Nijmegen breakage syndrome in Ukraine: diagnostics and follow-up

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Abstract

Nijmegen breakage syndrome (NBS) belongs to the group of chromosomal instability syndromes, is predominantly distributed in Slavic countries and is characterized by microcephaly, combined immunodeficiency and a high predisposition to lymphoid malignancies. A clinical-immunological characteristic of 21 patients with NBS from the Western Region of Ukraine is presented. Cytogenetic disorders were studied in sick children and their parents. The dominating 657del5 mutation of the NBS1 gene is found in all NBS patients in this region. An assessment algorithm for the examination of patients who are suspected of having NBS is proposed, and the main aspects of monitoring and treatment are presented in the article.

Key words: *Nijmegen breakage syndrome (NBS), primary immunodeficiencies, children.*

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Introduction

Nijmegen breakage syndrome (NBS) is a rare autosomal recessive disease belonging to the group of chromosomal instability syndromes. Its clinical picture includes microcephaly, facial dismorphism, growth retardation, immunodeficiency, hypersensitivity to radiation and a high predisposition to lymphoid tumors. *NBS1* disease causing gene is localised on the short arm of 8q21 chromosome.

NBS was first described by Wheemaes et al. (1981) in two siblings with microcephaly, growth and development retardation, abnormal skin pigmentation, immunodeficiency and a high frequency of rearrangments in the 7th and 14th chromosomes in lymphocyte culture [1]. In 1985 Seemanova et al. described a group of patients with a possibly new genetic disease, characterized by microcephaly with normal intellect, cellular and humoral immunodeficiency and a high predisposition to lymphoreticular tumor formation [2]. Later, after additional studies, these cases were identified as Nijmegen breakage syndrome.

Further studies carried out on the cell cultures of patients with NBS revealed changes similar to those found in patients with ataxia-teleangiectasia (AT). Namely, the phenomenon of spontaneous chromosomal instabilty, hypersensitivity to ionizing radiation and radioresistance to DNA synthesis. Clinical features of NBS and AT are also similar and previously NBS has been viewed as a clinical variant of AT (IVth type of AT) [3-5].

The product of the *NBS1* gene, Nibrin (p95) is responsible for the interaction between two proteins: hMre11 and Rad50, which control the repair of parallel breaks of double-strand DNA that are induced by ionizing radiation, or occur naturally (mejotic reactions and mitotic VDJ-rearrangments in mature lymphocytes) [6]. *NBS1* is one of the multicomplex gene systems responsible for telomerase reparation, regulating cell proliferation and apoptosis. The defects in this system, particulary those related to *ATM*, *NBS1*, *MRE11*, *BRCA1*, band p53, *CDS1*, and *CHK2* are considered to be among the causes of oncological diseases [4, 7, 8].

Over 200 cases of NBS have been identified in many countries of the world. The largest group of patients as of April 2005 were diagnosed in Poland, The Czech Republic, Slovakia and Germany. 95% of patients were of Slavic descent and carried the homozygous 657 del5 mutation, called "the Slavic mutation" [9-13]. Such genetic conservatism of NBS simplifies pre-and postnatal molecular-genetic

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diagnosis. Under an iniative of The Berlin Institute of Human Genetics (K. Sperling, R. Varon) the frequency of heterozygote occurrence in Western Ukraine was studied [14]. The established frequency of heterozygote occurrence was 1:182, lower than in The Czech Republic (1:130) but higher than in Poland (1:253). The expected frequency of NBS in the population of West Ukrainian is 1:133 000.

The purpose of our study was to elucidate the clinical features, and immunological and cytogenetic changes in NBS patients.

Materials and Methods

In 1999-2007 a total of 26 patients were diagnosed with NBS in Ukraine, 23 of whom resided in Western Ukraine and only three – in Eastern and Southern regions. The mean age at the time of diagnosis was between three months and 12 years. The follow up time was between 1 month and 8 years correspondingly. We assessed 21 of these patients personally.

The diagnosis of NBS for all the children was based on the phenotype, clinical and laboratory changes, and was verified by mutation analyses.

We studied in detail patients' individual and family histories, and the basic laboratory parameters in order to reveal possible chronic foci of infection and malignancies. Aquantitative study of the main lymphocyte populations and subpopulations was conducted by a flowcytometry method with monoclononal antibodies ("Becton Dickinson"). The Level of Ig A, M, G in blood plasma was studied by a kinetic nefelometry method on an ICS-2 ("Beckman", USA) analyser.

Cytogenetic analysis was conducted on the short-term FHA stimulated lymphocytes of the patients and their parents, with further fixation at the 70th hour, and the preparation of standard chromosome slides. We studied the frequency of chromosomal structural rearrangments, caryotype, aneuploidy, polyploidy and atypical centromer division, especially preterm division of the separate chromosomes (PCD), and 50-100% of all chromosomes of the set (preterm anaphase, PA). In each case 100 metaphase plates (MP) were analysed. The results obtained were compared with the cytogenetic study of 23 healthy controls.

For molecular-genetic diagnosis we performed DNA extraction from blood leukocytes by standard methods of phenol-chloroform purification or by a salting out procedure. 657del5 mutation detection was done by PCR, followed by electrophoresis in 10% PAGE. The primers for exon 6 amplification were: NBS6F 5'-CAGATAGTCACTCC GTTTACAA-3' and NBS6R 5'-TTACCTGTTTGGCATT CAAA-3'. In the absence of 657del5 mutation in a patient the product of size 234 b.p. was amplified; in homozygous carriers – a product of 229 b.p. In heterozygous carriers fragments of 229 b.p.and 234 b.p. are formed, as well as heteroduplexes that appear as a result of denaturation of normal DNA chains and the chains with deletions during PCR cycles (Fig. 1).

"Exel" software was used for statistical analysis.

Results and Discussion

Patients' family and individual history

Clinical data showed that in 4 cases pregnancy was complicated with gestosis and in 5 cases with a threat of miscarriage. In 3 children microcephaly was diagnosed prenatally by ultrasound. A few families had a history of infant death of the native sibs with a phenotype of NBS from measles (one case) and oncological diseases (three cases).

One of the patients (see pedigree in figure 2) was born to a consanguineous couple.

Nine out of 21 mothers (42.9%) had had spontaneous abortions during previous pregnancies.

All children were born at full term for pregnancy. Seventeen out of 21 were born too small for gestation with proportionally low weight and length (Table 1). In all cases the children were diagnosed with microcephaly, head circumference (HC) being between 29-33 cm (mean – 30,8 cm), correlating with other parameters of physical development.

In one case the delivery was induced and traumatic with subsequent development of cephalohematoma and heamolitic disease of the newborn caused by Rh-conflict. Intrauterine infection (high IgG levels to toxoplasma, cytomegalovirus, herpes simplex virus) was also diagnosed, and attribted to intrauterine developmental retardation. In other children the period of infancy was not significant, which corresponds to the data found in medical literature, stating that children with

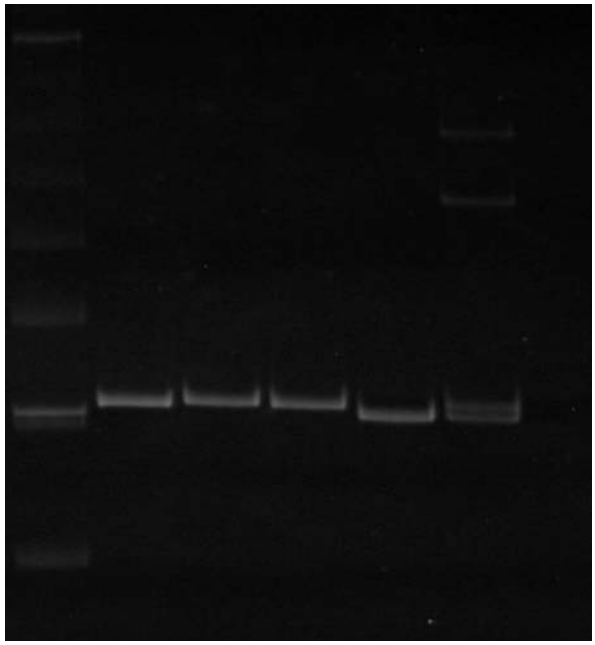

Fig. 1. Molecular-genetic analysis of 657del5 mutation in *NBS* gene. Columns from left to right: 1 – MW – 50 bp Ladder; 2, 3, 4 – wild type; 5 – homozygous for 657del5 mutation; 6 – heterozygous for 657del5 mutation

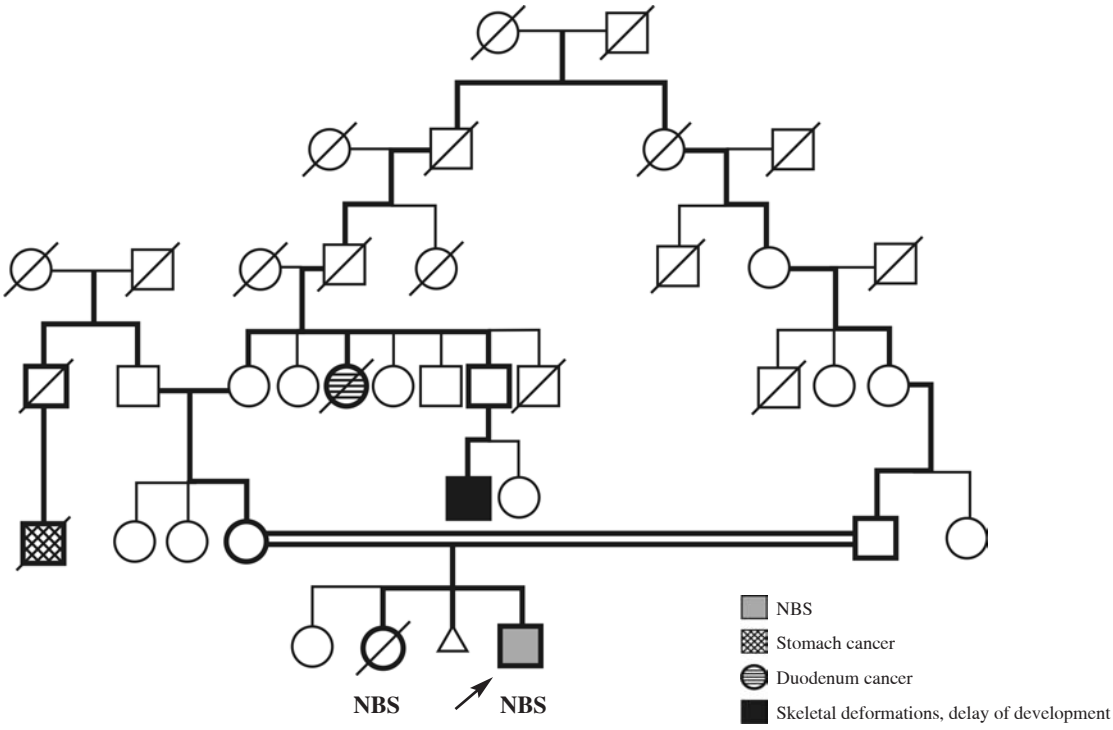

Fig. 2. Family tree of NBS patient

NBS are usually born at full term with a body weight ranging between 1900 and 3600 g for girls and 2170-3950 for boys, and a head circumference of 26.5-36 cm [9, 13].

Physical characteristics

During follow up all of the patients showed slow physical development. At the time of the initial diagnosis a body weight and height retardation (below -2 SD) was noted in 89.5% of patients (-3.7 SD for body weight, -4.2 SD for height and -6.3 SD for HC) (Table 1).

In the majority of children motor skills developed normally.

Besides microcephaly, NBS patients have other craniofacial abnormalities, described as a "birdlike" face, mowed forehead, mandibular hypoplasia, and a prominent profile with a relatively long nose. In the majority there is a mongoloid slant, comparatively long and displastic ears, a short neck, and in some patients – hypertelorism.

Patients with NBS also have other anomalies, usually minor skeletal defects: clynodactyly of the 5th finger and/or

Table 1. Retardation of physical development

Age	Parameters	Range	Mediane	Cases	
				abs.	[%]
At birth	Weight	1.8-3.8 kg	2.6 kg	18/21	85.7
	Height	44-52 cm	47.2 cm	18/21	85.7
	OFC	29-33 cm	30.8 cm	21/21	100
At the time of diagnosis	Weight	-1.5-4.7 SD	-3.7 SD	17/21	81
	Height	-1.3-5.6 SD	-4.2 SD	17/21	81
	OFC	-4.6-8.7 SD	-6.3 SD	21/21	100

OFC – occipito-frontal circumference.

partial sindactyly (in 28.6% of cases). In 5 children (23.8%) kidney abnormalities (horse-shoe kidney, hypoplasia of kidney, double kidney) were diagnosed upon additional examination. The following congenital malformations have also been reported: the small size of the brain is relatively often associated with corpus callosum agenesis, arachnoid cysts, and hydrocephaly. Tracheal hypoplasia, cleft lip and cleft palate, choanal atresia and cardiovascular defects are described in the literature [9, 15-17], although non of these defects were registered in our patients.

The skin malformations include abnormal pigmentation (data from previous reports suggests the presence of such abnormalities in close to 50% of the patients), spots of hypo-and hyperpigmentation (vitiligo and "cafe au late") usually between 2 and 6 of different size and localization. In our setting such malformations were detected in 15 children (71.4%). One girl also had psoriatic skin elements.

Immunological assessment

The main clinical features of NBS are recurrent infectious diseases that develop due to humoral and cellular immune defects. These were present in most of our patients (17 out of 21) at 2-3 years of age with frequent acute respiratory infections, recurrent pneumonias, and rhynosinusitises. Less often patients presented with recurrent otitis, enterocolitis, urinary tract infections and stomatitis. In three children we observed the development of chronic purulent endobronchitis, resistant to conservative treatment and requiring therapeutic bronchoscopies. Infectious agents are often represented by viruses and bacteria. In one child lung and cervical lymphatic nodes tuberculoses manifested (Table 2). Four children under 3 years of age did not have any infectious complications. In only one patient the infectious syndrome manifested at less than 1 year of age with otitis and recurrent bronchi-pulmonary diseases.

Immunological studies (Table 3) revealed strong or mild leuco-and lymphopenia in many patients (10/21, 47.6%).

Table 2. Frequency of infectious complications in patients with NBS

Infectious complications	Cases	[%]
RRI	16/21	76.2
Otitis media	8/21	38.1
Sinusitis	8/21	38.1
Recurrent bronchitis	9/21	42.9
Pneumonia	8/21	38.1
Chr.bronchitis/bronchoectasis	4/21	19.0
Reccurent stomatitis	4/21	19.0
Enterocolitis	3/21	14.2
Pyodermia	2/21	9.5
Pyelonephritis	3/21	14.2
Tuberculosis	1/21	4.8
Lambliosis	1/21	4.8

RRI – reccurent upper respiratory tract infections.

Table 3. Immunological indexes of patients with NBS

	Median	Minimum	Maximum	Lower quartile	Upper quartile
Leucocyte, $x10^9$/L	4.8	1	10	3.6	5.3
Lymphocyte, $x10^9$/L	1.26	0.69	3.14	0.89	2.35
$CD3^+$ [%]	51	24	70	36	60
$CD3^+$, abs	0.7	0.19	1.809	0.45	0.88
$CD4^+$ [%]	21	8	44	16	24
$CD4^+$, abs	0.31	0.06	0.683	0.174	0.461
$CD8^+$ [%]	23	6	39	16	33
$CD8^+$, abs	0.38	0.096	1.053	0.15	0.48
$CD19^+$ [%]	5	1	21	3	12
$CD19^+$, abs	0.12	0.013	0.875	0.034	0.189
$CD16/56^+$ [%]	30	1	55	21	38
$CD16/56^+$, abs	0.466	0.007	1.36	0.208	0.75
IgA [mg/dl]	10.5	0	171	0	29
IgM [mg/dl]	63	0	220	43.4	91
IgG [mg/dl]	393	80	1160	180	558

Table 4. Tumors in NBS patients

Patient	Age	Tumor	Therapy	Follow-up
1.	8 yr	Non-Hodgkin lymphoma with BM involvement	BFM	Remission 8 yr
2.	9 yr	B-cell lymphoma	–	Died at age 9 yr
3.	11 yr	B-cell lymphoma	modified BFM-NHL-90	Remission 6 yr
4.	7 yr	Non-Hodgkins lymphoma	–	Died at age 7 yr
5.	6 yr	Haemophagocytic lymphohystio-cytosis	DAL-HX83/90, LCH (I,II)	Died at age 6 yr
6.	5 yr	Diffuse large-cell B-lymphoma	modified BFM-NHL-90 ↓ R-ICE (salvage therapy)	Died at age 5.5 yr

In most of the cases we observed significant depletion of immunocompetent cells with interference of main cell populations and subpopulations. In the majority of cases we noted $CD3^+$-cells, $CD19^+$-cells, $CD4^+$-cells and helper-supressor index depletion. In 14 children we observed the phenomenon of "NK-cells expansion".

Statistical analysis using Tau Kendall correlation revealed that in NBS patients the percentage of $CD3^+$ ($p<0.005$), $CD4^+$ ($p<0.005$) and $CD8^+$ ($p<0.001$) cells as well as the absolute number of $CD19^+$ cells ($p<0.05$) decreases with age. At the same time the percentage of $CD16/56^+$ cells ($p<0.0001$) goes up. There was no significant correlation revealed between age and Ig A, M and G levels. Similar data are described in other reports [13, 18].

Many children had low IgA and IgG levels. In 8 children IgG levels were below critical (<3 g/L), which required immunoglobulin replacement therapy. Four more children required replacement therapy at the level of IgG 3-6 g/L because of significant infections. Due to the social-economic problems in Ukraine NBS patients only began to receive regular replacement IVIG therapy in 2006-2007 using preparations produced by Ukrainian companies (one product with a level of Ig A, M, G corresponding to the donors' plasma levels, and the second with a low IgA concentration). In two children with a long history of chronic bronchitis and sinusitis, despite the initiation of regular IVIG therapy (0.4-0.5 g/kg/month) the foci of infection were not cleared, necessitating the further extensive use of antibiotics. In two more children malignant tumors developed two months after the IVIG was started, hence we were not able to assess the efficacy of replacement therapy for them. In other cases IVIG treatment improved the course of the disease. We noted the absence of exacerbations of chronic infectious foci in children who started their replacement therapy before 3 years of age-no chronic foci of infection formed. In 9 out of 12 children (75%) moderate adverse reactions were noted when using IVIG with physiological levels of Ig A, M, G: shiver-8/9, vomiting-2/9, fever-8/9 and myalgia-4/9. Further only IVIG with low IgA concentration was used in these patients, no adverse rections were noted then.

Six out of 21 patients had autoimmune phenomena: sporadic neutropenia in 3 patients, oligoarthritis in 2, psoriasis

Table 5. Medium rate of metaphazal plates (mp) with signs of karyotype instability in cultivated lymphocytes

Object of investigation	Number of cases/MP	% MP with unstable karyotype				
		Specific abberations (7/14)	Nonspecific abberations	PA	PP	PDC
Controls	23/2300	0	1.2±0.2	0.04±0.04	0	1.4±0.3
NBS	5/433	7.8±1.4	5.6±2.2	2.3±1.0	1.2±0.7	2.8±1.5
Parents NBS	3/300	0	0.6±0.3	1.1±0.6	0.3±0.2	0.6±0.3
p_1		<0.001	<0.001	<0.001	<0.01	>0.05
p_2		>0.05	>0.05	<0.001	<0.01	>0.05
p_3		<0.001	<0.001	>0.05	>0.05	>0.05

PA – preliminary anaphase, PP – polyplois cells, PDC – preliminary division of centromers in certain chromosomes; p_1 – probability value of difference between control group and NBS patients; p_2 – probability value of difference between control group and patients' parents; p_3 – probability value of difference between the NBS patients and their parents.

in 1 patient. None of them required immunosuppressive therapy, and symptoms improved with the use of IVIG therapy.

Malignancies

In five cases (23.8%) a diagnosis of NBS was established upon manifestation of oncological diseases in children at age 5-11 years, and in two children malignant tumours developed during the follow up period (Table 4). In one of these patients a maxillar sinus tumor was diagnosed just recently (suspected neuroblastoma or lymphoma, although verification continues). Such a prevalence of tumors (7/21, 33%) corresponds with numbers described in the literature, although the frequency of malignant tumors of NBS reported in the Polish and Czech national registries is higher – 54% and 65% respectively, which might be due to the higher number of children under 6 years of age in our registry. It is known that most lymphoid organ tumors are reported in patients under 20 years of age (the mean-9 years). Usually these are non-Hodgkin lymphomas, lymphoblastic leukemia and Hodgkin's disease. Rare cases of acute myeloid leukemia, myoma, meningioma, medulloblastoma, intestinal cancer and Ewings sarcoma have been observed in NBS patients. The total risk of having oncological disease for NBS patients is 50 times higher then in the general population, being especially high for lymphomas – 1000 times exceeding population risk [9, 19, 20].

In six of our patients the malignancies involved lymphoid tissue – five cases of non-Hodgkin lymphoma, and one case of a rare type of hystiocytosis. Four children received specific treatment – polychemotherapy according to the BFM protocol for lymphomas, modified for Ukraine. One child also received salvage therapy with rituximab. During treatment in this programme all of the children had infectious complications: acute upper respiratory tract infections, mucositis, otitis, pneumonias, sinusitis and one case of paraproctitis. These complications required i.v. wide-sprectrum antbiotics and in the majority of cases IVIG therapy. Four children died from progressive oncological diseases and infectious complications, two of them were diagnosed in the terminal stages of the disease, hence did not receive any treatment. The other two children achieved remission (Table 4).

Karyotype

Cytogenetic studies were carried out on 11 patients with NBS and 7 of their parents. In one of the patients the study was conducted after the development of neoplastic complications – lymphoblastic lymphoma. In 3 patients a very low mitotic index was registered (below 10 mitotic plates). The study of 500 m.p. in 7 other cases of NBS without oncological complications comprised the experimental sample of the clinical group.

The data presented in Table 5 show a higher frequency of cells with chromosomal aberrations in the cell culture of NBS patients compared to the control group ($p<0.001$). There were no specific features of chromosomal instability with chromosomal transformation found for the control group, in patients' parents nor in 2 out of 7 children with NBS. Five other patients had typical manifestations of specific chromosomal transformations in 4-12% of m.p. The above-mentioned specific chromosomal transformations were: 3% of the cases – inv(7) (p13;q35), 2% – t(7;14)(q35;q11), 1% – t(7;14)(p13;q11), 2% – t(7;7)(p13;q35), t(7p13;14q32), del(7)(q35), del(14)(q11). An additional chromosome marker was found in 10% of the cells with translocations.

In the lymphocyte cell culture of patients in the control and study group, spontaneous chromosomal nonspecific anomalies (excluding 7^{th} and 14^{th} pairs) were also found. The mean number of cells with spontaneous nonspecific aberrations was significantly higher in patients with NBS and did not differ from control group one (the patients' parents). Nonspecific chromosomal aberrations in NBS patients were mostly represented by single-chromosome changes, although interchromosomal transformations were also registered in this group: t(1;3)(q22;p22), t(3;7)(p21;q11), t(2;10)(q21;p10), t(X; 5)(q26;qter), t(5;12)(q13;qter). Nearly 75% of all nonspecific chromosomal aberrations of NBS patient cells were found in chromosomes 1, 3, 8 and 9.

Besides chromosomal aberrations, the phenomenon of centromere instability-complete and partial centromere separation of metaphase chromosomes -is also seen in NBS patients' cell culture. The cells with complete centromere separation (PA) comprised 2.3%±1.0% while absent in the control ($p<0.001$). Asignificantly higher incidence of PA compared to the control group was also found in cell cultures of NBS patient parents.

Cells with a polyploid number of chromosomes were not detected in the control group, but found in 1.2±0.7% of m.p. of NBS patients ($p<0.01$) and in 0.3±0.2% of m.p. of parents.

The phenomenon of partial centromere separation was found in almost all of the control and study group chromosomes of similar frequency level.

Hence, cytogenetic abnormalities in NBS patients spread beyond the typical changes in and chromosomes 7 and 14 of the lymphocytes. A significant increase in the number of nonspecific chromosomal aberrations, cases of increased chromosomal breakage in centromere and precentromere regions, the number of cells with PA and polyploidy was detected.

Genetic analysis

NBS1 gene cloning, performed in 1998 enabled the molecular-genetic diagnosis of NBS not only in patients already sick, but also opened the window to prenatal diagnosis [22].

In the period 1999-2002 gene mutation studies for our first NBS patients were performed by R. Varon at Berlin Institute of Human Genetics. Next, we did 123 molecular-genetic tests of the 657del5 mutation *NBS1* gene on the

groups of patients and their family members suspected of having NBS. At present we have collected 22 NBS DNA samples with verified 657del5 mutation. All of the patients studied were found to be homozygotes for this mutation.

We have also performed 4 prenatal diagnostic tests aimed at early identification of NBS cases in families with high NBS risk. Two homozygous cases and two heterozygous carriers of the 657del5 mutation were detected. In the first 2 cases pregnancies were terminated, in one case a healthy child was born, and the forth pregnancy is a progress.

Conclusions

1. Taking into account the high prevalenceof NBS among Slavic nations, it is important to continue the study of NBS morbidity and the frequency of the heterozygote occurrence of the *NBS1* gene mutation in different regions of Ukraine.
2. 100% of our patients had 657del5 mutation of the *NBS1* gene in both chromosomes proving that this mutation prevails in Western regions of Ukraine.
3. The presence of the clinical "hallmarks" of this disease (microcephaly, retarded physical development, etc.) permits selective NBS screening for earlier and wider detection of the disease.
4. The doctor suspecting NBS should order immunological tests and cytogenetic analyses, which are of diagnostic and predictive value.
5. NBS patients should be followed up by several specialists: pediatric immunologist, oncologist and neurologist. Genetic counselling of the family is also necessary.

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