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Original Communication

Application of random amplified polymorphic DNA-PCR technique in early diagnosis of bladder cancer using urine samples

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ABSTRACT

The aim of this study is to examine the possible application of Random amplified polymorphic DNA (RAPD) technique based on the polymerase chain reaction (PCR) method, as a genetic test for identifying the significant genomic alterations in bladder cancer patients. DNAs were extracted from the exfoliated cells in the urine of 50 male bladder cancer patients, using the Phenol-Chloroform DNA extraction method, and the yielded DNAs were amplified with the BioA-09 random primer (5'-GGGTAACGCC-3'). The amplified PCR products from RAPD analysis were electrophoretically separated in agarose gels; banding profiles were visualized by ethidium bromide staining and demonstrated under ultraviolet light. The genomic alterations were clearly apparent in tumor RAPD-PCR patterns, by the loss of normal bands and the appearance of new tumor-related bands, as compared to the controls. The correlation between the genomic alteration and the histopathological subtypes was assessed using the X^2 test. In addition, bands with molecular sizes 500 ($p < 0.001$) and 1300 ($p = 0.033$) bp were used as molecular markers for transitional cell carcinoma, while squamous cell carcinoma type had two diagnostic bands with molecular sizes 1800 ($p < 0.001$) and 1400 ($p < 0.021$) bps, respectively. Our study suggests the potential of RAPD-PCR method to produce diagnostic markers for analysing the genomic instabilities in bladder tumors.

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KEYWORDS: RAPD-PCR, bladder cancer, Bio-A09 primer, genomic alterations, DNA, cancer

1. INTRODUCTION

Globally, urothelial bladder carcinoma ranks as the fifth most common cancer in men and the fifteenth in women, and the incidence rate increases in people older than 60 [1]. Egypt has one of the highest incidence rates of bladder cancer among men in the world, with an estimated incidence rate of 37.7 cases per 100,000 males [2]. Oncologists and pathologists in Egypt have suggested that the histopathological profile of bladder cancer has changed significantly over the past 26 years. Historically, squamous cell carcinoma was the predominant form in Egypt throughout the period of 1960-1982. Now, transitional cell carcinoma has become the most frequent type and this shows the change in the epidemiology of the disease [3]. Random amplified polymorphic DNA (RAPD) is a PCR based fingerprinting technique that amplifies random DNA fragments with single short primers of arbitrary nucleotides (10-12 bp). These primers anneal to complementary sequences at different genomic loci. If a mutation has occurred in the template DNA at the site that was previously complementary to the primer, a PCR product will not be produced, resulting in a different pattern of amplified DNA segments on the gel [4]. The main advantages of RAPD analyses are that they are fast, reliable and easy to perform. Also, they need minute quantities of initial genomic DNA (5–50 ng per reaction) and no prior information of the

nucleotide sequence is required. Consequently, the RAPD-PCR method has the potential to detect a wide range of genomic alterations [4, 5]. The Phenol-Chloroform DNA extraction method is one of the most useful and commonly used methods that yields high molecular weight DNA and therefore can be utilized in situations where PCR typing is performed. It is the preferred method of extraction for samples that are old or degraded because it consistently yields higher quantity of DNA [6]. The usefulness of this DNA fingerprinting method for the detection of genomic instability in bladder cancer should be evaluated.

2. MATERIALS AND METHODS

2.1. Patients and controls

The study was prospective and included 50 male patients admitted at Urology Nephrology center, Mansoura, Egypt. Their age ranged from 30-80 years (mean 56.7 ± 10.18). We used the hospital information system and medical record to collect data about these patients and their tumors; the transitional cell type (TCC) was the most frequently diagnosed (31/50 cases; 62%), followed by mixed tumors (9/50; 18%) and the squamous cell carcinoma (8/50; 16%). However, the small cell carcinoma and adenocarcinoma types had the lowest frequency (2%). In transitional cell carcinoma, grade-II (21/31 cases; 67.7%) tumors had a higher percentage than grade-III (10/31 cases; 32.3%) tumors, while grade-I was the predominant in the squamous cell type (Table 1). Pathologically, all mixed tumors were of a high grade and the transitional cell type was the predominant component. In addition, urine samples were collected from eight healthy individuals for use as a control (mean age 28.2 ± 2.8).

2.2. Chemicals

Tris-base and Tris-HCl were purchased from Molekula, England. Absolute isopropanol alcohol (99.7%) and ethylene diamine tetra-acetic acid (EDTA) were purchased from Riedel-de Haën, Switzerland. Ethidium bromide (EtBr) and sodium chloride (NaCl) were purchased from Sigma-Aldrich, Germany. Phenol: Chloroform: Isoamyl Alcohol (25:24:1) solution was purchased from BioFlux, Japan. Low melting agarose for gel electrophoresis was purchased from AxyGEN, USA. Gene Ruler™ 100 bp Plus DNA Ladder, proteinase K (20 mg/ml) and RNase (10 mg/ml) were purchased from Fermenats, Canada. Lambda DNA/*Hind* III ladder with blue/orange 6x loading dye was purchased from SibEnzyme, Russia. Glacial acetic acid was purchased from Ranbaxy, India. Red blood cell lysis buffer was purchased from Roche, Germany. PCR-GOLD Master-Mix Beads (each bead contains ~1.0 unit of *Taq* DNA polymerase, 10 mM Tris-HCl (pH 9.0), 30 mM KCl, 1.5 mM $MgCl_2$, 250 μM of each deoxy Nucleotide Tri Phosphate (dNTPs)

Table 1. Percentages of various types of bladder cancer diagnosed during the period of study.

Types of bladder cancer	No. of patients	Percentage (%)
1. Transitional cell carcinoma (TCC)	31	62
i) Grade III	10	20
ii) Grade II	21	42
2. Squamous cell carcinoma (SqCC)	8	16
i) Grade II	3	6
ii) Grade I	5	10
3. Mixed type (TCC + SqCC)	9	18
4. Small cell carcinoma (SmCC)	1	2
5. Adenocarcinoma	1	2

and stabilizers, including Bovine Serum Albumin) and the synthetic G-C rich Bio-A09 (5'-GGGTAACGCC-3') primer were purchased from BIORON, Germany.

2.3. Stock solutions

Tris-Acetate EDTA (50x) stock solution: 242 gm Tris-base, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA (pH 8.0) were mixed with 800 ml deionized water and the volume was completed to 1 L [7]. Digestion buffer: prepared by mixing 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 25mM EDTA (pH 8.0) and 0.5% sodium dodecyl sulphate (SDS) and was stored at room temperature [8]. Tris-Acetate EDTA (1x) working solution: 20 ml of 50x stock solution was taken and made up to 1 L by deionized H_2O [9].

2.4. Methodology

2.4.1 Sample collection and processing

Urine samples were collected from eight healthy individuals and 50 male bladder cancer patients. Samples were centrifuged at 1500xg for 20 min to pellet the exfoliated cellular material. In patients with hematuria, the cell pellet was resuspended with 400 µl red blood cell lysis buffer. A dry pellet containing the cells was transferred into an Eppendorf tube (1.5 ml) and centrifuged for 10 min at 10,000xg (4 °C).

2.4.2. Phenol-chloroform DNA extraction

Digestion buffer (500 µl) was added to the dry pellet, and the tube was gently inverted several times to resuspend the pellet. Proteinase K (50 µl of 20 mg/ml) was added, and then the tube was incubated in a water bath at 56 °C overnight. An equal volume of Phenol: Chloroform: Isoamyl alcohol solution (25:24:1) was added to the suspension and mixed gently by inverting the tube for a few minutes. Samples were centrifuged at 13,200xg (4 °C) for 15 min and the upper aqueous layer was transferred to a new sterile eppendorf. RNase (10 µl of 10 mg/ml) was added and kept for incubation at 37 °C for 4 hours. An equal volume of Phenol: Chloroform: Isoamyl alcohol solution was added, and the tube was centrifuged again at 10,000xg (4 °C) for 10 min. The upper aqueous layer was transferred into a fresh, sterilized microcentrifuge tube. 1 M sodium chloride solution (NaCl) was added and mixed well, then six-tenth volume of chilled isopropanol was added and mixed gently by inverting the tube several times, and chilled at -20 °C for 1 h for precipitation; then the sample was centrifuged at 10,000xg (4 °C) for 15 min. After decanting the supernatant, 600 µl 70% ethanol was added, and the pellet was dissolved; the mixture was centrifuged at 10,000xg for 10 min, and the supernatant was decanted gently. Furthermore, the pellet was air-dried under laminar air flow, and the dried pellet was resuspended in 50 µl nuclease-free water. The concentration of the extracted DNA was determined spectrophotometrically at 260 nm ($1OD_{260nm}$ = 50 µg/ml of dsDNA). On the other hand, the degree of purity of the extracted DNA was calculated by the ratio of 260 nm/280 nm readings; ratios between 1.8 and 2.0 indicate high-quality DNA, while lower values indicate protein contamination. Extracted DNA was then further separated by DNA electrophoresis on an agarose gel (0.7%) in 1x TAE, mixed with ethidium bromide (final concentration of 0.5 µg/ml) and demonstrated under ultraviolet light [8, 9].

2.4.3. RAPD-PCR

1 µg of genomic DNA extracted from urine samples of bladder cancer patients or healthy individuals were amplified in reaction mixture containing one PCR bead and 15 picomol of the BioA-09 primer in a final volume of 20 µl. Two drops of mineral oil were added, then the PCR reaction mixture was transferred into the thermal cycler. The PCR conditions used for the amplification of DNA were as follows: an initial denaturation (DNA strand separation) at 94 °C for 3 min, followed by 34 cycles of denaturation at 94 °C for 1 min, primer annealing with DNA at 40 °C for 1 min, new DNA extension (*Taq* DNA polymerase enzyme adds nucleotides at the 3′ ends of annealed primers) at 72 ºC for 2 min and finally, one cycle for 7 min at 72 ºC for final DNA extension. Then, the reaction was terminated by holding the tubes at 4 °C in the PCR machine. The PCR product was separated by electrophoresis on an 1.0% agarose gel prepared in 1x Tris Acetate EDTA (TAE) and stained with ethidium bromide (final concentration of 0.5 µg/ml) as recently described by El-Far *et al.* [9].

2.4.4. Statistical data analysis

The molecular weights of the amplified DNA fragments, which included the normal and the tumor-related bands, were calculated by using

Sambrook standard curve [10]. The correlation between the genomic instability and the histologic type and grade of bladder cancer were assessed by chi-squared analysis and Fisher's exact test (for less than 20 cases). The acceptable level was $p < 0.05$ as significant correlation. All the statistical analyses of data were carried out by SPSS software ver. 19 (IBM, US).

3. RESULTS

Genomic DNAs were extracted, purified from all studied urine samples and the unpurified DNAs were excluded. Following the steps of DNA extraction, samples were run in 0.7% agarose gels. The isolated genomic DNAs from urine samples of bladder cancer patients are indicated in Figures 1 and 2. In our previous work [9], we applied RAPD- PCR

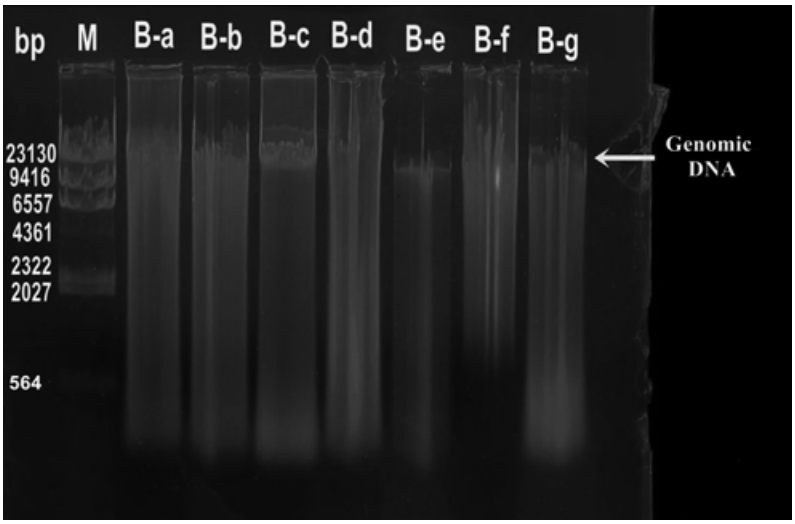

Figure 1. Gel electrophoresis of genomic DNA extraction of 7 bladder cancer samples (B-a to B-g) in 0.7% agarose gel in 1x TAE buffer at 100 V for 46 min. M: Lambda DNA Hind III-digested marker (23130-564) bp.

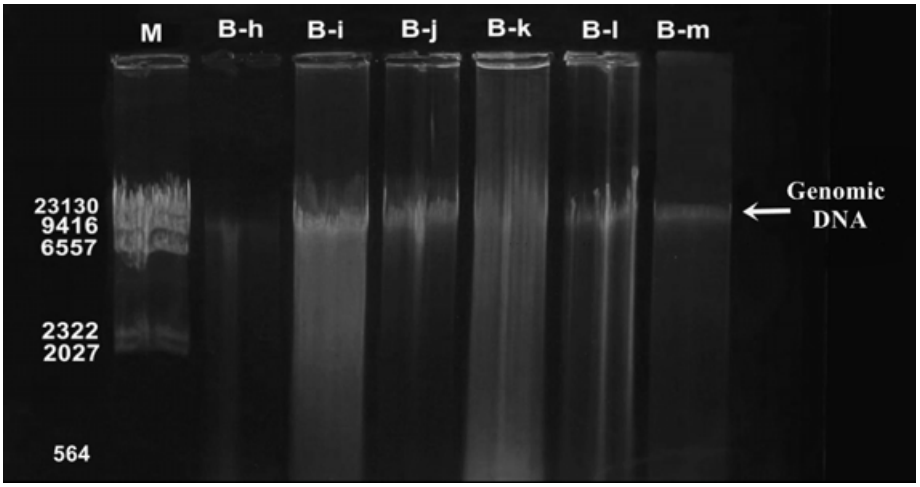

Figure 2. Gel electrophoresis of genomic DNA extraction of 6 bladder cancer samples (B-h to B-m) in 0.7% agarose gel in 1x TAE buffer at 100 V for 40 min. M: Lambda DNA Hind III-digested marker (23130-564) bp.

on the urine obtained from eight healthy individuals by using Bio-A09 primer. The primer produced five major bands; their molecular sizes were 650, 750, 1000, 1100 and 1500 bps, respectively (Figure 3 indicates the RAPD-PCR profile obtained from the DNAs extracted from the only two healthy individuals). The profiles of the amplified DNA generated by the primer Bio-A09 from each class of bladder carcinoma showed a wide range of genomic instabilities, which were expressed by the loss of normal bands and/or the appearing of new tumor-related bands (Table 2). Remarkably, the tumor RAPD-PCR profiles gained four common tumor bands, with molecular sizes 500, 1300, 1400 and 1800 bps (Figures 4-10). In both grades of transitional cell carcinoma, an insertion of two significant tumor-related bands was found, with molecular sizes 500 (X^2 = 23.53, p < 0.001) and 1300 (X^2 = 4.521, p = 0.033) bps in 93.5 and 61.3% of the tumor samples, respectively. In grade-III bladder transitional cell carcinoma (B1-B10) samples, the missing of normal bands with molecular sizes 650, 750, 1000, 1100 and 1500 bps in 90, 70, 90, 50 and 90% of the tumor samples, respectively, was seen. On the other hand, by comparing the pattern of grade-II bladder transitional cell carcinoma (B11-B31)

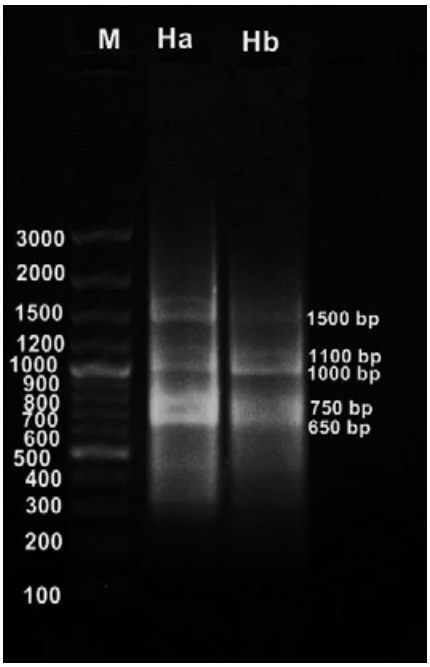

Figure 3. RAPD-PCR patterns of amplified DNA fragments amplified by primer Bio-A09 from genomic DNA of 2 healthy samples (Ha and Hb). Electrophoresis was performed on (1%) agarose gel in 1x TAE buffer and run with 80 volt for 2 hrs. M: Gene Ruler™ 100 bp Plus DNA ladder marker (3000-100) bp.

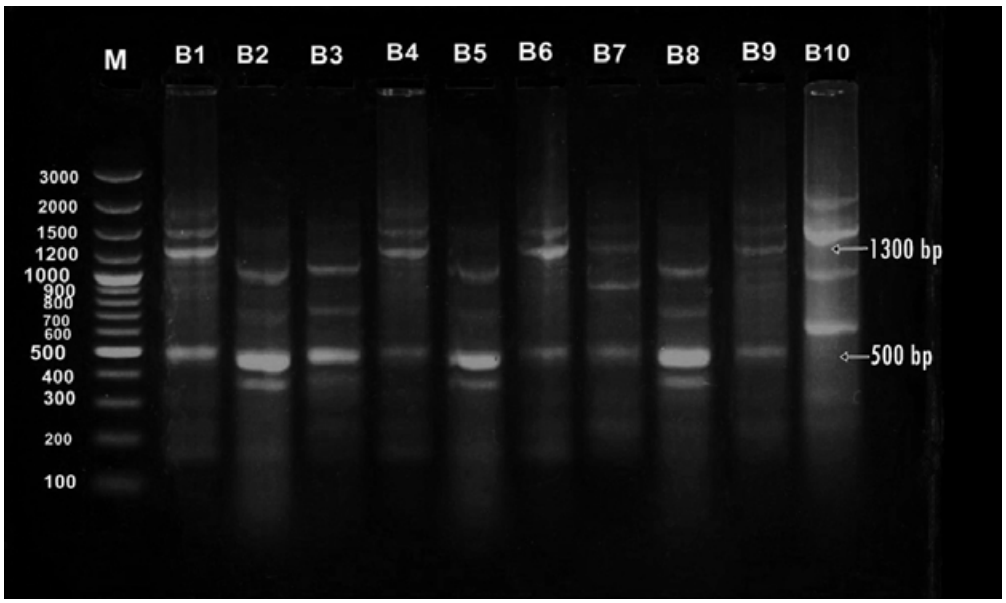

Figure 4. RAPD-PCR amplification pattern of TCC samples of grade III (B1-B10) amplified by primer Bio-A09. Electrophoresis was performed on (1%) agarose gel in 1x TAE buffer and run with 80 volt for 1.5 hrs. M: Gene Ruler™ 100 bp Plus DNA ladder marker (3000-100) bp.

Table 2. Percentages of amplified DNA fragments generated by Bio-A09 in bladder cancer patients and controls. Sizes of DNA bands are indicated in base pairs (bp).

		Percentages of amplified DNA bands								
		Tumor bands				**Normal bands**				
Histopathological types	**No. of Cases**	**500 bp**	**1300 bp**	**1400 bp**	**1800 bp**	**650 bp**	**750 bp**	**1000 bp**	**1100 bp**	**1500 bp**
TCC- Grade III	10	90.0	50.0	0.0	0.0	10.0	30.0	10.0	50.0	10.0
TCC- Grade II	21	95.2	66.6	9.5	0.0	19.0	38.1	38.1	47.6	23.8
Mixed Type (SqCC + TCC)	9	88.8	44.4	22.0	11.1	0.0	33.3	44.4	22.2	22.2
SqCC- Grade II	3	33.3	33.3	33.3	100.0	33.3	33.3	0.0	33.3	0.0
SqCC-Grade I	5	40.0	40.0	80.0	90.0	60.0	20.0	20.0	20.0	20.0
SmCC	1	0.0	0.0	100.0	0.0	100.0	0.0	0.0	100.0	0.0
Adenocarcinoma	1	100.0	100.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0
Healthy control	8	12.5	0.0	0.0	0.0	100.0	100.0	100.0	75.0	62.5

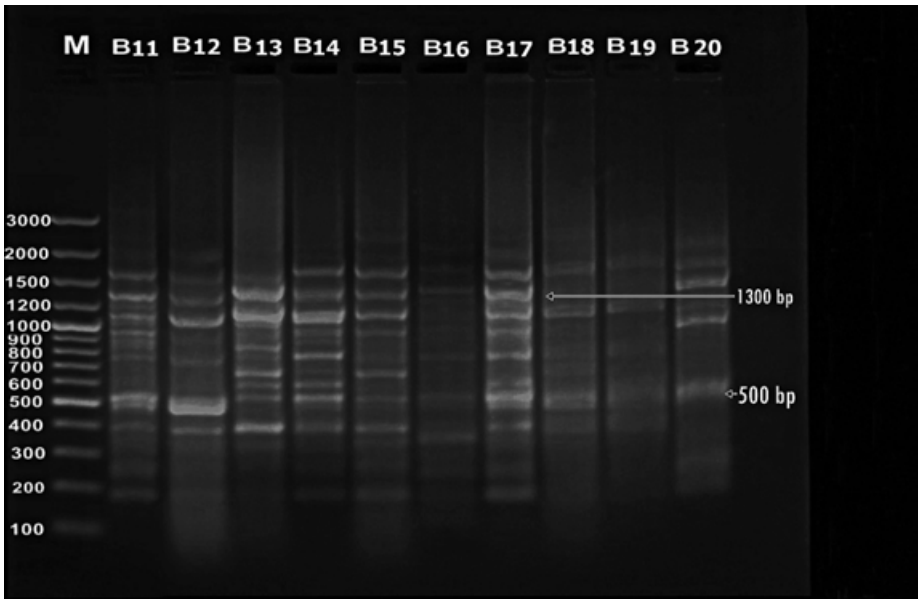

Figure 5. RAPD-PCR amplification pattern of TCC samples of grade II (B11-B20) amplified by primer Bio-A09. Electrophoresis was performed on (1%) agarose gel in 1x TAE buffer and run with 90 volt for 2 hrs. M: Gene Ruler™ 100 bp Plus DNA ladder marker (3000-100) bp.

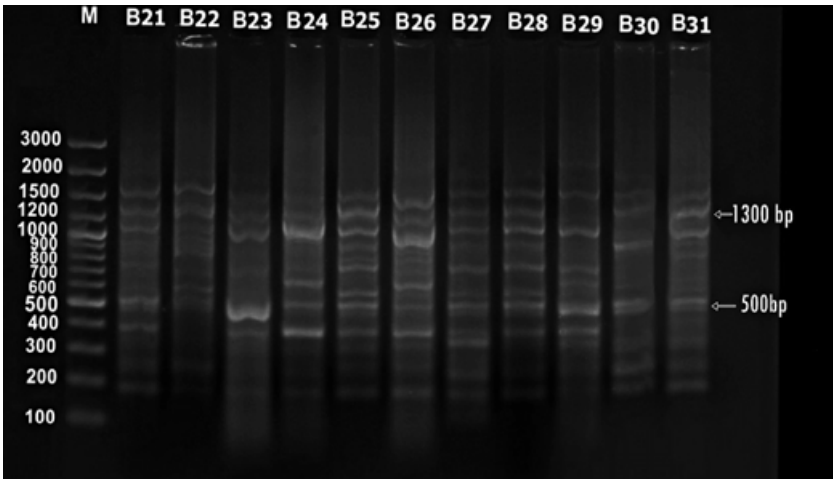

Figure 6. RAPD-PCR amplification pattern of TCC samples of grade II (B21-B31) amplified by primer Bio-A09. Electrophoresis was performed on (1%) agarose gel in 1x TAE buffer and run with 100 volt for 1.5 hrs. M: Gene Ruler™ 100 bp Plus DNA ladder marker (3000-100) bp.

samples with the healthy pattern, the loss of normal bands with molecular sizes 650, 750, 1000, 1100 and 1500 bps were seen in 81, 61.9, 61.9, 52.4 and 72.2% of the tumor samples, respectively (Table 2). In addition, the percentages of missing normal bands increased in grade-III as compared to grade-II. It is worth mentioning that Fisher's exact test showed non-significant relation between the missing of normal bands with molecular sizes 750 (p = 0.634), 1000 (p = 0.729), 1100 (p = 1.0) and 1500 (p = 0.379) bps and the difference between the two grades. Moreover, the present study showed the appearance

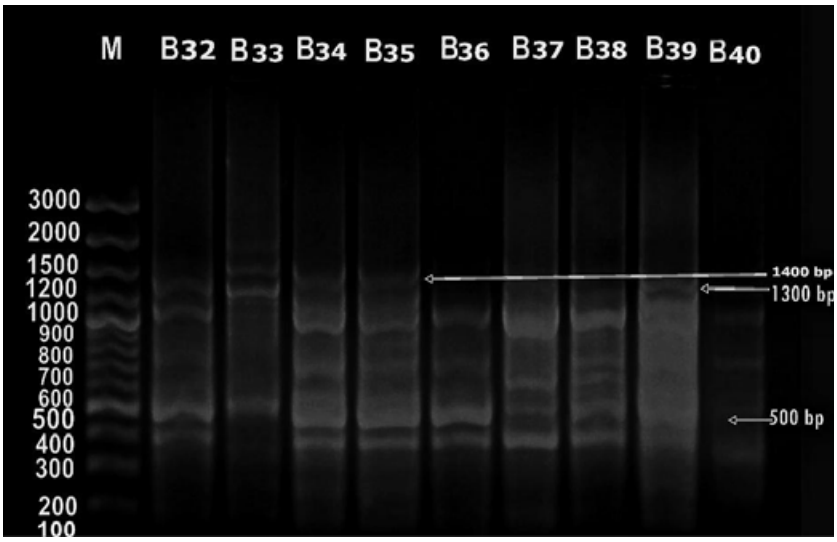

Figure 7. RAPD-PCR amplification pattern of Mixed type (TCC and SqCC) of samples (B32-B40) amplified by primer Bio-A09. Electrophoresis was performed on (1%) agarose gel and run with 100 volt for 1.5 hrs. M: Gene Ruler™ 100 bp Plus DNA ladder (3000-100) bp.

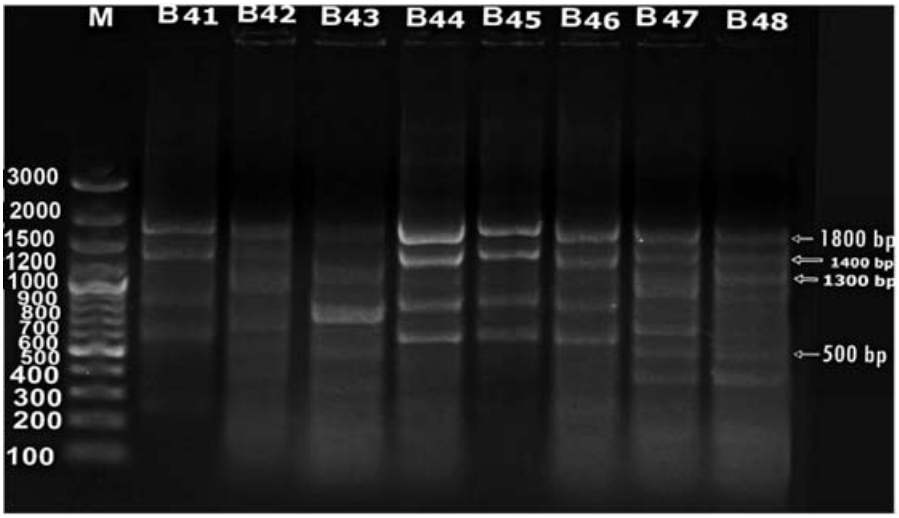

Figure 8. RAPD-PCR amplification pattern of SqCC; Grade II (B41-B43) and Grade I (B44 - B48) amplified by primer Bio-A09. Electrophoresis was performed on (1%) agarose gel in 1x TAE buffer and run with 80 volt for 1.5 hrs. M: Gene Ruler™ 100 bp Plus DNA ladder (3000-100) bp.

of the two significant tumor-related bands with molecular sizes 1800 (X^2 = 12.44, p < 0.001) and 1400 (X^2 = 5.33, p = 0.021) bps in the squamous cell type (Figure 8, Table 2). Additionally, bands with molecular sizes 500 (X^2 = 0.608, p = 0.435) and 1300 (X^2 = 3.692, p = 0.055) bp were not significant in squamous cell carcinoma, when compared to the transitional cell type. Moreover, Fisher's exact test finds no evidence of association among the presence and/or absence of 500, 1300, 1000, 1400 and 1500 bps bands and the grade variation of the squamous cell type (p-values 1.0, 1.0, 1.0, 0.464 and 0.231,

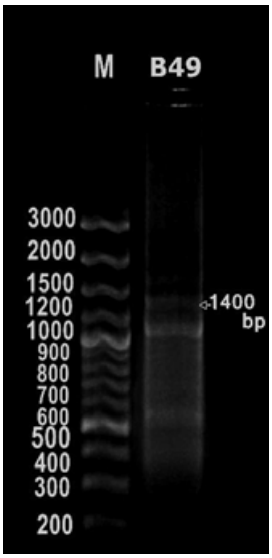

Figure 9. RAPD-PCR amplification pattern of small cell carcinoma (B49) amplified by primer Bio-A09. Electrophoresis was performed on (1%) agarose gel in 1x TAE buffer and run with 100 volt for 1.5 hrs. M: Gene Ruler™ 100 bp Plus DNA ladder (3000-100) bp.

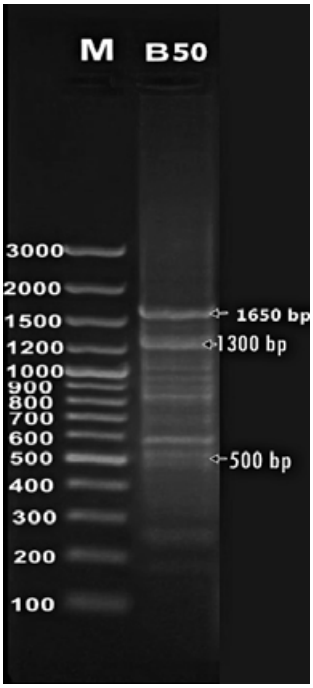

Figure 10. RAPD-PCR amplification pattern of adenocarcinoma (B50) amplified by primer Bio-A09. Electrophoresis was performed on (1%) agarose gel in 1x TAE buffer and run with 90 volt for 2 hrs. M: Gene Ruler™ 100 bp Plus DNA ladder (3000-100) bp.

respectively). In mixed tumors of transitional and squamous cell carcinoma (B12-B40) samples, new tumor-related bands with molecular sizes 500, 1300, 1400 and 1800 bps appeared in 88.8, 44.4, 22 and 11.1 % of the tumor samples, respectively (Table 2, Figure 8). Bands with molecular sizes 500 (X^2 = 9.92, p = 0.002) and 1300 (X^2 = 4.6, p = 0.031) bp, which are considered as marker bands for transitional cell carcinoma were found in a significant manner in the mixed type, whereas the bands related to the squamous cell component, with molecular sizes 1400 and 1800 were found insignificantly (p > 0.05). Remarkably, our results are compatible with the pathology reports of patient, which showed increasing predominance of transitional over squamous elements. It is also worth mentioning that we have only one sample of small cell carcinoma and also of adenocarcinoma, and so it is difficult to detect significant bands for these rare types.

4. DISCUSSION

Recently, the RAPD-PCR technique has been used for the detection of the genomic alterations in human cancers, revealing that genomic instability occurred frequently in most of renal tumors [9], brain tumors [11], lung cancer [12], breast cancer [13], colon cancers [14], head and neck squamous cell carcinoma [15], acute lymphoblastic leukemia [16], hepatocellullar carcinoma [17], skin cancers [18] and pituitary adenomas [19]. To the best of our knowledge, this is the first report on the analysis of genomic instability in bladder cancer using RAPD-PCR fingerprinting. In addition, the RAPD-PCR profiles of all patients and controls were generated by the same synthetic primer (Bio-A09). Statistically, the presence of tumor bands with molecular sizes 500 and 1300 bps together can be used as diagnostic markers for the transitional cell carcinoma. Higher percentages of normal bands in grade-II transitional cell carcinoma than grade-II give evidence to the utility of RAPD-PCR method in inspecting the tumor development. Unfortunately, this increment was not established statistically in our work. Hence, further work is required on larger samples to validate the results. Remarkably, the RAPD-PCR pattern of squamous cell carcinoma gained two significant tumor bands with molecular sizes 1800 and 1400 bps, which were not found in the other bladder cancers. Accordingly, these DNA fragments can be used as diagnostic markers for this histologic type. We need to mention

that our study was not successful in differentiating between the tumor grades according to the presence or absence of normal and tumor bands. El-Far *et al.* [9] very recently applied the RAPD-PCR method in identifying the genomic instability in renal cancer using the same primer, which we used in the present study. In comparison with the existing results, Bio-A09 primer has the ability to demonstrate the genomic difference between bladder and renal cancer. For instance, a band with molecular size 1300 bp, a significant band in transitional cell carcinoma, was shifted to 1250 bp in clear renal carcinoma. In addition, there is no significant relation between clear renal cell carcinoma and the presence of 1400 (X^2 = 1.21, p = 0.271) bp band, whereas this band is considered the diagnostic marker for bladder squamous cell carcinoma (X^2 = 5.33, p = 0.021). It is noteworthy that a band with molecular size 1300 bp is a significant band in transitional cell carcinoma of the bladder and renal pelvis, which is thought to be due to the similarity in the tumor origin; both of them originated in the urothelium layer. Interestingly, the RAPD profiles of bladder and renal cancer gained a common tumor-related band with molecular size 500 bp. Consequently, the present study suggests this band as a diagnostic marker for most of the urologic cancers.

5. CONCLUSION

RAPD decamer primer Bio-A09 produced 500 and 1300 bps DNA fragments as molecular markers for transitional cell carcinoma and mixed tumors. In addition, bands with molecular sizes 1800 and 1400 bps can be used as diagnostic markers in squamous cell carcinoma. The study gives new evidence of potential promise of RAPD-PCR analysis in cancer research for detecting and selecting novel genomic markers.

CONFLICT OF INTEREST STATEMENT

We certify that there is no actual or potential conflict of interest in relation to this article.

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