

The clinical and biological overlap between Nijmegen Breakage Syndrome and Fanconi anemia

A.R. Gennery^{a,*}, M.A. Slatter^b, A. Bhattacharya^b, D. Barge^c, S. Haigh^d, M. O'Driscoll^e,
R. Coleman^e, M. Abinun^a, T.J. Flood^b, A.J. Cant^a, P.A. Jeggo^e

^a*School of Clinical Medical Sciences, University of Newcastle-upon-Tyne, UK*

^b*Department of Paediatric Immunology and Bone Marrow Transplantation, Newcastle General Hospital, Newcastle-upon-Tyne Hospitals NHS Trust, UK*

^c*Department of Immunology, Royal Victoria Infirmary, Newcastle-upon-Tyne Hospitals NHS Trust, UK*

^d*Institute of Human Genetics, Newcastle-upon-Tyne, UK*

^e*Genome Damage and Stability Unit, University of Sussex, Brighton, East Sussex, UK*

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Abstract

Fanconi anemia (FA), an autosomal recessive chromosomal instability syndrome, is characterized clinically by developmental abnormalities, growth retardation, progressive bone marrow failure, pancytopenia, and pronounced cancer predisposition. Nijmegen Breakage Syndrome (NBS) is a related disorder that shares overlapping clinical features, principally, developmental delay, microcephaly, and cancer predisposition. The diagnosis has relied on chromosomal instability following exposure to DNA cross-linking agents in FA and to ionizing radiation (IR) in NBS. We describe two patients who clinically had FA, but showed sensitivity to both DNA cross-linking agents and ionizing radiation, and who were found to have a rare mutation in the NBS gene. The importance of genetic diagnosis with respect to treatment and prognosis is discussed.

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Introduction

Fanconi anemia (FA) is an autosomal recessive chromosomal instability syndrome characterized clinically by developmental abnormalities, growth retardation, progressive bone marrow failure, pancytopenia, and pronounced cancer predisposition [1,2]. Developmental abnormalities include skeletal malformations such as malformed digits and microcephaly. The most common malignancy observed in FA is myeloid leukemia [3,4], but myelodysplasia and anemia are also common features. A striking cellular feature is sensitivity to DNA cross-linking agents, such as Mitomycin C (MMC) and diepoxybutane (DEB) [1,2]. Indeed,

elevated chromosome instability following exposure to these agents is used to aid FA diagnosis [5]. Molecular analysis has identified six FANC genes that function in a common pathway that plays a role in the response to DNA cross-links [1].

Nijmegen Breakage Syndrome (NBS) is a related chromosomal instability disorder that shares overlapping clinical features with those of FA [6], principally, developmental delay, microcephaly, and cancer predisposition. However, immunodeficiency, particularly humoral immunodeficiency, is more common in NBS than in FA [7]. Myelodysplasia or aplastic anemia is rarely described [8]. Although a range of tumors has been described in NBS patients, B cell lymphomas represent the most common tumor type. Cytogenetically, NBS patients are characterized by elevated aberrations, typically inversions and translocations that preferentially involve chromosomes 7 and 14 [6]. Both primary and transformed NBS cell lines show characteristic sensitivity to ionizing radiation (IR) [9], a feature also found in cell lines

* Corresponding author. Paediatric Immunology, Ward 23, Newcastle General Hospital, Westgate Road, Newcastle-upon-Tyne NE4 6BE, UK. Fax: +44 191 2730813.

E-mail address: a.r.gennery@ncl.ac.uk (A.R. Gennery).

derived from patients with ataxia telangiectasia (A–T) and ataxia telangiectasia-like disorder (ATLD) [10]. Chromosomal sensitivity to IR is used to aid diagnosis of NBS and sensitivity to DNA cross-linking agents has not been considered a diagnostic tool. The gene defective in NBS has been identified as *NBS1* [11]. *NBS1* is a component of the Mre11 complex, which involves *MRE11*, *RAD50*, as well as *NBS1* [12]. Significantly, ATLD is conferred by mutations in *MRE11* [10]. Prevailing evidence suggests that the Mre11 complex functions in an ATM-dependent signaling pathway that responds to DNA double strand breaks (DSBs), the major lethal lesion induced by IR.

Taken together, the majority of past literature has suggested that FA and NBS are defective in mechanisms that respond to different forms of DNA damage, namely, DNA cross-links and DNA DSBs, respectively. However, recent evidence has suggested that there is overlap between the pathways in which NBS1 and the FANC proteins function. One study provided evidence for the convergence of the FA and ATM-dependent signaling pathways [13]. Firstly, FA-D2 cells were shown to display an aberrant response to IR, namely an inability to effect S phase checkpoint arrest. Significantly, this is a characteristic feature displayed by A–T and NBS cells. Additionally, it was shown that FANCD2 is phosphorylated by ATM in response to IR and that this phosphorylation is required for S phase checkpoint arrest [13]. More recently, it has also been shown that FANCD2 co-localizes with the Mre11 complex after DNA damage [14]. Cell lines from NBS patients display sensitivity to MMC as well as to IR and conversely FA-D2 cells display mild radiosensitivity [14]. To add to these overlapping cellular functions of the proteins, an unusual individual was reported who showed features common to both FA and NBS. Here, we report two patients who were diagnosed clinically as having Fanconi anemia, but who were subsequently found to have a rare mutation in *NBS1*.

Patients

Patients were referred to the United Kingdom Northern Supraregional Unit for Paediatric Immunology for assessment. Patient 1, the 6th child of consanguineous Pakistani parents developed respiratory syncytial virus (RSV)-positive bronchiolitis at 2 weeks of age requiring ventilation for 8 days. She continued to secrete RSV in nasopharyngeal secretions for over 5 weeks. There was no family history of unusual susceptibility to infection. She was small with microcephaly (birth weight 2.56 kg, occipitofrontal circumference 31.5 cm) but no digital anomalies (Fig. 1). She showed continuing evidence of failure to thrive with weight of 8.76 kg and extreme microcephaly (occipitofrontal circumference 40 cm) at 2.25 years of age. Her development was age appropriate, and she was walking, feeding herself, dressing, and had a few words, and good comprehension of three languages.



Fig. 1. Patient 1 at 1-month-old showing microcephaly. (Reproduced with permission of the Audiovisual Department, Newcastle upon Tyne University.)

Patient 2, the 2nd child of first cousin consanguineous Pakistani parents, was referred at 5 months of age; he had a mild RSV negative bronchiolitic episode at age 9 weeks, an episode of rotavirus positive enteritis at 11 weeks, and pneumonia with chest radiograph changes at aged 13 weeks. The patient was growth retarded at birth (weight 2.46 kg, occipitofrontal circumference 32 cm) and failed to thrive; by age 20 months, his weight was 6.6 kg, occipitofrontal circumference 38.5 cm. Developmental progress was severely delayed and although the patient could reach out for objects and transfer them, and roll from front to back and back to front, he was unable to sit, crawl, weight bear, or vocalize.

The phenotypic appearance and initial investigations in both patients suggested a diagnosis of Fanconi anemia, although such findings would also be consistent with NBS.

Methods

Serum immunoglobulins and specific antibodies

Serum immunoglobulins and IgG subclasses were measured by rate nephelometry. Specific anti-tetanus, anti-Hib, and anti-pneumococcal polysaccharide IgG were measured by ELISA and calibrated against international standards.

Lymphocyte phenotyping

Lymphocyte phenotypes were measured on whole blood using FITC-, PE-, PerCP-, or APC-conjugated antibodies by flow cytometry using a Becton Dickinson FACScan (Becton Dickinson UK Ltd, Oxford) as previously described [15].

T cell receptor repertoire analysis and genotypic analysis of TCRVB and IgH

Direct immunofluorescence was performed on whole blood using FITC- or PE-conjugated antibodies [16]. DNA was isolated from peripheral blood lymphocytes using the QIAGEN mini blood kit according to the manufacturer's protocol and amplified for *TCRB*, *TCRG*, and *IgH* gene analysis as previously described [17–20].

Lymphocyte chromosomal sensitivity analysis

Whole blood was incubated with Diepoxybutane or Mitomycin C according to standard protocols and analyzed for chromosomal instability. Radiation sensitivity was examined by exposing cultured lymphocytes to gamma irradiation and analyzing for chromosomal instability [21].

Western Blot protein analysis and DNA sequencing

Western blotting was performed using whole cell extract from each fibroblast cell line incubated with Anti-p95NBS1 (Ab-1) rabbit polyclonal antibody (Oncogene Research products, Merck KGaA, Darmstadt, Germany) using standard methods. Mutation analysis was performed on cDNA as previously described [8]. Genomic DNA was sequenced to confirm the presence of mutations.

Results

Both patients were lymphopenic; lymphocyte phenotype revealed pan lymphopenia (Table 1) with a marked CD8 lymphopenia, and an increased percentage of T cells

expressing the gamma delta receptor in patient 2. In both patients, four V beta T cell receptor (TCR) families were demonstrated to be absent by fluorescence-activated cell sorting (FACS), although normally detected in healthy controls [16]. Molecular DNA analysis demonstrated a polyclonal distribution of V beta T cell receptor families. Both patients showed reduced lymphocyte proliferation to phytohaemagglutinin (PHA) compared with a normal control (Table 1). Patient 1 had pan-hypogammaglobulinaemia while patient 2 had low IgG but normal IgM and IgA levels. Despite patient 1 completing the primary immunization schedule and a booster dose, the tetanus antibody response was very poor and the HiB antibody response was absent (Table 1). The tetanus vaccination antibody response was normal but HiB antibody response was absent in patient 2 (Table 1).

Chromosomal analysis of blood from patient 1 showed a normal 46XX karyotype with marked increase in spontaneous chromosome breakage; 11/163 cells showed inversions or translocations of chromosome 7 or 14. However, there was also a significant increase in chromosome breakage and rearrangement following exposure of lymphocytes to MMC, with patient lymphocytes showing a 10-fold increase compared with a normal control (Fig. 2). Increased sensitivity to DEB was also demonstrated, with patient lymphocytes showing a greater than 20-fold increase in chromosome breakage, compared to a normal control, findings more commonly associated with FA (Fig. 2). In patient 2, chromosomal analysis on blood was unsatisfactory because of poor growth of cells but limited examination showed a 46XY karyotype with an unusually high level of spontaneous chromosome breakage. None of seven cells examined demonstrated re-arrangement of chromosome 7 or 14. Fibroblast cytogenetic analysis demonstrated increased sensitivity to MMC with an 18-

Table 1
Immunophenotype of patients with 1089C→A mutation of the *NBS1* gene

Patient	Lymphocyte x10 ⁹ /L (4.0–10.5)	CD3 cells/μl (1400– 8000)	CD4 cells/μl (900– 5500)	CD8 cells/μl (400– 2300)	CD19 cells/μl (600– 3100)	TCRγδ (% of CD3) (1–10)	Missing TCR Vβ families	IgG g/L (2.9– 8.6)	IgA g/L (0.15– 0.70)	IgM g/L (0.33– 1.43)	Tetanus IU/ml (0.1– 10)	HiB mg/L (1–10)
1	2.4	449	336	67	333	10	3,4,23,24	1.81	<0.07	0.29	0.11	<0.1
2	1.22	261	151	117	147	13	4,13,23,24	1.84	0.13	0.79	0.48	<0.1

Phytohaemagglutinin (PHA) response
(5 ug/ml, 1 x 10⁶ lymphocytes/ml)

Patient	Background control	Background patient	PHA control	PHA patient
1	178	531	42349	19360
2	335	151	45135	4095

Notes. Number in parentheses represent the normal range. Specific antibody levels to tetanus and HiB are post the primary immunisation doses and a fourth booster dose.

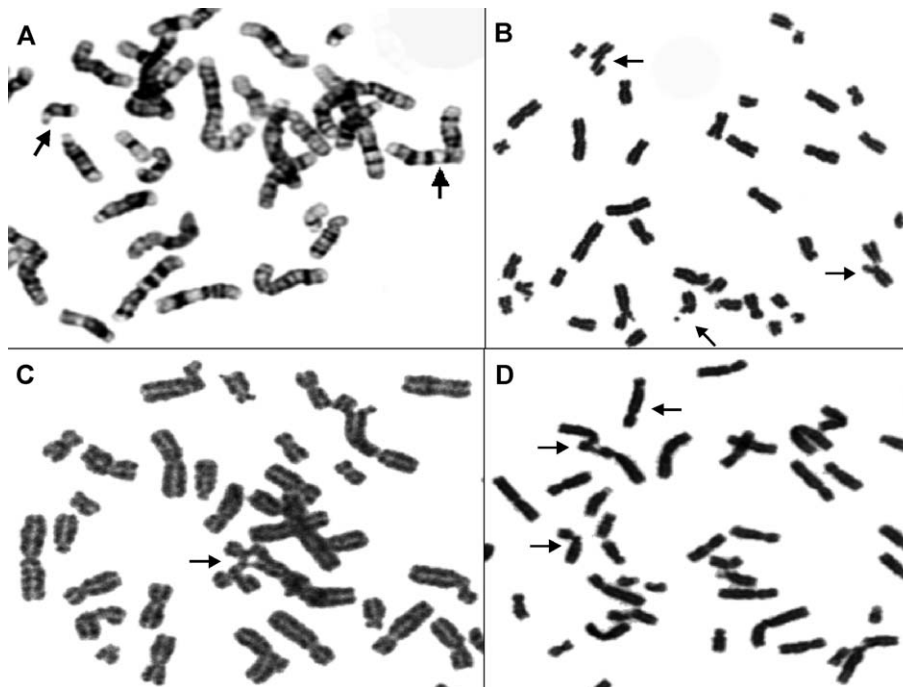
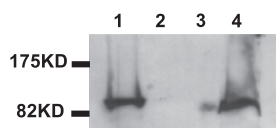


Fig. 2. Karyotype from patient 1 showing (A) Chromosome t(7;14) re-arrangement (arrow), (B) chromosomal breakage following exposure to 50 centigray ionizing radiation, (C) multiradial formation (arrow) after 72-h culture following exposure to Mitomycin C at 0.32 µg/ml for 60 min, (D) chromosome breakage (arrow) following lymphocyte culture with DEB for 72 h.

to 19-fold increase in chromosome breakage compared to control cells.

Although fibroblast cell lines from patient 1 grew very poorly and it was not possible to examine survival to IR, patient lymphocytes showed a 40- to 50-fold increase in chromosome aberrations following exposure to ionizing radiation compared with a normal control, an abnormality normally shown by NBS patients (Fig. 2). Full length NBS1 protein could not be detected by Western blotting (Fig. 3). Sequence analysis demonstrated a homozygous 1089C>A mutation resulting in Y363X, which, if translated, is predicted to generate a prematurely truncated NBS1 protein of 45 kDa, which was not however, detected by Western blotting. Full length NBS1 protein is approximately 95 kDa. A skin fibroblast cell line was established from patient 2, but the cells grew exceptionally poorly. Sequence analysis demonstrated the same homozygous 1089C>A mutation in NBS1 as observed in the patient described above, confirming a diagnosis of NBS rather than FA.



Lane 1. 249BR (WT primary fibroblast)
 2. F02/114 (Patient primary fibroblast)
 3. NBS1LBI (SV40 transformed fibroblast NBS Slavic mutation)
 4. NBS1LBI + p95 (retrovirally complemented)

Fig. 3. Absence of NBS protein in patient 1 (Lane 2) by Western blot compared with normal control (Lane 1) and NBS patient with a Slavic mutation (657del5) (Lane 3).

In view of the antibody deficiency demonstrated in both patients, both were commenced on intravenous immunoglobulin replacement and remain infection free.

Discussion

Both FA and NBS are chromosomal instability disorders characterized by developmental defects, microcephaly and cancer susceptibility, classically acute myeloid leukemia in FA, and lymphoid malignancies in NBS [1,6]. Recently, variants of FA have been described with more pronounced immune deficiency [14], and some patients with NBS have been described with aplastic anemia [8], a complication more commonly associated with FA. In addition to these clinical similarities, some FA cell lines show sensitivity to IR and, as is apparent from these cases and other reports, some NBS cell lines show sensitivity to DNA cross-link agents such as MMC and DEB [22]. Both NBS1 and FANCD2 proteins are phosphorylated by ATM (the protein defective in A-T patients) following exposure to IR [13,22]. Furthermore, both NBS1 and FANCD2 assemble in nuclear foci with the MRE11-RAD50 complex following MMC exposure. The molecular pathways involved in response to IR and DNA cross-links are, therefore, not separate but integrated. Our findings show that MMC sensitivity should not be taken as being diagnostic for just FA as it may be found in other chromosomal breakage syndromes, in particular NBS. Interestingly, this feature is also found in cells from ATLD patients who are defective in MRE11, a protein that interacts closely with NBS1 [14].

This report describes two patients with mutations in NBS1 at 1089C>A. The mutational change introduces a premature stop codon and is predicted to produce a truncated protein of 45 kDa. This mutational change has also been reported in one previous patient who also displayed abnormal features that were similar to those found in FA patients [14]. The clinical features of all these patients appear to be particularly severe compared to other NBS patients with early onset infection due to immune deficiency. Failure to thrive was also more marked than described in other NBS patients. This mutation has not been described in the NBS Registry of 55 patients and is distinct to the common 657del5 mutation described in patients of Slavic origin [7]. Mice lacking Nbs1 are embryonic lethal. The common founder Nbs1 mutation 657del5 was originally thought likely to represent a null mutation. However, recently, it has been shown that cells harboring the 657del5 mutation express a 70-kDa NBS1 protein lacking the native N terminus that arises by internal translation initiation within the NBS1 mRNA using an open reading frame generated by the deletion [23]. All other mutations in Nbs1 are likely to be hypomorphic mutations consistent with the notion that Nbs1 might also be essential in humans. We were, however, not able to detect any expression of the predicted 45-kDa protein. One possibility is that this truncated protein is not detected by our antibody. Alternatively, the mutant transcript or protein may not be stably expressed. Further work is required to determine whether there is some residual function in cells expressing the 1089C>A mutant protein and how this compares to any residual function attributable to the 657del5 mutation. The analysis of such hypomorphic changes can provide important insight into protein function.

As evidence mounts that the pathways responding to damage induced by DNA cross-linking agents and ionizing radiation are interwoven, these chromosomal instability disorders can no longer be confidently diagnosed simply from abnormal responses to IR or MMC sensitivity. Indeed, at least four syndromes have now been described with microcephaly and sensitivity to either IR or MMC, including DNA ligase IV deficiency (LIG4 syndrome) [24] and Seckel syndrome [25]. A precise genetic diagnosis is therefore important because it may provide important clues regarding prognosis and the risks and benefits of therapeutic intervention such as bone marrow transplantation. Our report described here provides important information in that context. Patients considered as candidates having FA or NBS who display early onset infection should be examined for the 1089C>A mutational change.

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