Ultrastructural study of the main epigenetic markers in hepatocyte nuclei

Maria Assunta Lacavalla,[1,2] Stella Siciliani[1]

[1]Laboratory of Cell Biology and Neurobiology, Department of Biology and Biotechnology, University of Pavia
[2]Department of Neurosciences, Biomedicine and Movement Sciences, Section of Anatomy and Histology, University of Verona, Italy

Corresponding author: Maria Assunta Lacavalla, Department of Neurosciences, Biomedicine and Movement Sciences, Anatomy and Histology Section, University of Verona, Strada Le Grazie 8, 37134 Verona, Italy. Tel. +39.045.8027561.
E-mail: mariaassuntalacavalla@univr.it

MAL and SS contributed equally to this work.

Key words: 5-methylcytosine; 5-hydroxymethylcytosine; DNA methylation; RNA methylation; transmission electron microscopy.

Non-commercial use only

SUMMARY

DNA methylation is one of the most frequently investigated epigenetic processes and is involved in many biological mechanisms. It consists in the addition of a methyl group, donated by the S-adenosyl methionine, on the cytosine carbon 5 to form 5-methylcytosine (5mC). Cytosine methylation is reversible, as 5mC may be converted into 5-hydroxymethylcytosine (5hmC) through an oxidation step. The presence of 5mC and 5hmC has been investigated also on RNA molecules and they have been found in tRNAs, rRNAs and in pre-mature and mature mRNAs. In this work, we studied the distribution of 5mC and 5hmc on DNA and RNA in mouse hepatocyte nuclei by using morphological and morphometric analyses at transmission electron microscopy and a semi-quantitative analysis on extracted mRNA. 5mC and 5hmC were found to occur in different amounts in heterochromatin and perichromatin region (*i.e.*, where euchromatin is located); moreover, their relative amounts differed in DNA and RNA. Our results support the hypothesis that the process of methylation plays a crucial role in gene silencing and expression. Methylation and de-methylation might also represent two preliminary steps in the processing of mRNA, maybe marking the mRNA fraction destined to degradation or that would never leave the nucleus for translation.

Received for publication: 13 March 2020. Accepted for publication: 1 April 2020.
©Copyright: the Author(s), 2020
Licensee PAGEPress, Italy
microscopie 2020; 31:8996
doi:10.4081/microscopie.2020.8996
This article is distributed under the terms of the Creative Commons Attribution Noncommercial License (by-nc 4.0) which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

Introduction

DNA methylation is one of the most frequently investigated epigenetic processes and is involved in different biological mechanisms, *e.g.* the normal embryonic development, genomic imprinting, X-chromosome inactivation, cell differentiation and cancer (Chen *et al.*, 2017).

DNA methylation consists in the addition of a methyl group, donated by the S-adenosyl methionine (SAM), on the cytosine carbon 5 to form 5-methylcytosine (5mC). This reaction is catalysed by a family of enzymes called DNA methyltransferases (DNMT): DNMT3A and DNMT3B are responsible for *de novo* methylation, while DNMT1 promotes the maintenance of the DNA methylation pattern during cell replication. CpG sites are the most involved sequences in the methylation process; since generally CpG sites locate in the promoter region (known as CpG islands), the major role of cytosine methylation could be related to the regulation of gene expression. However, CpG methylation may also occur in the gene body promoting the transcriptional process (Mendizabal *et al.*, 2017). It was also observed that, in the oocyte (Tomizawa *et al.*, 2011), embryonic stem cells (Ramsahoye *et al.*, 2000) or the brain (Xie *et al.*, 2012), methylation might also occur on non-CpG motifs, which suggests that the function of this epigenetic modification could be variable.

Cytosine methylation is reversible, as 5mC may be converted into 5-hydroxymethylcytosine (5hmC) through an oxidation step. A family of oxygenases called Ten-Eleven-Translocators (TETs) catalyze this reaction; these enzymes are also able to further oxidize 5hmC to form 5-formylcytosine (5fC) and 5-carboxycytosine (5caC). Different studies demonstrated that 5hmC could be considered as an intermediate of the demethylation process (Song and He, 2013). On the other hand, this modification is presently thought to have all the characteristic of an epigenetic signal itself: for instance, it may be present on the opposite strand of the same CG dinucleotide where CpG is located, or demethylation could occur unperturbed also in the absence of TET enzymes, contrarily to what happens in the case of DNMTs knock-out organisms, where the methylation status is completely abolished (Koh and Rao, 2013; Szyf *et al.*, 2016). Actually, there is not unanimous consensus as for the hypothetical role of DNA methylation even if a putative involvement as a positive marker of gene expression is strongly favored.

The widespread presence of 5mC and 5hmC on DNA encouraged scientists to investigate the distribution and function of these two modifications also on RNA molecules. Thus, 5mC was observed in tRNAs from a variety of organisms (yeast, Archaea and higher eukaryotes) and in prokaryotic rRNAs (Motorin *et al.*, 2010); recently, it was also described at electron microscopy on nascent RNA fibrils, as well as on pre-mature and mature mRNAs (Masiello and Biggiogera, 2017).

The process by which tRNA and rRNA molecules are methylated was associated to different methylases (MTases), while for mRNA the only identified enzyme was the NOP2/Sun RNA Methyltransferase Family Member 2 (NSUN2), which seems to be involved also in the mRNA export from the nucleus (Motorin *et al.*, 2010; Yang *et al.*, 2017). However, the function of 5mC on RNA still remains to be well elucidated.

The formation of 5hmC may occur on all kinds of RNA, being mediated by TET enzymes (Fu *et al.*, 2014). Even though in the literature the function of 5hmc on RNAs remains unclear, its presence on mRNAs seems to play a role in the regulation of the translation process, *i.e.* on protein synthesis.

The aim of the present investigation was to describe the distribution of 5hmc on DNA and RNA, and to add new information on the localization and function of 5mC on DNA. To do this, we performed a morphological and morphometric analysis at transmission electron microscopy (TEM) of the distribution of these two epigenetic modifications in the nuclei of mouse hepatocytes, and performed a semi-quantitative analysis of their amounts on extracted mRNA. The hepatocytes were selected as a typical example of functionally active cells.

Materials and Methods

For the ultrastructural analysis, we used liver samples from two-month-old mice already present in the tissue archive of the Laboratory of Cell Biology and Neurobiology of the University of Pavia. The tissue blocks had been fixed with 4% paraformaldehyde in 0.1 M phosphate buffer saline (PBS) pH 7.4, then incubated in 0.5 M NH_4Cl in PBS for 30 min at room temperature (to block the free aldehyde groups), dehydrated with graded ethanol, and embedded in LR White resin.

Ultrathin sections were cut by an ultramicrotome, collected on Formvar-carbon-coated nickel grids or naked gold grids (for sections to be stained with osmium ammine), and used for the immunocytochemical and morphometric analyses.

In order to investigate the distribution of the two epigenetic markers and TET2 enzyme in the hepatocytes nuclei, the liver sections were incubated with the following probes: rabbit polyclonal antibodies against (5mC); mouse monoclonal antibodies against 5hmC; rabbit polyclonal antibodies against TET2. In the case of double labeling reactions, sections were incubated with the primary antibodies revealed by secondary gold-conjugated probes with two differently-sized gold particles (12 and 6 nm). To reduce the chromatin contrast and preferentially reveal the nuclear RNP constituents, the sections were stained with the EDTA regressive technique (Bernhard, 1969). Other liver sections on naked gold grids were incubated with osmium ammine (as reported in Vàzquez-Nin *et al.*, 1995) to obtain a selective staining of DNA. The samples were observed in a Zeiss EM 900. For morphometric assessment, ten nuclei were randomly selected and two different operators counted the number of gold particles over two selected nuclear compartments: the heterochromatin (*i.e.*, the condensed chromatin lining the nuclear envelope), and the perichromatin region (facing the innermost part of the nucleus at the periphery of the condensed chromatin areas). Immunolabeling density was expressed as the number of gold particles per surface unit. An f-test was performed to check the variance followed by a t-test to check if the counting between the operators was concordant. Afterwards, the mean values and standard deviations were calculated, and f-test and *t*-test performed to assess possible quantitative differences in the amount of 5hmc and 5mC on DNA in the chromatin regions considered.

mRNA extraction and immuno dot-blot

mRNA extraction was performed by using a GeneElute™ Direct mRNA MiniPrep Kit (Sigma-Aldrich, St. Louis, MO, USA), starting from mouse liver homogenates previously stored at -80°C; polyadenylated mRNA was also quantified using Nanodrop. One µl of the extracted mRNA was spotted on different nitrocellulose membranes, which were incubated overnight with one of the following antibodies: a mouse monoclonal against 5hmC, a rabbit polyclonal antibody 5mC, or the mouse monoclonal H20 (an antibody recognizing the m3G-m7G RNA cap). After several washes with PBS, the membranes were incubated for 30 min with a secondary antibody conjugated with horseradish peroxidase (HRP). Immunoreactivity was detected with the reagent Luminata™ Crescendo and revealed on slabs. Each experiment was performed with three independent replicates, and the quantitative evaluations of the dots was made using ImageJ *via* densitometric analysis. The spot intensities of the immune dot-blot reaction were converted into spike graphs: the area under the curve for each antibody (corresponding to the amount of methylated or hydroxymethylated mRNA) was expressed as the percentage of the area of the H20 antibody (taken as the value for total mRNA). The following formula was used to calculate the percentage values of either epigenetic modification:

$$5mC \text{ (or } 5hmC) \text{ Area} : H20 \text{ Area} = X : 100\%$$

Results

Ultrastructural localization of 5mC and 5hmC on heterochromatin and perichromatin region

By high resolution analysis at TEM we studied the distribution of the two epigenetic modifications, 5mC and 5hmC, in two different sub-nuclear compartments: heterochromatin (close to the nuclear envelope) and the perichromatin region (*i.e.* the elusive 200-nm-thick area located at the periphery of condensed chromatin where several and important process take place, such as DNA replication and repair, RNA transcription and processing, and possibly the epigenetic modifications of both nucleic acids; Masiello *et al.*, 2018).

The immunolabeling for 5mC was abundant and heterogeneously distributed on the heterochromatin areas where the oxidized form was, instead, poorly present (Figure 1 a,b); the statistical analysis confirmed that the amount of 5mC on heterochromatin was larger than that of 5hmC (Figure 1d; $p<0.01$). On the contrary, in the perichromatin area the immunolabeling for 5mC tended to decrease in comparison to the one for 5hmC, although the difference was not significant (Figure 1e; $p>0.05$).

Non-commercial use only

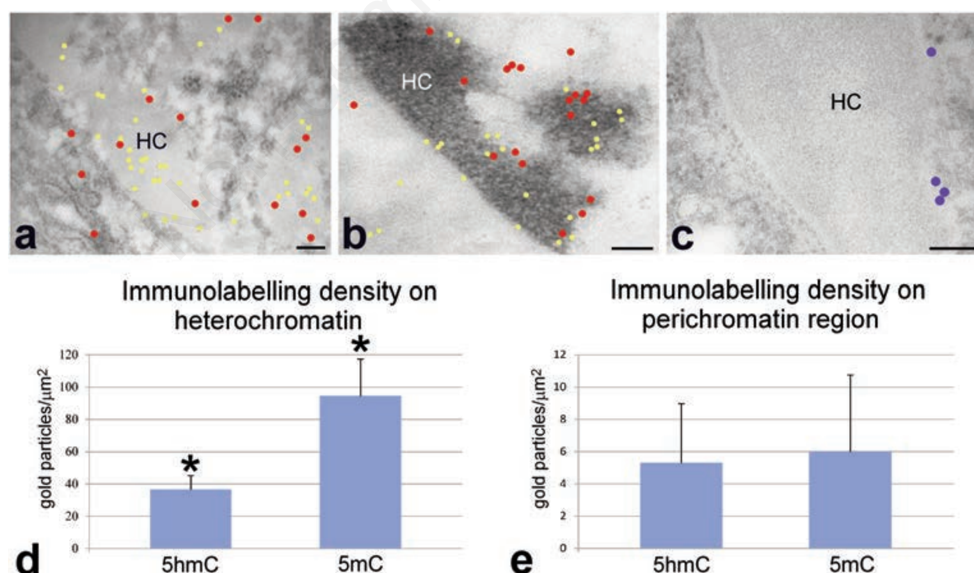

Figure 1. Transmission electron micrographs of mouse hepatocytes. a,b) Immunolabeling for 5mC (yellow spots) and 5hmC (red spots); EDTA staining (a) or osmium ammine staining (b); the signal for 5mC accumulates over heterochromatin clumps, while 5hmC is preferentially distributed in the perichromatin region. c) Immunolabeling for TET2 (blue spots). The signal is located along the border of heterochromatin clumps. d,e) Mean±SD values of immunolabeling density for 5mC and 5hmC on heterochromatin and perichromatin region; asterisks indicate statistically significant difference (P<0.01). HC, heterochromatin; scale bars: a-c) 200 nm.

Evaluation of the amount of 5mC and 5hmC on mRNA by immuno dot-blot

The 5mC and 5hmC may be post-trascriptional modifications of mature mRNAs, with possible involvement in the nucleus-to-cytoplasm traffic and protein synthesis. In order to estimate the amount of the 5mC and 5hmC on extracted mRNA, dot-blot experiments were performed using antibodies recognizing 5hmC, 5mC or the m3G-m7G RNA cap structure, the last antibody allowing to label the total mRNA. The oxidized form was more present on mature mRNA respect to the 5mC (Figure 2), contrary to what we observed at the ultrastructural level on heterochromatin DNA. Nevertheless, this result is consistent with the strong ultrastructural immunolabeling for 5hmC observed on peripheral heterochromatin, adjacent to the perichromatin region.

Distribution of TET2 enzyme

In the nucleus of mouse hepatocytes, TET2 localized predominantly on the perichromatin region (Figure 1c); therefore, the distribution of 5hmC and TET2 were comparable, stressing the idea that both enzyme and product of reaction co-localized where the conformational state of chromatin is opened to transcription.

Discussion

The study about spatial localization and distribution of the epigenetic markers 5mC and 5hmC in nuclei of murine hepatocytes suggests their possible functional role. In the data obtained with high resolution analysis at TEM, 5mC shows heterogeneous distribution on condensed chromatin. This result allows hypothesizing that the process of methylation plays a crucial role in gene silencing. This epigenetic marker, in parallel with histone modifications, participates in making chromatin inaccessible to the transcriptional factors. On the other hand, methylation of chromatin DNA is a dynamic process, since we observed that de-methylation may occur in the perichromatin area.

The different distribution of 5hmC in the nuclear domains considered prompts us to define it not only as an intermediate of the demethylation process of 5mC, but also a putative epigenetic marker. In fact, 5hmC localizes along the periphery of condensed chromatin, in the elusive zone of the nucleus (*i.e.*, the perichromatin region) where several fundamental nuclear processes, such as DNA repair and transcription take place. This evidence suggests a possible "positive" function of 5hmC in gene expression.

Taking into account previous studies on the functional role of the perichromatin region (Masiello and Bigioggera, 2017), the widespread presence of 5mC and 5hmC in chromatin DNA suggests that enzymes involved in methylation and de-methylation of mRNA might also be present. Recently, Masiello and Biggiogera (2019) demonstrated that the RNA processing, including 5'capping and 3'polyadenylation and splicing, occurs in the perichromatin region; here, DNMT enzymes have been detected, and in the present investigation we also found the occurrence of TET2. This allows hypothesizing that methylation and de-methylation might represent two preliminary steps in the processing of mRNA, although it is still necessary to elucidate when these processes take actually place.

The semi-quantitative analysis performed on mRNA from mouse liver showed that 5hmC is present in larger amount than 5mC, contrarily to what we observed on DNA at the ultrastructural level. The percentage of methylated mRNA was found to be about 40% of the total mRNA of liver cells: interestingly, similar results were also obtained for cerebellar extracts and HeLa cells (*unpublished results)*. These data extend the possible scenario as to the role of RNA methylation: for example, 5mC could mark the mRNA fraction destined to degradation or that would never leave the nucleus for translation.

On the contrary, the amount of de-methylated mRNA, in the above-mentioned tissues or cells was found to be variable: this could relate to their different transcriptional activity. Moreover, it is possible that de-methylation is an event which accompanies the RNA molecule through its journey out of the nucleus; this hypothesis is consistent with the immunofluorescence evidence that in HeLa cells TET2 localizes along the perimeter of condensed chromatin and, in particular, in close proximity of the nuclear pores (*data not shown*).

Future studies will be focused on the possible changes in the intranuclear distribution of methylation and de-methylation sites on DNA and RNA under experimental and pathological conditions.

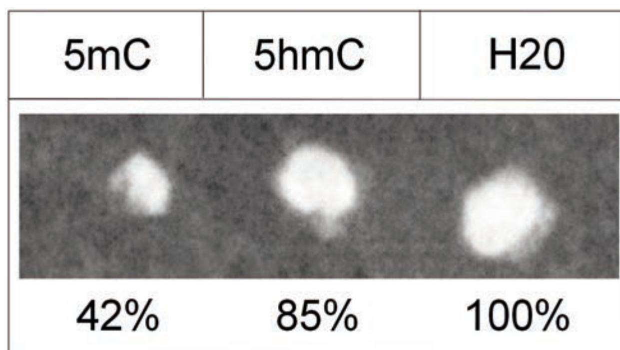

Figure 2. Results obtained with the dot-blot technique using antibodies against 5mC, 5hmC and H20.

Acknowledgements

We would like to express our gratitude to Prof. Marco Biggiogera who introduced us into the theoretical and practical bases of microscopy sciences.

MAL is a PhD student in receipt of a fellowship from the Doctoral Program in Nanoscience and Advanced Technologies, University of Verona.

SS is a PhD student in receipt of a fellowship from the Doctoral Program in Genetics, Molecular and Cellular Biology, University of Pavia.

Non-commercial use only

References

Bernhard W. A new staining procedure for electron microscopical cytology. J Ultrastruct Res 1969;27:250-65.

Chen Y, Hong T, Wang S, Mo J, Tian T, Zhou X. Epigenetic modification of nucleic acids: from basic studies to medical applications. Chem Soc Rev 2017;46:2844-72.

Fu L, Guerrero CR, Zhong N, Amato NJ, Liu Y, Liu S, et al. Tet-mediated formation of 5-hydroxymethylcytosine in RNA. J Am Chem Soc 2014;136:11582-5.

Koh KP, Rao A. DNA methylation and methylcytosine oxidation in cell fate decisions. Current Opinion Cell Biol 2013;25:152-61.

Masiello I, Biggiogera M. Electron microscope detection of 5-Methylcytosine on DNA and RNA. Methods Mol Biol 2019;1870:165-77.

Masiello I, Biggiogera M. Ultrastructural localization of 5-methylcytosine on DNA and RNA. Cell Mol Life Sci 2017;74:3057-64.

Masiello I, Siciliani S, Biggiogera M. Perichromatin region: a moveable feast. Histochem Cell Biol 2018;150:227-33.

Mendizabal I, Zeng J, Keller TE, Yi SV. Body-hypomethylated human genes harbor extensive intragenic transcriptional activity and are prone to cancer-associated dysregulation. Nucleic Acids Res 2017;45:4390-400.

Motorin Y, Lyko F, Helm M. 5-methylcytosine in RNA: detection, enzymatic formation and biological functions. Nucleic Acids Res 2010;38:1415-30.

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 U S A 2000;97:5237-42.

Song C-X, He C. Potential functional roles of DNA demethylation intermediates. Trends Biochem Sci 2013;38:480-4.

Szyf M. The elusive role of 5′-hydroxymethylcytosine. Epigenomics 2016;8:1539-51.

Tomizawa SI, Kobayashi H, Watanabe T, Andrews S, Hata K, Kelsey G, et al. Dynamic stage-specific changes in imprinted differentially methylated regions during early mammalian development and prevalence of non-CpG methylation in oocytes. Development 2011;138:811-20.

Vázquez-Nin GH, Biggiogera M, Echeverría OM. Activation of osmium ammine by SO2- generating chemicals for EM Feulgen-type staining of DNA. Eur J Histochem 1995;39:101- 6.

Xie W, Barr CL, Kim A, Yue F, Lee AY, Eubanks J, et al. Base-resolution analyses of sequence and parent-of-origin dependent DNA methylation in the mouse genome. Cell 2012;148:816-31.

Yang X, Yang Y, Sun B-F, Chen Y-S, Xu J-W, Lai W-Y, et al. 5-methylcytosine promotes mRNA export - NSUN2 as the methyltransferase and ALYREF as an m5C reader. Cell Res 2017;27:606-25.

Non-commercial use only