Germline \( p53 \) single-base changes associated with Balkan endemic nephropathy

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Abstract

The Balkan endemic nephropathy (BEN) is a significant clinical and scientific problem in need of novel effective therapies. Though many genetic and environmental factors have been investigated the basis, cause, and predisposition to BEN are still unclear. In this study, based on the hypothesis that the genetic pathways leading to BEN might be associated with \( p53 \) dysfunction, we screened for \( p53 \) gene mutations 90 Bulgarian BEN patients using optimized PCR-SSCP-sequencing analysis. Germline \( p53 \) single-base changes were found in blood samples in 10% of BEN cases. Three of them caused amino acid substitutions (p.Arg283Cys, p.Gln317His, and p.Lys321-Glu); the other six were either synonymous amino acid substitutions (p.Arg213Arg) or intron polymorphisms (T14766C). To the best of our knowledge, these are the first data investigating tumor suppressor gene mutations in patients with BEN. The obtained results are in support of our hypothesis that \( p53 \) gene alterations are possibly involved in BEN genetic pathways.

Keywords: Balkan endemic nephropathy; Tumor suppressor genes; \( p53 \); Mutation; Cancer; PCR-SSCP

The Balkan endemic nephropathy (BEN) is a chronic, slowly evolving kidney disease affecting some well-defined rural areas of Bulgaria, Romania, Croatia, Serbia, and Montenegro, and Bosnia and Herzegovina. The disease has no acute onset and may be asymptomatic for many years [1]. It is characterized by progressive renal failure and by rule has a fatal outcome. Uroepithelial cancers appear as a frequent complication of BEN [2]. Approximately 30–48% of BEN patients develop urinary tract tumors localized mainly in the renal pelvis and rarely in the ureter and bladder [3]. BEN is a familial disease without distinct Mendelian pattern of inheritance [4]. The etiology of BEN is still a mystery. The distinctive BEN characteristics (mosaic-like area distribution and familial clustering) have led to the concept that it arises as a multifactorial phenomenon in genetically susceptible individuals subjected to exposure to specific environmental agents. Several hypotheses on the etiology of BEN have been proposed. Thus, it has been assumed that the disease may result from long-term exposure to environmental agents, such as mycotoxins like ochratoxin A (the mycotoxin hypothesis) [5], polycyclic aromatic hydrocarbons, and other toxic organic compounds leaching into the drinking water from the coal deposits (the Pliocene lignite hypothesis) [6], herbs containing aristolochic acid [7], heavy metals [8], and others. The screening for chromosomal aberrations has led to the indication of a putative chromosomal region 3q24–3q26, which may contain a BEN candidate gene [9]. A recent cytogenetic study on three tumors of Bulgarian BEN patients found multiple chromosomal abnormalities including loss of heterozygosity at 3q24, genetic loss at 4q, and genetic gains at 17 other chromosomal regions [10]. An inherited metabolic susceptibility [11] and lecithin-cholesterol-acyl-transferase partial deficiency [12] were supposed to be involved in the pathogenesis of the disease. Recently, a study on 54 blood samples of Bulgarian BEN patients suggested that polymorphic glutathione \( S \)-transferase
variants could also play a role in the etiology and development of BEN [13].

Though many genetic and environmental factors have been investigated, no explicit environmental or reliable genetic agent involved in the etiopathogenesis of BEN was discovered. Numerous studies to date have led to the conception that the cancerogenic process is genetically determined, resulting from a disruption of the normal function of two classes of genes: tumor-suppressor genes (TSGs) and proto-oncogenes. Among all studied TSGs involved in human cancerogenesis, most general is the function of p53 [14]. Disruption in the normal p53 function was found in tumors of almost any histological type. The gene covers 20 kb of cellular DNA and maps to 17p13.1 [15]. It is composed of 11 exons and encodes a 53 kDa nuclear phosphoprotein. The functional molecule is a tetramer, composed of several functional domains: activation domain 1 and activation domain 2; five proline-rich growth-suppression motifs; a sequence-specific DNA-binding domain; a nuclear localization signal; an oligomerization domain; a basic C-terminal regulatory domain. p53 is involved in maintaining of normal cellular stability of cells via cell cycle arrest at G1/S, G2/M, and spindle checkpoint or apoptosis. p53 is also engaged in cell differentiation, morphogenesis, and in the control of cell cooperation. The incidence of point mutations (predominantly base substitutions) within the coding sequence of the gene is the main mechanism for loss of the normal p53 function. Most of p53 mutations are clustered in hot spots within the conserved regions of exons 4–8 and map to amino acid residues in or near the DNA-binding domain of the protein. Clinical studies have indicated that analysis of p53 status is of diagnostic and prognostic value. Mutational p53 status is associated with more aggressive tumor development, early metastasis, and a poor clinical outcome.

The status of the p53 gene in BEN patients was not thus far studied. Petronic and Savin examined apoptosis and p53 protein status of upper urothelial carcinomas (UUC) from BEN regions in former Yugoslavia. Using immunohistochemical labeling for measurement of p53 protein status on UUC tumor sections, they found 37% p53+ UUC from endemic and 38% p53+ UUC outside endemic regions [16].

Our study is based on the hypothesis that the genetic pathways leading to BEN might include p53 gene disorder. To address this, we screened for p53 gene mutations (exons 4–9) by PCR-SSCP-sequencing analysis 90 Bulgarian BEN patients. Single-base substitutions in p53 were found in 10% of the cases. Three of the mutations resulted in amino acid substitutions (p.Arg283Cys, p.Gln317His, and p.Lys321Glu), three caused synonymous amino acid substitutions (p.Arg213Arg), and three were intron polymorphisms (T14766C).

Materials and methods

Patients. Our study group included 90 BEN patients who were diagnosed following the criteria: birth and inhabitance of endemic region >20 years, mild proteinuria (0.5–1.0 g/day), impaired renal capacity to concentrate, lowered urine osmolality, reduced kidney ultrasound size and kidney insufficiency, non-iron deficient anemia, low arterial hypertension, and lack of tumefactions and of bacteruria. Patients inhabited 10 endemic villages of Vratza region—Gorno Pestene, Tischeviza, Veslez, Beli izarov, Pudria, Hubavene, Radovene, Roman, Kalen, and Tzakonotza. The selected group included unrelated individuals only. Of the patients, 21 were male and 69—female. Age ranged from 38 to 79 years (average 64 years) with one exception—a patient at the age of 19 years. Seven of the patients developed tumors on the kidneys or/and the bladder – 7.77%. Blood specimens of 50 individuals inhabiting the same endemic regions who were healthy at the time of sampling were used as negative controls. Informed consent was given by all participants.

DNA isolation. High molecular weight genomic DNA was isolated from 5 ml of peripheral blood by a modification of the standard proteinase K/phenol method [17].

PCR-SSCP analysis of p53 gene (exons 4–9). Previously optimized PCR-SSCP method was applied to detection of p53 mutations [18]. The studied p53 exons (4–9) were amplified in a total volume of 25 μl containing 150 ng of template DNA, 1× PCR buffer, 1.75 mM MgCl2, 0.2 mM of each dNTP, 2.5 pmol of each primer, and 0.3 U of Taq DNA polymerase (PCR Core kit, Boehringer, Mannheim; STS PCR kit, STS Ltd). The primers used for PCR amplification were as follows [19]: 4F 5’CATGGAAGCCAGCCCTCTAG3’ and 4R 5’TGCACCAAGCTCTACATC3’ for exon 4, yielding a product of 181 bp; 5F 5’GGGCAACAGCCGCCTTGC3’ and 5R 5’GTGCGCTGCTTTCACTCTG3’ for exon 5, 266 bp; 6F 5’CGTGCGACAGTTTCACTGAG3’ and 6R 5’CGTGTTAGCTTCCACG3’ for exon 6, 166 bp; 7F 5’CGTGGTGTCGTTTACG3’ and 7R 5’GTCGTAAGACGCGCTC3’ for exon 7, 205 bp; 8F 5’CTGGTGTCTTC3’ and 8R 5’ATTCTCTACGCTCTTTC3’ for exon 8, 218 bp and 9F 5’GACTGGAATTTCCGATTTG3’ and 9R 5’GCTCTAGATTTTTCTAG3’ for exon 9, 161 bp. The samples were incubated at 94 °C for five minutes and amplified for 32 cycles of 94 °C for 30 s denaturation, 30 s at the appropriate annealing temperature (exon 4, 64 °C; exon 5, 59 °C; exons 6–9, 63 °C) and 30 s at 72 °C elongation with a final extension cycle of 72 °C for 5 min (MJ Research thermal cycler, Techne Cyclogen thermal cycler).

An aliquot of 7 μl PCR product was added to 10 μl 95% formamide, 0.01 N NaOH, bromophenol blue. The mixture was denatured at 95 °C for 10 min, then immediately cooled on ice and loaded onto pre-run for 2 h on non-denaturing polyacrylamide gels. The polyacrylamide gel concentration varied between 12% and 18% depending on the studied exons. The electrophoresis was carried out on a 32 cm long Protein Dual Slab Gel apparatus (Bio-Rad) at 15 mA, 500–650 V, 4 °C for 16–21 h running time in 1× TBE buffer. Polyacrylamide gels were visualized using modification of the standard method for silver staining. The gels were soaked in 50% methanol for 1 h, then transferred to ammonium–silver solution, containing 0.02 N NaOH, 0.375% NH4OH, and 0.8% AgNO3 for 15 min and washed with 500 ml dH2O for 10 min. The staining reaction was developed by the use of 0.01% citric acid, 0.035% formaldehyde, and the gels were fixed in 0.5% acetic acid. Staining reactions were done by gentle shaking.

Sequencing analysis. The amplified products with p53 mutations were purified (GFX PCR DNA and Gel Band Purification Kit, Amersham Biosciences) and sequenced on ABI Prism™ 310 fluorescent sequencing analyzer (DYEnamic™ ET Terminator Cycle Sequencing Kit, Amersham Biosciences).

Results

PCR-SSCP analysis revealed aberrant migrating bands in 9 (10%) of the 90 blood DNA samples screened for mutations in exons 4–9 of the p53 gene. Three samples exhibited identical aberrant electrophoretic pattern in p53 exon 6 (Fig. 1A). Altered electrophoretic mobility in p53 exon 8 was registered in one of the patients (Fig. 2A). Five
mutations grouped in three aberrant patterns of electrophoretic mobility were found in exon 9 of p53 gene (Fig. 3A). Exons 4, 5, and 7 displayed no electrophoretic changes in any of the patients. No mutations in p53 gene were registered in the control group.

The results from the sequencing analysis of the detected p53 alterations are summarized in Table 1. All of the mutations were single-base substitutions. Of them, eight were transitions and one was a transversion. The three alterations in exon 6 corresponded to an identical base substitution A–G in codon 213 (CGA-CGG; Arg-Arg) (Fig. 1B). The mutation found in exon 8 mapped to codon 283 and represented a single base change C–T altering the amino acid composition of the p53 protein.

<table>
<thead>
<tr>
<th>Case</th>
<th>Codon n (exon)</th>
<th>w.t.</th>
<th>Mutated sequence</th>
<th>Amino acid change</th>
<th>Mutation type</th>
<th>n of patients (frequency)</th>
</tr>
</thead>
<tbody>
<tr>
<td>236</td>
<td>213 (6)</td>
<td>CGA</td>
<td>CGG</td>
<td>Arg-Arg</td>
<td>Synonymous amino acid change</td>
<td>3/90 (3.3%)</td>
</tr>
<tr>
<td>660</td>
<td>213 (6)</td>
<td>CGA</td>
<td>CGG</td>
<td>Arg-Arg</td>
<td>Synonymous amino acid change</td>
<td>3/90 (3.3%)</td>
</tr>
<tr>
<td>2457</td>
<td>213 (6)</td>
<td>CGA</td>
<td>CGG</td>
<td>Arg-Arg</td>
<td>Synonymous amino acid change</td>
<td>3/90 (3.3%)</td>
</tr>
<tr>
<td>930</td>
<td>283 (8)</td>
<td>CGC</td>
<td>TGC</td>
<td>Arg-Cys</td>
<td>Missense</td>
<td>1/90 (1.1%)</td>
</tr>
<tr>
<td>970</td>
<td>317 (9)</td>
<td>CAG</td>
<td>CAC</td>
<td>Gln-His</td>
<td>Missense</td>
<td>1/90 (1.1%)</td>
</tr>
<tr>
<td>993</td>
<td>321 (9)</td>
<td>AAA</td>
<td>GAA</td>
<td>Lys-Glu</td>
<td>Missense</td>
<td>1/90 (1.1%)</td>
</tr>
<tr>
<td>44</td>
<td>14766 bp (intron 9)</td>
<td>T</td>
<td>C</td>
<td>—</td>
<td>Intronic polymorphism</td>
<td>3/90 (3.3%)</td>
</tr>
<tr>
<td>1268</td>
<td>14766 bp (intron 9)</td>
<td>T</td>
<td>C</td>
<td>—</td>
<td>Intronic polymorphism</td>
<td>3/90 (3.3%)</td>
</tr>
<tr>
<td>1379</td>
<td>14766 bp (intron 9)</td>
<td>T</td>
<td>C</td>
<td>—</td>
<td>Intronic polymorphism</td>
<td>3/90 (3.3%)</td>
</tr>
</tbody>
</table>
The sequence analysis of the p53 mutations found in exon 9 revealed two mis-sense substitutions at positions 317 (CAG-CAC; Gln-His) and 321 (AAA-GAA; Lys-Glu) (Fig. 3B) and three T–C intron 9 polymorphisms at a position 14766. The distribution of the p53 alterations found in exons 4–9 is shown in Fig. 4.

Our results showed that the sequencing of different samples with identical aberrant SSCP pattern demonstrated identical nucleotide alteration. Application of that observation could reduce considerably the number of samples subjected to sequencing.

Comparison of the data on the p53 gene status with the clinical characteristics of patients revealed that only one patient with a p53 alteration (T14766C) developed the characteristic for the disease tumors. This is the only patient within the group who developed both kidney and bladder tumors.

Discussion

The p53 tumor-suppressor gene is the most frequent target of genetic alterations in human cancers. Mutations in p53 were found in approximately 50% of adult cancers of colon, stomach, liver, lung, breast, brain, and others. More than 85% of the registered to date p53 mutations are base substitutions, which cause the synthesis of a phosphoprotein with an altered composition. Most of the p53 mutations reported in different tumor types are clustered within exons 4–8, which encode a highly conserved region, containing the DNA binding domain of the protein. Within that region, at least four mutation hot spots corresponding to amino acid residues 175, 248, 273, 282 have been identified in a variety of neoplasms. In contrast, just a few mutations have been described in exons 2 and 3, encoding the trans-activation domain, and in exons 9–11, encoding the regulatory region. Mutations in p53 disrupt its normal function in maintaining of genetic stability of cells and predispose to neoplasia.

In view of the literature data available, we raised the hypothesis that p53 gene could be involved in BEN genetic pathways. To confirm or reject this assumption, we carried out PCR-SSCP-sequencing analysis in p53 in a group of 90 Bulgarian patients with BEN. Our study revealed alterations in p53 gene in blood samples of BEN patients with a frequency amounting to 10%. These are to the best of our knowledge the first data concerning presence of mutations in a tumor suppressor gene in BEN patients. The here-detected p53 alterations were clustered within exons 6, 8, and 9. None of them mapped to the known p53 mutation hot spots. Of the detected nine p53 alterations, only three caused amino acid changes. The other six were either synonymous amino acid substitutions or actual intron polymorphisms.

The three non-synonymous amino acid substitutions were found in three separate BEN cases and mapped to codons 283, 317, and 321. At the amino acid position 283, overall 102 mutations have been reported in different human carcinomas (UMD p53 database). The registered here CGC-TGC (p.Arg283Cys) base and amino acid change is found in 22 cases of human cancers including bladder [20,21], breast [22,23], colorectal [24,25], gastric [26,27], and others. The mutation leads to substitution of a highly conserved Arg residue with Cys, thus altering the amino acid composition in the DNA—binding domain of the p53 protein. The other two amino acid substitutions were found at unusual locations when considering the p53 mutation spectrum: the CAG-CAC (p.Gln317His) substitution is thus far reported only in one case of skin cancer [28], while AAA-GAA (p.Lys321Glu) is described in a study on rheumatoid arthritis [29]. Both mutations affect highly conserved amino acid residues within NLS-domain of the p53 protein.

Three identical synonymous amino acid substitutions were found in codon 213 in three unrelated BEN patients. Codon 213 is frequently mutated in human cancers. The UMD p53 database reports for the detection of 325 mutations at that amino acid position. The base substitution
CGA-CGG (p.Arg213Arg) is also found in three cases of breast carcinoma [30,31] and in one of colorectal carcinoma [32]. The mutation does not change the amino acid residue but an effect on mRNA splicing, stability or translation could not be excluded.

The other three p53 alterations, reported here, were polymorphisms in a non-coding region of p53, T14766C. The polymorphism mapped 12 bp downstream of the 3' end of exon 9 and was previously reported in a cohort of 103 Italian healthy blood donors [33]. Recently, the concept that intron along with promoter sites may be important in regulating gene expression is gaining more support. It has been suggested that intronic variants may function as dominant mutations similar to the mis-sense, non-sense, and splice-site mutations, thus altering specific biochemical cellular pathways. This finding does not exclude the p53 tumor suppressor gene [34]. At least 14 p53 intronic polymorphisms have been described [35]. Inherited p53 intronic polymorphisms were found to correlate with cancer phenotype, including breast, colon, and others [36,37] but their association with cancer risk and prognosis remains undefined. As with the synonymous amino acid substitutions (CGA-CGG, p.Arg213Arg), the three T14766C intronic polymorphisms were found in three separate unrelated BEN patients, inferring for three independent but identical genetic events leading to the same polymorphism.

The p53 mutations were found in leucocyte DNA, indicating for their germline character. The germline p53 mutations, so far registered, are concerned predominantly with the Li-Fraumeni syndrome; an autosomal dominant disorder characterized with the formation of diverse multiple neoplasms [38]. Germline p53 mutations have been reported to be associated with inherited cancer risk. Normally, individuals who develop a sporadic tumor inherit two wild type p53 alleles both inactivated during the ontogenesis in the same cell by a somatic mutation(s). When a mutant p53 allele is inherited, the risk of a somatic mutation in the only wild type allele, and therefore the initiation of carcinogenesis is considerably increased. As patients with germline mutations in p53 (Li-Fraumeni syndrome) develop neoplasms affecting many organs, logically enough a question arises: if p53 is involved in BEN induction mechanisms why are then carcinomas detected in BEN only in the urinary tract? Unambiguous answer to this question at that stage of BEN genetic base studies could not be given. Obviously the mechanisms of BEN induction are more complex. Many genes dysfunctions, acting alone or in combinations, are responsible for the establishment of genetic susceptibility to BEN, manifested at specific environmental conditions. Based on the obtained data, we cannot conclude directly that the mutant p53 gene status in itself is responsible for the initiation and the development of the carcinogenic process in BEN. But nevertheless, the presence of germline p53 mutations in BEN patients, the multifactorial nature of the disease, and the above considerations are indicative for the possible involvement of p53 in BEN etiology. Furthermore, considering the here-detected spectrum of mutations, the prospect of existing of p53 mutation hot spots specific for BEN patients could not be excluded. Our results are in good agreement with the proposed hypothesis that the genetic pathways leading to BEN might include p53 gene disorder. Dysfunction in p53 may lead to accumulation of mutations in other yet not known BEN-related genes (tumor suppressor genes, proto-oncogenes or others) thus provoking a disruption in the signaling mechanisms, which control the normal cellular pathways.

The evaluation of the potential impact of p53 gene dysfunction on the signaling pathways leading to BEN needs more elaborate studies on p53 gene and protein status, investigated in parallel with other p53 functionally related genes. Serious efforts have yet to be made in searching for other BEN-candidate genes, which alone or in combination provoke BEN induction and development. Genetic analysis on potential BEN-related genes will not only reveal the molecular mechanisms of the disease but will make its diagnosis, prophylaxis, and therapy more successful.

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