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THE RELEVANCE OF METHYLATION PROFILES OF EQUINE *ITGAL* GENE*

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Abstract

One of epigenetic features of mammalian genomes is methylation of DNA. This nucleotide modification might exert suppressive effect on gene transcription. We have described putative relevance of methylation of one of immune cells related gene (*ITGAL*) observed in the set of 11 equine tissues. Comparison between qualitative RT-PCR results and DNA bisulfite sequencing of investigated set of tissues pointed to potential correlations between tissue specific methylation and tissue specific transcription in *ITGAL* locus. These findings might be important for studies on genetic and epigenetic background of autoimmune disorders in the horse.

Key words: DNA methylation, transcription, horses

Wide DNA methylation is a common feature of the genomes of most eukaryotic organisms including plants, animals, and fungi (Tzuu-fen et al., 2010). This chemical modification is responsible for stabilization of genetic apparatus through methylation of pericentromeric chromosomal areas and silencing of foreign DNA derived from retroviral sequences (Hoelzer et al., 2008). Methylation mainly affects cytosines in CG context which can be grouped on short DNA stretches (CpG islands – CGIs). These types of CG enriched sequences might be a feature of promoter regions of genes (Deaton and Bird, 2011). Different states of methylation of CpG enriched promoters correlate with certain histone modifications and could play a role in the regulation of transcription. The suppressive function of methylation has been firstly observed during the study on imprinting and mammalian X chromosome inactivation (Bird, 2002). DNA methylation was discovered to be associated with the

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state of condensed chromatin blocking the availability of binding sites for transcription factors (TF) in the promoter area (Bird, 2002). Some other described mechanisms also included the recruitment of methyl binding domains (MBD proteins) to heavily methylated CpG promoters which lock the sites of activity of transcriptional complexes (Deaton and Bird, 2011). In addition it seems that this classical point of view is going to be challenged in relation to the recently conducted research (Wan et al., 2015). Namely, some studies have identified a group of TFs that preferentially bind to methylated CpG sites of promoters of active genes (Mann et al., 2013). These data suggest that the regulatory mechanism involving DNA methylation in gene transcription might be more complicated than it was previously thought (Wan et al., 2015). Nevertheless the application of NGS methylome studies revealed that around 80 percent of genes are characterized with hypomethylated state of their CpG promoters which are the exception to the wide hypermethylation of most of genomic areas not enriched by coding sequences (Varley et al., 2013). A significant proportion of these loci are the housekeeping genes active throughout the lifespan (Bird, 2002). In turn, when loci are activated under specific circumstances, the observed methylation patterns may vary dependently on tissue or cell type. Therefore a number of genes exist whose tissue-specific transcription might correlate with tissue specific methylation (Rao et al., 2013). In this work we have discussed the potential function of methylation patterns of equine *ITGAL* gene in relation to its transcript abundance in the group of 11 equine tissues originating from three embryonic layers. This gene encodes the integrin alpha L chain belonging to the leukocyte antigens. *ITGAL* is overexpressed in T lymphocytes and its role in autoimmune disorders is widely discussed (Balada et al., 2014). The role of methylation in transcriptional regulation of this locus has been described in study of Lu et al. (2002). They demonstrated that the *ITGAL* promoter is extensively methylated in fibroblasts, which do not express CD11a, but is largely demethylated in T lymphocytes, which expressed this gene. Because horses are animal species that are characterized by longevity, they suffer from a number of diseases typical for the old age. Therefore, investigations of *ITGAL* locus just like other genes involved in the immune response, may be important in order to find molecular basis of age-related autoimmune disorders in this species.

Material and methods

DNA samples were prepared from a set of three ectodermal (brain, pituitary gland, skin), three endodermal (liver, lungs, thymus) and five mesodermal tissues (heart, kidney, spleen, testis, blood leukocytes) derived from an adult horse (Zyagen, USA). DNA was isolated with the usage of Wizard Genomic DNA Purification Kit (Promega, Poland) and modified with sodium bisulfite using Epitec Bisulfite Conversion Kit (Qiagen, Poland). Detection of sequences with increased CG content was conducted with EMBOSS Cpgplot software (Larsen et al., 1992). Promoter regions were predicted using MatInspector software (Cartharius et al., 2005). Bisulfite-PCR

primers (BSPCR) were designed in the sites free of CG using Methyl Primer Express® Software v1.0 (Life Technologies, Poland) (Table 1). Two step BS-PCR reactions were implemented following conditions described in previous studies (Ząbek et al., 2015) (Table 1). Amplified bisulfite DNA was sequenced with the use of BSPCR primers. Bisulfite sequencing reads were then used for calculations of relative methylation percent based on method proposed by Leakey et al. (2008). To perform this, four-dye-trace value outputs generated by BioEdit software (Hall, 1999) were used to read and calculate primary and secondary peaks from the electropherograms (Excel). In order to confirm the relative PM results for selected samples, the BSPCR products were cloned with the use of TOPO TA Cloning kit (Invitrogen, Poland) to be subsequently sequenced with universal primers. PM values were calculated based on cytosine counts over all of the clones using BISMA software (Rohde et al., 2010). In order to discover potential relationships between methylation status in putative promoter region and the presence of transcripts, RT-PCR was performed using cDNA from a set of RNAs of investigated tissues derived from the horse that was a DNA donor for BSPCR examinations (Zyagen, USA). RT-PCR primers were designed in the splice sites using primer-BLAST (Ye et al., 2012) (Table 2).

Table 1. BS-PCR primers for amplification of bisulfite converted DNA in the predicted promoter region of *ITGAL* (gene annotated from the reverse strand)

CGI sequence	Start	Stop	CG %	BSPCR oligos (5'–3')	Amplicon size (bp)	Ta (°C)
ITGAL NC_009156	19212177	19212980	44.9	F:gtggtatttgattttgtttttga R:aatttccctaatcacaaacaca	906	60/58

Table 2. RT-PCR primers for detection of ITGAL transcript

mRNA sequence	RT-PCR oligos (5'–3')	Start	Stop	Exon junction	Size (bp)	Ta (°C)
ITGAL XM_001496070	F:gctgtgggagcccctctgga	1612	1631	2229/2230 (reverse primer)	629	60
	R:tccttgccctgcgcccttg	2240	2221			

Results

Application of Cpgplot and Matinspector software allowed determining predicted promoter region for *ITGAL* gene (annotated from the reverse strand) in form of CpG island spanning 15 CpG sites in the location of -698 to +208 base pairs (bp) relative to the transcription start site (TSS).

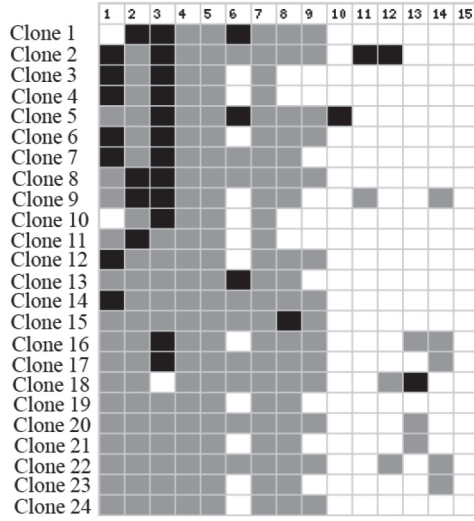

Figure 1. *ITGAL* methylation patterns of blood leukocytes using cloned BSPCR sequencing approach. Black boxes refer to methylated cytosines; gray boxes are unmethylated cytosines; white boxes represent lack of data. CpG 6 includes G>C polymorphic site

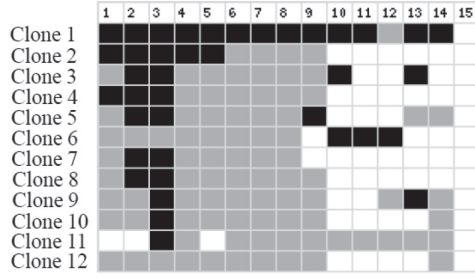

Figure 2. *ITGAL* methylation patterns of the spleen tissue using cloned BSPCR sequencing

The use of BSPCR primers for investigations of methylation percent of *ITGAL* promoter revealed informative data only for the partial number of CpG sites (Figures 1 and 2). The cycle sequencing reads were to some extent of lower quality due to the presence of long homopolymeric stretches generated after bisulfite conversion of DNA. As shown above (Figures 1 and 2), BSPCR amplicons of the two of investigated tissues (blood leukocytes and spleen) were subjected to TOPO TA cloning and sequencing in order to validate the application feasibility of calculation of the relative PM values (Leakay et al., 2008). 24 and 12 BSPCR clones respectively were sequenced with universal primers (Figures 1 and 2). Comparison between PM values obtained after BSPCR cloning and the relative approach proposed by Leakay et al. (2008) showed similar tendency and the most concordant PM results in the first 5 CpG sites (Table 3). Information about the methylation state of CpG 6 was dis-

turbed by G>C polymorphism. In turn, the quality of BSPCR sequencing reads spanning CpG positions from 7 till 14 was insufficient for calculations of relative PM values for the mentioned CpG sites. The limited data mentioned above were excluded from analysis. Differences of methylation percent at the first five CpG sites were detected (Table 3). Methylation percent calculated for particular CpG positions was substantially lower in the case of blood leukocytes, spleen and thymus in relation to the rest of tissue types in this study (Table 3). In these tissues hypomethylation or no methylation was observed. The exception was cytosine hypermethylation (PM > 50%) of CpG 3 detected in the spleen tissue (Table 3). The results of CpG 2 and CpG 3 in the spleen and blood leukocytes respectively were almost contradictory, pointing to putative hemimethylation at both CpG positions (Table 3). The average value of PM across 5 CpG sites was the lowest for the three above mentioned tissues (Table 3). In silico analysis of the sequences covering 5 CpG sites revealed homology to the binding sites of transcription factors mostly expressed in the blood cells (Table 4). The sequences of TF binding sites were in the reverse orientation being concordant with the annotation of *ITGAL* gene. One exception was the forward orientation of a single TF site covering CpG 4 (Table 4). The combination of the data from Tables 3 and 4 allowed showing a link between the occurrence of hypomethylated CpG sites in spleen, thymus and blood leukocytes, and the respective binding sites of certain transcription factors which undergo expression in mentioned tissues (as determined by Matinspector software). Sequence including CpG1 (hypomethylated in spleen tissue) (Table 3) revealed homology to the TF binding site of GATA group of transcription factors which undergo expression in erythrocytes (Table 4). CpG2 sites were hypomethylated in thymus and blood leukocytes (Table 3) and were located in the putative TF binding position of the fork head domain which is expressed in both mentioned tissues and also in the binding site of the heat shock factors being expressed ubiquitously (Table 4). CpG4 was hypomethylated in blood leukocytes (Table 3) and was located in the sequence of binding site specific to glucocorticoid responsive and related elements being expressed in antigen-presenting cells (Table 4). In turn, the nucleotide position of CpG5 (hypomethylated in blood leukocytes) (Table 3) corresponds to the TF anchor position of the nuclear factor of activated T-cells (NF-AT) undergoing expression in leukocytes (Table 4).

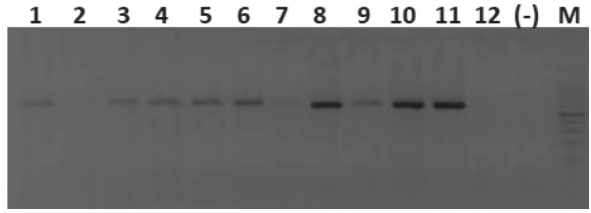

Figure 3. RT-PCR results of *ITGAL* transcripts in the set of 11 equine tissues; 1 – brain, 2 – heart, 3 – kidney, 4 – liver, 5 – lungs, 6 – pituitary gland, 7 – skin, 8 – spleen, 9 – testis, 10 – thymus, 11 – blood leukocytes, 12 – equine control DNA, (–) – negative control, M – 100 bp DNA ladder

Table 3. Percent of methylation (PM) calculated using method of Leakay et al. (2008) and bisulfite cloning approach (selected tissues)

CpG site	CpG position relative to TSS	Brain	Heart	Kidney	Liver	Lung	Pituitary gland	Skin	Spleen		Testis	Thymus	Blood leukocytes	
									relative PM	PM (clones)			relative PM	PM (clones)
1	–649	64.74	95.63	84.28	79.72	86.27	79.43	64.74	0	33.3	96.33	46.18	32.89	31.8
2	–616	77.2	76.19	66.49	55.21	47.25	74.06	77.2	27.19	57.1	75.27	0	1.38	16.7
3	–558	89.39	90.67	89.87	75.27	81.63	96.7	89.39	71.5	78.9	89.44	28.49	34.19	52.2
4	–502	84.6	91.85	84.86	60.74	82.58	90.49	84.6	24.05	11.8	84.9	9.79	2.14	0
5	–463	88.35	58.83	81.77	62.43	53.69	77.03	88.35	27.72	12.5	76.51	17.97	9.97	0
Mean PM		80.86	82.63	81.45	66.67	70.28	74.73	80.86	30.09	38.72	84.49	20.48	16.12	20.14

Table 4. The list of putative TF binding sites (in silico prediction using Matinspector) covering five CpGs of investigated island

CpG site	CpG position relative to TSS	Transcription factor (TF)	Tissue	TF binding site position relative to TSS		TF anchor position relative to TSS	Strand orientation	Sequence of TF binding site
				start	end			
1	–649	GATA transcription factors	Blood Cells, Bone Marrow Cells, Erythrocytes, Hematopoietic System, Immune System, Leukocytes, Lymphocytes	–658	–646	–652	(–)	gacaGATAagccg
2	–616	The fork head domain;	Antigen–Presenting Cells, Blood Cells, Endocrine System, Immune System, Leukocytes, Lymphocytes, Thymus Gland	–622	–606	–614	(–)	ctagaataTACAaagaa
		Heat shock factors	Ubiquitous	–620	–596	–608	(–)	ggactcgtatctAGAAtatacaaag
3	–558	Cellular and viral myb–like transcriptional regulators;	Blood Cells, Bone Marrow Cells, Hematopoietic System, Immune System, Leukocytes, Lymphocytes	–570	–556	–563	(–)	acagAACGggaggaa
		AT rich interactive domain factor	Antibody–Producing Cells, Blood Cells, Immune System, Leukocytes, Lymphocytes	–565	–545	–555	(–)	aaatAATAcccacagaacggg
4	–502	Glucocorticoid responsive and related elements	Antigen–Presenting Cells, Endocrine System, Immune System	–518	–500	–509	(+)	actagctatgcTGTTctgt
5	–463	Nuclear factor of activated T–cells	Blood Cells, Immune System, Leukocytes, Lymphocytes	–472	–454	–463	(–)	agcGGAAagatatccatga

RT-PCR assay with primers designed for mRNA sequence of *ITGAL* locus revealed the highest yield of amplification for the spleen, thymus and blood leukocytes. For the rest of tissues the intensity of RT-PCR band was substantially weaker or even non visible (Figure 3).

Discussion

A number of genes exist which are responsible only for particular biologic processes related to the specialized groups of cells. An example of which is *ITGAL* gene encoding integrin LFA-1 (CD11a/CD18, L 2) involved in inflammatory and immune responses (Lu et al., 2002). The present studies discuss methylation patterns and qualitative transcript abundance of equine *ITGAL* locus which has been previously found to undergo tissue specific transcription in humans (Lu et al., 2002). Unlike the already conducted research on the human cell lines (Lu et al., 2002), this study includes equine tissue samples and blood leukocytes representing heterogeneous mixture of cells. A collection of samples included tissues derived from all three embryonic layers in order to show potential differences. The aim of the present research was to show the magnitude of tissue related differences on the methylation and transcript level unbiased by inter-individual variability. Because of only single horse being the donor of investigated samples, presented results include the preliminary data which might be, however, very useful to answer some further questions about epigenetic background of physiology and pathology of the immune cells of the horse. Observed methylation state of CpG island covering 5' end of *ITGAL* gene was compared to its qualitative transcript abundance in order to find potential interrelations. There is an evidence that the methylation-free state of CGIs is related to their functions as promoters (Deaton and Bird, 2011). It is believed that for around of 70% of genes demethylation of CGI at the 5' end is necessary for their transcription (Day et al., 2013). In turn the hypermethylated state of regulatory regions has been described to be associated with reduced transcriptional activity of genes (Bird, 2002). Further evidence was provided by a number of studies concerning a single locus (Lu et al., 2002) or whole genome approaches (Varley et al., 2013) which revealed a number of genomic sites showing methylation dependent transcription. The comparison of results of 11 tissues in this study revealed certain interrelations between methylation and transcription of equine *ITGAL* gene. Hypermethylation of investigated CpG island observed in case of brain, heart, kidney, liver, lungs, pituitary gland, skin and testis of the horse coincides with qualitatively evaluated smaller amount of *ITGAL* mRNA in comparison to the rest of three other tissues included. This observation is consistent with the repressive effect of methylation on *ITGAL* transcription reported in the functional studies of Lu et al. (2002) conducted on human fibroblasts. In the present research, only three tissues were hypomethylated in the *ITGAL* promoter region parallel to the highest abundance of *ITGAL* mRNA assessed using a non-quantitative approach. *ITGAL* is strongly expressed in blood and blood producing organs, therefore its specificity of detected methylation patterns would be reason-

able. In addition, it was previously found that methylation and chromatin structure may contribute to tissue-specific expression of this locus (Lu et al., 2002). One of the proposed mechanisms which can alter methylation state of promoter regions is the influence of the regulatory elements acting in trans. Namely, a group of transcription factors has been described whose binding gave rise to the promoter hypomethylation and gene transcription (as reviewed by Marchal and Miotto, 2015). In this work, the in silico analysis of the investigated portion of *ITGAL* promoter revealed a couple of TF factors which could play a role in the transcriptional activity of the *ITGAL* in the equine blood leukocytes, T cell producing organs or organs involved in erythrocyte metabolism like spleen. In the case of mentioned equine tissues all of described TF factors possess potential binding affinity to the sequences covering hypomethylated CpG sites of the investigated island. In addition, they were in silico determined to be expressed in the mentioned tissues or cell types. Functional studies would probably provide the information which of them or which couple of them would be important for the *ITGAL* transcription. One of the examples was provided by the study on *IL-2* gene expression in activated naïve CD4 T cells (Murayama et al., 2006). One of the factors driving *IL-2* expression was *IL-2* promoter demethylation connected with the binding of a group of transcription factors including NF-AT (Murayama et al., 2006). Nuclear factor of activated T cells was also described in the conducted research to be potentially relevant for *ITGAL* regulation in equine blood leukocytes. It would be possible that its binding will contribute to the hypomethylated state of *ITGAL* promoter and *ITGAL* expression in equine white blood cells. Despite functional studies, disclosure of the direct interplay between DNA methylation and expression patterns is not straightforward. Methylation level might be also biased by environmental factors and variation of individual methylomes (Lam et al., 2012). Due to this, such study would require investigation of a large cohort of samples including different age and gender as additional factors. Despite that, the present study provides information about regulatory region of *ITGAL* locus in the horse. This knowledge would be important in order to find potential epigenetic mutations (e.g. those disrupting methylation marks in CG context) influencing proper function of equine *ITGAL* gene.

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