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A novel mutation and novel features in Nijmegen breakage syndrome

EDITOR—Nijmegen breakage syndrome (NBS) is a rare autosomal recessive disorder, characterised by microcephaly, bird-like face, growth retardation, immunodeficiency, cytogenetic abnormalities, increased radiosensitivity, and high susceptibility to lymphoid malignancy. The *NBS1* gene, mapped on chromosome 8q21¹ and recently cloned,^{2,3} codes for nibrin, a member of the hMre11/hRAD50 protein complex, involved in DNA double strand break repair. The NBS Registry in Nijmegen includes 55 patients. The majority of them are of eastern European origin and share a common haplotype, suggesting a founder effect, and a mutation consisting of a truncating 5 bp deletion in exon 6, 657-661 del ACAA.⁴ Five further mutations have been found in six patients with different haplotypes and of various ethnic origins.

We found a new mutation of the *NBS1* gene in a 2 year old girl from Morocco. The patient, a girl born at term in August 1997 (fig 1), is the third child of apparently non-consanguineous parents; the two brothers, aged 12 and 6 years, are healthy. The pregnancy was uneventful until the 33rd week, when growth retardation, dilatation of the cerebral ventricles, and agenesis of the corpus callosum was diagnosed by ultrasound examination.

Figure 1 (A) The patient at 18 months. (B) The right hand with preaxial polydactyly.

Birth weight was 2520 g (3rd centile), length 46 cm (<3rd centile), and head circumference 28.5 cm (<3rd centile). Physical examination showed a "bird-like" face with strabismus, downward slanting palpebral fissures, large and low set dysmorphic ears, and a high palate. The hands showed right preaxial polydactyly and bilateral clinodactyly of the fifth finger with a short middle phalanx and single flexion crease. The feet showed bilateral cutaneous syndactyly 4-5. Severe gastro-oesophageal reflux was also present. Normal findings were obtained on ultrasound examination of the heart and abdominal structures including the ovaries. Agenesis of the right 12th rib was also noted on chest x ray examination. MRI, performed when the patient was 1 month old, showed agenesis of the corpus callosum, dilatation of the ventricles, and cerebral hypotrophy, features not previously described in association with NBS.

TORCH complex analysis failed to show any evidence of viral infection and routine blood analysis was within normal limits, including serum AFP. When re-examined at 27 months, weight, height, and head circumference were below the 3rd centile. Clinical examination confirmed the findings observed at birth with a receding forehead, long nose, short philtrum, and large ears (4.5 cm) compared to head size; one hyperpigmented spot and several hypopigmented striae were noted on the back. Sparse hair, epicanthic folds, and a receding mandible, frequently observed in other NBS patients, were absent.

Serum immunoglobulin levels at 18 months were extremely low: IgA 1 mg/dl (normal 100-490), IgG 174 mg/dl (normal 800-1700), IgM 8 mg/dl (normal 50-320). A decrease in CD3 positive cells (24%, normal 60-87%) (267 C/μl, normal 860-2607), CD4 (14%, normal 32-61) (145 C/μl, normal 493-1666), and CD8 (10%, normal 14-43) (114 C/μl, normal 224-1112) was observed, while the number of NK cells was increased (64%, normal 4-28) (752 C/μl, normal 73-654). This pattern was confirmed six months later.

Neuropsychomotor development was assessed by the Brunet Lezine Developmental Scale when the patient was 27 months old. The full developmental quotient (DQ) gave a score of 68, corresponding to an age of 18 months. The lowest score was observed for the sub-item "Fine Motor Coordination" (DQ 52 = 15.5 months), the highest for "Gross Motor Coordination (DQ 82 = 22.5 months), while "Speech and Language" and "Social Ability" gave similar results (DQ 73 = 20 months and DQ 76 = 21 months, respectively). The overall evaluation of the patient during the last 15 months showed a slight improvement in neuromotor development, more pronounced for performance related to environmental stimuli.

No recurrent or severe infections were recorded up to 18 months, after which she suddenly started to develop infectious diseases, including three episodes of febrile enteritis and a stomatitis followed by candidosis. Substitutive IV immunoglobulin therapy was then established, which resulted in immediate weight gain and no further infections were recorded. At the last examination (2 years 4 months of age) no evidence of malignancy was found.

Chromosome analysis of the proband was carried out on 72 hour stimulated lymphocytes and on a lymphoblastoid cell line (LCL). Of the 152 metaphases analysed from the proband's peripheral blood, 77 (50.6%) showed aberrations, consisting mainly of chromosome or chromatid breaks (csb, ctb) and fragments. In 31 metaphases, one or more structural rearrangements were present: nine occurred in chromosomes 7 and 14 and were inversions and translocations with breakpoints at 7p15, 7q35, and 14q11, regions that are rearranged to produce active T cell receptor genes. Other anomalies (nine translocations, 15

deletions, three dicentrics, two rings, and two unidentified markers) involved various chromosomes at random. In the LCL, out of the 35 cells analysed, 19 (54.3%) showed chromosome abnormalities, eight with breaks and sporadic rearrangements and 11 with a clonal translocation t(1;18)(q32.1;q23).

A consistent number of cells with non-specific chromosome aberrations were also found in peripheral blood lymphocyte cultures of the parents. In the father, 12 metaphases with aberrations were found out of 49 analysed (24.5%). The majority of these showed chromatid breakages (13 breaks in seven cells). Five further cells had a chromosome breakage or a deletion. In the mother, 15 out of 50 (30%) cells had anomalies, of which 11 had a total of 15 ctb, one pericentric inversion, and one quadriradial configuration and four other cells had one csb each.

DNA analysis of the patient and her parents showed aberrant patterns on SSCP in exon 8 of the *NBS1* gene. Subsequent sequencing of the PCR products² showed that the patient was homozygous for a new *NBS1* mutation. The mutation was a large deletion of 25 bp designated 900del25, and leading to a premature termination of nibrin, six amino acids downstream of the deletion. Both parents were heterozygous for the same mutation (fig 2).

In accordance with the molecular data, nibrin was not detected in the proband by immunoblot analysis with an anti-nibrin polyclonal antibody (fig 3).

With the aim of characterising this new NBS mutation at the cellular level, chromosomal sensitivity, cell cycle disturbances, and induction of p53 and p21 (WAF1/Cip1) were evaluated after treatment with DNA damage inducing agents and compared with those of normal and ataxia-telangiectasia (AT) cells. To score chromosome aberrations, LCL from the patient, a normal control, and an AT patient were treated with 15-30 cGy x rays. The yield of chromatid type aberrations observed in G2 phase cells at the time of the treatment is shown in fig 4. Irradiation produced mainly chromatid breaks. NBS and AT cells appeared markedly sensitive to irradiation: values of induction of chromosome damage compared to that of normal cells were 3.8- and 2.9-fold in NBS and 4.7- and 4-fold in AT at 15 and 30 cGy, respectively.

In order to assess inhibition of mitotic entry, cells were either irradiated with 25-100 cGy or treated with 4-16 pg/ml calicheamycin-γ1 (kindly provided by Dr P R Hamann, Wyeth-Ayerst, Pearl River, NY, USA). Mitotic index was assessed scoring for 1000 cells per experiment in cells harvested two hours after treatment (fig 5A). Normal cells were strongly impaired in their progression from G2 to M phase in a dose related manner. However, irradiated NBS and AT cells sustained less reduction, in particular at the lowest dose. At 100 cGy, the mitotic entry was reduced to about 50% in NBS and AT patients compared to 24% in control cells. In experiments with calicheamycin, a compound known to cause DNA double strand breakage (dsb) at the TCCT/AGGA sequences,⁹ the pattern of mitotic inhibition in AT and control cells appeared similar to that observed after radiation, while NBS cells behaved differently, with mitotic entry inhibition intermediate between that observed in control and AT cells (fig 5B).

Fig 6 shows the results of 24 hour post-irradiation G2/M accumulation as evaluated in LCL irradiated with 50-200 cGy. The fraction of G2/M accumulated cells appeared dose dependent for all LCLs investigated. However, doses as low as 50 cGy caused a consistent accumulation only in AT cells (43.2 %), whereas normal and NBS appeared less sensitive (18.2% and 22.0%, respectively). At the highest dose, NBS cells showed an intermediate response between

Figure 2 (A) SSCP pattern of exon 8 of the NBS1 gene (P=patient, M=mother, F=father, C=control). (B) Segment of genomic sequence in exon 8 of the patient homozygous for mutation 900del25.

that observed in normal and AT cells (41.8%, 32.0%, and 55.2%, respectively for NBS, normal, and AT cells).

To assess p53 and p21 protein induction, cells were irradiated with 400 cGy α rays and harvested two and four hours later. Only slight differences were detected in the level of p53 induction in normal and NBS cells (data not shown). Similarly, the p21 radiation induced response showed a sensitivity higher than normal (1.9- and 4.0-fold induction at two and four hours, respectively) and NBS cells (1.9- and 4.4 -fold) compared to AT cells (1.0- and 1.75-fold).

In conclusion, all the cardinal features of NBS are present in our patient, who shows, in addition, less common features such as pigmentation abnormalities, clinodactyly, preaxial polydactyly, and syndactyly. She also had a high palate, agenesis of the corpus callosum, dilatation of the ventricles, and cerebral hypotrophy, malformations not previously described in NBS patients. At this stage, with only 55 patients reported in detail, it is still worth gathering information on previously unreported symptoms or malformations to improve the definition of the clinical picture and facilitate early diagnosis, which is helpful for clinical management and therapy.

According to Hiel *et al.*,⁴ cytogenetic aberrations are present in all cases with a frequency ranging from 10 to 45% of metaphases in PHA stimulated T cells; in our patient we found chromosome aberrations in 50.6% of T cells and 54.3% of LCL. In most of the published NBS cases, chromosomes 7 and 14 are preferentially involved in aberrations.⁶ In the present case, rearrangements of chromosomes 7 and 14 were found together with a high number of non-specific translocations and abnormalities, as in a previously described Italian patient,⁷ indicating a

general chromosome instability resulting from mutations of the NBS gene. Scanty and contradictory data are available on heterozygous NBS parents' karyotypes. Weemaes *et al.*⁸ described two NBS sons of second cousin parents; cytogenetic studies of the relatives showed a lower frequency of the same chromosome abnormalities found in the patients, in the father, and in three of the phenotypically normal sibs. In other families, no increase of spontaneous fragility in parents or relatives of NBS patients was

Figure 3 Nibrin in proteins extracted from LCLs of a normal subject (C), the NBS patient, and an AT patient, as evaluated by means of an anti-nibrin polyclonal antibody.

Figure 4 Frequencies of chromatid type aberrations induced by 15 and 30 cGy α rays in LCL treated three hours before harvesting.

Figure 5 Mitotic delay evaluated in cells harvested two hours after irradiation with 25-100 cGy x rays (A) or calicheamycin treatment (B). The mitotic fraction in treated samples was expressed as a percentage of the mitotic fraction in the same treated cultures.

Figure 6 Percentage of G₂/M phase cells in untreated and x irradiated cultures harvested 24 hours after treatment. DNA content was evaluated by propidium iodide staining and cytofluorimetry.

detected.⁷⁻⁹ Recently, Stumm *et al.*¹⁰ using a three colour chromosome painting test, found an increased translocation frequency in NBS heterozygotes. In our case, a consistent number of chromosome aberrations in the parents confirms possible chromosome instability in heterozygotes for NBS mutations. Since the chromosomal instabil-

ity is one of the leading causes for developing a malignancy, our data are compatible with the suggestion that NBS heterozygotes could also have an increased cancer risk,¹¹ although no malignancy has yet occurred in our patient's parents.

The parents of our patient, heterozygous for the same mutation, are apparently non-consanguineous and were born in two villages 900 km apart. This may suggest a high frequency of the new mutation in North African populations as reported for 657del5 in eastern Europe.

Our data on DNA damage inducing agents indicate a close similarity between NBS and AT cells in the G₂ chromosomal response after irradiation. This is in agreement with the few published data available on the radiation sensitivity of LCL established from NBS patients.¹²⁻¹³ A higher number of chromatid aberrations in AT and NBS cells, if compared with normal cells, might arise as a result of DNA repair defect or checkpoint dysfunction allowing damaged cells to proceed from G₂ to mitosis. The analysis of G₂ checkpoint activation, as evaluated by cell inhibition to entry to mitosis indicates that after irradiation both NBS and AT cells are less delayed than normal cells. Interestingly, this effect has not been observed in another four NBS lines with either the 657del5 or private mutations showing an intermediate response between normal and AT cells.^{2,12-13} We found such an intermediate response in our NBS cell line after incubation with calicheamycin and this seems to indicate a more specific sensitivity in checkpoint activation of these cells, compared to AT ones, in respect to this specific kind of damage. According to Carney *et al.*¹⁴ and Petrini *et al.*¹⁵ nibrin, the product of the *NBS1* gene, is a member of the hMre11/hRad50 complex involved in dsb repair, by transducing a signal originating from the sites of DNA damage. Moreover, a role of this complex in cell cycle checkpoint activity has been suggested.¹⁵⁻¹⁶ According to our and other published data, the relationship between DNA damage and cell cycle delay is not so straightforward. In fact, in spite of their similar chromosomal radiosensitivity, NBS and AT display strong differences in the percentage of G₂ accumulated cells after irradiation.^{12-13,17} Ionising radiation induced accumulation of p53 and p21, as evaluated at two and four hours after 400 cGy x rays, appears in this NBS line to be similar to that observed in normal cells. These observations are in close agreement with recent reports¹⁷⁻¹⁹ showing that NBS cells, from this point of view, are more similar to normal cells than to AT ones. In conclusion, our results confirm that the radiation mediated response is not identical in NBS and AT. In particular, the cell cycle response of NBS clearly differs from that of AT cells.

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Molecular cytogenetic characterisation of a complex 46,XY,t(7;8;11;13) chromosome rearrangement in a patient with Moebius syndrome

EDITOR—Carriers of de novo balanced reciprocal translocations and inversions have an increased risk of approximately 6% for developing multiple congenital abnormalities (MCA) and/or mental retardation (MR), compared to a 2-3% risk overall in newborn populations.^{1,2} Cytogenetically cryptic deletions or physical disruption or inactivation of a gene(s) in one or both breakpoint regions may account for the observed phenotypes.^{3,4} It seems plausible to assume that the risk for MCA/MR may be even higher in carriers of de novo complex chromosome rearrangements (CCRs), which involve at least three different chromosomes and breakpoint regions. Extreme cases involving up to seven chromosomes and 10 breakpoints have been described.⁵⁻⁷ Indeed, most reported CCRs are associated with MCA/MR.^{8,9} In addition, they have been found in infertile men¹⁰ and in women suffering from multiple miscarriages.^{11,12}

The complex nature of CCRs renders karyotype interpretation by classical chromosome banding alone difficult. In many cases fluorescence in situ hybridisation (FISH) will be the best method to delineate the underlying chromosome rearrangements.^{7,13} Here we have applied conventional FISH with chromosome painting probes and region specific large insert clones, comparative genomic hybridisation (CGH),^{14,15} and spectral karyotyping (SKY)^{16,17} to an apparently balanced and very complex rearrangement in a profoundly retarded patient with Moebius syndrome (MBS, MIM 157900).¹⁸ MBS is characterised by congenital paralysis of the seventh cranial nerve leading to facial diplegia.¹⁹ Other cranial nerves may also be affected. In addition, orofacial and limb malformations, defects of the musculoskeletal system, and MR may occur.

This patient, who has classical Moebius syndrome, has been reported previously.⁴ He was the third child born to a 35 year old father and a 32 year old mother. Because of paresis of the facial muscles as a newborn, he had feeding problems owing to inefficient sucking and swallowing. A more detailed examination at 17 years showed moderate to severe MR and deficient language development. At this time, his height was 1.56 m and his weight 39.5 kg (<3rd centile). Other clinical features included ptosis, blepharophthalmosis, atrophy of the optic nerve, and severe amblyopia. His mandible and facial muscles were hypoplastic. His metacarpals and metatarsals were short, limiting the mobility of his hands and feet. The pectoral muscles were hypoplastic.

Q band analysis²⁰ showed an apparently balanced de novo rearrangement between chromosomes 7, 8, 11, and 13. Breakpoints were assigned to chromosome bands 7q21.1, 8q21.3, 11p14.3, and 13q21.2 (fig 1). CGH did not detect any chromosomal imbalances (sensitivity: gain or loss of >10 Mb DNA) in the patient's karyotype (data not shown). Since an unambiguous interpretation of the rearranged karyotype was not possible by classical chromosome banding analysis, we applied chromosome specific and region specific FISH probes. A schematic representation of our molecular cytogenetic mapping results is shown in fig 2A.

Simultaneous visualisation of all 24 human chromosomes in different colours by SKY was very beneficial in dissecting the patient's karyotype (fig 2B). It confirmed the involvement of chromosomes 7, 8, and 13 in the CCR. The short derivative chromosome 7 was painted in its entirety by the chromosome 7 library. The material distal to 7q21 was translocated onto the der(8) and the der(13). The segment of the der(8) distal to 8q21 was translocated onto der(13). Inversely, the distal long arm 13q21-qter was moved to the der(8). Thus, the der(8) carried additional material from both chromosomes 7q and 13q, whereas the der(13) gained material from both chromosomes 7q and 8q. The chromosome 11 rearrangement, which had been suspected after Q banding, could not be characterised further by SKY. Some metaphases suggested a small insertion into 11p (data not