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Treatment of lymphoid cells with the topoisomerase II poison etoposide leads to an increased juxtaposition of *AML1* and *ETO* genes on the surface of nucleoli

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AML1 and ETO genes are known partners in the t(8,21) translocation associated with the treatment-related leukaemias in the patients receiving chemotherapy with DNA-topoisomerase II (topo II) poisons. ***Aim****. To determine whether the genes AML1 and ETO are in close proximity either permanently or temporarily in the nucleus.* ***Methods****. 3D FISH.* ***Results****. We found that in 5 % of untreated cells, alleles of AML1 and ETO are in close proximity. This number increased two-fold in the cells treated with the topo II poison etoposide. Surprisingly, in more than 50 % of the cases observed, co-localization of the genes occurred at the nucleoli surface. We found also that the treatment of cells triggers preferential loading of RAD51 onto bcr of the AML1 and ETO genes.* ***Conclusions****. Our results suggest that the repair of DNA lesions introduced by topoisomerase II poisons may be mediated simultaneously by multiple mechanisms, which may be the cause of mistakes resulting in translocations.*

Keywords: DNA-topoisomerase II, nucleoli, Rad51, AML1, ETO.

Introduction. Chromosomal translocations are a characteristic feature of leukaemia, and they are the underlying cause in a number of cases. Translocations are a consequence of the faulty repair of double-stranded DNA breaks (DSBs) originating in the course of various physiological and pathological processes. As a rule, leukaemogenic translocations affect key regulators of hemopoiesis and result in the formation of chimeric genes. In some cases, oncogenesis is caused by the products of these particular chimeric genes [1].

For the translocation between gene partners, certain conditions must be satisfied. These conditions concern both the localization of recombination partners in the nuclear space and the mode of action of DSB repair mechanisms. First of all, the direct contact between recombination partners should be possible and even probable. To fulfil this condition, the recombination partners should be located close to each other in the nuclear space either permanently or for a considerable time interval. The latter may happen when the genes are temporarily attracted to the same nuclear compartment, such as a common transcription factory [2], PML bodies [3], or a hormone receptor [4]. Localization of a pair of genes in the vicinity of the nucleolus may increase the probability of illegitimate recombination between these genes. The nucleolus appears to

be a storage place for many proteins that participate in DNA repair [5, 6]; moreover, the process of sliding along the nucleolus surface may help the heterologous DNA ends, generated as a result of double-strand breaks, to contact one another. Alternatively, rapprochement between distantly located genes could be a result of fundamental changes in nuclear architecture that may occur under some special conditions. However, simple rapprochement of genes in the nuclear space does not result in translocation. The presence of a DSB and physical contact between broken DNA ends appears to be absolutely necessary. Furthermore, the translocation partners should be damaged simultaneously or an unrepaired DSB should persist in the nucleus for a significant period of time. This increases the probability of contact between heterologous DNA ends.

A DNA DSB may be repaired by two different mechanisms [7]. In the post-replication stage, the most effective mechanism is the homologous recombination (HR). HR is normally considered to be an error-free system because the undamaged sister chromatid provides a perfect donor of homology [8]. In contrast to HR, the non-homologous end joining (NHEJ) system mediates direct ligation of DNA ends originating as a result of DSBs without looking for a homology donor. This system, which appears to be the main system of DSB repair in higher eukaryotes, is an error-prone system [9]. DSB repair by NHEJ often leads both to insignificant «misprints» in the immediate proximity of the breakpoint and to translocations of extended DNA regions due to the joining of «wrong» DNA ends [10]. Rapid disconnection of damaged DNA ends resulting in their separation within the nuclear space appears to be a condition for the subsequent joining of incorrect ends and therefore for a translocation event.

The translocation t(8,21) between loci containing the genes *AML1* and *ETO* is associated with the development of acute myeloid leukaemia [11]. That is especially characteristic for secondary leukaemias (*t-ANLL*, treatment-related leukaemia) originating as a consequence of anti-tumour chemotherapy with inhibitors of topo II. DSBs originating under the action of topo II poisons are distributed non-randomly in the sequences of translocation partners, and are grouped into so-called breakpoint cluster regions – bcr [12]. The translocations leading to the formation of chimeric genes, the products of which cause malignant transformation and t-ANNL, occur predominantly between bcr of different genes [13].

The aim of the present work was to determine whether the genes *AML1* and *ETO* are in close proximity either permanently or temporarily in the nucleus. To this end, we assessed the degree of mutual localization between these genes and their proximity to the nucleolus in both normal cells and cells treated with the topo II poison etoposide. In addition, we studied the distribution of repair proteins that may help to establish and maintain contact between heterologous DNA ends and thereby promote the recombination between *AML1* and *ETO*.

Materials and methods. *Cell culture and slide preparation*. A culture of Jurkat cells was received from the Institute of Medical Genetics RAMS. The cells were grown in RPMI with 10 % FBS at 37 °C in a 5 % CO_2. In the experiments with etoposide, the Jurkat cells were incubated for 1.5 h in the presence of 0.17 mM etoposide. Cells were attached to slides using Cell-Tak™ («BD Bioscience»). The slides were incubated for 1 min in 0.3 × PBS, fixed immediately in 4 % paraformaldehyde in 0.3 × PBS (pH 7.4) for 10 min at RT and washed in 1 × PBS. The cells were permeabilized at RT in 0.5 % (w/v) Triton X-100 in 1 × PBS for 10 min, incubated for 1 h in 20 % (v/v) glycerol in 1 × PBS followed by freezing-thawing in liquid nitrogen and washing in 1 × PBS. Cells were treated with 0.1 M HCl for 20 min at RT, washed in 1 × PBS, treated with RNAseA in 2 × SSC (200 μg/ml) for 30 min at 37 °C, washed with 2 × SSC and equilibrated in 50 % (v/v) deionized formamide in 2 × SSC for at least 2 h. The prepared slides were used immediately or stored at 4 °C for up to two weeks.

Visualization of the AML1 and ETO genes and nucleoli via 3D fluorescence in situ hybridization (3D FISH). The DNA in cells fixed on microscope slides was denatured in 70 % (v/v) deionized formamide in 2 × × SSC for 16 min at 75°C. One microliter of Vysis® LSI® *AML1*/*ETO* Dual Color, Dual Fusion Translocation Probe («Abbott Lab.») was mixed with 4 μl of Vysis® hybridization buffer, and the mixture was incubated for 5 min at 74 °C. Then the mixture was applied to the hot slides, and hybridization was carried out overnight at 37 °C (in a humid chamber).Then the samples were washed for 30 min in 50 % (v/v) formamide in 2 × SSC at 52 °C, for 10 min in 0.2 × SSC at 52 °C, for 5 min in 0.1 % (v/v) NP-40 in 2 × SSC at 52 °C and finally for 3 min in 2 × SSC at RT.

To visualize nucleoli, mouse monoclonal anti-nucleophosmin antibody («Chemicon Int.») was used with

subsequent signal acquisition via an Alexa 647-conjugated chicken anti-mouse antibody («Mol. Probes»). In all cases, DNA was counterstained with DAPI.

Immunostaining. Cells were precipitated to microscope slides using cytospin (0.5 million cells per slide), fixed (1 % paraformaldehyde, 2.5 % Triton-X100, 10 mM Pipes (pH 6.9), 100 mM NaCl, 1.5 mM $MgCl_2$, 300 mM Sucrose) for 20 min at RT and washed in 1 × × PBS. Then treated with blocking reagent solution (BR, «Roche») for 1 h at RT and incubated with primary antibody (dilution 1:50 (v/v) in BR) overnight at 4 °C. Then, the preparations were washed in washing solution (1 × PBS, 0.25 % BSA, 0.05 % Tween 20), incubated with secondary antibody (dilution 1:200 (v/v) in BR) for 30 min at RT, washed in washing solution, rinsed with 1 × PBS and counterstained with DAPI. The antibodies used: mouse monoclonal anti-nucleophosmin antibody («Chemicon Int.»), mouse monoclonal anti-fibrillarin antibody (Abcam) and Alexa 488-conjugated rabbit anti-mouse antibody («Mol. Probes»).

Chromatin immunoprecipitation and real-time PCR. Chromatin immunoprecipitation was performed as described previously [14]. Rabbit anti-nucleolin antibody («Sigma») and rabbit anti-Rad51 antibody («Sigma») were used. The amount of DNA bound to nucleolin or to Rad51 before and after etoposide treatment was determined by real-time PCR with the following primers and TaqMan probes (FAM – a fluorescent dye at the 5' end of samples; BHQ-1 – fluorescence quencher in the probe on T):

AML1_BCR3_direct: 5'CCAGCCCACAACAGG AGAC3'; AML1_BCR3_reverse: 5'ATACTTCTGAG GGAAAGGGATG3;

AML1_exon_5_direct: 5'TTCCTGCCTTCATTC TCTGC3; AML1_exon_5_reverse: 5'TGTCCCCAAA AGCCAAGAT3';

ETO_BCR2_direct: 5'TCTGATAGTGCCAATG CCTTTA3'; ETO_BCR2_reverse: 5'CTTGCTAGTG CCTATGTAGGAATCT3';

ETO_exon_1a_direct: 5'GCATCCTTGAATCCA GCGTA3'; ETO_exon_1a_reverse: 5'CCTCCACATT TCTGCTCCAA3';

AML1_BCR3: 5'(FAM)CGCAAACAGCCT (BH Q-1)GAGTCACGCA3'; AML1_exon_5: 5'(FAM)AA GAGGAAAGT(BHQ-1)GAGGTTCAGCAAGGC3';

ETO_BCR2: 5'(FAM)TTCATTTCCACCAACT (BHQ-1)TATTTTCAACGTCT3'; ETO_exon_1a: 5'(F AM)TGGAATGAGT(BHQ-1)GGCAGCAGAGAGG A3'.

Amplification was performed in 20 μl of PCR buffer (50 mM Tris (pH 8.6), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.1 % Tween 20) containing test DNA, 0.5 μM each primer, 0.25 μM TaqMan probe, 0.2 mM each dNTP and 0.75 units of hot-start Taq DNA polymerase («Sibenzyme»). Conditions: at 94 °C for 5 min (one cycle); and 94 °C for 15 s, 60 °C for 60 s, fluorescence detection (49 cycles). The results were analysed using Opticon Monitor 3.1 («Bio-Rad Lab»).

Results and discussion. *Analysis of the mutual localization of the AML1 and ETO genes in the nucleus*. We first studied the mutual localization of the *AML1* and *ETO* genes in nuclear space in cells under normal conditions and in cells treated with the topo II poison etoposide. The 3D immuno-FISH technique was employed in these experiments using commercially available fluorescent probes, regularly used in clinical practice for analysis and diagnosis of t(8;21) translocations in leukaemia. We were particularly interested in enumerating the cases where *AML1* and *ETO* loci are in contact with each other. In addition, the relative positions of these loci with respect to the nucleolus were registered.

The FISH results were analysed using an LSM 510 («Carl Zeiss, Inc.») confocal microscope. Confocal sections of the cells under study were processed using ZEN 2009 LE software («Carl Zeiss, Inc.») to make 3D reconstructions. Typical examples of the 3D reconstructions showing various juxtapositions of the *AML1* and *ETO* genes together with the nucleolus are presented in Fig. 1, *A–C* (see inset). In Fig. 1, *A*, the situation is shown where one of the *AML1* alleles is located very close to one of the *ETO* alleles (the signals touch each other but do not overlap). In Fig. 1, *B*, the situation is shown where *AML1* and *ETO* alleles touch each other and the surface of the nucleolus. Finally, in Fig. 1, *C*, the situation is shown where all the *AML1* and *ETO* alleles are located at a considerable distance from one another. The results of an analysis are presented in a table (Fig. 1, *D*, see inset).

To characterize the mutual localization of these genes, we used the open-source software NEMO (an upgraded version of the well known ImageJ plug-in «Smart 3D-FISH»). NEMO is designed to detect automatically various objects (e. g., spots, genes, nuclei or chromosomal regions) located in individual cells and to analyse the distances between these objects [15]. We defined hybridization signals as being in close vicinity (or in contact as shown in Fig. 1, *A1*) in cases where the

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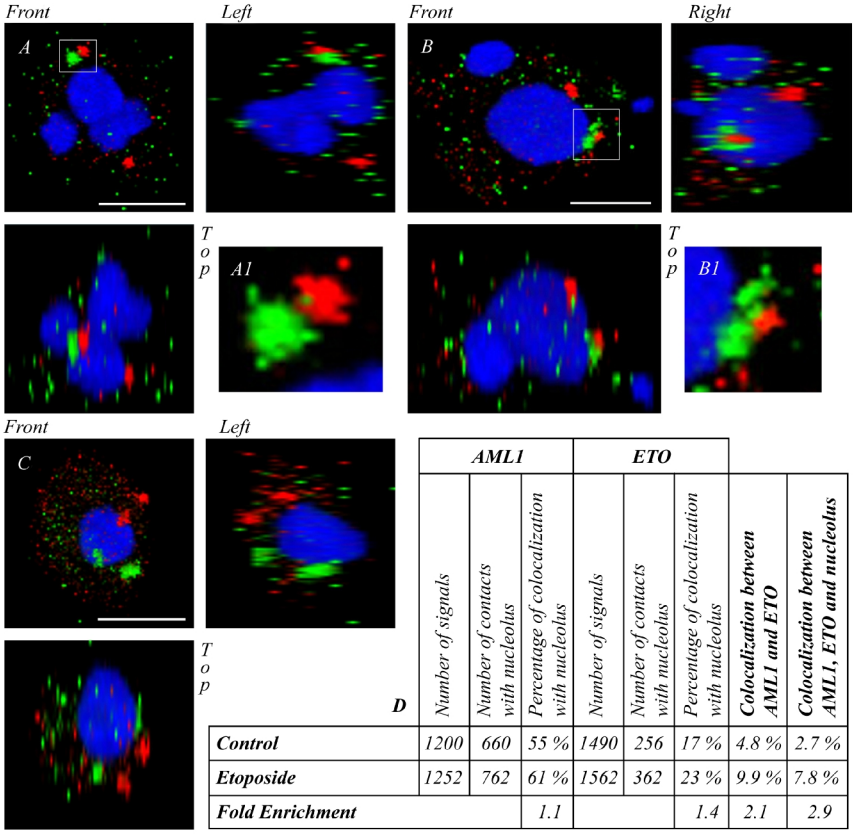

D

	AML1			ETO				
	Number of signals	*Number of contacts with nucleolus*	*Percentage of colocalization with nucleolus*	*Number of signals*	*Number of contacts with nucleolus*	*Percentage of colocalization with nucleolus*	***Colocalization between AML1 and ETO***	***Colocalization between AML1, ETO and nucleolus***
Control	*1200*	*660*	*55 %*	*1490*	*256*	*17 %*	*4.8 %*	*2.7 %*
Etoposide	*1252*	*762*	*61 %*	*1562*	*362*	*23 %*	*9.9 %*	*7.8 %*
Fold Enrichment			*1.1*			*1.4*	*2.1*	*2.9*

Fig. 1. Three distinct juxtapositions observed during mutual localization analysis of the genes AML1 and ETO and nucleoli inside Jurkat cell nuclei. Blue – nucleoli immunostained for nucleophosmin (B23), green and red – hybridization signals corresponding to AML1 and ETO genes, respectively. Bar: 5 μm. For each case, three projections of the 3D model are shown. *A* – AML1 and ETO are in contact with each other but not with a nucleolus; *B* – AML1 and ETO are in contact with each other and with a nucleolus (*A1*, *B1* – scaled-up excisions); *C* – AML1 and ETO are distant from each other; *D* – results of signal counting. In the line designated «Etoposide» the results of counting signals in etoposide-treated cells are presented. Given results represent an aggregated values obtained in three independent experiments having total samplings indicated in «Number of signals» column. All the values expressed as a percentage possess standard error (SE) of less than 0.85 %

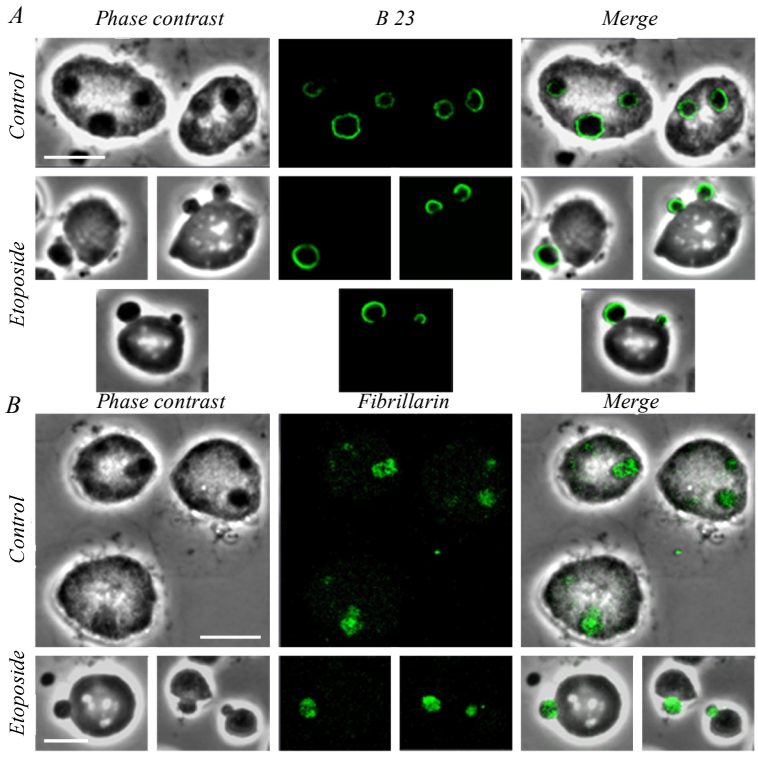

Fig. 2. Immunostaining of Jurkat cells using antibodies against nucleolin (*A*) and fibrillarin (*B*) is shown. Nucleolar extrusion from the nuclei of cells treated with etoposide (Etoposide) is apparent. Staining of nucleoli with the same antibodies in non-treated cells (Control) is shown for a comparison. Bar: 5 μm

estimated NEMO «colocalization percentage» (which the authors define as «the percentage of the smallest object into the biggest object colocalization») was more than 1 %. To this end it should be mentioned that we have never observed significant overlapping of the *AML1* and *ETO* signals. In our case it has never exceeded 5 %. In spite of its peri-telomeric position in chromosome 21, at least one AML1 signal was located at the surface of one of the nucleoli in 55 % of cells. An analysis of the spatial distribution showed that almost 5 % of all the *AML1* and *ETO* hybridization signals were located in close proximity (Fig. 1, *A1*). Furthermore, almost half of the juxtaposed *AML1* and *ETO* signals were located at the border of the nucleolus (Fig. 1, *B1*).

We demonstrated previously that treatment of primary human fibroblasts with etoposide causes relocation of the *ETO* gene in nuclear space [16]. As a result of this relocation, the average distance between *AML1* and *ETO* alleles decreased. However, in this previous work, we did not observe the cases in which *AML1* and *ETO* alleles were in direct contact. Perhaps this does not happen in primary fibroblasts. In the present work, we performed the same analysis using human lymphoid cells (Jurkat). Remarkable rapprochement of the *AML1* and *ETO* genes occurred after treatment of the cells with etoposide. The percentage of juxtaposed *AML1* and *ETO* signals doubled as a result of etoposide treatment, reaching almost 10 % of the total number of calculated signals (Fig. 1, *D*). The number of juxtaposed *AML1* and *ETO* alleles located on the surface of nucleoli increased approximately three-fold (Fig. 1, *D*).

Role of the nucleolus in the rapprochement of the AML1 and ETO genes induced by etoposide. In humans, *AML1* is located on chromosome 21, which harbours a nucleolar organizer. Thus, the relocation of *AML1* in nuclear space may be a consequence of nucleolar relocation. In this regard, it may be of importance that treating cells with etoposide causes the extrusion of the nucleolus beyond the border of the nucleus, as shown in Fig. 2 (see inset). We treated Jurkat cells with etoposide and then fixed and stained them with antibodies against nucleophosmin and fibrillarin. This revealed that nucleoli are extruded from the cells treated with etoposide. The nucleolar extrusion is likely to occur when the apoptotic program is triggered. However, reversion is still likely to be possible during the initial steps of this program. To this end, it should be noted that under conditions of cell treatment used in our experiments a complete extrusion of nucleoli was observed only in a very small portion of cells. In control experiment, when after 1.5 h treatments with etoposide the cells were placed in a fresh medium without etoposide, the population continued to grow (not shown). Nonetheless, the movement of nucleoli toward the nuclear periphery may constitute a driving force for the repositioning of *AML1* from the central to the peripheral part of the nucleus. It has been shown before that treatment of cells with other cytotoxic agents also causes nucleolar extrusion without separation of the nucleolus from the surrounding chromatin layer [17]. Thus, under certain conditions, the nucleolus may serve as a «carrier» that conveys proximal genes (including AML1) to the periphery, where *ETO* is predominantly located.

One of the major nucleolar proteins is nucleolin. In addition to participating in the biogenesis of ribosomes, nucleolin performs many other functions, being involved in the regulation of cell growth and proliferation, stress responses, DNA replication and repair [18]. Taking into account the fact that *AML1* and *ETO* alleles were frequently observed to be in close proximity on the nucleoli, we looked for association of nucleolin with the bcr of *AML1* and *ETO*.

Using the chromatin IP approach, we found that nucleolin interacts not only with the bcr of both *AML1* and *ETO* but also with other regions of these genes. Moreover, the degree of association between nucleolin and both *AML1* and *ETO* increased two-fold in response to etoposide treatment (data not shown). One can speculate that this increased association of both *ETO* and *AML1* with nucleolin after etoposide treatment contributes to their retention at the border of the nucleolus and increases the probability that these heterologous DNA ends would interact.

Direct participation of nucleolin in the repair of DSBs (including those introduced by topo II) should not be ruled out. Nucleolin interacts with a variety of proteins that participate in the repair, replication and recombination of DNA, and it possesses helicase, strand-annealing and strand-pairing activities [18]. It has been previously shown that electroporation of antibodies against nucleolin into cells increases their sensitivity to the topoisomerase poison amsacrine [19].

Increased association of RAD51 with both AML1 and ETO in cells treated with etoposide. We have previously demonstrated that stalled topo II complexes are recognized by cells as DSB and are repaired via NHEJ [20]. Moreover, we have found that treating cells with etoposide caused the proteins mediating NHEJ to

accumulate at the bcr of both *AML1* and *ETO* [21]. DSB repair by NHEJ is accompanied by numerous mistakes that may be a cause of translocations. However, the participation of the HR pathway in the repair of DNA DSB caused by inhibitors of topo II ligation should not be ruled out. HR mechanisms can facilitate replication fork passage of the damaged region and the resumption of normal replication [22]. One of the key players in the HR repair system is RAD51 [23]. RAD51 forms a complex with DNA close to breakpoints [24].

We analysed the level of association between RAD51 and the bcr of both *AML1* and *ETO* in control cells and cells treated with etoposide. The results obtained (Fig. 3) allowed us to conclude that treatment of cells with etoposide results in a significant (up to 100 fold) increase in the levels of association between RAD51 and all the loci, and this effect was especially prominent at the bcr. Thus, HR may participate in the repair of topo II induced DSB. In this regard, it may be of interest that the bcr of AML1 and ETO possess relatively long (up to 17 bp) regions of homology that may be «considered» as homology donors by the HR repair mechanism, resulting in the joining of heterologous chromosomal fragments and the generation of a leukaemogenic chimeric gene.

Whatever the reason for the recruitment of RAD51 to bcr in the cells treated with topo II poisons, this recruitment is likely to destabilize these regions in conjunction with the topo II-mediated DSB and collapsed replication forks. Consequently, the mobilization of broken DNA ends is likely to occur there. Indeed, the normal function of RAD51 is to form a filament with single-stranded DNA and to invade undamaged homologous sister chromatids. In the conditions when recombination partners are in close proximity, their simultaneous damaging and simultaneous destabilization of breaks may increase the probability of translocations between them. A search for the homologous sequences carried out by RAD51 in complex with DNA may facilitate the rapprochement of heterologous DNA ends, and the presence of microhomologies increases the probability of translocations.

The observations presented allow one to propose a prospective way to prevent leukaemogenic translocations, in particular by using chemotherapeutic agents which does not lead to significant changes in nuclear architecture. In case of topo II poisons, it should be those that do not cause accumulation of topo II stalled complexes.

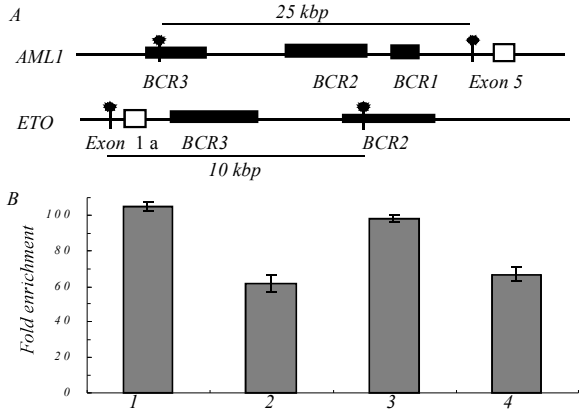

Fig. 3. Increased association of RAD51 with *AML1* and *ETO* after treatment of cells with etoposide: *A* – map of the genomic regions under study showing positions of test amplicons (vertical lines with asterisks); *B* – a diagram illustrating the results of ChIP with antibodies against Rad51 (fold-enrichment of DNA fragments containing test-amplicons in immunoprecipitated material obtained from cells treated with etoposide relative to that in material from untrea ted cells). 1-AML1-BCR3: 2-AMl1-exon5 3-ETO-BCR2 4-ETO-exon1a. Bars represent the standard deviation

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Обробка лімфоїдних клітин інгібітором топоізомерази II етопозитом спричиняє зростання колокалізації генів *AML1* і *ETO* на поверхні ядерця

Резюме

Гени AML1 і ETO відомі як партнери по транслокації t(8,21), асоційованої з розвитком вторинних лейкозів у пацієнтів, які піддавалися хіміотерапії із застосуванням інгібіторів топоізомерази II. ***Мета****. Оцінити частоту взаємної колокалізації генів AML1 і ETO у культурі лімфоїдних клітин людини.* ***Методи****. 3D FISH.* ***Результати****. У 5 % необроблених клітин лінії Jurkat алелі AML1 і ETO знаходяться в безпосередній близькості один від одного. У клітинах, оброблених інгібітором топоізомерази II етопозитом, частота подій колокалізації AML1 і ETO зростає в два рази. При цьому більш ніж у 50 % випадків колокалізація генів відбувається на поверхні ядерця. Показано, что обробка клітин етопозидом спричиняє посилення зв'язування білка RAD 51 з кластерами точок розриву (bcr) генів AML1 і ETO.* ***Висновки****. Репарація розривів ДНК, індукованих інгібіторами топоізомерази II, вірогідна за одночасної участі різних механізмів, що може бути причиною помилок, які викликають транслокації.*

Ключові слова: ДНК-топоізомераза II, ядерця, Rad51, AML1, ETO.

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Обработка лимфоидных клеток ингибитором топоизомеразы II этопозитом приводит к возрастанию колокализации генов *AML1* и *ETO* на поверхности ядрышка

Резюме

Гены AML1 и ETO известны как партнеры по транслокации t(8,21), которая ассоциирована с развитием вторичных лейкозов у пациентов, подвергшихся химиотерапии с применением ингибиторов ДНК-топоизомеразы II. ***Цель****. Оценить частоту взаимной колокализации генов AML1 и ETO в культуре лимфоидных клеток человека.* ***Методы****. 3D FISH.* ***Результаты****. В 5 % необработанных клеток линии Jurkat аллели AML1 и ETO находятся в непосредственной близости друг от друга. В клетках, обработанных ингибитором ДНК-топоизомеразы II этопозидом, частота событий колокализации AML1 и ETO увеличивается в два раза. При этом в более чем 50 % наблюдаемых случаев колокализация генов происходит на поверхности ядрышка. Показано, что обработка клеток этопозидом приводит к увеличению связывания белка RAD 51 с кластерами точек разрыва (bcr) генов AML1 и ETO.* ***Выводы****. Репарация разрывов ДНК, индуцированных ингибиторами ДНК-топоизомеразы II, вероятна при одновременном участии различных механизмов, что может являться причиной ошибок, приводящих к транслокациям.*

Ключевые слова: ДНК-топоизомераза II, ядрышка, Rad51, AML1, ETO.

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