Detection of Genomic Instability in Renal Cancer by Random Amplified Polymorphic DNA Analysis from Urine Sample as a Non-invasive Method: Potential Use in Diagnosis

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Abstract

The study applied random amplified polymorphic DNA (RAPD) analysis including PCR technique in detection of genomic instability of renal cancer from urine samples as an early detective and a non-invasive method.

DNAs extracted, using manual method, from exfoliated cells in urine of 52 renal cancer patients. All yielded DNAs were amplified with the same 10-base pair random primer (BioA-09) with GC rich sequence (5'-GGGTAACGCC-3'). PCR products from RAPD analysis were electrophoretically separated in agarose gels and banding profiles were visualized by ethidium bromide staining and viewed under ultraviolet light.

Genomic alteration has been shown and included the disappearing of normal bands and appearing of new bands related to the tumor. The correlation between the genomic alteration and the histopathological types was assessed by using the χ^2 test. Bands with molecular sizes 180 ($\chi^2 = 13.15$, $p < 0.001$), 250 ($\chi^2 = 9.524$, $p = 0.002$), and 1250 ($\chi^2 = 5.357$, $p = 0.021$) bp can be utilized as molecular markers for conventional renal cell carcinoma in addition to 1700 bp utilized as molecular marker for transitional cell carcinoma of renal pelvis ($P = 0.005$). Also there is an association between the presence of 500 bp and renal tumor as general (90.4%).

The results of current investigation support the availability of using of RAPD-PCR analysis for screening of specific molecular markers by using Bio-A09 primer. These markers might be useful in diagnosis of renal cancer types and inspecting the tumor development.

Keywords: RAPD-PCR; Renal cancer; Bio-A09 primer; Genomic alterations, DNA

Introduction

Renal cell carcinoma (RCC) takes the third place in incidence among urologic tumors following prostate carcinoma and transitional cell carcinoma of bladder. This malignancy takes the tenth and fourteenth place among men and women, respectively.

Among urologic tumors, it is the worst in cancer specific mortality, since more than 40% of the patients with RCC die of the disease, opposite to the 20% mortality observed in prostate cancer or bladder carcinomas [1]. In Egypt, the two predominant types of renal cancers are renal cell carcinoma and Wilms' tumor in children of all malignant renal tumors, accounting as 56.78% and 37.28% respectively [2]. The organic DNA extractions like Phenol: Chloroform: Isoamyl Alcohol method is the preferred method of DNA extraction because it consistently yields higher quantity of DNA [3]. RAPD analysis is a PCR-based technique that based on enzymatic amplification of target or random DNA segments by using synthetic primer of arbitrary nucleotide sequence (10-12 bp). This primer anneal to complementary sequences at different genomic loci. Amplified products (usually within the 0.5-5 kb size range) are separated on agarose gels in the presence of ethidium bromide and demonstrated by ultraviolet transilluminator. The presence and absence of bands will be observed. 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. The variable bands are commonly called polymorphic DNA [4]. The main advantages of RAPDs are that they are quick and easy to perform. Because PCR is involved, only low quantities of template DNA are required, usually 5-50 ng per reaction. Since random primers are commercially available, no sequence data for primer construction are needed and, radioisotopes not necessary. Moreover, RAPDs have a very high genomic abundance and large number of loci that can be screened quickly [4]. So, RAPD have also been applied in gene mapping studies to fill gaps not covered by other markers [5].

Materials and Methods

Patients and controls

The study was conducted in a prospective nonrandomized pattern. Voided urine samples were obtained from 52 patients

with renal tumors admitted in Urology and Nephrology center, Mansoura University, Egypt, for treatment. Their ages ranging from 2-74 (mean 51.07 ± 16.84). The female-to-male ratio is estimated as 1:3. Eight healthy individuals gave a urine sample to be used as a control (mean age 28.2 ± 2.8 ; 5 female, 3 male). Table 1 showed that conventional renal cell carcinoma comprised the highest percentage (80.8%) compared to the other types.

Chemicals

Tissue Digestion buffer preparation: 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) to each other and stored at room temperature [6]. Lambda DNA/Hind III marker: was mixed with blue/orange 6x loading dye with a ratio 5:1 respectively and kept in -20°C. Proteinase-K preparation: the lyophilized proteinase K were reconstituted in sterile deionized water to a final concentration of 20 mg/ml and stored in small vials at -20°C until use [7]. RNase preparation: 10 mg/ml RNase solution was prepared by dissolving 10 mg pancreatic RNase in 10 mM Tris-Cl (pH 7.5) and 15 mM NaCl to a final concentration of 10 mg/ml; boiled at 100°C for 15 minutes and allowed to cool slowly to room temperature before being dispensed into small aliquots and stored at -20°C [7]. Tris-Acetate EDTA (50x) stock solution preparation: A 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 liter. Tris-Acetate EDTA (1x) working solution preparation: A 20 ml of 50x stock solution was taken and completed to 1L by deionized H₂O [7].

Methodology

Urine sample collection

Voided urine specimens were collected from all patients and controls into a 50 ml sterile urine specimen container. 1-2 drops of glacial acetic acid (98%) was added to lyse the red blood cells. Samples were centrifuged at 1500 xg for 15 minutes to pellet the exfoliated cellular material. The supernatant was decanted and the pellet was transferred to a 1.5 ml Eppendorf.

Genomic DNA extraction

A 700 µl of digestion buffer containing 20 mg/ml proteinase K was added to all Eppendorfs containing pellets (to a final conc. 0.1mg/ml), thumbing for 3 min until no visible cell clumps remained and incubated overnight at 55-65°C in water bath till

the solution became clear. Equal volumes of Phenol: Chloroform: Isoamyl Alcohol (PCI) was added and mixed well until the phases were completely mixed. Samples were centrifuged at 13200 xg for 10 min and the upper clear layer was transferred to a new sterile Eppendorf. To the clear aqueous layer, RNase solution was added to a final concentration of 20µg/ml, mixed thoroughly and incubated in a water bath at 37°C for 4 hours. An equal volume of PCI was added, mixed well and centrifuged again at 13200 xg for 10 min and the upper clear aqueous layer was transferred to a new sterile Eppendorf. Sodium chloride solution was added to a final concentration of 1M and mixed well, then 0.6 volume of Isopropanol was added and mixed by inversion several times and kept at room temperature until DNA appeared as white floating filaments. DNA was spooled out and centrifuged at 13200 xg for 5 minutes. The supernatant was decanted, and the DNA pellet was washed twice with 600 µl of room temperature 70% ethanol. The ethanol was aspirated and the DNA pellet was air dried for 15 minutes or dried in oven at 60°C for overnight to obtain a dry lyophilized DNA pellet [7]. The DNA pellet rehydrated in 10-30 µl deionized water or TE buffer (according to the pellet size) for 1 hour at 65°C or overnight at 4°C. 5µl of extracted genomic DNA was mixed with 1995 µl sterile deionized water; DNAs concentration and purity were assessed spectrophotometrically at 260 and 280 nm. The concentration was calculated according to the standard value ($1OD_{260nm} = 50 \mu\text{g/ml}$ of dsDNA). The degree of purity of the DNA preparation was determined by the ratio of 260nm/280nm readings, which lies between 1.8-2.0 [7]. The yielded DNA integrity was demonstrated by DNA electrophoresis on an agarose gel (0.7%) in 1x TAE and mixed with ethidium bromide (final concentration of 0.5 µg/ml).

RAPD-PCR amplification protocol

In a total volume 20 µl, 1µg of genomic DNA extracted from urine samples of renal carcinomas or corresponding normal individuals were amplified in reaction mixture containing one PCR bead_ each bead contains ~1.0 unit of *Taq* DNA polymerase, 10 mM Tris-HCl, (pH 9.0 at room temperature), 30 mM KCl, 1.5 mM MgCl₂, 250 µM of each deoxy Nucleotide Tri Phosphate (dNTPs) and stabilizers, including Bovine Serum Albumin (BSA)_ and 15 picomol of BioA-09(5'-GGGTAACGCC-3'). The reaction mixture was overlaid with 25µl of mineral oil. PCR reaction mixture was then transferred into thermal cycler programmed for 35 cycles of 1 minute at 94°C for DNA denaturing, 1 minute at 40°C for primer annealing with DNA and 2 minutes at 72°C for new DNA extension. A second program as one cycle for 7 minutes at 72°C for final DNA extension was performed. Finally, the reaction was terminated by holding the tubes at 4°C in the PCR machine as a third program. The PCR product was separated by electrophoresis on an agarose gel (1.0 %) prepared in 1x Tris Acetate EDTA (TAE) and stained with ethidium bromide (final concentration of 0.5 µg/ml).

Data analysis

The banding pattern obtained by RAPD-PCR was noted in the photograph. Each change observed in RAPD profiles (appearance/ disappearance of bands compared with control)

Histopathological subtypes	NO	%
A. Conventional renal cell carcinoma (RCC)	42(total)	80.8
i. Clear renal cell carcinoma	22	42.3
ii. Chromophobe renal cell carcinoma	2	3.8
iii. Papillary renal cell carcinoma	18	34.6
B. Transitional cell carcinoma of renal pelvis	5	9.6
C. Wilms' tumor (Nephroblastoma)	2	3.8
D. Renal sarcoma	1	1.9
E. Renal oncocytoma	2	3.8

Table 1: Patients histopathological subtypes.

were scored by using Sambrook standard curve [8]; Standard curves were constructed between molecular weight of the DNA marker bands(bp) and their corresponding electrophoretic mobilities (mm) (The electrophoretic mobility is the distance in mm between mid well and mid zone of the specific DNA band). The relationships between the presence of new bands (tumor related bands) and the histopathological subtypes were assessed using chi squared analysis for 20 samples and more, while Fisher' 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. 17 (IBM, US)

Results

Genomic DNAs were extracted from all studied urine samples including cancer patients and negative controls and electrophoresed in 0.7% agarose gels. Figures 1, 2 and 3 shed light upon different examples of gel electrophoresis of extracted DNA from renal cancer and healthy controls respectively.

RAPD-PCR analysis was carried out on the selected DNA samples obtained from exfoliated cells in urine of both renal cancer and healthy using Bio-A09 primer. The primer Bio-A09 produced five major normal bands of amplified fragments and were appeared in normal RAPD pattern; their molecular sizes

Figure 1: Gel electrophoresis of genomic DNA extraction of 8 healthy samples (H1 to H8) in 0.7% TAE buffer agarose gel at 100 V for 1.5 hours. M: Lambda DNA Hind III-digested marker (23130-564 bp).

Figure 2: Gel electrophoresis of genomic DNA extraction of 7 renal cancer samples (R-a to R-g) in 0.7% agarose gel in 1x TAE buffer at 100 V for 40 minutes. M: Lambda DNA Hind III-digested marker (23130-564 bp).

Figure 3: Gel electrophoresis of genomic DNA extraction of 6 renal cancer samples (R-h to R-m) in 0.7% agarose gel in 1x TAE buffer at 100 V for 40 minutes. M: Lambda DNA Hind III-digested marker (23130-564 bp).

Figure 4: Gel electrophoresis of RAPD-PCR product of healthy DNA (H1-H8) amplified by primer Bio-A09. Electrophoresis was performed on (1%) agarose gel in 1x TAE buffer at 80 volt for 2 hrs. M: Gene Ruler™ 100 bp plus DNA ladder marker (3000-100) bp.

were 650, 750, 1000, 1100, 1500 bp respectively (Figure 4). The primer produced thirteen different tumor related bands, and there molecular sizes were 180, 250, 350, 400, 500, 550, 900, 1250, 1300, 1400, 1650, 1700 and 1900 bp (Table 2, Figure 5-13). However normal white blood cells and epithelial cells which present in patients urine are potentially in a very low quantities if compared to the exfoliated tumor cells quantities. So; the genomic DNA and RAPD-PCR DNA product of that normal white blood cells and epithelial cells is significantly negligible in relation to that of the tumor cells. The RAPD-PCR analysis of renal cancer patterns revealed loss of normal bands and appearance of new tumor related bands (Table 2).

Conventional renal cell carcinoma (RCC)

Clear renal cell carcinoma (clear RCC): Eight new tumor related bands appeared with molecular sizes around 180, 250, 350, 400, 500, 900, 1250 and 1400 bp. The incidence of them were 95.5%, 77.2%, 54.5%, 54.5%, 100%, 50%, 45.5% and 13.6% respectively (Figures 5,6). By running the statistical analyses (Chi-square tests) there were a significant association between the presence of 180 ($X^2 = 25.5$, $p < 0.001$), 250 ($X^2 = 14.26$, $p < 0.001$), 350 ($X^2 = 7.273$, $p = 0.007$), 400 ($X^2 = 7.273$, $p = 0.007$),

Histo-pathological types	No. of tested	Percentages of amplified DNA bands																	
		Tumor bands									Normal bands								
		180 bp	250 bp	350 bp	400 bp	500 bp	550 bp	900 bp	1250 bp	1300 bp	1400 bp	1650 bp	1700 bp	1900 bp	650 bp	750 bp	1000 bp	1100 bp	1500 bp
Clear RCC	22	95.5	77.2	54.5	54.5	100.0	9.0	50.0	45.5	4.5	13.6	0.0	0.0	0.0	45.4	72.7	72.7	59.1	33.3
Papillary RCC	18	44.4	44.4	16.7	27.8	100.0	5.6	16.6	44.4	5.6	0.0	0.0	0.0	33.3	22.2	11.0	27.8	33.3	44.4
Chromophobe RCC	2	0.0	0.0	0.0	0.0	0.0	100.0	50.0	0.0	0.0	100.0	100.0	0.0	0.0	0.0	50.0	100.0	50.0	0.0
TCC	5	0.0	0.0	60.0	40.0	100	0.0	0.0	0.0	60.0	0.0	0.0	100.0	40.0	0.0	40.0	60.0	100.0	0.0
Wilms' tumor	2	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	100.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0	0.0
Renal sarcoma	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0
Renal oncocyoma	2	0.0	100.0	100.0	100.0	100.0	0.0	0.0	0.0	50.0	0.0	0.0	0.0	0.0	50.0	100.0	100.0	100.0	100.0
Healthy controls	8	0.0	0.0	0.0	0.0	12.5	0.0	0.0	0.0	0.0	0.0	12.5	0.0	0.0	100	100	100	75.0	62.5

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

500 ($X^2 = 25.12, p < 0.001$), 900 ($X^2 = 6.32, p = 0.012$) and 1250 ($X^2 = 5.455, p = 0.02$) bp bands and this clear RCC histosubtype. On the other hand, there is no significant association between this histosubtype and the presence of 1400 ($X^2 = 1.21, p = 0.271$) bp band.

Papillary renal cell carcinoma (PRCC)

The study results showed the incidence of eight tumor related bands of 180, 250, 350, 400, 500, 900, 1250 and 1900 bps in the genomic PRCC were 44.4%, 44.4%, 16.7%, 27.8%, 100%, 16.65, 44.4% and 33.3% respectively (Figures 7,8). By running the statistical analyses (Chi-square tests) there were a significant association between the presence of 180 ($X^2 = 5.136, p = 0.023$), 250 ($X^2 = 5.136, p = 0.023$), 500 ($X^2 = 21.55, p < 0.001$), and 1250 ($X^2 = 5.136, p = 0.023$) bp bands and PRCC histosubtype. On the other hand, there is no significant association between this histosubtype and the presence of 350($X^2 = 1.507, p = 0.22$), 400($X^2 = 2.751, p = 0.097$), 900 ($X^2 = 3.46, p = 0.063$) and 1900 ($X^2 = 3.46, p = 0.063$) bp bands.

Figure 6: Gel electrophoresis of RAPD-PCR product of clear renal carcinoma samples (R12-R22) amplified by primer Bio-A09. Electrophoresis was performed on (1%) agarose gel in 1x TAE buffer at 80 volt for 1.5 hrs. M: Gene Ruler™ 100 bp plus DNA ladder marker (3000-100 bp).

Figure 7: Gel electrophoresis of RAPD-PCR product of papillary renal carcinoma (R23-R31) amplified by primer Bio-A09. Electrophoresis was performed on (1%) agarose gel in 1x TAE buffer at 90 volt for 1.15 hrs. M: Gene Ruler™ 100 bp plus DNA ladder marker (3000-100 bp).

Figure 5: Gel electrophoresis of RAPD-PCR product of clear renal cell carcinoma samples (R1-R11) amplified by primer Bio-A09. Electrophoresis was performed on (1%) agarose gel in 1x TAE buffer at 80 volt for 1.5 hrs. M: Gene Ruler™ 100 bp plus DNA Ladder (3000-100 bp).

Chromophobe renal cell carcinoma

Three major significant bands ($p = 0.02$, using Fisher's exact test) with molecular sizes 550, 1400 and 1650 bps appeared in all of the samples (Figure 9). On the other hand band 900 bp not considered to be a significant band ($p = 0.2$).

Transitional cell carcinoma of renal pelvis (renal TCC)

Five new tumor related bands appeared with molecular sizes 350, 400, 500, 1300, 1700 and 1900 bp. The incidence of them were 60%, 40%, 100%, 60%, 100%, and 40% respectively. Fisher's exact test finds an evidence of association between the presence of 350, 500, 1300, and 1700 bp bands and TCC (p -values 0.035, 0.005, 0.035 and 0.001 respectively). Both 400 ($p = 0.128$) and 1900 ($p = 0.128$) bp bands considered to be non significant bands (Figure 10).

Wilms' tumor (Nephroblastoma)

Three significant bands with molecular sizes 550, 1400 and 1700 bp (p values 0.022, 0.022 and 0.022, using Fisher's exact test) are appeared (Figure 11).

Renal Sarcoma

It was found an absence of all the normal bands. It is also worth mentioning that we have only one sample of this histotype, so it is difficult to detect significances or a marker bands. A tumor related band with molecular size 1900 bp appeared in this sample (Figure 12).

Renal oncocytoma

The pattern of renal oncocytoma expressed high similarity to the healthy pattern in their major normal bands 650(50%), 750(100%), 1000(100%), 1100(100%) and 1500(100%). In addition, Fisher's exact test finds no evidence of association between tumor related bands 250 ($p = 0.022$), 350($p = 0.022$), 400($p = 0.022$), 500($p = 0.022$) and 1300 bp ($p = 0.2$) and renal oncocytoma tumor (Figure 13).

Discussion

The randomly amplified polymorphic DNA (RAPD) protocol was reliable, simple to set up, fast and large areas of genomic DNA

Figure 8: Gel electrophoresis of RAPD-PCR product of papillary RCC (R32 – R40) amplified by primer Bio-A09. Electrophoresis was performed on (1%) agarose gel in 1x TAE buffer at 80 volt for 1.5 hrs. M: Gene Ruler™ 100 bp plus DNA ladder marker (3000-100 bp).

Figure 9: Gel electrophoresis of RAPD-PCR product of chromophobe renal cell carcinoma (R41-42) amplified by primer Bio-A09. Electrophoresis was performed on (1%) agarose gel in 1x TAE buffer at 80 volt for 2 hrs. M: Gene Ruler™ 100 bp plus DNA ladder marker (3000-100 bp).

Figure 10: Gel electrophoresis of RAPD-PCR product of transitional renal cell carcinoma (R43-47) amplified by primer Bio-A09. Electrophoresis was performed on (1%) agarose gel in 1x TAE buffer at 80 volt for 2 hrs. M: Gene Ruler™ 100 bp plus DNA ladder marker (3000-100 bp).

Figure 11: Gel electrophoresis of RAPD-PCR product of Wilms' tumor (R48-R49) amplified by primer Bio-A09. Electrophoresis was performed on (1%) agarose gel in 1x TAE buffer at 80 volt for 2 hrs. M: Gene Ruler™ 100 bp plus DNA Ladder Marker (3000-100 bp).

Figure 12: Gel electrophoresis of RAPD-PCR product of renal sarcoma (R50) amplified by primer Bio-A09. Electrophoresis was performed on (1%) agarose gel in 1x TAE buffer at 80 volt for 2 hrs. M: Gene Ruler™ 100 bp plus DNA ladder (3000-100 bp).

Figure 13: Gel electrophoresis of RAPD-PCR amplification pattern of renal oncocytoma (R51-R52) amplified by primer Bio-A09. Electrophoresis was performed on (1%) agarose gel in 1x TAE at 90 volt for 1.15 hrs. M: Gene Ruler™ 100 bp plus DNA ladder (3000-100 bp).

screened. The RAPD-PCR amplification with a single decamer primer is affected essentially with Primer, DNA template and reaction conditions [9]. So that these advantages in comparison to other DNA fingerprinting method, such as restriction fragment length polymorphism (RFLP) that need larger quantity of pure DNA, target gene or few loci of the gene and some information's about DNA restriction sites and restriction enzymes. So the RAPD method has the potential to detect a wide range of DNA

damage as well as mutations [10]. To our knowledge, our study is the unique study that applying the RAPD-PCR test to detect the genomic instability in renal cancer histopathological types. Other studies have been successfully used RAPD assay to detect the genomic instability in brain tumors [11], lung cancer [12], breast cancer [13], colon cancers [14], head and neck squamous cell carcinoma [15], acute lymphoblastic leukemia [16], hepatocellular carcinoma [17], astrocytic tumors [18], skin cancers [19] and pituitary adenomas [20]. The study of Ibrahim et al. (2010) used the same primer, which we already used in our study, in diagnosis of acute lymphoblastic leukemia. It was found that, a band which represents DNA fragment of 980 bp was absent in normal individual but it was present in 55.6% of all leukemia patients. In our study we used the same synthetic primer (5'-GGGTAACGCC-3') and showed the possibility of using this primer to identify the genomic DNA instability of renal cancer patients when compared with normal individuals. The observation of newly appeared DNA fragment of 500 bp (marker band) in the tumor samples was of 90.4% of the screened cases. In other words, a 500 bp band was significant band in clear RCC ($P < 0.001$), papillary RCC ($P < 0.001$), renal TCC ($P = 0.005$) and renal oncocytoma ($P = 0.022$). Moreover, the appearing of 180 ($X^2 = 13.15, p < 0.001$), 250 ($X^2 = 9.524, p = 0.002$), and 1250 ($X^2 = 5.357, p = 0.021$) bp bands in RAPD-PCR product of conventional RCC might suggest the possibility of using these DNA fragments as molecular markers for detection of the genomic instability of this major type of renal carcinoma. Also we suggested 1700 bp band as a marker band for both renal TCC ($p = 0.001$) and Wilms' tumor ($p = 0.022$). The pattern of renal oncocytoma (benign tumor) expressed high similarity to the healthy pattern. So, our present study agrees to the study of Liping et al. (2003) in proving the role of RAPD-PCR in diagnosis of tumor development generally. In addition, the gender difference by using RAPD-PCR method was not one of our study goals in the present study that is why it was not addressed or discussed. Moreover, further work is required on larger sample of normal individuals and cancer patients to calculate the specificity and the sensitivity for such novel diagnostic biomarkers using other large scale oriented statistical methods. Furthermore, cloning and sequencing of appeared marker bands would have been nice to know which specific genes are changed in urine samples from cancer patients compared to the controls and compare or combine it with some kinds of diagnostic biochemical tools done in other tumors [21,22].

Conclusion

RAPD decamer primer Bio-A09 produced 500 DNA fragments in conventional RCC (clear and papillary) and renal TCC type in addition to 1700 DNA fragments in in Wilms' tumor and renal TCC, these DNA fragments were not detected in tested normal individuals. Also, convention RCC characterized by three bands with molecular sizes 180, 250 and 1250bp not found in other types. The amplified DNA fragment might be proved useful for further development of molecular markers for diagnosis of renal cancer. The study gives new evidence of potential promise of RAPD-PCR analysis in cancer research for detecting and selecting

novel genomic markers and proving the role of RAPD-PCR in diagnosis of tumor development generally.

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