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Constitutive 53BP1/γH2AX foci are increased in cells of ALL patients dependent on BCR-ABL and TEL-AML1 preleukemic gene fusions

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Childhood leukemia arises from hematopoietic stem cells by induction of mutations. Quite often chromosomal translocations arise prenatally as first key event in multistage process of leukemogenesis. These translocations result in so called preleukemic gene fusions (PGFs), such as BCR-ABL and TEL-AML1, which generate hybrid proteins with altered properties. Critical DNA damage resulting in translocations are DNA double-strand breaks (DSBs). BCR-ABL and TEL-AML1 were shown to be associated with increased constitutive DSBs in various model systems. We analyzed cells from peripheral blood and CD34-/CD34+ cells from bone marrow of pediatric acute lymphoblastic leukemia (ALL) patients harboring BCR-ABL or TEL-AML1. We used sensitive technique that is based on automated enumeration of DSB co-localizing proteins γH2AX and 53BP1, which form so called DNA repair foci. We found that level of constitutive γH2AX/53BP1 foci is significantly higher in cells of ALL pediatric patients than in healthy subjects. There was also significant increased level of constitutive γH2AX/53BP1 foci in cells from ALL patients harboring BCR-ABL or TEL-AML1 compared to patients without PGFs. The same increase was observed regardless cell type for both PGFs. Our data on increased DSB levels in the BCR-ABL/TEL-AML1 patient's cells support a model where BCR-ABL/TEL-AML1 induces DNA instability through facilitating mutagenesis and appearance of additional genetic alterations driving leukemogenesis.

Key words: leukemia, BCR-ABL, TEL-AML1, 53BP1, γH2AX

The European data show acute lymphoid leukemia (ALL) at 81%, to be the most frequent leukemia; followed by acute myeloid leukemia (AML), which represents 15% of cases; and the other three, markedly rarer subgroups of chronic myeloid (1,5%), unspecified (1,3%) and other specified (under 0,5%) leukemia [1]. Leukemia is a clonal disease arising from the transformation of a single cell. The cell, which undergoes transformation, is likely to be a pluripotent hematopoietic stem cell (HSC) or a more mature progenitor cell [2-5]. Over 200 genetic alterations, including point mutations, deletions and insertions, have been linked to leukemia, resulting in gross chromosomal changes such as hyperdiploidy and translocations. Nonrandom chromosomal translocations and deletions are the hallmark of human leukemias [6]. Most leukemia-associated translocations are reciprocal translocations, with arms from different chromosomes switching places. These translocations result in so called preleukemic gene fusions (PGFs), which generate hybrid proteins with altered properties, such as activated kinase activity or novel transcriptional regulation [7, 8]. The number of chromosomal translocations resulting in PGFs is constantly growing with usage of new powerful screening approaches [9].The most common chromosomal translocations with corresponding PGFs and incidences in childhood ALL are: t(12;21)(p13;q22) TEL-AML1 (24-26%), t(1;19)(q23;p13) E2A-PBX (5-6%), t(9;22)(q34;q11) BCR-ABL p190 (3-5%) and t(4;11)(q21;q23) MLL-AF4 (~5%). The translocations are usually not sufficient for manifestation of disease. Acute leukemia requires at least two genetic lesions, one which blocks differentiation of HSC/progenitor, usually a translocation that predates the formation of leukemia, and one that occurs later and stimulates proliferation, usually a point mutation [10].

DNA damage is a key element in the accumulation of genetic changes associated with cell transformation. Double strand breaks (DSBs) is the most important type of DNA damage. DSBs can occur as a result of endogenous insults, such as attack by radical oxygen species (ROS) produced during metabolism. Chromosomal translocations are initiated by DSBs and each requires at least two DSBs in separate chromosomes. Inherited mutations in DSB repair components in humans generate a predisposition to leukemia and other cancers [11]. Thus, coincidence of both increased DSBs and ineffective

repair could create the environment for the acquisition of genetic alterations.

Recently, innovative technique for analysis of DSB-co-localizing discrete foci (referred to as DNA repair foci) that are formed by several proteins such as γ-H2AX, 53BP1, ATM, and NBS1 at the sites of DSB localization has been developed [12-14]. Analysis of DNA repair foci is currently accepted as most sensitive and specific technique for measuring DSB in both untreated cells and cells exposed to genotoxic agents [15].

Elevated constitutive levels of DNA repair foci were found in various human cancer cell lines, premalignant lesions and solid tumors suggesting that increased DNA damage is a general characteristic of cancer development [16-18]. Seo et al. found a >8-fold increase in H2AX expression in Jurkat cell line, a model for acute T-cell leukemia [18]. Yu et al. observed γH2AX foci in 17 different tumor cell lines and demonstrated that cell lines possessing overall higher numbers of γH2AX foci tended to have more structural chromosomal rearrangements [19].

While nonrandom chromosomal translocations and deletions are the hallmark of human leukemias, the data on DSB assessment in pathological cells from leukemic patients is very sparse. Myeloid leukemia cell lines (HL60 and K562) and primary cells from two patients with chronic myeloid leukemia (CML) showed increased levels of constitutive DNA damage compared with normal hematopoietic cells [20]. Primary cell populations from four patients with CML contained more γH2AX foci–positive cells than normal cells from three healthy donors [17]. Similarly, CD34+ cells from patient with chronic myeloid leukemia in chronic phase (CML-CP) were characterized by higher γH2AX level than cells from healthy donor [21]. Walters et al. reported significantly increased level of constitutive γH2AX foci in malignant plasma cells from patients with multiple myeloma (MM) [22]. To our knowledge, whether pathological cells from ALL patients exhibit higher level of DNA repair foci has not yet been reported.

Noteworthy, BCR-ABL and TEL-AML1 PGFs were shown to be associated with increased level of constitutive DNA damage. Involvement of BCR-ABL in occurrence of ROS, constitutive DNA damage and effects on genomic instability has recently been reviewed [23]. Overall data support concept that BCR-ABL expression either affects fidelity of DNA repair or alters accumulation of DNA damage [23]. A novel mouse model where ETV6(TEL)-RUNX1(AML1) expression is restricted to CD19+ B lymphoid cells has recently been introduced [24]. Using this model, the authors have shown association of TEL-AML1 expression with increased levels of ROS and subsequent accumulation of DSBs [24]. These findings suggested that the expression of TEL-AML1 triggers mutagenesis through enhanced ROS production. Whether the same associations of TEL-AML1 and BCR-ABL with increased DNA damage are observed in human cells from leukemic patients remain to be investigated.

In this study, lymphocytes from peripheral blood (PB) and bone marrow (BM) of ALL pediatric patients with and without PGFs were analyzed for their constitutive DSBs, in comparison to constitutive DNA damage in cells from healthy subjects. We quantified γH2AX and 53BP1 DNA repair foci as sensitive molecular markers co-localizing with DSB. We aimed to analyze possible correlation of constitutive DNA damage with risk group and presence of BCR-ABL and TEL-AML1.

Table 1. Basic information on leukemic patients enrolled to this study.

Patient	Age (months)	Diagnosis	Risk group	Leukemic gene fusion
PL1	83	BCP-ALL	HRG	BCR-ABL
PL2	204	BCP-ALL	IRG	
PL3	36	BCP-ALL	SRG	TEL-AML1
PL4	24	BCP-ALL	IRG	
PL5	72	BCP-ALL	HRG	BCR-ABL
PL6	36	BCP-ALL	SRG	
PL7	54	BCP-ALL	SRG	TEL-AML1
PL8	48	BCP-ALL	SRG	TEL-AML1
PL9	122	BCP-ALL	IRG	
PL10	101	BCP-ALL	HRG	
PL11	65	BCP-ALL	SRG	
PL12	138	BCP-ALL	HRG	BCR-ABL
PL13	182	BCP-ALL	IRG	
PL14	22	BCP-ALL	SRG	TEL/AML 1

Notice: BCP-ALL – B-cell precursor acute lymphoblastic leukemia. SRG- standard risk group, IRG- intermediate risk group, HRG- high risk group.

Patients and methods

Patients. Group of patients consisted of nine boys and five girls (Table 1). Mean age in the group was 85±58 months (from 22 to 204 months). Seven patients were without PFG, three with BCR-ABL and four with TEL-AML1 fusion gene. Patients were treated in the Department of Pediatric Hematology and Oncology in Bratislava. This study was approved by the local ethics committee, children's parents gave written informed consent to participate in the study.

Diagnostic tools. Diagnosis of ALL was based on the French-American-British classification and flow cytometric immunophenotyping using a standard set of monoclonal antibodies according to the European Group for Immunological Characterization of Leukemia [25]. FISH, immunophenotyping and PCR screening for preleukemic gene fusions (PGFs) were routinely performed on samples from each patient. To decrease heterogeneity of the study group, only patients with prognostically important PGFs TEL-AML1 and BCR-ABL with immunophenotype CD34+, CD10+, CD20-, CD19+, CD22+ with exception patient PL2 with immunophenotype CD34+, CD10-, CD20-, CD19+, CD22+ were chosen. Majority of pathological cells were CD34+, mean value 83,8±18,4% (from 40% to 100%).

Treatment. The elements of this treatment protocol were previously published [26, 27]. According to the protocol, the patients received a 7-day prednisone regimen with one

treatment of intrathecal methotrexate, followed by an 8-week induction therapy consisting of eight agents. For SRG (standard risk group) and IRG (intermediate risk group), the consolidation phase was comprised of four courses of high-dose methotrexate (2 g/m^2). The reinduction phase was randomized such that SRG patients received one treatment of protocol II or two treatments of protocol III. IRG patients were randomized to receive one treatment of protocol II or three treatments of protocol III. HRG (high risk group) patients received three treatments of protocol III, six treatments of protocol III plus one treatment of protocol II or three treatments of protocol III and two treatments of protocol II. Maintenance therapy with a total treatment duration of 24 months consisted of daily mercaptopurine and a weekly treatment with methotrexat. BM was classified as M1 (less than 5% blasts), M2 (5-24% blasts), or M3 (over 25% blasts) using standard morphological criteria. The bone marrow sampling for monitoring the minimal residual disease (MRD) was performed at the end of induction phase 1 (day 33) and preconsolidation at week 12 (day 78). Response to treatment was evaluated according to a non-MRD-based protocol with cytomorphology. All ALL patients were with complete remission (CR) of disease. Treatment response is listed in Table 1.

Healthy controls. Healthy controls group included seven children (three boys and four girls) with mean age 139±29 months (from 121 to 204 months).

Cells. Lymphocytes from PB and BM of pediatric leukemic patients and peripheral blood from healthy controls were collected as part of routine clinical examination before treatment and isolated by the density gradient centrifugation in Lymphocyte Separation Medium (LSM) (PAA laboratories GmbH, Austria). The cells were transported to the 90% basal medium RPMI 1640 (Gibco, Life Technologies, Grand Island, NY 14072, USA) with 10% Fetal Bovine Serum (FBS, Gibco, Life Technologies, Grand Island, NY 14072, USA) and incubated at cell density of 1-2x10^6 cells/ml in a humidified incubator at 5% CO_2 and 37°C for 2 h to remove adherent monocytes. Viability of cells was about 97% as measured by the 0,4% trypan blue (Gibco, Life Technologies, Grand Island, NY 14072, USA). If there was isolated more than 60 millions of lymphocytes from bone marrow (4 samples), progenitor stem cells CD34+ were isolated from suspension by immunomagnetic separation using CD34 MicroBead Kit and MidiMACS® (Miltenyi Biotec, Germany). Purity generally exceeded 95%.

DNA repair foci analysis. After incubation, 1,5x10^5 cells in 200 µl phosphate-buffered saline (PBS, 3,2 mM NA_2HPO_4, 0,5 mM KH_2PO_4, 1,3 mM KCl, 135 mM NACl, pH 7,4) were spinning down on double cytoslides coated with polysine (Menzel Glaser, Thermo Fisher Scientific, USA) at 85 g for 5 min by Cytospin 2 cytocetrifuge (Shandon, Thermo Fisher Scientific Inc., USA). Cells were fixed in cold 3% paraformaldehyde (P6148, Sigma Aldrich, St. Luis) for 15 min, washed in PBS and day after permeabilized with 0,2% Triton X-100 (93426, Fluka Analytical, St. Luis). After washing, cells were blocked in 3% FBS for 30 min at room temperature (RT) and then stained with primary antibodies anti-γH2AX phosphospecific (ser 139) mouse monoclonal (1:400, NB100-78356, Novus Biologicals, Germany) and anti-53BP1 rabbit polyclonal (1:800, NB100-305, Novus Biologicals, Germany) diluted in 3% FBS for 1 h at RT in wet chamber. Slides were washed in PBS and incubated 1 h in wet chamber at RT with the secondary antibodies, Alexa Fluor® 488 goat anti-rabbit IgG (H+L) (1:200, A11034, Molecular Probes, Invitrogen, Life Technologies, NY, USA) and Alexa Fluor ® 555 goat anti-mouse IgG (H+L) (A-21422, Molecular Probes, Invitrogen, Life Technologies, NY, USA). After washing cover slides were mounted with antifade DAPI Vectashield (H-1500, Vector Laboratories, Burlingame, CA) and sealed to cytoslides. We had prepared two independent slides from each subject with few exceptions where only one slide could be prepared for technical reasons. Zeiss Axio Imager Z1 (Zeiss Microscopy, Jena, Germany) microscope combined with Metafer system MetaCyte (MetaSystems, Germany) was used to analyze constitutive DSB foci from 1000 cells randomly acquired by the Metafer using custom made classifier and blinded to patient identity. Main parameters of analysis were: objective magnification (63x), number of focus planes (7), focus plane distance (28/40 µm). Cells with non circular shape were excluded from analysis.

Statistical analysis. The data were analyzed using the two-tailed Student's t-test. Statistical significance was set at a p value of <0,05.

Results

Significant difference in constitutive DNA damage as measured by all endpoints, 53BP1, γH2AX foci, and their co-localization was observed between cells of healthy donors and ALL patients (Figure1, Table 2).

The same difference with control subjects retained if ALL patients were divided into two subgroups with or without

Table 2. Statistical analysis of constitutive DSBs in PB cells from healthy donors and leukemic patients. Bold font designates statistically significant differences compared to cells of healthy donors.

P-values, Healthy donors vs.	ALL patients	Patients without PGFs	Patients with PGFs	BCR-ABL	TEL-AML1
53BP1	**0.000004**	**0.000003**	**0.00000003**	**0.000000004**	**0.000002**
γH2AX	**0.000363**	**0.001248**	**0.000009**	**0.0000003**	**0.000196**
Co-localization	**0.017030**	0.597968	**0.000108**	**0.00012**	**0.000680**

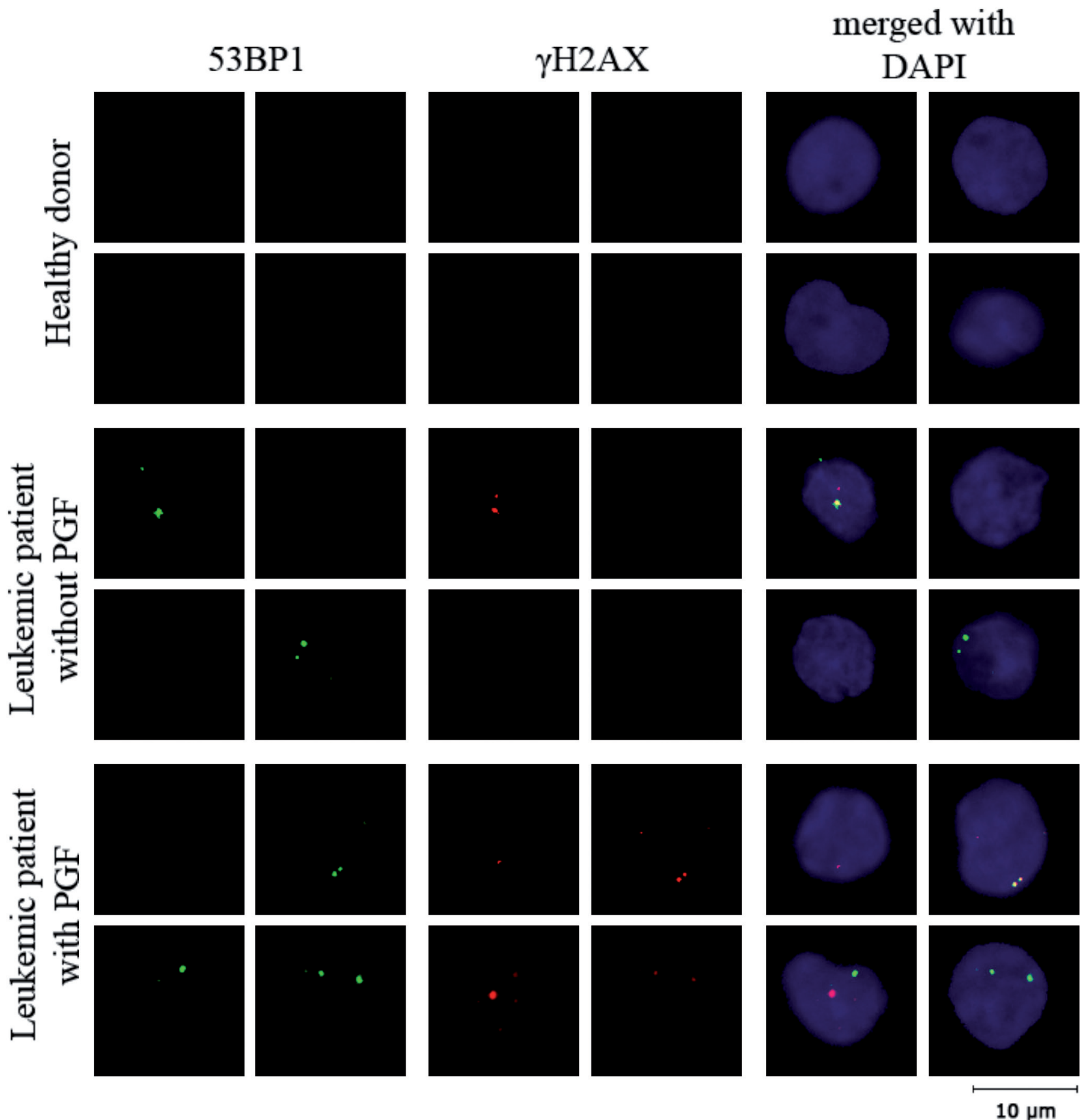

Figure 1. Representative nuclei of PB cells from healthy donor H1, leukemic patient PL10 without PGF, and leukemic patients with PGF (BCR-ABL, PL12): green (53BP1), red (γH2AX) and blue (DAPI), objective magnification 63x.

PGFs. Significantly lower level of constitutive 53BP1 and γH2AX foci was observed in PB of control donors in comparison with both subgroups (Table 2).

Noteworthy, DNA damage was significantly different between subgroups of ALL patients, with and without PGFs (Figure 2, 3). This difference was consistently observed regardless cell type (PB or BM) or end-point (53BP1, γH2AX foci, their co-localization) (Table 3). The only exception was the same constitutive DNA damage in CD34+ and CD34- cells, which might be accounted for by low number of subjects for this comparison, two versus two (Figure 3) (Table 3). Constitutive DNA damage did not vary significantly between cell

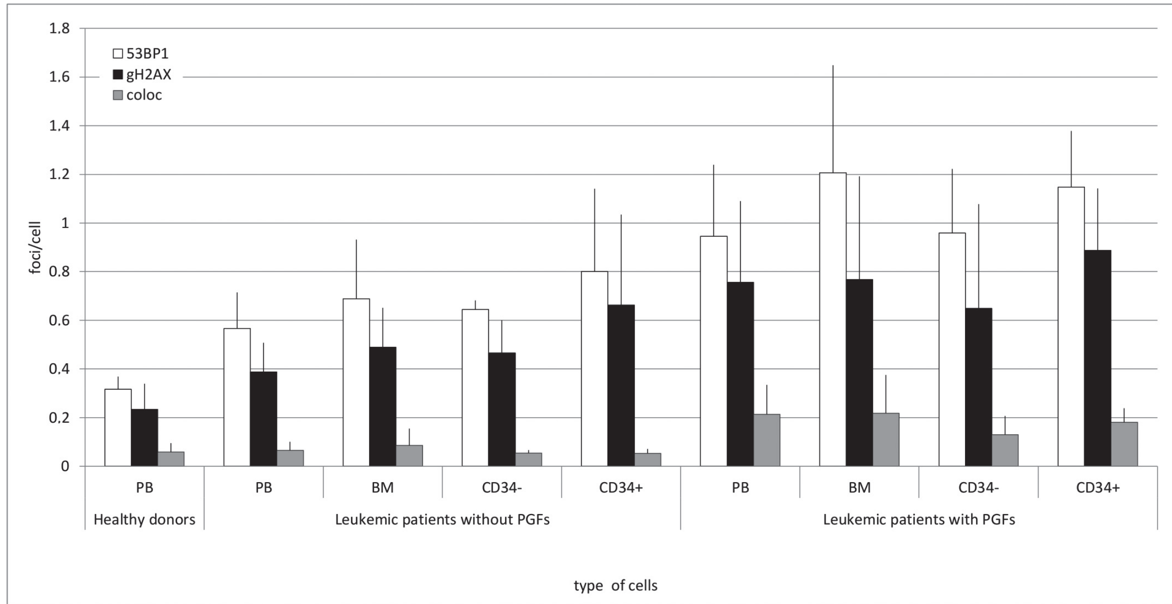

Figure 2. Number of DNA repair foci in cells from healthy donors and ALL patients. Data are shown as mean values +SD obtained from seven healthy donors, seven patients with BCR-ABL or TEL-AML1 gene fusions, and seven patients without PGFs except for CD34- and CD34+ cells where data from two patients are shown in each data point.

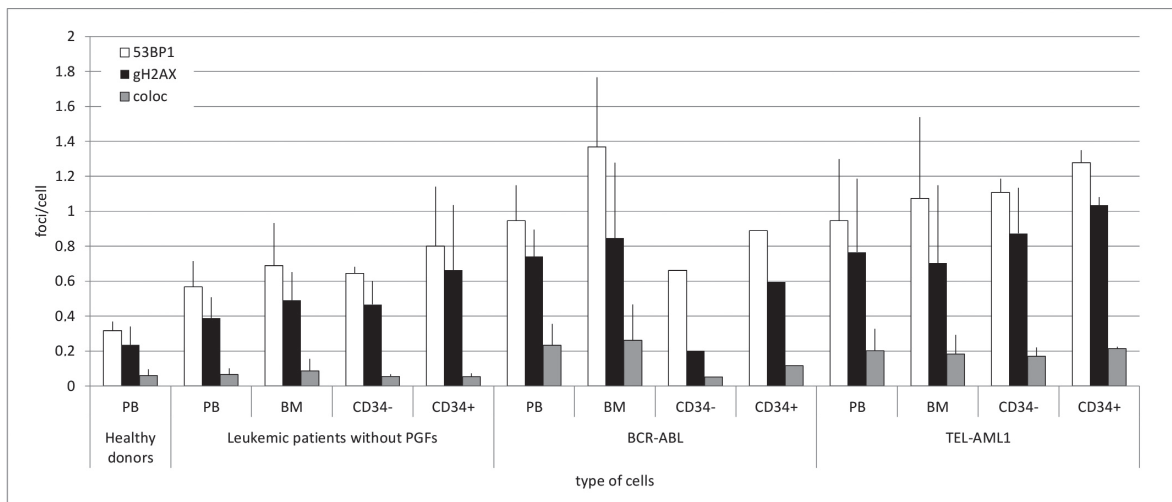

Figure 3. Number of DNA repair foci in cells from leukemic patients with BCR-ABL or TEL-AML1. Data are shown as mean values +SD obtained from PB of three leukemic patients with BCR-ABL, and four patients with TEL-AML1 preleukemic gene fusion. CD34+ and CD34- data are shown for two patients without PGFs, one patient with TEL-AML1, and one patient with BCR-ABL (only one sample was taken from this donor for CD34+/CD34- analysis and thus SD is missing). Data for healthy donors and leukemic patients without PGFs from Figure 2 are shown in the same scale for illustration purpose.

Table 3. Comparison of constitutive DSBs in cells from leukemic patients without and with PGFs. Bold font designates statistically significant differences compared to cells of ALL patients without PGFs.

P-values, Leukemic patients	Leukemic patients with PGFs				TEL-AML1				BCR-ABL	
without PGFs vs.	PB	BM	CD34-	CD34+	PB	BM	CD34-	CD34+	PB	BM
53BP1	**0.000247**	**0.001467**	0.059282	0.191093	**0.001995**	**0.028175**	**0.000471**	0.137132	**0.000353**	**0.000408**
γH2AX	**0.000702**	**0.039168**	0.447058	0.413432	**0.004681**	0.137552	0.055727	0.255591	**0.000061**	**0.018296**
Co-localization	**0.000181**	**0.011779**	0.102569	**0.007594**	**0.001041**	**0.031337**	**0.008876**	**0.000426**	**0.000169**	**0.013021**

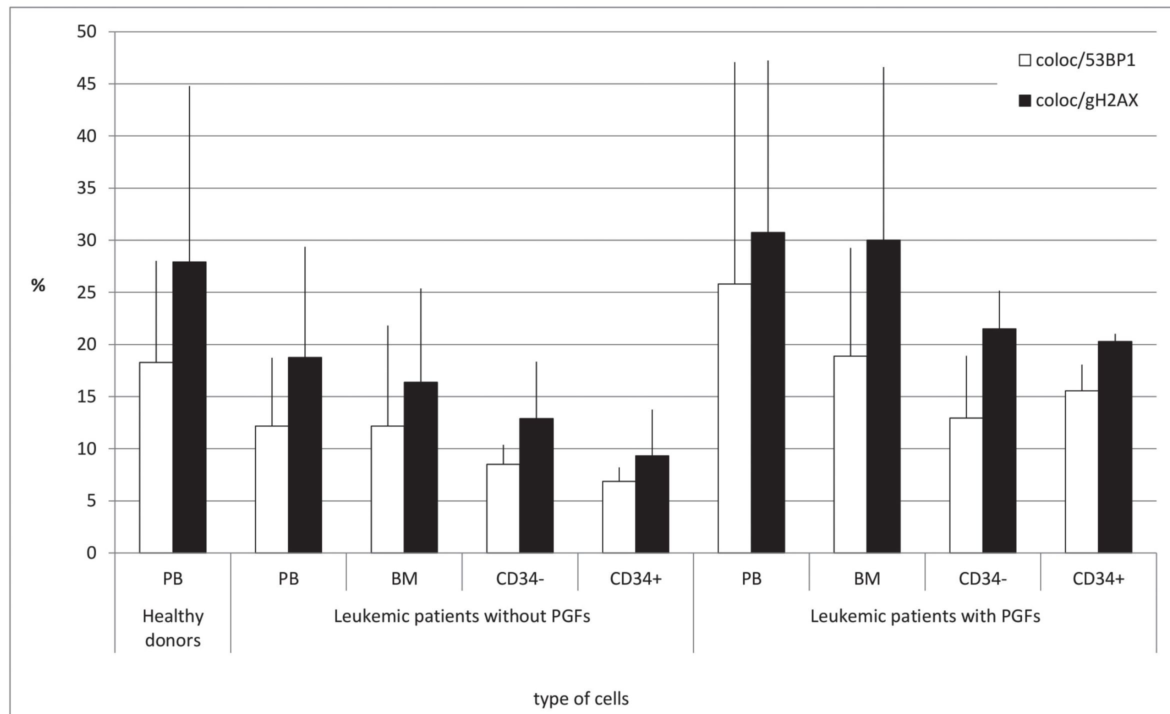

Figure 4. Percentual co-localization of 53BP1 and γH2AX foci in different cell types. Co-localization was normalized to either 53BP1 or γH2AX foci.

types (PB, BM, CD34+, CD34-) within each group of patients (Figure 3, Table 3).

Of note, co-localization of γH2AX and 53BP1 proteins in DNA repair foci did not exceed 30% (Figure 4). Relatively low co-localization of constitutive foci is explained by various kinetics for persistence of γH2AX and 53BP1 at locations of DSBs and corresponds well to previously reported studies where co-localization of γH2AX and 53BP1 in exogenous DNA repair foci in hematopoietic cells has been quantified [28-30]. Percentual co-localization was the same regardless cell type (compare CD34+ vs. CD 34-) or cell origin (PB vs. BM) (Figure 4). Thus, persistence of γH2AX and 53BP1 at locations of DSB did not vary between differentiated lymphocytes and stem/progenitor cells and was the same in PB and BM. On the other hand, co-localization was slightly higher in cells from leukemic patients with PFGs possibly indicating that γH2AX and 53BP1 proteins remain longer in DNA repair foci.

Statistical analysis (data not shown) did not reveal any consistent differences between leukemic patients divided into three subgroups depending on risk of disease (SRG, IRG, HRG) (Figure 5). Thus, constitutive DNA repair seems not to be a valuable endpoint for assessment of risk group.

Discussion

Accumulated DNA damage in hematopoietic cells is believed to be a principal mechanism for leukemogenesis. However, the data on DNA damage in hematopoietic cells of ALL patients has not yet been reported. Therefore, we have compared constitutive DNA damage in hematopoietic cells from ALL patients and healthy subjects in this study. We have used most sensitive technique to estimate DSBs which is based on measurement of DNA repair foci using antibodies to γH2AX and 53BP1. Constitutive DNA repair foci seems to be an appropriate endpoint for genetic instability in cancer cells of various origin including leukemia, because cancer cell lines with higher level of constitutive γH2AX foci exhibit more chromosomal aberrations than those with fewer constitutive foci [19] and leukemic cells are also prone to chromosomal rearrangements [31]. We report here that constitutive γH2AX/53BP1 foci are more often formed in hematopoietic cells of ALL patients as compared to healthy subjects. Both molecular markers, γH2AX and 53BP1, and their co-localization have consistently shown statistically significantly increased constitutive level of DSBs in hematopoietic cells of pediatric ALL patients. This data are in line with results showing increased constitutive DNA damage in variety of cancer cell lines [19, 32] and tumors [16, 33] by analyzing constitutive γH2AX. Similarly, normal cells express less 53BP1 constitutive foci than variety of cancer cells [34, 35].

One of the key event in leukemogenesis is formation of a specific chromosomal translocations and correspondent PGFs [31]. Because formation of chromosomal translocations/PGFs requires DSBs, it may be hypothesized that hematopoi-

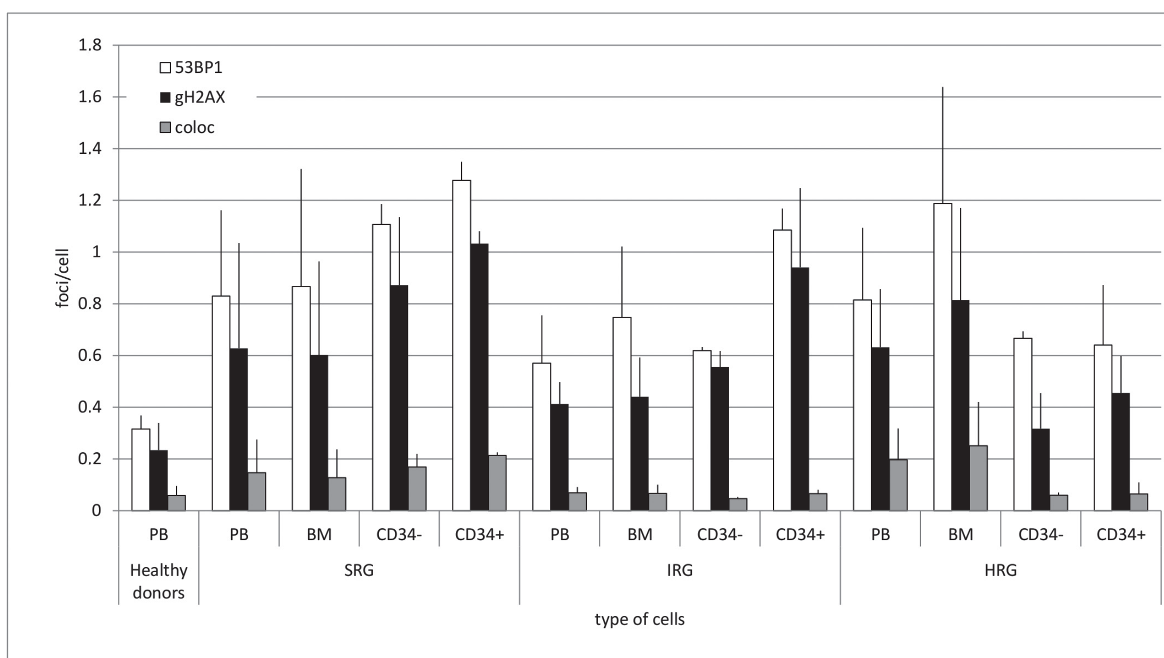

Figure 5. Comparison of leukemic patients according to risk of disease. Mean values and SD are shown for leukemic patients divided into three groups according to risk of disease: six SRG, four IRG, and four HRG patients.

etic cells from leukemic patients with specific PGF would have increased level of constitutive DSBs. We verified this hypothesis by comparing constitutive DSBs in cells from pediatric ALL patients with and without PGFs. All measured endpoints, namely γH2AX, 53BP1, and γH2AX/53BP1 co-localization have consistently shown increased level of constitutive DNA damage in ALL patient's cells harboring either TEL-AML1 or BCR-ABL gene fusions. To our knowledge, this is the first evidence demonstrating increased constitutive γH2AX/53BP1 foci in primary ALL patient's cells harboring PGFs. This data suggest increased genomic instability in leukemic hematopoietic cells with PGFs. Comparison of data between groups of patients with either TEL-AML1 or BCR-ABL has not shown statistically significant difference suggesting similar effects of these PGFs on genomic instability in hematopoietic cells. It is widely accepted that constitutive DNA damage can occur as a result of endogenous reactive oxygen species (ROS) produced during metabolism. Increased ROS may underlie the increased level of constitutive DSBs in variety of leukemic patients including ALL [36-39]. Recent study has shown that BCR-ABL-transformed cell lines, and also leukemia stem cells contained 2–6 times more ROS in comparison with their normal counterparts [40]. It is likely that BCR-ABL kinase enhances oxidative DNA damage and stimulates genomic instability by inhibiting uracil DNA glycosylase UNG2 [41]. Our data on increased DSB levels in the BCR-ABL patient's cells support a model where BCR-ABL induces DNA instability through oxidative DNA damage resulting in DSBs and thereby facilitating progression of leukemia [17].

ETV6/TEL-RUNX1/AML1 expression was also associated with elevated endogenous ROS levels and an increase in DSBs in transgenic mouse model where the expression of the fusion protein is restricted to CD19+ B cells [24]. Our data represent first experimental evidence for increased DSB levels in the TEL-AML1 patient's cells supporting a model where TEL-AML1 induces DNA instability through ROS deregulation, thereby facilitating mutagenesis and the appearance of additional genetic alterations driving leukemogenesis [24].

Our data have also provided evidence that the level of constitutive DSBs is the same in PBL and BM cells (Figure 2, 3). CD34- and CD34- cells did not differ in constitutive damage either (Figure 2, 3). Thus, the increased level of constitutive DNA damage is observed in hematopoietic cells of pediatric ALL patients versus healthy subjects, or PGF-harboring patients versus patients without PGFs regardless cell type suggesting that increased genetic instability is generated in whole hematopoietic system including bone marrow and peripheral blood.

The authors are aware that the group of patients should be increased and more endpoints should be followed for better description of the proposed correlation between PGFs and increased constitutive DNA damage in cells from ALL pediatric patients.

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