Global DNA methylation in rats´ liver is not affected by hypercholesterolemic diet

Short communication

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Abstract

Increased plasma cholesterol levels are listed between the major atherosclerosis risk factors. The final plasma cholesterol levels results from the interplay between the genetic and environmental (diet, physical activity) factors. Little is known, how dietary factor influence epigenetics. We have analyzed, if an over-generation feeding of rat with cholesterol influences total liver-DNA methylation; and if total liver-DNA methylation differ between the different rat strains (Prague hereditary hypercholesterolaemic rats, Prague hereditary hypertriglyceridemic rats and Wistar Kyoto rats). The animals were feed with high fat (additional 5% over normal capacity) high cholesterol (2%) diet for 14 days. DNA methylation in the liver tissue in different generations was analyzed using the liquid chromatography coupled with tandem mass spectrometry. We have not observed any significant changes in total liver-DNA methylation over the 9 generations of animals feed by fat/cholesterol enriched diet. Additionally, there were no differences in DNA methylation between different rat strains. In animal model, the dietary changes (hypercholesterolemic diet) not significantly influence the total DNA methylation status within the liver.

Key words: Rat, cholesterol, diet, liver, DNA methylation

Atherosclerosis is a chronic inflammatory disease, developing for decades by an accumulation of lipids and fibrous elements and activation of monocyte-macrophages in the arterial vascular wall (Lusis 2000). Although recent results are conflicting (Hubacek *et al.* 2017a; Pikhart *et al*. 2015), dyslipidemia (total cholesterol over 5 mmol/l and triglycerides over 1.7 mmol/l) is believed to be a one of the major risk factors of atherosclerosis development. Excess of low density lipoprotein (LDL) particles initiate the cascade of inflammatory response in large and medium-sized arteries (Hansson 2005).

Predisposition to atherosclerosis has multifactorial background, results from interactions between various genes and between genes and environmental factors. It was approved that environment, behavior and therapeutic are important epigenetic determinants (Segal *et al*. 2017; Lévesque *et al*. 2014). The factors like smoking (Breitling *et al.* 2011), unhealthy diet, drugs (Bahl *et al*. 2015) and also aging (Bollati *et al*. 2009; Wong *et al.* 2010) were linked with aberrant DNA epigenetic pattern. Epigenetic focus on DNA non-sequence heredity and comprises alterations in chromatin structure, such as DNA methylation (most intensively studied), histone modification, nucleosome positioning and regulatory RNAs (Portela & Esteller, 2010, Dlouha & Hubacek 2017). In contrast to stable DNA sequence, epigenetic modifications are dynamic and occur during development and differentiation of the cells. They possibly result in alterations in transcriptional and post-transcriptional gene expression among the cell types (Ferguson-Smith 2011).

In mammals is DNA methylation catalyzed by DNA methyltransferases (DNMTs), which transfer a methyl group from S-adenosylmethionin to the 5-carbon of cytosine residue forming 5-methylcytosine (Hermann *et al*. 2004). DNA demethylation is more complicated process occurring passively through the inhibition of DNMTs or

actively by several mechanisms including enzymatic catalyzes and DNA repair (for review see Wang *et al*. 2014). DNA methylation associated with the transcriptional silencing occurs mainly in CpG islands (Vinson & Chatterjee 2012) of the promoters and CpG island shores (Irizarry *et al*. 2009), since less common methylation of gene body leads to increase of transcription of highly expressed genes (Jjingo *et al*. 2012). Though, CpG islands of the genes´ promoters occupy only 2% of the genome and are usually unmethylated. The main portion of CpG methylation in the genome represents transposable elements (TEs – DNA sequences with the ability to move through the genome, sources of transcriptional factors and non-coding regulatory miRNAs) (Chuong *et al.* 2017); TEs comprise at least 50% of the genome and are heavily methylated (Slotkin & Martienssen 2007).

Animal models in atherosclerosis of hypercholesterolemia in rat had been developed in the 80s after long term brother-sister inbreeding of Wistar rat and testing to the high fat high cholesterol diet (HCD). A new phenotype appeared in the 8th generation (Befekadu *et al.* 1992, Kovář *et al.* 2009, Poledne & Jurčíková-Novotná 2017). The hypercholesterolemia in these Prague hereditary hypercholesterolaemic (PHHC) rats is induced only by HCD without any addition of bile salts and/or antithyroid drugs and reaches the cholesterol concentration between 8-10 mmol/l. The identical experiment was repeated in 90s with the similar results (Poledne, unpublished data). The microarray analysis of hepatic transcriptome of PHHC and Wistar rats cannot explain the hypercholesterolemia in PHHC rats (Vlachová *et al*. 2014) albeit some single genes, as cholesterol 7 α-hydroxylase (Hubacek & Bobkova 2006) seems to be of importance (Hubacek *et al*. 2008). According to previous results and our knowledge, we have suggested that HCD diet

in rats may alter the epigenome of the offspring and therefore contribute to hypercholesterolemia development.

Here we report longitudinal analysis of global liver DNA methylation in animal rat model. The animals were fed with high-fat high-cholesterol (HCD) diet and positive selected according to increasing total serum cholesterol in nine generations.

Wistar rats (AnLAb, Prague, Czech Republic) were fed with the hypercholesterolemic diet (HCD) obtaining 2% cholesterol and 5% palm kernel oil for 14 days. The total cholesterol (TC) in serum was measured twice, before HCD feeding and one day prior to return to standard chow diet. Parent generation (6 males and 6 females) was selected from Wistar rats (N = 50) with the highest induced cholesterolemia, followed by brother-sister inbreeding up to 9^{th} generation.

Further, age-matched of Prague hereditary hypercholesterolaemic rats (PHHC, N = 6), Prague hereditary hypertriglyceridemic rats (HTG, N = 6) and Wistar Kyoto rats (WKY, N = 6) were used for comparisons. These animals were on standard laboratory diet (chow without any thyroid toxic or hepatotoxic factors).

All animals were sacrificed by rapid decapitation; their blood was collected directly from opened carotid arteries for TC analysis. Livers were immediately excised and frozen and stored at -20°C. The experimental protocol of the study and all animal care were approved by the Institutional ethics committee and were in compliance with the State Veterinary Administration.

Total cholesterol was measured in serum using enzymatic kits (Roche Diagnostics, Basel, Switzerland). Genomic DNA was isolated from the frozen liver. Briefly 50 mg of rat liver were homogenized and incubated with RNase A and proteinase K and lysis buffer overnight followed by standard salting out method (Miller *et al*. 1988). DNA concentration was determined on NanoDrop

spectrophotometer, the *A*260/*A*280 ratio was between 1.8 and 2.0. One µg of genomic DNA samples were enzymatically hydrolyzed at least 3 hours to individual deoxyribonucleosides by DNA Degradase Plus (Epigentek, Farmingdale, NY, USA) followed manufacturer protocol. We used the high sensitivity method for the determination of the global DNA methylation levels, the liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Both calculation modes, the ratio of 5-methyl-2´-deoxycitidine (5mdC) to 2´-deoxyguanosine (dG) and the ratio of 5mdC versus sum of 5mdC and 2´-deoxycitidine (dC), were used for the quantification of DNA methylation levels. Chromatographic separation was achieved with hydrophilic interaction liquid chromatography using an Ultimate 3000 RS (ThermoFisher Scientific, MA, USA). The aminopropyl column (Luna NH2 3 µm 100 Å, 100 x 2 mm, Phenomenex, Torrance, USA) was maintained at 35˚C. The mobile phase consisted of 20 mM ammonium acetate in water at pH 9.75 (mobile phase A) and acetonitrile (mobile phase B). The gradient elution was performed as follows: t = 0.0-3.0, 80% B; t = 5.0–6.8, 10% B; t = 7.8-10.0, 95% B. The flow rate was set to 0.3 ml/min, and the injection volume was 5 µl. MS measurement was carried out a electrospray ionization in polarity switching mode of +5500/-4500 V, temperature of ion source was 400°C, curtain gas 40 psi, collision gas at 8 psi and both ion source gases 40 psi. Data acquisition and quantitation were performed using Analyst 1.6.2 and MultiQuant 3.0 software (Sciex, Foster City, CA, USA).

Contrary to the original experiment from 90s (Befekadu *et al.* 1992) when stimulated cholesterolemia increased moderately and continuously up to 4th generation in both genders, in this time the effect of HDC was immediately in generation F1, but did not increased significantly after that and only slightly differ in selected females with the highest levels in generations three (9.37 ± 1.16 mmol/l) and

six (8.86 ± 0.72 mmol/l). The global DNA methylation (expressed as % of methylated C - % 5mdC) in Wistar rat feed with HCD measured in first F1 and last F9 generations and in generations with highest cholesterol after HCD diet F3 and F6 did not significantly differ as well (Figure 1).

The average cholesterolemia in different rat strains without HCD was 2.23 ± 0.19 mmol/l in PHHC (N = 5), 2.34 ± 0.09 mmol/l in WKY (N = 6) and 2.00 ± 0.17 mmol/l in HTG (N = 6) and do not significantly differ between the strains. The differences were not observed among the compared animals in global liver-DNA methylation likewise (Figure 2).

The LC-MS/MS method is accurate and suitable method for the global DNA methylation measuring. The calculation models differ constantly in each measurement of each sample with the ratio 0.71 ± 0.07. The difference is due to different ion suppression effects on the fast 5mdC and slow eluting dG compounds (Li & Franke 2011).

DNA methylation is the most studied epigenetic modification. The associations between global DNA methylation and hypercholesterolemia were studied in humans with different methods, and with very inconsistent results (Pearce *et al*. 2012; Nicoletti *et al*. 2016; Kato *et al*. 2012; Cash *et al*. 2011). Also atherosclerosis and cardiovascular disease are associated with global DNA methylation changes (Baccarelli *et al*. 2010, Khyzha *et al*. 2017; Wang *et al*. 2017).

The dietary impact on atherosclerosis development was observed in offspring of hypercholesterolemic mothers (Napoli *et al*. 2002). Also, maternal undernutrition during the fetal development leads to atherosclerosis in progeny (Yates *et al*. 2009) and it was shown, that protein restriction up-regulates the cholesterol transport in

placenta (Daniel *et al*. 2016). Therefore, it was suggested that the different diets may affect an epigenetic modification of the genome.

Our experimental study did not support the idea, that a high fat/high cholesterol diet could extensively affect total liver-DNA methylation and influence plasma lipid levels through this regulatory mechanism.

Further, as the total liver-DNA methylation was almost identical within the different rat strains, we suggest that the total DNA analysis is not sufficiently precise to distinguish between the potential different DNA methylation or to be associated with different phenotypes. Likely the DNA methylation of all genes involved in lipid metabolism needs to be examined separately to descry its potential link between diet DNA methylation and plasma cholesterol levels.

In comparison with the most studies published so far, we have analyzed DNA methylation within the liver tissue (and not in leucocytes) – which is the major source of lipoprotein particles – thus, the liver is expected to be target organ for the dietary induced hypercholesterolemia. But we cannot exclude, that there could be some changes in DNA methylation occurring in other tissues, as there are significant DNA methylation differences between the different tissues (Voisin *et al*. 2015, Leenen *et al*. 2016).

As the previous experiments exclude also the major role of differences in transcriptome (Vlachová *et al*. 2014) as a determinant of PHHC-rat phenotypes, the last possibility how the cholesterol in these animals is influenced is a role of accumulation of many single nucleotide polymorphisms, similarly as in humans (Hubacek *et al*. 2017b, Talmud *et al*. 2013, Paththinige *et al*. 2017), as suggested previously (Befekadu *et al*. 1992). If high number of cholesterol increasing SNPs is

accumulated, the inbreeding crossing could conserve the sufficient negative genetic background which could be transferred into the next generations.

We conclude, that the over-generational feeding the rats with high-fat high-cholesterol diet is not influencing the total DNA methylation in rats livers.

Conflict of Interest

There is no conflict of interest

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Figure legends

Figure 1

Liver DNA global methylation in Wistar Kyoto rats on high fat – high cholesterol diet. DNA methylation in each animal sample of generations P0, F1, F3, F6 and F9 is expressed as % 5mdC.

Figure 2

Liver DNA global methylation in different rat strains (Wistar Kyoto rats - WKY, Prague hereditary hypercholesterolaemic rats - PHHC and Prague hereditary hypertriglyceridemic rats - HTG). DNA methylation in each animal sample is expressed as % 5mdC.