

Identification of *p53* Gene Mutations among Iranian Patients Involved with Esophageal Squamous Cell Carcinoma: the Efficiency of the Two Different Methods

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Abstract

Mutation in *p53* tumor suppressor gene is highly frequent in different cancers. It was reported that the efficiency of Microarray and ABI 310 system in identification all types of *p53* gene mutations (for fresh tissues) are 95% and 91%, respectively and detection of point mutations by Microarray and ABI 310 system are 100% and 92%, respectively. In the present study, Microarray and ABI 310 analysis were used to detect *p53* gene mutations in esophageal carcinoma from archived tissues. For this purpose, formalin-fixed, paraffin-embedded esophageal tissues from cancer patients diagnosed with esophageal squamous cell carcinoma (ESCC) were collected. DNA was extracted by Microdissection method (with and without laser) and was purified with Microcon 50 filters (Millipore) before performing PCR. *p53* gene mutations were identified in 9 analyzed samples by ABI 310 system. For these samples, the genomic DNA were obtained from microdissected-samples without laser. Microarray could detect mutations in 3 of 9 analyzed ESCC specimens from Iranian patients, which were identified *p53* gene mutations by ABI 310 system. In addition, in laser-microdissected samples mutations were identified by ABI 310 system. The extracted DNA obtained from laser-microdissected samples was insufficient for the assessment of *p53* gene mutations with Microarray. It was determined that Microarray was dependent on the amount of tissues used in DNA extraction. The results indicated that the choice of method for extracting DNA from test samples to assess mutation in *p53* gene is very important. The efficiency of ABI 310 system and Microarray in detection of *p53* gene mutations (for exons 5-8) was 100% and 30%, respectively for archived samples. Therefore, it is recommended to use fresh tissues for Microarray analysis.

Keywords: *p53* Gene; Mutation; Microarray; Microdissection; Esophageal squamous cell carcinoma

Introduction

It is well known that most cancers carry *p53* gene mutations [1-4]. The *p53* protein is considered as a key

combination in countering stress messages such as DNA damage [2,5]. More than 16,371 mutations for *p53* gene have been identified [3]. Prevalence of somatic mutations in *p53* gene has been reported in many

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tumors, and hereditary mutations in this gene increase the chance of developing a wide range of cancers [6]. As nucleotide sequence determination with microcapillary electrophoresis is a time consuming method, a modern one has been developed for determining nucleotide sequence with *p53* GeneChips. This method makes it possible to study all the exons of *p53* gene, in one day and simultaneously, for the presence of mutations [8-16]. The probes on Chips in Microarray are designed according to the nucleotide sequence of exons 2 to 11 of *p53* gene [11].

The frequency of esophageal squamous cell carcinoma (ESCC), the predominant esophageal cancer, is very high in northern Iran [17-19] and identification of *p53* gene mutation is very important to find out the major causes of this type of cancer in Iran [19]. In the present study, microdissected esophageal squamous cell carcinoma samples, with and without laser, from archived tissues were analyzed to detect *p53* gene mutations among exons 2-11 using Microarray, and the results were compared with ABI 310 sequencing system. The results indicated that the choice of method for extracting DNA from test samples to assess mutation in *p53* gene was very important. Also, the efficiency of ABI 310 system in detection of *p53* gene mutations (exons 5-8) was 100% for archived samples and the efficiency of Microarray in detection of *p53* gene mutations was 30% for archived samples. Therefore, it will be necessary to use fresh tissues for Microarray analysis.

Materials and Methods

Tissue Collection and Dissection for DNA Preparation

12 Formalin-fixed, paraffin-embedded esophageal tissues (surgically resected) from cancer patients diagnosed with ESCCs in the Cancer Institute of Tehran were collected for analysis. No patient had been given chemotherapy or radiotherapy before the operation. Serial sections of 10- μ m thickness were prepared for DNA extraction. Tissue areas with high neoplastic cellularity (>50%) were dissected from dewaxed slides, and the materials were digested by proteinase K in SDS-containing buffer for 3-5 days at 50°C to release DNA suitable for PCR.

Laser Microdissection and DNA Extraction

Two esophageal tumors, samples 8 and 9 (Table 1), diagnosed with SCC were used for laser microbeam microdissection to extract DNA. These samples were selected from those who had *p53* gene mutations in

exon 5-8 using ABI 310 system. Serial sections of 3- μ m thickness were prepared for this purpose. Tissue areas with high (>70%) neoplastic cellularity were selected from dewaxed, eosin-stained slides under visual control. These areas were isolated from the surrounding tissue by the focused nitrogen laser beam (Fig. 1) with a microdissection apparatus (PALM Laser MicroBeam System; P.A.L.M., Wolfrathausen, Germany). The materials were harvested with the increased energy of the laser and the microdissected area was catapulted by a single laser shot as described by Cohen *et al.* [21]. The detached tissue samples were then collected in a microfuge cap coated with the PCR oil and mounted above the object slide. The cap containing samples was placed on a microfuge tube filled with 100- μ L SDS-containing buffer. DNA was isolated using digestion of Proteinase K, Phenol/Chloroform extraction and ethanol precipitation.

Uniplex PCR and DNA Sequencing

Genomic DNA was mixed with PCR buffer containing 1.5 mM MgCl₂, 10 mM Tris-HCl and 500 mM KCl with pH=8.3, 0.2 mM of each dNTP, 3 ng/ μ L of each primer and 2.5 Unit Taq polymerase (from Roche company) in a final volume of 50 μ L. Exons 5-8 of the *p53* gene were amplified by a single 40-Cycle PCR using primers described in Table 2. Cycling conditions were as follows: primer annealing at 57°C for 1 min, polymerization at 72°C for 30 s and strand separation at 95°C for 1 min in a Thermocycler Model PTC-150-16 & 25 from MJ Research Co. PCR products were purified with Microcon 100 filters (Millipore) and sequenced directly by BigDyeTM fluorescent dye dideoxy sequencing and microcapillary electrophoresis with an ABI 310 Genetic Analyzer according to the supplier's Instructions (Applied Biosystems International) (Fig. 2).

Multiplex PCR and p53 GeneChip Array

For this purpose, only 9 samples were selected from those had *p53* gene mutations in exon 5-8 using ABI 310 system, as it was showed in Table 1 (nine samples out of 12 had *p53* gene mutations). Multiplex polymerase chain reaction (PCR) was performed by a single 35 cycle using 20 primer pairs covering exon 2 to 11 of the *p53* gene. The oligonucleotide sequences of the primers used and the lengths of the fragments are given in Table 2. Genomic DNA was mixed with 10 mM MgCl₂, 200 mM of each dNTP, 5 μ L of mixed 20 primers and 2 Unit/ μ L of Ampli Taq Gold in a final volume of 50 μ L. Cycling conditions were as follows:

Table 1. Comparison of *p53* gene mutations identified in ESCCs from Iran by ABI 310 system (conventional DNA sequencing analysis) and *p53* GeneChip Array, the extracted DNA obtained from microdissected samples (with and without laser), also, refer to Figure 5.

No.	Patient code	Age/Sex	Diagnosis	Detection of <i>p53</i> gene mutation		Type of <i>p53</i> Mutation	
				ABI 310 system	Microarray	Codon	Base
1	GC-F14R	58/M	SCC	+	+(score 16)	248	CGG →TGG
2	GC-F17R	50/M	SCC	+	ND	152	CCG →CTG
3	GC-F18R	48/F	SCC	+	+(score 23)	152	CGG →CTG
4	GC-F15R	50/F	SCC	+	ND	213	CGA →TGA
5	GC-F27R	66/M	SCC	+	+(score 29)	248	CGG →TGG
6	GC-F59R	60/F	SCC	+	ND	242	TGC →TTT**
7	GC-F14R	63/M	SCC	+	ND	258	GAA →AAA
						282	CGG→TGG
8	GC-F37R*	49/F	SCC	+	ND	248	CGG →TGG
9	GC-F39R*	65/M	SCC	+	ND	273	CGT →CAT
10	GC-F55	66/F	SCC	+	NT		no mutation
11	GC-F88	40/F	SCC	+	NT		no mutation
12	GC-F34	58/F	SCC	+	NT		no mutation

ND: not detectable by *p53* GeneChip Array

NT: not tested

*: DNA extraction was done after laser microbeam microdissection

**: a tandem mutation

Table 2. Primers used for polymerase chain reaction of *p53* gene (Affymetrix, Inc., Santa Clara, CA). All of primers were used in Microarray analysis and multiplex PCR. Primers for axons 5 to 8 were used in ABI 310 system (conventional DNA sequencing analysis) and uniplex PCR protocol

Exon	Primer	Sequence	Product length
2	GCEX2F	5'-TCATGCTGGATCCCCACTTTTCCTCTTG-3'	164 bp
	GCEX2R	5'-TGGCCTGCCCTTCCAATGGATCCACTCA-3'	
3	GCEX3F	5'-AATTCATGGGACTGACTTCTGCTCTTGTC-3'	90 bp
	GCEX3R	5'-TCCAGGTCCCAGCCCAACCCTGTCC-3'	
4	GCEX4F	5'-GTCTCTGACTGCTCTTTTCACCCATCTAC-3'	368 bp
	GCEX4R	5'-GGGATACGGCCAGGCATTGAAGTCTC-3'	
5	GCEX5F	5'-CTTGTGCCCTGACTTTCAACTCTGTCTC-3'	272 bp
	GCEX5R	5'-TGGGCAACCAGCCCTGTCGTCGTCCTCCA-3'	
6	GCEX6F	5'-CCAGGCCTCTGATTCCTCACTGATTGCTC-3'	294 bp
	GCEX6R	5'-GCCACTGACAACCACCCTTAACCCCTC-3'	
7	GCEX7F	5'-GCCTCATCTTGGGCCTGTGTTATCTCC-3'	175bp
	GCEX7R	5'-GGCCAGTGTGCAGGGTGGCAAGTGGCTC-3'	
8	GCEX8F	5'-GTAGGACCTGATTTCTTACTGCCTCTTGC-3'	241bp
	GCEX8R	5'-ATAACTGCACCCTTGGTCTCCTCCACCGC-3'	
9	GCEX9F	5'-CACTTTTATCACCTTTCTTGCCTCTTCC-3'	146 bp
	GCEX9R	5'-AACTTTCCACTTGATAAGAGGTCCCAAGAC-3'	
10	GCEX10F	5'-ACTTACTTCTCCCCCTCTGTGCTGCTC-3'	210 bp
	GCEX10R	5'-ATGGAATCCTATGGCTTCCAACCTAGGAAG-3'	
11	GCEX11F	5'-CATCTCTCCTCCCTGCTTCTGCTCCTAC-3'	225 bp
	GCEX11R	5'-CTGACGCACACCTATTGCAAGCAAGGGTTC-3'	

primer annealing at 60°C for 30 s, polymerization at 72°C for 45 s and strand separation at 95°C for 30 s with a final extension of 10 min at 72°C in a Master Cycler from Eppendorf Co. The multiple PCR product was analyzed in 4% agarose gel in a standard electrophoretic apparatus (Horizon 58 Life Technologies Co.). The protocol was continued when bands presented on the gel (Fig. 3). The samples were rejected with missing bands or bands of decreased intensity on the gel. 45 µL of the whole multiple PCR product fragmented with 0.25 Unit of DNase I and 2.5-µL of 1 Unit/µL Alkaline Phosphatase at 25°C for 15 min and at 95°C for 10 min. fragmented samples labeled with 1 mM of Fluorescin-N6-ddATP, 1.4 µL of 25 Unit/µL Terminal transferase and 5 mM of CoCl₂ at 37°C for 45 min and at 95°C for 5 min. 250 µL of 12X SSPE+10% Triton X-100 (containing 1.8 M NaCl, 0.12 M NaH₂PO₄, 0.012 M EDTA and 100 ml Triton X-100), 50 µL of 20 mg/ml Acetylated BSA, 10 µL of 100 nM Control Oligonucleotide F1 (5'-Fluorescin-CTGAACGGTAGCATCTTGAC-3') and 90 µL distilled water were added to each fluorescent labeled fragmented DNA sample. Finally, these samples were washed over the Chip and allowed to bind to complementary oligonucleotide probes on a *p53* GeneChip Array at 45°C for 30 min in a DNA-Chip Machine (Affymetrix, Inc., Santa Clara, CA). Hybridized probe arrays were then read using the GeneArray Scanner. All sequences were compared with the control human placental DNA (Fig. 4a,b).

Results

Analysis of Microdissected Samples (without Laser) by ABI 310 System

p53 gene mutations were identified in 9 out of 12 analyzed samples by ABI 310 system.

Detected mutations were as follows:

1. A missense mutation at codon 248 (CGG→TGG) in sample GC-F14R,
2. A missense mutation at codon 152 (CGG→CTG) in sample GC-F17R,
3. A missense mutation at codon 152 (CGG→CTG) in sample GC-F18R,
4. A missense mutation at codon 213 (CGA→TGA) in sample GC-F15R,
5. A missense mutation at codon 248 (CGG→TGG) in sample GC-F27R,
6. Tandem mutation at codon 242 (TGC→TTT) in sample GC-F59R;
7. Missense mutations at codon 258 (GAA→AAA) and at codon 282 (CGG→TGG) in sample GC-F14R;



Figure 1. Tumor cells were isolated from the surrounding tissue by focused nitrogen laser beam in tumor sample GC-F37R.

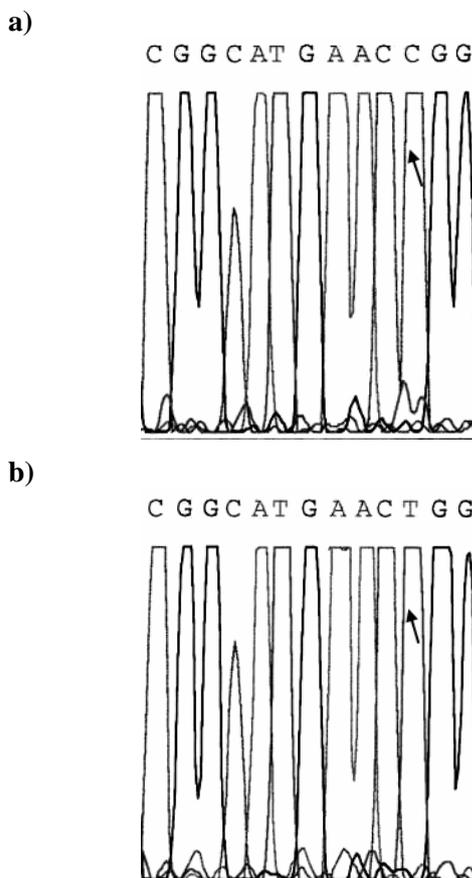


Figure 2. a. Electropherogram of DNA sequencing (5'→3') showing normal sequence at codon 248 (CGG) in a sample without mutation. b. Electropherogram of DNA sequencing (5'→3') showing base substitution mutation at codon 248 (CGG→TGG); see also Table 1, tumor sample GC-F37R. Laser microbeam microdissection was used to extract DNA in this sample.

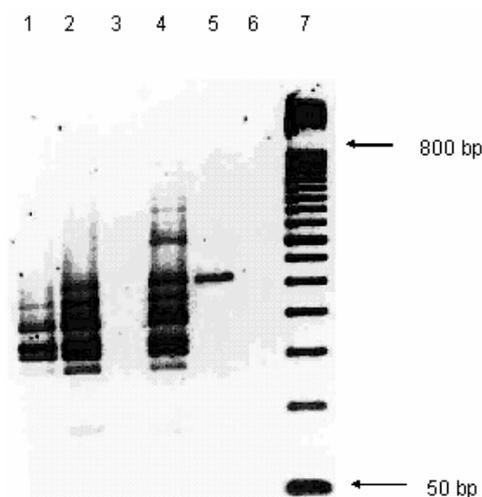


Figure 3. Gel electrophoresis of PCR product from multiplex and uniplex PCR method. The genomic DNA flanking exons 2-11 was amplified using set of primers which showed in Table 2. Lanes 1, 2 product of multiplex PCR for samples GC-F17R and GC-F18R, respectively. The sample GC-F17R was rejected because of missing bands. The sample GC-F18R was identified *p53* mutation with score 23 by Microarray analysis, refer to Table 1; Lane 4, product of multiplex PCR for Reference DNA; Lane 5, PCR product of exon 5 from uniplex PCR; Lanes 3 and 6 are controls; Lane 7, 50-800 bp molecular marker (from lifetech).

8. A missense mutation at codon 248 (CGG→TGG) in sample GC-F37R;

9. A missense mutation at codon 273 (CGT→CAT) in sample GC-F39R

Analysis of Laser-Microdissected Samples by ABI 310 System

For two samples, (samples 8 and 9 or GC-F37R and GC-F39R), DNA was extracted from esophageal neoplastic cells by laser microdissection. For these samples (cases 8, 9, or samples GC-F37R and GC-F39R, Table 1), mutations could be detected by ABI 310 system (see also Fig. 2b). As Figure 1 shows, by using laser in DNA extraction, cancer cells have been separated from other cells and in this way mutation in codon 248 (CGG→TGG) was shown clearly and without the presence of the normal tissue peak (Fig. 2b). Figure 2a shows sequence of normal codon 248 (CGG).

Analysis of Microdissected Samples (without Laser) by Microarray

Microarray could detect mutations in 3 of 9 ESCC specimens in which *p53* gene mutations were identified

by ABI 310 system. *p53* gene mutations were detected in three microdissected samples by Microarray and the scores of mutations was recorded 16-29. The score was calculated automatically by the Affymetrix Inc. software. Microarray detected a missense mutation for *p53* gene at codon 248 (CGG→TGG) in sample GC-F14R with score 16, a missense mutation at codon 152 (CGG→CTG) in sample GC-F18R with score 23, a missense mutation for *p53* gene at codon 248 (CGG→TGG) in sample GC-F27R with score 29 (Table 1, Fig. 4b). Other mutations out of exons 5-8 were not found in these specimens. The tandem mutation (in sample 6 or GC-F59R, TGC→TTT) Table 1, could not be detected by Microarray.

In Figure 4a and in the top row the normal nucleotide sequence of the codon 247 (AAC) is recorded. The score was zero for this codon. In Figure 4b and in the top row the normal and mutant nucleotide sequence of the codon 248 (CGG) is recorded. For this sample, score was 29. The antisense strand was converted automatically to sense strand. Figure 4b records the mutation in codon 248 (CGG→TGG) with a score of 29.

Three samples (GC-F55, GC-F88, GC-F34) had no mutations using ABI 310 system (Table 1), therefore they were not selected for Microarray and ABI system analysis. The results from assessing *p53* gene mutations with Microarray were compared with the results of nucleotide sequence determination by ABI system (Table 1, Fig. 5).

Analysis of Laser-Microdissected Samples by Microarray

For two samples, (sample 8 and 9 or GC-F37R and GC-F39R), DNA was extracted from esophageal neoplastic cells by laser microdissection, but the amount of DNA was not enough for Microarray.

Discussion

An interesting point in nucleotide sequence determination with Microarray method in this research is the use of formalin-fixed, paraffin-embedded tissue; other researchers prefer to use fresh tissue in order to assess mutation in *p53* gene with this method [11,12]. DNA breaks into pieces during the processes of formalin fixation and paraffin embedding. The quality of extracted DNA from formalin-fixed, paraffin-embedded samples is dependant on the quality of the starting materials including formalin buffer. Keeping tissues in formalin after operation or biopsy for short period, is effective in preventing breakage of DNA. Therefore, the extracted DNA was purified with

Microcon 50 filters (Millipore) before performing PCR to remove the fragmented DNA. By this measure, we could detect only 3 point mutations for *p53* gene by Microarray analysis (samples 1, 3, 5 in Table 1). The results indicate that the choice of method for extracting DNA from test samples to assess mutation in *p53* is very important. In addition, the thickness of the tissue in each DNA extraction test has its own importance in achieving precise results (the optimal thickness of the tissue for laser microbeam microdissection is 3- μ m). It was determined that Microarray is dependent on the amount of tissue in DNA extraction and the extracted DNA which obtained from laser-microdissected samples cannot be sufficient for the assessment of *p53* mutation with Microarray (cases 8, 9, Table 1). However, this amount of DNA is perfectly suitable for determining nucleotide sequence with microcapillary method. It was reported that the efficiency of Microarray and ABI 310 system in identification of all types of *p53* gene mutations (for fresh tissues) are 95% and 91%, respectively [11]. Detection rate of point mutations by Microarray and ABI 310 system are 100% and 92%, respectively [11]. The efficiency of Microarray in detection of *p53* gene mutations was 30% for archived samples. Therefore, using fresh tissues to detect *p53* gene mutations is recommended.

Although the accuracy of Microarray in identifying mutations of *p53* gene between exons 2 and 11 has been proven, a number of mutation types can not be identified by this method. Concerning the structure and design of *p53* Chip probes, in which only point mutations or one nucleotide base deletion is identifiable, tandem mutation or frameshifts (insertions or deletions other than single base deletions) are not identifiable with Microarray. As it was explained in the results section, tandem mutation (case 6, Table 1) is not identifiable with Microarray. Tandem mutation is very rare in esophageal squamous cell carcinoma [3]. Tandem mutation and complex deletion and insertion are not common in *p53* gene for tumor tissues.

It was found that *p53* gene mutations are significant predictors of treatment response and for survival in patients [7,20,22]. In order to determine the success rate of cancer treatment methods in hospital, detection of *p53* gene mutations is necessary. Identification of mutations along exons 2 to 11 for *p53* gene using Microarray happens in a shorter period (4/5 h) compared to the conventional DNA sequencing analysis. Therefore, the application of Microarray to identify mutation for *p53* gene, in tumor tissues, will be necessary for central hospitals, where the fresh tissue samples are available easily.

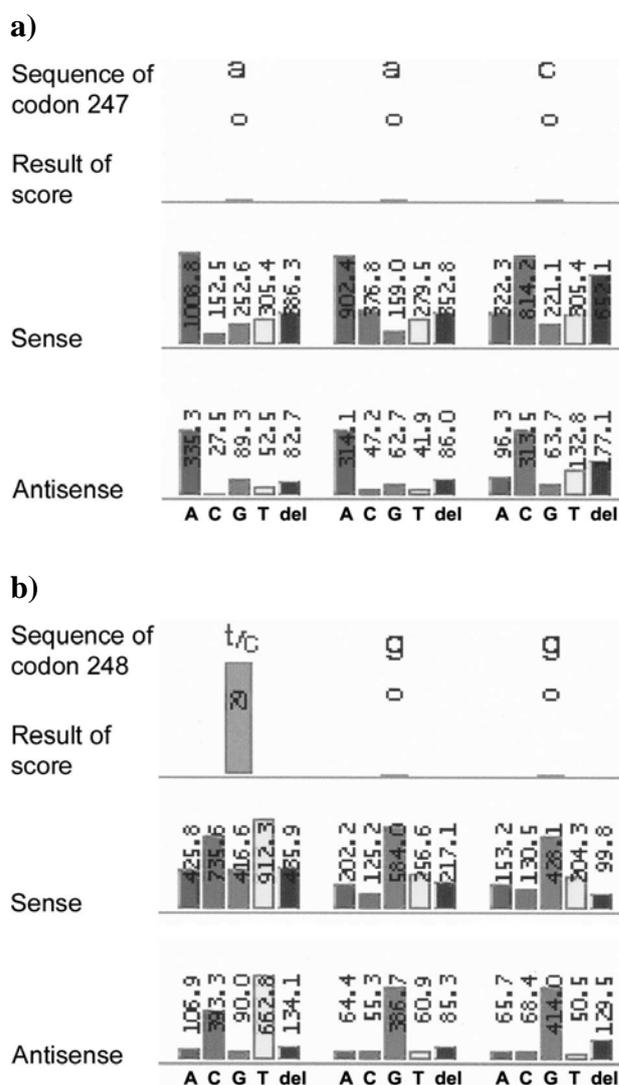


Figure 4. a. The normal nucleotide sequence of the codon 247 (AAC) is shown. The second row is the result of the comparison of the nucleotide sequence of the control sample and the test sample; the same row also presents no score. The third and fourth rows present the nucleotide sequence in sense and antisense strands related to the test sample. b. Mutation analysis by a *p53*-Chip showing base substitution mutation at codon 248 (CGG→TGG) in tumor GC-F27R with score 29. For each nucleotide 5 different columns were shown to identify different nucleotides and del for 1-bp deletion. The intensity of hybridization appeared with a number on each column. For each nucleotide existed only one column with high intensity hybridization. Presence of two columns with high intensity hybridization indicated the existence of gene mutation. In this figure, presence of column for T and C with complete hybridization indicates the existence of base substitution C→T. Each sequence mismatch was designated with a special score (column at the second row). In this sample, score of mutation was 29. Cases with scores between 16 and 36 indicate the presence of mutation.

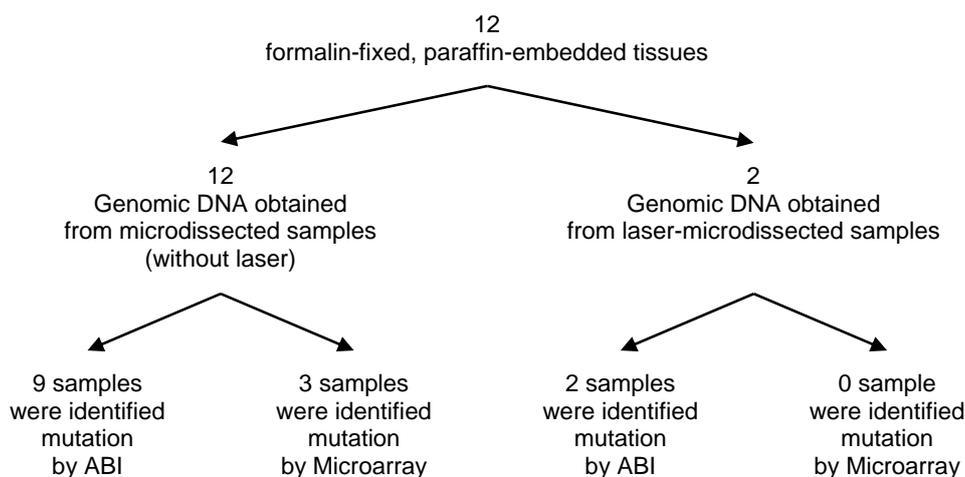


Figure 5. The summary of results shows in this chart.

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