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ПРИЛОЗИ. Одд. за мед. науки, XXXVI 2, 2015 **МАНУ**
CONTRIBUTIONS. Sec. of Med. Sci., XXXVI 2, 2015 **MASA**

10.1515/prilozi-2015-0058 ISSN 1857-9345
UDC: 616.61-036.21-056.7(497)

GENETIC, GENOMIC AND EPIGENOMIC STUDIES OF BALKAN ENDEMIC NEPHROPATHY (BEN)

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Abstract

BEN is a primary, chronic tubulointerstitial nephritis characterized with chronic anemia, absence of edema, xantoderma, normal blood pressure and normal findings on the fundus oculi. The disease is distributed in restricted areas in Bulgaria, Romania, Croatia, Bosnia, Former Yugoslavia. Despite numerous studies on genetic and environmental factors and their possible involvement in BEN, its etiopathogenesis still remains elusive.

Our recent study aim to elucidate the possible epigenetic component in BEN development. Whole genome DNA array methylation analysis was applied to compare the methylation profiles of male and female BEN patients from endemic regions in Bulgaria and Serbia and healthy controls.

All three most prominent candidate genes with aberrations in the epigenetic profile discovered with this study are involved in the inflammatory/immune processes and oncogenesis. These data are in concordance with the reported pathological alterations in BEN. This research supports the role of epigenetic changes in BEN pathology.

Exome sequencing of 22.000 genes with Illumina Nextera Exome Enrichment Kit revealed three mutant genes (CELA1, HSPG2, and KCNK5) in BEN patients which encode proteins involved in basement membrane/extracellular matrix and vascular tone, tightly connected to process of angiogenesis. We suggest that an abnormal process of angiogenesis plays a key role in the molecular pathogenesis of BEN.

Key words: next generation sequencing (NGS), exome sequencing, methylation analysis, gene mutations, single nucleotide polymorphisms, Balkan Endemic Nephropathy.

BEN is a primary, chronic tubulointerstitial nephritis characterized with chronic anemia, absence of edema, xantoderma, normal blood pressure and normal findings on the fundus oculi. The most fascinating and mysterious part of this disease is not the clinical or histological picture but its epidemiology. The disease is distributed in restricted areas in Bulgaria, Romania, Croatia, Bosnia, Former Yugoslavia [1].

Typical of BEN is the presence of affected individuals from several generations and the accumulation of sickness in a family, which indicates the presence of family history [2]. The emergence of Balkan endemic nephropathy in people who have left since childhood the endemic regions supports the theory for the existence of a genetic predisposition to BEN. One of the most striking epidemiological characteristics of BEN is the high incidence of uroepithelial tumors in the affected individuals [3–5]. About 30–40% of patients develop tumors with a typical involvement of the upper urinary tract.

The etiology of BEN remains unclear. Two groups of factors may contribute and may explain the endemic distribution of BEN – environmental agents and hereditary factors [6]. Different hypotheses were proposed about the emergence and development of the disease.

Exogenous hypothesis. It connects the etiology of BEN with the effect of nephrotoxins such as: lead, copper, bismuth, silicon, phenolic compounds, PAH; zink, selen; mycotoxins – ochratoxin A and plants toxins – acid from Aristolochia Clematis. Until now, no conclusive and generally accepted data exist for their leading role in the pathogenesis of the disease.

Studies have been conducted in regard to the viral hypothesis for BEN such as: West Nile virus, Leptospira, Picorna virus, Herpes simplex, Hepatitis B etc. Using electron microscopy, the presence of virus particles was demonstrated in the cytoplasm of tumor cells from patients with BEN. They were morphologically identical to the viruses from the family Bunyaviridae, such as Hantaviruses [7] (Fig. 1). These electronic microscopic studies proved in 27.7% of patients the presence of antibodies against the Hanta viruses.

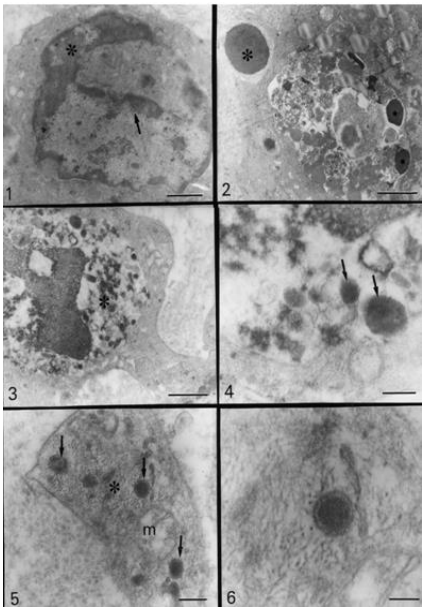

Fig 1 – Electron microscopy study of BEN tumors

Genetic hypothesis is based on the epidemiological studies and pedigree analysis, genetic and genomic studies [8, 9]. The data revealed that the disease are "familial" without a distinctive pattern of transmission within a single family. The proportion of offspring is signifycantly higher when two parents are sick as compared to the offspring with one affected parent and progressively decreased in the cases with healthy parents (Fig. 2).

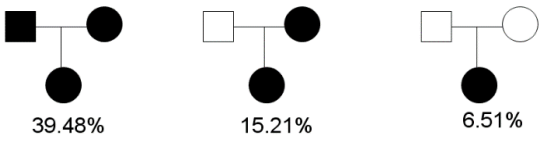

Figure 2 – Genealogical studies in families with Balkan Endemic Nephropathy

The frequency of the disease is proportional to the degree of relatedness. The risk is two times lower for second-degree than for the first-degree relatives and decreases rapidly for more remote relatives (Fig. 3).

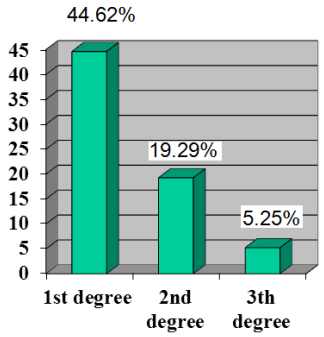

Figure 3 – Incidence of BEN in different degree's relatives

Genetically determined liability for the disease was supposed because BEN is not found in the Gypsy population living in the endemic areas and the disease appears in individuals from at-risk families who left the endemic regions in their early childhood. A large scale investigation in BEN patients on the role of genetic polymorphism in 9 genes from detoxification system was done (*CYP2D6 – alleles *3, *4, *5, *L; CYP3A4 – *1, *V; CYP3A5 – variants 6986G>A, 14690G>A, 27289C>A; 3705C>T, 3709-10insG; NQO1 – *1, *2, *3; NAT1 – C559T, G560A, T640G, T1088A, C1095A; NAT2 – T341C u C282T; GSTT1 – *1, *0; GSTM1 – *1, *0*), TGFβ, MDR1 (*C3435T, G2677T*) etc. were investigated. A higher risk for BEN (OR 2.41) in individuals caring the CYP3A5*1 allele G6986A was revealed. A modifying effect of NAT gene variants on the BEN risk was not found [10–17] (Fig. 4).

Polymorphisms in transporters may also contribute to inter individual differences in the response to the environmental factors. The haplotype pair 11/22 is associates with BEN (OR 2.5).

Exome sequencing of 22.000 genes with Illumina Nextera Exome Enrichmen kit was performed on 22 DNA samples (11 Bulgarian and 11 Serbian patients). Software analysis was performed via NextGene, Provean and PolyPhen. We focused on non-annotated variants and nominated 3 mutant genes in Bulgarian and Serbian BEN patients [18] (Fig. 5).

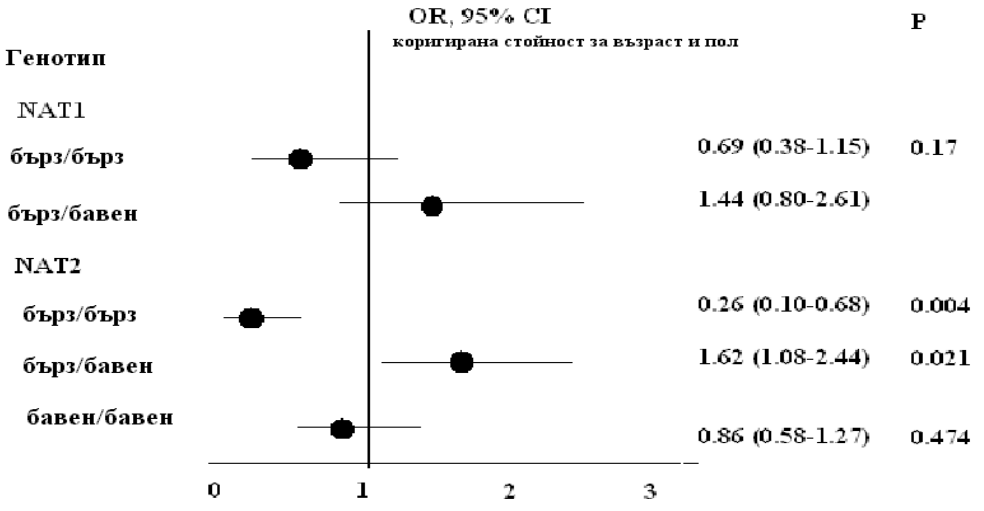

Figure 4 – Relative risk for the disease in patients with different NAT1 and NAT2 genotype

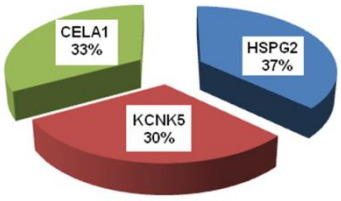

Figure 5 – Frequency of the nominated genes with non-annotated variants in BEN patients

The genes were selected as related to the pathogenesis of BEN based on their mutation frequency, their similar incidence in both patients groups, lack of information about the established variants in European population and non-incidence in healthy Bulgarians. Analysis of their function sheds light on the possible pathophysiology in BEN (Fig. 6).

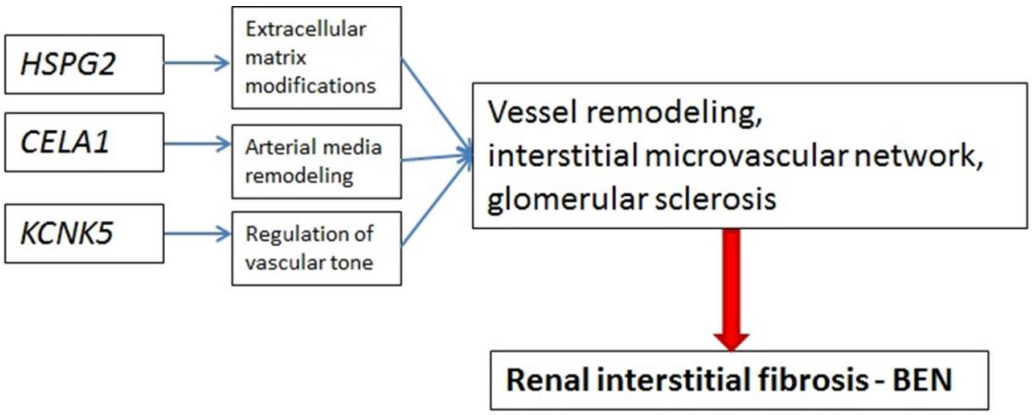

Figure 6 – Proposed model for molecular pathogenesis of BEN kidney pathology

Epigenetic hypothesis

Despite numerous studies on genetic and environmental factors and their possible involvement in BEN, its etiopathogenesis still remains elusive. None of the previously described factors can fully explain the complex clinical presentation of BEN and can't be named as sole predisposing factor or unique causative agent. This stirred the research on BEN in new directions that can shed new light on its pathogenetic mechanisms.

Recent years saw vast amount of research in the field of epigenetics. It may prove to be the key to unlocking the unsolved problems of genetic predisposition to many major disorders. Epigenetics studies the heritable characteristics of gene function and their alterations which are not coded by the primary DNA sequence. The epigenome is flexible – it facilitates stable transmission of "genetic programs" through cell generations as well as allows the cell to perform different roles (differentiation) and to adapt to external stimuli [19]. Thus major alterations in the cellular state can occur without any mutations in the primary DNA sequence. More and more studies are emerging that confirm the role of epigenetic alterations in broad spectrum of disorders – cancer, cognitive impairment, respiratory, cardiovascular, reproductive and immunomediated diseases [20]. Despite the plasticity of the epigenome epigenetic profiles show family clustering, which might explain the higher prevalence of certain disorders in some families [21]. Thus epigenetics may have a role in transmitting "genetic predisposition" through generations.

DNA methylation represents one of the three major mechanisms of epigenetic regulation. DNA methylation consists of addition of a methyl group at 5^{th} carbon atom in the cytosine molecule (Fig.7). DNA methylation is one of the best recognized epigenetic signals for repression of gene expression levels [22]. Aberrations in the DNA methylation process are implicated in the pathogenesis of cancer and autoimmune disorders [23–26].

A recent study was conducted to elucidate the possible epigenetic component in BEN development. 159 patients from two of the most affected countries were enrolled in the study. The clinical diagnosis was based on an internationally approved panel of diagnostic criteria [27].

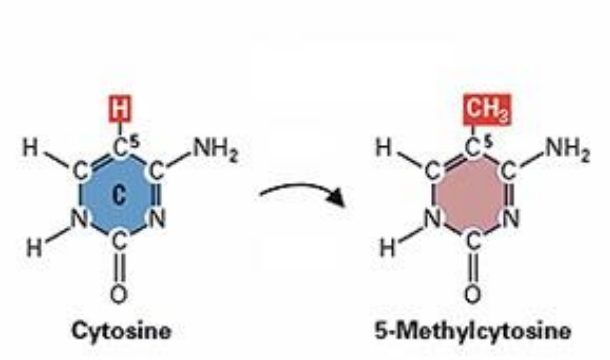

Figure 7 – Methylation of cytosine
(adapted from http://www.hgu.mrc.ac.uk)

Whole genome DNA array methylation analysis was applied to compare the methylation profiles of male and female BEN patients from endemic regions in Bulgaria and Serbia and healthy controls [28]. Patients and respecttive controls differed by the methylation status of ca. 2,84% of the analyzed CpG islands-differently methylated regions (DMRs). In all patients – controls pairs the greater part of the DMRs were hypomethylated in patients and hypermethylated in controls (Fig. 8).

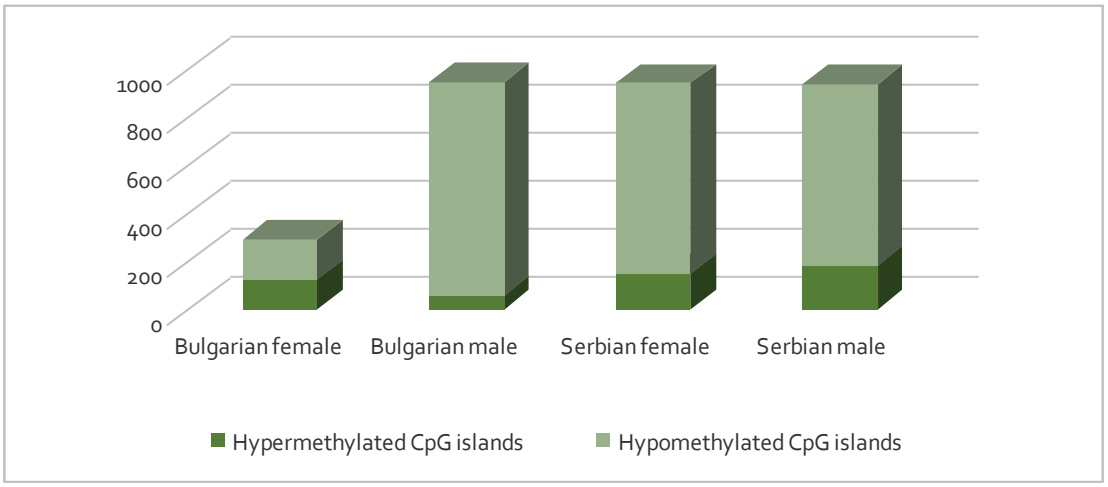

Figure 8 – Methylation status of the discovered DMRs in the four patient-control pairs (Bulgarian male patients-Bulgarian male controls, Bulgarian female controls-Bulgarian female patients, Serbian female patients-Serbian female controls, Serbian male patients-Serbian male controls)

Comparison of the differently methylated regions (DMRs) across patients-controls pairs revealed that the affected CpG islands are mostly associated with genes involved in inflamation, immune response and oncogenesis. Only 3 CpG islands are private to all four patient-control pairs – *HDAC11, IL17RA, SEC61 [10]*. They appear to be the most likely candidat-genes for epigenetic deregulation in BEN (Fig. 9).

CpG-island (hg19)	Starting nucleotide	End nucleotide	Chromosomal band	Gene
CpG: 60	13521516	13522227	3p25.1	*HDAC11*
CpG: 158	17565498	17566950	22q11.1	*IL17RA*
CpG: 56	54826556	54827168	7p11.2	*SEC61G*

Figure 9 – CpG-islands associated with BEN

HDAC11 codes class IV hystone deacetylase. Hystone deacetylases are involved in transcription, cell cycle, cell diferentiation and cell death regulation. *HDAC11* expression aberrations are considered to be due to mechanisms different than conventional DNA mutation. *HDAC 11* deregulation is a cofactor of tumorogenesis – higher expression levels of the enzyme are reported in various cancers. HDAC11 is involved in maintaining the immune ballance between proinflamatory and antiinflamatory signals. It is likely that aberrant expression of HDAC11 may lead to abnormal immune

response in BEN. Thus HDAC11 deregulation could play a role in immune response aberrations in BEN and could potentially induce immune tolerance to cancer cells. HDAC11 inhibitors are proved to have antifibrotic and antiinflammatory effect and are promissing drugs for treating kidney disease due to impaired inflamatory processes and fibrosis.

SEC61G is a member of protein transport apparatus of the endoplasmic reticulum. Its function is not well known. Available data show that SEC61G is a player in the adaptive immune response. Its overexpression may lead to disproportionate immune response against viral and other antigens that are presented by MHC-I molecules. It has a possible significance for autoimunnity.

IL17RA codes a receptor of a member of the interleukin signalling family. Its activation is responsible for inducing synthesis of proinflamatory cytokines. Literature overview reveals that IL17 signalling pathway deregulation is reported in many inflamatory disorders such as rheumatoid arthritis and Chron's disease. Furhtermore this signalling pathway is involved in regulation of the extracellular matrix and abnormal development of fibrosis. The processes of excessive fibrogensis are part of the BEN pathogenesis. This supports the hypothesis for IL17RA in its development. IL17RA dysregulation is also reported in cancer – prostate cancer, osteosarcome and pituitary adenoma.

All three most prominent candidate genes with aberrations in the epigenetic profile dicovered with this study are involved in the inflammatory/immune processes and oncogenesis. These data are in concordance with the reported pathological alterations in BEN. This research supports the role of epigenetic changes in BEN pathology.

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Р е з и м е

ГЕНЕТСКИ, ГЕНОМСКИ, И ЕПИГЕНОМСКИ СТУДИИ НА БАЛКАНСКАТА ЕНДЕМСКА НЕФРОПАТИЈА

Рада Г. Станева*[1], Л. Балабански*[2], И. Димова[1], Б. Рукова[1], С. Хаџидекова[1], П. Димитров[3], В. Симеонов[4], С. Иванов[2], Р. Вајарова[2], М. Малинов[2], Р. Чукурановиќ[5], В. Стефановиќ[5], М. Поленаковиќ[6], В. Ѓонов[7], А. Галабов[8] и Д. Тончева[1]

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Балканската ендемска нефропатија (БЕН) е примарен, хроничен тубулоинтерстицијален нефритис што се карактеризира со хронична анемија, отсуство на едем, ксантодерма, нормален крвен притисок и нормални наоди на фундус окули. Болеста е дистрибуирана во ограничени области во Бугарија, Романија, во поранешна Југославија – Хрватска, Босна и Херцеговина. И покрај бројните студии за генетски и еколошки фактори и нивната можна вклученост во БЕН, нејзината етиопатогенеза останува нејасна.

Нашата скорешна студија има цел да ја расветли можната епигенетска компонента во развојот на БЕН. Беше применета анализа на метилациониот статус на геномот на ДНК да се споредат метилациските профили на машки и на женски пациенти со БЕН од ендемските региони во Бугарија и во Србија и здрави контроли.

Сите три најзначајни гени-кандидати со аберации во епигенетскиот профил откриени со ова истражување се вклучени во воспалителните/имунолошките процеси и онкогенезата. Овие податоци се во согласност со соопштените патолошки нарушувања кај БЕН. Ова истражување ја поддржува улогата на епигенетските промени во патологијата на БЕН.

Егзом секвенционирањето на 22.000 гени со Illumina Nextera Exome Enrichment Kit откри три мутантни гени (CELA1, HSPG2 и KCNK5) кај пациентите со БЕН кои кодираат протеини вклучени во базалната мембрана/екстрацелуларниот матрикс и васкуларниот тонус, тесно поврзан со процесот на ангиогенеза. Ние укажуваме на тоа дека абнормалниот процес на ангиогенеза игра клучна улога во молекуларната патогенеза на БЕН.

Клучни зборови: следна генерација секвенционирање (NGS), егзом секвенционирање, метилациска анализа, генетски мутации, полиморфизам на единечен нуклеотид, Балканска ендемска нефропатија.