Investigation of p53 Tumor Suppressor Gene Mutations in Patients with a Lung Mass Using Sequence Analysis

Akciğer Kitlesi Olan Hastalarda p53 Tümör Baskılayıcı Gen Mutasyonlarının Sekans Analizi ile Araştırılması

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ABSTRACT Objective: The p53 tumour suppressor gene plays an important role in the regulation of cell proliferation. It is located on the short arm of the 17th chromosome. It has 11 exons and encodes for a tumor suppressor protein called p53 which is 53kD in weight and 393 amino acids in length. This protein, a transcription factor, is an important regulator of cell cycle. Up to date, a number of mutations (75 % of which are found between codon 26 and 332) have been detected on p53 gene. Recent researches showed that lung neoplasm resulting from the mutations of p53 gene varied between 33% (adenocarcinoma) and 70% (small cell lung cancer), and it is reported that the hot spots were mainly found at the codons 175, 248, and 273. **Material and Methods:** In this study, the exons, exon-intron junctions, and some intron regions, which are located between exon 4-9 of p53 gene, of 24 patients who had a surgical operation due to a lung mass were examined by automatic DNA sequencing in University of Leipzig. **Results:** 53 missense and 7 frameshift mutations were detected between 4th and 9th exons (Codons 36-318) of 18 samples among the 24 samples. Fifty five of these mutations were heterozygous, and five of them were homozygous. Similarly, 12 missense mutations detected as a result of the serial analyses of the region between introns 4-9, and seven of them were heterozygous and 5 were homozygous. **Conclusion:** Some research regarding p53 gene reported that codon 175, 248, and 273 were hot spots and mutations were frequent in these codons. However we have not seen any mutations in any of these codons in our study. Nucleotide changes at the positions 13432 (5' beginning) and 13999 (3' ending) of 6th intron, which are very important regions, may result in the formation of an abnormal protein. We suppose that other nucleotide changes are not very important due to their heterozygous nature and location.

Key Words: Lung neoplasms; genes, p53; sequence analysis, DNA

ÖZET Amaç: p53 tümör baskılayıcı geni hücre çoğalmasının düzenlenmesinde önemli rol oynar. 17. kromozomun kısa kolunda yerleşmiştir. 11 eksonu vardır ve 53 kD ağırlığında ve 393 aminoasit uzunluğunda olan p53 şeklinde isimlendirilen bir tümör baskılayıcı geni şifreler. Bir transkripsiyon faktörü olan bu protein hücre döngüsünün önemli bir düzenleyicisidir. Bugüne kadar p53 geninde (%75'i kodon 26 ve 332 arasında olan) birtakım mutasyonlar saptanmıştır. Son zamanlarda yapılan araştırmalar göstermiştir ki p53 genindeki mutasyonlardan kaynaklanan akciğer kitleleri %33 (adenokarsinom) ile %70 (küçük hücreli akciğer kanseri) arasında değişir ve sorunlu bölgelerin başlıca kodon 175, 248 ve 273'de olduğu bildirilmiştir. **Gereç ve Yöntemler:** Bu çalışmada akciğerde kitle tanısıyla cerrahi girişim uygulanan 24 hastanın p53 geninin 4-9. eksonlarına lokalize eksonlar, ekson-intron bileşkeleri ve bazı intron bölgeleri Leipzig Üniversitesi'nde otomatik DNA sekansı ile değerlendirildi. **Bulgular:** Yirmi dört örneğin 18'inin 4. ve 9. eksonları arasında (kodon 36-318) 53 yanlış anlam mutasyonu ve 7 çerçeve kayması mutasyonu saptandı. Bu mutasyonların 55'inin heterozigot, 5'inin homozigot olduğu bulundu. Benzer şekilde, 4-9. intronlar arasındaki bölgenin seri incelemeleri sonucunda saptanan 12 anlam kayması mutasyonundan 7'si heterozigot, 5'i homozigottu. p53 geni ile ilgili bazı araştırmalarda kodon 175, 248 ve 273'ün sorunlu bölgeler olduğu ve bu kodonlarda mutasyonların sık görüldüğü bildirilmiştir. Fakat biz çalışmamızda bu kodonların hiçbirinde mutasyona rastlamadık. **Sonuç:** Çok önemli bölgeler olan altıncı intronun 13432 (5' başlangıç) ve 13999. (3' bitiş) pozisyonlarında nükleotid değişiklikleri anormal bir proteinin ortaya çıkmasıyla sonuçlanabilir. Heterozigot olmaları ve yerleşimleri nedeniyle diğer nükleotid değişikliklerinin çok önemli olmadığını düşünüyoruz.

Anahtar Kelimeler: Akciğer tümörleri; genler, p53; dizi analizi, DNA

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It is reported that more than 80% of the cancer cases in the USA are primarily resulted from environmental factors.[1] Most of the cancer deaths are due to lung neoplasm in the USA. Furthermore lung neoplasm is the most prevalent cancer type all over the world.[2] According to the Ministry of Health Data, lung neoplasm is the leading cause of cancer deaths among male in Turkey.[3]

Tobacco is the most effective factor in the formation of lung tumours in addition to industrial pollution.[2,4] For this reason, it is reported that lung neoplasm cases are frequently seen among industrial workers and groups of people who smoke a lot. The fact that cigarette and industrial wastes are certainly cancerous is shown by the animal studies and in vitro studies.[5,6]

It is reported that one of the reasons for different malignant tumour formation in humans is the mutations at p53 tumour suppressor gene.[7-11] p53 tumour suppressor gene has 11 exons and spans approximately 20.000 bp length on the short arm of 17th chromosome. It produces a nuclear protein, which functions as a cell cycle regulator and as a transcription factor.[12-14]

Mutations at p53 tumour suppressor gene cause a production of an inactive protein which can not function and at the end lead to neoplasias such as leukaemia, lymphoma, sarcoma, and many tissue tumours mainly seen in lungs, breast, and intestines (most of the human cancers). p53 tumour suppressor protein translated from this gene regulates the transition from G_1 to S phase during cell cycle. Under normal conditions, it is found that cells contain low level of p53 protein. However, when the cell is radiated by UV which induces DNA damage, then p53 is activated and its production is increased. p53 stimulates the production of another gene, p21, which is an inhibitory protein for Cdk (Cyclin dependent kinase). Then p21 protein recruits Cyclin/Cdk complex leading to the cell cycle arrest. In particular, p21 binds to proliferating cell nuclear antigen, which is a subunit of DNA polymerase d. Thus, p21 plays a dual role in cell cycle arrest induced by DNA damage.[15-21]

Up to date, more than 500 mutations have been reported in p53 gene as a result of studies on approximately 10.000 tumour tissue samples and it was reported that lung cancer is in the second prevalent cancer type after colon cancer.[22] It is also reported that, more than 90% of the mutations seen on p53 gene were between exons 4-9 where the functional domains that are necessary for the activity of the p53 protein reside.[8,23]

While frameshift mutations or gene deletions in p53 gene are effective on the gene more than 90%, missense mutations result in the accumulation of mutant p53 in the tumour cell. Furthermore, these mutations are related to the loss of second allele of the gene.[24-26]

It is reported that (according to the analysis of mutations on p53 gene), 42% of the mutations were formed due to transversion of GC to AT, and 63% of this is affected from the CpG nucleotides.[27] For instance, codons 175, 248 and 273 are known as hot spot codons.[28-30] As these codons contain CG bases, it is stated that Cytosine, 5-methyl cytosine by connecting methyl group, is transformed into Thymine in the further phase.[31-33]

In this study, our aim is to perform a serial analysis on exons 4-9 of p53 gene at the molecular level by sampling cancerous tissue from patients with lung mass diagnosis who were diagnosed as cancer and were undergone a surgery at the University of Çukurova, Faculty of Medicine, Thoracic and Cardiovascular Surgery Department.

MATERIAL AND METHODS

TISSUE SAMPLES

Informed consent was obtained from each patient (The study was approved by the Çukurova University Ethics Committee on March 4, 2003, Assembly number: 3). Cancerous lung tissue samples from 24 patients, who were diagnosed by lung mass and operated surgically at University of Çukurova, Faculty of Medicine, Thoracic and Cardiovascular Surgery Department, were used. The samples were put into 70% ethanol and stored at -20°C. DNA isolations from the tissue samples were performed according to the modified method of Sambrook et

al.[34] DNA pellet was dissolved in 200 µl Tris-EDTA and stored at 4°C.

POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION

Primers used separately amplify exons 4 to 9 of p53 gene and synthesized using a Applied Biosystem UK (Warrington) (Table 1).

PCR amplifications of the exons were carried out according to reaction mixture conditions given in Table 2. Cycle conditions of the reaction mix were carried out with a thermal cycle (Ependorf Mastercycler Personal) using the thermal profile given below; initial denaturation at 94°C for 5 minutes, annealing (at 57°C for exons 8 and 9, 58°C for exons 5 and 6, and 60°C for exons 4 and 7) for 30 seconds, and primer extension at 72°C for 1 minute during 30 cycles, and final extension at 72°C for 5 minutes.

DNA SEQUENCING

All PCR products were purified with Qiagen purification kit and sequenced by Applied Biosystem model 377 automated sequencer (PE Biosystems, Foster City, CA, USA) using 2 µl of the products. The sequencing results were controlled in comparison with reference serials obtained from p53 data bank (GI:35213) employing "align two sequences" software at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

RESULTS

The list of the mutations, which are determined as a result of sequence analysis, was given in Table 3 and 4. Missense mutations were found between codons 36 and 123 of exon 4 (Table 3). Fourteen mutations were found between codons 134-149 of exon 5, of these mutations 11 were heterozygote missense and 3 were homozygote frameshift mutations (Codon 145 CTG →-TG, C →del) (Table 3, Figure 1). Four mutations were found between codons 187-218 of exon 6, 3 of them were heterozygote missense and one of them was a homozygote frameshift mutation (Codon 189 GCC → -CC, G → del) (Table 3). Five mutations were found between codons 225-252 of exon 7, 4 of these mutations were heterozygote missense and one of them was a heterozygote frameshift mutation (Codon 245 GCC → AGGC, A→ ins) (Table 3, Figure 1). Nine mutations were found between codons 261-262 of exon 8, 7 of these mutations were heterozygote missense, and 2 of them were heterozygote frameshift mutations (Codon 262 GGT → GG-, T →del) (Table 3). For exon 9, only one heterozygote missense mutation was found in codon 318 (Table 3, Figure 2).

TABLE 1: Forward (F) and reverse (R) primers for polymerase chain reaction amplification of exons 4 to 9 of p53 gene.

Primers	Sequence
Exon 4 5-3'F	ACC TGG TCC TCT GAC TGC TC
Exon 4 5-3'R	AAT CAA ACC TTG AAA CCC TA
Exon 5 + 6 5-3'F	CCG TGT TCC AGT TGC TTT AT
Exon 5 + 6 5-3'R	AAT TGG GGA GGA GGG TCT
Exon 7 5-3'F	TGC TTG CCA CAG GTC TCC
Exon 7 5-3'R	TGG AGA GAT GTG TAA AGG CC
Exon 8 + 9 5-3'F	TTCCTTACTGCCTCTTGCTT
Exon 8 + 9 5-3'R	CACTCAAAATGCCGTTTTCT

TABLE 2: Polymerase chain reaction conditions for exon 4-9 amplification and PCR products.

Reaction components (stock)	Exon 4	Exon 5 + 6	Exon 7	Exon 8+9
Water (bi-distilled)	38.5 µl	38.5 µl	37.5 µl	37.5 µl
MgCl2 (25 mM)	3 µl	3 µl	4 µl	4 µl
PCR buffer (10X)	5 µl	5 µl	5 µl	5 µl
dNTP mix (25 mM)	0.4 µl	0.4 µl	0.4 µl	0.4 µl
Forward primer (20 pmol/µl)	1 µl	1 µl	1 µl	1 µl
Reverse primer (20 pmol/µl)	1 µl	1 µl	1 µl	1 µl
Genomic DNA (500 ng /µl)	1 µl	1 µl	1 µl	1 µl
Taq polymerase (5U/µl)	0.25 µl	0.25 µl	0.25 µl	0.25 µl
Total volume	50 µl	50 µl	50 µl	50 µl
PCR product (bp)	402 bp	363 bp	301 bp	411 bp

TABLE 3: Mutation types at exon 4-9.

Exon	Codon	Codon change	Base change	Amino acid change	No of sample	Mutation type	Genetic status
4	36	CCG→GTT	CCG→GTT	Pro→Val	1	Missense	Heterozygous
	37	TCC→TGC	C>G	Ser→Cys	2	Missense	Heterozygous
	41	GAT→CAT	G>C	Asn→His	2	Missense	Heterozygous
	50	ATT→CTT	A>C	Ile→Leu	1	Missense	Heterozygous
	54	TTC→CTT	T>C, C>T	Phe→Leu	2	Missense	Heterozygous
	54	TTC→CTC	T>C	Phe→Leu	3	Missense	Heterozygous
	69	GAG→GAT	G>T	Glu→Asp	1	Missense	Heterozygous
	74	GCC→GTC	C>T	Ala→Val	2	Missense	Heterozygous
	81	ACA→TCA	A>T	Thr→Ser	3	Missense	Heterozygous
	81	ACA→TCT	A>T, A>T	Thr→Ser	2	Missense	Heterozygous
	81	ACA→ACT	A>T	Thr→Thr	1	Silent	Heterozygous
	90	TCC→TAC	C>A	Ser→Tyr	2	Missense	Heterozygous
	110	CGT→CGA	T>A	Arg→Arg	2	Silent	Heterozygous
	120	AAG→AAT	G>T	Lys→Asn	1	Missense	Heterozygous
	121	TCT→ACT	T>A	Ser→Thr	1	Missense	Heterozygous
	122	GTG→GTA	G>A	Val→Val	1	Silent	Heterozygous
	123	ACT→TCT	A>T	Thr→Ser	2	Missense	Heterozygous
5	134	TTT→ATC	T>A, T>C	Phe→Ile	1	Missense	Heterozygous
	134	TTT→ATT	T>A	Phe→Ile	1	Missense	Heterozygous
	134	TTT→TTC	T>C	Phe→Phe	1	Silent	Heterozygous
	136	CAA→CAT	A>T	Gln→His	1	Missense	Heterozygous
	140	ACC→TCC	A>T	Thr→Ser	1	Missense	Heterozygous
	144	CAG→CTG	A>T	Gly→Leu	2	Missense	Heterozygous
	145	CTG→-TG	C>del		3	Frameshift	Homozygous
	147	GTT→GGT	T>G	Val→Gly	2	Missense	Heterozygous
	148	GAT→GTT	A>T	Asp→Val	1	Missense	Heterozygous
	149	TCC→TTT	CC>TT	Ser→Phe	1	Missense	Heterozygous
6	187	GGT→GAGT	A>ins,T>del	Gly→Glu	1	Missense	Heterozygous
	189	GCC→-CC	G>del		1	Frameshift	Homozygous
	217	GTG→GGG	T>G	Val→Gly	1	Missense	Heterozygous
	218	GTG→GGG	T>G	Val→Gly	1	Missense	Heterozygous
7	225	GTT→GCT	T>C	Val→Ala	2	Missense	Heterozygous
	237	ATG→ATT	G>T	Met→Ile	1	Missense	Heterozygous
	245	GGC→AGGC	A>ins		1	Frameshift	Heterozygous
	252	CTC→TTC	C>T	Leu→Phe	1	Missense	Heterozygous
8	261	AGT→AGA	T>A	Ser→Arg	1	Missense	Heterozygous
	262	GGT→GG-	T>del		2	Frameshift	Heterozygous
	266	GGA→AGA	G>A	Gly→Arg	1	Missense	Heterozygous
	267	CGG→CGT	G>T	Arg→Arg	1	Silent	Heterozygous
	268	AAC→TAC	A>T	Asp→Tyr	1	Missense	Heterozygous
	282	CGG→CGT	G>T	Arg→Arg	1	Silent	Heterozygous
	282	CGG→CCT	G>C	Arg→Arg	1	Silent	Heterozygous
	288	AAT→TAT	A>T	Asn→Tyr	1	Missense	Heterozygous
9	318	CCA→CCG	A>G	Pro→Pro	1	Silent	Heterozygous

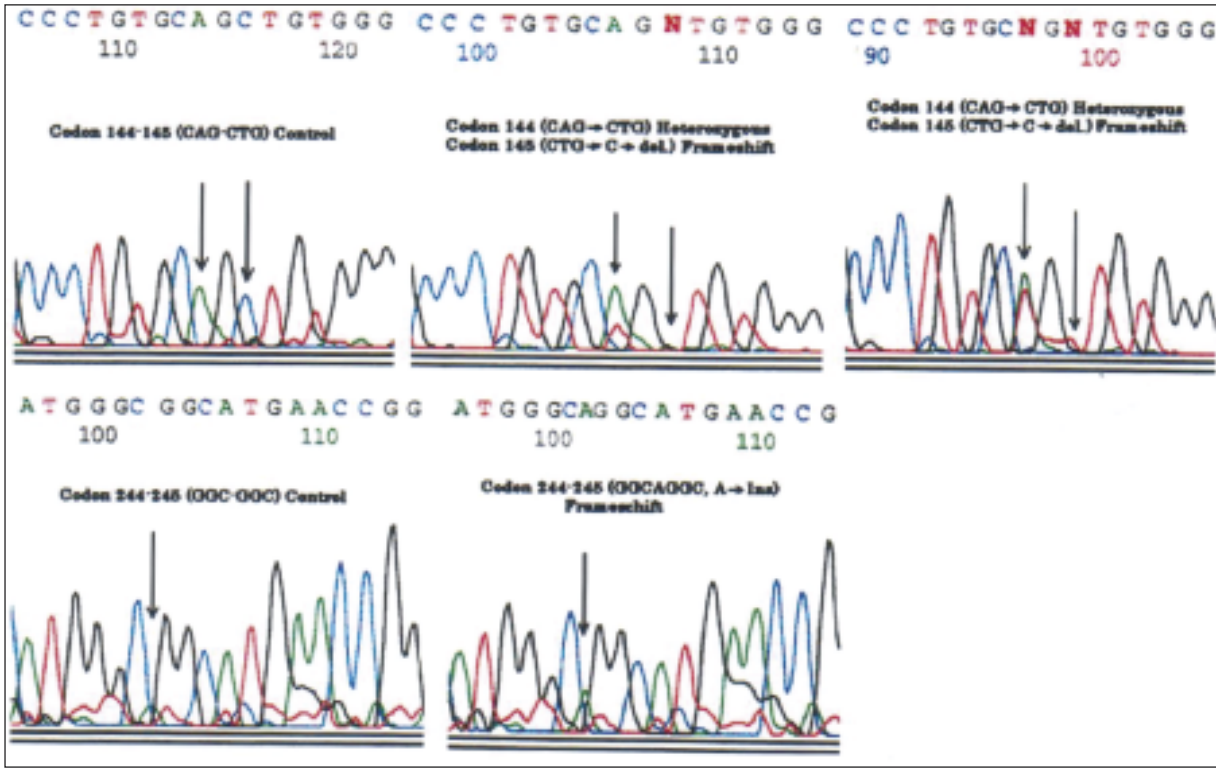

FIGURE 1: Heterozygous missense mutation at exon 5 codon 144 (CAG→ CTG), Homozygous frameshift mutation at codon 145 (CTG→-TG, C→del) and exon 7 codon 244-245 (GGCAGGC) A→ins.

In this study, intron regions (intron 4-8) were also analysed in terms of mutations. It was found that there were totally 12 nucleotide changes in various parts of 4th and 7th intron. Although, 7 of the changes were found as heterozygote and 5 of them were found as homozygote form. It was found that nucleotide changes at the positions 13432th and 13999 were the beginning and ending points of the 6th intron. It was found that the other nucleotide changes detected were located at the inner parts of the intron (Table 4).

DISCUSSION

Many study shown 75 % of p53 mutations occurs in single missense mutations (parallel with ours study results). The majority of p53 mutations in cancer are missens mutations occurring throughout the central domain of the coding region exon 5-9.

A missence point mutation in one of the two p53 alleles in a cell can abrogate almost all p53 ac-

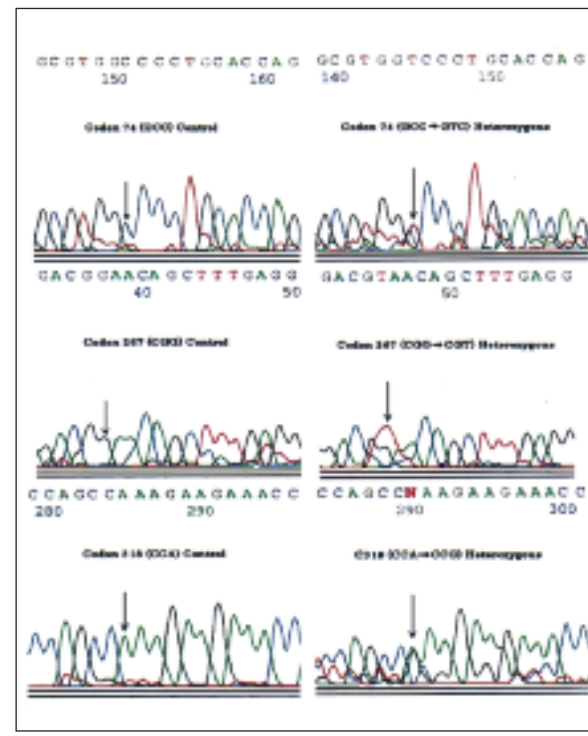

FIGURE 2: Heterozygous missense mutation; at exon 4 codon 74: GCC→ GTC, exon 8 codon 267: CGG→CGT and exon 9 codon 318: CCA→CCG.

TABLE 4: Change of nucleotides at intron 4-8.

Intron	Nucleotide	Nucleotide change	No of sample	Genetic status	Importance
IVS4	Nt. 12305	t→g	1	Heterozygous	
	Nt. 12312	g→t	1	Heterozygous	
	Nt. 13053	a→c	1	Heterozygous	
	Nt. 13054	g→c	1	Heterozygous	
IVS5	Nt. 13256	g→t	1	Homozygous	
IVS6	Nt. 13432	gt→gc	1	Homozygous	5' nucleotide change
	Nt. 13989	t→a	2	Homozygous	
	Nt. 13999	ag→at	1	Homozygous	3' nucleotide change
IVS7	Nt. 14141	c→a	1	Heterozygous	
	Nt. 14201	t→g	1	Heterozygous	
	Nt. 14613	t→a	1	Heterozygous	

tivity, because virtually all the oligomers will contain at least one defective subunits and such oligomers can not function as transcriptions factors. Oncogenic p53 mutations act dominantly in negative allele carries for the point mutation.[36]

Due to the fact that, the mutations found in the exon 4 were heterozygote missense mutations; we suppose that they are not the primary effect on the formation of the tumour. However, they are significant, because it is reported that this region is very important part (DNA binding part) of p53 protein, therefore, allele loss is effective in the formation of a tumour.[24-26,35] Thus, we believe that it is of great importance as it is reported that mutations occurred in this region are effective in the loss of p53 function.[1-6] In some patients homozygous frameshift mutation occured because of Cytosine deletion (CTG→-TG, C→del) in the 145th position of codon site leading to a stop codon at position 157. The reason is that this mutation results in frameshift, and thus, the p53 protein to be formed will be rather different. Although, mutation rate in codon 144 was reported to be higher in the previous studies[37,38] we have found 2 mutations in heterozygote forms in this codon in our study. Nevertheless, 9 mutations between codon 144-149 show that this region is exposed to mutations and therefore it is also very important. Formation of heterozygote missense mutation in codon 187 of exon 6 (GGT→ GAG, T→del A→ins) may not be as important as the homozygote frameshift mutation. In addition the other homozygous frameshift mutations occured because of Guanine deletion (GCC→-CC, G→del) at position 189 of the codon site which does not lead to any stop codon but different aminoacid sequence until the end of exon only for one patient. Likewise, we believe that insertion of Adenine between codons 244 and 245 in exon 7 (GGC GGC→ GGC A GGC, A→ins) and deletion of Timine in codon 262 of exon 8 (GGT→GG-, T→del) results in frameshift, though it was in heterozygote formation, which is an important factor in the formation of the tumour. Although, some researches regarding p53 gene reported that codon 175, 248, and 273 are hot spots and mutations are frequently seen in these codons,[28,30] we have not seen any mutations in any of these codons in our study.

Nucleotide changes at the positions 13432 (5' beginning) and 13999 (3' ending) of 6th intron, which are very important regions, may result in the formation of an abnormal protein. We suppose that other nucleotide changes are not very important due to their heterozygous nature and location.

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