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► **To cite this version:**

Raymonda Varon, Véronique Dutrannoy, Ilja Demuth, Kateryna Konrat, Heidemarie Neitzel, et al.. Clinical Variability and Novel Mutations in the NHEJ1 Gene in Patients with a Nijmegen Breakage Syndrome-like Phenotype. *Human Mutation*, 2010, 31 (9), pp.1059. 10.1002/humu.21315 . hal-00556036

HAL Id: hal-00556036

<https://hal.science/hal-00556036>

Submitted on 15 Jan 2011

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**Clinical Variability and Novel Mutations in the NHEJ1 Gene
in Patients with a Nijmegen Breakage Syndrome-like
Phenotype**

Journal:	<i>Human Mutation</i>
Manuscript ID:	humu-2009-0583.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	27-May-2010
Complete List of Authors:	<p>Varon, Raymonda; Charité University Medical Center, Institute of Human Genetics Dutrannoy, Véronique; Charité University Medical Center, Institute of Human Genetics Demuth, Ilja; Charité University Medical Center, Institute of Human Genetics Konrat, Kateryna; Charité University Medical Center, Institute of Human Genetics Neitzel, Heidemarie; Charité University Medical Center, Institute of Human Genetics Radszewski, Janina; Charité University Medical Center, Institute of Human Genetics Rothe, Susanne; Charité University Medical Center, Institute of Human Genetics Sperling, Karl; Charité University Medical Center, Institute of Human Genetics Digweed, Martin; Charité University Medical Center, Institute of Human Genetics Baumann, Ulrich; Medical School Hannover, Department of Pediatric Pulmonology and Neonatology Schindler, Detlev; University of Würzburg, Department of Human Genetics Gillessen-Kaesbach, Gabriele; Universität zu Lübeck, Institute of Human Genetics Schellenberger, Mario; Charité University Medical Center, Institute of Human Genetics Keng, Wee; Jalan Resideni, Hospital Pulau Pinang Nallusamy, Revathy; Jalan Resideni, Hospital Pulau Pinang Reis, André; University Hospital Erlangen, Institute of Human Genetics Nürnberg, Gudrun Nürnberg, Peter; University of Cologne, Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases</p>

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Key Words:	Nijmegen Breakage Syndrome -like, microcephaly, NHEJ1 gene, clinical variability, large genomic deletions



For Peer Review

Humu-2009-0583

Research Article

Supporting Information for this preprint is available from the
Human Mutation editorial office upon request (humu@wiley.com)

Clinical Variability and Novel Mutations in the *NHEJ1* Gene in Patients with a Nijmegen Breakage Syndrome-like Phenotype

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ABSTRACT

We have previously shown that mutations in the genes encoding DNA *Ligase IV* (*LIGIV*) and *RAD50*, involved in DNA repair by non-homologous-end joining (NHEJ), lead to clinical and cellular features similar to those of Nijmegen Breakage Syndrome (NBS). Very recently, a new member of the NHEJ repair pathway, *NHEJ1*, was discovered and mutations in patients with features resembling NBS were described. Here we report on 5 patients from 4 families of different ethnic origin with the NBS-like phenotype. Sequence analysis of the *NHEJ1* gene in a patient of Spanish and in a patient of Turkish origin identified homozygous, previously reported mutations, c.168C>G (p.Arg57Gly) and c.532C>T (p.Arg178Ter), respectively. Two novel, paternally inherited truncating mutations, c.495dupA (p.Asp166ArgfsTer20) and c.526C>T (p.Arg176Ter) and two novel, maternal genomic deletions of 1.9 and 6.9 kb of the *NHEJ1* gene, were found in a compound heterozygous state in two siblings of German origin and in one Malaysian patient, respectively. Our findings confirm that patients with NBS-like phenotypes may have mutations in the *NHEJ1* gene including multi-exon deletions and show that considerable clinical variability could be observed even within the same family.

Key Words: Nijmegen Breakage Syndrome –like; NBS; microcephaly; *NHEJ1* gene; clinical variability; large genomic deletions

Introduction

It has been repeatedly demonstrated that mutations in genes involved in the repair of DNA double-strand breaks (DSBs) lead to diseases combining immunodeficiency, radiosensitivity, neurodegeneration and cancer predisposition. One such condition is Nijmegen breakage syndrome (NBS), an autosomal recessive disorder, characterized by microcephaly, facial dysmorphism, growth retardation, immunodeficiency, hypersensitivity to ionizing radiation and a highly increased risk for lymphoreticular malignancy (The international NBS Study Group (2000). The underlying gene is *NBN* (previously *NBS1*) (MIM# 602667) and its product, nibrin, forms a complex with *MRE11* (MIM# 600814) and *RAD50* (MIM# 604040) which is involved in the repair of DNA DSBs, cell cycle control, telomere maintenance and homologous recombination (Carney et al. 1998; Varon et al. 1998).

Already with the identification of the *NBN* gene, genetic heterogeneity for NBS was indicated since some patients closely resembling this phenotype showed no mutations in the gene (Varon et al. 1998). Since then, a number of patients with similar clinical and cellular features but no *NBN* mutations have been reported in the literature, thus supporting the concept of genetic heterogeneity in NBS (Berardinelli et al. 2007; Hiel et al. 2001; Maraschio et al. 2003; Varon et al. 1998).

Recently we were able to show that some patients with features similar to the NBS phenotype have hypomorphic mutations in the DNA *Ligase IV* (MIM# 601837) (O'Driscoll et al. 2001) and *RAD50* genes (Bogdanova et al. 2008). While already a number of patients with mutations in *LIGIV* gene have been reported (Ben-Omran et al. 2005; Buck et al. 2006b; Enders et al. 2006; van Gent and van der Burg 2007), only one patient with mutations in the *RAD50* gene is known so far (Bogdanova et al. 2008). It was shown that the *LIGIV* gene product functions in a complex

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2
3 with the XRCC4 protein and directly mediates DNA strand joining during non-homologous end
4 joining (NHEJ). The inactivation of the *LIGIV* or *XRCC4* (MIM# 194363) genes in mouse cells
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8 has been shown to result in radiosensitivity and a defect in NHEJ (Barnes et al. 1998; Frank et al.
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10
11 1998). However, no human phenotype has been assigned to the *XRCC4* gene so far.

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13 In an attempt to identify new factors involved in DNA repair, whose existence has been
14 suggested before (Dai et al. 2003), two groups, Ahnesorg and colleagues and Buck and
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16
17 colleagues, 2006, simultaneously reported a novel gene involved in NHEJ, named *XRCC4-like*
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19
20 *factor (XLF) /Cernnunos/NHEJ1* gene (MIM# 611290). Here, we will use the official acronym
21
22 of the HGNC, *NHEJ1*. The *NHEJ1* gene product displays structural similarity to *XRCC4*, the
23
24 binding partner of *LIGIV* (Chang et al. 2009), and interacts directly with the *LIGIV-XRCC4*
25
26 complex (Ahnesorg et al. 2006; Fernet et al. 2005; Revy et al. 2006; Yano et al. 2008). It was
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28 shown that mutations in the *NHEJ1* gene lead to profound immunodeficiency due to T- and B-
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30 lymphocytopenia, microcephaly, developmental delay and cellular sensitivity to ionizing
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32 radiation, features common with the ones observed in patients with NBS and *LIGIV* syndromes.
33
34 However, in contrast to NBS, deficiency of the *NHEJ1* gene does not lead to impaired cell cycle
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36 checkpoints and none of the known patients so far has developed malignancy (Buck et al. 2006a;
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38 Carney et al. 1998; Dai et al. 2003). Until now, only 6 patients with (recessive) mutations in the
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41 *NHEJ1* gene have been described (Ahnesorg et al. 2006; Buck et al. 2006a). Here we report on
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44 further 5 patients from 4 families with *NHEJ1* deficiency due, in 3 of them, to novel mutations in
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47 this gene.
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53 **Materials and Methods**

54 55 **Patients**

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3 Five patients from 4 families of Spanish, Turkish, German and Malaysian origin are described in
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5 this paper. DNA was extracted from EDTA-blood according to a standard procedure from all
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7
8 five patients, their parents and healthy siblings after written informed consent. The Spanish and
9
10 the Turkish families were consanguineous. All patients were referred to our laboratory for
11
12 mutation analysis of the *NBN* gene to confirm a prior clinical diagnosis of NBS. Analyses of the
13
14 *NBN*, *LIGIV* and *RAD50* genes, showed no mutation. The main clinical and mutational data of all
15
16 five patients are summarized in Table 1. A detailed clinical description of the patients is
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18 available as Supporting Information.
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24 **Homozygosity Mapping**

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26 After negative molecular analysis for the *NBN* and *LIGIV* genes in the consanguineous Spanish
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28 family, we carried out a genome-wide homozygosity mapping with the 10K Affymetrix SNP
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30 Chip. Multi-Point analysis showed significant linkage in the family with a LOD score of 2.05 on
31
32 chromosome 2q35. The flanking SNP markers defined a region of about 17 cM.
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39 **Cell Cycle Analysis**

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41 Cell-cycle analysis was performed in PHA-stimulated lymphocytes in P2 and P3 and in cultured
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43 fibroblasts in P1 with and without irradiation using 5-bromo-2'-deoxyuridine (BrdU)-Hoechst
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45 33258 flow cytometry. Irradiated and non-irradiated patients' cells and control cultures were
46
47 harvested after 72 h. The cells were stained with 1.2 µg/ml Hoechst for 15 min and subsequently
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49 with 1.5 µg/ml propidium iodide (PI) for another 15 min at 4 °C in the dark. Bivariate flow
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51 histograms were recorded on an analytical, triple laser-equipped
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3 flow cytometer (LSRII, Becton Dickinson) using UV excitation of the Hoechst dye and 488-nm
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5 excitation of PI. The resulting cell cycle distributions reflecting cellular DNA content and cell
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7 cycle progression were quantified using the MPLUS AV software package (Phoenix Flow
8
9 Systems). A correction for cells having divided once, twice, or more times was introduced to
10
11 truly reflect the fate of the cells initially placed in culture independent of their proliferative
12
13 capacity. The results were compared with cells derived from a normal control subject and
14
15 patients with AT and LIGIV deficiency.
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22 **Chromosome Analysis**

23
24 Chromosome analysis was performed in cultured skin fibroblasts for P5 using standard
25
26 techniques. Patient 4 was analyzed elsewhere. Irradiation of cells was carried out using the X-ray
27
28 apparatus Muller MG 150 (UA = 100 kV, I = 10 mA, filter 0.3 mm Ni, dose rate: 2.1 Gy/min)
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30 with doses 0.5, 1.0, and 2.0 Gy. Radiosensitivity was determined by analysing the number of
31
32 breaks per cell in 75 metaphases per irradiation dose. The results were compared with fibroblasts
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34 derived from a normal control subject and a patient with NBS.
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41 **Molecular Analysis**

42
43 After no mutations were found in *NBN*, *LIGIV* and *RAD50* genes, all patients were analyzed for
44
45 mutations in the recently identified *NHEJ1* gene by means of PCR and direct sequencing.. The
46
47 sequences were compared to the NCBI reference sequence MN_024782.2 for the *NHEJ1* gene.
48
49 The nucleotide numbering of the mutations reflects cDNA numbering with +1 corresponding to
50
51 the A of the ATG translation initiation codon in the reference sequence, according to
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53 (www.hgvs.org/mutnomen) To identify the second *NHEJ1* mutation in the German family,
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3 introns 1, 2 and 3 of the gene were amplified and sequenced with overlapping primers covering
4
5 the whole sequence (primer sequences are available on request). All samples were run on the
6
7
8 ABI PRISM 3730 analyzer. Haplotypes, based on the polymorphisms found for the German and
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10
11 Malaysian family were constructed.

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13 In addition, we also sequenced the *XRCC4* gene as a potential modifying gene to explain the
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clinical variability observed in the German family.

RT-PCR

After written informed consent, EBV-immortalized lymphoblastoid cell lines (LCL) of all members of the Spanish, German and Malaysian families were set up according to standard procedures. Several attempts to establish LCLs from patients P1, P2, P4 and P5 failed. For P1 and P5, fibroblast cell lines could be established. RNA was extracted from cultured LCLs (Trizol) from all members of the German family with the exception of P2, and from the parents of the Malaysian patient. A small amount of RNA from P2 was extracted by using the PAX gene blood RNA-System following the manufacturer's instruction.

For first-strand cDNA synthesis, 2 µg RNA of each sample, M-MLV reverse transcriptase and random hexamer primers were used. The subsequent RT-PCR reaction used 2 µl of the cDNA product and specific *NHEJ1* gene primers, covering the whole mRNA in overlapping fragments: exons 1–4 (RT_Ex1F CGCGGCCGCTGACGGTCTCCCAACATTTGAT and RT_Ex4R GTCTTTCATATGAAGTAACGT and exons 4–8 (RT_Ex4F CTGATGGGCATGAGTCTGGCA and RT_Ex8R GCAGCTGAGGCCACAACAGAT). The

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3 resulting PCR-product(s) were cloned with the TOPO Cloning Kit, sequenced with M13
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5 universal primers and run on the ABI PRISM 3730 analyzer.
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10 **Quantitative Genomic PCR**

11
12 To detect the genomic deletions in the German and Malaysian patients suggested by the SNP
13
14 haplotypes, we performed quantitative PCR at the DNA level. Walking primers were designed
15
16 covering the regions suspected to be deleted (primer sequences are available as Supporting
17
18 Information). DNA copy number determination by quantitative PCR was performed by using
19
20 “Power SYBR Green PCR Master Mix” (ABI) and the ABI 7500 Real-Time PCR System. All
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22 samples were analyzed in duplicate and the Ct-values were compared with these of the control
23
24 samples run in parallel. Relative gene copy numbers were calculated by using the $\Delta\Delta C_t$ method.
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27 The exact positions of the deletion break points in the German and Malaysian patients were
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29 confirmed by amplification and sequencing of the junction fragments.
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36 **SNP-Analysis**

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38 In order to check whether the transcripts carrying the truncating mutations found in the German,
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40 Malaysian and Turkish patients are eliminated selectively by the mRNA surveillance mechanism
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42 nonsense-mediated mRNA decay (NMD), we carried out a SNP-analysis for the paternally
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44 inherited mutations in the corresponding families, comparing the mutant and normal alleles at the
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46 DNA and RNA level. We used the ABI SNaPshot Multiplex kit and the samples were run on the
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48 ABI PRISM 3730 analyzer.
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Western Blot Analysis

Proteins were isolated from LCLs of P3, his healthy brother and parents according to standard procedures. Blots were probed with a rabbit polyclonal antibody (Novus Biologicals) directed against the C-terminal portion of the *NHEJ1* gene product (residues 250-299). Antibodies to MRE11 (Novus Biologicals) and actin (Amersham) were used as controls.

Repeat Masker Program

Searching for a potential explanation for the occurrence of the genomic deletions found in P2, P3 and P4, we analyzed the breakpoints flanking sequences for repetitive elements by using Repeat masker program (<http://www.repeatmasker.org>).

Results

Homozygosity Mapping

The striking clinical and cellular similarities between the Spanish patient and NBS patients together with the fact that no mutations were detected in the *NBN*, *LIGIV* or *RAD50* genes, prompted us to search for an alternative gene locus in this family. Since the parents were consanguineous, we carried out a homozygosity mapping with the 10K Affymetrix SNP Chip and found linkage to chromosome 2q35 with a Lod score of 2.05. Fine mapping with microsatellites narrowed further the initial critical region of 17 cM to the 12 cM between markers D2S164 and D2S439. A total of 6 candidate genes in this region, all functionally relevant for the NBS phenotype, were sequenced and excluded as disease causing genes in this family.

Molecular Analysis

Ahnesorg et al. and Buck et al. (Ahnesorg et al. 2006; Buck et al. 2006a) reported the identification of a novel gene, *NHEJ1*, involved in DNA repair through the non-homologous end joining pathway (NHEJ). This newly described gene, designated as FLJ12010 in the database (<http://www.ncbi.nlm.nih.gov/>), was located in our region of interest on chromosome 2q35.

Sequencing of the *NHEJ1* gene in the Spanish patient (P1) revealed the homozygous missense mutation, c.168C>G (p.Arg57Gly) in exon 2, also reported by Buck et al. (2006a). Segregation analysis confirmed the presence of the mutation and the variant in the family (data not shown).

After the *NHEJ1* gene mutation was detected in the Spanish family, we screened a total of 120 patients with an NBS-like phenotype, collected over the last 10 years for mutations in *NHEJ1*.

The analysis revealed two novel paternally inherited heterozygous mutations in two patients of German (P2) and Malaysian (P4) origin. The mutation in P2 was a one base duplication of

adenine in exon 4 of the *NHEJ1* gene at position 495 leading to a frameshift and stop codon 20 amino acids downstream; c.495dupA (p.Asp166ArgfsTer20) (Fig. 1A), and in P4 - a

heterozygous nonsense mutation, also in exon 4, leading to a truncated protein, c.526C>T

(p.Arg176Ter) (Fig. 2A). In one further patient of Turkish origin with typical NBS phenotype

recently referred to us, a known homozygous nonsense mutation in exon 5 was detected,

c. 532C>T (p.Arg178Ter), (data not shown).

Further sequencing of the *NHEJ1* gene failed to detect the second maternal mutation in P2 and

P4. However, the presence of deleterious mutations on one allele in both patients and their NBS

appearance, suggested the existence of a second mutation, not detectable through conventional

mutation screening at the DNA level.

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3 Segregation analysis in the German family showed that the healthy brother and the newly born
4 third child were both heterozygous for the paternal *NHEJ1* c.495dupA (p.Asp166ArgfsTer20)
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6 mutation.
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10 In an attempt to identify the second mutation in P2, we continued analysis at the RNA level.
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12 RT-PCR was carried out with 2 sets of primers covering the whole coding sequence of the
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14 *NHEJ1* gene. Two transcripts were detected in P2, his mother and the new born child (P3) by
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16 amplifying the cDNA with primers located in exons 1 and 4. In addition to the expected product
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18 of 576 bp, a smaller product of 186 bp was seen in the agarose gel. The smaller PCR product was
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20 present neither in the father nor the healthy child, nor in control samples run in parallel (Fig. 1B).
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22 To define exactly the nature of this aberrant transcript, the RT-PCR products were cloned and
23
24 analysed by sequencing. The sequence analysis of the products spanning exons 1-4 identified an
25
26 aberrantly spliced transcript lacking exons 2 and 3 in P2, the newborn child (P3) and their
27
28 mother (Fig. 1C shown only P2). This transcript was not seen in more than 40 control samples
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30 additionally analyzed (data not shown). This finding confirmed that this aberrant transcript
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32 reflects the second, maternally inherited mutation in this family.
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39 The molecular basis for the occurrence of the aberrant transcript in the German family was
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41 unknown. We hypothesised that an intronic mutation, destroying a putative cryptic splice site
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43 might be responsible for the aberrant splicing and to prove this, we sequenced in their entirety
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45 introns 1, 2 and 3 of the gene, which encompass approx. 10 kb. We found only polymorphisms
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47 which could not explain the presence of the aberrant transcript. However, we noticed that the
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49 mother, who showed homozygosity for 13 of 14 variants, was heterozygous for a SNP in intron 3
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51 (rs55728979), close to exon 4. P2 and the newborn child P3 were homozygous for this particular
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53 polymorphism while the healthy child of the family was heterozygous (Fig. 1D). This finding
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3 suggested that the second mutation in the German family might be a large genomic deletion
4 rather than a splicing mutation. The deletion was predicted to encompass introns 1 to 3 without
5 affecting the accurate splicing of exon 1 to exon 4.
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10 To check this prediction we designed genomic primers covering intron 1, exon 2, intron 2, exon
11 3, intron 3, exon 6 and exon 7 and carried out quantitative PCR. We found a deletion of 1.9 kb
12 from intron 1 to intron 3. The break point at the 5' end was located 805 bp before exon 2, and the
13 3'-end break point was located 188 bp downstream of exon 3 (Fig. 1E). The deletion was
14 confirmed by competitive-PCR with 3 primers, 2 forward primers located in intron 1 and exon
15 3, and a common reverse primer in intron 3. The 494 bp band found in both patients and their
16 mother covers the breakpoints of the deletion and derives from the mutant allele. The 663 bp
17 fragment is generated from the wild type allele (Fig. 1F, G).
18
19

20 Haplotype analysis (data not shown) in the Malaysian family based on the intragenic SNPs
21 detected during the mutation screening strongly suggested that the second mutation here could
22 also be a genomic deletion. Indeed, quantitative PCR with genomic primers revealed an even
23 larger, maternally inherited, genomic deletion leading to a loss of approximately 6.9 kb of
24 genomic sequence (Fig. 2B). We were able to amplify a fragment of 649 bp with primers
25 flanking the deletion. The amplified and sequenced junction fragment confirmed the deletion
26 breakpoints, (Fig. 2C, D). The deletion encompasses 1295bp upstream of exon 1, including the
27 region of the minimal promoter of the *NHEJ1* gene, 3330bp spanning the sequence between
28 exon 1 to exon 3 and 2329 bp of intron 3.
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31 We sequenced in addition the *XRCC4* gene in the German family searching for modifying factors
32 which could possibly explain the phenotypic differences in this family. We detected two
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3 heterozygous polymorphisms - c.921T>G (p.Ser307Ser) (rs1056503) and c.894-7G>A
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5 (rs1805377) and in P2 but not in P3.
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10 **SNP-Analysis**

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12 Transcripts carrying premature stop codons are frequently eliminated by the NMD mechanism.
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14 We found truncating mutations in all patients described here with the exception of P1. To
15
16 quantify the alleles carrying the truncating mutations we performed comparative analysis at the
17
18 DNA and RNA level by the SnapShot method by using the samples of the corresponding
19
20 heterozygous parents in order to eliminate the influence of the second mutant allele in the
21
22 patients. Fig. 3(A and B) shows the results of the SnapShot analysis of the paternal mutation in
23
24 the German family (Fig. 3A) and the mutation in the Turkish family (Fig. 3B) (data for P4 not
25
26 shown). While both alleles are almost equally represented at the DNA level, at cDNA level the
27
28 expression of the allele carrying the truncating mutation is reduced to ~30%. Essentially the
29
30 same observations were made by comparing the profiles from genomic sequencing with cDNA
31
32 sequencing (data not shown). These results are consistent with the expected degradation of the
33
34 mutated mRNA.
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44 **Western Blot Analysis**

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46 Western blot analysis was carried out for the German family and showed clearly less NHEJ1
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48 protein in P3 as compared to the parents and the healthy brother. Unfortunately, we were not able
49
50 to include P2 in the protein analysis (Fig. 1H).
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55 **Cell Cycle and Chromosome Analysis**

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3 The example of patient P3 shows how radiosensitivity can block progression of NEHJ1-deficient
4 lymphocytes through the cell cycle. In 72-h-cultures, non-irradiated cells are distributed within
5 four consecutive cycles (Fig. 4A). Following irradiation with 1.5 Gy prior to PHA-stimulation of
6 the same cells, a prominent track of debris is detected, an elevated cell fraction remains in a
7 resting state (G0/G1) and the majority of cycling cells are arrested in the G2 phase of the first
8 cycle with only few cells reaching the G1 phase of the second cycle (G1') such that a
9 dramatically different cell cycle distribution emerges (Fig. 4B, arrows). The extent of increase of
10 G2 phase accumulation relative to growth fraction is shown in Fig. 4C. Patients P1, P2 and P3
11 reveal very high radiosensitivity with great differences of non-irradiated cells and cells irradiated
12 with 1.5 Gy, resulting in G2 ratios at the upper limit of the ataxia telangiectasia range. Indeed,
13 initial recognition of cellular radiosensitivity in patients P1, P2 and P3 was based on flow
14 cytometric cell cycle studies. Fig. 5D demonstrates the steep increment of G2 phase
15 accumulation with irradiation of NHEJ1-deficient lymphocytes. Radiosensitivity of cells from
16 patients P1, P2 and P3 as assessed by flow assay was even greater than that of cells from A-T
17 patients. G2 phase blockage of lymphocytes from a patient with DNA ligase IV deficiency fell in
18 the mean range of A-T cells (Fig. 4C, D).

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20 Radiosensitivity was assessed by chromosomal breakage test in fibroblasts of P5 in response to
21 0.5 and 1.0 Gy radiation (Fig. 5). The chromosomal damage following radiation was compared
22 to that of a normal control cell line and a cell line of an NBS patient. The cells of P5 showed a
23 high spontaneous breakage rate (0.38; normal range: 0.00 to 0.06) and extremely high levels of
24 damage after IR which were higher than those of an NBS patient. The percentage of multi-
25 aberrant cells (>8 breaks per cell) was increased to 28% at 0.5 Gy and 80% at 1.0 Gy.
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Analysis of the Sequences Surrounding the Genomic Deletions

In order to obtain clues about the mechanism underlying the genomic deletions found in our patients, we examined precisely the sequences surrounding the break points by using Repeat Masker Program (<http://www.repeatmasker.org>).

The analysis failed to reveal repetitive sequences directly in the deletion breakpoints in either patient. However, examination of the sequences across the deletion breakpoints of patient 2 revealed a 4bp homologous sequence (GAGT) at the 5' and 3' breakpoints. The 4bp short repeat was present once more, 3 bp downstream of the 3' breakpoint. Interestingly, these 3bp were inverted to the last 3 bp of the GAGT repeat (Supp. Figure S2A). In addition, the sequence downstream of the 3' deletion breakpoint in P2 showed low homology to 4 LINEs and a SINE repetitive element, the nearest of them located 22 bp from the deletion junction. No such sequences were detected in the vicinity of the 5' deletion breakpoint.

Similar to P2, a short sequence homology of 2bp (AC), potentially responsible for the deletion, was present at the 5' and 3' deletion breakpoints in the Malaysian patient (Supp. Figure S2B). Low homology to an MER1 repetitive element was found at the 5' breakpoint; however, it is located 623bp proximal to the deletion.

Discussion

Two major pathways are mainly responsible for the repair of DSBs in eukaryotes, homologous recombination (HR) and non-homologous-end joining (NHEJ) (Cahill et al. 2006; Takata et al. 1998). A number of genes, involved in DSB repair, both by HR and NHEJ, have already been assigned to different human chromosomal instability disorders such as AT, NBS, LIGIV-

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3 Syndrome, ATLD, RS-SCID etc. (Buck et al. 2006b; Neveling et al. 2007; O'Driscoll et al. 2001;
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5 Savitsky et al. 1995; Stewart et al. 1999; Varon et al. 1998).

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8 The most recently identified member of the NHEJ pathway, *NHEJ1*, located on chromosome
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10 2q35, was shown to be mutated in patients with clinical features resembling NBS (Ahnesorg et
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12 al. 2006; Buck et al. 2006a).

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15 Sequencing of the *NHEJ1* gene allowed us to identify the causative mutations in 5 patients from
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17 4 families, referred to us for molecular diagnosis of NBS. Apart from the two previously
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19 reported mutations found in P1 and P5 (Buck et al. 2006a), we identified four novel mutations in
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21 the *NHEJ1* gene in 3 further patients. These patients, two of them siblings of German origin and
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23 one child of Malaysian origin were compound heterozygous for a paternally inherited truncating
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25 mutation, c.495upA (p.Asp166ArgfsTer20) and c.526C>T (p.Arg176Ter), and a large maternal
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27 deletion of 1.9 kb and 6,9 kb genomic sequence, respectively. The consequence of the deletion in
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29 the German family was an aberrant transcript lacking sequences of exons 2 and 3.
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34 It has been shown that large genomic deletions, although not as common as the other mutations,
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36 could significantly contribute to the number of pathogenic mutations (Cavalieri et al. 2008;
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38 Cavalieri et al. 2006; Engert et al. 2008; Masson et al. 2006; Niel et al. 2006; Savino et al. 2003;
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40 Taulan et al. 2007). Large genomic deletions usually occur as a result of errors during
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42 homologous or non-homologous recombination and involve DNA sequences with short
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44 homology flanking the breakpoints, such as A-T rich sequences, palindromes or topoisomerase I
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46 and II consensus sites (Bacolla et al. 2004; Chen et al. 2005; Dai et al. 2003; Saran et al. 2006;
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48 Yeoh et al. 2006); (Cavalieri et al. 2006; Guenther et al. 2004).

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51 To better understand the possible mechanisms for the occurrence of the two deletions found in
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53 our patients, we analyzed the DNA sequences flanking the deletion breakpoints. The analysis
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3 showed that neither deletion has occurred directly between repetitive sequences. However, the
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5 deletion breakpoints in both patients did reveal short sequence homologies adjacent to the
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7 junction – GAGT in P2 (3) and AC in P4. Such short repetitive elements are often located at the
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9 5' and 3' breakpoints flanking a deletion and might be generated by slipped mispairing during
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11 DNA replication (Chen et al. 2005; Efstratiadis et al. 1980; Magnani et al. 1996). We suggest
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13 that such a scenario could be the underlying mechanism leading to the occurrence of the
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15 deletions described in this study.
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19 The genomic deletions described here are the first reported for patients with autosomal recessive
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21 NHEJ1 deficiency. Recently, a balanced chromosomal translocation t(2;7)(q35;p22), leading to a
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23 haploinsufficient disruption of the *NHEJ1* gene was found in a patient with polymicrogyria, a
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25 common malformation of the human cerebral cortex (Cantagrel et al. 2007). The authors showed
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27 that *NHEJ1* is the only gene affected by the translocation and that a truncated transcript,
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29 containing only exons 1-5 is produced. In addition, they showed that the *NHEJ1* gene is
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31 preferentially expressed in the telencephalic ventricular and subventricular zones of human
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33 embryos and suggest a role of the gene in the development of the human cerebral cortex.
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35 However, we feel that the polymicrogyria in the patient described cannot be explained merely by
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37 haploinsufficiency, but rather, by a contiguous condition, since the heterozygous parents of
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39 patients with NHEJ1 deficiency are healthy.
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46 At the time we carried out the molecular analysis in P2, the third child, P3, of the German family
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48 was born. Subsequent analysis of the newborn revealed that the boy was also a compound
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50 heterozygote for the mutations found in his affected brother and thus would also be expected to
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52 be affected. These findings were quite surprising because the newborn child was clinically
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54 healthy, apart from a mild lymphopenia, detected during episodes of several pulmonary
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3 infections shortly after birth. In contrast to his older brother, the child had no apparent
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5 microcephaly and no profound immunodeficiency. At the same time, at the cellular level P3
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7 showed a similarly high radiosensitivity as his brother, typical for patients with *NHEJ1* gene
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9 mutations (Ahnesorg et al. 2006; Buck et al. 2006a). These findings indicated that there may be
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11 great intrafamilial clinical variability despite relatively uniform cellular features. In addition,
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13 difficulties are often experienced in establishing of LCL's from patients with chromosomal
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15 instability disorders and while we were unable to establish an LCL of P2, it was no problem to
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17 establish the cell line for the newborn child, P3. These striking phenotypic differences between
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19 P2 and P3 prompted us to search for an explanation.
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24 The aberrant transcript, lacking exons 2 and 3, a consequence of the deletion between intron 1
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26 and intron 4 found in P2 and P3, is predicted to remain in-frame. The regular start codon for
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28 *NHEJ1* is in exon 2, however, an alternative initiation of translation could theoretically occur
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30 from an ATG codon at position 150, 10 amino acids downstream of the beginning of exon 4.
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32 However, in a conventional Western blot analysis we observed a decreased amount of full length
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34 NHEJ1 protein in P3, but no truncated protein corresponding to the putative alternative
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36 translation. It has already been mentioned that the NHEJ1 protein is involved in NHEJ required
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38 for DNA DSB repair and V(D)J recombination (Ahnesorg et al. 2006; Buck et al. 2006a; Tsai et
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40 al. 2007) by interacting with the XRCC4-LigIV complex (Andres et al. 2007; Hentges et al.
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42 2006; Lu et al. 2007). It was shown, that while the N-terminal fragment of the NHEJ1 protein,
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44 residues 1-170, is highly conserved and responsible for the interaction with XRCC4, the C-
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46 terminal 75 amino acid residues are required for stimulation of mismatched DNA ligation and
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48 DNA binding (Andres et al. 2007). If the aberrant transcript found in the German family did
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50 produce a truncated protein in very low amounts, it might have some residual activity
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3 considering its intact C-terminal portion and preservation of 20 of the amino acids involved in
4 the interaction with XRCC4-LIGIV. The presence of such a transcript, with partially retained
5 function in a higher amount in P3 than in P2 could be an explanation for the phenotypic
6 differences observed in the family. Unfortunately, such direct comparison between P2 and P3
7 was not possible because of lack of RNA of P2. Another explanation for the milder clinical
8 course of P3 could be modifying genetic factors which could, for example, influence the binding
9 of the NHEJ1 protein to the LIGIV-XRCC4 complex. It has recently been shown that patients
10 with the same homozygous *LIGIV* mutation could have different disease courses due to two
11 polymorphisms, A3V and T9I. The additional presence of these two polymorphisms negatively
12 modulates the activity of the LIGIV protein and the affected individual has developmental delay
13 including microcephaly, while the patient carrying only the mutation is developmentally normal
14 (Girard et al. 2004) . Since these modifying polymorphisms were not found in the German
15 family during the sequencing of the *LIGIV* gene we sequenced additionally the other binding
16 partner of the *NHEJ1* gene, *XRCC4*. Different variations in this gene have repeatedly been
17 associated with a number of cancer predispositions (Chang et al. 2009; Hsu et al. 2009; Liu et al.
18 2008; Yen et al. 2008). Surprisingly, we found two heterozygous variants in the *XRCC4* gene,
19 namely c.921T>G (p.Ser307Ser) (rs1056503) and c.894-7G>A (rs1805377) in P2 but not in P3.
20 The SNP rs1056503, together with other SNPs has recently been associated with a risk of glioma
21 (Liu et al. 2008). Of special interest is the second polymorphism in intron 7, c.894-7G>A
22 (rs1805377), which leads to alternative in frame splicing and is a common allele. Taking into
23 consideration that the excluded codons from the transcripts produced by the G allele occur within
24 the evolutionary conserved XRCC4 binding domain and the important role which this gene plays
25 in NHEJ, (Nalla and Rogan 2005) speculated that this allele could negatively influence the repair
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3 of double strand breaks. The presence of the two *XRCC4* polymorphisms in P2, and especially
4 the (rs1805377) could modify the function of the NHEJ1 protein and thus influence the clinical
5 course. One possible explanation for the effect of the polymorphisms is that they might disturb
6 the efficient binding of the *XRCC4* to the partially preserved 20 N-terminal domain of the
7 NHEJ1 protein. In contrast, the binding capacity in P3 would not be disturbed, possibly
8 explaining the milder clinical course and lack of microcephaly.
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There are a number of examples in the literature where clinical variability among carriers of the same mutation, even within one family, has been documented. We described recently also clinical variability in two siblings with NBS, carrying the same mutation (Varon et al. 2006).

Similar strong phenotypic intrafamilial variability has been documented in patients with Cystic Fibrosis. Different, genetically determined factors, referred to as gene modifiers, have been made responsible for the phenotypic variability in some conditions (Genin et al. 2008).

As already mentioned, with the exception of P3, all patients described in this study, as well as all 6 previously reported patients with *NHEJ1* gene deficiency, presented with microcephaly at birth, an accompanying characteristic in a number of genetic disorders with mutations in genes involved in the DNA damage response, assumed to be a consequence of reduced proliferation and/or an increased rate of apoptosis (O'Driscoll and Jeggo 2008). Microcephaly is a hallmark of NBS. However, Chrzanowska et al. have described an atypical NBS patient without microcephaly (Chrzanowska et al. 2001). Actually, cranial MRI analysis showed an abnormal parieto-occipital cerebrospinal fluid collection in the brain of this patient, as being responsible for the apparently normal head circumference. A similar situation could be assumed for P3 in our study. Follow-up of the patient and MRI analysis of the brain will probably shed more light on the pathogenesis of the disease in this patient. Interestingly, absence of microcephaly and

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3 developmental abnormality but fertility defects and chromosomal instability has recently been
4 reported for two adult siblings, compound heterozygote for two truncating *NBN* mutations. The
5 authors hypothesized that the very mild phenotype of these patients must be due either to a
6 partially preserved *NBN* functions or to certain modifier factors (Warcoin et al. 2009).
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8 Heterogeneity in terms of developmental delay, including microcephaly has also been observed
9 in patients with ATLD (Fernet et al. 2005).
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12 As in the German family, comparison of the wild type allele and the allele carrying the
13 truncating mutations at the DNA and RNA level in P4 and P5 indicated that the mutant allele is
14 partially, degraded by NMD. On the other hand, we showed by analyzing maternal RNA that the
15 maternal transcript in P4 (data not shown) is not expressed due to the deletion of the promoter
16 region, thus predicting a severe clinical course in the Malaysian patient. Actually, the child is in
17 a stable condition, due to regular immunotransfusion and antibiotic prophylaxis, however, the
18 presence of digital abnormalities and hearing loss could reflect the extremely reduced transcript
19 amount in this patient. The patient of Turkish origin (P5) is the only one of all the patients
20 described here for whom an autoimmune hemolytic anaemia was detected at the age of 1 and
21 treated with transfusion. In addition, the patient had severe immunodeficiency and his fibroblasts
22 showed extreme radiosensitivity, similar to other patients with *NHEJ1* deficiency. P5 died
23 recently due to a septic shock after bone marrow transplantation at the age of 18 months.
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25 Similarly, two of the patients described by Buck et al., (Buck et al. 2006a), one of French origin
26 and one of Turkish origin (this patient carried the same mutation as P5) also died due to a septic
27 shock.
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30 Our study confirms that patients with an NBS-like phenotype can have mutations in the *NHEJ1*
31 gene, including multi-exon deletions and that analysis of this gene must be considered for
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3 patients for whom molecular diagnosis of NBS and LIGIV deficiency has been excluded. One of
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5 the families described here is of particular interest because of the high degree of phenotypic
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7 variability observed in the affected children which might be associated with the identified
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9 XRCC4 gene polymorphisms.
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12 13 14 15 **Web Resources**

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17 Accession numbers and URL's for the data presented herein are as follows: NCBI,
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19 <http://www.ncbi.nlm.nih.gov/>; Online Mendelian Inheritance in Man (OMIM),
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21 <http://www.ncbi.nlm.nih.gov/Omim/>; Repeat masker program <http://www.repeatmasker.org>
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29 **Acknowledgments**

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31 We are indebted to all patients and their families who participated in this study. We thank
32
33 Christian Becker for expert technical assistance. Our special thanks go to the anonymous Referee
34
35 2 for the excellent suggestion that modifying factors might affect the phenotypic variability in
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37 one of the families.
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43 **References**

44
45 Ahnesorg P, Smith P, Jackson SP (2006) XLF interacts with the XRCC4-DNA ligase IV
46
47 complex to promote DNA nonhomologous end-joining. *Cell* 124: 301-13
48
49 Andres SN, Modesti M, Tsai CJ, Chu G, Junop MS (2007) Crystal structure of human XLF: a
50
51 twist in nonhomologous DNA end-joining. *Mol Cell* 28: 1093-101
52
53
54
55
56
57
58
59
60

- 1
2
3 Bacolla A, Jaworski A, Larson JE, Jakupciak JP, Chuzhanova N, Abeysinghe SS, O'Connell CD,
4
5 Cooper DN, Wells RD (2004) Breakpoints of gross deletions coincide with non-B DNA
6
7 conformations. *Proc Natl Acad Sci U S A* 101: 14162-7
8
9
10 Barnes DE, Stamp G, Rosewell I, Denzel A, Lindahl T (1998) Targeted disruption of the gene
11
12 encoding DNA ligase IV leads to lethality in embryonic mice. *Curr Biol* 8: 1395-8
13
14
15 Ben-Omran TI, Cerosaletti K, Concannon P, Weitzman S, Nezarati MM (2005) A patient with
16
17 mutations in DNA Ligase IV: clinical features and overlap with Nijmegen breakage
18
19 syndrome. *Am J Med Genet A* 137A: 283-7
20
21
22 Berardinelli F, di Masi A, Salvatore M, Banerjee S, Myung K, De Villartay JP, Revy P, Plebani
23
24 A, Soresina A, Taruscio D, Tanzarella C, Antoccia A (2007) A case report of a patient
25
26 with microcephaly, facial dysmorphism, chromosomal radiosensitivity and telomere
27
28 length alterations closely resembling "Nijmegen breakage syndrome" phenotype. *Eur J*
29
30 *Med Genet* 50: 176-87
31
32
33
34 Bogdanova N, Feshchenko S, Schurmann P, Waltes R, Wieland B, Hillemanns P, Rogov YI,
35
36 Dammann O, Bremer M, Karstens JH, Sohn C, Varon R, Dork T (2008) Nijmegen
37
38 Breakage Syndrome mutations and risk of breast cancer. *Int J Cancer* 122: 802-6
39
40
41 Buck D, Malivert L, de Chasseval R, Barraud A, Fondaneche MC, Sanal O, Plebani A, Stephan
42
43 JL, Hufnagel M, le Deist F, Fischer A, Durandy A, de Villartay JP, Revy P (2006a)
44
45 Cernunnos, a novel nonhomologous end-joining factor, is mutated in human
46
47 immunodeficiency with microcephaly. *Cell* 124: 287-99
48
49
50 Buck D, Moshous D, de Chasseval R, Ma Y, le Deist F, Cavazzana-Calvo M, Fischer A,
51
52 Casanova JL, Lieber MR, de Villartay JP (2006b) Severe combined immunodeficiency
53
54
55
56
57
58
59
60

- 1
2
3 and microcephaly in siblings with hypomorphic mutations in DNA ligase IV. *Eur J*
4
5 *Immunol* 36: 224-35
6
7
8 Cahill D, Connor B, Carney JP (2006) Mechanisms of eukaryotic DNA double strand break
9
10 repair. *Front Biosci* 11: 1958-76
11
12 Cantagrel V, Lossi AM, Lisgo S, Missirian C, Borges A, Philip N, Fernandez C, Cardoso C,
13
14 Figarella-Branger D, Moncla A, Lindsay S, Dobyns WB, Villard L (2007) Truncation of
15
16 NHEJ1 in a patient with polymicrogyria. *Hum Mutat* 28: 356-64
17
18
19 Carney JP, Maser RS, Olivares H, Davis EM, Le Beau M, Yates JR, 3rd, Hays L, Morgan WF,
20
21 Petrini JH (1998) The hMre11/hRad50 protein complex and Nijmegen breakage
22
23 syndrome: linkage of double-strand break repair to the cellular DNA damage response.
24
25 *Cell* 93: 477-86
26
27
28
29 Cavalieri S, Funaro A, Pappi P, Migone N, Gatti RA, Brusco A (2008) Large genomic mutations
30
31 within the ATM gene detected by MLPA, including a duplication of 41 kb from exon 4 to
32
33 20. *Ann Hum Genet* 72: 10-8
34
35
36 Cavalieri S, Funaro A, Porcedda P, Turinetto V, Migone N, Gatti RA, Brusco A (2006) ATM
37
38 mutations in Italian families with ataxia telangiectasia include two distinct large genomic
39
40 deletions. *Hum Mutat* 27: 1061
41
42
43 Chang CH, Chang CL, Tsai CW, Wu HC, Chiu CF, Wang RF, Liu CS, Lin CC, Bau DT (2009)
44
45 Significant association of an XRCC4 single nucleotide polymorphism with bladder
46
47 cancer susceptibility in Taiwan. *Anticancer Res* 29: 1777-82
48
49
50 Chen JM, Chuzhanova N, Stenson PD, Ferec C, Cooper DN (2005) Complex gene
51
52 rearrangements caused by serial replication slippage. *Hum Mutat* 26: 125-34
53
54
55
56
57
58
59
60

- 1
2
3 Chrzanowska KH, Stumm M, Bekiesiska-Figatowska M, Varon R, Biaecka M, Gregorek H,
4
5 Michakiewicz J, Krajewska-Walasek M, Jowiak S, Reis A (2001) Atypical clinical
6
7 picture of the Nijmegen breakage syndrome associated with developmental abnormalities
8
9 of the brain. *J Med Genet* 38: E3
10
11
12 Dai Y, Kysela B, Hanakahi LA, Manolis K, Riballo E, Stumm M, Harville TO, West SC,
13
14 Oettinger MA, Jeggo PA (2003) Nonhomologous end joining and V(D)J recombination
15
16 require an additional factor. *Proc Natl Acad Sci U S A* 100: 2462-7
17
18
19 Efstratiadis A, Posakony JW, Maniatis T, Lawn RM, O'Connell C, Spritz RA, DeRiel JK, Forget
20
21 BG, Weissman SM, Slightom JL, Blechl AE, Smithies O, Baralle FE, Shoulders CC,
22
23 Proudfoot NJ (1980) The structure and evolution of the human beta-globin gene family.
24
25 *Cell* 21: 653-68
26
27
28 Enders A, Fisch P, Schwarz K, Duffner U, Pannicke U, Nikolopoulos E, Peters A, Orłowska-
29
30 Volk M, Schindler D, Friedrich W, Selle B, Niemeyer C, Ehl S (2006) A severe form of
31
32 human combined immunodeficiency due to mutations in DNA ligase IV. *J Immunol* 176:
33
34 5060-8
35
36
37
38 Engert S, Wappenschmidt B, Betz B, Kast K, Kutsche M, Hellebrand H, Goecke TO, Kiechle M,
39
40 Niederacher D, Schmutzler RK, Meindl A (2008) MLPA screening in the BRCA1 gene
41
42 from 1,506 German hereditary breast cancer cases: novel deletions, frequent involvement
43
44 of exon 17, and occurrence in single early-onset cases. *Hum Mutat* 29: 948-58
45
46
47
48 Fernet M, Gribaa M, Salih MA, Seidahmed MZ, Hall J, Koenig M (2005) Identification and
49
50 functional consequences of a novel MRE11 mutation affecting 10 Saudi Arabian patients
51
52 with the ataxia telangiectasia-like disorder. *Hum Mol Genet* 14: 307-18
53
54
55
56
57
58
59
60

- 1
2
3 Frank KM, Sekiguchi JM, Seidl KJ, Swat W, Rathbun GA, Cheng HL, Davidson L, Kangaloo L,
4
5 Alt FW (1998) Late embryonic lethality and impaired V(D)J recombination in mice
6
7 lacking DNA ligase IV. *Nature* 396: 173-7
8
9
10 Genin E, Feingold J, Clerget-Darpoux F (2008) Identifying modifier genes of monogenic
11
12 disease: strategies and difficulties. *Hum Genet*
13
14
15 Girard PM, Kysela B, Harer CJ, Doherty AJ, Jeggo PA (2004) Analysis of DNA ligase IV
16
17 mutations found in *LIG4* syndrome patients: the impact of two linked polymorphisms.
18
19 *Hum Mol Genet* 13: 2369-76
20
21
22 Guenther UP, Schuelke M, Bertini E, D'Amico A, Goemans N, Grohmann K, Hubner C, Varon
23
24 R (2004) Genomic rearrangements at the *IGHMBP2* gene locus in two patients with
25
26 *SMARD1*. *Hum Genet* 115: 319-26
27
28
29 Hentges P, Ahnesorg P, Pitcher RS, Bruce CK, Kysela B, Green AJ, Bianchi J, Wilson TE,
30
31 Jackson SP, Doherty AJ (2006) Evolutionary and functional conservation of the DNA
32
33 non-homologous end-joining protein, *XLFI/Cernunnos*. *J Biol Chem* 281: 37517-26
34
35
36 Hiel JA, Weemaes CM, van Engelen BG, Smeets D, Ligtenberg M, van Der Burgt I, van Den
37
38 Heuvel LP, Cerosaletti KM, Gabreels FJ, Concannon P (2001) Nijmegen breakage
39
40 syndrome in a Dutch patient not resulting from a defect in *NBS1*. *J Med Genet* 38: E19
41
42
43 Hsu NY, Wang HC, Wang CH, Chang CL, Chiu CF, Lee HZ, Tsai CW, Bau DT (2009) Lung
44
45 cancer susceptibility and genetic polymorphism of DNA repair gene *XRCC4* in Taiwan.
46
47 *Cancer Biomark* 5: 159-65
48
49
50 Liu Y, Zhou K, Zhang H, Shugart YY, Chen L, Xu Z, Zhong Y, Liu H, Jin L, Wei Q, Huang F,
51
52 Lu D, Zhou L (2008) Polymorphisms of *LIG4* and *XRCC4* involved in the NHEJ
53
54 pathway interact to modify risk of glioma. *Hum Mutat* 29: 381-9
55
56
57
58
59
60

- 1
2
3 Lu H, Pannicke U, Schwarz K, Lieber MR (2007) Length-dependent binding of human XLF to
4
5 DNA and stimulation of XRCC4.DNA ligase IV activity. *J Biol Chem* 282: 11155-62
6
7
8 Magnani C, Cremonesi L, Giunta A, Magnaghi P, Taramelli R, Ferrari M (1996) Short direct
9
10 repeats at the breakpoints of a novel large deletion in the CFTR gene suggest a likely
11
12 slipped mispairing mechanism. *Hum Genet* 98: 102-8
13
14
15 Maraschio P, Spadoni E, Tanzarella C, Antoccia A, Di Masi A, Maghnie M, Varon R, Demuth I,
16
17 Tiepolo L, Danesino C (2003) Genetic heterogeneity for a Nijmegen breakage-like
18
19 syndrome. *Clin Genet* 63: 283-90
20
21
22 Masson E, Le Marechal C, Chen JM, Frebourg T, Lerebours E, Ferec C (2006) Detection of a
23
24 large genomic deletion in the pancreatic secretory trypsin inhibitor (SPINK1) gene. *Eur J*
25
26 *Hum Genet* 14: 1204-8
27
28
29 Nalla VK, Rogan PK (2005) Automated splicing mutation analysis by information theory. *Hum*
30
31 *Mutat* 25: 334-42
32
33
34 Neveling K, Bechtold A, Hoehn H (2007) Genetic instability syndromes with progeroid features.
35
36 *Z Gerontol Geriatr* 40: 339-48
37
38
39 Niel F, Legendre M, Bienvenu T, Bieth E, Lalau G, Sermet I, Bondeux D, Boukari R, Derelle J,
40
41 Levy P, Ruzniewski P, Martin J, Costa C, Goossens M, Girodon E (2006) A new large
42
43 CFTR rearrangement illustrates the importance of searching for complex alleles. *Hum*
44
45 *Mutat* 27: 716-7
46
47
48 Nijmegen breakage syndrome (2000) The International Nijmegen Breakage Syndrome Study
49
50 Group. *Arch Dis Child* 82: 400-6
51
52
53 O'Driscoll M, Cerosaletti KM, Girard PM, Dai Y, Stumm M, Kysela B, Hirsch B, Gennery A,
54
55 Palmer SE, Seidel J, Gatti RA, Varon R, Oettinger MA, Neitzel H, Jeggo PA, Concannon
56
57
58
59
60

- 1
2
3 P (2001) DNA ligase IV mutations identified in patients exhibiting developmental delay
4 and immunodeficiency. *Mol Cell* 8: 1175-85
5
6
7
8 O'Driscoll M, Jeggo PA (2008) The role of the DNA damage response pathways in brain
9 development and microcephaly: insight from human disorders. *DNA Repair (Amst)* 7:
10 1039-50
11
12
13
14
15 Revy P, Malivert L, de Villartay JP (2006) Cernunnos-XLF, a recently identified non-
16 homologous end-joining factor required for the development of the immune system. *Curr*
17 *Opin Allergy Clin Immunol* 6: 416-20
18
19
20
21
22 Saran A, Abeysinghe DC, Boyd JT (2006) Microelectromechanical system pressure sensor
23 integrated onto optical fiber by anodic bonding. *Appl Opt* 45: 1737-42
24
25
26
27 Savino M, Borriello A, D'Apolito M, Criscuolo M, Del Vecchio M, Bianco AM, Di Perna M,
28 Calzone R, Nobili B, Zatterale A, Zelante L, Joenje H, Della Ragione F, Savoia A (2003)
29 Spectrum of FANCA mutations in Italian Fanconi anemia patients: identification of six
30 novel alleles and phenotypic characterization of the S858R variant. *Hum Mutat* 22: 338-9
31
32
33
34
35
36 Savitsky K, Bar-Shira A, Gilad S, Rotman G, Ziv Y, Vanagaite L, Tagle DA, Smith S, Uziel T,
37 Sfez S, Ashkenazi M, Pecker I, Frydman M, Harnik R, Patanjali SR, Simmons A, Clines
38 GA, Sartiel A, Gatti RA, Chessa L, Sanal O, Lavin MF, Jaspers NG, Taylor AM, Arlett
39 CF, Miki T, Weissman SM, Lovett M, Collins FS, Shiloh Y (1995) A single ataxia
40 telangiectasia gene with a product similar to PI-3 kinase. *Science* 268: 1749-53
41
42
43
44
45
46
47
48 Stewart GS, Maser RS, Stankovic T, Bressan DA, Kaplan MI, Jaspers NG, Raams A, Byrd PJ,
49 Petrini JH, Taylor AM (1999) The DNA double-strand break repair gene hMRE11 is
50 mutated in individuals with an ataxia-telangiectasia-like disorder. *Cell* 99: 577-87
51
52
53
54
55
56
57
58
59
60

- 1
2
3 Takata M, Sasaki MS, Sonoda E, Morrison C, Hashimoto M, Utsumi H, Yamaguchi-Iwai Y,
4
5
6 Shinohara A, Takeda S (1998) Homologous recombination and non-homologous end-
7
8 joining pathways of DNA double-strand break repair have overlapping roles in the
9
10 maintenance of chromosomal integrity in vertebrate cells. *Embo J* 17: 5497-508
11
12
13 Taulan M, Girardet A, Guittard C, Altieri JP, Templin C, Beroud C, des Georges M, Claustres M
14
15 (2007) Large genomic rearrangements in the CFTR gene contribute to CBAVD. *BMC*
16
17 *Med Genet* 8: 22
18
19
20 Tsai CJ, Kim SA, Chu G (2007) Cernunnos/XLF promotes the ligation of mismatched and
21
22 noncohesive DNA ends. *Proc Natl Acad Sci U S A* 104: 7851-6
23
24
25 van Gent DC, van der Burg M (2007) Non-homologous end-joining, a sticky affair. *Oncogene*
26
27 26: 7731-40
28
29
30 Varon R, Dutrannoy V, Weikert G, Tanzarella C, Antoccia A, Stockl L, Spadoni E, Kruger LA,
31
32 di Masi A, Sperling K, Digweed M, Maraschio P (2006) Mild Nijmegen breakage
33
34 syndrome phenotype due to alternative splicing. *Hum Mol Genet* 15: 679-89
35
36
37 Varon R, Vissinga C, Platzer M, Cerosaletti KM, Chrzanowska KH, Saar K, Beckmann G,
38
39 Seemanova E, Cooper PR, Nowak NJ, Stumm M, Weemaes CM, Gatti RA, Wilson RK,
40
41 Digweed M, Rosenthal A, Sperling K, Concannon P, Reis A (1998) Nibrin, a novel DNA
42
43 double-strand break repair protein, is mutated in Nijmegen breakage syndrome. *Cell* 93:
44
45 467-76
46
47
48 Warcoin M, Lespinasse J, Despouy G, Dubois d'Enghien C, Lauge A, Portnoi MF, Christin-
49
50 Maitre S, Stoppa-Lyonnet D, Stern MH (2009) Fertility defects revealing germline
51
52 biallelic nonsense NBN mutations. *Hum Mutat* 30: 424-30
53
54
55
56
57
58
59
60

1
2
3 Yano K, Morotomi-Yano K, Wang SY, Uematsu N, Lee KJ, Asaithamby A, Weterings E, Chen
4
5 DJ (2008) Ku recruits XLF to DNA double-strand breaks. *EMBO Rep* 9: 91-6
6
7

8 Yen CY, Liu SY, Chen CH, Tseng HF, Chuang LY, Yang CH, Lin YC, Wen CH, Chiang WF,
9
10 Ho CH, Chen HC, Wang ST, Lin CW, Chang HW (2008) Combinational polymorphisms
11
12 of four DNA repair genes XRCC1, XRCC2, XRCC3, and XRCC4 and their association
13
14 with oral cancer in Taiwan. *J Oral Pathol Med* 37: 271-7
15
16

17 Yeoh EE, Holloway RH, Fraser RJ, Botten RJ, Di Matteo AC, Butters J, Weerasinghe S,
18
19 Abeysinghe P (2006) Hypofractionated versus conventionally fractionated radiation
20
21 therapy for prostate carcinoma: updated results of a phase III randomized trial. *Int J*
22
23 *Radiat Oncol Biol Phys* 66: 1072-83
24
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32 **Legends to the figures**

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41 **Fig. 3.** SNP-analysis. To quantify the alleles carrying the truncating mutations we performed
42 comparative analysis at the DNA and RNA level by the SnapShot method. We used the samples
43 of the corresponding heterozygous parents in order to eliminate the influence of the second
44 mutant allele in the patients. (A) SnapShot analysis of the paternal mutation in the German
45 family; G-Guanosine, A-Adenosine. (B) SnapShot analysis of the mutation in the Turkish
46 family; C-Cytosine, T-Thymidine. While both alleles are almost equally represented at the DNA
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3 level, at the cDNA level the expression of the allele carrying the truncating mutation is reduced
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5 to ~30% showing that the mRNA from this allele is likely to be eliminated by NMD.
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10 **Fig. 4.** Cell cycle analysis. Extraordinary sensitivity of cells with *NHEJ1* gene defects towards
11 ionizing radiation. (A) Cell cycle distribution in a 72-h PHA-stimulated lymphocyte culture from
12 patient P3 without irradiation. Bivariate BrdU-Hoechst 33258 and PI flow cytometry shows
13 scattering of the cells over four cell cycles, I (G₀/G₁-G₂), II (G₁'-G₂'), III (G₁''-G₂'') and IV
14 (G₁'''). (B) Exposure of these cells to 1.5 Gy irradiation prior to culture initiation results in a
15 collapse of cell cycle progression mainly due to G₂ phase accumulation in cycle I such that only
16 very few lymphocytes reach the G₁ phase of cycle II (G₁'). Arrows indicate increased debris and
17 elevated cell fractions in a resting state (G₀/G₁) and in G₂, respectively. (C) G₂ phase relative to
18 growth fraction ratios of normal control (small gray circles) and ataxia telangiectasia lymphocyte
19 cultures (small gray diamonds) after exposure to 1.5 Gy. The dashed line separates normal
20 control from elevated ranges. The exaggerated responses of lymphocytes from *NHEJ1*-deficient
21 patients P1 (Δ), P2 (□), and P3 (∇) towards 1.5 Gy irradiation are indicated by the large shifts of
22 their G₂ phase relative to growth fraction ratios (dotted lines) between 0 and 1.5 Gy irradiation.
23 The G₂ ratio of irradiated lymphocytes from a patient with DNA ligase IV deficiency (*LIG4*, O)
24 falls in the mean range of ataxia telangiectasia cells. (D) Dose-response curves reveal greatly
25 increased radiosensitivity of ataxia telangiectasia (A-T, gray circles) and DNA ligase IV-deficient
26 (*LIG4*, open circles) over normal control lymphocytes (CON, gray diamonds) in 72-h cultures.
27 Denoted are means ± 1 SD. Radiosensitivity of lymphocytes from *NHEJ1*-deficient patients P1
28 (Δ), P2 (□), and P3 (∇) towards 1.5 Gy irradiation is even greater than that of A-T and *LIG4*
29 lymphocytes (t-test, p<0.005).
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6 **Fig. 5.** Analysis of chromosomal instability after irradiation of fibroblasts from P5.
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8 Radiosensitivity was assessed by the chromosomal breakage test in fibroblasts of P5 in response
9 to 0.5 and 1.0 Gy radiation. The cells derived from P5 showed a high rate of spontaneous
10 breakage (0.38; normal range: 0.00 to 0.06) and extremely high levels of damage after IR which
11 were even higher than those of an NBS patient. The percentage of multi-aberrant cells (>8 breaks
12 per cell) was increased to 28% at 0.5 Gy and 80% at 1.0 Gy.
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Table 1. Clinical and molecular data of five patients with *NHEJ1* gene mutations

	P1	P2*	P3*	P4	P5**
Age(years)	~12	6	2	8	1
Origin	Spanish	German	German	Malaysian	Turkish
Consanguinity	yes	no	no	no	yes
Microcephaly^a	yes	yes	no	yes	yes
Clinical features	Facial dysmorphism			Facial dysmorphism Clinodactyly Left hearing loss	Autoimmune haemolytic anaemia
Radiosensitivity^c	yes-extreme	yes-extreme	yes-extreme	n.i.	yes-extreme
Immunodeficiency^d	yes	yes	mild	yes	yes –severe died due to sepsis
Mutation^b -paternal	c.168C>G(p.Arg57Gly)	c.495dupA(p.Asp166ArgfsTer20)	495dupA(p.Asp166ArgfsTer20)	c.526C>T(p.Arg176Ter)	c.532C>T (p.Arg178Ter)
Mutation^b -maternal	c.168C>G(p.Arg57Gly)	1.9kb deletion	1.9kb deletion	6.9kb deletion	c.532C>T (p.Arg178Ter)

*P2 and P3 are siblings

**A picture of P5 at the age of 41/2 months is shown in Supp. Figure S1

n.i.: no information available

^a Head circumference - P2 [32 cm (-4.0 SD)]; P5 [32 cm (-4.0 SD)]; for P1 and P4 – no data available.

^bThe nomenclature is following Journal guidelines (www.hgvs.org/mutnomen), NHEJ1 RefSeq accession number MN_024782.2. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG initiation codon in the reference sequence.

^c Detailed data is available in Supporting information.

^d Detailed data is available in Supporting information.

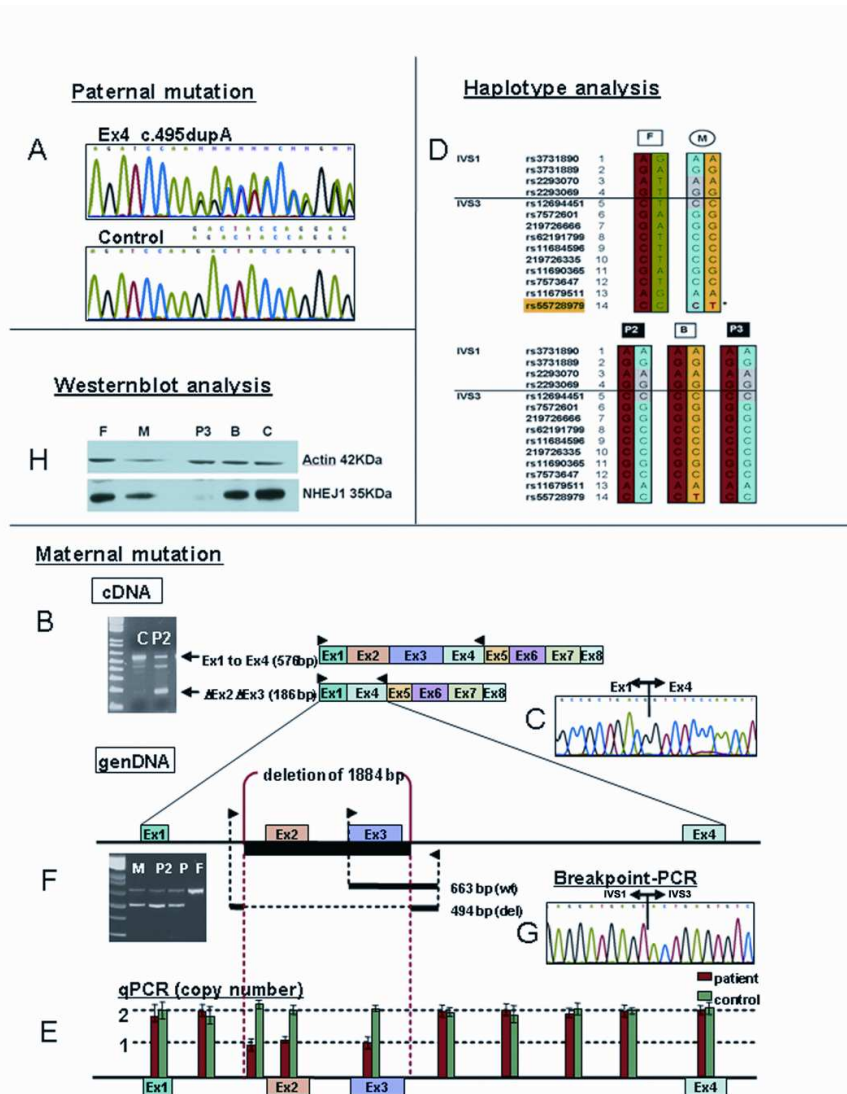


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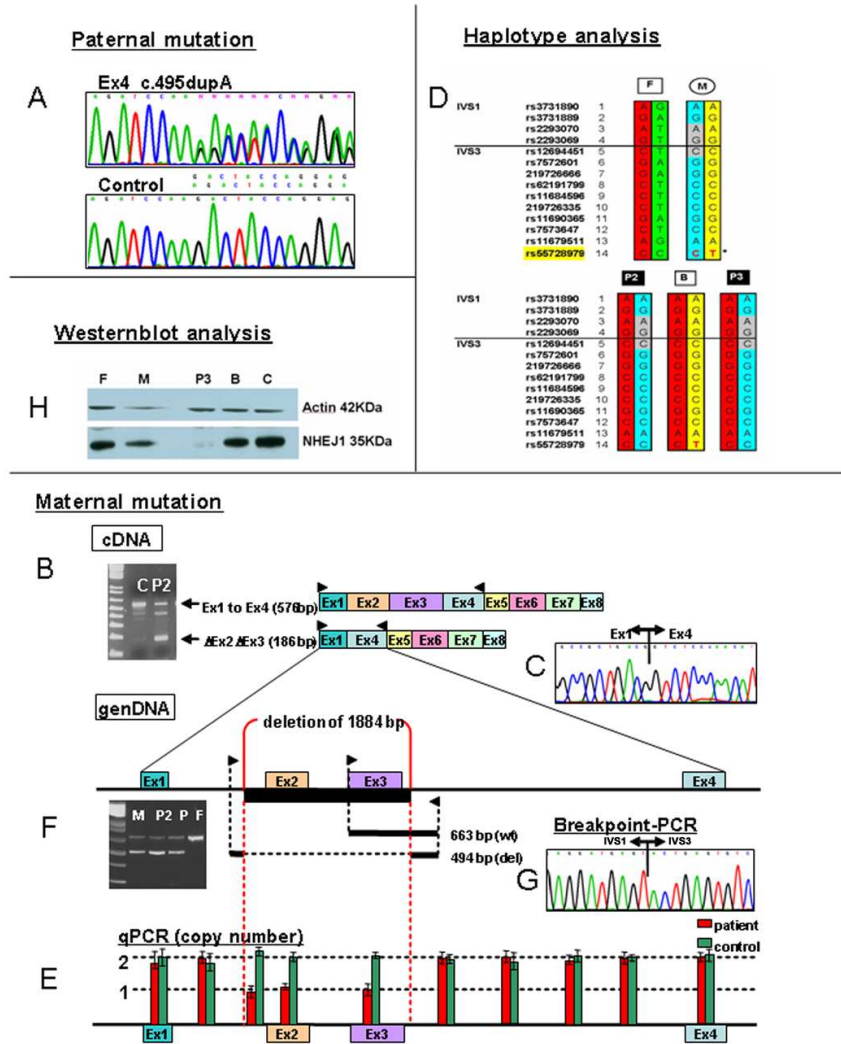


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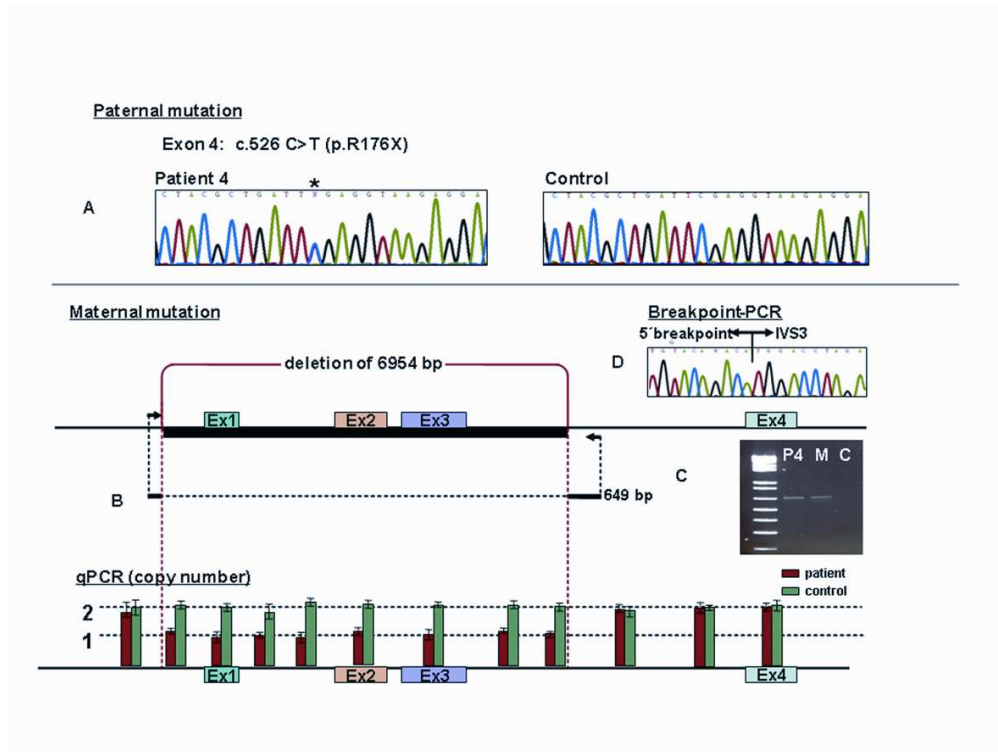


Fig. 2: Molecular analyses in patient 4

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Amplification of the deletion breakpoint. The deletion was confirmed by PCR with a forward primer located upstream of exon 1 and a reverse primer in intron 3. The junction fragment of 649 bp could be amplified in DNA from P4 and his mother but not in DNA from the control individual. P4, M – mother, C – control. (D) Sequence analysis of the deletion breakpoints. Shown is the segment of the sequence analysis of the deletion breakpoint. The deletion starts 1295bp upstream of exon 1 of the NHEJ1 gene, 3330bp spanning the sequence between exon 1 and exon 3 and 2329 bp of intron 3.

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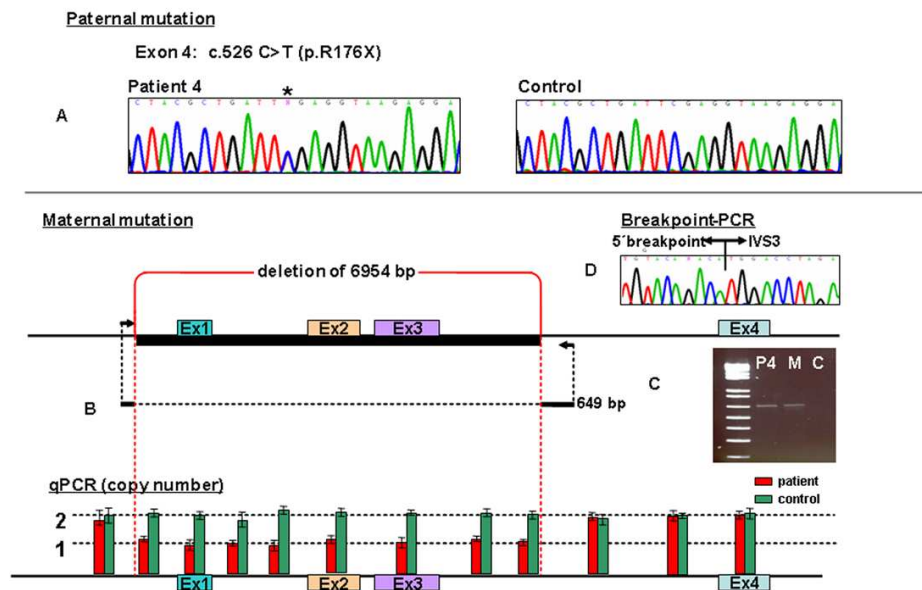


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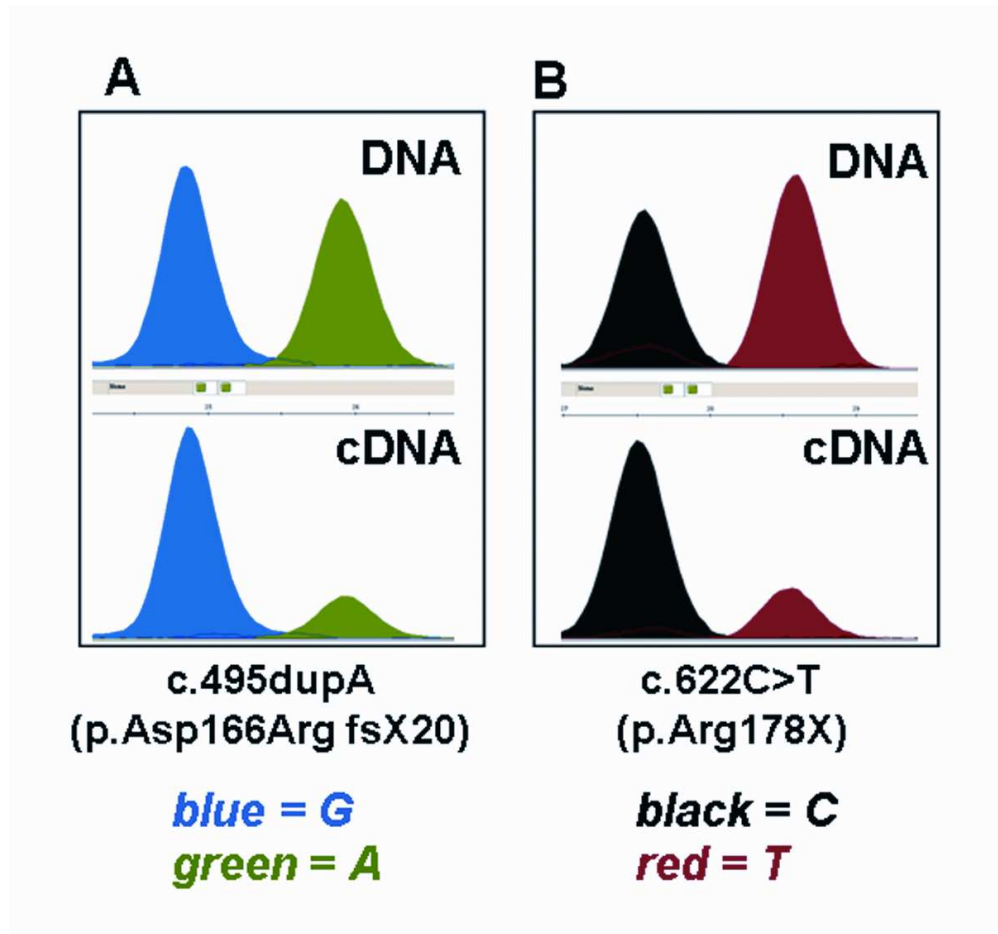


Fig. 3: SNP-analysis

To quantify the alleles carrying the truncating mutations we performed comparative analysis at the DNA and RNA level by the SnapShot method. We used the samples of the corresponding heterozygous parents in order to eliminate the influence of the second mutant allele in the patients. (A) SnapShot analysis of the paternal mutation in the German family; G-Guanosine, A-Adenosine. (B) SnapShot analysis of the mutation in the Turkish family; C-Cytosine, T-Thymidine. While both alleles are almost equally represented at the DNA level, at the cDNA level the expression of the allele carrying the truncating mutation is reduced to ~30% showing that the mRNA from this allele is likely to be eliminated by NMD.

153x143mm (200 x 200 DPI)

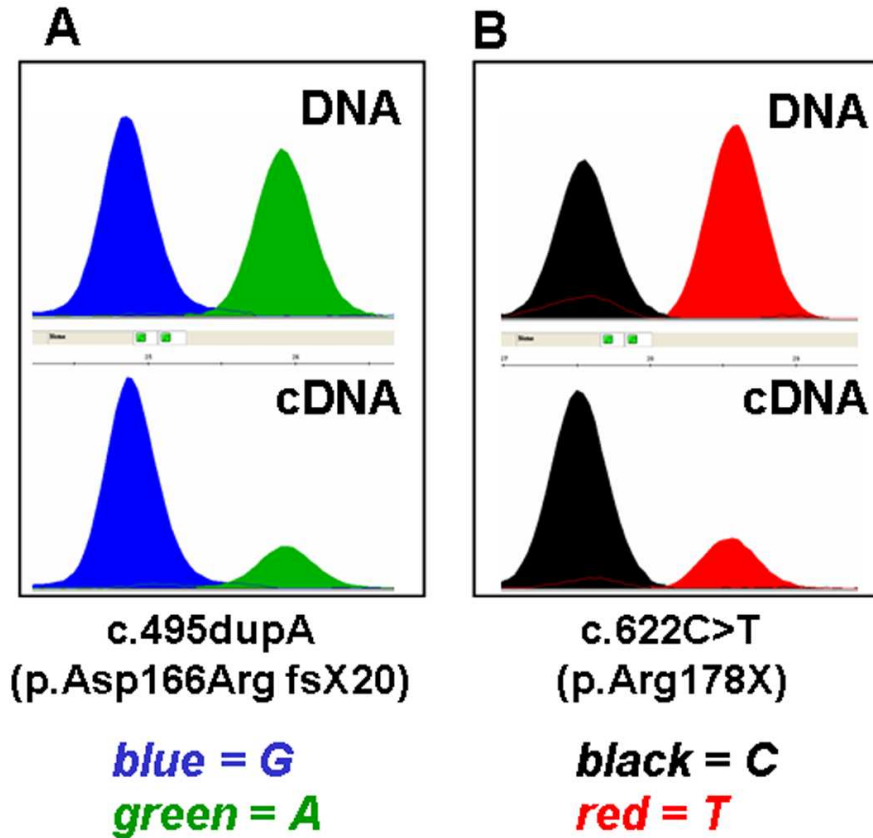


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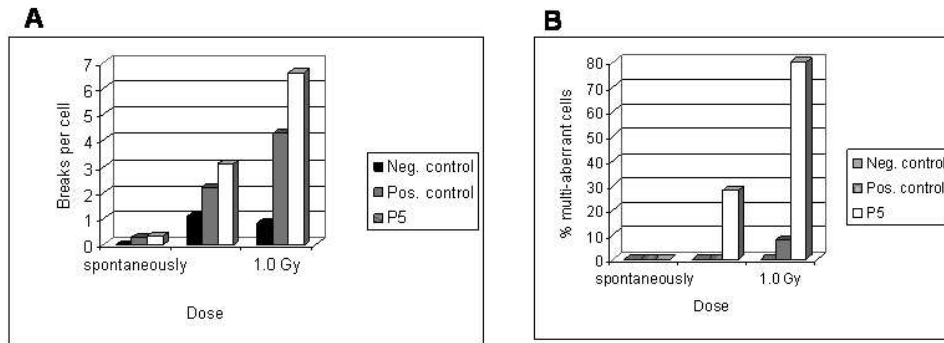


Fig. 5: Analysis of chromosomal instability after irradiation of fibroblasts from P5. Radiosensitivity was assessed by the chromosomal breakage test in fibroblasts of P5 in response to 0.5 and 1.0 Gy radiation. The cells derived from P5 showed a high rate of spontaneous breakage (0.38; normal range: 0.00 to 0.06) and extremely high levels of damage after IR which were even higher than those of an NBS patient. The percentage of multi-aberrant cells (>8 breaks per cell) was increased to 28% at 0.5 Gy and 80% at 1.0 Gy.

102x38mm (200 x 200 DPI)

Supporting Information

Patients – Clinical Description

Patient 1 (P1)

The patient was born in a consanguineous Spanish family, the parents are 2nd degree cousins. The family also has two healthy children. The affected boy presented with microcephaly, developmental delay, immunodeficiency, and recurrent respiratory infections. The patient's cells showed radiosensitivity.

Patient 2 (P2) and Patient 3 (P3)

P2 was the first liveborn male child in a German family. The family pedigree contained 5 stillbirths, most probably due to a balanced paternal translocation;

46, XY t (11;22)(q23;q11.2). The pregnancy was uneventful and P2 was delivered at term. The boy had a birth weight of 2480 g (-3,6 SD), length of 44 cm (-4,2 SD) and head circumference of 32 cm (-4,0 SD). The diagnosis NBS was suspected at the age of 4 due to microcephaly, urinary tract infection, diarrhoea, persistent lymphopenia (lymphocyte count 600/ μ l), neutropenia, hypogammaglobulinemia with B-cell deficiency. DNA of the child was referred to our laboratory at the age of 4 years 9 months for molecular diagnosis of NBS. At the age of 6 11/12 he presented with a height of 117 cm (-1,25 SD) , weight of 19.6 kg (-0,3 SD) and head circumference of 47 cm (-3,8 SD). Regular immunoglobulin replacement therapy keeps the child in relative good health and even allows him to attend a regular school.

A healthy brother was born three years later and at the time the molecular analysis for P2 was carried out, the family was expecting their third child. Since no mutations were detected either in the *NBN* or *LIGIV* genes the family continued the pregnancy. The third child was born at 39th week of gestation after an uneventful pregnancy with normal weight (3170g) and length (49 cm) and no microcephaly (head circumference 38 cm). Shortly after birth, several episodes of respiratory infections revealed a slight but persistent lymphopenia (lymphocyte count 1200/ μ l) and low IgA and IgG levels, suggesting that the newborn might suffer from the same disease as his older brother. Follow-up at the age of 1 confirmed the slight combined immunodeficiency and the absence of microcephaly. The child receives no immunoglobulin replacement therapy and is currently in a good health.

Patient 4 (P4)

An 8-year-old boy of Malaysian origin with the clinical features of NBS was referred to our laboratory for molecular analysis. The child presented with microcephaly, facial dysmorphism, and immunodeficiency due to panhypogammaglobulinemia. In addition, clinodactyly and profound left sided hearing loss were present. At the age of 2 years, thrombocytopenia was detected, which has been cured after 2 years of treatment. Cytogenetic analysis showed increased sensitivity towards mitomycin C which initially suggested Fanconi anemia. The child's condition remains relatively good due to regular immunoglobulin transfusions and Bactrim prophylaxis.

Patient 5 (P5)

P5 was born to consanguineous parents of Turkish origin as a first child. The pregnancy was uneventful; the boy was delivered at term with Apgar scores of 9/10/10. His birth weight was 2290g (-4.2 SD), length 49cm (-1,7 SD) and head circumference 32 cm (-4.0 SD). Supp. Figure

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2
3 S1 shows the patient at the age of 41/2 months. At the age of 9 months the child was hospitalized
4 because of dystrophy and mouth lesions. At 10 months the weight was 5200 g (-3.3 SD), length
5 64 cm (-2.8 SD) and head circumference 38.5 cm (-5.3 SD). Based on microcephaly, failure to
6 thrive and severe immunodeficiency, due to B- and T- cell deficiency and absolute lymphopenia,
7 the diagnosis NBS was suspected. At the age of 12 months severe autoimmune haemolytic
8 anaemia (HB:2,5g%) was detected and treated by blood transfusions and, later, bone marrow
9 transplantation was carried out. The child died at the age of ~18 months due to sepsis.
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For Peer Review



Supp. Figure S1. The picture shows the patient at the age of 41/2 months, note the typical facial NBS-like appearance.

A: P2

IVS1 TTGGTGGCAGTGCACCTGGAGTGGGAATGTGGAGGATGAGTTAAGTGGAGTTAATTCAGTC TTTACCTCT
 Mutant TTGGTGGCAGTGCACCTGGAGTGGGAATGTGGAGGATGAGTACTGAGTGTCTTTATTTCATTAATTAAT TTA
 IVS3 TTCTTGACTGTTAAATCCTTTCCTTGCAAAGGAGCAGAGTACTGAGTGTCTTTATTTCATTAATTAAT TTA

A: P4

5' End AATGCCTCAGAAGCCCAGGGAGAAGGCTGCTCAAGTGTACAGACTTCTTTCCCATCCAAAACCTCTGTCCC
 Mutant AATGCCTCAGAAGCCCAGGGAGAAGGCTGCTCAAGTGTACAGACATGGACCTAGAAATGCGGAATATGTAGT
 IVS3 GCCTTTGGGAATCCCTAGCAATAGCTAATCTCATTTCATTACATGGACCTAGAAATGCGGAATATGTAGT

Supp. Figure S2. Nucleotide sequences of the deletion junction and the corresponding 5' and 3' breakpoints in P2 and P4. (A) A short 4bp region of homology, **GAGT**, at the breakpoints and the deletion junction is underlined. A second **GAGT** repeat is located 3 bp after the 3' breakpoint. The 3 bp separating the two **GAGT** repeats – ACT are inverted to the last 3 bp of GAGT. (B) A 2bp short region of homology AC at the breakpoints and the deletion junction is underlined.

Supp. Table S1. Primers used for quantitative PCR to detect genomic deletions within the *NHEJ1* gene

Primer	Sequence
qPCRXLf_Ex1F	TATCCCAAGACCCTGCCTCC
qPCRXLf_Ex1R	ACTCTCTTAGCCGCACGCAC
qPCRXLf_Ex2F	TTTTATCACCAAGCAGGGCTATG
qPCRXLf_Ex2R	TCGCTGGCTGACCACACTAG
qPCRXLf_Ex3F	CTCCTTCGCCCATTGTTGAA
qPCRXLf_Ex3R	AATCAGTGCATCTGCCACAC
qPCRXLf_Ex6F	GGCATGCAGCATTGGTGAT
qPCRXLf_Ex6R	CTTGATGCTTCTGTCCCCTTG
qPCRXLf_Ex7F	AGATCCTCATACTCAAACAGTGCTT
qPCRXLf_Ex7R	GGCTGAGGAGACCAGTTGTTCT

Exon numbering according to transcript ENST00000356853, Ensembl vs57