Exp Oncol 2008
30, 3, 230–234

Experimental ONCOLOGY

ALTERATIONS OF CONSTITUTIVE PERICENTROMERIC HETEROCHROMATIN IN LYMPHOCYTES OF CANCER PATIENTS AND LYMPHOCYTES EXPOSED TO 5-AZACYTIDINE IS ASSOCIATED WITH DNA HYPOMETHYLATION

*L.P. Shvachko**

Department of Molecular Genetics, Institute of Molecular Biology and Genetics of NAS of Ukraine, 03143 Kiev, Ukraine

Background: **DNA hypomethylation plays a key role in carcinogenesis. The malignant transformation of cells as well as tumor progression is accompanied with increasing DNA hypomethylation in cancer cells. Nevertheless, the evolution of dis-epigenetic genomic alteration in the somatic cellular malignant transformation has not yet been clear.** ***Aim:*** **To study the relationship between the pattern of genomic DNA hypomethylation and DNA hyperploidy.** ***Methods:*** **The model of 5-azacytidine demethylating DNA treatment of the mitogen-stimulated lymphocytes in parallel with patients with solid cancer has been explored. DNA content was measured by quantitative DAPI and Hoechst 33258 fluorescence *in situ* hybridization on interphase nuclei. The conventional mitogen-stimulated blood lymphocyte culture development was performed in metaphase chromosomes and interphase nuclei assay. The light and fluorescent cytomorphological microscopy was performed.** ***Results:*** **The model 5-azacytidine induced DNA demethylation results in increased DNA hyperploidy accompanying major pericentromeric heterochromatin (Alu) DNA repeats amplification similar to those during DNA hypomethylation-associated cancer events, and both contributed to nuclear heteropycnosis development. The constitutive pericentromeric heterochromatin consequent morphological disturbance to the latent polytene chromomerization and heteropycnosis development both in cancer patients and in model 5-azacytidine exposured lymphocytes are associated whith DNA hypomethylation.** ***Conclusion:*** **We have observed that the induced global DNA hypomethylation triggers dis-epigenetic morphological reprogramming of constitutive pericentromeric heterochromatin on the extrachromosomal organization pathway as seen during the heterochromatin latent polytene features development, which is of importance as one of the mechanism involving DNA hypomethylation in initiation and progression of cancer.**

Key Words: **DNA hypomethylation, 5-azacytidine, latent polyteny, constitutive pericentromeric heterochromatin.**

The alterations in DNA methylation patterns [1–3], in particular the pattern of the global genomic DNA hypomethylation [4–6] and genomic regional CpG-promoter DNA hypermethylation of the tumor suppressor genes [7–10] are the characteristic general features of carcinogenesis in various models [11] focusing on the importance of epigenetic events in oncogenic transformation and tumor progression [12–17].

Genome-wide DNA hypomethylation is a consistent finding in human tumors, but the importance of this change for human tumorigenesis remains an open question. The biological significance of DNA hypomethylation in carcinogenesis is far from being clearly elucidated in details. In particular, the mechanisms of genomic instability caused by hypomethylation remain unclear. This hypomethylation of the genome largely affects the intergenic and intronic regions of the DNA, particularly repeat sequences and transposable elements [18], and is believed to result in chromosomal instability and increased mutation events [19]. Previously, we have revealed the association between the aberrant pattern of genomic DNA hypomethylation in peripheral blood lymphocytes of the patients with various solid cancers and the increase in genomic DNA content (hyperploidy); the same effects have been evident upon 5-azacytidine treatment in the culture of normal lymphocytes [20]. The aim of the present study was to analyze the association between genomic DNA hypomethylation in peripheral blood lymphocytes of cancer patients and normal lymphocytes treated with 5-azacytidine and the alterations in chromatin organization in these cells. We suggest that the induced global DNA hypomethylation triggers dis-epigenetic morphological reprogramming of constitutive pericentromeric heterochromatin on the extrachromosomal organization pathway. This may be one of the mechanisms involving DNA hypomethylation in initiation and progression of cancer.

Received: July 2, 2008.

*Correspondence: E-mail: l.p.shvachko@imbg.org.ua

MATERIALS AND METHODS

The peripheral blood lymphocytes were obtained from blood samples taken from the patients with solid tumors: (thyroid gland cancer, n = 35; colorectal cancer, n = 26; breast cancer, n = 5; neuroblastoma, n = 8; Wilms tumor, n = 6). Lymphocytes from healthy donors were used as a control (n = 20). The permission from Bioethyc committee concerning the investigations conduction has been obtained.

The satellite DIG-pUC(Alu)- DNA repeats dot-hybridization with cancer-associated genomic DNA hypomethylation and 5-azacytydine exposed genomic DNA was performed. The mitogen-stimulated peripheral blood lymphocytes were cultured in RPM1640 medium supplemented with 10% fetal serum. 5-Azacytidine used as DNA demethylation reagent was added at the correct concentration of 10^{-5} M for 72 h.

Cytomorphological study of metaphase chromosomes (n = 500, $P < 0.001$) and interphase nuclear lymphocytes (n = 500, $P < 0.001$) was conducted

using light and fluorescent microscopy with Giemsa, DAPI, and Hoechst 33258 staining, using microscope "Axiotar FL" (ZEISS, Germany) and "Scion Image" program (ZEISS, Germany).

The statistic Program "Origin 6.1" was used to analyze the data obtained [20].

RESULTS AND DISCUSSION

As shown in Fig. 1, DNA content in the peripheral blood lymphocytes of the patients with various histological forms (n = 5, thyroid cancer, colorectal cancer, breast cancer, neuroblastoma and Wilms tumor) of cancer increases similarly to that in the lymphocytes of the healthy donors (n = 5) cultured in the presence of 5-azacytidine (10^{-5} M) as DNA demethylation reagent (Table 1). Simultaneously, the targeted major pericentromeric (Alu)-DNA repeats amplification in genomic lymphocyte DNA of cancer patients as well as in 5-azacytidine exposed cultured healthy lymphocyte DNA have been revealed (Fig. 2, *a*, *b*). This suggests that pericentromeric/centromeric Alu-repeats amplification feature is the sensitive molecular sensor associating with targeted genomic DNA demethylation and evidently contributs to nuclei pericentromeric heterochromatinization both in cancer patients and upon experimental 5-azacytidine exposed lymphocytes.

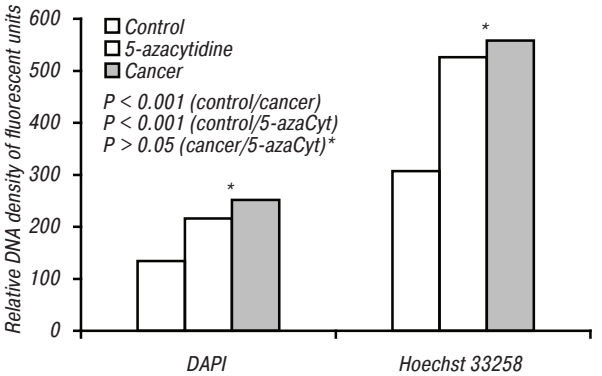


Fig. 1. The increase in nuclear DNA content of interphase peripheral blood lymphocytes from the patients with solid cancer (red), healthy (gray) and healthy lymphocytes after 5-azacytidine treatment (stroke) using DAPI and Hoechst 33258 staining
*No difference.

Table 1. The increasing heterochromatin fluorescense of the interphasic lymphocytes from solid cancer patients (A) and upon 5-azacytidine treatment (B) using DAPI and Hoechst staining DAPI

Variant	M ± m	> % (C)	*P*
Control (C)	134 ± 11.86; n = 5		*P1* < 0.001
Cancer (A)	252 ± 32.08; n = 5	88.06 ± 7.04	*P2* < 0.001
5-AzaCyt (B)	216 ± 19.32; n = 5	61,19 ± 4,88	*P3* > 0.05 (t = 0.961)
	Hoechst 33258		
	M ± m	> % (C)	*P*
Control (C)	307 ± 21.34; n = 5		*P1* < 0.005
Cancer (A)	558 ± 47.13; n = 5	81.75 ± 5.32	*P2* < 0.005
5-AzaCyt (B)	526 ± 36.95; n = 5	71. 3 ± 6.85	*P3* > 0.05 (t = 0.021)

Notes: 500 lymphocytes from every person were analysed. *P1* – *P* value between (C) and (A); *P2* – *P* value between (C) and (B); *P3* – no difference between (A) and (B).

The analysis of heterochromatin features in the metaphase chromosomes of the blood lymphocytes taken from the patients with solid cancer (Table 2) demonstrated the crucial decondensation of constitutive pericentromeric/centromeric heterochromatin (Fig. 3, *a*). At the same time, we have found the exact association of the decondensed pericentromeric heterochromatin regions with their morphological abnormality, in particular the latent polytene chromomerization events in the metaphase chromosomes of the peripheral blood lymphocytes of cancer patients.

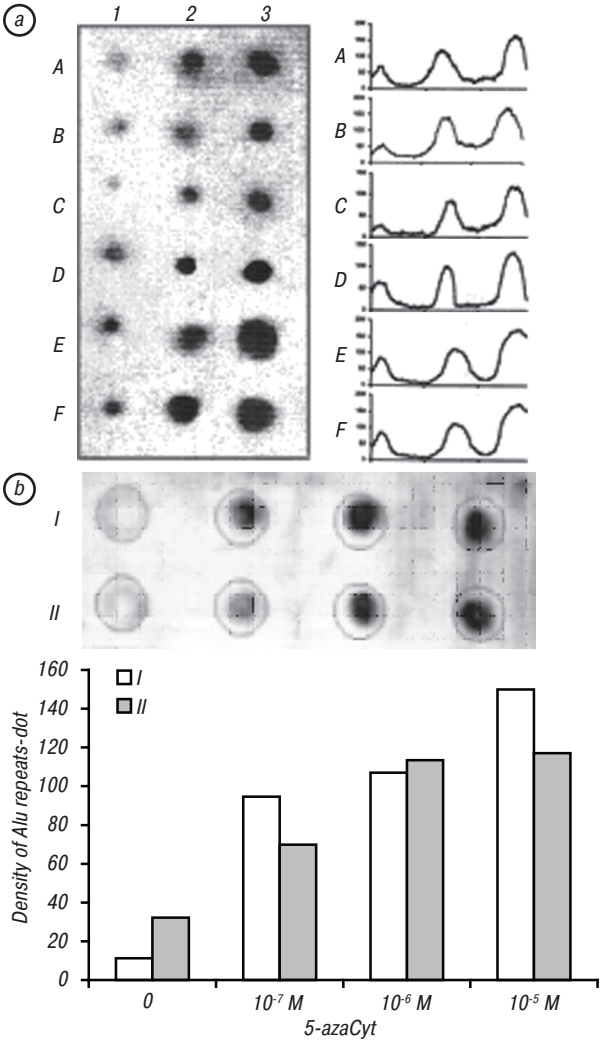


Fig. 2. Dot-DIG (Alu)-repeats amplification in cancer patients (*a*) and in cultured healthy lymphocytes in the presence of different concentrations of 5-azacytidine (*b*). DIG-pUC(ALU) was used as a probe. *a*, 1 — DNA from healthy lymphocytes; 2 — the embryonic DNA; 3 — DNA from the solid cancer patient lymphocytes; A — neuroblastoma; B — breast cancer; C — Ewing sarcoma; E — colorectal cancer; F — thyroid gland cancer. *b*, I — 50 ng lymphocyte DNA; II — 25 ng lymphocyte DNA

Table 2. The crucial patterns of mitotic pericentromeric heterochromatin in cancer patients lymphocytes

Type of tumors	Metaphase decondensation heteropycnisis	Metaphase latent chromomerization	Interphase nuclei
Thyroid gland cancer (n = 35)	++++	+++	+++
Colorectal cancer (n = 26)	++++	+++	+++
Breast cancer (n = 5)	++++	+++	+++
Children`s neuroblastoma (n = 8)	++++	+++	++
Children's Wilms tumor (n = 6)	++++	++	++
Control (n = 20)	–	–	–

Notes: 500 metaphases and 500 interphasic nuclei were investigated from each patient. [+; +++++] – the relative cytomorphological heterochromatin phenotype index.

Fig. 3, *b*, 1 demonstrates typical chromomerization of pericentromeric/centromeric heterochromatin at the metaphase stage of the patient with the advanced thyroid cancer. The cancer-associated latent polyteny of pericentromeric/centromeric heterochromatin regions also ap-

peared in its distinctive tendency to bilateral ectopic contacts between metaphase sister chromatids (Fig. 3, *b*, 2).

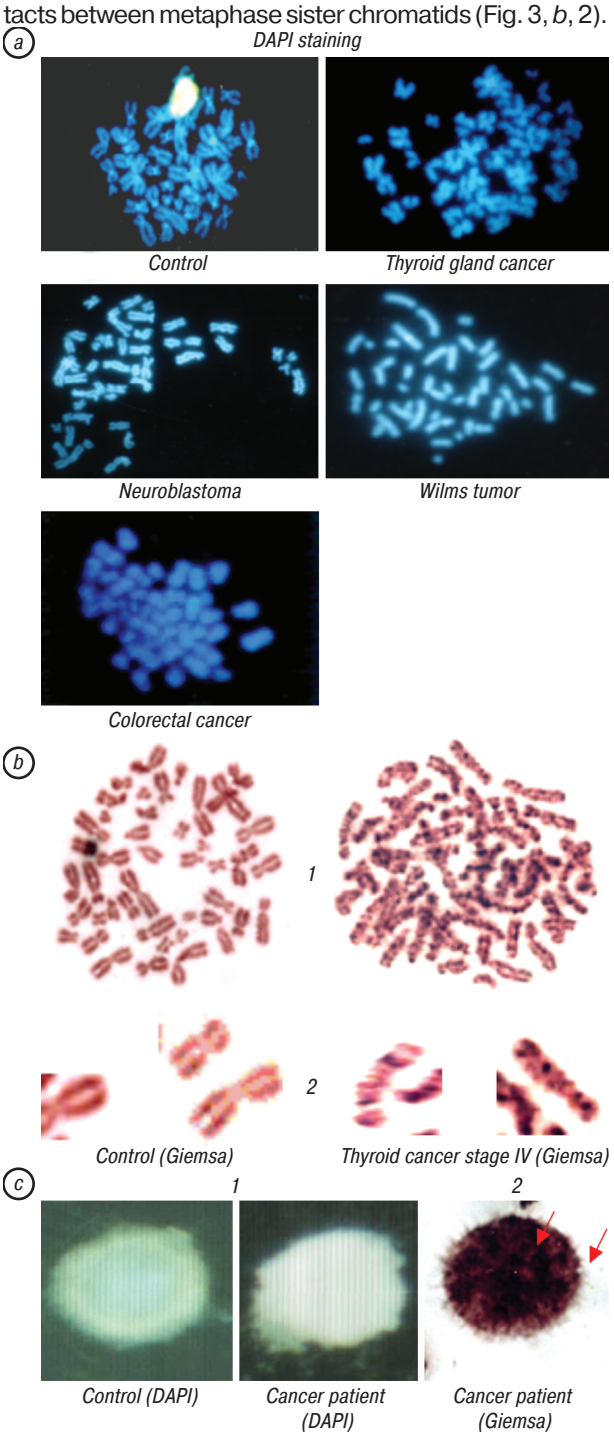
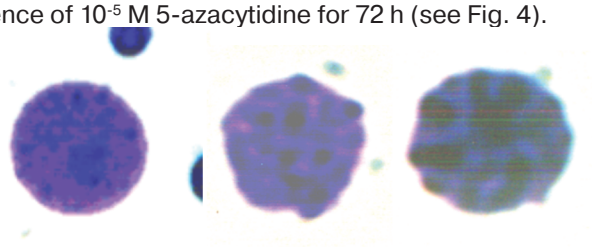


Fig. 3. *a,* Decondensation of pericentromeric/centromeric heterochromatin in metaphase chromosomes of the blood lymphocytes taken from the patients with solid cancers. *b*, 1 — the latent polytene chromomerization of pericentromeric heterochromatin of metaphase chromosomes taken from the blood lymphocytes of patient with the thyroid cancer stage IV. 2 — the bilateral ectopic conjugation of chromomeric heterochromatin regions between the sister chromatids. *C*, 1 — cancer-associated nuclear heteropycnosis of constitutive interphasic heterochromatin by DAPI staining. 2 — interphasic nuclear polytene chromomerization of constitutive heterochromatin with α-dense and β-looping heterochromatin formation by Giemsa staining

Cytomorphological analyses of DAPI and Giemsa stained cells has shown that the development of heteropycnosis in interphasic nuclei of lymphocytes is evident both in cancer patients (Fig. 3, *c*,1, 2) and upon exposure of the normal mitogen-stimulated lymphocytes to 5-azacytidine as demethylating agent (Fig. 4). In the case of the lymphocytes of cancer patients, the interphasic nuclear heterochromatinization comprises the formation of massive heterochromatinic granules, chromocenters, composed of dense α-heterochromatin and friable reticular β-heterochromatin (Fig. 3, *c*, 2) as distinctive elements of the latent polyteny features. At the same time, 5-azacytidine directly induced the irreversible nuclear heteropycnosis by forming massive chromocenters in normal interphasic lymphocytes, cultured in the presence of 10^{-5} M 5-azacytidine for 72 h (see Fig. 4).



Control (Giemsa) (interphasic lymphocyte of healthy donor)

Control + 5-azacytidine (Giemsa) (lymphocyte of healthy donor upon 5-azacytidine demethylation treatment)

Fig. 4. The formation of the massive chromocenters of interphasic constitutive heterochromatin induced by 5-azacytidine DNA demethylating agent in the normal lymphocytes cultured for 72 h (Giemsa staining)

We believe that the morphological alterations of pericentromeric/centromeric heterochromatin may be the evidence of the causal connection of aberrant genomic DNA hypomethylation/demethylation with the genomic latent polyteny events, as it was shown on targeted metaphase chromomerization and interphasic nuclear heteropycnosis using the overreplication mechanism of the constitutive pericentromeric heterochromatin.

We have earlier revealed that genomic hypomethylation in different solid cancers at the level of peripheral blood lymphocytes reflects the global pericentromeric (Alu) DNA satellites demethylation [20]. As shown in the present study, the hypomethylation may run in parallel to the critical decondensation of constitutive pericentromeric/centromeric heterochromatin of metaphase chromosomes representing the significant cytoepigenetic deregulation in tumor progression [21]. The association between the metaphase chromosome decondensation and distinctive chromomere organization of heterochromatin regions of lymphocytes on the one hand and hypomethylation state induced by 5-azacytidine as demethylating agent has been demonstrated also by other authors [22]. It could be suggested that genomic hypomethylation is associated with the induction of dis-epigenetic morphological constitutional pericentromeric/centromeric heterochromatin alteration as the latent polyteny events. We have observed that the characteristic morphological features of the nuclei of normal lymphocytes treated with 5-azacytidine are similar to that observed in the lymphocytes of cancer patients. It is interesting that in several studies 5-azacytidine treatment of tumor cell lines has been shown to increase their metastatic potential [23, 24].

Therefore, we have observed three discrete latent polyteny features of mitotic constitutive heterochromatin in lymphocytes of cancer patients: a) the targeted metaphase heterochromatin chromomerization; b) the ectopic conjugation events between sister chromatids in chromomerized heterochromatin regions; and c) the targeted nuclear heteropycnosis during interphasic heterochromatinization along with massive chromocenters formation.

We suppose that genomic DNA methylation implies the strategic protection from the latent polyteny "invasion" of mitotic genome with the development of the global genomic, chromosomal and genetic imbalances. Therefore, the aberrant epigenetic DNA hypomethylation may be one of the neoplastic factosr initiating the latent polyteny "invasion" of mitotic genome during the somatic cellular malignant transformation.

The formation of the friable reticular β-heterochromatin may result in the disordered gene expression and DNA replication (Fig. 5). As is well known, β-heterochromatin loops can be characterized by very active gene expression and by DNA amplification, mainly, by satellite and mobile genetic DNA sequences [25], contributing to the global mitotic disturbances leading to the somatic cellular malignant transformation. Chen T. et al. recently stated that global DNA hypomethylation in carcinogenesis leads to "mitotic catastrophe" but the details of this process have not yet been explained [26]. The induction of DNA hypomethylation may represent the molecular basis of the progressive latent polyteny events of pericentromeric/centromeric heterochromatin, which may be regarded as one of the factors contributing cancer progression. The findings might be important regarding the potential use of the substances preventing DNA hypomethylation for the therapy of the malignancies [27, 28].

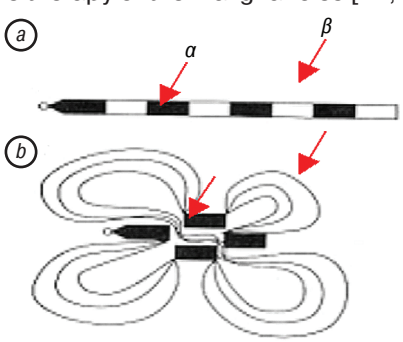


Fig. 5. The schematic organization of pericentromeric heterochromatin in mitotic (*a*) and polytene chromosomes (*b*); black regions — α-heterochromatin, white regions — β-heterochromatin [25]

ACKNOWLEDGEMENTS

The autor is grateful to Drs. A.P. Stepanenko and M.V. Guljchy — from Kiev Thyroid Gland Centre; Prof. V.S. Procyk, Head of Dep. of Head and Neck Oncology, Dr. G.I. Klimnjuk, Head of Dep. of Children's Oncology, Prof. V.A. Kikotj, Head of Dep. of Colorectal Oncology — from the Institute of Oncology AMS of Ukraine, for the peripheral blood samples, kindly given for investigations in our study. The author is grateful to Dr. I.V. Alekseeva from the Institute of Molecular Biology & Genetics of NAS of Ukraine for the invaluable 5-azacytidine reagent synthesis. This work was supported by the National Ukrainian Scientific Program "Genomics".

REFERENCES

1. **Jones PA.** DNA methylation and cancer. Oncogene 2002; **21**: 5358–60.

2. **Szyf M.** Targeting DNA methylation in cancer. Bull Cancer 2006; **93**: 961–72.

3. **Shames DS, Minna JD, Gazdar AF.** DNA methylation in health, disease, and cancer. Curr Mol Med 2007; **7**: 85–102.

4. **Feinberg AP, Vogelstein B.** Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature 1983; **301**: 89–92.

5. **Baylin SB, Bestor TH.** Altered methylation pattern in cancer cell genomes: cause or consequence? Cancer cell 2002; **1**: 299–305.

6. **Paz MF, Fraga MF, Avila S, *et al.*** A systematic profile of DNA methylation in human cancer cell lines. Cancer Res 2003; **63**: 1114–21.

7. **Baylin SB, Herman JG.** DNA hypermethylation in tumorigenesis: epigenetics joins genetics. Trends Genet 2000; **16**: 168–74.

8. **Herman JG, Baylin SB.** Promoter-region hypermethylation and gene silencing in human cancer. Curr Top Microbiol Immunol 2000; **249**: 35–54.

9. **Garinis GA, Patrinos GP, Spanakis NE, Menounos PG.** DNA hypermethylation: when tumour suppressor genes go silent. Hum Genet 2002; **111**: 115–27.

10. **Sherr CJ.** Principles of tumor suppression. Cell 2004; **116**: 235–46.

11. **Fraga MF, Herranz M, Espada J, *et al.*** A Mouse skin multistage carcinogenesis model reflects the aberrant DNA methylation patterns of human tumors. Cancer Res 2004; **64**: 5527–34.

12. **Baylin SB, Herman JG, Graff JR, *et al.*** Alterations in DNA methylation — a fundamental aspect of neoplasia. Adv Cancer Res 1998, **72**: 141–96.

13. **Wajed SA, Laird PW, DeMeester TR.** DNA methylation: an alternative pathway to cancer. Ann Surg 2001; **234**: 10–20.

14. **Worm J, Guldberg P.** DNA methylation: an epigenetic pathway to cancer and a promising target for anticancer therapy. J Oral Pathol Med 2002; **31**: 443–9.

15. **Clark SJ, Melki J.** DNA methylation and gene silencing in cancer: which is the guilty party? Oncogene 2002; **21**: 5380–7.

16. **Ballestar E, Esteller M.** The epigenetic breakdown of cancer cells: from DNA methylation to histone modifications. Prog Mol Subcell Biol 2005; **38**: 169–81.

17. **Jones PA, Baylin SB.** The epigenomics of cancer. Cell 2007; **128**: 683–92.

18. **Howard G, Eiges R, Gaudet F, *et al.*** Activation and transposition of endogenous retroviral elements in hypomethylation induced tumors in mice. Oncogene 2008; **27**: 404–8.

19. **Wilson AS, Power BE, Molloy PL.** DNA hypomethylation and human diseases. Biochim Biophys Acta 2007; **1775**: 138–62.

20. **Shvachko LP.** The correlation relationships between cancer-associated pattern genome DNA hypomethylation and mitotic interphase nuclear heterochromatinization contributing to carcinogenesis. Proc Biol Med 2008; **2**: 38–45. (In Ukrainian).

21. **Shvachko LP, Buck IG, Stepanenko AP, *et al.*** The method of early detection of cancer. Declaration Patent of Ukraine 2006, **UA 13616**, Byul. № 4. (In Ukrainian).

22. **Stefanova VN, Chyryaeva OG, Smirnov AF.** The influence of 5-azacytidine on the structure-function organization of

mitotic chromosomes. IV All-Union symposium "Molecular mechanisms of genetics processes", Proceedings, Moscow, 3–6 February 1987; 49. (In Russian).

23. **Habets GG, van der Kammen RA, Scholtes EH, Collard JG.** Induction of invasive and metastatic potential in mouse T-lymphoma cells (BW5147) by treatment with 5-aza-cytidine. Clin Exp Metastasis 1990; **8**: 567–77.

24. **Szyf M, Pakneshan P, Rabbani SA.** DNA demethylation and cancer: therapeutic implications. Cancer Letters 2004; **211**: 133–43.

25. **Zhimulev IF.** The chromomere organization of polytene chromosomes. Novosybirsk, VO "Science"1994; 565 p. (In Russian).

26. **Chen T, Hevi S, Gay F, *et al.*** Complete inactivation of DNMT1 leads to mitotic catastrophe in human cancer cells. Nat Genet 2007; **39**: 391–6.

27. **Momparler RL.** Epigenetic therapy of cancer with 5-aza-2'-deoxycytidine (decitabine). Semin Oncol 2005; **32**: 443–51.

28. **Szyf M.** Therapeutic implications of DNA methylation. Future Oncol 2005; **1**: 125–35.

ИЗМЕНЕНИЯ КОНСТИТУТИВНОГО ПЕРИЦЕНТРОМЕРНОГО ГЕТЕРОХРОМАТИНА В ЛИМФОЦИТАХ ОНКОБОЛЬНЫХ И ЛИМФОЦИТАХ, ОБРАБОТАННЫХ 5-АЗАЦИТИДИНОМ, АССОЦИИРОВАННЫЕ С ДНК ГИПОМЕТИЛИРОВАНИЕМ

ДНК-гипометилирование играет ключевую роль в канцерогенезе. Маллигнизация, как и прогрессия опухоли, сопровождается увеличением гипометилирования ДНК в опухолевых клетках. Тем не менее до сих пор вопросы эволюции дис-эпигенетических геномных изменений в соматической клеточной злокачественной трансформации, связанной с ДНК-гипометилированием, остаются невыясненными. *Цель:* исследовать взаимоотношения между характером геномного ДНК-гипометилирования и ДНК-гиперплоидностью. *Методы:* в модельной системе ДНК-деметилирования 5-азацитидином обрабатывали митоген-стимулированные лимфоциты и лимфоциты периферической крови онкобольных с солидными опухолями. Содержание ДНК определялось на основе количественной DAPI и Hoechst 33258 флуоресценции интерфазных лимфоцитов. Фитогемагглютинин-стимулированная культура лимфоцитов использовалась для исследования метафазных хромосом и интерфазных ядер с помощью световой и флуоресцентной микроскопии. *Результаты:* модельное действие 5-азацитидина увеличивает сомати-ческую ДНК-гиперплоидность, которая сопровождается направленной амплификацией основных перицентромерных Alu ДНК-повторов, подобно таковой при онкологическом процессе, ассоциированном с геномным гипометилированием, что вносит вклад в развитие ядерного гетеропикноза. *Выводы:* выявлено, что индуцированное глобальное гипометилирование ДНК запускает дис-эпигенетическое репрограммирование конститутивного перицентромерного гетерохроматина по пути экстрахромосомной организации, как показано в развитии латентной политении гетерохроматина, которая является одним из ключевых механизмов вовлечения гипометилирования в инициацию развития и прогрессию опухолей.

Ключевые слова: **ДНК-гипометилирование, 5-азацитидин, латентная политения, конститутивный перицентромерный гетерохроматин.**