms of copper homeostasis. Front Biosci 2009;14:4878–4903

[5] Nicholas AV, Ann PG, Richard B P, Kipros G, James C. The multi-layered regulation of copper translocating P-type ATPases. Biometals 2009;22:177–190

[6] Linder MC, Wooten L, Cerveza P, Cotton S, Shulze R, Lomeli N. Copper transport. Am J Clin Nutr 1998;67(5):965S-971S

[7] Lutsenko S, Erik S, Shane L, Shinde U. Biochemical basis of regulation of human copper-transporting ATPases. Arch Bioch Biophy 2007;463(2):134-148

[8] Bie P, Muller P, Wijmenga C, Klomp LWJ. Molecular pathogenesis of Wilson and Menkes disease: correlation of mutations with molecular defects and disease phenotypes. J Med Gen 2007; 44: 673-688

[9] Hellman NE, Kono S, Mancini GM, Hoogeboom AJ, DeJong GJ, Gitlin JD. Mechanisms of copper incorporation into human ceruloplasmin. J Biol Chem 2002;277:46632–8

[10] Wijmenga C, Klomp LW. Molecular regulation of copper excretion in the liver. Proc Nutr Soc 2004;63:31–9

[11] Langner C, Denk H. Wilson disease. Virchows Arch 2004; 445:111–8.

[12] Bartee MY, Lutsenko S. Hepatic copper-transporting ATPase *ATP7B*: function and inactivation at the molecular and cellular level. Biometals 2007;20:627–637

[13] Guo Y, Nyasa, L, Braiterman LT, Hubbard AL. NH2-terminal signals in *ATP7B* cu-ATPase mediate its Cu-dependent anterograde traffic in polarized hepatic cells. Am J Physiol Gastr Liver Phy 2005; 289:G904–16.

[14] Tanzi RE, Petrrukhin K, Chernov I, Pellequer JL, Wasco W, Ross, B. The Wilson disease gene is a copper transporting ATPase with homology to Menkes disease gene. Nat Genet 1993;5:344-350

[15] Yamaguchi Y, Heiny ME, Gitlin JD. Isolation and characterization of a human liver cDNA as a candidate gene for wilson disease. Biochem. Biophys. Res. Commun 1993;197:271–277

[16] Ferenci P, Caca K, Loudianos G, Mieli-Vergani G, Tanner S, Sternlieb I, Schilsky M, Cox D, Berr F. Diagnosis and phenotypic classification of Wilson disease. Liver Int 2003 Jun; 23(3):139-42.

[17] Merle U, Schaefer M, Ferenci P, Stremmel W. Clinical presentation, diagnosis and long-term outcome of Wilson's disease: a cohort study. Gut 2007; 56(1):115-20.

[18] El-Youssef M. Wilson disease. Mayo Clin Proc 2003 Sep; 78(9):1126-36.

[19] Sharma S, Toppo A, Rath B, Harbhajanka A, Lalita Jyotsna P. Hemolytic Anemia as a Presenting Feature of Wilson's Disease: A Case Report. Indian J Hematol Blood Transfus 2010 Sep; 26(3):101-2.

[20] Singh P, Ahluwalia A, Saggar K, Grewal CS. Wilson's disease: MRI features. J Pediatr Neurosci 2011 Jan; 6(1):27-8.

[21] Medici V, Mirante VG, Fassati LR, Pompili M, Forti D, Del Gaudio M, Trevisan CP, Cillo U, Sturniolo GC, Fragiuoli S,Monotematica AISF. Liver transplantation for Wilson's disease: The burden of neurological and psychiatric disorders. Liver Transpl 2005 Sep; 11(9):1056-63.

[22] Sahoo MK, Avasthi A, Sahoo M, Modi M, Biswas P. Psychiatric manifestations of Wilson's disease and treatment with electroconvulsive therapy. Indian J Psychiatry 2010 Jan; 52(1):66-8.

[23] Benhamla T, Tirouche YD, Abaoub-Germain A, Theodore F. The onset of psychiatric disorders and Wilson's disease. Encephale 2007 Dec; 33(6):924-32.

[24] Ala A, Borjigin J, Rochwarger A, Schilsky M. Wilson disease in septuagenarian siblings: raising the bar for diagnosis. Hepatology 2005;41:668–670

[25] Naveed AK, Majeed A, Mansoor S. Spectrum of *ATP7B* gene mutations in Pakistani wilson disease patients: A novel mutation is associated with severe hepatic and neurological complication. Inter J Biol 2010;2(1):117-122

[26] Sambrook J. Russell D. Molecular Cloning: A laboratory Manual 3rdedition. Cold Spring Harbor laboratory press ;2001

[27] Scheinberg I, Sternlieb I. Wilson disease.In: Lloyd H, Smith J, editors. Major problems in Internal medicine. Philadelphia: Saunders; 1984. pp 23

[28] Loudianos G, Kostic V, Solinas P, Lovicu M, Dessi V, Svetel M, Major T, Cao A. Delineation of the spectrum of Wilson disease mutations in the Greek population and the identification of six novel mutations. Genet Test 2000;4:399–402.

[29] Perri RE, Hahn SH, Ferber MJ, Kamath PS. Wilson disease keeping the bar for diagnosis raised. Hep 2005;42:974

[30] Wilson DC, Phillips MJ, Cox DW, Roberts EA. Severe hepatic Wilson's disease in preschool-aged children. J Pediatr 2000;137:719–722

[31] Caca K, Ferenci P, Kuhn HJ. High prevalence of the H1069Q mutation in East German patients with Wilson disease: rapid detection of mutations by limited sequencing and phenotype-genotype analysis. J Hepatol 2001; 35:575–581

[32] Olivarez L, Caggana M, Pass KA, Ferguson P, Brewer GJ. Estimate of the frequency of Wilson's disease in the US Caucasian population: a mutation analysis approach. Ann Hum Genet 2001; 65:459–463

[33] Li XH, Lu Y, Yun L, Fu QC, Xu J, Zang GQ, Zhou F, Yu D, Han Y, Zhang D, Gong QM, Lu ZM, Kong XF, Wang JS, Zhang XX. Clinical and molecular characterization of Wilson's disease in China: identification of 14 novel mutations. BMC Med Gen 2011; 12:6

[34] Wang LH, Huang YQ, Shang X, Su QX, Xiong F, Yu QY, Lin HP, Wei ZS, Hong MF, Xu XM. Mutation analysis of 73 southern Chinese Wilson's disease patients: identification of 10 novel mutations and its clinical correlation. J Hum Genet 2011;56(9):660-665

[35] Park HD, Ki CS, Lee SY, Kim JW. Carrier frequency of the R778L, A874V, and N1270S mutations in the *ATP7B* gene in a Korean population. Clin Genet 2009; 75:405-7

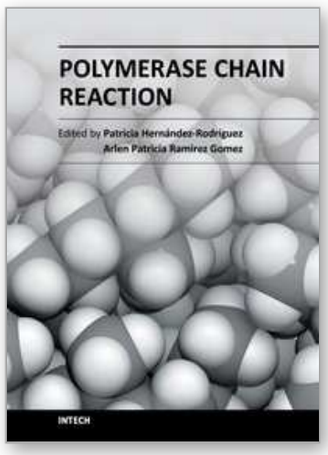
[36] Yoo HW. Identification of novel mutations and the three most common mutations in the human *ATP7B* gene of Korean patients with Wilson disease. Genet Med 2002; 4: 43S-48S.

[37] Wan L, Tsai CH, Tsai Y, Hsu CM, Lee CC, Tsai FJ. Mutation analysis of Taiwanese Wilson disease patients. Biochem Biophys Res Commu 2006; 345:734-8.

[38] Wan L, Tsai CH, Hsu CM, Huang CC, Yang CC, Liao CC, Wu CC, Hsu YA, Lee CC, Liu SC, Lin WD, Tsai FJ. Mutation analysis and characterization of alternative splice variants of the Wilson disease gene *ATP7B*. Hepatol 2010; 52(5):1662-1670.

[39] Zappu A, Lepori BM, Incollu S, Noli CSa, De Virgiliis S, Cao A, Loudianos G. Development of TaqMan allelic specific discrimination assay for detection of the most common Sardinian Wilson's disease mutations. Implications for genetic screening. Mol Cell Prob 2010; 24:233-235.

[40] Gupta A, Maulik M, Nasipuri P, Chattopadhyay I, Das KS, Gangopadhyay KP. The Indian Genome Variation Consortium,3 and Kunal Ray1Molecular Diagnosis of Wilson Disease Using Prevalent Mutations and Informative Single-Nucleotide Polymorphism Markers Clin Chem 2007;53:9:1601–1608.



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