Biomarkers, 2012; 17(3): 201–208

ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.3109/1354750X.2011.651157

informa
healthcare

RESEARCH ARTICLE

Biomarkers for genome instability in some genetic disorders: a pilot study

Nataliya V. Savina[1], Marharyta P. Smal[1], Tatyana D. Kuzhir[1], Tatyana M. Egorova[2], Olga M. Khurs[2], Anna D. Polityko[2], and Roza I. Goncharova[1]

[1]*Institute of Genetics and Cytology, National Academy of Sciences of Belarus, Minsk, Republic of Belarus and* [2]*The Republican Scientific and Practical Center "Mother and Child", Minsk, Republic of Belarus*

Abstract

Context: The study of genome integrity in some genetic disorders has diagnostic and prognostic importance because of the evident relationship between genome instability and both DNA repair deficiencies and cancer predisposition.

Objective: The objective was to compare the chromosomal and DNA damage responses in lymphocytes from patients with Nijmegen breakage syndrome (NBS), Fanconi anemia (FA) and Williams–Beuren syndrome (WBS) to find additional biomarkers of genome instability.

Methods: The cytogenetic approaches were combined with the alkaline Comet assay to estimate genome integrity in cultured or freshly isolated and H_2O_2-treated lymphocytes.

Results: Basal frequencies of chromosome aberrations were significantly increased in NBS/FA probands and NBS heterozygous carriers. The NBS diagnosis was confirmed by detecting site-specific rearrangements, while the mitomycin C (MMC)-stress test was highly positive in a FA patient. Among patients with suspected WBS, 12 individuals had a 7q11.23 microdeletion. In the Comet assay, genome instability was revealed in all three disorders, impaired capacity to repair oxidative damage being observed in NBS and WBS in contrast to FA and controls.

Conclusion: The results indicate that the estimates of DNA damage response may be proposed as efficient biomarkers for detecting and characterizing genome instability in the genetic disorders under study.

Keywords: Genome instability, chromosome aberrations, DNA damage, DNA repair, genetic disorders

Introduction

Some genetic disorders including rare autosomal recessive diseases, viz., Nijmegen breakage syndrome (NBS, OMIM #251260) and Fanconi anemia (FA, OMIM #227650), are characterized by increased frequency of spontaneous chromosome aberrations and hypersensitivity to certain clastogenic factors. Along with some others, they belong to the group of chromosome instability syndromes (CISs), which are also accompanied by multiple congenital malformations, immunologic/hematopoietic deficiencies and cancer predisposition (Taylor 2001). Unlike monogenic diseases, Williams–Beuren syndrome (WBS, OMIM #194050) arises from a microdeletion of the critical region within band 7q11.23 having an autosomal dominant inheritance and cardiac defects and a specific neurodevelopmental/behavioral profile as predominant phenotypic manifestations (Tassabehji 2003; Martens et al. 2008).

The autosomal recessive chromosome instability and hyper-radiosensitivity NBS was shown to arise from a mutation in the *NBSI* gene at 8q21; an unexpectedly high carrier frequency of the major 657del5 mutation (1/177) was found in the three Slavic populations (in the Czech Republic, Poland and the Ukraine) (Varon et al. 2000; Seemanová et al. 2006) that greatly exceeds its prevalence in the German population (1:866) (Carlomagno et al. 1999). The estimated carrier rate for mutations associated with FA is approximately

Address for Correspondence: Mrs. Natalya Savina, Institute of Genetics and Cytology, National Academy of Sciences of Belarus Akademicheskaya St. 27, Minsk, 220072 Republic of Belarus. Fax: +375-17-284-19-17. Tel: +375-17-294-91-79. E-mail: n.savina@igc.bas-net.by

(Received 17 October 2011; revised 14 December 2011; accepted 15 December 2011)

1:300 in the American and European populations, and as high as 1:90 among Ashkenazi Jews (Peleg et al. 2002; Kook 2005). These mutations have been reported to affect DNA double-strand break (DSB) repair leading to chromosomal breakage and cell malignancies (Williams et al. 2007; Li & Heyer 2008) and increasing cancer risk in heterozygous carriers (Seemanová et al. 2006; Berwick et al. 2007; di Masi & Antoccia 2008). Microarray analysis of cells from NBS carriers and normal controls revealed significant differences in expression profiles of 520 genes, and a combination of 16 genes allowed 100% correct classification of individuals as either NBS carriers or non-carriers (Cheung & Ewens 2006). Among the down-regulated pathways, there was homologous recombination repair, in which, the *NBSI* gene product, nibrin, forms a complex with RAD50 and MRE11 at double-strand breaks. In addition, two other repair pathways (the MAD1- and MAD2-mediated mitotic spindle checkpoint, and the XPG- and ERCC1-dependent nucleotide excision repair) were also down-regulated. These findings suggested that although NBS is a recessive disorder, carriers have distinctive features in their DNA repair patterns compared to non-carriers that may be important prognostic indicators of malignancies (Cheung & Ewens 2006).

WBS usually occurs sporadically with an estimated prevalence of 1 in 7500–20000 newborns (Tassabehji 2003). Available literature has put the main emphasis on WBS-associated microdeletion, this chromosomal defect being directly related to the genomic structure of the critical region of chromosome 7 (Bayés et al. 2003; Schubert 2009).

Cytogenetic approaches are generally accepted in diagnostics of CISs with detecting site-specific chromosome aberrations and hypersensitivity to cross-linking agents in NBS and FA cells, respectively (Hiel et al. 2000; Cohen et al. 1982), whereas the fluorescence in situ hybridization (FISH) test is still remaining the widespread and effective tool to verify WBS (Shaffer et al. 2007). The objective of the present pilot study was to compare the cytogenetic data with the DNA damage response in lymphocytes from patients with NBS, FA and WBS as well as from NBS or FA heterozygous carriers to find additional effective biomarkers of genome instability, which would be helpful to clarify mechanisms and/or events possibly contributing to pathogenesis of these genetic disorders and could be used as potential predictors of cancer risk.

Materials and methods

Study subjects

From patients of the Republican Scientific & Practical Center (RSPC) "Mother and Child", the following groups were selected: (1) five patients with clinical manifestations of NBS; (2) one patient clinically suspected of FA; (3) relatives of the patients with suspected CISs (eight subjects); (4) twelve patients with WBS.

Clinically healthy residents of Belarus, belonging to Eastern Slavic population, were recruited to the control group, which included 35 donors-volunteers aged from 22 to 63 years old. The previous study demonstrated no correlation between age and levels of DNA damage in lymphocytes of healthy people (Smal et al. 2010; Savina et al. 2011); therefore this group was used for comparison with groups of patients examined. Informed consent was obtained from each subject for inclusion in the study and before the collection of blood samples. All subjects completed a questionnaire covering medical, residential and occupational history as well as lifestyle habits. It was ensured that subject anonymity was kept. It should be emphasized that controls were exposed neither occupationally nor environmentally to mutagenic pollutants as well as they maintained a healthy lifestyle (e.g. non-tobacco cigarette smoking).

Blood sampling, lymphocyte isolation and treatment

Blood sampling was performed by the staff of the RSPC "Mother and Child" by means of venous puncture. For the Comet assay, 1–2 mL peripheral blood was collected into the heparinized vacutainer tubes and kept at 4°C for not longer than 2 h.

Lymphocytes were isolated from whole blood samples by centrifugation over 2.5 mL Histopaque at 1500 rpm for 30 min. Then lymphocytes were washed twice with Roswell Park Memorial Institute medium (RPMI) 1640, suspended in cold PBS, and exposed to oxidative stress. For this purpose, lymphocytes were treated with hydrogen peroxide (100 μM H_2O_2) at 4°C 1 min, and the chemical was removed by washing with cold PBS. Intact and treated lymphocytes were incubated in RPMI 1640 with 10% fetal bovine serum (FBS) during a 3-h period at 37°C. Cell viability was traditionally evaluated with the trypan blue exclusion test.

Estimation of the endogenous and mutagen-induced DNA damage, DNA repair kinetics and efficiency

All the reagents and procedures for the alkaline Comet assay (single cell gel electrophoresis) were used as described elsewhere (Tice et al. 2000; Collins 2004). The procedures included slide preparation; lysis of cell membranes for DNA elution by keeping the slides in the cold lysing solution (2.5 M NaCl, 10 mM EDTA, 10 mM Tris, pH 10) for 1 h; DNA unwinding in fresh electrophoresis buffer (1 mM EDTA, 300 mM NaOH) for 20 min and horizontal electrophoresis for 20 min at 1 V/cm, 300 mA and pH > 13. After electrophoresis, slides were washed twice for 5 min with 0.4 M Tris buffer (pH 7.5) for neutralization and then fixed in ice-cold 96% ethyl alcohol for 10 min. Slides were stained with ethidium bromide and analysed with a fluorescence microscope Olympus BX-50 equipped with a 100 W mercury lamp and an excitation filter of 510–550 nm. Visual estimation of DNA damage in arbitrary unites (a.u.) was carried out according to published recommendations (Collins et al. 1994). Two slides were prepared for each point of analysis, and at least 100

cells were scored per each of two replicate slides by one researcher that provided the concordance between the results. The levels of DNA damage were calculated as average values.

Endogenous DNA damage was evaluated after 180 min incubation of intact lymphocytes in RPMI 1640 with 10% FBS. The initial level of induced DNA damage was estimated immediately after mutagenic treatment of lymphocytes, i.e. at 0 min, and the residual level of oxidative DNA damage was measured 180 min after exposure.

To estimate DNA repair kinetics, samples of the H_2O_2-treated lymphocyte suspension were collected 0, 15, 30, 60, 120 and 180 min after mutagenic exposure.

DNA repair efficiency (RE) was calculated as percentage of DNA lesions eliminated relative to their initial level, i.e:

$$RE_t = [(IF_0 - IF_t)/IF_0] \times 100$$

Where RE_t is the repair efficiency at the given time point; IF_0 and IF_t are the frequencies of induced DNA damage at 0 min and subsequent time points, respectively.

Cytogenetic analysis

Heparinized venous blood samples were used to prepare phytohemmaglutinin-stimulated cell cultures according to the standard procedures. For karyotype examination, 72-h lymphocyte cultures were used for GTG-banding technique with a resolution of 600 bands per haploid karyotype according to International System for Human Cytogenetic Nomenclature (Shaffer et al. 2009). At least 25 metaphases were routinely analysed for each patient. To analyse the frequency and spectrum of chromosome aberrations, 48-h cultures of peripheral blood lymphocytes were used for standard chromosome preparations. No less than 100 metaphases per individual were scored for different types of chromosome abnormalities.

Lymphocytes collected from a patient with suspected FA and his mother were additionally investigated using the MMC-stress test, which measures the frequencies of chromosome aberrations induced by mitomycin C (MMC) as compared to normal cells. This method is a widespread cytogenetic approach to diagnose this disease because of chromosomal hypersensitivity to cross-linking agents in FA cells (Cohen et al. 1982; Rosendorff & Bernstein, 1988; Talwar et al. 2004; Tootian et al. 2006). For this purpose, the cross-linking agent MMC was added to cultured lymphocytes (48-h culture) for 24 h at a final concentration of 0.1 μg/mL. Routinely, 100 metaphases per culture (MMC-treated and untreated) were scored and the clastogenic effects of MMC in FA homo- and heterozygous cells were compared with those in lymphocytes of healthy subjects.

A molecular cytogenetic approach was chosen to detect the microdeletion in the critical region of chromosome 7 in patients with clinically suspected WBS. Two-colour FISH, applying the locus-specific DNA probes LSI Williams–Beuren Region Probe (Q-biogene, Germany) and Williams Region Probe (Vysis Abbott Molecular, USA), was performed according to accepted procedures (Rautenstraus & Liehr 2001). Analysis of probe hybridization on metaphase chromosomes was carried out with a DMLB fluorescence microscope (Leica, Germany) equipped with the necessary fluorescence filters.

Statistical analysis

The frequencies of chromosome aberrations in various groups of individuals were compared with the control data by means of Student's *t*-test, whereas the individual values were compared using a Fisher's exact test. In the Comet assay, the results shown are the means ± standard error (SE) in each group of individuals. The statistical significance of differences between these values was determined according to Student's *t*-test. Group data on DNA repair kinetics were plotted as power-behaved dependence of DNA lesion yields on time of strand break rejoining. The data concerning the control and patient groups in the logarithmic coordinates were compared by means of regression analysis, and significant differences between the linear regression coefficients were determined with the *t*-test as described in a previous report (Savina et al. 2011).

Results

Diagnostics of NBS, FA and WBS was based on both clinical manifestations and results of laboratory cytogenetic and molecular cytogenetic analysis of cultured peripheral blood lymphocytes. According to the results of these investigations, NBS was diagnosed in five cases and FA was confirmed in one patient. Diagnosis of WBS was verified by metaphase FISH-testing for a 7q11.23 microdeletion, which was identified in 12 patients with clinical manifestations of this syndrome. Cytogenetic characteristics of lymphocytes from all patients as well as from their relatives are presented in Table 1.

The standard karyotyping technique showed normal karyotypes in all cases. Cytogenetic analysis of blood samples collected from donor-volunteers showed individual variations in chromosome aberration frequencies from 1% up to 6%, with an average rate of 2.50 ± 0.35%. Frequencies of chromosomal abnormalities were usually increased in NBS and FA probands, especially in patients 3/632, 4/635 and 13/638. A similar tendency was observed in their parents. In NBS probands, the spectrum of aberrations was shifted towards chromosomal type of rearrangements, which constituted 57% versus 30% in the controls. Additionally, 100 GTG-banded metaphases per each patient with clinically suspected NBS were scored for site-specific chromosome aberrations in chromosomes 7 and/or 14. Diagnostically significant cytogenetic markers were identified in all five patients, viz. inv(7)(p15q35) and del(7)(q35) in patients 1/512; t(7;7)(p13;q35) in patient 2/542; inv(7)(p15q35) and t(14;14)(q12;q32) in patient

Table 1. Data on chromosomal aberration analysis in individuals involved in the study.

Number/code	Metaphases (*n*)	Frequency, %		Rearrangement types, %	
		Aberrant cells	Aberrations	Chromosomal	Chromatid
Healthy donors					
Control	2000	2.50 ± 0.35	2.50 ± 0.35	0.75 ± 0.19	1.75 ± 0.29
NBS probands					
1/512	100	5	5	3[a]	2
2/542	100	7[a]	7[a]	3[a]	4
3/632	100	21[b]	21[b]	15[b]	6[a]
4/635	100	22[b]	23[b]	12[b]	11[b]
5/759	100	15[b]	16[b]	8[b]	8[b]
Total (5)	500	14.0 ± 3.49[d]	14.4 ± 3.63[d]	8.2 ± 2.4[d]	6.2 ± 1.56[c]
NBS parents					
6/643 (mother)	100	6[a]	6[a]	0	6[a]
7/633 (mother)	100	5	5	2	3
8/634 (father)	100	8[b]	8[b]	1	7[a]
9/636 (mother)	100	8[b]	8[b]	4	4
10/637 (father)	100	5	5	0	5
11/759 (mother)	100	4	4	2	2
12/759 (father)	100	3	3	1	2
Total (7)	700	5.57 ± 0.72[d]	5.57 ± 0.72[d]	1.43 ± 0.53	4.14 ± 0.74[c]
FA proband					
13/638	100	38[b]	56[b]	8	48[b]
FA parent					
14/639 (mother)	100	6[a]	6[a]	1	5
MMC-test in lymphocytes from FA proband and a carrier of FA-associated mutations					
Control	550	17.45 ± 1.62	21.64 ± 1.76	3.64 ± 0.8	18.0 ± 1.64
13/638	20	100[b]	245[b]	5	44[b]
14/639	100	10	10	1	9
WBS probands					
Total (12)	1300	3.83 ± 0.37[c]	3.83 ± 0.37[c]	0.96 ± 0.28	2.88 ± 0.26

For NBS obligatory heterozygotes: No. 6 is a patient 2/542 mother; No. 7, 8 are patient 3/632 parents; No. 9, 10 are patient 4/635 parents; No. 11, 12 are patient 5/759 parents.
Statistically significant differences according to a Fisher's exact test ([a]$p < 0.05$; [b]$p < 0.01$) or Student's *t*-test ([c]$p \leq 0.05$; [d]$p \leq 0.01$).

3/632; inv(7)(p15q35), t(7;14)(p15;q12), and del(14)(q12) in patient 4/635; t(7;14)(p15;q12), t(7;14)(q35;q11) and t(1;14)(p35;q11) in patient 5/759. Increased chromosome breakage was also observed in obligatory NBS heterozygotes. In this case, aberrant cells occurred at a frequency of 5.57% that exceeded the control level by more than two-fold. The MMC-stress test was highly positive in cultured lymphocytes from patient 5/638 with clinically suspected FA, since the frequency of MMC-induced chromosome aberrations reached 245%, whereas chromosome breakage in response to MMC in an obligatory carrier was within the ordinary range. In WBS patients, the average frequency of chromosome aberrations was slightly higher than in the control sample.

Briefly, pronounced chromosome instability was determined in NBS and FA patients and, to a lesser degree, in NBS heterozygous carriers. In WBS patients, chromosomal instability concerned only the 7q11.23 critical region resulting in a specific microdeletion.

Simultaneously, DNA damage response was estimated according to the approach proposed for revealing genome instability in various risk groups (Smal et al. 2010), i.e. the same blood samples were tested for endogenous and H_2O_2-induced DNA damage, and for DNA repair capacity using the alkaline Comet assay.

As shown in Table 2, the levels of endogenous DNA damage in lymphocytes from patients with NBS and WBS as well as in NBS heterozygous carriers were significantly higher than in the controls. The levels of H_2O_2-induced DNA damage (initial and/or residual) were increased in all samples, significant differences from the control values being established for the residual level in lymphocytes from NBS probands and their parents, and overall in WBS cells. Only one patient with FA was examined in the present study, therefore any conclusion may be premature in respect of the data obtained. Nevertheless, the initial level of induced DNA damage seems indicative of the high sensitivity of lymphocytes to oxidative stress in this case. It should be also noted that the residual levels of oxidative DNA damage significantly exceeded the basal levels in NBS cells, whereas a similar trend was not statistically proved in WBS lymphocytes ($p = 0.065$), possibly because of wide individual variations of these values (21–71 a.u.). Nevertheless, RE indices indicated an appreciable reduction in DNA repair capacity in both NBS and WBS cells.

Table 2. DNA damage response in lymphocytes from individuals involved in the study.

Groups	Endogenous DNA damage (a.u.)	DNA damage induced by H_2O_2 (a.u.)				DNA repair efficiency (%)		
	180 min	0 min	15 min	60 min	180 min	15 min	60 min	180 min
Control	9.13 ± 0.79	85.63 ± 4.38	39.17 ± 5.78	22.79 ± 2.05	13.32 ± 1.07	52.67 ± 3.52	72.09 ± 2.25	83.19 ± 1.48
NBS (children)	24.00 ± 1.53[a]	90.67 ± 12.81	60.17 ± 10.65	45.33 ± 8.67	34.25 ± 3.20[a,b]	36.35 ± 6.74	53.94 ± 4.13[a]	60.79 ± 6.74[a]
NBS (parents)	22.67 ± 2.19[c]	110.57 ± 14.65	62.14 ± 10.35	30.67 ± 7.09	21.33 ± 2.73[c]	42.91 ± 8.25	74.34 ± 2.38	80.14 ± 2.99
FA (child)	17.0	143.0	57.25 ± 2.25	37.0	21.0 ± 2.68	59.58 ± 1.28	74.13	84.20 ± 1.87
FA (mother)	13.0	101.0	76.0	20.0	12.0	24.75	80.20	88.12
WBS	28.20 ± 4.62[d]	104.88 ± 8.69	69.88 ± 5.39[d]	54.13 ± 5.41[d]	48.86 ± 8.66[d]	38.19 ± 5.16[d]	52.87 ± 5.88[d]	53.89 ± 9.56[d]

Statistically significant differences according to Student's *t*-test: between controls and NBS homozygotes ([a]$p = 0.0033$ and 0.0034 for endogenous and residual levels of DNA damage; $p = 0.0062$ and 0.0043 for DNA repair efficiency 60 and 180 min after exposure); between residual and background levels of DNA damage in NBS homozygotes ([b]$p = 0.044$); between controls and NBS heterozygotes ([c]$p = 0.001$ and 0.029 for endogenous and residual levels of DNA damage); between controls and WBS cells ([d]$p = 0.002$ for endogenous damage; 0.0009, 0.0004 and 0.007, for H_2O_2-induced DNA damage 15, 60 and 180 min after exposure; $p = 0.039$, 0.016 and 0.024 for DNA repair efficiency 15, 60 and 180 min after exposure).

When measuring DNA strand breaks and their rejoining, some peculiarities of DNA repair process were revealed in lymphocytes from patients compared to healthy individuals. Although overall yields of H_2O_2-induced lesions depending on time of sampling fit well to the power equations (Figure 1A), a comparison of the curves shows well-marked differences between NBS homozygotes and controls, between NBS and FA homozygotes and between WBS cells and normal lymphocytes. These patterns are more visible in the semi-logarithmic plot (Figure 1B), since the slopes of repair kinetics closely resemble each other in NBS homozygotes and WBS cells being clearly different from those in FA and control samples. Notably, the DNA repair profile in FA lymphocytes is identical to that in normal lymphocytes. When comparing the coefficients of linear regression (−0.36 for controls, and −0.17, −0.2 and −0.37 for WBS, NBS and FA, respectively), a significant delay in DNA repair in WBS and NBS cells was also confirmed ($p < 0.05$). Analysis of the results shows an impaired capacity to repair oxidative DNA damage in NBS and WBS cells in contrast to control and FA samples. These data fully conform to RE values (Table 2), which have a tendency to decrease in the following order: Control = FA > WBS = NBS.

In summary, lymphocytes from patients with established diagnoses of NBS and FA were used to model conditions promoting chromosome/genome instability. Genome integrity in these cells was compared to that in lymphocytes from patients with WBS, which was not directly associated with chromosomal breakage. Nevertheless, the proposed approach allowed detection of hallmarks of genome instability in all three disorders. Based on these data, we may conclude that DNA oxidative damage repair is delayed in lymphocytes from NBS patients, whereas both high sensitivity to oxidative stress and the functional competence in repair of oxidative DNA damage appear to be distinctive of FA cells. Increased levels of endogenous and residual H_2O_2-induced DNA damage indicate somewhat genome destabilization in NBS heterozygous carriers. An abnormal DNA damage response has been unexpectedly found in WBS patients.

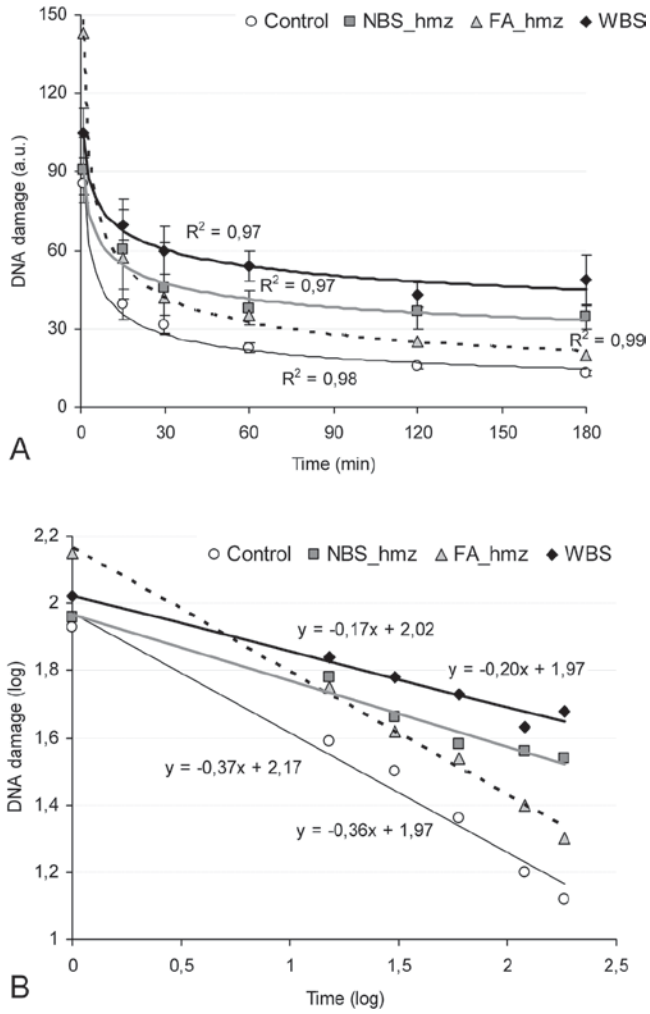

Figure 1. DNA repair kinetics in H_2O_2-treated lymphocytes from patients with NBS, FA and WBS as compared to healthy individuals. (A) Experimental observations and (B) Logarithmic transformation.

Discussion

Genome integrity was estimated in such genetic disorders as NBS, FA and WBS that have different underlying molecular mechanisms. Admittedly, two first diseases are associated with DSB repair deficiencies leading to genome destabilization that, in turn, triggers malignancies in hematopoietic and other tissues. The impact of NBS and FA heterozygosity on carcinogenesis, on the one hand (see Introduction), and the strong relationships between genome instability and cancer risks, on the other hand (Streffer 2010), have stimulated a search for more biomarkers of genome instability in patients with clinically suspected diseases and their families.

It should be noted that the spontaneous frequencies of chromosome aberrations (2.50 ± 0.35%) in the control group exactly corresponded to values determined earlier in the large groups of Belarusian children and adults (Polityko & Egorova 2001, Polityko et al. 2002). Pronounced chromosome instability was determined in NBS and FA patients due to significantly increased frequencies of spontaneous chromosome aberrations, the presence of site-specific clonal rearrangements in NBS cells and chromosomal hypersensitivity to MMC in FA cells. Significantly elevated chromosomal breakage was detected in NBS heterozygous carriers. A slight increase in the average rate of chromosome aberrations was observed in WBS patients; however in the latter case, individual frequencies of chromosomal abnormalities remained within the limits of variability in a healthy population (Savina et al. 2011).

Along with generally accepted cytogenetic markers, the cellular response to DNA damage and DNA repair capacity were studied as described above. The results of both approaches usually coincided with each other, and cytogenetic data were in-line with findings, suggesting diagnostic relevance of increased chromosome aberration rates in NBS homo- and heterozygotes (Hiel et al. 2000; Tanzanella et al. 2003), whereas the MMC-stress test, being principal in defining FA (Cervenka et al. 1981; Rosendorff & Bernstein 1988; Talwar et al. 2004; Tootian et al. 2006), failed to discriminate between obligatory heterozygous carriers of FA-related mutations and healthy individuals (Rosendorff & Bernstein 1988; Pearson et al. 2001; Mohseni Meybodi et al. 2007). Unlike these disorders, routine cytogenetic analysis of lymphocytes did not reveal obvious chromosome instability in patients with WBS, whereas an abnormal DNA damage response was initiated by a contiguous gene deletion within WBS-critical region of chromosome 7 (Savina et al. 2011).

Interestingly, studies of genome integrity in cells from FA and NBS homo- and heterozygotes, mainly dealing with evaluation of cellular radiosensitivity by the Comet assay, demonstrated increased baseline levels of DNA damage in both disorders, but provided rather conflicting data concerning cell responses to ionizing radiation. High initial DNA damage rates and delayed DNA repair were found in irradiated cells from NBS and FA patients (Djuzenova et al. 2001; Djuzenova & Flentje M 2002; Bürger et al. 2006), although the similar approach did not allow discrimination between FA patients, healthy donors and heterozygotes in other investigation, suggesting that MMC-induced DNA damage was the better index for diagnosis of FA compared to γ-rays (Mohseni Meybodi & Mozdarani 2009). At the same time, monitoring the repair kinetics 4 h after cell irradiation indicated a reduced capacity to repair DNA damage in leukocytes from FA patients as opposed to cells from healthy people and heterozygous carriers (Mohseni Meybodi et al. 2009).

Specific features of genome instability have been revealed in all three disorders due to our approach. Presumably, oxidative base damage and single strand breaks are predominantly measured under the proposed experimental conditions. Some diversity in the Comet data between NBS and FA homozygotes appears to be caused by unique molecular mechanisms and non-equivalent contribution of oxygen metabolism to pathogenesis of these diseases. The *NBS1* gene product, nibrin, is known to be closely associated with DSB repair and to interact with ATM-kinase (Lee & Lim 2006), which is an important sensor of reactive oxygen species in mammalian and human cells (Ito et al. 2007, Guo et al. 2010) and probably promotes base excision and single strand break repair through post-translational modification of DNA ligase IIIα and/or the scaffold protein XRCC1 (Dong & Tomkinson 2006; Chou et al. 2008). However, proteomic analysis of *Nbn* null mutant murine cells has recently demonstrated an increased production of reactive oxygen species following induction of DSBs by ionizing radiation (Melchers et al. 2009). It appears likely that a blockage in both signaling cascades might result in accumulation of DNA lesions in NBS lymphocytes due to somewhat decline in DNA repair or alterations in cellular redox homeostasis. The former assumption seems to be in-line with our data on the DNA repair kinetics and efficiency. In contrast to NBS, oxidative stress greatly contributes to a FA phenotype (Degan et al. 1995; Pagano et al. 2005). Reactive oxygen species as well as oxidative DNA damage are accumulated due to incompetence of antioxidant defense (Pagano & Youssoufian 2003; Thompson et al. 2005) rather than due to base excision repair deficiency in FA cells (Will et al. 1998, Zunino et al. 2001) that is also supported by our single observation. As to WBS patients, we have already discussed the relationship between a 7q11.23 microdeletion and genome instability in their lymphocytes (Savina et al. 2011) that may be interpreted in terms of losses of WSTF and/or RFC2 proteins involved in DNA repair, ATR-related DNA damage response and apoptosis (Yoshimura et al. 2009; O'Driscoll et al. 2007; Stucki 2009).

In the context of advanced diagnostic/prognostic approaches combining multiple biomarkers, the increased levels of endogenous and H_2O_2-induced DNA damage, alterations in DNA repair kinetics and efficiency may be considered the additional markers of genome

instability in the genetic disorders mentioned. In fact, these do not substitute for the cytogenetic markers, especially diagnostically specific ones. Nevertheless, estimates of the DNA damage sensitivity and DNA repair capacity strongly suggest genome destabilization in all disorders including WBS. The approach as a whole would be helpful in specification of mechanisms underlying pathogenesis of these diseases, e.g. impaired DNA repair and/or cellular intolerance to oxidative stress. Taking into account the role of genome instability in carcinogenesis, the biomarkers proposed here might also serve as potential predictors of cancer risk in both homo- and heterozygous carriers.

Conclusion

Cytogenetic analysis of chromosomal integrity in lymphocytes from patients with two rare monogenic disorders and a representative group of individuals with a chromosomal disease showed the greatly increased frequency of chromosome aberrations in NBS/FA probands and in potential NBS heterozygotes in contrast to WBS patients. The diagnoses of diseases were verified by detecting site-specific rearrangements in chromosomes 7 and 14 in NBS, the 7q11.23 microdeletion in WBS, and highly positive MMC-stress test in FA. DNA integrity was simultaneously estimated in the alkaline Comet assay with respect to the levels of endogenous and exogenous DNA damage, DNA repair kinetics and efficiency in lymphocytes exposed to hydrogen peroxide *in vitro*. Somewhat, advantage of this approach lies in the possibility to identify an abnormal DNA damage response in lymphocytes from WBS patients as well as to reveal some distinctive features of DNA damage response in lymphocytes from NBS and FA probands, likely due to different molecular mechanisms involved. The latter conclusion needs further investigation to be confirmed. However, the results of this pilot study indicate that the estimates of DNA strand breakage and capacity to repair oxidative damage can be proposed as additional and efficient biomarkers for detecting and characterizing genome instability in the genetic disorders under study.

Acknowledgements

The authors are grateful to Professor Irena Szumiel, DSc (Center of Radiobiology & Biological Dosimetry, Institute of Nuclear Chemistry and Technology, Poland) and to Dr. Gregory Nikiforovich, DSc (MolLife Design LLC, St. Louis, USA) for critical reading of the manuscript and helpful remarks as well as to Boris Anoshenko, PhD (the Central Botanical Garden, the National Academy of Sciences of Belarus) for assistance with statistical analysis of the data. The authors express sincere gratitude to the Presidents of the Association "Enfants de Tchernobyl Belarus" (France), Professor Michel Fernex and Yves Lenoir for support of investigations of genome instability in the Belarusian population.

Declaration of interest

The authors report no conflict of interest.

References

Bayés M, Magano LF, Rivera N, Flores R, Pérez Jurado LA. (2003). Mutational mechanisms of Williams-Beuren syndrome deletions. *Am J Hum Genet* 73:131–151.

Berwick M, Satagopan JM, Ben-Porat L, Carlson A, Mah K, Henry R, Diotti R, Milton K, Pujara K, Landers T, Dev Batish S, Morales J, Schindler D, Hanenberg H, Hromas R, Levran O, Auerbach AD. (2007). Genetic heterogeneity among Fanconi anemia heterozygotes and risk of cancer. *Cancer Res* 67:9591–9596.

Bürger S, Schindler D, Fehn M, Mühl B, Mahrhofer H, Flentje M, Hoehn H, Seemanová E, Djuzenova CS. (2006). Radiation-induced DNA damage and repair in peripheral blood mononuclear cells from Nijmegen breakage syndrome patients and carriers assessed by the Comet assay. *Environ Mol Mutagen* 47:260–270.

Carlomagno F, Chang-Claude J, Dunning AM, Ponder BA. (1999). Determination of the frequency of the common 657Del5 Nijmegen breakage syndrome mutation in the German population: no association with risk of breast cancer. *Genes Chromosomes Cancer* 25:393–395.

Cervenka J, Arthur D, Yasis C. (1981). Mitomycin C test for diagnostic differentiation of idiopathic aplastic anemia and Fanconi anemia. *Pediatrics* 67:119–127.

Cheung VG, Ewens WJ. (2006). Heterozygous carriers of Nijmegen Breakage Syndrome have a distinct gene expression phenotype. *Genome Res* 16:973–979.

Chou WC, Wang HC, Wong FH, Ding SL, Wu PE, Shieh SY, Shen CY. (2008). Chk2-dependent phosphorylation of XRCC1 in the DNA damage response promotes base excision repair. *EMBO J* 27:3140–3150.

Cohen MM, Simpson SJ, Honig GR, Maurer HS, Nicklas JW, Martin AO. (1982). The identification of fanconi anemia genotypes by clastogenic stress. *Am J Hum Genet* 34:794–810.

Collins AR. (2004). The comet assay for DNA damage and repair: principles, applications, and limitations. *Mol Biotechnol* 26:249–261.

Collins AR, Fleming IM, Gedik CM. (1994). *In vitro* repair of oxidative and ultraviolet-induced DNA damage in supercoiled nucleoid DNA by human cell extract. *Biochim Biophys Acta* 1219: 724–727.

Degan P, Bonassi S, De Caterina M, Korkina LG, Pinto L, Scopacasa F, Zatterale A, Calzone R, Pagano G. (1995). *In vivo* accumulation of 8-hydroxy-2′-deoxyguanosine in DNA correlates with release of reactive oxygen species in Fanconi's anaemia families. *Carcinogenesis* 16:735–741.

di Masi A, Antoccia A. (2008). NBS1 Heterozygosity and Cancer Risk. *Curr Genomics* 9:275–281.

Djuzenova CS, Rothfuss A, Oppitz U, Spelt G, Schindler D, Hoehn H, Flentje M. (2001). Response to X-irradiation of Fanconi anemia homozygous and heterozygous cells assessed by the single-cell gel electrophoresis (comet) assay. *Lab Invest* 81:185–192.

Djuzenova CS, Flentje M. (2002). Characterization of Fanconi anemia fibroblasts in terms of clonogenic survival and DNA damage assessed by the Comet assay. *Med Sci Monit* 8:BR421–BR430.

Dong Z, Tomkinson AE. (2006). ATM mediates oxidative stress-induced dephosphorylation of DNA ligase IIIalpha. *Nucleic Acids Res* 34:5721–5729.

Guo Z, Kozlov S, Lavin MF, Person MD, Paull TT. (2010). ATM activation by oxidative stress. *Science* 330:517–521.

Hiel JA, Weemaes CM, van den Heuvel LP, van Engelen BG, Gabreëls FJ, Smeets DF, van der Burgt I, Chrzanovska KH, Bernatowska E, Krajewska-Walasek M, Bialecka M, Abramczuk D, Gregorek H, Michalkiewicz J, Perek D, Midro AT, Seemanová E, Belohradsky BH, Sölder B, Barbi G, Wegner RD, Sperling K, Dixon J, Maraschio P, Marseglia GL, Green A, Taylor AM,

Der Kaloustian VM, Komatsu K, Matsuura S, Conley ME, Concannon P, Gatti RA. (2000). Nijmegen breakage syndrome. *Arch Dis Child* 82:400–406.

Ito K, Takubo K, Arai F, Satoh H, Matsuoka S, Ohmura M, Naka K, Azuma M, Miyamoto K, Hosokawa K, Ikeda Y, Mak TW, Suda T, Hirao A. (2007). Regulation of reactive oxygen species by Atm is essential for proper response to DNA double-strand breaks in lymphocytes. *J Immunol* 178:103–110.

Kook H. (2005). Fanconi anemia: current management. *Hematology* 10 Suppl 1:108–110.

Lee JH, Lim DS. (2006). Dual role of Nbs1 in the ataxia telangiectasia mutated-dependent DNA damage response. *FEBS J* 273:1630–1636.

Li X, Heyer WD. (2008). Homologous recombination in DNA repair and DNA damage tolerance. *Cell Res* 18:99–113.

Martens MA, Wilson SJ, Reutens DC. (2008). Research Review: Williams syndrome: a critical review of the cognitive, behavioral, and neuroanatomical phenotype. *J Child Psychol Psychiatry* 49:576–608.

Melchers A, Stöckl L, Radszewski J, Anders M, Krenzlin H, Kalischke C, Scholz R, Jordan A, Nebrich G, Klose J, Sperling K, Digweed M, Demuth I. (2009). A systematic proteomic study of irradiated DNA repair deficient Nbn-mice. *PLoS ONE* 4:e5423.

Mohseni-Meybodi A, Mozdarani H, Vosough P. (2007). Cytogenetic sensitivity of G0 lymphocytes of Fanconi anemia patients and obligate carriers to mitomycin C and ionizing radiation. *Cytogenet Genome Res* 119:191–195.

Mohseni Meybodi A, Mozdarani H. (2009). DNA damage in leukocytes from Fanconi anemia (FA) patients and heterozygotes induced by mitomycin C and ionizing radiation as assessed by the comet and comet-FISH assay. *Iran Biomed J* 13:1–8.

Mohseni-Meybodi A, Mozdarani H, Mozdarani S. (2009). DNA damage and repair of leukocytes from Fanconi anaemia patients, carriers and healthy individuals as measured by the alkaline comet assay. *Mutagenesis* 24:67–73.

O'Driscoll M, Dobyns WB, van Hagen JM, Jeggo PA. (2007). Cellular and clinical impact of haploinsufficiency for genes involved in ATR signaling. *Am J Hum Genet* 81:77–86.

Pagano G, Degan P, d'Ischia M, Kelly FJ, Nobili B, Pallardó FV, Youssoufian H, Zatterale A. (2005). Oxidative stress as a multiple effector in Fanconi anaemia clinical phenotype. *Eur J Haematol* 75:93–100.

Pagano G, Youssoufian H. (2003). Fanconi anaemia proteins: major roles in cell protection against oxidative damage. *Bioessays* 25:589–595.

Pearson T, Jansen S, Havenga C, Stones DK, Joubert G. (2001). Fanconi anemia. a statistical evaluation of cytogenetic results obtained from South African families. *Cancer Genet Cytogenet* 126:52–55.

Peleg L, Pesso R, Goldman B, Dotan K, Omer M, Friedman E, Berkenstadt M, Reznik-Wolf H, Barkai G. (2002). Bloom syndrome and Fanconi's anemia: rate and ethnic origin of mutation carriers in Israel. *Isr Med Assoc J* 4:95–97.

Polityko AD, Egorova TM. (2001). Cytogenetic analysis of individuals migrated from Gomel region to Minsk. *Int J Rad Med* 3:274 (in Russian).

Polityko AD, Egorova TM, Pisarik IV, Khurs OM. (2002). Cytogenetic monitoring database: the assessment of the tendencies and dynamics of mutagenesis in children. Abs of 14th Meeting of the German Society of Human Genetics, Leipzig, Germany. *Medizinische Genetik*, No3:290.

Rautenstraus B, Liehr T. (2002). FISH Technology. Springer Lab Manual, 494 p.

Rosendorff J, Bernstein R. (1988). Fanconi's anemia–chromosome breakage studies in homozygotes and heterozygotes. *Cancer Genet Cytogenet* 33:175–183.

Savina NV, Smal MP, Kuzhir TD, Egorova TM, Khurs OM, Polityko AD, Goncharova RI. (2011). Chromosomal instability at the 7q11.23 region impacts on DNA-damage response in lymphocytes from Williams-Beuren syndrome patients. *Mutat Res* 724:46–51.

Seemanová E, Jarolím P, Seeman P, Varon R, Sperling K. (2006). Increased risk of malignancies in heterozygotes in families of patients with Nijmegen breakage syndrome. *Cas Lek Cesk* 145:138–143.

Schubert C. (2009). The genomic basis of the Williams-Beuren syndrome. *Cell Mol Life Sci* 66:1178–1197.

Shaffer LG, Bejjani BA, Torchia B, Kirkpatrick S, Coppinger J, Ballif BC. (2007). The identification of microdeletion syndromes and other chromosome abnormalities: cytogenetic methods of the past, new technologies for the future. *Am J Med Genet C Semin Med Genet* 145C:335–345.

Shaffer LG, Slovak ML, Campbell LJ. (2009). An International System for Human Cytogenetic Nomenclature, (ISCN 2009): *Recommendations of the International Standing Committee on Human Cytogenetic Nomenclature*. Basel: Karger. pp 138.

Smal MP, Savina NV, Kuzhir TD, Goncharova RI. (2010). Estimation of genome instability by the comet assay in different risk groups, Rapid Diagnosis in Populations at Risk from Radiation and Chemicals/*NATO Science for Peace and Security Series E: Human and Societal Dynamics*, Vol.73. In: A., Cebulska-Wasilewska, A., Osipov, F., Darroudi, eds. Washington, DC: IOS Press. pp. 201–210.

Streffer C. (2010). Strong association between cancer and genomic instability. *Radiat Environ Biophys* 49:125–131.

Stucki M. (2009). Histone H2A.X Tyr142 phosphorylation: a novel sWItCH for apoptosis? *DNA Repair (Amst)* 8:873–876.

Talwar R, Choudhry VP, Kucheria K. (2004). Differentiation of Fanconi anemia from idiopathic aplastic anemia by induced chromosomal breakage study using mitomycin-C (MMC). *Indian Pediatr* 41:473–477.

Tanzanella C, Antoccia A, Spadoni E, di Masi A, Pecile V, Demori E, Varon R, Marseglia GL, Tiepolo L, Maraschio P. (2003). Chromosome instability and nibrin protein variants in NBS heterozygotes. *Eur J Hum Genet* 11:297–303.

Tassabehji M. (2003). Williams-Beuren syndrome: a challenge for genotype-phenotype correlations. *Hum Mol Genet* 12 Spec No 2:R229–R237.

Taylor AM. (2001). Chromosome instability syndromes. *Best Pract Res Clin Haematol* 14:631–644.

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. (2000). Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ Mol Mutagen* 35:206–221.

Thompson LH, Hinz JM, Yamada NA, Jones NJ. (2005). How Fanconi anemia proteins promote the four Rs: replication, recombination, repair, and recovery. *Environ Mol Mutagen* 45:128–142.

Tootian S, Mahjoubi F, Rahnama M, Hormozian F, Mortezapour F, Razazian F, Manoochehri F, Zamanian M, Nasiri F, Soleymani S, Seyedmortaz L. (2006). Cytogenetic investigation in Iranian patients suspected with Fanconi anemia. *J Pediatr Hematol Oncol* 28:834–836.

Varon R, Seemanova E, Chrzanowska K, Hnateyko O, Piekutowska-Abramczuk D, Krajewska-Walasek M, Sykut-Cegielska J, Sperling K, Reis A. (2000). Clinical ascertainment of Nijmegen breakage syndrome (NBS) and prevalence of the major mutation, 657del5, in three Slav populations. *Eur J Hum Genet* 8:900–902.

Will O, Schindler D, Boiteux S, Epe B. (1998). Fanconi's anaemia cells have normal steady-state levels and repair of oxidative DNA base modifications sensitive to Fpg protein. *Mutat Res* 409:65–72.

Williams RS, Williams JS, Tainer JA. (2007). Mre11-Rad50-Nbs1 is a keystone complex connecting DNA repair machinery, double-strand break signaling, and the chromatin template. *Biochem Cell Biol* 85:509–520.

Yoshimura K, Kitagawa H, Fujiki R, Tanabe M, Takezawa S, Takada I, Yamaoka I, Yonezawa M, Kondo T, Furutani Y, Yagi H, Yoshinaga S, Masuda T, Fukuda T, Yamamoto Y, Ebihara K, Li DY, Matsuoka R, Takeuchi JK, Matsumoto T, Kato S. (2009). Distinct function of 2 chromatin remodeling complexes that share a common subunit, Williams syndrome transcription factor (WSTF). *Proc Natl Acad Sci USA* 106:9280–9285.

Zunino A, Degan P, Vigo T, Abbondandolo A. (2001). Hydrogen peroxide: effects on DNA, chromosomes, cell cycle and apoptosis induction in Fanconi's anemia cell lines. *Mutagenesis* 16:283–288.