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RESEARCH ARTICLE

TLC-based detection of methylated cytosine: application to aging epigenetics

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Abstract 5-Methylcytosine (m^5C) has a plethora of functions and roles in various biological processes including human diseases and aging. A TLC-based fast and simple method for quantitative determination of total genomic levels of m^5C in DNA is described, which can be applicable to aging research with respect to rapid and high throughput screening and comparison. Using this method, an example of the analysis of global alternations of m^5C in serially passaged human skin fibroblasts is provided, which shows age-related global hypomethylation during cellular aging in vitro. This method can be useful for screening potential modulators of aging at the level of epigenetic alterations.

Keywords Aging · DNA · Cytosine methylation · Hypomethylation

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Introduction

In addition to the four major nucleosides, DNA from various sources has been shown to also contain the methylated bases, 6-methyladenine (m^6A), 4-methylcytosine (m^4C) and 5-methylcytosine (m^5C), (Ehrlich 2002, 2003; Shiraishi et al. 2002). These methylated bases are natural components of DNA, and distinguish them from a large variety of chemically modified bases that can be formed by alkylation or oxidative damage of the DNA. Specific DNA methylation is catalysed by different DNA methyltransferases (DNMTs) which use *S*-adenosylmethionine (SAM) as the substrate. The methyl group binds to a base through C–N bond (m^6A and m^4C) and with C–C bond in m^5C. Although SAM is a very effective donor for methyl groups, methylation of cytosine residues at position 5 is not a simple reaction. This is because cytosine is an electron-poor heterocyclic aromatic ring system and position 5 of cytosine is not capable of making a nucleophilic attack on the methyl group of SAM. The enzyme DNMT facilitates the nucleophylic attack on the C6 atom by transient protonation of the cytosine ring at the endocyclic nitrogen atom N3 and therefore position 5 of cytosine is strongly activated what facilitates an attack of the methyl group. The covalent enzyme-DNA complex is resolved by deprotonation at position 5, which leads to the elimination of cysteine SH group and reestablishes aromaticity of the base.

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In metazoa only m^5C, but not other methylated cytosines, has been found in DNA. It is a rare but normal component of cellular DNA (Ehrlich et al. 1982; Szyf et al. 2004). It always, but not only, occurs in the dinucleotide sequence CpG. That modification occurs only in cytosines in which 3′ carbon is linked by a phosphodiester bond to 5′ carbon atom of a guanine (CpG dinucleotide). Most of CpG dinucleotides are clustered in small stretches of DNA known as CpG islands and those are protected from methylation in normal cells by mechanisms that are unclear at present. CpG islands are found at promotor regions where lack of methylation is essential to switch the genes on. However, about 70% of CpG dinucleotides located elsewhere in the genome are methylated. Few CpG sequences are found within the coding region of transcribed genes (Shiraishi et al. 2002; Cox 2003; Giles et al. 2003). It is assumed that 3–8% of all cytosine residues in DNA are methylated (Pfeifer 2000).

Usually, m^5C elicits transcriptional repression by blocking the binding of transcriptional activators or stimulates the binding of sequence-specific proteins to DNA. m^5C forms a complementary base pair with guanine. It is rather easily deaminated to thymine, which is a source for CG → TA transition. Because thymine is a normal DNA base, the spontaneous deamination of m^5C is not so easily detected by a cell's DNA repair system. Thus, m^5C residue constitutes mutational hotspot, which occurs within a sequence of a structural gene or in regulatory regions (Cao and Wang 2007).

Modified bases along with basic components of DNA are the targets for DNA oxidative damage processes which result in the appearance of new nucleotide derivatives. Macromolecules, including nucleic acids are targets for reactive oxygen species (ROS), which are formed within cells as by-products of normal cellular metabolism or by external sources such as ionising radiation, redox-active drugs and sensitising dyes. ROS react with DNA and RNA bases to form various genotoxic lesions. Furthermore many products of m^5C reaction with hydroxyl radical have been identified (Kamiya et al. 2002; Hori et al. 2003).

Although the pattern of DNA (m^5C) methylation is stable over cell divisions it can be edited either by de novo methylation or by demethylation. It makes DNA methylation a unique way to encode information and control cellular differentiation and development processes. So, DNA methylation is a central mechanism in epigenetic inheritance (Holliday 2005; Aas et al. 2003; Jeltsch 2002). Cells which have accumulated m^5C in promoter regions of genes needed for adequate response to carcinogenic signals are prone to become tumor cells. Once CpG sequences are methylated, they are inherently mutagenic.

The amount of m^5C in the genomic DNA can be measured by a wide range of methods designed to yield quantitative and qualitative information on genomic DNA methylation. The first approaches were concentrated on the study of global level of m^5C, but recent studies have focused on the study of methylation of specific DNA sequences (Fraga and Esteller 2002; Kok et al. 2007; Oakeley 1999; Oakes et al. 2003).

All methods for detection of m^5C in DNA can be divided into two groups: HPLC analysis and PCR-based identification (Dahl and Guldberg 2003; Esteller 2007; Wojdacz and Hansen 2006). HPLC technique is one dimensional column chromatography, which needs a substantial amount of tissue for DNA isolation. Furthermore, DNA has to be RNA free because 5-methylribocytosine with very similar column retention time to deoxy counterpart can increase the content of that modified nucleoside. Although the PCR-based methods are very sensitive and precise, and they provide a high resolution information on methylation sites of DNA, specific primer sequences for a gene of interest are required.

The method described here combines advantages of both approaches: a high sensitivity, and a genomic scale methylation level determination. This method can also be used as a quality and purity control of DNA samples. We have used cellulose thin layer chromatography (TLC) for separation of m^5C and other nucleotides labelled with [^{32}P]ATP and T4 polynucleotide kinase. The amount of m^5C was calculated as spot intensities ratio of [$m^5C/m^5C + C + T] \times 100$ and expressed as R coefficient which gives a quantitative measurement.

Materials and methods

Isolation of DNA

DNA was isolated from different cells with the method described by Miller et al. (1988) or with a

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commercial kit (A&A Biotechnology, Gdynia, Poland). The key issue here is to avoid random oxidation of DNA during extraction e.g., phenol presence of divalent metal ions, such as Fe, Zn.

DNA hydrolysis and [γ-^{32}P]ATP labelling

DNA (dried, 1 μg) was dissolved in 20 mM succinate buffer pH 6 containing 10 mM $CaCl_2$ and digested with 0.001 U spleen phosphodiesterase II and 0.02 U micrococcal nuclease in the total volume of 3.5 μl for 5 h at 37°C. DNA digest was (0.17 μg) labelled with 2 μCi [γ-^{32}P]ATP (4,500 Ci/mmol, ICN) and 1.5 U T4 polynucleotide kinase in 3 μl of 10 mM bicine-NaOH pH 9.7 buffer containing 10 mM $MgCl_2$, 10 mM dithiothreitol and 1 mM spermidine. After incubation for 35 min at 37°C, 3 μl of apyrase in 10 mM bicine-NaOH, pH 9.7 buffer (10 U/ml) was added and incubation was continued for 35 min. 3′ phosphate was cleaved off with 0.2 μg RNase P1 in 500 mM ammonium acetate buffer pH 4.5.

TLC chromatography of 5′ [γ^{32}P] labelled nucleotides

Separation and identification of [γ^{32}P] m^{5}dC was performed with two directional chromatography on cellulose TLC plates (Merck) using isobutyric acid: NH_4OH: H_2O (66:1:17 vol/vol) in the first dimension and 0.1 M sodium phosphate pH 6.8—ammonium sulfate—*n*-propanol (100 ml/60 g/1.5 ml) in the second dimension. Analysis was made on Phosphoimager Typhoon (Pharmacia) with Image Quant Software (Zukiel et al. 2004). The analysis was repeated five times and data were evaluated with Statistica Annova Test (University of Medical Sciences) or are represented as mean (SD).

Results and discussion

We have applied two-dimensional TLC separation method of 5′ labelled nucleotides from enzymatic hydrolysate of DNA. Spots of m^{5}C, C and T which also include products of m^{5}C damage with very similar chromatographic mobility were quantified (Fig. 1). Using these data we have calculated the R coefficient, which is equal to the ratio of [m^{5}C/m^{5}C + C + T] × 100. It is very important to take care about the quality of DNA isolated from cells. It should be RNA free and intact. While pure DNA provides only 4 (A, G, T, C) + 1 (m^{5}C) spots (Fig. 1A), sample contaminated with RNA shows also ribonucleotides (Fig. 1B).

We measured the amount of m^{5}C in relation to pyrimidines C and T (Fig. 1). Demethylation of m^{5}C to C can be done by enzymatic hydroxylation or the hydroxyl radical damage of the methyl group. The oxidized m^{5}C derivatives are unstable and release formaldehyde, which results in the removal of the methyl group from m^{5}C. On the other hand deamination of m^{5}C forms T, which as a naturally occurring DNA base is difficult to detect and repair with DNA repairing enzyme. Therefore, thymine is included in calculation as a direct m^{5}C deamination product as well as cytosine. Chromatographic mobility of the last two compounds on TLC are very similar and overlap with that of thymine.

Although our approach has some similarity to other methods (Oakeley 1999; Kok et al. 2007), it also differs significantly. DNA was purified with the isolation kit which provides DNA of the highest quality. Apyrase application removes excess of ATP after post labelling. The R parameter used in this paper is very reliable because it includes not only m^{5}C and C as the parent nucleoside, but also some of m^{5}C decomposition products e.g., thymine. Although their amounts in DNA are relatively small in comparison to basic nucleotides, it is a high enough to see differences. It is evident that small chemical changes in DNA are summed up and provide better global information on DNA quality. So, these improvements make this method very reliable and applicable to a very small amount of tissue.

Currently global methylation analysis can be done with HPLC method. However there are some limitations: it requires substantial amount of the starting material. DNA has to be RNA free, because it is difficult to differentiate with the column chromatography ribo and deoxy nucleoside of 5-methylcytosine. That approach is focused only on measuring of m^{5}C but products of its damage (m^{5}C modified in aged cells) are not visible on a column.

All other methods employ bisulfite-mediated deamination of DNA. They require information on

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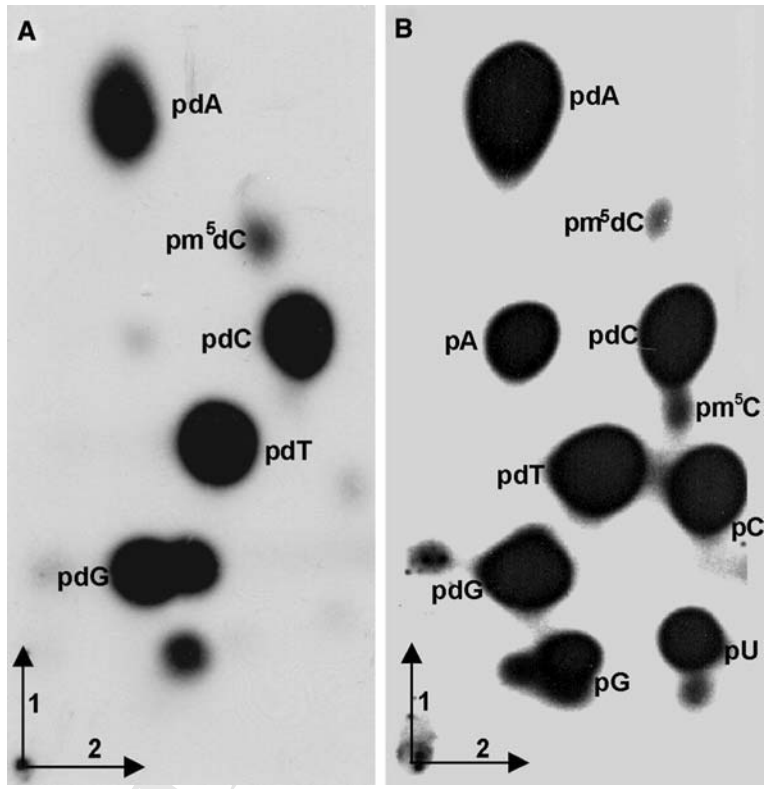

Fig. 1 Two-dimensional cellulose thin layer chromatography (TLC) analysis of [5′-^{32}P] labelled deoxynucleotides obtained by enzymatic hydrolysis of DNA from different types of cells. Hydrolysate of pure DNA sample (**A**) and DNA sample contaminated with RNA (**B**)

a gene which is involved in particular process as well as a proper primer sequence in promoter or a structural gene. Bisulfite induced deamination introduces a basic sites that generate a significant number of single-strand breaks in DNA.

To summarize our method of analysis of genomic cytosine methylation has several advantages. It is two-dimensional chromatography, is very sensitive and suitable for analysis of limited amount of sample, it monitors not only ratio of m^5C but it is a quality control of sample to be analysed. A contamination of DNA with ribonucleotide counterparts makes results not clear. It relates actual amount of m^5C with its oxidative damage products. No other method can do it. Slight changes in level methylation can be monitored.

We have applied this method to compare the global methylation levels of DNA extracted from serially passaged human adult skin fibroblasts at various stages in their replicative lifespan. The life history and culturing method details of the cells used in this study were similar to those published previously (Beedholm et al. 2004; Fonager et al. 2002). In this series of experiments, human adult skin fibroblasts reached a final cumulative population doubling level of 59, which is considered as 100% replicative lifespan completed. Figure 2 shows that there is a significant decrease in DNA methylation during cellular aging in vitro. The results are presented in three age groups: early passage young cells with less than 15% lifespan completed; middle-aged cells with 50–80% lifespan completed; and late passage senescent cells with more than 98% lifespan completed. The highest levels of m^5C were detected in young cells, followed by about 50% reduced levels in middle-aged cells, and with another 20% reduction in the senescent cells. These data are similar to the results published before showing that there is a global decrease in m^5C content during ageing (for cross-references, see: Holliday 2004, 2005; Lu et al. 2006; Finkel et al. 2007; Fraga et al. 2007).

Finally, the method described in this paper is simple, reliable and easy to apply, using a limited amount of the starting material. A quick determination of global levels of methylation can be very useful for screening the beneficial or harmful effects of natural and synthetic molecules with respect to modulation of aging at the epigenetic level.

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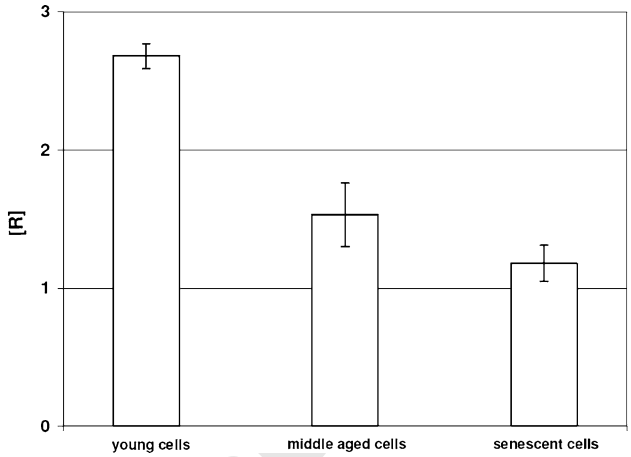

Fig. 2 A comparison of amount m^5C in DNA samples from three age groups of serially passaged human skin fibroblasts: young (less than 15% lifespan completed), middle aged (between 50 and 80% lifespan completed) and senescent (more than 98% lifespan completed). R is the coefficient, which is equal to the ratio of $[m^5C/m^5C + C + T] \times 100$

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