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CLINICAL TRIAL

I171V germline mutation in the *NBS1* gene significantly increases risk of breast cancer

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Abstract Nijmegen Breakage Syndrome (NBS) is a rare autosomal, recessive disease caused by homozygous mutations in the *NBS1* gene. The most common deletion of 5 bp (657del5) in exon 6, which affects mostly the population of Central Europe is observed. Among the typical features of this disorder is that NBS patients experience a high incidence of lymphoid malignancies as well. An increased risk of solid tumors development for 657del5 carriers was the reason to investigate the role of *NBS1* gene as a susceptible one for the breast cancer. The purpose of this work is to identify mutations in all 16 exons of the *NBS1* gene in the group of the patients with diagnosed breast cancer and the control group of healthy individuals. In the group of 270 women with breast cancer, seven cases of mutated *NBS1* gene were revealed. In the subgroup presenting mutated *NBS1* gene, the mutation I171V in 5th exon occurred in five cases. It is the first such a discovery concerning breast cancer patients because this mutation had been previously observed only in the course of lymphoid or hematological malignancies. The rate of I171V mutation in the group of breast cancer patients was significantly higher than in the controls (OR: 9.42; 95% CI: 1.09–81.05; $P = 0.02$). The conclusion is that heterozygous germline mutation I171V in *NBS1* gene is a significant risk factor for breast cancer development. It concerns especially the women whose first degree relatives had a previously diagnosed breast cancer (OR: 6.00; 95% CI: 0.98–38.07; $P = 0.04$). The histopathological and clinical features of breast cancer with I171V mutation suggest accumulation of the negative prognostic factors. The treatment's results however were unexpectedly satisfactory, that is why further investigations are necessary to assess the role of I171V mutation in *NBS1* gene as a prognostic and predictive factor for breast cancer.

Keywords Breast cancer · I171V mutation · *NBS1* gene

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Introduction

NBS1 gene transcript—nibrin is a protein involved in many essential intracellular processes responsible for maintaining genome stability. One of its roles is cooperation with other proteins like MRE11, RAD50, ATM and BRCA in initiating reparation of spontaneous or induced (e.g., by ionization) DNA damages. The carriers of homozygous mutations of *NBS1* gene have a characteristic appearance and anatomical features [1]. They are oversensitive to radiation, and are at a much higher risk of different malignancy development. Mostly lymphoid and hematological disorders are observed [2]. However, the susceptibility to solid tumors, both in homozygotes and heterozygotes of the mutated *NBS1* gene, is noted as well [3, 4].

Since the beginning of this decade several studies have been conducted to assess the role of *NBS1* gene as a susceptibility gene for the breast cancer. Most of them described the significance of mutated *NBS1* gene in Slavic populations. The highest rate of heterozygous carriers of *NBS1* gene mutations is noted in Central Europe, especially

in Poland [5, 6]. The recently published data proved that *NBS1* gene mutations contribute significantly to the occurrence of not only familial but also consecutive breast cancer among Polish women. All these studies however were focused only on the most frequent mutation of *NBS1* gene—657del5 located in 6th exon [7].

Since the *NBS1* gene identification in 1998 many other mutations in this gene have been discovered [8]. Varon et al. [9] described a novel mutation in 5th exon of *NBS1* gene—I171V, and pointed at its role in relapsed and resistant acute lymphoblastic leukemia (ALL). Mosor et al. [10] proved that heterozygous carriers of I171V mutation are at a significantly higher risk of ALL development in childhood.

The results of the studies mentioned gave us the assumption to screen all 16 exons of *NBS1* gene in search of the new mutations. The point was to assess their significance in the group of women with confirmed breast cancer diagnosis in comparison with the healthy probants.

Patients and methods

Biological material for genetic analysis was obtained from 270 non-selected patients of Clinic of Oncology at the University School of Medical Sciences in Poznań (Wielkopolska region, Poland) with cytological or histological confirmation of breast cancer diagnosis. The data concerning clinical and histopathological features of breast cancer were obtained from all patients. The information concerning the disease course and the pedigree were precisely collected. The study was approved by the local Bioethics Committee at University School of Medical Sciences in Poznań and all patients signed the Informed Concerned Form.

The control group involved anonymous healthy newborns from Wielkopolska. DNA was isolated from Guthrie's test cards, routinely taken for fenyloketonuria screening.

Genomic DNA from peripheral blood leukocytes was isolated with the use of Genomic Mini (A&A Biotechnology).

For preliminary selection of abnormal sequences in all 16 exons of *NBS1* gene, single strand conformation polymorphism method was used (PCR-SSCP). Sensitivity of PCR-SSCP decreases with an increased length of DNA fragments. The highest rate of the true positive results is obtained with DNA fragments of 100–300 bp length [11]. For this reason 20 pairs of specific starters for all 16 exons of *NBS1* gene were constructed to obtain products 206–305 bp length. For the long exons: 7, 10 and 11, two or even three pairs of starters were prepared to avoid appearance of products longer than 300 bp. The products of electrophoresis were visualized by a modified Sanguinetti method of silver staining [12]. All samples giving different pattern during electrophoresis from control track were directly analyzed with the use of automatic ABI PRISM® 310 Sequencer.

The data obtained were verified by an exact Fisher's test and Fisher–Freeman–Halton's test. The choice of the test depended on the quantity of the probes. The results were assessed as statistically significant at *P-value* lower than 0.05.

Results

In the group of 270 non-selected patients with breast cancer (BC), seven cases of *NBS1* gene mutation were found. Additionally, in cases of another 22 patients, rare intronic sequences were identified. One of them was for the first time discovered deletion of 7 bp in 4th intron (IVS4-73TTATAAC). In the control group only three cases of mutated *NBS1* gene were revealed (2.59 vs. 1.02%).

All kinds of pathological changes in *NBS1* gene, found in breast cancer patients and comparison rates of particular mutations between the analyzed and the control group are shown in the Tables 1 and 2.

The most frequent pathology discovered in *NBS1* gene was mutation I171V in 5th exon. It was identified in five cases (patients BC 18, 47, 80, 147, 207). So far the presence of this mutation was described only in cases of patients with hematological malignancies. The studies analyzing mutations in *NBS1* gene, have not described I171V mutation in the cases of breast cancer so far. This mutation is a transition A > G in 511 position. The consequence of this missense mutation is an exchange izoleucin for valin in position 171st of nibrin. The effect of I171V mutation is a change in this protein's structure. One of its N-terminal domains—Breast Cancer Terminal domain (BRCT) is affected [13]. The BRCT domain is

Table 1 Mutations and other changes in *NBS1* gene identified in 270 the breast cancer patient group

Exon/intron	Mutations and rare intronic sequences of *NBS1* gene	Number of cases
Ex 5	I171V	5 (1.85%)
	IVS4-73TTATAAC	2 (0.74%)
Ex 6	657del5	2 (0.74%)
Ex 7a	IVS6-18A > A	1 (0.37%)
	IVS6 + 18G > A	7 (2.59%)
Ex 8	IVS8-42G > C	1 (0.37%)
Ex 15	IVS15 + 88C > G	11 (4.07%)
Total		29 (10.74%)

Table 2 The frequency of mutations in *NBS1* gene in the breast cancer group and the control group

Exon	Mutation	Breast cancer group		Control group		Statistical significance
		Number	Percentage	Number	Percentage	
Ex 5	I171V	5/270	1.85	1/500	0.2	$P = 0.02$[a]
Ex 6	657del5	2/270	0.74	2/295	0.68	$P = 0.65$

[a] Result statistically significant ($P < 0.05$)

responsible for a proper interaction with other proteins in processes of DNA reparation and cell cycle regulation [14].

In two cases (BC 87 and 261) the presence of "Slavic" type of *NBS1* gene mutation (657del5 in 6th exon) was confirmed. The consequence of this frameshift mutation is synthesis of two different transcripts. One of them ($NBS1^{p26}$) contains N-terminal of nibrin with its Fork Head Associated (FHA) domain and BRCT domains. This fragment in correct nibrin is responsible for the interaction with histone H2AX and a proper recognition DSB sites recognition in DNA, and in this way initiates its repair. The other product ($NBS1^{p73}$) contains C-terminal of nibrin, responsible for binding MRE11 protein. Due to the presence of this truncated protein $NBS1^{p70}$, mutation 657del5 does not have lethal character [15].

The heterozygous germinal mutation I171V, for the first time identified in breast cancer patients, was found significantly more often in breast cancer group than in controls. The extension of control's group quantity to 500 probants was necessary because in the first set of 295 healthy probants no case of mutation in 5th exon of *NBS1* gene was found. The most important result of the analysis performed was the fact that in case of heterozygous carriers of I171V mutation in *NBS1* gene the odds ratio was estimated at 9.42 (95% CI: 1.09–81.05). It means that the carriers of this mutation are at an over 9-fold higher risk of developing breast cancer than the women with a correct *NBS1* gene (Table 3).

The analysis of "Slavic" 657del5 mutation in 6th exon of *NBS1* gene did not show significant differences in occurrence between two groups: patients with breast cancer and healthy controls.

The histopathological data were also acquired and analyzed to compare differences between the carriers of *NBS1* gene mutation and the patients with breast cancer presenting a wild type of this gene.

Table 3 Statistical analysis: number of NBS1 gene mutations in the breast cancer group and the control group

Mutation	Breast cancer group	Control group	OR (95% CI)	*P*
I171V	5/270	1/500	9.42 (1.09–81.05)	0.02[a]
657del5	2/270	2/295	1.11 (0.15–7.90)	1.00

[a] Result statistically significant ($P < 0.05$)

Although without statistical significance for the carriers of this mutation, breast cancer was typified by a higher histological grade G3, more often presented overexpression of HER2 receptor and a lower expression of estrogen receptor ER. These facts suggest that breast cancer with underlying I171V mutation should have more aggressive course and worse prognosis. Indirect confirmation of this hypothesis was a statistically significant observation that the diagnosis of breast cancer for carriers of I171V mutation was established at a more advanced clinical stage of the disease—IIIA.

The pedigrees obtained from all breast cancer patients gave interesting conclusions as well. Breast cancer patients with I171V mutation more often claimed presence of this malignancy in first degree relatives than patients with regular *NBS1* gene. This difference was statistically significant—OR: 6.00 [95% CI: 0.98–38.07], $P = 0.04$.

Discussion

The lack of proper functional and structural nibrin cases a high incidence of different malignancies in patients with Nijmegen Breakage Syndrome, as it has been proved in many in vitro studies. Nibrin is responsible for the reparation of double strand breaks (DSBs) of DNA via of cooperation with other proteins [16]. DSBs might be the consequence of the exposure to irradiation, but it can also appear during natural processes in the course of the cell cycle.

The most important result of our study was the revealing five cases of a new I171V mutation in 5th exon, which has been so far described in patients with lymphoid malignancies. Varon et al. [9] confirmed the presence of this mutation in four cases of children with Acute Lymphoblastic Leukemia (ALL). The hypothesis that germline I171V mutation is one of the risk factors for ALL occurrences was established. The results of Taylor's et al. [17] work were contrary. Also Shimada et al. [18], during the analysis of aplastic anemia did not find such a dependency. Moreover, he found five healthy carriers of I171V mutation in 413 probants group. Only Mosor et al. [10] presented the results of the trial, providing a high, statistically significant dependency between germline I171V mutation in *NBS1* gene and incidences of childhood ALL.

The mutation analyzed concerns 511 position of 5th exon in *NBS1* gene and results in A > G transition. The

effect of this change is the replacement of izoleucin by valin in 171st position of encoded by *NBS1* gene protein, nibrin. This change takes place in one of nibrin's N-terminal domains—BRCT (Breast Cancer Carboxyl Terminal). This domain, together with the neighboring FHA domain, is a highly conservative amino acid sequence appearing in many prokaryotes and eukaryotes. They are responsible for a proper interaction with other proteins in DSB reparation on the way of homological recombination, also in cell cycle regulation and telomeres stability [14]. A > G transition as a point mutation does not influence the nibrin's expression. *NBS1* gene carrying such mutation is transcribed to nibrin of proper length of 754 amino acids. The replacement of izoleucin by valin in 171st position results however in disorder in proper protein's activity during the most vital cell functions. As a consequence it may cause genome instability and increase the risk of carcinogenesis initiation [19]. In case of solid tumors for carcinogenesis initiation one of the basic factors is chromosomal instability. It is observed in the course of such inherited chromosomal instability syndromes like the Bloom syndrome, Fanconi anemia, ataxia-teleangiectasia (AT) and NBS. In a high percentage of patients with AT and NBS syndrome an increased level of spontaneous chromosomal aberration was observed, mostly concerning chromosomes 7th and 14th [20]. Such aberrations like breaking of the whole chromosomes, chromatides breakages or acentric fragments were found not only in peripheral blood lymphocytes but in other cells and tissues, mostly in fibroblasts [21]. Introducing new cytogenetic method of analysis: three-color whole chromosome paints (WCPs), Stumm et al. [21] proved statistically higher appearances of spontaneous chromosomal translocations in AT and NBS patients. As a result of this kind of aberration activation of protooncogenes might take place. Translocations are stable chromosomal aberrations and may be transferred to descendant cells, promoting in this way the process of carcinogenesis. Monoclonal growth of such cells consolidates a malignant genotype.

The similar role in initiation of carcinogenesis may have a disorder in stability and integrity of telomeres, both in homo- and heterozygous carriers of mutated *NBS1* gene. The most frequent aberration untypical for normal cells is telomeres association [22]. Although direct influence of this aberration on carcinogenesis process is not entirely known, it is found not only in NBS patients' cells but in AT syndrome and in numerous cancer cells including breast cancer ones [23]. In normal cells a correct nibrin, together with telomere binding protein (TRF2) protects telomeres for the associations [24].

Zang et al. [22] observed this phenomenon examining cell lines in which the function of nibrin was blocked by a small interfering RNA. His researches proved without doubts that the role of nibrin in maintaining stability and integrity of telomeres is completely separated from nibrin's action in process of DSBs reparation and regulation of the cell cycle.

The next proof for the increased incidence of solid tumors in case of *NBS1* gene mutation are researches carried out on cell lines derived from human breast and ovarian cancer. Tessitore et al. [15] revealed three new mutations in this gene leading to decreasing of DSBs reparation via of homologous recombination. In these cells alternatively non-homologous recombination of ends binding took place. Both situations lead to mistakes in reparation process resulting in arising of new mutations, transferred and cumulated in next generations of neoplasmatic cells.

Since the beginning of 90th last century, population studies were conducted to identify susceptibility genes for solid tumors development, parallely to laboratory researches. After identifying the high penetrating genes such as *BRCA1, BRCA2, P53*, responsible for most of familial breast cancer cases, the genes responsible for genome integrity and reparation of DNA breakages became the focus of studies and researches. The first gene from this group was the *ATM* gene, because a high incidence of breast cancer was noted in the families of patients with AT syndrome. Among heterozygous carriers of *ATM* gene mutation risk of developing breast cancer is 2 to 4-fold higher than in the rest of the population [25].

There were many similarities in clinical course of AT and NBS syndromes, and similar as well as supplemental role in a clinical manifestation of these syndrome's genes. That is why there followed the researches which aimed at revealing the role of heterozygous mutations in *NBS1* gene in breast cancer development. From the start however, the attention was mostly focused on the "Slavic" mutation—657del5 in 6th exon. It caused limitations because of unequal distribution of this mutation in populations of different origins. The results of a few studies investigating the role of *NBS1* gene mutations in lymphoid malignancies were inconsistent, it also refers to similar studies concerning breast cancer. In German and Russian populations higher percentage of heterozygous carriers of 657del5 mutations among patients with diagnosed breast cancer was not proved [26, 27].

An unequal distribution of heterozygous *NBS1* gene mutation carriers was confirmed by a study of Varon et al. [5]. In three Slavic populations—Polish, Czech and Ukrainian, a higher frequency of heterozygous *NBS1* "Slavic" mutation was found and described as one case for 177 healthy individuals. For the Polish population this rate was one case for 190 probants. But the following studies showed that also on the territory of Poland the occurrence of this mutation depends on a region. For example in

Mazowsze region 657del5 mutation can be found in one for 162 probants, whereas in some parts of Wielkopolska region its frequency increases to one in 77 examined probants [7].

That is why Polish oncologists and geneticians are extremely interested in the quickest assessment of breast cancer risk for heterozygous carriers of *NBS1* gene mutations. So far however, the results of Polish studies concerning this problem were incoherent. The data of the first such a study were published by Górki et al. [28]. The conclusion of this study was that 657del5 mutation in *NBS1* gene has a small but significant contribution to familial breast cancer incidence. The results of Steffen's et al. [3] work confirmed a high rate of "Slavic" mutation in breast cancer patients but without a statistical significance. Another investigation of this group, published in 2006, proved a significant contribution of 657del5 mutation in breast cancer development in patients from Central Poland. Another conclusion of this study was that the carriers of this mutation present sporadic breast cancer more often, whereas this mutation does not influence a higher incidence of familial breast cancer [7].

In all studies concerning the incidence of mutations in *NBS1* gene and, what follows, the risk of developing breast cancer, only the "Slavic" 657del5 mutation in 6th exon of *NBS1* gene was analyzed.

For the first time an attempt to assess the rate and role of mutations in all 16 exon of *NBS1* gene in breast cancer patients was undertaken in our study. The most important achievement of this work is revealing I171V mutation in 5th exon of *NBS1* gene, which has been detected only in lymphoid and hematological malignancies so far. This mutation was identified in five cases of breast cancer patients, which makes 1.85% of all examined group. The I171V mutation occurred significantly more frequently in the breast cancer group than in the control one (OR: 9.42 [1.09–81.05], $P = 0.02$). The conclusion may be that the I171V mutation in 5th exon of *NBS1* gene is a significant risk factor for breast cancer development. It concerns especially these patients with breast cancer whose first degree relatives also had diagnosis of that malignancy. In the group of five women with confirmed I171V mutation, three declared incidences of breast cancer in first degree relatives and one of them claimed also a case of breast cancer in second degree relative (consequently mother and grand mother), while Steffen et al. [7] put the emphasis on a sporadic character of breast cancer in carriers of *NBS1* gene mutations, especially 675del5 one.

Taking into account the results obtained, concerning much more frequent appearance of I171V mutation in breast cancer patients, introducing to genetic consulting detection of heterozygous carriers of this mutation in first degree relatives of breast cancer patients seems appropriate. Such testes may prove very important for our knowledge concerning the groups of higher breast cancer risk. It concerns mostly Wielkopolska region where the percentage of heterozygous carriers of *NBS1* gene mutations is one of the highest in Poland and in Europe [6]. Further investigations regarding this problem are necessary and they may prove a much bigger role of *NBS1* gene mutations in breast cancer development than it has been claimed so far.

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