Nijmegen Breakage Syndrome (NBS) with neurological abnormalities and without chromosomal instability

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ABSTRACT

**Background:** Nijmegen breakage syndrome (NBS) is an autosomal recessive chromosomal instability disorder with hypersensitivity to ionizing radiation. The clinical phenotype is characterized by congenital microcephaly, mild dysmorphic facial appearance, growth retardation, immunodeficiency, and a highly increased risk for lymphoreticular malignancy. Most NBS patients are of Slavic origin and homozygous for the founder mutation 657del5. Recently, another NBS1 mutation, 643C>T(R215W), was found to have a higher frequency among tumor patients than in the control population. This alteration results in the substitution of the basic amino acid arginine with the nonpolar tryptophan in the NBS1 protein, nibrin.

**Methods:** Since the identification of the NBS1 gene, children with congenital microcephaly are routinely tested for the 657del5 mutation in the Czech and Slovak Republics. Follow-up analyses included: spontaneous and radiation-induced chromosomal breakage, nibrin expression and radiation-induced phosphorylation of ATM, p53 and nibrin itself.

**Results:** We describe monozygotic twin-brothers with profound congenital microcephaly and developmental delay who are compound heterozygotes for the 657del5 and 643C>T(R215W) NBS1 mutations. Both children showed severely reduced expression of full length nibrin. Radiation response processes such as phosphorylation of ATM and phosphorylation/stabilisation of p53, which are promoted by NBS1, are strongly reduced in cells from these patients. However, there is no increase in chromosome breakage in these patients.

**Discussion:** The compound heterozygous 657del5/643C>T(R215W) NBS patients described here are more severely affected than the majority of NBS patients who are homozygous for 657del5. We postulate that homozygosity for the 643C>T(R215W) mutation will also lead to a, possibly very, severe disease phenotype.
INTRODUCTION
Nijmegen breakage syndrome (NBS) is an autosomal recessive chromosomal instability disorder characterized by congenital microcephaly, growth retardation with pre- or postnatal onset, immunodeficiency, hyperradiosensitivity, and cancer predisposition [1][2]. Most NBS patients are of Slavic origin and are homozygous for the founder NBS1 mutation 657del5 [2][3][4]. Congenital microcephaly is a hallmark of NBS (2) and could be used, together with the identification of the responsible NBS1 mutations as a signal criteria for an early diagnosis of NBS in the Czech and Slovak Republics. Recently, we showed that the proportion of NBS patients among Czech infants with congenital microcephaly was 13%. In the Slovak Republic, this incidence was found to be even as high as 50% among infants with both microcephaly and increased chromosomal instability [5][6]. Among infants with primary microcephaly, screened for the 657del5 mutation, we diagnosed monozygotic twin-brothers at the age of 2 months to be heterozygous for this mutation. The severe congenital microcephaly and hypotrophy were indications for further analysis of the NBS1 gene. Another NBS1 mutation, namely 643C>T(R215W), was detected on the second allele of the twin-brothers. This mutation has been previously described in patients with ALL (acute lymphoblastic leukemia) [7], non-Hodgkin’s lymphoma [8], and at a high frequency among tumour patients of Slavic origin [9]. Further clinical investigations of the children revealed partial lissencephaly and epileptic seizures, respiratory and feeding problems were also noted. Based on the clinical and molecular findings, we propose that the compound heterozygosity, 657del5/643C>T(R215W), of the twin-brothers is the primary cause of their clinical phenotype which seems to be more severe than the “classical” NBS form.

Clinical report
The propositi – monozygotic twin-boys, JR and PR, were born to a 27-year-old primigravid mother and a 29-year-old father. The couple is healthy, non-consanguineous, non-endogameous and had no contributory family history. The pregnancy was uncomplicated until the 31st+1 week of gestation when fetal ultrasound examination showed an abnormal head shape and growth retardation of both fetuses. Two weeks later oligohydramnion occurred in one of the twins after amniotic fluid disruption; spontaneous delivery took place the next day for both children from head position.

Twin A – a boy, PR (Fig.1 A), was born after 33+1 weeks of gestation with an Apgar score of 7-9-9. His birth weight was 1520 g, length 40 cm, OFC 26 cm. Immediately after birth, an abnormal skull shape with a small anterior fontanel (0,5 x 0,5) without small fontanel and with synostosis of coronal and sagital sutures were recognized. The sutura metopica was prominent, occiput was flat and the skull developed a microturricephalic shape. Ultrasound investigation after birth showed enlarged, mild asymmetric lateral ventriculus, enlarged subarachnoidal areas (4mm) and poor gyrification of the brain. No signs of intraventricular bleeding were found. Phototherapy was applied at 5 days because of icterus. Enteral feeding was performed for the first 5 days, after that the child was breast fed and could be discharged home at the age of 33 days with 2095 g weight, 43 cm length and OFC 29,8 cm (all parameters below the 3th percentile, OFC very low, corresponding to 3 sigma). PR showed mild muscle hypertonia and brisk reflexes particularly involving the legs. A first attack of myoclonic seizures was documented at the age of 3 months and progression to the status epilepticus at 5 months was rapid. During his stay in
the clinic of neurology, the status epilepticus was medicated and improved but some 15 attacks of seizures per day were common and feeding per sonda was necessary. Feeding and respiratory problems were progredient and therefore gastrostomy was necessary at the age of 7 months. Psychomotoric development was severely retarded and correlated with development in infants in the 1st trimenon. The OFC at the age of 1 year was 37.6 cm, weight 8750 g, and length 69 cm (3rd percentile except OFC, which corresponds to -5 SD). The child was last seen at the age of 15 months and his somatic development due to feeding by gastrostomy and despite rare respiratory problems was satisfactory (9500 g weight, 74 cm length, OFC 39 cm). His microturricephaly with prominent sutura metopica was severely pronounced. The frequency of seizures was 20-30 per day.

**Twin B** – the boy, JR, was born with a birth weight of 1600 g, length of 40 cm, OFC 26 cm. His Apgar score was 8-9-9 and the clinical findings of microcephaly were identical with his twin brother, PR. He was discharged home with a weight of 2380 g, length of 45 cm and OFC of 30.5 cm. Testes were descended as they were in his brother, but he had also bilateral hydrocele and diastasis m. recti abdominis. The development was identical to that of his brother although his weight was always somewhat higher than PR’s (Fig.1 B).

**Family history**
The father is a policemen and the mother is a nurse in a paediatric clinic. Both parents have an increased contact with infections (homeless and drug addicted persons, respiratory infects). Both were born as the second child of three healthy sibs (brothers on the fathers side and sisters on the mothers side). The paternal line comes from Moravia and the maternal from Bohemia.

**METHODS**

**Mutation analysis**
DNA was isolated from peripheral blood and lymphoblastoid cell lines (LCLs) were established according to standard procedures. Informed consent was obtained from all individuals analysed in this study. Because of their congenital microcephaly and Slavic origin both children were tested for the *NBS1* mutation 657del5 at the age of 2 months. The mutation analysis was carried out as previously described [5] and it was found that both children were heterozygous for the Slavic mutation. In an attempt to find a second mutation, further analysis of the *NBS1* gene was carried out. The DNA samples were analyzed by PCR amplification and sequencing of *NBS1* exons and flanking intronic sequences on an ABI 3100 DNA Analyzer.

**Western Blot analysis**
Proteins were isolated from LCLs of both patients according to standard procedures and examined in comparison with control cells and cells heterozygous for the 657del5 or 643C>T(R215W) mutations. Blots were probed with a rabbit polyclonal antibody (Novus Biologicals) directed against the C-terminal portion of nibrin. Antibodies against MRE11 (Novus Biologicals) and actin (Amersham) were used as a controls.

**Phosphorylation assays**
For examination of nibrin phosphorylation LCLs were irradiated with 12Gy and proteins extracted after one hour further cultivation at 37°C. A portion of each extract was treated with lambda phosphatase (New England Biolabs) before separation of all
probes on polyacrylamide gels, blotting and immunodetection using anti nibrin antibody. Phosphorylated nibrin runs more slowly during gel electrophoresis and is visible as an upwardly shifted band. Loss of the mobility shift after treatment with lambda phosphatase indicated that it is indeed due to phosphorylation.

Phosphorylation of ATM was measured at 30 minutes after 0, 0.5, 1 and 2Gy irradiation. Proteins were separated on polyacrylamide gels and blots were probed with an anti-ATM serine-1981p antibody (Abcam, Cambridge, UK), stripped and reprobed with an anti-ATM antibody (Santa Cruz Biotechnology, Santa Cruz USA). Phosphorylation of p53 after IR was detected using the anti-p53 serine-15p antibody (Cell Signalling Technology, Beverly, USA) and, after stripping, an anti-tubulin antibody as a loading control (Abcam, Cambridge, UK).

Zygosity testing
To determine whether the twin-brothers were mono- or dizygotic we performed microsatellite analyses using markers on chromosome 1 and 14 (D1S80, D14S63, D14S68, D14S70, and D14S72). The analysis was done by means of PCR amplification with fluorescently labelled primers and fragment length analysis on an ABI 3100 DNA Analyzer.

Chromosome analysis
Chromosome analyses from peripheral blood lymphocytes and lymphoblastoid cell lines of the twins were performed using standard techniques. Irradiation of cells was carried out using the X-ray apparatus Muller MG 150 (U = 100 kV, I = 10 mA, filter 0.3 mm Ni, dose rate: 2.1 Gy/min) with doses 0.5, 1.0, and 2.0 Gy. The radiosensitivity was determined by analysing the number of breaks per cell in 75 metaphases per irradiation dose. We also performed spectral karyotyping (SKY) [10] as an additional and highly sensitive method for identifying translocations in patients cells. The results were compared with a control LCL and LCLs derived from a Fanconi anaemia patient. FISH analysis of the LIS1 gene was performed using a commercially available probe (VYSIS).

RESULTS
The severe congenital microcephaly and developmental delay of the twin-brothers reported here were indications for analysing them for the major NBS1 mutation, 657del5 in exon 6. Figure 2a shows the domain structure of NBS1 and its protein product, nibrin. The mutation 657del5 leads to premature termination of translation and reinitiation at a cryptic start codon. Partially functional proteins of 26kDa and 70kDa are the products of the hypomorphic 657del5 NBS1 mutation. The sequence analysis showed that both children were heterozygous for this mutation (Figure 2b). Further mutation screening of the NBS1 gene, illustrated in Figure 2b, revealed that they carry on their second allele another NBS1 exon 6 mutation, namely 643C>T(R215W). This confirmed the suspected diagnosis of NBS in both children. Analysis of parents’ DNA samples showed that the mother carries the 657del5 and the father the 643C>T(R215W) mutation. Further investigation of this family showed that the two grandfathers were the carriers of the respective NBS1 mutations. A microsatellite analysis confirmed that the twins were monozygotic (data not shown).

The chromosome analysis indicated normal karyotypes in both patients. Because of their partial lissencephaly, a microdeletion analysis of the LIS1 gene in 17p13.3 was
performed by FISH with the LSI LIS1 probe. The FISH showed regular signals on both chromosomes 17 (data not shown). Thus, a Miller-Dieker lissencephaly syndrome could be excluded with a probability of 90%.

Chromosome breaks were not elevated in the lymphocytes of PR, particularly, the translocations or inversions involving chromosomes 7 and 14, which are characteristic of AT and NBS, were not observed. In comparison, generally 10-45% of NBS patient lymphocytes are found to be chromosomally aberrant, of these aberrations, 66% involve chromosome 7 and/or chromosome 14 [2]. In addition to standard cytogenetics, the particularly sensitive SKY technique for detection of translocations was used to examine the lymphoblastoid metaphases. In 60 metaphases, no evidence for an increased translocation rate for either twin was found in comparison to control cells (data not shown).

Radiosensitivity was assessed by counting chromosome breakage in lymphoblastoid cell lines of both twins in response to 0.5, 1.0, and 2.0 Gy radiation (Fig.3). The lymphoblastoid cell lines from the twins show an increase in chromosome damage with radiation dose comparable to a control cell line. NBS cells generally show high levels of damage after IR: 64 - 71% aberrant cells after 2Gy [11]. The NBS lymphoblastoid cell line examined here in parallel shows a comparably high level of chromosome damage after IR (Fig 3). In conclusion, chromosomal instability is not a feature of the patients presented here.

We performed a Western blot analysis to compare the amount of nibrin in lymphoblastoid cell extracts from both patients with control cell lines and heterozygotes for the 657del5 or 643C>T(R215W) mutations. This analysis showed a striking difference in the amount of nibrin in both patients when compared with a control, a heterozygote for the 657del5 or a heterozygote for the 643C>T(R215W) mutations (Fig. 4). Nibrin-Trp\textsuperscript{215} is clearly much less abundant than the wild type nibrin-Arg\textsuperscript{215}.

We next examined three essential functional aspects of nibrin, firstly, its role in the activation of ATM (ataxia telangiectasia mutated), measured as ATM-phosphorylation after IR, secondly, nibrin’s own IR-induced phosphorylation by ATM and, finally, the phosphorylation of p53 by ATM after IR, which is promoted by nibrin. For examination of ATM phosphorylation we used a specific antibody for ATM phosphorylated on serine-1981. As shown in Figure 5a, phosphorylation of ATM is seen in wild type cells 30 minutes after irradiation with just 0.5Gy and with a dose dependent increase at 1 and 2Gy. In contrast, in 657del5 homozygous cells, ATM-phosphorylation is first detected at 2Gy, indicating a four fold reduction in ATM activation in these cells. In cells compound heterozygous for 657del5 and 643C>T(R215W), ATM-phosphorylation is barely detectable at 2Gy. The nibrin-Trp\textsuperscript{215} protein is apparently even less able to sustain IR-induced ATM activation than the 70kDa carboxy terminal protein fragment.

For evaluation of nibrin phosphorylation a specific gel mobility assay was employed [12]. As shown in Figure 5b, both wild type nibrin-Arg\textsuperscript{215} and nibrin-Trp\textsuperscript{215} show slower electrophoretic mobility after irradiation. This mobility shift is lost after phosphatase treatment of proteins from irradiated cells indicating that it is due to phosphorylation. The extremely reduced level of nibrin-Trp\textsuperscript{215} in patient’s cells is again clearly shown in Figure 5b. Anti-actin was used as a loading control.
Finally we examined the phosphorylation and stabilisation of p53 in response to IR using immunoblots and an antibody directed against p53 phosphorylated on serine-15. As can be seen in Figure 5c, the phosphorylation of p53 is abrogated in cells homozygous for 657del5 and also in the cells compound heterozygous for 657del5 and 643C>T(R215W). Anti-tubulin antibody was used as a loading control.

DISCUSSION

The frequency of the common NBS1 mutation 657del5 is relatively high (0.5 - 1%) in the Slavic population [3] and in homozygotes leads to the classical manifestation of NBS. The twin-brothers described here are compound heterozygotes for two NBS1 mutations – the major truncating NBS1 mutation, 657del5, and the missense mutation, 643C>T(R215W). Originally, the 657del5 mutation was regarded as a null mutation, however, Maser et al., 2001 showed that the NBS1 657del5 mutation is actually a hypomorphic mutation and a truncated protein is produced, at least in lymphoblastoid patient cells [13]. Similar alternative translation products have been shown for two further truncating NBS1 mutations [13] [14]. The relatively mild clinical course of patients with NBS contrasts strongly to the lethality of null-mutation of the murine NBS1 homologue [15] [16]. We have recently been able to confirm the hypomorphic nature of the major human NBS1 mutation by complementation of an inducible murine null-mutation [17].

Unlike the 657del5 mutation, the pathogenicity of the 643C>T(R215W) mutation has been uncertain for some time. Arginine is conserved at position 215 of the mouse protein and the substitution of a basic amino acid, arginine, with a nonpolar amino-acid, tryptophan, can obviously be postulated to have an effect on function. The 643C>T(R215W) mutation has been shown to be more common among tumour patients of Slavic origin than in the general Slavic population [9], a further finding pointing to a possible involvement of this mutation in cellular metabolism and cancerogenesis.

We find nibrin-Trp215, the product of the 643C>T(R215W) allele, to be much less abundant than wild type nibrin-Arg215 as shown by Western blot analysis of lymphoblastoid cell extracts (compare lanes 3, 4, 5 with lane 1 in Figure 4 and control and patient lanes in Figure 5c). This difference may indicate a lower expression level of the 643C>T(R215W) allele, a shorter half-life of the mRNA or, more likely, reduced stability of the nibrin-Trp215 protein. This might in turn reflect an inability of the mutant protein to associate correctly with its cellular partners, Mre11 and Rad50, and subsequent degradation of non-bound nibrin monomer. In cells with only a truncated nibrin as an alternative, the 643C>T(R215W) mutation might therefore lead to a reduction in active trimeric complex below a critical level.

It has been hypothesized that missense ATM mutations might act by dominantly interfering with the function of the second allele, thus resulting in additional phenotypic effects beyond what might be expected from an absence or reduction of the protein, as normally occurs with truncating mutations [18]. A similar argument could also be made here, with a missense NBS1 mutation interfering with the residual activity of the truncated protein from the 657del5 allele. This hypothesis, however, awaits experimental verification.

It is becoming clear that NBS1 plays a role not only in the active repair of DNA
double-strand breaks (DSBs), but also within the cellular signal cascades responding to changes in the status of the genome. Thus, \textit{NBS1} is required not only for the activation of \textit{ATM} after DNA damage [19] [20] but also for the efficient phosphorylation by \textit{ATM} of downstream targets, most of which are concerned with the assembly of cell cycle checkpoints rather than DNA repair \textit{per se} [21] [22]. Nibrin-Trp\textsuperscript{215} is strongly affected in its capacity to activate ATM as measured by extremely reduced phosphorylation of ATM in patient cells after IR. Indeed, phosphorylation is even poorer than in 657del5 homozygous cells. Interestingly a similar finding was reported for AT-like disorder (AT-LD) in which different MRE11 mutations led to more or less severe abrogation of ATM phosphorylation [19]. In the case of MRE11, ATM activation correlated with disease severity with the poorly activating mutations associated with a more severe phenotype [19].

In contrast to its ability to mediate ATM activation, nibrin-Trp\textsuperscript{215} is itself efficiently phosphorylated by ATM and, indeed, chromosomal breakage after IR is not increased in cells from the patients described here. Phosphorylation of a critical downstream target of ATM, p53, is promoted by nibrin [23]. As in cells from patients with the 657del5 mutation, the compound heterozygous cells also phosphorylate p53 poorly (Figure 5c) and this is accompanied by a failure to stabilize p53 after irradiation.

We recently described the effects of targeted Nbs1 null mutation in mice neuronal precursors. In these animals, the cerebellum was drastically diminished in size and foliation was also greatly reduced [24]. We were able to demonstrate that this cerebellar phenotype was due to a p53-dependent reduction in proliferation of neuronal stem cells and increased p53-dependent apoptosis in post-mitotic neurons [24]. Since these mice had a tissue specific null mutation, the neuronal precursors were devoid of nibrin. The partial lissencephaly in the patients described here might perhaps stem from a reduced cellular level of full-length nibrin, due to the destabilizing 643C>T(R215W) mutation. In patients homozygous for the 657del5 mutation, the truncated 70kD protein may uphold aspects of nibrin function sufficient to prevent this neurological anomaly. Alternatively, a dominant-negative function of the 643C>T(R215W) mutation might be responsible for the phenotypic differences between the patients described here and 657del5 homozygotes.

The clinical picture of our patients is similar to the classical NBS phenotype due to homozygosity for the Slavic mutation with respect to the severe congenital microcephaly and growth retardation with prenatal onset. However, there are substantial differences in several other aspects. Firstly, cranial investigations on over 16 NBS patients aged from 1.75 to 19 years of age has indicated decreased size of the frontal lobes and narrowing of the frontal horns of the lateral ventricles [25] [26]. Even the only NBS patient described without microcephaly has these neurological features [26]. This clinical picture differs from that of enlarged lateral ventricles, enlarged subarachnoidal areas and poor gyrification found in the patients described here.

Secondly, mental development is severely retarded in these patients but is nearly normal in „classical“ NBS. Thirdly, with respect to sexual development there is ovarian dysgenesis in females and frequent cryptorchism in males with the „classical“ type of NBS. Finally, the immunodeficiency is generally more pronounced in „classical“ NBS than in our patients, although clearly this may change with age.
Severe developmental delay, unusual for NBS, has been reported in patients diagnosed initially as Fanconi anaemia but later found to have a homozygous truncating mutation (1089C>A / Y363X) in NBS1 [27] [28].

Compound heterozygosity in patients with NBS is not a rare event, especially in non-Slavic populations [4] [29]. The only difference to our patients is, however, that most compound heterozygotes carry two truncating mutations. Despite the monozygosity of the twins described here, their symptoms with respect to developmental delay, respiratory and feeding problems are not absolutely identical. Twin B (JR) is progressing better than his brother and his mental development is also more satisfactory.

Patients with classical NBS have an elevated cancer risk as a result of chromosomal instability and radiosensitivity. Considering their age, evaluation of an increased risk for malignancy in the twin-boys described here is obviously premature. However, we propose that the two NBS1 mutations found in our patients are responsible for their clinical phenotype which deviates from the classical NBS and seems to be even more severe. Apparently, the combination of a truncated and a missense NBS1 mutation leads to a partially different form of the disease. The presence of congenital microcephaly and the absence of chromosomal instability and radiosensitivity found in our patients suggest that the development of microcephaly might even be independent of IR sensitivity and perhaps more related to other factors, for example, low cell proliferation.

In conclusion, with this study we provide the first data on a novel variant of NBS, characterized by severe congenital microcephaly and neurological features unusual for the “classical” form and due to compound heterozygosity for the truncating 657del5 and the missense 643C>T(R215W) NBS1 mutations. The clinical phenotype as a result of homozygosity for the 643C>T(R215W) mutation is so far unknown, despite the moderate frequency (1:234) of 643C>T(R215W) heterozygotes among Czech newborns [30]. Therefore, we postulate that homozygosity for the 643C>T(R215W) mutation may lead to a disease phenotype, which might even be lethal early in development.

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Fig. 1: Twin brothers PR and JR. The twin boys are shown at the age of 5 months (A) and 7 months (B). Note microturicephaly, micrognathia and large auricles.

Fig. 2: NBS1 Mutation analysis
A: The domain structure of NBS1 and its protein product, nibrin. The intron/exon structure and the 11 NBS1 mutations are indicated on the cDNA with the 657del5 and 643C>T mutations underlined. The full length nibrin protein and the two protein fragments arising from the 657del5 mutation are shown. The serines phosphorylated by ATM in response to irradiation are indicated as is the site of interaction with MRE11. BRCT: Breast Cancer 1 Carboxy Terminal Domain; FHA: Fork Head Associated Domain.
B: Sequence analysis of NBS1. A segment of genomic sequence from exon 6 in a control DNA and the patient, JR, described here. The region contains the 657del5 and the 643C>T (R215W) mutations.

Fig. 3: Analysis of chromosomal instability after irradiation. Lymphoblastoid cells of the two probands, JR and PR, were irradiated with the indicated dose and chromosomal damage was scored in preparations of mitotic cells. Data are shown as breaks per cell and in comparison to the mean values of 18 normal control subjects (controls) and those of 4 NBS patients homozygous for the 657del5 mutation. 75 metaphases were counted for each preparation.

Fig. 4: Immunoblot blot analysis of nibrin expression. The Western blot shows the expression levels of nibrin and MRE11 in lysates of lymphoblastoid cell lines with the given NBS1 genotypes. The same blot was reprobed with an antibody directed towards actin as a loading control. The protein bands are labelled on the right of the blot.

Fig. 5: Cellular response to ionising irradiation in 657del5/643C>T compound heterozygous cells
A: ATM activation after ionising irradiation
An immunoblot of cell lysates with the indicated genotypes 30 minutes after 0, 0.5, 1.0 and 2Gy ionising irradiation is shown. The blot was probed first with an antibody directed against ATM-serine-1981p then stripped and reprobed with an antibody directed against ATM. The protein bands are labelled on the right of the blot.
B: Nibrin phosphorylation after ionising irradiation.
Nibrin phosphorylation after ionising irradiation was examined using a gel-shift assay. An immunoblot of cell lysates from unirradiated cells and from cells one hour after 12Gy, probed for nibrin is shown. Genotypes of the cells are given above the lanes. A portion of the irradiated lysates was treated with λ-phosphatase as indicated to demonstrate that the shift in nibrin mobility after irradiation is due to phosphorylation. The same blot was reprobed with an antibody directed towards actin as a loading control. The protein bands are labelled on the right of the blot.
C: p53 phosphorylation after ionising irradiation
An immunoblot of cell lysates with the indicated genotypes 30 minutes after 0, 0.5, 1.0 and 2Gy ionising irradiation is shown. The blot was probed with an antibody directed against p53-serine-15p then reprobed with an antibody directed towards tubulin as a loading control. The protein bands are labelled on the right of the blot.
References:


Figure 1
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Figure 5
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