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Received on July 7, 2011; revised on August 16, 2011; accepted on October 4, 2011

Nibrin, product of the NBN gene, together with MRE11 and RAD50 is involved in DNA double-strand breaks (DSBs) sensing and repair, induction of apoptosis and cell cycle control. Biallelic NBN mutations cause the Nijmegen breakage syndrome, a chromosomal instability disorder characterised by, among other things, radiosensitivity, immunodeficiency and an increased cancer risk. Several studies have shown an association of heterozygous c.657-661del, p.I171V and p.R215W mutations in the NBN gene with a variety of malignancies but the data are controversial. Little is known, however, whether and to what extent do these mutations in heterozygous state affect nibrin functions. We examined frequency of chromatid breaks, DSB repair, defects in S-phase checkpoint and radiosensitivity in X-ray-irradiated cells from control individuals, NBS patients and heterozygous carriers of the c.657-661del, p.I171V and p.R215W mutations. While cells homozygous for c.657-661del displayed a significantly increased number of chromatid breaks and residual γ-H2AX foci, as well as abrogation of the intra-S-phase checkpoint following irradiation, which resulted in increased radiosensitivity, cells with heterozygous c.657-661del, p.I171V and p.R215W mutations behaved similarly to control cells. Significant differences in the frequency of spontaneous and ionising radiation-induced chromatid breaks and the level of persistent γ-H2AX foci were observed when comparing control and mutant cells heterozygous for c.657-661del. However, it is still possible that heterozygous NBN mutations may contribute to cancer development.

Introduction

Nibrin, product of the NBN gene, is involved in several cellular processes essential for maintaining genomic stability. Together with MRE11 and RAD50, nibrin forms a protein complex (the so called MRE11/RAD50/nibrin (MRN) complex) playing a crucial role in the detection and repair of DNA double-strand breaks (DSBs), a particularly dangerous type of DNA damage (1–3). Other functions of the MRN complex include control of S and G2/M cell cycle checkpoints (4–8), induction of apoptosis (9,10), regulation of DNA replication (11,12) and centrosome duplication (13), recombination during meiosis (14), telomere maintenance (15,16) and V(D)J recombination of immunoglobulin genes (17,18).

Nibrin is a 754-amino acid protein encoded by the NBN gene located on chromosome band 8q21.3. It interacts with MRE11 through the C terminus, and at the N-terminal end, nibrin contains a forhead-associated (FHA) domain and two BRCA1 carboxy-terminal (BRCT) domains responsible for the interaction of the MRN complex with MDC1 that guides the complex to the sites of DSBs (19–21). In mice, null mutations in NBN result in embryonic lethality (22), whereas in humans, biallelic NBN mutations lead to the Nijmegen breakage syndrome (NBS, OMIM #251260), a rare autosomal-recessive disorder characterised by immunodeficiency, microcephaly, growth retardation and increased risk of malignancies, especially leukaemias and lymphomas (23). The NBN-defective cells display chromosome instability, a defect in correct rejoining of DSBs, hypersensitivity to ionising radiation (IR) and radioresistant DNA synthesis (RDS) (24–26). The majority of NBS patients are homozygous for the c.657-661del mutation (the so called Slavic mutation) which results in translation of two truncated proteins maintaining some functions of the full-length nibrin: a short N-terminal fragment with FHA/BRCT domains interacting with MDC1 and a C-terminal protein capable of binding to MRE11 (27). Recently, a severe form of NBS has been diagnosed in compound heterozygotes for c.657-661del and p.R215W (c.643C > T) mutations (28).

The involvement of nibrin in cellular processes crucial for maintaining genomic stability suggests that mutations disrupting its functionality may lead to carcinogenesis. Evidence from NBS patients and their relatives, carriers of the heterozygous Slavic mutation, supports this supposition (29). In recent years, several studies have examined the association of heterozygous NBN mutations and molecular variants with tumours. Most commonly analysed mutations were c.657-661del, p.I171V (c.551A > G) and p.R215W (c.643C > T) and the majority of studies concerned leukaemias/lymphomas and breast cancer. However, the results are inconclusive: while some studies indicated a strong association of heterozygous NBN mutations with various types of cancer, such connections did not emerge from other studies [reviewed in ref. (30)]. This raises the question whether and to what extent do heterozygous c.657-661del, p.I171V and p.R215W mutations affect nibrin functions. The mutations cause structural changes in the functional domains of nibrin. The Slavic mutation leads to production of two truncated fragments of the protein and is known to have a deleterious impact on nibrin functions when in homozygous state, causing the NBS. NBS also manifests in compound heterozygotes for c.657-661del and p.R215W (28). The latter mutation lies between the two BRCT domains and alters their relative geometry (31). p.I171V is located in the first BRCT domain and a case of aplastic anaemia in a child homozygous for p.I171V was reported (32). So far, however, little is known about the effect that these mutations in the heterozygous form exert on nibrin
functions. To answer that question, we examined lymphoblastoid cell lines (LCLs) derived from four control individuals, patients with NBS and heterozygous carriers of the c.657-661del, p.I171V and p.R215W mutations. DNA damage was induced by ionising radiation and nibrin functioning was assessed by evaluating the cells ability to repair the damage, efficiency of the intra-S-phase checkpoint and radiosensitivity.

Materials and methods

Cell lines, immortalisation and culture conditions

We used LCLs derived from B lymphocytes of four control individuals, heterozygous carriers of p.I171V (BC60 and BC147), p.R215W (B177B) and c.657-661del (GM15811 and GM15841) mutations and nibrongytes for the Slavic mutation (GM15812 and NL). GM15811, GM15812 and GM15841 were purchased from Coriell Institute for Medical Research, USA. The remaining cell lines were established in our laboratory from individuals with NBS mutations recognised in the course of other studies (33,34). The research protocol was approved by the Ethics Committee at Poznan University of Medical Sciences.

Peripheral blood lymphocytes were isolated on a Ficoll/Uroprofile gradient, washed two times in phosphate-buffered saline (PBS) and resuspended at 2 × 10⁶ cells/ml in RPMI-1640 medium (Pan Biotech) supplemented with 20% foetal bovine serum (FBS), 2 μg/ml cyclosporin A and 10% Epstein-Barr virus-containing medium. Cells were maintained at 37°C in a 5% CO₂ atmosphere. After 2–3 weeks, the cells clumps indicative of a successful transformation were visible and cells were henceforth cultured in RPMI-1640 supplemented with 15% FBS.

Irradiation

Cells were X-ray irradiated using a Gammacell 1000 137Cs irradiator (dose rate of 3.5 Gy/min) at the Greater Poland Cancer Centre.

Chromatid breakage analysis

Chromatid breaks were assayed following premature chromosome condensation (PCC). Following irradiation, cells were cultured for 24 h to allow for break rejoining. After that, colcemid (Pan Biotech) was added at a concentration of 0.1 μg/ml for 1 h. Thirty minutes before the end of colcemid incubation, calcyclin A (Wako) was added at a final concentration of 50 nM (stock solution 0.1 mM in ethanol). Cells were then centrifuged and incubated in hypotonic solution (0.4% KCl) for 10 min at 37°C and fixed three times in ice cold 3:1 methanol:acetic acid. Following spreading, cells were stained with 5% Giemsa in PBS. Slides were coded and 100 G2 and M cells were analysed for each sample. Chromatid breaks and gaps were scored as any clear discontinuity in the chromatid (35). The number of breaks in unirradiated controls was subtracted from breaks scored in irradiated samples.

Immunofluorescence analysis of γ-H2AX foci

γ-H2AX foci formation was analysed as an indirect measure of DSB repair in cells irradiated with 0.75, 2, 5 and 8 Gy. At indicated time points after IR (1, 6, 24 and 48 h), LCLs were fixed in 4% paraformaldehyde, permeabilised in ice cold 3:1 methanol:acetic acid. Following spreading, cells were stained with fluorescein-conjugated anti-BrdU antibody and propidium (BrdU) was added for 1 h and after that, the cells were fixed in 70% ethanol. Staining with fluorescein-conjugated anti-BrdU antibody and propidium iodide was performed according to the kit manufacturer’s protocol. Data from PCC, immunofluorescence, RDS and clonogenic survival assay were statistically analysed by two-sample t-test. Flow cytometry data (percentage of cells in S phase) were compared using χ² test. Differences with a value of P < 0.05 were considered statistically significant.

Results

Cells with heterozygous c.657-661del mutation have an increased frequency of spontaneous and IR-induced chromatid breaks

The frequency of chromatid breaks was determined 24-h post-irradiation (0–5 Gy). We found that the frequency of spontaneous breaks in unirradiated cells was significantly higher in cells from NBS patients and heterozygous carriers of the c.657-661del mutation. It is noteworthy that while one NBS cell line (GM15812) clearly stood out, the other one (NL) had the mean number of spontaneous breaks in the range of c.657-661del heterozygotes. Furthermore, the number of breaks per cell 24 h after irradiation with 1.5–5 Gy was several times greater in NBS cells than in control cells. Cell lines with a heterozygous c.657-661del mutation had a significantly higher frequency of breaks after 5 Gy of irradiation, although levels were still much lower than in homozygous NBS mutants. Cell lines with heterozygous p.I171V and p.R215W mutations showed no significant differences compared to control cells (Figure 1).

A significantly higher number of residual γ-H2AX foci is observed in cells with heterozygous c.657-661del mutation after high radiation doses

γ-H2AX immunostaining at various times (1–48 h) after irradiation (0–8 Gy) was used as an indirect measure of DSB repair. No significant differences were found in the basal levels of foci in untreated cells (data not shown). NBS cells showed a delay in γ-H2AX foci formation 1 h after irradiation with 0.75 and 2 Gy (it was impossible to distinguish single foci at this time after higher IR doses) but at later time points, the number of persistent foci was significantly higher compared to control cells. In cells with heterozygous c.657-661del, γ-H2AX foci disappeared at a slower rate but 48 h after IR, they reached the level of control cells for 0.75 and 2 Gy. 5 and 8 Gy resulted in a significantly higher number of residual γ-H2AX foci, though much less than in NBS cells. One cell line with heterozygous p.I171V (BC80) and one with p.R215W (B177B) mutation showed a similar delay in disappearance of γ-H2AX foci to heterozygous c.657-661del cells but only after 0.75 Gy; at higher doses, no differences were observed compared to control cells (Figure 2).

The intra-S-phase checkpoint is undisturbed in cells with heterozygous NBN mutations

Another feature of NBS cells is the impairment of the intra-S-phase checkpoint whose role is to inhibit DNA synthesis until damage caused by irradiation is repaired. To assess the activity of the intra-S-phase checkpoint, the cell lines were irradiated (0–8 Gy) and evaluated by two methods: [³H]thymidine incorporation for the measurement of DNA synthesis...
inhibition and EZ-BrdU test to determine the percentage of actively replicating cells. NBS cells showed significantly lower rates of DNA synthesis inhibition as well as much more active S-phase cells than the control cell lines. In contrast, cells with heterozygous NBN mutations did not differ from the control cells (Figure 3).

Heterozygous NBN mutations do not increase radiosensitivity of the cells
Radiosensitivity of the studied cell lines was examined by the clonogenic survival assay 14 days after exposure to X-rays (0–8 Gy). NBS cells showed markedly decreased survival rates after all radiation doses in comparison to the control cells. In contrast, cells with heterozygous NBN mutations did not differ from the control cell lines (Figure 3).
contrast, in the cell lines with heterozygous c.657-661del, p.I171V and p.R215W mutations, no differences in radiosensitivity were observed compared to the control cells (Figure 4).

Discussion

In the present study, we report on the response to DNA damage caused by ionising radiation of cells with heterozygous mutations in the NBN gene. We performed several assays to analyse nibrin functioning in the following aspects: DSB repair, intra-S-phase checkpoint activation and sensitivity to ionising radiation. Our results indicate that heterozygous c.657-661del, p.I171V and p.R215W mutations in the NBN gene do not significantly impair main nibrin functions. Whereas cells containing a homozygous c.657-661del displayed a significantly higher number of chromatid breaks, residual γ-H2AX foci and intra-S-phase checkpoint defects following irradiation, which resulted in increased radiosensitivity, cells with

Fig. 3. Inhibition of DNA synthesis after irradiation in four control cell lines, p.I171V heterozygotes (BC80 and BC147), p.R215W heterozygote (B177B), c.657-661del heterozygotes (GM15811 and GM15841) and c.657-661del homozygotes (GM15812 and NL). (A) Following irradiation, cells were cultured for 3 h with [3H]thymidine. Data from three independent experiments, each performed in triplicate, are shown; values are expressed as the percentage of an unirradiated control. (B) Cells after irradiation were cultured for 1 h with BrdU and stained with fluorescein ~anti-BrdU antibody. Fluorescence was measured by flow cytometry in 2 x 10^4 cells per each sample. Data are shown from two independent experiments; values are expressed as the percentage of an unirradiated control. **P < 0.01.

Fig. 4. Clonogenic survival assay in four control cell lines, p.I171V heterozygotes (BC80 and BC147), p.R215W heterozygote (B177B), c.657-661del heterozygotes (GM15811 and GM15841) and c.657-661del homozygotes (GM15812 and NL). Cells were counted, exposed to X-rays and plated in sextuplicate onto a 96-well plate. After 14 days, colonies of >50 cells were counted. Data are shown from two independent experiments; values are expressed as the percentage of an unirradiated control. **P < 0.01.
heterozygous c.657-661del, p.I171V and p.R215W mutations behaved similarly to control cells. The only significant differences we observed regarded the frequency of spontaneous and X-ray-induced chromatid breaks and γ-H2AX foci after high radiation doses (>5 Gy) in c.657-661del heterozygotes. The results obtained for c.657-661del heterozygotes were between the controls and the homozygotes, which would be expected as the heterozygotes express also the full-length nibrin (p95) in addition to the truncated protein (p70) transcribed from the mutated allele. Studies have previously noted that the amount of the p70 protein can modulate the phenotypic effects, with individuals having higher p70 levels showing a reduced cancer incidence (36). In addition, another study noted considerable variability in the levels of p70 in c.657-661del heterozygotes (37).

Increased frequency of spontaneous breaks and translocations in c.657-661del heterozygotes was reported earlier (38,39). In our assay, one NBS cell line (GM15812) clearly stood out, while the other one (NL) had the mean number of breaks in the range of c.657-661del heterozygotes. Similar interindividual variability was observed by Stummb et al. (39) regarding the frequency of spontaneous translocations. In c.657-661del heterozygotes, using the PCC technique, we also found significantly more chromatid breaks induced by the highest irradiation dose (5 Gy). In contrast, Tanzarella et al. (38) did not observe differences in the frequency of IR-induced chromatid-type aberrations in two c.657-661del carriers, but IR doses used in their experiment were much lower (0.15 and 0.3 Gy). In this study, we observed higher levels of residual γ-H2AX foci in c.657-661del heterozygotes 48 h after high doses (5 and 8 Gy) of irradiation. At lower IR doses, this mutation delayed the DSB repair, as judged by the rate of disappearance of γ-H2AX foci, but eventually, no significant differences were observed 48-h post-IR. However, it only means that the IR-induced breaks were joined but does not tell us whether this was done properly. Previous work has revealed that NBS cells show only a slight defect in total DSB repair, yet are defective in correct rejoining of DNA ends (26). In our assay, we did not examine translocations and it is possible that some breaks were mis-joined. Indeed, Neubauer et al. (40) and Distel et al. (41) found an increased frequency of translocations in c.657-661del heterozygotes following irradiation with 0.7 and 2 Gy.

An impaired DNA damage response was noted in NBS patients and their relatives carrying heterozygous c.657-661del mutation by means of the alkaline comet assay (42). These results are in opposition to other studies in which the fraction of activity released assay revealed none or only a subtle DSB repair defect in NBS cells (25,26,43). However, the alkaline comet assay—in contrast to the neutral version which is more specific for DSBs—detects a variety of DNA lesions, including single-strand breaks (SSBs) (44). Taking into account the recent finding that the base excision repair mechanism is deficient in NBN-depleted cells and cells from NBS patients (45), the extent of DNA damage observed by Burger et al. (42) could be in large part attributable to SSBs and not DSBs. In our study, we observed a delay in γ-H2AX foci formation and higher levels of residual foci in NBS cells, likely indicative of a defect in DSBs signalling and repair. However, although phosphorylated histone H2AX is widely used as a molecular marker of DSBs, it was also shown that the rate of disappearance of γ-H2AX foci does not always reflect the DSB repair as they can remain at the sites of chromosomal aberrations even after these aberrations have been formed. Also, DSB-unrelated γ-H2AX foci are found in cells after irradiation [reviewed in ref. (46)]. Thus, the higher levels of persistent γ-H2AX foci observed in our study in NBS cells and c.657-661del heterozygotes treated with high IR doses may not necessarily indicate a defect in DSB repair but could result from slower dephosphorylation/degradation of γ-H2AX or mark chromosome aberrations.

Another nibrin function, the activation of the intra-S-phase checkpoint, was not disturbed in any of the cell lines carrying heterozygous c.657-661del, p.I171V and p.R215W mutations. Nibrin participates in one of the two parallel branches of the intra-S-phase checkpoint, both of which involve the ataxia telangiectasia mutated kinase. Cells from NBS patients display an intermediate RDS phenotype compared to ataxia telangiectasia cells and control cells (4). In our assays, c.657-661del homozygotes showed a significant abrogation of the intra-S-phase checkpoint, while cells with heterozygous c.657-661del, p.I171V and p.R215W mutations inhibited DNA synthesis as efficiently as the control cells. Also, survival rates of NBN heterozygotes following irradiation were similar to those of the control cells. So far, there have been no reports on the impact of heterozygous c.657-661del, p.I171V and p.R215W mutations on the intra-S-phase checkpoint and sensitivity to IR. Our results obtained with assays routinely used for the assessment of these nibrin functions indicate that, though located in the domains crucial for nibrin structure and functioning, these mutations in the heterozygous form are insufficient to adversely affect the activation of the intra-S-phase checkpoint and do not increase the cells radiosensitivity.

There is experimental evidence that p.R215W mutation decreases nibrin stability (28,47,48). It was also shown to impair γ-H2AX binding but only in compound c.657-661del/p.R215W heterozygote; in p.R215W heterozygote, nibrin co-localised with γ-H2AX and γ-H2AX foci disappeared completely 24 h after IR (31). Our results showing no increase in the number of chromatid breaks and unchanged rate of disappearance of γ-H2AX foci are in agreement with the latter report.

To date, a substantial number of studies have been published on the potential association of heterozygous NBN mutation with a variety of cancers. The c.657-661del mutation was found to be associated with leukaemia and lymphoma (49–51), breast cancer (48,52–54) and prostate cancer (55). However, this was not confirmed in other studies of leukaemia and lymphoma (33,56–59), nor breast cancer (34,60–62). p.I171V was significantly more frequent in patients with leukaemia (33,63), breast cancer (34), head and neck cancer (64) and colorectal cancer (65). But again, other groups did not find such association in breast cancer (62,66), leukaemia and lymphoma (59) nor solid (67) or astrocytic (68) tumours in children. For p.R215W, association with cancer was found in colon and rectum cancer (60) but not in leukaemia and lymphoma (33,50,56,58,59), paediatric astrocytic tumours (68), breast (48,54), prostate (69) or head and neck cancer (64).

In the context of conflicting data regarding association of the heterozygous NBN mutations with various types of cancer, our results do not exclude that these mutations may increase cancer risk in heterozygous carriers. Recently, a concept of ‘conditional haploinsufficiency’ has been developed (70). It assumes that heterozygous mutations in genes involved in DNA damage repair which do not cause obvious defects under normal conditions are unmasked in early pre-malignant lesions with increased demand for efficient DNA repair and cell cycle control. Previous studies have demonstrated that loss of heterozygosity, a classical mechanism of action for tumour suppressor
genes, is very rare in humans and mice carrying heterozygous NBN mutations. This suggests that reduced gene dosage is the probable cause underlying tumour development in NBN heterozygotes [reviewed in ref. (71)]. Our results, showing an increased number of chromatid breaks and persistent γ-H2AX foci in c.657-661del heterozygotes after higher radiation doses, are in line with this concept. Genetic background in which heterozygous mutations exist is also of importance. In some compounds, compound heterozygosity within one gene, as in c.657-661del/p.R215W in NBN (28), or of different genes involved in a common process, e.g. DNA repair, can contribute significantly to tumour development, even though none of the heterozygous mutations alone would manifest itself (72,73). Thus, although the only significant defect in cellular response to IR observed in the present study was an increased number of IR-induced chromatid breaks and residual γ-H2AX foci following high IR doses in c.657-661del heterozygotes, the contribution of heterozygous c.657-661del, p.I171V and p.R215W NBN mutations to cancer initiation and/or development cannot be ruled out.

Funding
Ministry of Science and Higher Education (N N407 128836).

Acknowledgements
The authors thank Dr K. Różnowski for providing material for establishing the BC30 and BC147 LCLs, Drs E. Kwiatkowska and D. Kowalczyk for assistance with cell irradiation and Dr M. Kaczmarek for assistance with flow cytometry.

Conflict of interest statement: None declared.

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