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Research paper

Genome-wide DNA methylation analysis of the regenerative and non-regenerative tissues in sika deer (*Cervus nippon*)☆

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ABSTRACT

Deer antlers, the secondary organs of deer, are a unique model to study regeneration of organ/tissue in mammals. Pedicle periosteum (PP) is the key tissue type for antler regeneration. Based on our previous study, the DNA methylation was found to be the basic molecular mechanism underlying the antler regeneration. In this study, we compare the genome-wide DNA methylation level in regenerative tissues (the potentiated PP of antler, muscle, heart and liver) and non-regenerative tissue (the dormant PP) of deer by the fluorescence-labeled methylation-sensitive amplified polymorphism (F-MSAP) method. Our results showed that DNA methylation level was significantly lower in the regenerative tissues compared to the non-regenerative tissue ($P < 0.05$). Furthermore, 26 T-DMRs which displayed different methylated status in regenerative and non-regenerative tissues were identified by the MSAP method, and were further confirmed by Southern blot analysis. Taken together, our data suggest that DNA methylation, an important epigenetic regulation mechanism, may play an important role in the mammalian tissue/organ regeneration.

1. Introduction

The tissue regeneration continues to be a growing concern in recent years. Epigenetic mechanisms play a crucial role in maintaining the regeneration capacity and remodeling the regenerative response. It is well known that all tissues originate from a zygote in individual. It also clearly indicates that a single genome contains the information needed for tissues growing, and possibly for tissue regeneration. However, it is gradually repressed due to the DNA methylation during development. In animals, cytosine methylation of genomic DNA is an important epigenetic modification implicated in many biological processes (Bird, 2002), such as regulation of gene expression (Cedar and Bergman, 2012), cellular proliferation (Wu and Zhang, 2010), differentiation (Alvaro et al., 2007), and genomic imprinting (Gornikiewicz et al., 2013).

Currently, some studies indicate that DNA methylation is critical for the tissue regeneration. It demonstrates that the *sonic hedgehog* gene expression is correlated with the methylation status of the limb-specific *sonic hedgehog* enhancer in *Xenopus* limb regeneration (Yakushiji et al., 2007). And the neonatal murine heart is able to regenerate after severe injury, however, the heart will loses the regeneration capacity when the DNA methylation level and patterns changed (Gornikiewicz et al., 2016). These findings may provide evidences for the possible correlation between the level of DNA methylation and tissue regeneration. However, there is few reports focus on comparing of genome-wide DNA methylation profiles related to tissue regeneration due to the absence of a suitable animal model in mammals.

In recent years, deer antlers have been used as a novel model to study tissue regeneration in mammals (Li and Suttie, 2003). Generally, antlers regenerating from the permanent cranial bony protuberances

Abbreviations: ABI, Applied Biosystems; AFLP, amplified fragment length polymorphism; CG, cytosine-guanine; MSAP, methylation sensitive amplification polymorphism; F-MSAP, fluorescent methylation sensitive amplification polymorphism; PCR, Polymerase Chain Reaction; PP, pedicle periosteum; PPP, potentiated pedicle periosteum; DPP, dormant pedicle periosteum; T-DMRs, tissue-specific differentially methylated regions

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are called pedicles (Li et al., 2009). The pedicle periosteum (PP) plays a key role in antlers regeneration. The PP of the distal pedicle stump (PPP), but not the PP of the proximal pedicle stump (DPP) could regenerate an antler (Rolf et al., 2008). In our previous study (Yang et al., 2016), we found that the DNA methylation level of the PPP was significantly lower than that of the DPP ($P < 0.05$), which was further verified by functional analysis. Our results indicated that DNA methylation was associated with antler regeneration. We demonstrated that DNA methylation was the basic of molecular regulatory for deer antler regeneration, and provided evidence for the correlation between the level of DNA methylation and mammalian tissue regeneration. In mammals, studies have demonstrated some organs had potential regenerative capability, such as muscle, heart and liver (Ferrari et al., 1998; Laflamme and Murry, 2011; Mao et al., 2014). Thus, deer antlers provide a valuable model to evaluate the relationship between DNA methylation and regenerative capability.

With the development of new techniques, DNA methylation can be studied through a variety of methods, such as bisulfite sequencing (BS-Seq), methylated DNA immunoprecipitation (MeDIP), methylation-sensitive amplified polymorphism (MSAP), and others. Among these methods, MSAP is a stable, and high-throughput method to investigate cytosine methylation at the genomic level without a priori knowledge of the genomic sequence (Herrera and Bazaga, 2010; Verhoeven et al., 2010; Wang et al., 2016; Yang et al., 2018). F-MSAP, an improvement of MSAP, is based on fluorescently labeled primers and capillary gel electrophoresis with the internal lane size standard instead of traditional denaturing acrylamide gel electrophoresis and silver staining (Yang et al., 2011). This method has been proven to be more sensitive, safer and more effective than the original MSAP (Wang et al., 2016; Yang et al., 2018).

The purpose of this study was to assess the correlationship between the level of DNA methylation and regenerative capability of tissues. By method of F-MSAP, the genome-wide DNA methylation between the regenerative tissues and the non-regenerative tissue of sika deer was compared and analyzed. Furthermore, several fragments that are differentially methylated in regenerative and non-regenerative tissue types were isolated, sequenced and verified. It may be useful for further investigations into DNA methylation on regulating gene expression during tissue regeneration.

2. Materials and methods

2.1. Ethics statement

Animal experiments were performed in accordance with the guidelines on animal care and use established by Chinese Academy of Agricultural Sciences (CAAS) Animal Care and Use Committee.

2.2. Animal material and genomic DNA preparation

Five tissues (PPP, DPP, muscle, heart and liver) were collected separately from four 2-year-old healthy male sika deer and stored at −20 °C. The detailed protocol for the collection of the PPP and DPP tissues were described by Li and Suttie (2003). Genomic DNA was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's protocols. The quality and concentration of DNA were measured by both agarose gel electrophoresis (1%) and spectrophotometric assays (Thermo Scientific NanoDrop 2000c, USA).

2.3. Fluorescence-labeled methylation-sensitive amplified polymorphism (F-MSAP) procedure

F-MSAP experiments were performed according to the Xiong's protocols (Xiong et al., 1999) with minor modifications. DNA samples were separately digested with the enzyme combinations *Eco*RI/*Hpa*II or *Eco*RI/*Msp*I (TaKaRa, Dalian, China). The digestion reaction was performed in a volume of 20 μl containing 300 ng DNA template, 3 U *Eco*RI, 3 U *Hpa*II (or *Msp*I). The reaction mixture was incubated at 37 °C for overnight and then inactivated at 65 °C for 10 min. The ligation reaction was performed in a volume of 25 μl containing 20 μl digestion mixture, 5 U T4 DNA ligase (TaKaRa, Dalian, China), 5 pmol *Eco*RI adapter, 50 pmol *Hpa*II/*Msp*I adapter and 2.5 μl 10 × T4 ligase buffer. The ligation mixture was incubated at 16 °C for 12–16 h. Pre-amplification reaction was performed in a volume of 50 μl containing 5 μl of ligation product, 40 ng of each pre-amplification primer, 40 ng H-M + 1 primer (Table 1), 0.5 U *Ex* Taq polymerase, 1.6 μl of dNTPs (2.5 mM), 1.2 μl of $MgCl_2$ (25 mM), 2 μl of 10× PCR buffer. Pre-amplifications were programmed as 25 cycles of 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 56 °C for 1 min and 72 °C for 1 min; and extension at 72 °C for 10 min. Selective amplification reaction was performed in a volume of 20 μl containing 5 μl of 20-fold diluted pre-amplification product, 10 ng E + 3 primer, 40 ng H-M + 3 primer labeled with fluorescence dye (Table 1), 0.5 U *Ex* Taq polymerase, 1.6 μl of dNTPs (2.5 mM), 1.2 μl of $MgCl_2$ (25 mM) and 2 μl of 10 × PCR buffer. Selective amplification were programmed as: 13 touch-down cycles of 94 °C for 30 s, 65 °C (subsequently reduced each cycle by 0.7 °C) for 30 s and 72 °C for 1 min; 23 continued cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min; and extension at 72 °C for 7 min.

Table 1
Sequences of adapters and primers used in F-MSAP.

Adapters/primers	Sequence (5′-3′)
*Eco*RI adapter	5′-CTCGTAGACTCGTACC-3′
	3′-CATCTGACGCATGGTTAA-5′
*Hpa*II and *Msp*I adapter	5′-GACGATGAGTCTAGAA-3′
	3′-CTACTCAGATCTTGC-5′
E + 1 primers (PreAmp)	5′-GACTGCGTACCAATTC + A-3′
HM + 1 primers (PreAmp)	5′-GATGAGTCTAGAA[1]CGG + T-3′
E + 3 primers	5′-GACTGCGTACCAATTC + AAC-3′
	5′-GACTGCGTACCAATTC + AAG-3′
	5′-GACTGCGTACCAATTC + ACA-3′
	5′-GACTGCGTACCAATTC + AGT-3′
	5′-GACTGCGTACCAATTC + ACT-3′
	5′-GACTGCGTACCAATTC + AGA-3′
	5′-GACTGCGTACCAATTC + ATG-3′
	5′-GACTGCGTACCAATTC + ATC-3′
HM + 3 primers	5′-FAM[a]-GATGAGTCTAGAACGG + TAA-3′
	5′-FAM-GATGAGTCTAGAACGG + TAT-3′
	5′-FAM-GATGAGTCTAGAACGG + TAG-3′
	5′-FAM-GATGAGTCTAGAACGG + TAG-3′

[a] Primer was labeled with the blue fluorescent dye 5′-FAM (5′-carboxy-fluorescein).

2.4. Sequencing and analysis

The products of selective amplification were sequenced and analyzed according to the Zhao's protocols (Zhao et al., 2015) with minor modifications. Based on the differential methylation sensitivity of isoschizomers *Hpa*II and *Msp*I, cleaved band patterns were divided into three types (Fig. 1): Type I, which represents both bands for *Hpa*II and *Msp*I digestion, indicating no-methylation or inner methylation of single-stranded DNA. In order to simplify the analysis, Type I bands in our study were considered as no-methylation; Type II bands, which represent bands only for *Hpa*II digestion, indicating outer methylation of a single stranded DNA and hemi-methylation at the outer cytosine nucleotide in the CCGG sequence; Type III bands, which represent bands only for *Msp*I digestion, indicating inner methylation of double stranded DNA and full methylation of the CCGG sequence (Table 2).

The methylation ratio was calculated using the following formula:

$$\text{Methylation ratio} = (\text{Type II bands} + \text{Type III bands}) / (\text{Type I bands} + \text{Type II bands} + \text{Type III bands})$$