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Analysis of Genomic Instability and Tumor-Specific Genetic Alterations by Arbitrarily Primed PCR

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1. Introduction

It is now widely accepted that cancer development is a multistage process that results from an accumulation of mutations (Lengauer et al., 1998). Since spontaneous mutation rates in human cells are considerably lower then the large number of mutations observed in cancer cells, cancer cells must be a manifestation of the mutator phenotype. The mutator phenotype, also referred to as genomic instability, designates the increased mutation rate that occurs in neoplastic cells (Loeb, 1991). The induction of the genomic instability phenotype is emerging to be a crucial early event in carcinogenesis that enables an initiated cell to evolve into a cancer cell by achieving a greater proliferative capacity and genetic plasticity, which can overcome host immunological resistance, localized toxic environments and a suboptimal supply of micronutrients (Loeb, 1991; Cahill et al., 1999; Fenech 2002). Two distinct forms of genomic instability have been identified, microsatellite instability (MIN) and chromosomal instability (CIN). They probably encompass most characterized malignances (Lengauer et al., 1998; Breivik & Gaudernack, 1999). Genomic instability is present in all stages of cancer, from precancerous lesions to advanced cancers (Negrini et al., 2010; Markovic et al., 2008)

Measurements of instability have been performed by a variety of techniques, including flow cytometry, comparative genomic hybridization (CGH), allelotyping, and analysis of gene amplification rates (Vogelstein et al., 1989; Kallioniemi et al., 1994; Jass et al., 1994). These approaches, although informative, are generally cumbersome and somewhat impractical for widespread clinical use. Unlike these techniques, DNA fingerprinting methods, RAPD (Random Amplified Polymorphic DNA) and AP-PCR (Arbitrarily Primed Polymerase Chain Reaction) are rapid and simple procedures that examine the whole genome and detect the propensity of a tumor to undergo genomic rearrangements (Peinado et al., 1992; Perucho et al., 1996).

AP-PCR is a PCR-based DNA fingerprinting method that utilizes arbitrarily chosen primers to co-amplify multiple and independent sequences under low stringency conditions during the first cycles. It was first described by Welsh and McClelland (1990), who designed it to amplify multiple DNA fragments from anonymous regions of the genome. Initial cycles of

the reaction are performed under low stringency conditions which are achieved with low temperatures during the annealing step of PCR and/or high magnesium concentration in the reaction. Under these conditions the arbitrary primer anneals to the best matches in the template. The priming events during the initial low stringency cycles are arbitrary since they depend on the nucleotide sequence of the PCR primer, which is arbitrarily chosen. Competition between these annealing events results in reproducible and quantitative amplification of many discrete bands. Further amplification of these sequences (discrete bands) under high stringency conditions produces a complex fingerprint which can be visualized by gel electrophoresis. The obtained band pattern is characteristic and representative of the genome used as template.

The large number of bands amplified with a single arbitrary primer generates a complex fingerprint that can be used to detect differences in the arbitrary amplified DNA sequences from two different but closely related genomes, like DNA from normal and cancer cells. Such differences correspond to somatic genetic alterations. In addition, AP-PCR method permits direct cloning and identification of altered variant bands i.e. altered DNA sequences. Therefore, this unbiased methodology allows for molecular karyotyping of somatically acquired genomic abnormalities, comparing related genomes, whereby one is a derivative of the other emerging via undefined and abnormal genomic events. Indeed, AP-PCR has been successfully used as a molecular alternative for cancer cytogenetics since it has proved to be capable of detecting chromosomal gains and losses as well as point mutations associated with carcinogenesis (Perucho et al., 1996; Chariyalertsak et al., 2005). This is based on the following favorable properties of the method: (i) the amplified bands usually originate from single copy sequences rather then from repetitive elements; (ii) there is no apparent bias for the chromosomal origins of the amplified bands, and therefore, fingerprints representative of the full chromosomal complement can be obtained by using a few arbitrary primers; (iii) the amplification is semi-quantitative, that is, the intensities of the amplified bands are almost proportional to the concentration of the corresponding template sequences.

Taking into account the potential and advantages of AP-PCR method, it seems as a reasonable approach to use this method to detect and quantify the level of genomic instability in various cancer samples. Therefore, we applied AP-PCR to measure genomic instability in samples of patients with Non Small Cell Lung Carcinoma (NSCLC) of various stages and grades, samples of patients with Malignant Gliomas of various grades (Anaplastic Astrocytomas and Glioblastomas) and samples of patients with Head and Neck Squamous Cell Carcinoma (HNSCC) and their premalignant lesions leukoplakias. Moreover, we aimed to identify some of these genomic alterations associated with the process of carcinogenesis in these types of tumors.

Here we describe the procedure for analyzing the level of genomic instability and identifying specific genetic alterations that occur during the tumorigenic process by Arbitrarily Primed PCR. This procedure involves the following steps: (i) comparative AP-PCR analysis of matching normal and tumor tissue and determination of the frequency of DNA alterations, a measurement of genomic instability; (ii) correlation between the level of genomic instability and histological grade and stage of each tumor; (iii) isolation and identification of altered amplified bands. Obtained results are presented and discussed in terms of the evolution of these types of tumors.

2. Materials and methods

2.1 Tissue samples and DNA extraction

Paired tumor and normal tissue samples (adjacent normal lung tissue and blood for malignant gliomas, HNSCC and leukoplakias) were analyzed. Specifically, 30 malignant glioma patients who underwent surgical resection at Clinic for Neurosurgery, Clinical Center of Serbia, 30 NSCLC patients who underwent surgery at the Institute for Lung Diseases and Tuberculosis, Clinical Centre of Serbia, 32 leukoplakia patients and 30 HNSCC patients who underwent surgery at the Clinic of Maxillofacial Surgery, School of Dentistry, University of Belgrade. Freshly excised tissue samples were partitioned for histopathology and DNA analyses. The specimens for DNA analyses were frozen in liquid nitrogen until DNA extraction. The samples were collected and used after obtaining informed consents and approval from the Ethics Committee, in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

DNA was extracted using the phenol/chloroform/isoamyl alcohol method (Sambrook et al., 1989). The quality of the DNA was verified by electrophoresis on 0.8% agarose gel. The DNA concentration was assessed spectrophotometrically.

2.2 AP-PCR DNA fingerprinting

Genomic instability was determined by comparing the AP-PCR profiles of paired tumor and normal DNA samples of the same patient. Altogether, twenty primers were tested for the ability to generate informative fingerprints that distinguish tumor from normal tissue. Optimization of AP-PCR reactions was done for each primer according to Cobb (1997) and included the search for conditions that would yield profiles of moderate complexity in order to simplify the analysis (McClelland & Welsh, 1994). Normally, optimization of AP-PCR DNA fingerprinting would require each variable to be tested independently. An experiment investigating the effects and interactions of four critical reaction components (dNTPs, $MgCl_2$, primer and DNA), each at three concentrations, would require 81 (i.e., 3^4) separate reactions. However, using modified Taguchi method (Taguchi & Wu, 1980, as cited in Cobb, 1997) only nine reactions are required to perform the same optimization. Here an estimate of the effect of individual components is achieved by looking at the effects that component interactions have on the fingerprint. These interactions are determined by arranging those components that are likely to affect the reactions into an orthogonal array. The product yield for each reaction is used to estimate the effects of individual components on amplification. We varied the PCR components in the following final concentrations: dNTPs (0.2 mM, 0.4 mM, 0.6 mM), $MgCl_2$ (1.5 mM, 2.5 mM, 3.5 mM), primer (1.5 μM, 3.0 μM, 5.0 μM) and DNA (50 ng, 100 ng, 150 ng). DNA concentration did not affect AP-PCR fingerprints and it was used to validate the method. Namely, after optimal reaction conditions were established, each experiment included the analysis of two template concentrations (25 ng and 50 ng in a final volume of 25 μL) for each individual in order to exclude artifacts arising from impurities in the DNA preparations.

Twelve out of twenty primers produced informative profiles differentiating normal from tumor tissue. Primer sequences, AP-PCR conditions and reaction mixtures are given in Table 1.

Table 1. Primer sequences, AP-PCR conditions and reaction mixtures

Primer	Primer sequence	AP-PCR low-stringency conditions	AP-PCR high-stringency conditions
CCNA1	5′-AAG AGG ACC AGG AGA ATA TCA-3′	95°C 30″ 45°C 2′ 72°C 1′	95°C 30″ 60°C 1′ 72°C 1′
LRP-A	5′-GCT TCC GAG GTC TCA AAG C-3′	95°C 30″ 40°C 2′ 72°C 1′	95°C 30″ 58°C 1′ 72°C 1′
MDR-A	5′-GTT CAA ACT TCT GCT CCT GA-3′	95°C 30″ 40°C 2′ 72°C 1′	95°C 30″ 58°C 1′ 72°C 1′
E8S p53	5′-TAA ATG GGA CAG GTA GGA CC-3′	95°C 30″ 40°C 2′ 72°C 1′	95°C 30″ 58°C 1′ 72°C 1′
GAPDH-S	5′– CGG AGT CAA CGG ATT TGG TCG TAT– 3′	95°C 30′′; 50°C 2′; 72°C 1′	95°C 30′′; 70°C 1′; 72°C 1′
GAPDH-A	5′–AGC CTT CTC CAT GGTGGT GAA GAC–3′	95°C 30″; 50°C 2′; 72°C 1′	95°C 30″; 72°C 1′; 72°C 1′
E5A p53	5′–CAG CCC TGT CGT CTC TCC AG–3′	95°C 30″; 40°C 2′; 72°C 1′	95°C 30″; 55°C 1′; 72°C 1′
p53 A	5′–TTG GGC AGT GCT CGC TTA GT–3′	95°C 30″; 40°C 2′; 72°C 1′	95°C 30″; 60°C 1′; 72°C 1′
H61-5′	5′-AGG TGG TCA TTG ATG GGG AG-3′	94°C 1′; 45°C 2″, 72°C 2′	94°C 1′; 62°C 1′; 72°C 2′

The reactions consisted of an initial denaturation step (95ºC for 5 min), 4 cycles at low-stringency conditions (specific for each primer), 35 cycles at high-stringency conditions (specific for each primer), and a final extension (72ºC for 7 min) in a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, California, USA).

The AP-PCR products were separated on 6 – 8% non-denaturing polyacrylamide (PAA) gels and visualized by silver staining. Silver- staining procedure creates permanent record of the electrophoresis results and includes several steps: fixing, silver impregnation, development and stopping the reaction. In the fixing step, the gel is treated with 1% nitric acid solution to render the macromolecules in the gel insoluble and prevent diffusion during the subsequent staining steps. In the silver impregnation step, soluble silver ion (Ag+) derived from the silver nitrate, 12mM $AgNO_3$ solution, binds to nucleic acid bases fixed in gel. Generally, DNA bases promotes the reduction of silver ion to metallic silver (Ag0), which is insoluble and visible, allowing nucleic acid-containing bands to be seen. In order to prevent reduction of silver ion to metallic silver before the end of silver impregnation, this step is often performed in mildly acidic acid conditions. During the development step, formaldehyde reduces silver ions to metallic silver in process that only proceed at high pH, approximately 12. For that reason, sodium carbonate is included as one of the main component that render development solution alkaline. Stopping reaction step imply prevention of any further silver ion reduction by soaking the gels in the 10 % acetic acid solution. Finally, it should be emphasized that water washes are also included between some of the above mentioned steps in the silver staining procedure (detailed procedure is given in Table 2).

Step	***Solution***	***Time***
Fixation Pretreatment	10% Ethanol	10 minutes
Fixation	1% Nitric Acid Solution	3 minutes
Water Washing	Destiled H2O	2 x 1 minute
Silver Impregnation	12 mM Silver Nitrate Solution	30 minutes
Water Washing	Destiled H2O	3 x 1 minutes
Developing - Reduction	0.28 M Sodium Carbonat with 0.019 % Formaldehyde	Until desired images appear
Stopping Reduction	10 % Acetic Acid	5 – 10 minutes

Table 2. In-house procedure for silver- staining of PAA gels.

Gel images were acquired with the Multi-Analyst/PC Software Image Analysis System (Bio Rad Gel Doc 1000). Digitized images were loaded into the specialized public software Image J (National Institute of Health, USA, www.rsb.info.nih.gov/ij) and analyzed by the image enhancement function 'adapthisteq'. This function performs contrast-limited adaptive

histogram equalization on small regions of the image, called tiles. Contrast of each tile is enhanced so that the histogram of the output region approximately matches a specified histogram. After equalization, adapthisteq combines neighboring tiles using bilinear interpolation to eliminate artificially induced boundaries.

2.2.1 Reproducibility

The problem of reproducibility of AP-PCR has been a matter of concern for quite some time (Meunier and Grimont, 1993; McClelland and Welsh, 1994). In our study, reproducibility was verified by at least three independent reactions and a reaction with a two-fold higher template concentration. Occasional irreproducibility was found to be due to template quality, where additional round of purification solved the problem. Template carry-over was routinely monitored by systematic incorporation of "no-template reaction" in each set of experiments. Day to day variation was found only in respect of band intensities. This variability was in the range of less than 10% (± 5%) as estimated by integration of densitometric scans. Interlab variation was not assessed but we presume that it does not affect the interpretation of data from this report.

2.3 Isolation, cloning and DNA sequencing of variant bands obtained by AP-PCR

Selected variant DNA bands, bands with altered mobility, were further characterized. The PCR amplicons resolved on the silver stained gels were gently removed with a hypodermic 22-gauge needle pre-wetted with the PCR master mix solution. The needle was dipped in the PCR master mix for 2 min and then discarded. The PCR products were reamplified with the same primers used for AP-PCR reactions at high-stringency conditions specific for each particular primer. The reamplified material was administrated on 1.5% agarose gels, purified using DNA Extraction Kit (Fermentas Life Sciences, Lithuania) and cloned with GeneJetTM PCR Cloning Kit (Fermentas Life Sciences, Lithuania) according to manufacturers' instructions. Plasmids were purified using GeneJetTM Plasmid Miniprep Kit (Fermentas Life Sciences, Lithuania).

Cloning process consisted of setting up the blunting and ligation reactions. Blunting reaction allows the conversion of PCR products generated with non-proofreading Taq DNA polymerase to DNA fragments with blunt ends using thermostable DNA Blunting Enzyme provided with the kit. The reaction consists of 10 µL of 2x Reaction Buffer, 2 µL of non-purified PCR product, 5 µL of nuclease free water and 1 µL of DNA Blunting Enzyme in 18 µL reaction mixture. The resulting blunt-ended DNA can be ligated efficiently into a vector, pJET1.2/blunt, using the included DNA Ligation Kit Solutions: 1 µL of pJET1.2/blunt Cloning Vector (50ng/ µl) and 1 µL of T4 DNA Ligase (5u/µl). The vector contains a lethal restriction enzyme gene that is disrupted by ligation of a DNA insert into the cloning site. As a result, only bacterial cells with recombinant plasmids are able to form colonies. Recircularized pJET1.2/blunt vector molecules lacking an insert express a lethal restriction enzyme which kills the host *E.coli* cell after transformation. This positive selection drastically accelerates the process of colony screening and eliminates additional costs required for blue/white selection. The reactions can be used directly for bacterial transformation and in vitro packaging procedures without further purification. All common laboratory *E.coli* strains can be directly transformed with the ligation product.

Before the transformation procedure, the preparation of competent bacteria of *E. coli* GM2163 strain was performed using TransformAid™ Bacterial Transformation Kit (Fermentas Life Sciences, Lithuania) according to the manufacturer instruction.

The next step was to recover plasmid DNA from recombinant *E.coli* cultures using GeneJET™ Plasmid Miniprep Kit (Fermentas Life Sciences, Lithuania). A single colony was picked from a freshly streaked selective plate for inoculation of 5 mL of LB liquid medium (Fermentas Life Sciences, Lithuania) supplemented with the ampicillin. A bacterial culture is harvested and lysed. The lysate is then cleared by centrifugation and applied on the silica column to selectively bind DNA molecules at a high salt concentration. The adsorbed DNA is washed to remove contaminants, and the pure plasmid DNA is eluted in a small volume of elution buffer or water. The purified DNA is ready for immediate use in all molecular biology procedures such as automated sequencing. Before sequencing, the ligation of DNA fragment into the plasmid was verified using restriction enzymes HindIII and EcoRI (Sigma-Aldrich Chemie GmbH, Germany). The fragments obtained after restriction were analyzed on 1% agarose gels. The sequencing was performed only after the presence of the DNA fragment in plasmid was confirmed by comparing the molecular weight of recombinant plasmid with DNA ladder.

Sequences were determined on ABI Prism 3130 Genetic Analyzer automated sequencer (Applied Biosystems, Foster City, CA, USA) using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Sequencing was performed in both directions on several clones for each selected DNA band. The obtained sequences were analyzed using BLAST software in the NCBI GenBank and EBI (Sanger Institute) database.

The sequencing procedure itself involved: 1) two independent cycle sequencing PCRs, each with one primer only (5′ and 3′), for the sequencing in both directions; 2) precipitation of the amplicons; 3) their denaturation and 4) automatic electrophoresis. Cycle sequencing PCRs were performed on the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA) using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the final concentration of 100-300 ng of the plasmid DNA and 4pmol of the primer under the following conditions: initial denaturation at 96°C for 1 min, 25 cycles at 96°C for 10 s, 50°C for 5 s, 60°C for 4 min and at 4°C indefinitely. The obtained PCR products were precipitated and EDTA (25 mM final) and EtOH (70-75% final) added.The mixture was incubated for 15 min at RT and then centrifuged 30-45 min at 6000 rpm and +4°C. The supernatant was removed, a new quantity of 70% EtOH added, followed by centrifugation for 25 min at 5000 rpm and +4°C. Supernatant was removed again and the obtained pellet dried at 90°C. Then, 15 µl of Hi-DI™ Formamide (Applied Biosystems, Foster City, CA, USA) was added for the denaturation at 95°C. 10 µl of the amplicons dissolved in formamide were subjected to the automatic electrophoresis and sequence reading on ABI Prism 3130 Genetic Analyzer automated sequencer (Applied Biosystems, Foster City, CA, USA). The obtained sequences were analyzed using BLAST software in the NCBI GenBank and EBI (Sanger Institute) database.

3. Results and discussion

Genomic instability was determined by comparing the AP-PCR profiles of DNA isolated from paired normal and tumor tissues of patients with non small cell lung cancer (NSCLC),

malignant glioma, head and neck squamous cell carcinoma (HNSCC) and leukoplakia (L). Twelve out of twenty tested primers produced informative amplification profiles differentiating normal from tumor tissue or normal from leukoplakia (Table 1). Specifically, five primers produced informative sequence alterations that distinguish NSCLC from normal tissue, a set of four primers produced informative fingerprints differentiating malignant gliomas from normal tissue and another set of four primers produced informative sequence alterations that distinguish HNSCC and leukoplakias from their normal counterparts. The AP-PCR products were separated on 6-8% nondenaturing polyacrylamide (PAA) gels and visualized by silver staining. Typical fingerprints are shown in Figure 1.

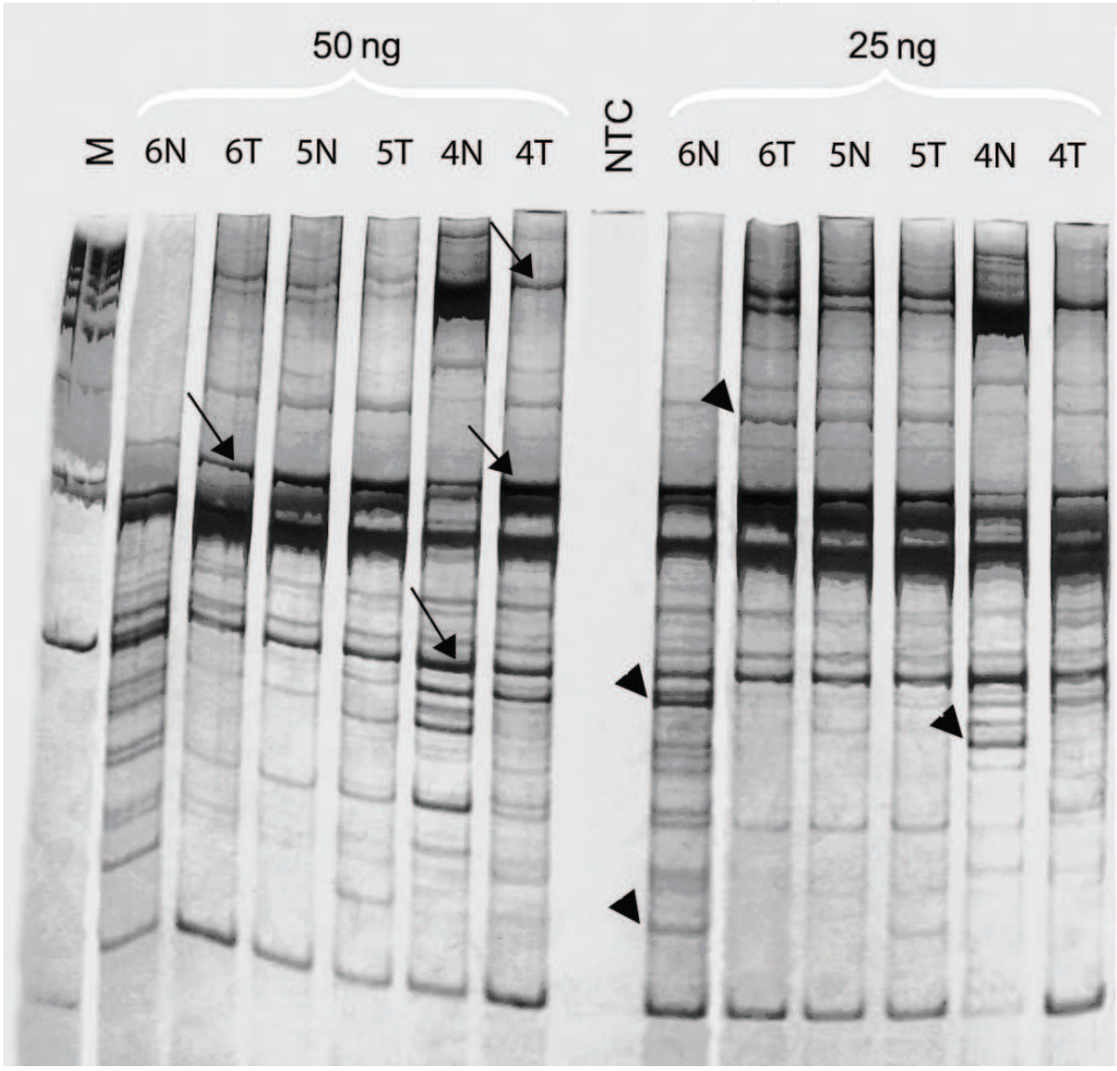

Fig. 1. AP-PCR fingerprint profiles of tumor (T) and normal (N) tissues from patients with NSCLC obtained with GAPDH AS primer. Reactions were performed in duplicate with 25 ng and 50 ng of DNA. Numbers 1–5 represent the patients; M–the DNA ladder; NTC–no template control. Arrows and arrowheads indicate examples of quantitative and qualitative changes, respectively.

This type of analysis differentiates individuals and, thus, displays the cardinal feature of the DNA profile analysis. Additionally, some bands are characteristic for the human genome, being common to all analyzed patients. Importantly, some electrophoretic bands were present in DNA profiles of tumor but not in normal tissue, and vice versa, indicating the mutational like events. The unbiased nature of AP-PCR profiling allows for the screening of anonymous regions of a genome without any prior knowledge of its structure (Welsh and McClelland, 1990; Williams et al., 1990) and provides information about two distinct types of DNA alterations: qualitative and quantitative. Qualitative differences, which represent microsatellite instability (MIN), are detected as mobility shifts in the banding pattern, i.e., the presence or absence of specific bands in tumor and control samples. Quantitative differences appear as altered band intensities and represent amplifications or deletions of existing chromosomal material as manifestations of chromosomal instability (CIN). Observed changes should be cautiously regarded as semi-quantitative and semi-qualitative due to the competitive nature of AP-PCR where sequence context may play unpredictable role. This situation may present a serious problem for simple to moderate patterns but not for complex patterns. Unfortunately, the former are preferred due to simplicity of interpretation. Since the profile is the result of a competition between many PCR products, the problem may appear with very simple profiles in analysis of similar but non-identical genomes. For this reason, it had been suggested to use profile pattern with more than 10 prominent PCR products of moderate complexity (McClelland and Welsh, 1994). We followed this reasoning and the necessary precautions for reproducibility and reliability of DNA profiling analysis in comparing DNA fingerprints of paired normal – tumor samples. We identified significant genomic instability in most cases as qualitative and quantitative electrophoretic changes. The qualitative alterations represented as a loss or a gain of a band are the result of mutations at the primer-template interaction sites leading to a mobility shift of a band. Quantitative changes were observed as bands of either decreased or increased intensity. Allelic losses, which may occur as a result of their linkage to suppressor genes, produce bands with decreased intensity. Gene amplification or chromosomal aneuploidy appears as bands with increased intensity.

For each type of DNA change, as well as for the total number of changes, the frequency of DNA alterations, a measurement of genomic instability, was calculated as the number of altered bands in the AP-PCR profile of tumor tissue divided by the total number of amplicons in the fingerprint of normal tissue from each patient. AP-PCR fingerprints were analyzed and qualitative and quantitative changes determined using image enhancement function ‘adapthisteq’ of the specialized public software Image J (Figure 2).

DNA alterations were detected in all analyzed samples with the frequency varying among different types of tumors (Table 3). The largest variation of the frequency of total DNA alterations was in NSCLC patients ranging from 8% to even 68%. The contribution of qualitative changes to overall genomic instability was significantly greater than the contribution of quantitative changes. This large range of instability raised the question of its distribution among samples of NSCLC patients. In other words we were interested to see if there was association between the level of genomic instability and any clinicopathological parameter.

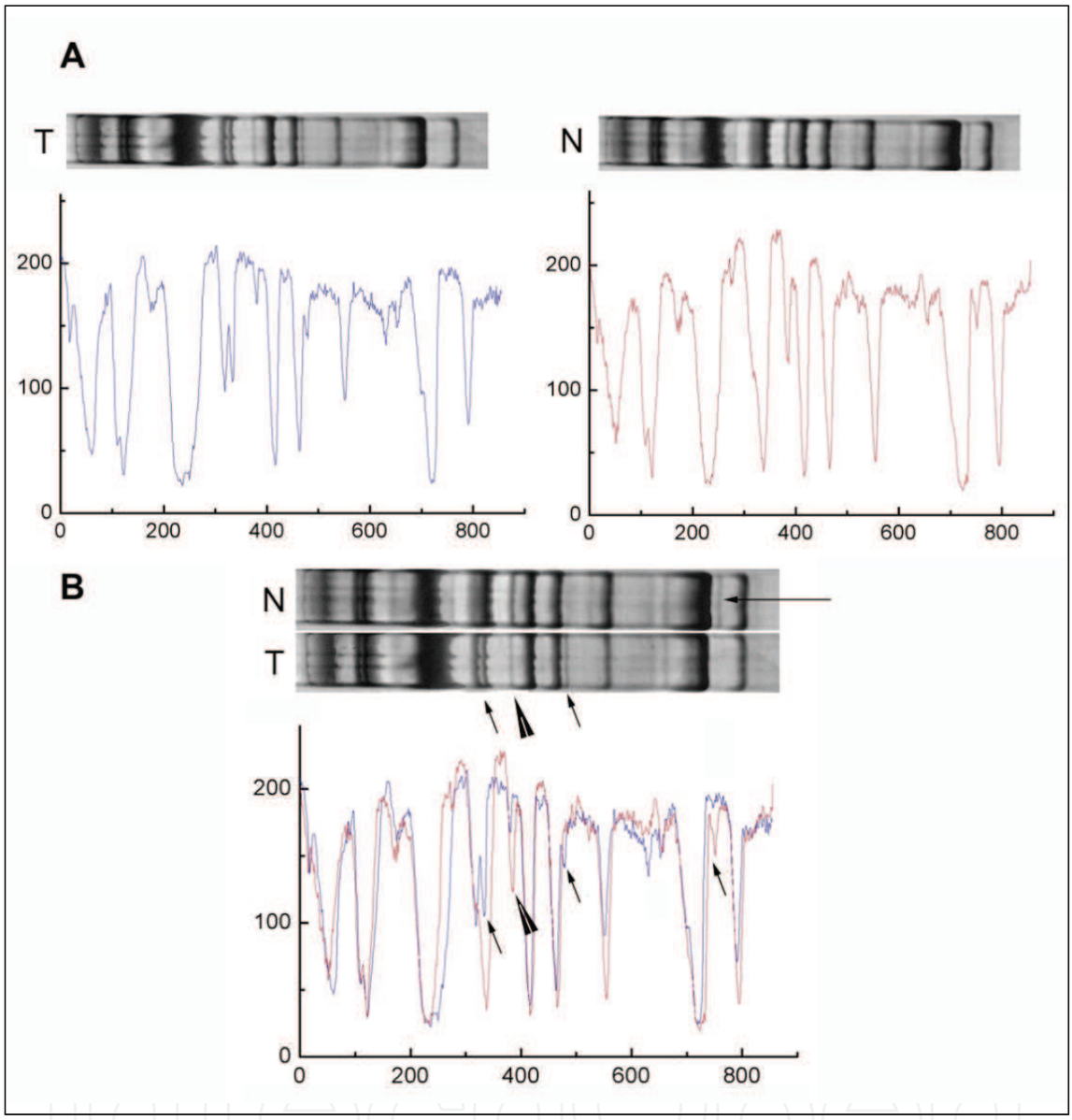

Fig. 2. AP-PCR fingerprinting analysis of genomic instability in glioma samples. AP-PCR profiles of tumor (T) and blood (N) tissues from the same patient obtained using MDRa primer, separated on 6% non-denaturing polyacrylamide (PAA) gel and corresponding contrast-limited adaptive histograms obtained using image enhancement function 'adapthisteq' of the specialized public software Image J (**A**). Arrows and arrowheads indicate examples of qualitative and quantitative electrophoretic changes respectively, clearly seen on the overlap of tumor and blood histograms (**B**).

The most noteworthy finding of this study was the association between the level of genomic instability and histological grades of NSCLC. Namely, we found the significant decrease of the total number of DNA alterations with increasing histological grade of the NSCLC. The same pattern was found for quantitative changes alone – the frequency of alterations

decreased with the increase of the histological grade (Figure 3). These results support the idea that mutational alterations conferring genomic instability and the mutator phenotype occur early during tumor formation. The mutator phenotype hypothesis proposes that such phenotypes result from mutations in genes that maintain genomic stability in normal cells. Instability promotes mutations in other genes, oncogenes and tumor suppressor genes, providing the tumor cell with a selective growth advantage. These findings strongly support the increasingly popular explanation of neoplastic transformation in terms of Darwinian evolutionary mechanisms (Breivik, 2001; Breivik & Gaudernack, 1999; Cahill et al., 1999). Evolution through natural selection depends on two essential elements, the availability of

type of DNA alteration	frequency of DNA alterations			
	NSCLC	glioma	leukoplakia	HNSCC
qualitative	0.07 – 0.53	0.06 – 0.27	0.18 – 0.41	0.05 – 0.21
quantitative	0.01 – 0.16	0.05 – 0.27	0.07 – 0.16	0.07 – 0.21
TOTAL	0.08 – 0.68	0.14 – 0.49	0.30 – 0.48	0.12 – 0.31

Table 3. Measurement of genomic instability in various types of tumors.

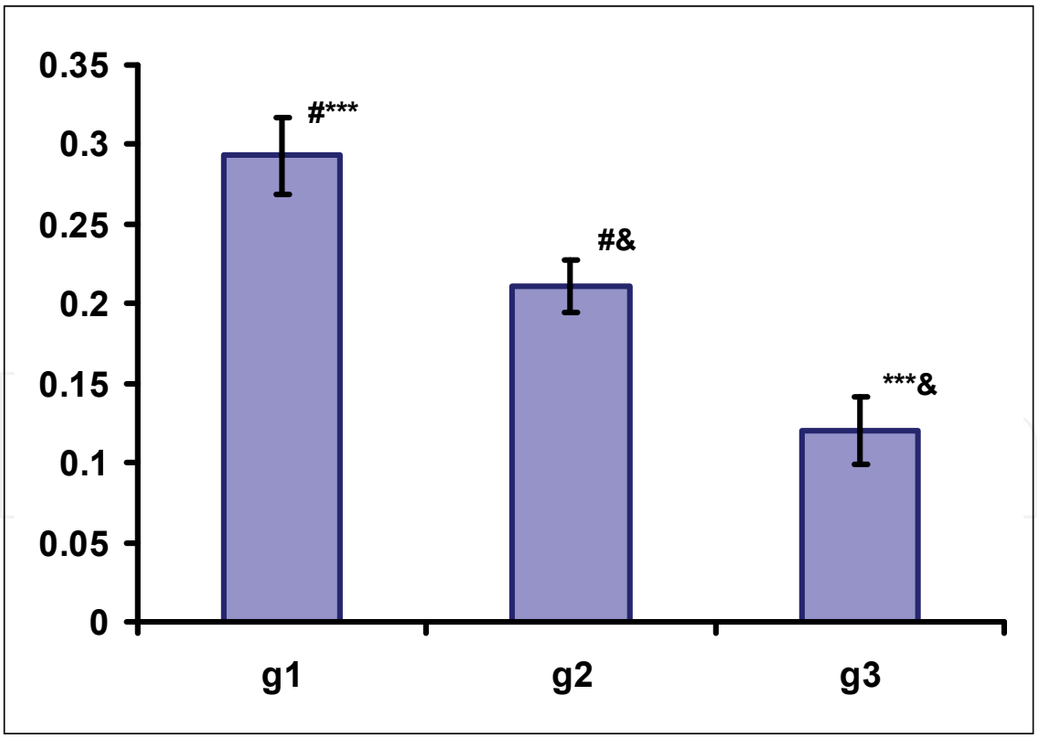

Fig. 3. The relationship between the total frequency of DNA alterations and the histological grades of the lung tumors. All values are presented as means ± SEM. # $p < 0.05$ when grade 1 was compared to grade 2; *** $p < 0.005$ when grade 1 was compared to grade 3; & $p < 0.05$ when grade 2 was compared to grade 3.

genetic variation and selection pressure (Dawkins, 1989.). In general evolutionary terms, it could be said that genomic instability accelerates the somatic evolutionary process by promoting genetic variation in an organism. Extensive genomic instability is thus expected in early phases of cancer progression (histological grade 1 in this study). At the same time, an increased mutation rate is expected to cause mutations that are deleterious or lethal at higher frequencies rather than mutations that have favorable effects on cellular proliferation. Consequently, elevated mutation rates must generally be regarded as disadvantageous to cellular growth (Tomlinson et al.,1996). Theoretical arguments suggest that the accumulation of large numbers of mutations can exceed the error threshold for cell replication and viability (Eigen, 1993). Only cells carrying reasonable number of mutations with favorable effects on cell growth would survive. Therefore, it seems probable that the expression of the mutator phenotype could be decreased and lost in the late phases of tumor progression. As a result, tumors may no longer exhibit a mutator phenotype but will nevertheless reveal its history, i.e. random mutations, throughout their genome (Loeb, 2001). In other words, the result showing the lower degree of genomic instability in advanced NSCLCs (grades 2 and 3) is not unexpected in the light of these arguments and could be considered as a marker of poor prognosis.

Following the study of genomic instability in NSCLC tissue samples, we made an attempt to identify some of detected DNA changes in order to identify genes that alter during NSCLC promotion and progression (Bankovic et al., 2010). Selected DNA bands with altered mobility were further characterized. Twenty one unique bands present only in tumor but not in normal tissue were retrieved from the gels and cloned. Variant bands that appeared in more than one sample (new bands with the same mobility), were chosen in order to identify DNA alterations common to as many NSCLC patients as possible. Bands (amplicons) with the same electrophoretic mobility were isolated and characterized from at least two patients in order to confirm that they represent the same DNA sequence. Three clones of each band were sequenced. Obtained sequences were submitted to homology or identity search in NCBI GenBank and EBI (Sanger Institute) databases. Following genes were identified: tetraspanin 14 (TSPAN14), cadherin 12 (CDH12), retinol dehydrogenase 10 (RDH10), cytochrome P450, family 4, subfamily Z, polypeptide 1 (CYP4Z1), killer cell immunoglobulin-like receptor (KIR), E2F transcription factor 4 (E2F4), phosphatase and actin regulator 3 (PHACTR3), PHD finger protein 20 (PHF20), PRAME (preferentially expressed antigen in melanoma) family member and solute carrier family 2 (facilitated glucose transporter), member 13 (SLC2A13). Moreover, we were able to identify types of mutations in revealed genes according to sequence data and BLAST search results and to examine their presence in relation to NSCLC subtype, histological grade and stage of the tumor, lymph node invasion and patients' survival. Examining their relation to the patients' clinicopathological parameters and survival we concluded that TSPAN14, SLC2A13 and PHF20 could have a role in NSCLC promotion, CYP4Z1, KIR and RDH10 would possibly play a role in NSCLC progression, while E2F4, PHACTR3, CDH12 and PRAME family member probably play important role in NSCLC geneses. Patients with altered E2F4 and PHACTR3 lived significantly shorter.

Unlike NSCLC samples, all leukoplakias demonstrated extensive instability in a relatively small range (Table 3). The frequency of total DNA alterations ranged from 0.30 to 0.48 and

clearly distinguished two groups of leukoplakias: a group of six leukoplakias had a frequency of DNA alterations of 0.3–0.34 and was denoted as leukoplakias with a moderate degree of instability while the other group of 26 leukoplakias had a frequency of DNA alterations of > 0.4 and was denoted as leukoplakias with a high degree of instability (Tanic et al., 2009). However, such high levels of genomic instability in leukoplakia samples were a surprise mainly because they are defined as white patches or plaques of oral mucosa that cannot be rubbed off and cannot be diagnosed clinically or pathologically as other specific diseases and have been considered premalignant lesions only since recently (Neville & Day, 2002; Hunter et al., 2005). It is impossible to state, with precision, the proportion of leukoplakias that undergo malignant transformation. For oral mucosa, in general, up to 20% of leukoplakias exhibit dysplasia. Dysplastic leukoplakias have a greater probability of developing into cancer, although leukoplakias without evidence of dysplastic changes may also progress to highly aggressive squamous cell carcinoma. Still, the majority of leukoplakias fail to undergo malignant transformation. The frequency of malignant alterations in oral leukoplakia varies from study to study and ranges from 8.9 to 17.5% (summarized in Neville & Day, 2002). These facts and our finding were the reasons to include samples of Head and Neck Squamous Cell Carcinoma patients, identify and quantify genomic instability in these samples and compare obtained results with those of leukoplakia samples.

Obtained frequency of DNA alterations in HNSCC samples was significantly lower than that of leukoplakia samples, as shown in Table 3. When comparing mean frequencies of DNA alterations the result is even more convincing. Namely, mean frequency of total DNA changes was 0.42 for leukoplakia samples vs. 0.28 for HNSCC samples. Interestingly, contribution of quantitative changes to the total instability in HNSCC samples is significantly higher (0.21) than the contribution of qualitative changes (0.16) which is quite opposite in leukoplakia samples. In other words, the level of genomic instability decreased during HNSCC promotion from premalignant lesions but more serious alterations, quantitative changes as manifestations of chromosomal instability, were selected. These results fit nicely into Darwinian evolutionary theory of neoplastic transformation. High instability is present at the very beginning of HNSCC genesis, providing genetic variability in the population of premalignant cells, which is absolutely necessary for the evolution by natural selection. During tumor progression the level of instability decreases due to selection of genotypes that are better adapted to the micro-environment in which natural selection took place. However, the question remains: why the majority of leukoplakias with such a huge instability fail to undergo malignant transformation? The answer may be in exceeding the error threshold for cell replication and viability (Eigen, 1993) with so many mutations. In other words, it seems that leukoplakias with a high degree of genomic instability have less chance to develop into HNSCC, whereas leukoplakias with a lower (moderate) degree of genomic instability have a better chance of transforming, probably because they carry a certain number of mutations that have favorable effects on cell growth (Tanic et al., 2009).

Following the same reasoning as in the case of NSCLC we attempted to identify some of detected DNA changes in leukoplakias, with the aim of identifying tumor-specific alterations (Peinado et al., 1992) that could lead to the development of potential diagnostic

markers involved in the genesis of HNSCC. To that end, nine variant bands present in leukoplakias but not in normal tissue, were selected. Unexpectedly, two different amplicons, originating from distinct leukoplakias, were identified as altered part of the TIMP-3 gene (tissue inhibitors of metalloproteinases 3), two were identified as mutated DNMT 3A gene (DNA (cytosine-5)-methyltransferase 3 alpha) and two represented copies of the Ty1-copia-like retrotransposon.

Further investigations of the detected genes in both, leukoplakia and NSCLC samples, on larger sample size, with special emphases on tumor promoting genes, are underway. We expect more detailed profile of their involvement in NSCLC and HNSCC after extensive analyses of their mutational status and detailed analyses of their expression profile at RNA and protein level in a larger sample. We expect that some of them might prove to be a good prognostic biomarkers for NSCLC or HNSCC patients.

Finally, we analyzed malignant gliomas, tumors that originate from glia, the most common and deadly brain tumors. All patients had histologically confirmed diagnosis of anaplastic astrocytoma (AA) or glioblastoma multiforme (GBM) according to the new World Health Organisation (WHO) classification. Anaplastic astrocytomas (WHO grade III) and glioblastomas (WHO grade IV) are two major groups of malignant gliomas. Glioblastomas are further classified as primary and secondary. Distinction between them is based on different genetic pathways leading to their development (Ohgaki & Kleihues, 2007; Van Meir et al., 2010). Primary glioblastoma develop rapidly *de novo*, without clinical or histological evidence of a less malignant precursor lesion. Secondary glioblastoma develop slowly progressing from low-grade diffuse astrocytoma (WHO grade II) or anaplastic astrocytoma (WHO grade III).

Examination of the extent of genomic instability revealed that samples of patients with anaplastic astrocytoma had similar level of total, microsatellite and chromosomal genomic instability as patients with glioblastoma multiforme, with very high values in both histological subtypes (Table 4). It was unexpected and, at first sight, looked like these results contradicted the expectation and results obtained from NSCLC and HNSCC samples. However, all analyzed grade IV glioblastomas were classified as primary glioblastomas (because glioblastoma diagnosis was made at the first biopsy, without clinical or histopathologic evidence of a less malignant precursor lesion), which are considered to be *de novo* tumors and not the progressive form of grade III astrocytomas. Therefore, obtained results are still consistent with the evolutionary theory of neoplastic transformation and the decrease of the level of genomic instability could be expected in secondary glioblastomas. In other words, extensive genomic instability might be used as diagnostic character where pathology cannot provide unambiguous distinction between primary and secondary GBM. Similar results were obtained by Nishizaki et al. (2002) who demonstrated that there was no significant difference in FISH heterogeneity between malignant gliomas of WHO grades III and IV. We expect that further research involving secondary glioblastomas will confirm our hypothesis and will provide additional confirmation for the evolutionary theory of tumor progression. Moreover, we hope that cloning and sequencing of amplified DNA bands showing genetic alterations specific for glioma genome, will allow the detection of new genes implicated in glioma pathogenesis and progression.

instability	Mean frequency		
	Anaplastic Astrocytoma	vs.	Glioblastoma Multiforme
microsatellite	0.15	vs.	0.16
chromosomal	0.19	vs.	0.16
TOTAL	0.34	vs.	0.33

Table 4. Mean frequency of DNA alterations in malignant glioma samples.

Finally, it is worth mentioning that measurements of genomic instability could be performed by another DNA fingerprinting technique, RAPD (Random Amplified Polymorphic DNA). Wang et al. (2002) measured genomic instability in various cancer types using RAPD and the instability they detected was in average higher than 40% for lung cancer tissues. In another study (Ong et al., 1998), DNAs from 20 lung cancer (18 non-small cell lung cancers and two small cell lung cancers) and their corresponding normal tissues were amplified individually by RAPD with seven different 10-base arbitrary primers. PCR products from RAPD were electrophoretically separated in agarose gels and banding profiles were visualized by ethidium bromide staining. The ability to detect genomic instability in 20 cancer tissues by each single primer ranged from 15 to 75%. DNA changes were detected by at least one primer in 19 (95%) cancer tissues. They concluded that these results seem to indicate that genomic rearrangement is associated with lung carcinogenesis and that RAPD analysis is useful for the detection of genomic instability in lung cancer tissues.

Misra A. et al. (2007) used RAPD to attempt to quantify the number of clonal mutations in primary human gliomas of astrocytic cell origin . They targeted genomic loci of a different nature and estimated that the number of overall alterations in tumor genome seemed to be greater than expected. They also observed a higher number of genetic changes in tumors of lower grade and suggested that it could be a consequence of an increased mutation rate in early tumorigenesis due to acquisition of a mutator phenotype. The increased extent of alterations occurring in tumors of a lower grade is consistent with our study. The results of Misra et al. showed the acquisition of a mutator phenotype early in tumorigenesis and support the mutator hypothesis proposed by Loeb (1991, 2001).

4. Conclusions

AP-PCR DNA fingerprinting is an efficient tool to quickly and easily screen a very large number of loci for possible DNA alterations in cancer cells. It has several advantages: first, minor amounts of template DNA are sufficient for analysis; second, it allows for the screening of anonymous regions of a genome without any prior knowledge of its structure; third, two types of DNA alterations could be detected in single reaction, chromosomal rearrangements and random mutations dispersed over the genome; and forth, possibility of reamplification, cloning and sequencing of variant bands enables the rapid identification of the genes probably linked to tumor progression. Here, we demonstrated the use of AP-PCR DNA fingerprinting in detection and quantification of genomic instability (microsatellite, chromosomal and total) in three types of tumors as well as in search for molecular biomarkers for cancer promotion and progression. Therefore, we conclude that AP-PCR

DNA fingerprinting is important and practically feasible technique for elucidating the genetic background of various tumors. Accordingly, we believe that this technique is rather neglected in contemporary research and should make a comeback because it still has a particularly promising future in experimental oncology.

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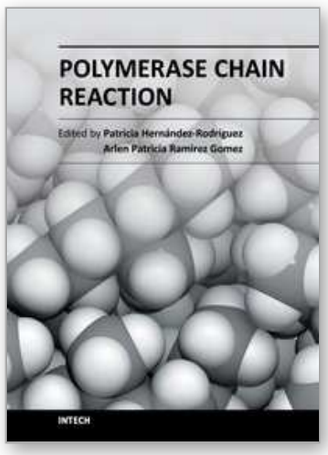

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