Praca oryginalna *Original paper*

Association between body weight and age of dogs and global DNA methylation

MAGDALENA GRYZIŃSKA, ANDRZEJ JAKUBCZAK, PIOTR LISTOS*, PAULINA DUDKO**, KATARZYNA ABRAMOWICZ, GRAŻYNA JEŻEWSKA-WITKOWSKA

Department of Biological Basis of Animal Production, Faculty of Biology and Animal Breeding, University of Life Sciences, Akademicka 13, 20-950 Lublin, Poland
*Department of Pathological Anatomy, Faculty of Veterinary Medicine, University of Life Sciences, Głęboka 30, 20-033 Lublin, Poland
**Department of Small Ruminants and Agriculture Advisory, University of Life Sciences in Lublin, Akademicka 13, 20-950 Lublin, Poland

Received 26.03.2015 *Accepted 20.05.2015*

Gryzińska M., Jakubczak A., Listos P., Dudko P., Abramowicz K., Jeżewska-Witkowska G.

Association between body weight and age of dogs and global DNA methylation

Summary

The aim of the study was to analyze whether the total DNA methylation level in dogs is dependent on age and body weight, using global DNA methylation techniques modeled on the ELISA assay. The material for the study consisted of blood collected from dogs of varying age and body weight. The experimental group consisted of 16 individuals with body weights ranging from 3.2 kg to 70 kg and aged from 2 months to 14 years. Total DNA methylation was determined using a kit for quantitative analysis of DNA methylation manufactured by MDQI, Imprint Methylated DNA Quantification Kit (Sigma Aldrich). The total DNA methylation level in the dog genome increases with age. The technique of analyzing the DNA methylation level based on the ELISA enzyme immune assay is an efficient and effective method for determining relative DNA methylation level.

Keywords: dog, body weight, age, DNA methylation

Ageing of the organism, which comprises numerous processes associated with indisposition and reduced ability to respond to environmental stress, is linked to epigenetic mechanisms (10). The geriatric process in dogs has a similar course as in humans and is associated with numerous ailments and infirmities (16). Ageing begins at the moment of birth, although its first signs, such as a general decline in activity, a tendency towards longer and deeper sleep and loss of enthusiasm, do not become visible for several years. The most frequent effects are hearing loss, cataracts, weight gain, impairment of the immune system, inflammation, and consequently also cancer (4).

DNA methylation is a species-specific and tissue--specific process. It consists in the addition of a CH3 group to the cytosine of selected CpG islands (22). Results obtained for various organisms support the hypothesis that the global DNA methylation level can be regarded as a type of clock based on which the life expectancy of an individual can be predicted (23). The gene methylation pattern is currently a safe diagnostic index for detection of tumors, even in the early stages of malignancy.

DNA methylation is an ideal parameter for comprehensive diagnostics. Methylation patterns provide information concerning the current state of activity of genes and potential means by which they are activated or inhibited, as well as "molecular age", the effect of numerous environmental factors, as well as individual variation, which is important in terms of both diagnostics and treatment (17).

The aim of the study was to analyze whether the total DNA methylation level in dogs is dependent on age and body weight, using global DNA methylation techniques modeled on the ELISA assay.

Material and methods

The material for the study consisted of blood collected from dead dogs of varying age and body weight. The experimental group consisted of 16 individuals with body weights ranging from 3.2 kg to 70 kg and aged from 2 months to 14 years. Dogs were divided into age groups (pups up to

9 months, adolescent dogs aged 9 to 15 months, adult dogs aged 15 months to 8 years, and old dogs over 8 years of age) on the basis of dog show regulations by FCI (Federation Cynologique Internationale (AISBL)), and into weight groups on the basis of dog body weight ranges used in veterinary prophylaxis (small – up to 10 kg; medium – 11-25 kg; large – 26-45 kg; and giant – over 45 kg). DNA was isolated using a QIAamp DNA Blond Mini Kit (QIAgen). A 100-µl volume of the DNA isolate obtained was suspended in AE buffer. Total DNA methylation was determined using a kit for quantitative analysis of DNA methylation manufactured by Sigma (MDQI, Imprint Methylated DNA Quantification Kit, Sigma Aldrich) according to the protocol included with the kit. The quantity of DNA collected for analysis was calculated so that the final DNA concentration following dilution in binding solution was 150 ng/µl. The total DNA methylation level was expressed as the relative methylation level in the samples with respect to the methylated control. Methylation level was calculated by the formula

$$((A_{450}S - A_{450}B)/(A_{450}MC - A_{450}B)) \times 100,$$

where: $A_{450}S$ – mean absorbance of the sample; $A_{450}B$ – mean absorbance of the blank sample; $A_{450}MC$ – mean absorbance of the methylated control.

Calculations were performed for each period of development and body weight range using the mean of two measurements.

Statistical analysis of the results (Duncan's test) was performed using SAS software. The significance of differences was determined at $P \leq 0.05$.

Results and discussion

The dogs were divided into groups according to age (pups, adolescents, adults and old dogs) (Tab. 1) and body weight (small, medium, large and giant) (Tab. 2), with 4 individuals in each group.

The average age in the first age group (pups) was 3 months, and in the remaining groups 13 months (adolescents), 6 years (adults) and 13 years (old dogs). A statistically significant difference was found in the global DNA methylation level between pups and adolescent dogs and between adult and old dogs (over 8 years) (Tab. 1).

The average body weight in each of the weight groups was as follows: 1.7 kg (small dogs), 13 kg (medium-sized dogs), 35 kg (large dogs) and 60 kg (giant dogs). No statistically significant difference was found in the global DNA methylation level between weight groups.

As DNA methylation is linked to age, changes in it have been associated with complex age-related diseases (3). Hannum et al. (13) developed a quantitative model of ageing by measuring the rate of ageing of the methylome of a given individual. About 450,000 CpG markers from the blood of 656 people aged 19 to 110 were used for the analysis. A quantitative measurement of the methylation level was made. Methylation patterns for the entire genome were found to be a strong and predictable biomarker of the rate of biological ageing (13). Despite the significant diagnostic potential of measurements of gene methylation, previous research has primarily involved model species (e.g. mice, rats, chickens and bees). There are no reports in the literature of research on the degree of gene methylation in dogs. Some of the most important studies of the last decade have demonstrated a link between methylation and life span in mammals (5, 8). Differences in patterns of gene expression were first observed in salmon during ontogenesis in an analysis conducted in 1996 (23), when methylation was found to decrease with age, which other authors have confirmed in independent research on mammals. A study by Kwabi-Addo et al. (15) put the previous view into question, finding that DNA methylation increased with age in certain genes or tissues (15). Gryzińska et al. (11) found a correlation between the stage of individual development in chicken embryos and global DNA methylation. The global level of 5-methylocytosine in DNA during pulmonary respiration was higher than during allantoic respiration (11). A study on embryonic (tissues) and post-embryonic (blood) development in chickens showed variation depending on the level of development. The DNA methylation level increased with the age of the embryos, and decreased in the case of one-day-old and 32-week-old chickens. The differences are probably due to the different tissue sources and indicate that the type of tissue affects the level of methylation. The results are evidence of the tissue--specificity of this epigenetic mechanism, but primarily of the dependence of the methylation level on age (12). An analysis of cytosine methylation in DNA at the gene level in Japanese quail by the MSP method showed

Tab. 1. Results of measurement of relative DNA methylation level in dogs in different age groups

Age group	Number of dogs	Age	Relative methylation level (%)
Pups	5	up to 9 mos.	$43.53^{a} \pm 20.22$
Adolescent	2	9-15 mos.	$53.60^{b} \pm 32.85$
Adult	4	15 mos. to 8 years	$61.55^{ab} \pm 22.95$
Old	5	over 8 years	$81.29^{a} \pm 9.79$

Explanations: a, b – means marked with different superscript letters differ significantly ($p \leq 0.05$)

Tab. 2. Results of measurement of relative DNA methylation level in dogs in different body weight groups

Weight group	Number of dogs	Body weight (kg)	Relative methylation level (%)
Small	4	up to 10	$56.88^{a} \pm 17.44$
Medium	5	11-25	$70.19^{a} \pm 20.00$
Large	3	25-45	$60.60^{a} \pm 17.39$
Giant	4	over 45	$54.01^{a} \pm 22.05$

Explanations: a – means with this same superscript letter not differ significantly ($p \leq 0.05$)

that the 28S rRNA gene is methylated and silenced in both 15- and 52-week-old quails, which indicates that methylation of this gene may be associated not only with age, but also with the cell cycle (1).

Age is currently known to affect the level of DNA methylation in most organisms. Correlations have also been found between DNA methylation and the age of the organism in both animals and plants. Boks et al. (6) observed that DNA methylation was correlated with age and sex in humans. The level of DNA methylation can be an indicator of age, and abnormal DNA methylation can lead to premature ageing (5).

Gas chromatography was used to determine a number of factors causing increased mortality in bees (2). Strachecka et al., (21) analyzed the effect of amphotericin B (AmB) on the level of DNA methylation in bees. Amphotericin B is an antifungal medication used to treat mycosis in humans. Amphotericin B was found to increase the global DNA methylation level in bees, and accelerated ageing can be regarded as a side effect of treatment with AmB (21).

The significant potential of methylation as an indicator led to measures taken by Horvath (14), who conducted statistical analyses of methylation using samples collected from various types of healthy human tissue from different age groups (including both fetuses and people aged 110 years). The study identified 353 specific genome regions in which the methylation level increased or decreased with age in nearly all the tissues. One of the samples analyzed was a woman's breast tissue whose "methylation age" was found to be 2-3 years higher than that of the other body tissues. The same algorithm was used to evaluate material from the heart, which in both sexes was 9 years younger than the tissues of the rest of the body. Breast cell genes are believed to have a higher methylation age due to their constant exposure to hormones, while the surprisingly low age in the case of the heart is probably due to the presence of stem cells. The fact that increasing age is one of the risk factors in cancerogenesis suggests that premature ageing of breast tissue may explain why tumors in women occur most frequently in this part of the body. Cancer tissues appear to be 36 years older than samples taken from healthy organs. The algorithm created by the researchers was found to be two times more accurate a technique than measurement of telomere length for estimating "methylation age". The use of this tool for estimating methylation levels may find application in analysis of tissue obtained from biopsies to detect accelerated ageing, which could indicate the development of a neoplastic process (14).

Previous research on the association between body weight and epigenetic changes has primarily focused on humans (19). Relton et al. (18) studied the relationship between the methylation pattern of DNA isolated from umbilical blood and childhood body weight. The results of the study confirmed the association between the degree of DNA methylation at birth and body weight in childhood. Genes that exhibited impaired expression in children with a high body mass index (BMI) had abnormal regulation at birth. This suggests that a change takes place in the DNA methylation pattern of genes at birth that influence body weight in childhood.

A study determining the level of DNA methylation in the skin and lipid tissue confirmed the link between BMI and methylation of CpG islands. Evaluation of methylation of the gene *HIF3A* in whole blood and lipid tissue found that the methylation process contributed to an increase in body weight and thus to obesity (7).

Slomko et al. (20) investigated the relationship between variably methylated regions (VMR) and BMI by analyzing 4.5 million CpG sites in the entire genome. Four VMR were found to be associated with BMI. Obesity is a disease with many underlying causes and is influenced by genetic, epigenetic and environmental factors. Despite the many studies that have investigated factors influencing obesity, research aimed at elucidating how epigenetic factors can contribute to an increase in body weight has only recently begun.

The DNA methylation profile is also affected by diet, single nucleotide polymorphisms and exposure to environmental factors. Deficiencies of folic acid, methionine or selenium can lead to hypomethylation of DNA, leading to impairment of gene expression and genetic instability (9). Analysis of DNA methylation profiles and the methylome is an important tool enabling a systemic approach to investigating and improving our understanding of genome function in states of health and disease.

The results of the study provide a basis for further research on the phenomenon of methylation in dogs. The association found between this mechanism and the age of individuals contributes to knowledge in the area of epigenetics and underscores the important role of methylation as an indicator of age and a tool for predicting life span, which in the future may be helpful not only in the case of dogs but in humans as well.

Research on the influence of the age of dogs on the degree of DNA methylation expands knowledge of the epigenome of this species and of mechanisms of epigenetic phenomena. The total DNA methylation level in the dog genome increases with age.

References

1. *Andraszek K., Gryzińska M., Wójcik E., Knaga S., Smalec E.*: Age-dependent change in the morphology of nucleoli and methylation of genes of the nucleolar organizer region in the Japanese quail model Coturnix japonica (Temminck and Schlegel, 1849) (Galliformes: Aves). Fol. Biol. (Krakow) 2014, 62, 293-300.
2. *Bańka K., Buszewicz G., Listos P., Mądro R.*: Usefulness of GC-MS method for the determination of DDT, DMDT, and gamma-HCH in bees (bodies) for legal purposes. Bull. Vet. Inst. Pulawy 2010, 54, 655-659.
3. *Barres R., Zierath J. R.*: DNA methylation in metabolic disorders. Amer. J. Clinic. Nutrition 2011, 93, 897-900.

4. *Błaszczyk J. W., Czerwosz L.*: Stabilność posturalna w procesie starzenia. Gerontol. Pol. 2005, 13, 25-36.
5. *Bocklandt S., Lin W., Sehl M. E., Sanchez F. J., Sinsheimer J. S., Horvath S., Vilain E.*: Epigenetic predictor of age. PLoS 2011, 6, 1-6.
6. *Boks M. P., Derks E. M., Weisenberger D. J., Strengman E., Janson E., Sommer I. E., Kahn R. S., Ophoff R. A.*: The relationship of DNA methylation with age, gender and genotype in twins and healthy controls. PLOS one, 2009, 8, 1-8.
7. *Dick K., Nelson Ch. P., Tsaprouni L., Sandling J. K., Ad'ssi D., Wahl S., Meduri E., Morange P. E., Gagnon F., Grallert H., Waldenberger M., Peters A., Erdmann J., Hengstenberg Ch., Cambien F., Goodall A. H., Ouwehand W. H., Schunkert H., Thompson J. R., Spector T. D., Gieger Ch., Trégouët D. A., Deloukas P., Samani N. J.*: DNA methylation and body mass index: a genome-wide analysis. Lancet 2014, dx.doi.org/10.1016/S0140-6736(13)62674-4
8. *Dunican D. S., Ruzov A., Hackett J. A., Meehan R. R.*: XDnmt1 regulates transcriptional silencing in pre-MBT Xenopus embryos independently of its catalytic function. Development 2008, 135, 1295-1302.
9. *Fenech M., Baghurst P., Luderer W., Turner J., Record S., Ceppi M., Bonassi S.*: Low intake of calcium, folate, nicotinic acid, vitamin E, retinol, beta-carotene and high intake of pantothenic acid, biotin and riboflavin are significantly associated with increased genome instability – results from a dietary intake and micronucleus index survey in South Australia. Carcinogenesis 2005, 26, 991-999.
10. *Gopisetty G., Ramachandran K., Singal R.*: DNA methylation and apoptosis. Mol. Immunol. 2006, 43, 1729-1740.
11. *Gryzińska M., Andraszek K., Jeżewska-Witkowska G.*: Estimation of global content of 5-methylcytosine in DNA in chicken embryos in critical moment during the hatching process. Fol. Biol. (Krakow) 2014, 62, 97-101.
12. *Gryzinska M., Błaszczak E., Strachecka A., Jezewska-Witkowska G.*: Analysis of Age-Related Global DNA Methylation in Chicken. Biochem. Genetics 2013, 51, 554-563.
13. *Hannum G., Guinney J., Zhao L., Zhang L., Hughes G., Sadda S., Klotzle B., Bibikova M., Bing J. F., Gao Y., Deconde R., Chen M., Rajapakse I., Przyjaciel S., Ideker T., Zhang K.*: Genome-wide Methylation Profiles Reveal Quantitative Views of Human Aging Rates. Molecular Cell. 2013, 49, 1-9.
14. *Horvath S.*: DNA methylation age of human tissues and cell types. Genome Biol. 2013, 14, R115. doi:10.1186/gb-2013-14-10-r115
15. *Kwabi-Addo B., Chung W., Shen L., Ittamann M., Wheeler T., Jelinek J., Issa J. P.*: Age-related DNA methylation changes in normal prostate tissues. Clin. Cancer Res. 2007, 13, 3796-3802.
16. *Piórkowski J., Nozdryn-Plotnicki Z., Listos P.*: Evaluation of pathomorphological splenic lesions in dogs from the Lublin region in 2005-2008. Bull. Vet. Inst. Pulawy 2010, 54, 243-246.
17. *Plass B., Soloway D.*: DNA methylation, imprinting and cancer. Europ. J. Hum. Gen. 2002, 10, 6-16.
18. *Relton C. L., Groom A., Pourcain B. St., Sayers A. E., Swan D. C., Embleton N. D., Pearce M. S., Ring S. M., Northstone K., Tobias J. H., Trakalo J., Ness A. R., Shaheen S. O., Smith G. D.*: DNA Patterns in Cord Blood DNA and Body Size in Childhood. PLoS One 2012, DOI: 10.1371/journal.pone.0031821
19. *Rokholm B., Andersen C. S., Sørensen T. I. A.*: Developmental Origins of Obesity- Genetic and Epigenetic Determinants. Open Obesity J. 2011, 3, 27-33.
20. *Slomko H., Heo H. J., Einstein F. J.*: Epigenetics of Obesity and Diabetes in Humans. Endocrinology 2012, 153, dx.doi.org/10.1210/en.2011-1759.
21. *Strachecka A., Borsuk G., Olszewski K., Paleolog J., Gagoś M., Chobotow J., Nawrocka A., Gryzińska M., Bajda M.*: The effect of amphotericin B on the lifespan, body-surface protein concentrations and DNA methylation levels in the honey bee (Apis mellifera). J. Apicult. Sci. 2012, 56, 107-113.
22. *Szyf M.*: Targeting DNA methylation in cancer. Ageing Res. Rev. 2003, 2, 299-328.
23. *Vanyushin B. F.*: Enzymatic DNA methylation is an epigenetic control for genetic functions of the cell. Biochem. (Mosc.) 2005, 70, 488-499.

Corresponding author: Dr hab. Magdalena Gryzińska, ul. Akademicka 13, 20-950 Lublin; e-mail: magdalena.gryzinska@up.lublin.pl