August 30, 2018

PharmacologyOnLine

Archives • 2018 • vol.2 • 149-160

POLYMORPHISMS IN CELL CYCLE REGULATOR GENE P27KIP1 AND P16INK4A AND LUNG CANCER RISK IN A BANGLADESHI POPULATION

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Abstract

Eccentricity of the proteins implicated in cell cycle checkpoints is awfully common among almost all cancers including lung cancer. The grail of the present study was to inquest the role of codon 109 of p27KIP1 and codon 148 of p16INK4A polymorphism as susceptible cell cycle regulator and to harmonize between genetic polymorphism and risk factors of lung cancer in a Bangladeshi population. Two hundred subjects were selected to explore single nucleotide polymorphisms of allelic variants of selective genes using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). Risk of lung cancer was reckoned as odds ratio (OR) at 95% confidence interval (CI) using logistic regression models adjusting for age, sex, and tobacco use. The frequency of Val/Gly heterozygosity of codon 109 was found to be significantly associated with lung cancer (OR=3.38, 95 % CI=1.64 - 6.96, p=0.0010). In contrary, both heterozygous Ala/Thr and mutant Thr/Thr form of codon 148 were significantly linked with lung cancer risk (OR=3.86, 95 % CI= 1.45 to 10.23, p=0.0067; OR=3.86, 95 % CI = 1.19 to 12.48, p=0.0242, respectively). Also inconsequential association was found between smoking and polymorphism of codon 109 of p27KIP1, codon 148 of p16INK4A. Accordingly present findings propound that both codon 109 of p27KIP1 and codon 148 of p16INK4A may contribute to susceptibility of lung cancer in a Bangladeshi population

Keywords: *p27KIP1, p16INK4A, lung cancer, cell cycle regulators, polymorphism, risk factors*

Introduction

Cancer is one of the leading causes of death worldwide. Lung cancer was the second most common cancer contributing 14% of the total number of new cases diagnosed in 2016 worldwide. According to the registry report (2014-2015) by National Institute of Cancer Research and Hospital (NICRH) of Dhaka, Bangladesh, lung cancer (18.1%) is the leading cancer in both sex of Bangladeshi population. Lung and breast cancer are the most prevalent forms of cancers among male and female patients, respectively in Bangladesh, with 28.39% male cancer patients fighting lung cancer and 26% female patients battling breast cancer. This report also shows that about 78.4% male patients are smokers during their lives and 63.9% are current smokers. Among female patients, these rates are 3.5% and 3.3%, respectively [1]. Not only smoking along with other environmental factors but also genetic factors possibly responsible for the development of lung cancer [2].

Genetic alteration at the chromosomal, nucleotide or at the epigenetic level could result in the activation of oncogenes and/or the inactivation of tumor suppressor genes that leads to cancer. Cell cycle dysregulation is a critical aspect of malignancies and arises through the mutation, deletion, amplification or abnormal regulation of a set of critical genes. Cell cycle progression is controlled by a group of proteins called cyclins that modulate the activity of cyclin-dependent kinases (CDKs), which in turn regulate the activity of specific transcription factors. The CDKs are also regulated by CDK inhibitors including p16INK4A, p15INK4B, p21Cip1 and p27Kip1, which show variable expression in different tumors [3].

p27Kip1, a member of the Cip/Kip family, is a universal CDK inhibitor, which is able to arrest cell cycle progression by complexing CDKs. Loss of expression or down regulation of p27Kip1 protein has been shown to be an independent prognostic factor in a variety of human malignancies [4,5]. A total of 21 single nucleotide polymorphism (SNP) of p27KIP1 have been reported (Gene Bank accession number AF480891) [6,7]. Among these SNPs a transversion of T>G at 326, C>A at 838 and T>A at 673 and transition of C>T at 79 and of G>A at 502, (Gene Bank accession number AB003688) have been previously analyzed [8, 9]. Among these, polymorphisms at codon 109 (326T>G) is of particular importance because it is the only SNP in p27KIP1 that changes in amino acid (Valine to Glycine) and consequently reduces cytosolic translocation and increase nuclear stability of protein [10, 11]. Polymorphism of p27KIP1 at codon 109 contributes to the risk of prognosis of oral squamous cell carcinoma, breast cancer, prostate cancer, thyroid cancer etc [12-15]. Controversial results also have been reported [16-18].

The tumor suppressor gene p16INK4A, encodes the CDK inhibitor p16 protein, is frequently inactivated in several cancers including lung cancer [19-20]. In contrast, p16-deficient mice develop spontaneous tumors at an early stage and are highly sensitive to carcinogens [21,22]. The p16INK4A gene is genetically altered by homozygous deletion, hypermethylation, point mutation or insertions [23-24]. However, genetic polymorphisms in this gene have been shown to be associated with the development and progression of various cancers in previous studies [25-27]. Several polymorphisms including Arg24Pro, Met53Ile, Ala57Val, Val59Gly, Arg99Pro, and Ala148Thr have been reported [28-31]. A G to A polymorphism leading to an amino substitution of alanine to threonine at codon 148 is broadly cited as a common polymorphism [32] in melanoma [33-34], pancreatic and breast cancer [35].

Hitherto, no report has been published yet on Val 109 Gly of p27 and Ala 148 Thr of p16 polymorphism and its association with lung cancer in Bangladeshi population. So, the present study was carried out to investigate the genotype distribution of Val 109 Gly of p27KIP1 and Ala 148 Thr of p16INK4A SNPs using the Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP) approach and the possible relevance of these polymorphisms in susceptibility to lung cancer.

Materials and methods

Patients and blood samples

A total of 100 lung cancer patients who reported to National Institute of Cancer research and Hospital (NICRH), Mohakhali, Dhaka, Bangladesh during the period of July 2014 to March 2015 formed the study

group. The guidelines of International Association of Lung Cancer were followed and the patients were histologically diagnosed. A detailed history containing demographic particulars, history of smoking, family history of lung cancer or any other cancer were collected in pretested proforma. The interviews of the patients and/or guardians were done by trained nurses in the presence of expert physicians. Peripheral venous blood samples from 100 healthy control subjects matched for age, sex, and smoking habits were also collected for comparison. Current smokers had been smoking regularly and nonsmokers had never smoked during his/her lifetime. Those smokers who renounce for more than 1 year before the recruitment were considered as ex smokers.

DNA extraction and primer design

Three milliliters of venous blood was collected from all patients and control subjects in EDTA-Na2-containing sterile tubes. Then the samples kept at −80°C until DNA extraction. Genomic DNA was extracted using chemical method [36]. One hundred and fifty microliters of blood was mixed with 1 mL cell lysis buffer, centrifuged at 3,000 rpm for 10 min (4°C), and the nucleus found as precipitate was mixed with 100 µL nuclear lysis buffer and 25 µL of 5M sodium per chlorate. The mixture was incubated at 37°C for 30 min to dissolve the nucleus properly. Chloroform (125 µL) was added to precipitate the protein, and after precipitation, the mixture was centrifuged at 1,500 rpm for 5 min at room temperature. Clear aqueous supernatant was collected and was mixed with double amount of ethanol to reveal the three-dimensional folded DNA. DNA was collected, dissolved in TE buffer, and kept at −20°C until PCR has performed. The quantity and purity of isolated DNA from blood samples were assessed by spectrophotometric assay using a UV Spectrophotometer (UV Prove v2.1). Absorbance was measured at wavelengths of 260 and 280 nm (A260 andA280, respectively). The absorbance quotient (OD260/OD280) provided an estimate of DNA purity. An absorbance quotient value of 1.8<ratio (R) <2.0 was considered to be good, purified DNA.

Polymerase Chain Reaction- Restriction Fragment Length Polymorphism

The target regions of the p27KIP1 Val 109 Gly and p16INK4A Ala148Thr were amplified using previously reported primers [37-38] (Table 1). The primer sequences for p27KIP1 were: forward, 5'–TGCGAGTGTCTAACGGGAGC-3'; and reverse, 5'-TTACCGTCGGTTGCAGGTC-3' and for p16INK4A forward primer 5'- CTGGACGTGCGCGATGCCTG-3'; reverse, 5'- GCAGGGCGATAGGGAGACTC-3'. Briefly, 25 µL PCR mixture consisted of 1µl genomic DNA samples (50–70 ng/µL), 2.5 µL of 10× standard Taq reaction buffer (with MgCl2), 0.5 µL dNTPs (2.5mM each), 2.0 µL of each primer (10µM), 0.13 µL Taq DNA polymerase (5 U/µL), and 20.37 µL nuclease free water. For PCR amplification, the standard program was used as follows: one initial denaturation step at 94 °C for 2 min, followed by 35 denaturation cycles of 1min at 94 °C, 45 sec of annealing at 56°C (for p16INK4A), 57°C (for p27KIP1), and 1min of extension at 72 °C. PCR products were analyzed on a 2% agarose gel by staining with ethidium bromide. For RFLP, the PCR products of p16INK4A and p27KIP1 SNPs were digested with BstuI (1U at 37 °C for 16 h) and BglI (1U at 37 °C for 16 h) (Thermo scientific, USA), respectively. BstUI recognizes a restriction site at p16INK4A (CG↓CG) and generates major fragments (133, 50, 48 bp), while the BglI (recognition site GCCNNNN↓NGGC) for p27KIP1 generate two fragments of 156 and 313 bp. Digested fragments were electrophoresed through 3.5 % agarose gel for resolution. The genotypes of more than 25 % of the samples were double blindly reassessed to confirm the results by two independent researchers.

Statistical analysis

Distributions of demographic variables were compared between cases and controls using chi-square tests and two sided unpaired t tests. The distribution of genotype frequency was also compared by chi-square test to verify whether they were in Hardy-Weinberg equilibrium. The significance of associations between different variables was determined using the statistical software package SPSS version 16.0 (SPSS, Inc., Chicago, IL). $p<0.05$ was considered statistically significant.

ISSN: 1827-8620

Results

Case and control characteristics

Of the 100 lung cancer patients, 86 patients were males and 14 were females (M/F ratio=6.14), and the control subjects comprised of 82 males and 18 females with an M/F ratio of 4.55 (Table 1). In this study, we found that genotype frequencies in cases and controls were in Hardy-Weinberg equilibrium.

Smoking status

The rate of ex smoking was 64% in the patient group compared to 68 % in the control subjects. And the current smokers in the patient group and control group were 22 % and 14 %, respectively. On the other hand nonsmokers in the patient group were 14% and 18% in control group (Table 1).

Histological subtypes of lung cancer

Among 100 cases, the histological subtypes of lung cancer were squamous cell carcinoma (44 %), adenocarcinoma (34%), small cell carcinoma (16 %), large cell carcinoma (4 %), and adenosquamous cell carcinoma (2 %) (Table 1).

The results of electrophoresis of PCR products confirmation the desired size (469 bp and 307 bp for p27KIP1and p16INK4A respectively) on 2% agarose gel and digestion patterns obtained by BstuI and BglI restriction enzymes on agarose gel 3.5% showed the desired bands (Figure 1a, 1b).

P27KIP1 Val 109 Gly polymorphism

In case of Val 109 Gly polymorphism, distribution of the three genotypes NH (Val/Val), HE (Val/Gly), and MH (Gly/Gly) was 52, 30, and 18% in lung cancer patients and 82, 14, and 4% in control subjects, respectively (Table 2). Based on the above results, the relative frequency of Val allele was 0.67 in patients and 0.89 in control subjects and that of Gly allele was 0.33 in patients and 0.11 in control subjects, indicating a significant difference. The frequency of Val/Gly heterozygosity was found to be significantly associated with lung cancer (OR=3.38, 95 % CI=1.64 - 6.96, p=0.0010). Further analysis revealed that the Gly/Gly genotype was considerably higher in patients than in control subjects (OR=7.09, 95%CI=2.27 - 22.14, p=0.0007). The combined frequency of Val/Gly + Gly/Gly genotype was also significantly related to lung cancer risk (OR=4.21, 95 % CI=2.21–8.00, p<0.0001). The study also reveals that the Glycine allele frequency is elevated in lung cancer patients.

P16INK4A Ala 148 Thr polymorphism

The percentage of NH (Ala/Ala) or MH (Thr/Thr) and HE (Ala/Thr) in 100 lung cancer patients was found to be 70, 12, and 18, respectively, as compared to 90, 4, and 6 in the control subjects (n=100) respectively. Significant difference was found between the Thr/Thr allele frequency distribution between the case and control groups (Table 2). In comparison with the Ala/Ala genotype, Ala/Thr and Thr/Thr genotypes were found to be significantly associated with lung cancer (OR=3.86, 95 % CI= 1.45 to 10.23, p=0.0067; OR=3.86, 95 % CI = 1.19 to 12.48, p=0.0242, respectively).

Association between tobacco consumption and p27KIP1 Val 109 Gly and p16INK4A Ala148 Thr polymorphism

The study reveals inconsequential association between smoking and p27KIP1 Val 109 Gly, p16INK4A Ala148Thr polymorphism when comparing smoking status in the lung cancer patient group ($\chi 2$=2.89, p=0.577 and $\chi 2$=7.9792, p=0.092345) (Table 3, 4).

Discussion

Single Nucleotide Polymorphism in DNA is related to tumor progression [39]. The pathogenesis of lung cancer is characterized by the polymorphism of the cell cycle regulator genes. Genetic alteration due to polymorphism in these genes causes uncontrolled cell growth and consequently the cancer. There has been great attention in understanding the role of cell cycle negative regulators p27KIP1 and p16INK4A polymorphism in lung cancer risk and prognosis. In our study the role of two specific polymorphisms in these genes has been tried to reveal in a Bangladeshi population.

In the case of p27KIP1 Val 109Gly SNP, we found that wild type Val/Val frequency was 52 % in cases whereas 82 % in control subjects and mutant Gly/Gly phenotype was 18 and 4 % in cases and controls, respectively. Several studies have been reported the association of P27 Val 109Gly polymorphism and several cancers. p27KIP1 V109G polymorphism was positively associated in prostate cancer in Chinese

population, pancreatic cancer in European-American population [40] , pituitary adenoma group of Brazilian patients [41], endocrine neoplasim [6], thyroid, squamous cell, prostate and breast cancers in different racial groups ([10,17]. But, controversial results also have been reported in Chinese population [6,11]. A recent meta-analysis (encompassing 3799 controls and 3591 patients) in eight studies of oral squamous cell, prostate, breast cancer, and pancreatic cancer found no correlation between p27KIP1 Val 109Gly polymorphism and the overall cancer risk in the general population [9].

In the present study, the relative frequency of Val allele was 0.67 in patients and 0.89 in control subjects and that of Gly allele was 0.33 in patients and 0.11 in control subjects. Variable Gly allele frequencies of Val109Gly of p27KIP1 due to mutations and polymorphism have been reported in different collections of cancer in Nigeria (0.789), Italy (0.26), Kenya (0.764), Japan (0.035), China (0.044), Colorado (0.037), Texas (0.302), African ancestry in Southwest USA (0.711) [42]. Our findings are consistent with most of the preceding studies in other ethnic groups. This study reveals that the glycine allele has a statistically significant association with lung cancer development (OR=7.09, 95% CI=2.27 - 22.14, p=0.0007).

On the other hand, p16INK4A Ala148Thr SNP, in comparison with the Ala/Ala genotype, Ala/Thr and Thr/Thr genotypes were found to be significantly associated with lung cancer (OR=3.86, 95 % CI= 1.45 to 10.23, p=0.0067; OR=3.86, 95 % CI = 1.19 to 12.48, p=0.0242, respectively). It is a polymorphism, which has been reported in lung carcinogenesis in central Europe [32], 8% of the Jewish population [43], 4% of the population in Utah [44] and 5% in the UK population [45] and also in a population of Poland [35]. But our finding is in contrast to the findings of M. Whirl-Carrillo et al., who have reported a lowered frequency in African ancestry in Southwest USA, Toscani in Italia, Luhya in Webuye, Kenya and Han Chinese in Beijing, China. These inconsistent results may be due to differences between studies of cancer types, patient population, and different risk factors for various types of cancers, small sample size, and gene-gene or gene-environment interactions.

Furthermore, the study reveals inconsequential association between smoking and p27KIP1 Val 109 Gly, p16INK4A Ala148Thr polymorphism when comparing smoking status in the lung cancer patient group ($\chi 2$=2.89, p=0.577 and $\chi 2$=7.9792, p=0.092345) (Table 4, 5). Also no significant correlation between different clinicopathological characteristics with p27KIP1 Val 109 Gly and p16INK4A Ala148Thr polymorphism in lung cancer in a Bangladeshi population was found.

Conclusion

Therefore, our study suggests that p27KIP1 Val 109 Gly, p16INK4A Ala148Thr polymorphism may contribute to the pathogenesis of lung cancer. Also, there is no significant correlation between p27KIP1 Val 109 Gly, p16INK4A Ala148Thr polymorphism and clinicopathological characteristics of lung cancer risk in a Bangladeshi population. Large-cohort studies focusing on p27KIP1 Val 109 Gly, p16INK4A Ala148Thr polymorphism are needed to better understand genetic factors and their role in lung cancer progression.

Acknowledgements: The authors are thankful to the Pharmacy Discipline of Khulna University, Bangladesh for providing laboratory facilities, instrumental support. We also acknowledge our deepest thanks to FMRT Discipline of Khulna University for their laboratory support.

Conflict of interest: The authors declare no conflict of interest.

Ethical approval: The study was approved by the Ethics Committee of Institute of Cancer Research and Hospital (NICRH), Dhaka, Bangladesh and the Ethics Committee of Khulna University, Khulna-9208, Bangladesh. All the procedures performed in this study involving human participants were in accordance with ethical standards of the ethics committee of Institute of Cancer Research and Hospital (NICRH), Dhaka, Bangladesh and WMA declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects amended, October 2008.

Informed consent: Informed consent was obtained from all individual participants included in this study.

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Variables	Cases (n=100) (%)	Controls (n=100)	p value
Gender			
Male	86	82	0.4404[a]
Female	14	18	
Age (years)			
Mean age, n (±SD)	56.3173 (±11.0964)	48.88 (± 15.75)	
Range	17-80	18-92	
Smoking status, n (%)			
Current smoker	22	14	
Ex-smoker	64	68	0.3013 [a]
Never smoker	14	18	
Histological type, n (%)			
Adenocarcinoma	34		
Squamous cell carcinoma	44		
Small cell carcinoma	16		
Large cell carcinoma	4		
Adenosquamous cell carcinoma	2		

[a] Chi-square test

Table 1. Distribution of demographic variables of the lung cancer patients and controls

	Genotypes	Cases	Control	P	OR (95% CI)
p^{27KIP1} Val109Gly T>G	Val/Val	52	82	-	1.00
	Val/Gly	30	14	0.0010	3.38 (1.64 - 6.96)
	Gly/Gly	18	4	0.0007	7.09 (2.27 - 22.14)
	Val/Gly+ Gly/Gly	48	18	< 0.0001	4.21 (2.21 - 8.00)
	Glycin Allele frequency	0.33	0.11		
$p^{16INK4A}$ Ala148Thr C>T	Ala/Ala	70	90	-	1.00
	Thr/Thr	18	6	0.0067	3.86 (1.45 - 10.23)
	Ala/Thr	12	4	0.0242	3.86(1.19 - 12.48)
	Thr/Thr + Ala/Thr	30	10	0.0166	3.86 (1.77 - 8.42)
	Threonine Allele frequency	0.21	0.07		

Table 2. Genotype frequencies of p27KIP1, p16INK4A gene polymorphisms in patients and controls

Variables	Cases				χ^2	p
	N=100	Val/Val	Val/Gly	Gly/Gly		
Age group						
≤50 years	24	14	6	4	0.5379	0.764167
>50 years	76	38	24	14		
Gender						
Male	86	43	27	16	0.996	0.607755
Female	14	9	3	2		
Smoking status						
Ex Smoker	64	36	18	10	2.89	0.577
Current Smoker	22	10	6	6		
Never Smoker	14	6	6	2		
Histological type						
Adenocarcinoma	34	21	6	7	10.2549	0.24759
Squamous cell carcinoma	44	25	13	6		
Small cell carcinoma	16	5	8	3		
Large cell carcinoma	4	1	2	1		
Adenosquamous cell carcinoma	2	0	1	1		

Table 3. Association between p27KIP1 phenotypes and clinicopathological characteristics

Variables	Cases				χ^2	p
	N=100	Ala/Ala	Ala/Thr	Thr/Thr		
Age group						
≤50 years	24	13	7	4	3.8917	0.142868
>50 years	76	57	11	8		
Gender						
Male	86	64	15	10	0.3407	0.843386
Female	14	9	3	2		
Smoking status						
Ex Smoker	64	49	7	8	7.9792	0.092345
Current Smoker	22	11	8	3		
Never Smoker	14	10	3	1		
Histological type						
Adenocarcinoma	34	27	4	3	12.1424	0.144961
Squamous cell carcinoma	44	29	9	6		
Small cell carcinoma	16	12	2	2		
Large cell carcinoma	4	2	1	1		
Adenosquamous cell carcinoma	2	0	2	0		

Table 4. Association between p16INK4A phenotypes and clinicopathological characteristics

ISSN: 1827-8620

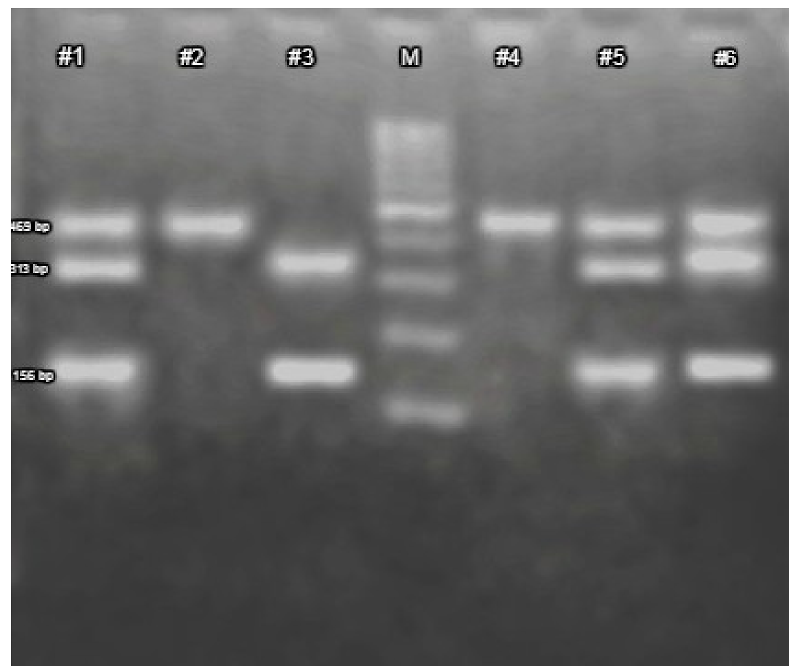

Figure 1. RE digestion of *p27KIP1* following electrophoresis in 3.5 % agarose gel. Lane M: DNA ladder; Lane 2,4: Val/Val (normal homozygous); Lane 1,5,6: Val/Gly (heterozygous); Lane 3: Gly/Gly (mutant homozygous)

ISSN: 1827-8620

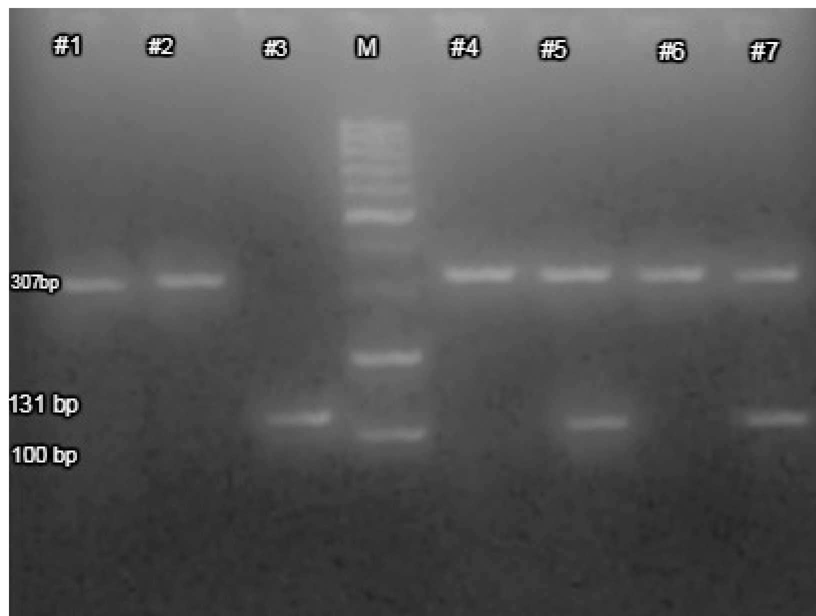

Figure 2. RE digestion fragments of *p16INK4A* following electrophoresis in 3.5 % agarose gel; Lane M: DNA ladder, Lane 1,2,4,6: Ala/Ala ((normal homozygous); Lane 5,7: Ala/Thr (heterozygous); Lane 3: Thr/Thr (mutant homozygous).

ISSN: 1827-8620