EXPERIMENTAL WORKS

UDC 577.323:576.08 doi: https://doi.org/10.15407/ubj97.03.042

RELATIONSHIP BETWEEN CpG AND NON-CpG DNA METHYLATION IN HUMAN LYMPHOCYTES ASSESSED WITH COMET ASSAY

M. CHOPEI, A. PIVEN, K. AFANASIEVA, A. SIVOLOB✉

ESC "Institute of Biology and Medicine",
Taras Shevchenko National University of Kyiv, Ukraine;
✉*e-mail: sivolob@knu.ua*

Received: 21 April 2025; **Revised:** 26 May 2025; **Accepted:** 11 June 2025

Tissue-specific DNA methylation plays an important role in the regulation of many functional processes. The methylation level in single cells can be assessed using the comet assay (single-cell gel electrophoresis), a simple and cost-effective technique. The methyl-sensitive comet assay approach has been widely used under the assumption that methylation in the context of CpG dinucleotides is the only type of this modification. However, although CpG is the main methylation target, non-CpG methylation is also widespread. We used the methyl-sensitive comet assay to demonstrate that, in human lymphocytes, non-CpG methylation significantly contributes to the global methylation level. The activation of lymphocyte proliferation results in an increase in non-CpG methylation, and the methyl-sensitive comet assay can be used to assess the ratio between CpG and non-CpG methylation levels.

K e y w o r d s: DNA methylation, non-CpG methylation, comet assay, lymphocytes, lymphoblasts.

Methylated cytosine base in DNA is a major epigenetic mark in vertebrates [1]. DNA methylation, among other consequences of this modification, directly affects the mechanical properties and stability of the double helix [2]. Cell-type-specific methylation profiles [3] and their changes play an important role in gene regulation, chromatin condensation [4], cell differentiation [5], tumorigenesis [6], and other processes.

The main targets of methylation, which have been the focus of research in this field for a long time, are cytosine bases in the context of CpG dinucleotides. However, cytosine methylation also occurs in other sequence contexts, and such non-CpG methylation, whose role is still not well understood, has been attracting increasing attention recently [7-9]. This type of methylation in mammals depends on the *de novo* methyltransferases DNMT3a and DNMT3b and not on the maintenance methyltransferase DNMT1 [10, 11]. Non-CpG methylation has been found to be tissue-specific and, in particular, enriched in embryonic stem cells and neurons [12, 13]. Associations of non-CpG methylation with diabetes [14], Alzheimer's disease [15] and cancer [8] have been reported.

Alongside other methods, single-cell gel electrophoresis (the comet assay) can be used to assess the global DNA methylation level in single cells. To perform the assay, cells are embedded in a thin layer of agarose on a microscope slide and then lysed to obtain nucleoids. Under an electric field, the nucleoid DNA migrates towards the anode, forming an electrophoretic track, which resembles a comet tail [16]. The migration parameters help reveal features of chromatin loop organization [17], although the main common application of the comet assay is to assess the level of single- and double-stranded breaks in DNA [16, 18-20]: an accumulation of breaks results in a significant increase in the migration rate [16, 18-22].

In the methylation-sensitive comet assay (sometimes called "methy-sens"), the rationale for evaluating the methylation level is to digest the nucleoids with methyl-sensitive restriction endonucleases to induce breaks. In one of the commonly used techniques, the isoschizomeric enzymes *Hpa*II

and *Msp*I are used [23-25]. The two enzymes recognize the same tetranucleotide site CCGG and cleave it in the non-methylated form but display different sensitivity to the methylation pattern. *Msp*I cleaves the CCGG sites if the second, but not the first, C is methylated or hemimethylated [26, 27]. *Hpa*II was initially thought to cut the CCGG sites if the first, but not the second, C is methylated [26, 28]. However, it was later established that this enzyme could cleave only the sites hemimethylated at the first C introducing nicks [27, 29], i.e., *Hpa*II cleavage reflects non-CpG methylation anyway. If, as is usually assumed, non-CpG methylation is negligible, then the difference in the relative amounts of DNA in the comet tails between *Hpa*II- and *Msp*I-digested nucleoids will give an estimate of CpG methylation level [23-25].

In fact, non-CpG methylation is not taken into account in the commonly applied methyl-sensitive comet assay: usually, it is performed in alkaline conditions when single-stranded DNA fragments, which appeared after DNA breaks, give the major contribution to the tail formation [21, 22]. Hence, nicks introduced by HpaII in hemimethylated non-CpG sites may be just invisible in the alkaline comet assay. In contrast, when the assay is performed at neutral pH, the tail is mainly formed by relaxed (as a consequence of nicks) DNA loops [30]. In other words, the neutral comet assay may help to discriminate between CpG and non-CpG methylation.

In this paper, we apply the *Hpa*II/*Msp*I digestion technique, using the comet assay at neutral pH, to investigate DNA methylation in human lymphocytes and lymphoblasts obtained through lymphocyte activation. Our results show that non-CpG methylation is far from negligible in lymphocytes, and increases considerably upon activation. When non-CpG methylation is significant, the methyl-sensitive comet assay at neutral pH can reveal the ratio between CpG and non-CpG methylation levels.

Materials and Methods

Cell isolation, cultivation and sample preparation. Human blood (finger-prick samples) was obtained from healthy donors between the ages of 25–35 (males and females, non-smokers). All procedures were performed in compliance with relevant laws and institutional guidelines. This has been approved by the Ethic Committee of Taras Shevchenko National University of Kyiv. Lymphocytes were isolated by centrifugation in a Histopaque-1077 density gradient (Sigma, USA) according to the manufacturer's recommendations and then washed in RPMI 1640 culture medium (Gibco, USA). To transform the lymphocytes, isolated cells were cultivated for 24 or 44 h at 37°C in the same medium supplemented with antibiotics and 10% fetal bovine serum (Gibco, USA). Phytohemagglutinin P (Sigma, USA) was added to the cell culture at a final concentration of 10 μg/ml to induce lymphocyte activation and proliferation (so-called blast transformation). After that, the cells were washed in Hanks' salt solution, and aliquots of the 24- or 44-hour cell culture were used to assess the efficiency of blast transformation as described earlier [31].

50 μl of cell suspension (lymphocytes or transformed lymphocytes) was mixed with 100 μl of 1% low-melting-point agarose (Sigma, USA) at 37°C. 20 μl of the mixture was applied to a microscope slide and covered with a coverslip. After agarose polymerization, slides were treated with an ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl (pH 8.0), 1% Triton X-100 (Ferak, Germany)) for several hours. After the lysis slides were washed twice with TBE buffer (89 мM Tris-borat, 2 mM EDTA, pH 7.5).

Restriction. Two isoschizomeric restriction endonucleases, *Hpa*II or *Msp*I, were used for nucleoid DNA digestion. For this, slides were incubated for 10 min at 4°C in TNE buffer (5 mM Tris-HCl, 5 mM NaCl, 0.5 mM β-mercaptoethanol, 1 mM EDTA, pH 7.5) and then placed in a humid chamber.

100 μl (1.5 units) of *Hpa*II or *Ms*pI solution in ONE restriction buffer (EURX, Poland) was applied to the slides, covered with a cover glass and incubated for 20 min at 37°C. To stop the digestion, the slides were washed in TBE buffer and immediately electrophoresed.

Electrophoresis, microscopy and statistical analysis. Slides (treated or not treated with the restriction enzymes) were electrophoresed in TBE buffer at 4°C for 5 min (1 V/cm, 300 mA). After electrophoresis, the slides were stained with 1.3 μg/ml of DAPI (4′,6-diamidino-2-phenylindole, Sigma, USA) and immediately analyzed with a fluorescent microscope connected with a camera Canon EOS 1000 D. Over 100 randomly selected nucleoids per slide were examined using image processing program ImageJ (imagej.net) with OpenComet plugin to assess the relative amount of DNA in the tails. Five to ten replicates were performed for each experiment. The mean values and standard errors for

all independent experiments were then calculated. Student's *t*-test was used to assess the *P*-values for pairwise comparisons.

Results and Discussion

We have measured the average relative amounts of DNA in the comet tails after electrophoresis at neutral pH of lysed lymphocytes and lymphoblasts before and after the treatment of the nucleoids with *Msp*I or *Hpa*II, the restriction enzymes, which are sensitive to different methylation patterns of CCGG sites. Several representative comet images are shown in Fig. 1, *A*. The changes in the percentage of DNA in the tails after the treatments (Fig. 1, *B*) highlight two observations: (i) the total methylation level decreases during lymphocyte transformation; and (ii) the different possible methylation patterns essentially contribute to this level.

Both restriction enzymes cleave non-methylated CCGG sites. Additionally, *Hpa*II cleaves CCGG hemimethylated at the first C, while *Msp*I cleaves the sites methylated or hemimethylated at the second C, i.e., in the CpG context [26, 27, 29]. In other words, *Hpa*II and *Msp*I are sensitive to non-CpG and CpG methyations, respectively. It follows that the DNA amount in the tails after *Hpa*II digestion (designated as *hpa*) can be used to estimate the sum of the fractions N and M_1, where N is the fraction of CCGG sites in the non-methylated form and M_1 is the fraction of the sites hemimethylated at the first C. Then, $N + M_2$, where M_2 is the fraction of sites methylated or hemimethylated at the second C, should be proportional to the DNA amount in the tails after *Msp*I digestion (designated as *msp*). Since, by definition, $N + M_1 + M_2 = 1$, the *hpa* fraction is proportional to $1 - M_2$ while the *msp* fraction is proportional to $1 - M_1$. Hence, the simple equation $M_2/M_1 = (1 - hpa)/(1 - msp)$ can be used to estimate the ratio of CpG to non-CpG methylation levels.

If the non-CpG methylation level is low (or absent, as it is often assumed) the ratio M_2/M_1 would obviously be very large. Fig. 2 shows that this is not the case: in lymphocytes, the two methylation levels are comparable, although the level of CpG methylation is approximately twice that of non-CpG methylation, as expected for terminally differentiated cells. Typically, in the methyl-sensitive comet assay, the global methylation level is assessed using the value $1 - hpa/msp$, which is valid provided that the global methylation level equals the CpG methylation level

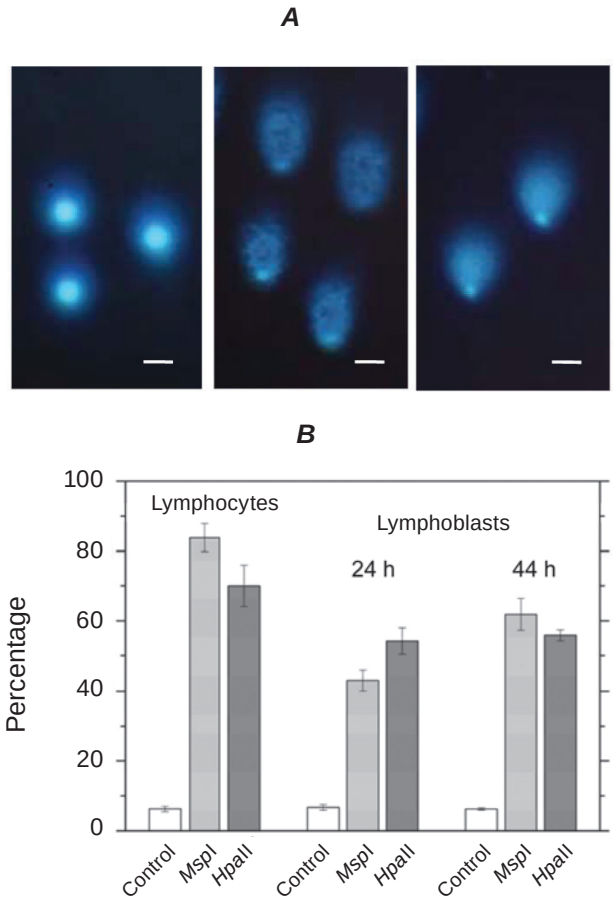

*Fig. 1. Representative examples of comet images for (from left to right) control nucleoids and nucleoids treated with HpaII or MspI (**A**, bars: 10 μm) and the percentages of the average relative DNA amounts in the tails (**B**) for nucleoids obtained from lymphocytes and lymphoblasts cultured for 24 and 44 h, non-treated (control) and treated with MspI or HpaII*

(i.e., non-CpG methylation is negligible and *hsp* is proportional to N). This approach works also if non-CpG methylation is considerable but invisible, since the comet assay is performed at alkaline pH. In lymphocytes, non-CpG methylation contributes significantly, and can be registered at neutral pH. It can be concluded that the possibility for the *Hpa*II digestion reflecting non-CpG methylation should be considered for other cell types as well.

The relative contribution of non-CpG methylation more than doubles in lymphoblasts cultured for 24 h (Fig. 2). Our observations are consistent with the significant role of non-CpG methylation and its dependence on functional status, as shown for B

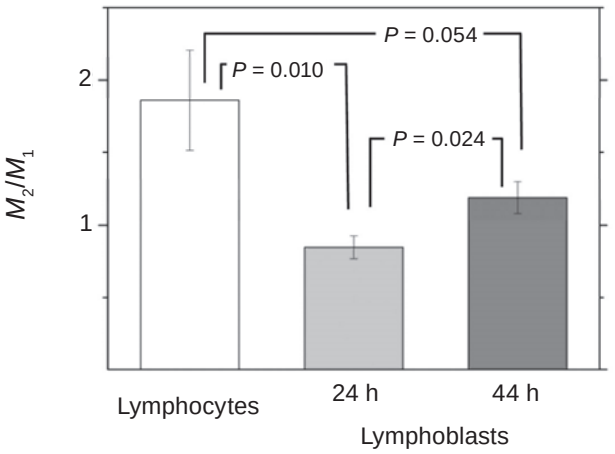

Fig. 2. The ratios of CpG (M_2) to non-CpG (M_1) methylation levels in lymphocytes and lymphoblasts cultured for 24 and 44 h

lymphocytes [32]. After 44 h of cultivation, there is a tendency for the M_2/M_1 ratio to become closer to that of non-activated lymphocytes (Fig. 2).

Conclusions. Our findings can be summarized as follows. In human lymphocytes, non-CpG methylation significantly contributes to the global methylation level, approximately half of the CpG methylation level. The activation of lymphocyte proliferation results in an increase in non-CpG methylation, so that the two types of DNA methylation become approximately equal. Our main conclusion is that the methyl-sensitive comet assay at neutral pH can be used to assess the ratio between CpG and non-CpG methylation levels.

Conflict of interest. The authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wpcontent/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

Funding. This study was conducted without financial support.

Acknowledgements. We are grateful to Liubov Stoliar, Biocorp LLC, for her support in providing some materials for our research work.

СПІВВІДНОШЕННЯ МІЖ МЕТИЛУВАННЯМ ДНК CPG І NON-CPG У ЛІМФОЦИТАХ ЛЮДИНИ, ОЦІНЕНЕ ЗА ДОПОМОГОЮ КОМЕТНОГО ЕЛЕКТРОФОРЕЗУ

М. Чопей, А. Півень, К. Афанасьєва, А. Сиволоб✉

ННЦ «Інститут біології та медицини» Київського національного університету імені Тараса Шевченка, Україна;
✉e-mail: sivolob@knu.ua

Тканино-специфічне метилування ДНК відіграє важливу роль у регуляції багатьох функціональних процесів. Рівень метилування в індивідуальних клітинах може бути оцінений за допомогою кометного електрофорезу (електрофорезу ізольованих клітин) – простої та дешевої техніки. Метил-чутливий кометний електрофорез широко застосовується на основі припущення, що метилування в контексті динуклеотидів CpG є єдиним типом цієї модифікації. Проте, хоча CpG є головною мішенню метилування, метилування в інших контекстах (“non-CpG”) також є поширеним явищем. У цьому дослідженні ми застосовували метил-чутливий кометний електрофорез для демонстрації того, що в лімфоцитах людини метилування non-CpG робить суттєвий внесок у загальний рівень метилування ДНК. Крім того, активація проліферації лімфоцитів приводить до підвищення такого метилування. Отже, метил-чутливий кометний електрофорез може бути використаний для оцінки співвідношення між рівнями метилування CpG і non-CpG.

Ключові слова: метилування ДНК, метилування non-CpG, кометний електрофорез, лімфоцити, лімфобласти.

References

1. Dor Y, Cedar H. Principles of DNA methylation and their implications for biology and medicine. *Lancet.* 2018; 392(10149): 777-786.
2. Zaichuk T, Marko JF. Single-molecule micro-manipulation studies of methylated DNA. *Biophys J.* 2021; 120(11): 2148-2155.
3. Loyfer N, Magenheim J, Peretz A, Cann G, Bredno J, Klochendler A, Fox-Fisher I, Shabi-Porat S, Hecht M, Pelet T, Moss J, Drawshy Z, Amini H, Moradi P, Nagaraju S, Bauman D, Shveiky D, Porat S, Dior U, Rivkin G, Or O, Hirshoren N, Carmon E, Pikarsky A, Khalaileh A, Zamir G, Grinbaum R, Abu Gazala M, Mizrahi I, Shussman N, Korach A, Wald O, Izhar U, Erez E, Yutkin V, Samet Y, Rotnemer Golinkin D, Spalding KL, Druid H, Arner P, Shapiro AMJ, Grompe M, Aravanis A, Venn O, Jamshidi A, Shemer R, Dor Y, Glaser B, Kaplan T. A DNA methylation atlas of normal human cell types. *Nature*. 2023; 613(7943): 355-364.
4. Han H, Cortez CC, Yang X, Nichols PW, Jones PA, Liang G. DNA methylation directly silences genes with non-CpG island promoters and establishes a nucleosome occupied promoter. *Hum Mol Genet.* 2011; 20(22): 4299-4310.
5. Greenberg MVC, Bourc'his D. The diverse roles of DNA methylation in mammalian development and disease. *Nat Rev Mol Cell Biol*. 2019; 20(10): 590-607.
6. Nishiyama A, Nakanishi M. Navigating the DNA methylation landscape of cancer. *Trends Genet*. 2021; 37(11): 1012-1027.
7. Jang HS, Shin WJ, Lee JE, Do JT. CpG and Non-CpG methylation in epigenetic gene regulation and brain function. *Genes (Basel).* 2017; 8(6): 148.
8. Ramasamy D, Deva Magendhra Rao AK, Rajkumar T, Mani S. Non-CpG methylation-a key epigenetic modification in cancer. *Brief Funct Genomics.* 2021; 20(5): 304-311.
9. Titcombe P, Murray R, Hewitt M, Antoun E, Cooper C, Inskip HM, Holbrook JD, Godfrey KM, Lillycrop K, Hanson M, Barton SJ. Human non-CpG methylation patterns display both tissue-specific and inter-individual differences suggestive of underlying function. *Epigenetics.* 2022; 17(6): 653-664.
10. Ramsahoye BH, Biniszkiewicz D, Lyko F, Clark V, Bird AP, Jaenisch R. Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. *Proc Natl Acad Sci USA.* 2000; 97(10): 5237-5242.
11. Arand J, Spieler D, Karius T, Branco MR, Meilinger D, Meissner A, Jenuwein T, Xu G, Leonhardt H, Wolf V, Walter J. In vivo control of CpG and non-CpG DNA methylation by DNA methyltransferases. *LoS Genet.* 2012; 8(6): e1002750.
12. Laurent L, Wong E, Li G, Huynh T, Tsirigos A, Ong CT, Low HM, Kin Sung KW, Rigoutsos I, Loring J, Wei CL. Dynamic changes in the human methylome during differentiation. *Genome Res.* 2010; 20(3): 320-331.
13. Lister R, Mukamel EA, Nery JR, Urich M, Puddifoot CA, Johnson ND, Lucero J, Huang Y, Dwork AJ, Schultz MD, Yu M, Tonti-Filippini J, Heyn H, Hu S, Wu JC, Rao A, Esteller M, He C, Haghighi FG, Sejnowski TJ, Behrens MM, Ecker JR. Global epigenomic reconfiguration during mammalian brain development. *Science*. 2013; 341(6146): 1237905.
14. Barrès R, Osler ME, Yan J, Rune A, Fritz T, Caidahl K, Krook A, Zierath JR. Non-CpG methylation of the PGC-1alpha promoter through DNMT3B controls mitochondrial density. *Cell Metab.* 2009; 10(3): 189-198.
15. Monti N, Cavallaro RA, Stoccoro A, Nicolia V, Scarpa S, Kovacs GG, Fiorenza MT, Lucarelli M, Aronica E, Ferrer I, Coppedè F, Troen AM, Fuso A. CpG and non-CpG Presenilin1 methylation pattern in course of neurodevelopment and neurodegeneration is associated with gene expression in human and murine brain. *Epigenetics.* 2020; 15(8): 781-799.
16. Ostling O, Johanson KJ. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. *Biochem Biophys Res Commun*. 1984; 123(1): 291-298.
17. Afanasieva K, Sivolob A. Physical principles and new applications of comet assay. *Biophys Chem*. 2018; 238: 1-7.
18. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res.* 1988; 175(1): 184-191.

19. Olive PL, Banáth JP. The comet assay: a method to measure DNA damage in individual cells. *Nat Protoc*. 2006; 1(1): 23-29.
20. Collins AR, Oscoz AA, Brunborg G, Gaivão I, Giovannelli L, Kruszewski M, Smith CC, Stetina R. The comet assay: topical issues. *Mutagenesis*. 2008; 23(3): 143-151.
21. Shaposhnikov SA, Salenko VB, Brunborg G, Nygren J, Collins AR. Single-cell gel electrophoresis (the comet assay): loops or fragments? *Electrophoresis*. 2008; 29(14): 3005-3012.
22. Afanasieva K, Zazhytska M, Sivolob A. Kinetics of comet formation in single-cell gel electrophoresis: loops and fragments. *Electrophoresis*. 2010; 31(3): 512-519.
23. Wentzel JF, Gouws C, Huysamen C, Dyk Ev, Koekemoer G, Pretorius PJ. Assessing the DNA methylation status of single cells with the comet assay. *Anal Biochem*. 2010; 400(2): 190-194.
24. Lewies A, Van Dyk E, Wentzel JF, Pretorius PJ. Using a medium-throughput comet assay to evaluate the global DNA methylation status of single cells. *Front Genet*. 2014; 5: 215.
25. Perotti A, Rossi V, Mutti A, Buschini A. Methy-sens Comet assay and DNMTs transcriptional analysis as a combined approach in epigenotoxicology. *Biomarkers*. 2015; 20(1): 64-70.
26. Waalwijk C, Flavell RA. MspI, an isoschizomer of hpaII which cleaves both unmethylated and methylated hpaII sites. *Nucleic Acids Res*. 1978; 5(9): 3231-3236.
27. Walder RY, Langtimm CJ, Chatterjee R, Walder JA. Cloning of the MspI modification enzyme. The site of modification and its effects on cleavage by MspI and HpaII. *J Biol Chem*. 1983; 258(2): 1235-1241.
28. Sneider TW. The 5′-cytosine in CCGG1 is methylated in two eukaryotic DNAs and Msp I is sensitive to methylation at this site. *Nucleic Acids Res*. 1980; 8(17): 3829-3840.
29. Korch C, Hagblom P. *In-vivo*-modified gonococcal plasmid pJD1. A model system for analysis of restriction enzyme sensitivity to DNA modifications. *Eur J Biochem*. 1986; 161(3): 519-524.
30. Afanasieva K, Chopei M, Zazhytska M, Vikhreva M, Sivolob A. DNA loop domain organization as revealed by single-cell gel electrophoresis. *Biochim Biophys Acta*. 2013; 1833(12): 3237-3244.
31. Afanasieva K, Chopei M, Lozovik A, Semenova A, Lukash L, Sivolob A. DNA loop domain organization in nucleoids from cells of different types. *Biochem Biophys Res Commun*. 2017; 483(1): 142-146.
32. Jhuang KF, Hsu ML, Chen YC, Chang JG, Zouali M. DNA methylation trajectories during innate and adaptive immune responses of human B lymphocytes. *Immunology*. 2023; 169(3): 344-357.