## Heterozygous carriers of germline c.657_661del5 founder mutation in *NBN* gene are at risk of central nervous system relapse of B-cell precursor acute lymphoblastic leukemia.

Isolated central nervous system (CNS) relapse is observed in 3-8% of patients with acute lymphoblastic leukemia (ALL) and accounts for 30-40% of all disease recurrence.[1,2] Although introduction of intensive CNS-directed therapy significantly improved 5-year overall survival of patients diagnosed with leukemia CNS relapse,[3] such aggressive treatment results in long-term side effects.[4] Therefore, more accurate risk stratification of CNS relapse in newly-diagnosed ALL pediatric patients is needed. In contrast to somatic gene defects, only a small number of studies have investigated the host genetic factors and their association with leukemia relapse.[5] Since germline *NBN* c.657_661del5 mutation increases the risk of developing pediatric ALL and other types of cancers in Slavic populations, and there is a high frequency in the Polish population (1:190 individuals[6,7]), we investigated its possible effect on leukemia development and progression among heterozygous carriers. *NBN* germline c.657_661del5 mutation leads to an impaired function of the nibrin protein, which acts as an element of the Mre11-Rad50-Nbs1 (MRN) complex in DNA double-strand breaks (DSB) repair processes.[8] Patients with a homozygous deletion in the *NBN* gene, diagnosed with Nijmegen-breakage syndrome (NBS, OMIM #251260), are known to often present CNS involvement at diagnosis of pediatric ALL.[9] In contrast to patients with NBS, who mainly develop lymphoma or T-cell ALL, heterozygous carriers of the c.657_661del5 deletion in the NBN gene are mainly diagnosed with B-cell precursor ALL (BCP-ALL).[10] Therefore, in this unique group of patients, it is possible to investigate whether germline c.657_661del5 heterozygous mutation in the NBN gene has an impact on the biology and course of childhood BCP-ALL.[11]

We retrospectively evaluated 578 pediatric patients diagnosed with BCP-ALL (median age 4.51 years, range 0.13-17.99 years; details are available in the *Online Supplementary Methods*). In total, 17 (2.94%) patients harbored the c.657_661del5 heterozygous mutation in the NBN gene. There was no significant difference in baseline clinical and biological features of leukemia and risk group

**Table 1.** Comparisons of clinical and molecular characteristics of c.657_661del5 patients and wild-type cohort.

| | NBN c.657_661del5 heterozygotes (n=17) | NBN wild-type (n=561) | *P* |
|---|---|---|---|
| Age at diagnosis, years (median, 25-75%) | 4.28 (2.76-5.56) | 4.58 (2.78-8.19) | 0.2539[a] |
| Sex (M/F) | 9/8 | 299/262 | 0.9719[b] |
| WBC at diagnosis, x10$^9$/L (median, 25-75%) | 10.1 (3.4-63.4) | 10.5 (4.1-31.5) | 0.6634[a] |
| Risk group (SR/IR/HR) | 3/12/2 | 121/286/102 | 0.4922[b] |
| MRD at day 15, % (median, 25-75%) | 0.50 (0.00-2.60) | 0.30 (0.04-2.70) | 0.7709[a] |
| MRD at day 33, % (median, 25-75%) | 0.00 (0.00-0.24) | 0.00 (0.00-0.01) | 0.3192[a] |
| CNS status at onset (CNS 1/2/3) | 17/0/0 | 468/29/24 | 0.3753[b] |
| CNS relapse (yes/no) | 3/14 | 12/549 | 0.0064[b] |
| Bone marrow relapse (yes/no) | 4/13 | 48/513 | 0.0371[b] |
| Testicular relapse (yes/no) | 1/7 | 4/264 | 0.1377[b] |
| Poor steroid response[c] (yes/no) | 1/16 | 44/469 | 1.0000[b] |
| Median (25-75%) follow up | 2.70 (1.97-3.50) | 3.30 (1.68-4.32) | 0.4003[a] |
| Hypodiploidy (yes/no) | 0/17 | 17/414 | 1.000[c] |
| Hyperdiploidy (yes/no) | 6/11 | 115/322 | 1.000[c] |
| MLL rearrangement (yes/no) | 3/14 | 26/535 | 0.026[c] |
| ETV6-RUNX1 fusion (yes/no)[e] | 0/15 | 76/343 | 0.084[c] |
| BCR/ABL1 fusion (yes/no) | 0/17 | 10/551 | 1.000[c] |
| IKZF1 deletion (yes/no) | 2/15 | 88/442 | 0.8612[c] |

n: number; M: male; F: female; WBC: white blood cell count; SR: standard risk; IR: intermediate risk; HR: high risk; MRD: minimal residual disease; CNS: central nervous system. [a]Mann-Whitney U test. [b]$\chi^2$ test. [c]Fisher's exact test (two-tailed). [d]Group allocation is described in the *Online Supplementary Appendix*. [e]Data available for only 434 (75.1%) patients. NA: not available.

allocation between these patients and those observed in the remaining 561 (97.06%) BCP-ALL patients with the wild-type NBN allele (wt-NBN). However, MLL rearrangements were more frequently observed in patients with NBN deletion [3 of 14 *vs.* 26 of 535, Odds Risk (OR)=4.41, 95%CI: 1.19-16.30; *P*=0.026]. Interestingly, *ETV6-RUNX1* fusion was not found in any of the heterozygous carriers of the c.657_661del5 mutation in the *NBN* gene; however, this difference did not reach statistical significance (0 of 15 *vs.* 76 of 343, OR=0.14, 95%CI: 0.01-2.45; *P*=0.085). The clinical and biological characteristics of both groups are presented in Table 1. Separate data for patients treated according to the 2002 and 2009 ALL-IC BFM protocols are listed in *Online Supplementary Tables S1-S4*.

To assess the level of baseline accumulation of DNA double-strand breaks (DNA DSB) according to *NBN* genotype, we investigated the expression of γ-H2AX in Epstein-Barr virus-immortalized lymphocytes derived from wt hetero- and homozygous carriers of 657del5 deletion in the *NBN* gene. (For details of the procedure see the *Online Supplementary Methods*). Mean percentages of cells positive for γH2AX in wt cell lines, cell lines with heterozygous and homozygous 657del5 deletion in the *NBN* gene were as follows: 1.92±0.13%; 4.61±1.54% and 5.94±1.48% (*P*=0.039, one-way ANOVA). The mean level of spontaneous DNA DSBs in cells with any (heterozygous or homozygous) deletion in the NBN gene was found to be significantly higher than wt cell lines (4.74±1.57% *vs.* 1.92±0.13%; *P*=0.022). Although this effect was mainly generated by the difference between wt cell lines and double mutants, spontaneous DNA DSBs were increasing gradually in wt hetero- and homozygous cell lines, respectively. Results stratified by genotype and by dominant mode of deletion c.657_661del5 are shown in Figure 1A and B. Dot plots of representative flow cytometry analysis are presented in *Online Supplementary Figure S1*.

We observed 60 relapses (10.4%) in the study group, 4 in patients with the c.657_661del5 mutation in the *NBN* gene: 3 combined [bone marrow (BM) and CNS] and one isolated BM relapse. Fifty-six relapses were observed in carriers of wt-NBN: 4 isolated CNS, 4 isolated testicular, 39 isolated BM and 9 combined (n=8 BM and CNS, n=1 BM and testicular). Univariate analysis found that heterozygous carriers of the c.657del5 mutation in the *NBN* gene had a poorer outcome, both in terms of overall survival (OS) and relapse-free survival (RFS), in comparison with patients with wt NBN (HR=3.34, 95%CI:1.20-9.31, *P*=0.022 and HR=3.44, 95%CI:1.23-9.59, *P*=0.026, respectively). Kaplan-Meier (KM) curves for RFS and OS are presented in *Online Supplementary Figure S2*. However, in multivariate analysis, only a high level of minimal residual disease (MRD) at day 15, the presence of *MLL* gene rearrangement, and poor steroid response acted as significant factors with an independent influence on RFS (*Online*

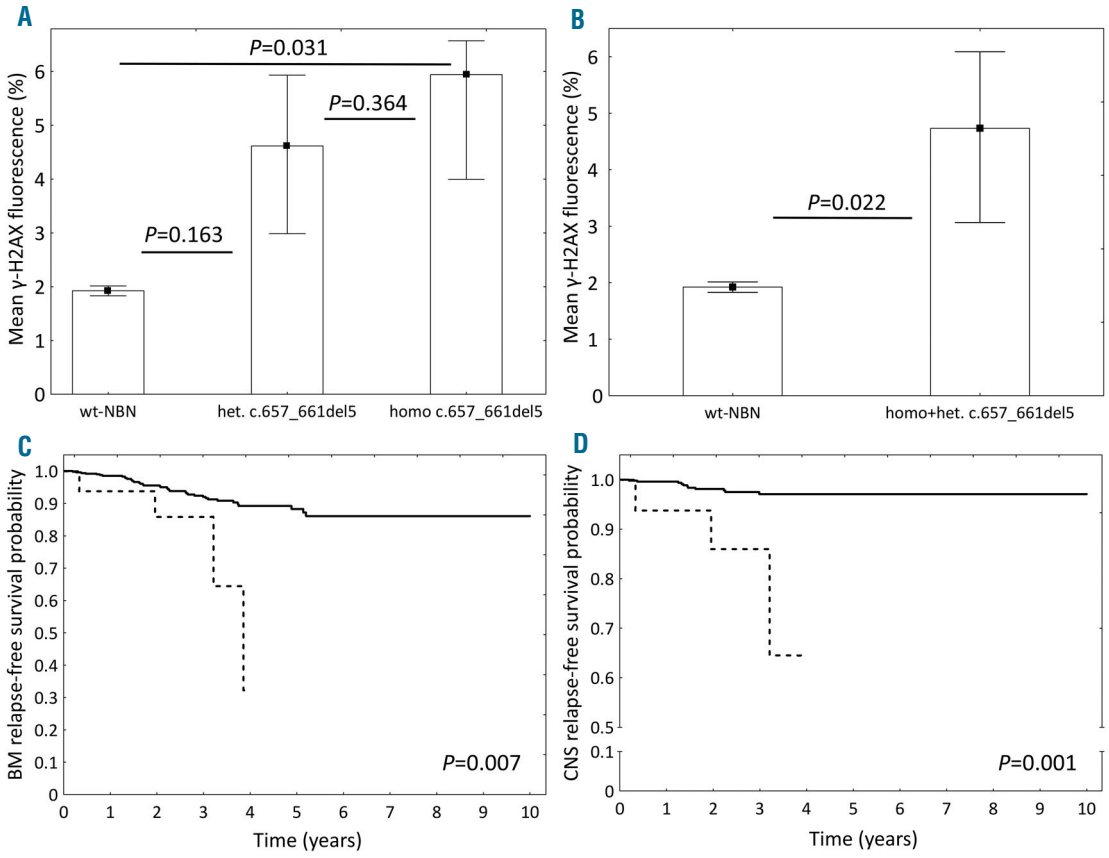


**Figure 1. Comparison of the mean percentage of cells positive for γH2AX with respect to the genotype variant in the *NBN* gene.** (A) Genotype stratification. (B) Dominant mode stratification. *P* values according to (A) Tukey's post-hoc tests and (B) *t*-test. Kaplan-Meier curves for (C) bone marrow relapse-free survival and (D) central nervous system (CNS) relapse-free survival. Patients with 657del5 heterozygous deletion in the *NBN* gene are represented by dashed line; wild-type patients are represented by black solid line (C and D).

*Supplementary Table S5*). Carriers of the heterozygous c.657del5 mutation were found to have a worse prognosis both in terms of "BM RFS" (HR=4.16, 95%CI: 1.48-11.71; *P*=0.007) and "CNS RFS" (HR=9.44, 95%CI: 2.62-33.94; *P*=0.001) (Figure 1C and D).

Bone marrow RFS was significantly affected by MRD level at day 15, poor steroid response, age at diagnosis, white blood cell count (WBC) at diagnosis, the presence of MLL rearrangement, and a germline c.657_661del5 mutation in the *NBN* gene. However, stepwise Cox's proportional hazard regression model confirmed an independent impact of high level of MRD at day 15, MLL rearrangement and high WBC at diagnosis on the risk of BM relapse (*Online Supplementary Table S6*). CNS RFS was significantly associated with MLL gene rearrangement and heterozygous germline c.657_661del5 mutation in the *NBN* gene. Moreover, both variables demonstrated an independent negative impact on CNS relapse risk in the multivariate model (*Online Supplementary Table S7*).

In the analysis according to protocol, we found the following factors affecting RFS: WBC (*P*=0.018), age at diagnosis (*P*=0.0002), and heterozygous c.657_661del5 mutation in the *NBN* gene (*P*=0.047) in the ALL-IC BFM 2002 protocol. A multivariate model showed that the presence of the heterozygous c.657_661del5 mutation in the *NBN* gene remained an independent risk factor for ALL relapse (*Online Supplementary Table S8*). In the ALL-IC BFM 2009 protocol, MRD at day 15 was the strongest predictive factor for relapse and its level above 10% at day 15 significantly affected the RFS in our study cohort (*P*=0.009). In contrast, the presence of the heterozygous c.657_661del5 mutation in the *NBN* gene was not associated with ALL recurrence in univariate analysis (*P*=0.291). In the multivariate analysis, MRD at day 15 remained the only significant factor predicting ALL relapse (HR=2.90, 95%CI: 1.24-6.79; *P*=0.014).

The aim of the study was to investigate biological and clinical features of BCP-ALL in heterozygous carriers of *NBN* deletion. Among somatic molecular defects, MLL/11q23 rearrangements were the only lesions more frequently observed in heterozygous carriers of *NBN* deletion. Sequencing studies of breakpoint junctions of MLL rearrangements in ALL cases revealed the presence of microhomologous sequences indicative of NHEJ repair.[12] Moreover, MLL/AF4 fusion does not compromise the recognition and/or repair of DNA damage itself, therefore we speculate that the dysfunction of *NBN* due to heterozygous c.657_661del5 mutation might predispose to somatic MLL rearrangements.[13] This hypothesis is also supported by our report describing secondary acute monocytic leukemia positive for 11q23 rearrangement in a homozygous carrier of the c.657_661del5 mutation in the *NBN* gene.[14] In addition, we observed a similar level of DNA DSB accumulation in lymphocytes from heterozygous and homozygous carriers of *NBN* deletion.

Regarding the clinical course of BCP-ALL, we observed a higher risk of relapse among heterozygous carriers of the c.657_661del5 mutation in the *NBN* gene compared to *NBN* patients, which is particularly interesting in terms of isolated CNS relapse. However, sensitive molecular testing has revealed submicroscopic BM involvement by leukemia ($\geq 10^{-4}$) in 80% of patients who were initially diagnosed with isolated CNS relapse.[1] Therefore, we cannot exclude the possibility that *NBN* deletion does not contribute specifically to the recurrence of leukemia in CNS. Interestingly, none of the *NBN* deletion carriers had any clinical signs of CNS involvement at leukemia diagnosis.

Although *NBN* deletion carriers treated according to the ALL-IC BFM 2002 protocol also had increased susceptibility to develop BM relapse, a similar effect was not seen in patients enrolled into the ALL-IC BFM 2009 protocol. None of the clinical features or genetic abnormalities increasing the risk of BM relapse was found in carriers of *NBN* deletion, except for one case positive for MLL rearrangements. Wessendorf *et al*. demonstrated that nibrin deficiency resulted in increased basal mutation frequency *in vivo*.[15] Therefore, it is reasonable to assume that leukemic cells harboring defective DNA repair and following chromosomal instability due to *NBN* gene mutation may be even more prone to the cytotoxic effect of chemotherapeutics.

To summarize, our results indicate that heterozygous carriers of the c.657_661del5 mutation in the *NBN* gene are at risk of BCP-ALL relapse within the CNS. In our opinion, identification of the c.657_661del5 mutation in the *NBN* gene prior to therapy should be considered, at least in countries with a high frequency of this genetic defect. However, further replication studies among BCP-ALL patients from populations with high frequencies of *NBN* deletion are needed to confirm these associations and formulate specific clinical indications.

*Bartłomiej Tomasik,[1,2,3*] Agata Pastorczak,[1*] Wojciech Fendler,[1,2] Marcin Bartłomiejczyk,[1] Marcin Braun,[1,3,4] Marcin Mycko,[5] Joanna Madzio,[1,3] Ewa Polakowska,[1] Edyta Ulińska,[6] Michał Matysiak,[6] Katarzyna Derwich,[7] Monika Lejman,[8] Jerzy Kowalczyk,[8] Wanda Badowska,[9] Bernarda Kazanowska,[10] Tomasz Szczepański,[11] Jan Styczyński,[12] Nina Irga-Jaworska[13] and Wojciech Młynarski[1] on behalf of the Polish Pediatric Leukemia/Lymphoma Study Group (PPLLSG)*

**BT and AP contributed equally to this work.*

[1]*Department of Pediatrics, Oncology, Hematology and Diabetology, Medical University of Lodz;* [2]*Department of Biostatistics & Translational Medicine, Medical University of Lodz;* [3]*Postgraduate School of Molecular Medicine, Medical University of Warsaw;* [4]*Department of Pathology, Chair of Oncology, Medical University of Lodz;* [5]*Department of Neurology, Medical University of Lodz;* [6]*Department of Pediatric Hematology and Oncology, Medical University of Warsaw;* [7]*Department of Pediatric Oncology, Hematology and Transplantology, Medical University of Poznan;* [8]*Department of Pediatric Oncology and Hematology, Medical University of Lublin;* [9]*Division of Pediatric Hematology and Oncology, Children Hospital, Olsztyn;* [10]*Department and Clinic of Pediatric Oncology, Hematology and Bone Marrow Transplantation, Wroclaw Medical University;* [11]*Department of Pediatric Hematology and Oncology, Medical University of Silesia, Zabrze;* [12]*Department of Pediatrics, Hematology and Oncology, Nicolaus Copernicus University, Bydgoszcz and* [13]*Department of Pediatrics, Hematology and Oncology, Medical University of Gdansk, Poland*

*Correspondence: wojciech.mlynarski@umed.lodz.pl*

*doi:10.3324/haematol.2017.181198*

*Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org*

## References

1. Hagedorn N, Acquaviva C, Fronkova E, et al. Submicroscopic bone marrow involvement in isolated extramedullary relapses in childhood acute lymphoblastic leukemia: a more precise definition of "isolated" and its possible clinical implications. Blood. 2007;110(12):4022-4029.
2. Longo DL, Hunger SP, Mullighan CG. Acute Lymphoblastic Leukemia in Children. N Engl J Med. 2015;373(16):1541-1552.
3. Nguyen K, Devidas M, Cheng S-C, et al. Factors influencing survival after relapse from acute lymphoblastic leukemia: a Children's Oncology Group study. Leukemia. 2008;22(12):2142-2150.
4. Temming P, Jenney MEM. The neurodevelopmental sequelae of childhood leukaemia and its treatment. Arch Dis Child.

2010;95(11):936-940.
5. Yang JJ, Cheng C, Devidas M, et al. Genome-wide association study identifies germline polymorphisms associated with relapse of childhood acute lymphoblastic leukemia. Blood. 2012;120(20):4197-4204.
6. Pastorczak A, Szczepanski T, Mlynarski W, International Berlin-Frankfurt-Munster (I-BFM) ALL host genetic variation working group. Clinical course and therapeutic implications for lymphoid malignancies in Nijmegen breakage syndrome. Eur J Med Genet. 2016;59(3):126-132.
7. Varon R, Seemanova E, Chrzanowska K, et al. Clinical ascertainment of Nijmegen breakage syndrome (NBS) and prevalence of the major mutation, 657del5, in three Slav populations. Eur J Hum Genet. 2000;8(11):900-902.
8. Cilli D, Mirasole C, Pennisi R, et al. Identification of the Interactors of Human Nibrin (NBN) and of Its 26 kDa and 70 kDa Fragments Arising from the NBN 657del5 Founder Mutation. PLoS One. 2014;9(12):e114651.
9. Pastorczak A, Stolarska M, Treliński J, et al. Nijmegen breakage syndrome (NBS) as a risk factor for CNS involvement in childhood acute lymphoblastic leukemia. Pediatr Blood Cancer. 2011;57(1):160-162.
10. Pastorczak A, Górniak P, Sherborne A, et al. Role of 657del5 NBN mutation and 7p12.2 (IKZF1), 9p21 (CDKN2A), 10q21.2 (ARID5B) and 14q11.2 (CEBPE) variation and risk of childhood ALL in the Polish population. Leuk Res. 2011;35(11):1534-1536.
11. Mosor M, Ziółkowska-Suchanek I, Nowicka K, Dzikiewicz-Krawczyk A, Januszkiewicz-Lewandowska D, Nowak J. Germline variants in MRE11/RAD50/NBN complex genes in childhood leukemia. BMC Cancer. 2013;13(1):457-464.
12. Meyer C, Hofmann J, Burmeister T, et al. The MLL recombinome of acute leukemias in 2013. Leukemia. 2013;27(11):2165-2176.
13. Castaño J, Herrero AB, Bursen A, et al. Expression of MLL-AF4 or AF4-MLL fusions does not impact the efficiency of DNA damage repair. Oncotarget. 2016;7(21):30440-30452.
14. Pastorczak A, Szczepanski T, Trelinska J, et al. Secondary acute monocytic leukemia positive for 11q23 rearrangement in Nijmegen breakage syndrome. Pediatr Blood Cancer. 2014;61(8):1469-1471.
15. Wessendorf P, Vijg J, Nussenzweig A, Digweed M. Deficiency of the DNA repair protein nibrin increases the basal but not the radiation induced mutation frequency in vivo. Mutat Res. 2014;769:11-16.