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Original research article

Bioinformatic analysis of the ssc-miR-146b upstream promoter region

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ABSTRACT

Sus Scrofa microRNA-146b-5p (ssc-miR-146b) was found to be one of differentially expressional microRNAs (miRNA) in our previous study. Not only it is highly expressed but also it maintains the largest up-regulated differences on the expressional level at different time points in the small intestinal mucosa of weaned piglets. To further explore the regulation mechanism of microRNA-146b-5p (miR-146b) during the stressful progress in weaned piglets, the present study predicted the functions of the ssc-miR-146b upstream promoter region using biological analysis. The analytical results showed that ssc-miR-146b is an intergenic miRNA. The length of the promoter region of ssc-miR-146b was predicted to be 2,249 bp using the Ensemble database. The length of the CpG island in the ssc-miR-146b promoter region was found to be 167 bp and it was located from 464 to 630 bp. Twenty six binding sites of 9 transcription factors in the upstream promoter region, including the sites of genes such as *Sp*1, *AP*-1, *MyoD*, *GATA* etc, were discovered using different kinds of analytical software. The predictions of the CpG island and transcription factor binding sites provided significant information for further studying the transcriptional regulation mechanism of ssc-miR-146b on the small intestinal injury due to weaning stress.

1. Introduction

Weaning is one of the most stressful events in a pig's life. Several studies have shown weaning stress mainly targets the small intestine of piglets. Intestinal mucosa injury and inflammatory response induced by weaning stress is one of the most reasons of diarrhea (Campbell et al., 2013; Smith et al., 2010; McLamb et al., 2013). Stress signaling pathways that activated by weaning mediate the process of intestinal mucosal barrier impairment and result in harmful inflammation (Lambert, 2009; Moeser et al., 2007). MicroRNAs (miRNA) have been proved to play an important role in regulating stress signaling pathways. They control intestinal epithelial differentiation, architecture, barrier function, and to some extent, determine the fate of intestinal epithelial cells (Mendell and Olson, 2012; Dalmasso et al., 2010; Mckenna et al., 2010). To date some special miRNA have been found to differentially express during the intestinal physiology and pathology such as pathogen infection (Al-Quraishy et al., 2011), stress-induced injury (Yu et al., 2011), intestinal mucosa immunity (Goto and Kiyono, 2011), intestinal homeostasis (Singh et al., 2012), colorectal cancer (Schetter et al., 2012), and inflammatory disease (Coskun et al., 2012) etc.

Studies confirmed the influences of weaning stress on intestinal injury mainly occur within the first week after weaning (Campbell et al., 2013). Using Solexa high-throughput sequencing technology, our previous research found a large number of differentially expressional miRNA in the small intestinal mucosa of piglets at different time points in the first week after weaning. One of the miRNA is microRNA-146b-5p (miR-146b). It was highly expressed, and it maintained continual and largest differences (Tao and Xu, 2013). These results suggested Sus Scrofa microRNA-146b-5p (ssc-miR-146b) probably plays a vital role in regulating intestinal injury due to weaning stress. The microRNA-146 (miR-146) family includes two members: one is miR-146b located on chromosome 14, and the other is microRNA-146a-5p (miR-146a) located on chromosome 16. They have similar target genes with only two different bases (Kozomara and Griffiths-Jones, 2014). There are more reports related to the regulation functions of miR-146 in human. Especially in recent years, studies have revealed that miR-146 regulates the inflammatory response of human cells,

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including retinal pigment epithelial cells, adipocytes, and monocytes etc (Curtale et al., 2013; Kutty et al., 2013; Shi et al., 2014). However, the role of miR-146b in the intestinal epithelial cells of piglets has been unclear. Therefore, we expect to obtain the information of methylation and transcription factor binding sites by predicting the ssc-miR-146b promoter using biological analysis. The present study provided a fundamental basis for further illuminating the function of miR-146b in the small intestinal injury of weaning piglets.

2. Materials and methods

2.1. The sequence of ssc-miR-146b

MicroRNA-146b-5p is an intergenic miRNA located on porcine chromosome 14 ranging from 123,301,752 to 123,301,850 bp. The precursor sequence is 5′-GAACUUUGGCCACCUGGCUC UGAGAACUGAAUUCCAUAGGCUGUGAGCUCUAGCAAAUGCCCUAGGAACUCAGUU CUGGUGCCCGGCUGUGCUAUAGUC-3′, and the mature sequence from the 5′ arm of the precursor is 5′-UGAGAACUGAAUUCCAUAGGC-3′ (Reddy et al., 2009).

2.2. The methods of biological analysis

The promoter region of miR-146b was obtained using the Ensemble database. Then methylation analysis was done using the MethPrimer software, and potential transcription factor binding sites were predicted using several kinds of software of promoter analysis for promoter region.

2.3. Programs and databases

The databases and programs included miRBase (http://www.mirbase.org/), Ensemble (http://asia.ensembl.org/index.html), TRANSFAC (a database on transcription factors and their DNA binding sites, http://www.biobase-international.com/product/transcription-factor-binding-sites), MethPrimer (http://www.urogene.org/methprimer/index.html), Promoter 2.0 (http://www.cbs.dtu.dk/services/Promoter/), Neural Network Promoter Prediction (http://fruitfly.org:9005/seq_tools/promoter.html) and Promoter SCAN (http://www-bimas.cit.nih. gov/molbio/proscan/).

3. The results of biological analysis

3.1. The ssc-miR-146b promoter region

The precursor sequence of the ssc-miR-146b, ranging from 1 to 99 bp, was obtained using the Ensemble database, but the predicted information of the ssc-miR-146b promoter region was not obtained. Therefore, according to the methods of Zhou and Wu (Zhou et al., 2007; Wu, 2011), we predicted the length of promoter region is 2,249 bp (from 2, 000 bp upstream to 150 bp downstream of the precursor).

3.2. The analysis of the CpG island in the ssc-miR-146b promoter region

The position of one CpG island was predicted using the online program, MethPrimer with default parameters (Criteria used: Island sized > 100 bp, GC% > 50.0%, Obs/Exp > 0.6). As shown in Fig. 1, the full length of the promoter was 2,249 bp; the length of the CpG island, located from 464 to 630 bp, was 167 bp. The predictions suggested the presence of this CpG island can inhibit the ssc-miR-146b promoter transcription.

3.3. The predicted results of the ssc-miR-146b promoter region

The two critical promoter regions of the ssc-miR-146b were predicted using the online program of Neural Network Promoter Prediction with cutoff score 0.80. Their positions of transcription sites are listed in Table 1.

The TATA box of the ssc-miR-146b was found to be missing using the software of Promoter 2.0 with cutoff score 2.0. Therefore, by determining the locations and distances of the GC box, CCAAT box and the cap site using the Neural Network method, we found that the two transcriptional start sites were located on 200 bp and 1,500 bp upstream, respectively, as shown in Table 2.

The ssc-miR-146b promoter was analyzed using the software of Promoter SCAN 1.7 with cutoff score 1.0. The results showed that the position on the forward strand was from 1,075 to 1,325 bp and the predicted score was 58.57 with the cutoff value of promoter 53.00. The location of transcription factor binding sites was from 1,131 to 1,317 bp, and these transcription factors mainly included genes such as *Sp1*, *AP-2* and

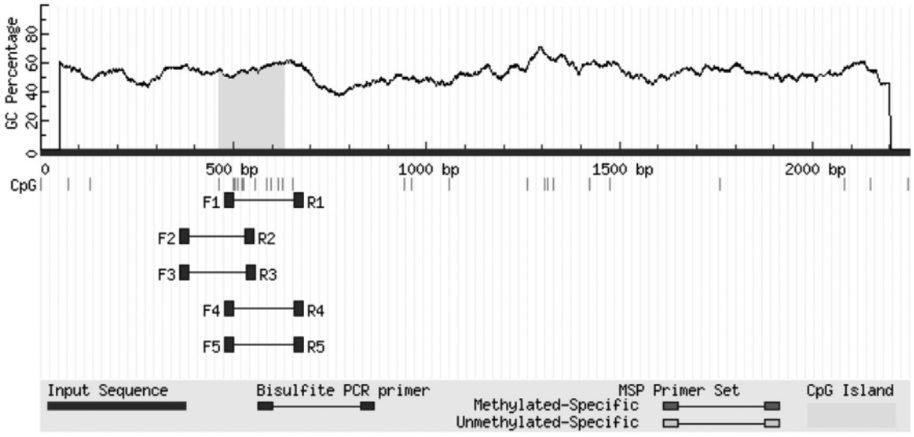

Fig. 1. The methylation predication of ssc-miR-146b promoter region. ssc-miR-146b = Sus Scrofa microRNA-146b-5p; PCR = polymerase chain reaction; MSP = methylated-specific PCR; F = forward; R = reverse; bp = base pair.

Table 1
Promoter predictions of the ssc-miR-146b sequence using cutoff score 0.80.[1]

Start	End	Score	Promoter region
22	72	0.99	TGCCAGCCCTCTATAGATGTGGCCATTCTTCCCCTCCCCCAGCTCCCATC
1,335	1,385	0.95	GTGGGGGCCCTATTTAAGAAGCACTCTCTGGGAGCATCTCTGCAGATCCA

ssc-miR-146b = Sus Scrofa microRNA-146b-5p.
[1] Cutoff score 0.80 was a condition selected using on line Neural Network Promoter Prediction (http://fruitfly.org:9005/seq_tools/promoter.html).

Table 2
Promoter predictions of the ssc-miR-146b sequence using the cutoff score 2.0.[1]

Position	Score	Likelihood
200	0.586	Marginal prediction
1,500	0.638	Marginal prediction

ssc-miR-146b = Sus Scrofa microRNA-146b-5p.
[1] Cutoff score 2.0 was a condition selected using the software Promoter 2.0 (http://www.cbs.dtu.dk/services/Promoter/).

KROX24 etc, as shown in Table 3. Similarly, the position on the reverse strand was from 1,310 to 1,560 bp and the predicted score was 61.24. The location of transcription factor binding sites was from 1,131 to 1,317 bp, and these transcription factors mainly included genes such as *Sp1*, *AP-2* etc, as shown in Table 4.

Table 3
Promoter predictions of the ssc-miR-146b sequence with cutoff score 1.0 on the forward strand.[1]

Gene names and TFD No.	Strand	Location	Weight
JCV_repeated_sequenc			
S01193	+	1,131	1.427
PuF			
S02016	+	1,131	1.082
AP-2			
S01936	−	1,137	1.091
NF-κβ			
S01498	+	1,199	1.080
UCE.2			
S00437	−	1,267	1.216
T-Ag			
S00974	+	1,305	1.086
Sp1			
S00979	+	1,305	6.023
S00326	+	1,305	3.129
S00978	+	1,306	3.013
JCV_repeated_sequenc			
S01193	+	1,306	1.427
Sp1			
S00781	+	1,307	3.191
EGR-1			
S01623	+	1,308	2.151
Sp1			
S00857	+	1,310	4.589
S00802	−	1,311	3.061
S00801	−	1,312	3.119
AP-2			
S01936	−	1,313	1.091
Sp1			
S01187	−	1,313	6.819
S00956	−	1,314	3.129
EGR-1			
S01956	−	1,316	2.294
S01624	−	1,316	1.912
AP-2			
S00346	−	1,317	1.672
EGR-2			
S01957	−	1,317	3.442

TFD = transcription factors database.
[1] Cutoff score 1.0 was a condition selected using the software Promoter SCAN 1.7 (http://www-bimas.cit.nih.gov/molbio/proscan/).

A lot of software can predict promoter region, but each has different algorithms. Therefore, the prediction results of the same gene promoter are different using different software. In the present study, because we predicted the information of the ssc-miR-146b promoter region using three software programs simultaneously, the prediction accuracy of the results could be effectively improved.

3.4. The predictions of transcription factor binding sites

We successfully predicted that the position of the highly reliable ssc-miR-146b promoter region was 22 to 1,560 bp, and it was located on chromosome 14 ranging from 123,299,774 to 123,301,312 bp according to the predicted results using three

Table 4
Promoter predictions of the ssc-miR-146b sequence with cutoff score 1.0 on the reverse strand.[1]

Gene names and TFD No.	Strand	Location	Weight
AP-2			
S01936	−	1,454	1.108
PuF			
S02016	+	1,447	1.391
JCV_repeated_sequenc			
S01193	+	1,447	1.658
T-Ag			
S00974	−	1,428	1.086
UCE.2			
S00437	−	1,426	1.278
GCF			
S01964	+	1,421	2.284
AP-2			
S00346	−	1,341	1.355
S01936	−	1,340	1.108
Sp1			
S01187	−	1,335	8.117
S00801	−	1,334	2.755
PuF			
S00802	+	1,333	1.391
JCV_repeated_sequenc			
S01193	+	1,333	1.658
Sp1			
S02016	−	1,333	3.292
S00781	+	1,329	2.772
S00978	+	1,328	3.361
S01542	+	1,327	3.608
S00979	+	1,327	5.934
S00327	+	1,327	17.211
S00064	+	1,327	6.023
EGR-2			
S01957	−	1,317	10.327
EGR-1			
S01624	−	1,316	9.559
S01956	−	1,316	5.736
Sp1			
S00956	−	1,314	9.386
S00857	+	1,310	4.876

ssc-miR-146b = Sus Scrofa microRNA-146b-5p; TFD = transcription factors database.
[1] Cutoff score 1.0 was a condition selected using the software Promoter SCAN 1.7 (http://www-bimas.cit.nih.gov/molbio/proscan/).

software programs. The predictions of the promoter region showed 323 binding sites of 138 transcription factors on the basis of the transcription factor binding matrix provided by TRANSFAC. Finally, only 26 binding sites of 9 transcription factors were preserved by screening the pig genome, as shown in Table 5.

4. Discussion

The miR-146 family has been much prospectively, widely and deeply studied. Studies on human subjects found that it is involved in some physiological and pathological processes, such as immunity, tumor and inflammation etc (Hulsmans et al., 2012; Monteys et al., 2010; Chassin et al., 2010). Therefore, the change of the ssc-miR-146b transcriptional level is probably related to the functions of the body. Currently, it has been proved that all intergenic miRNA and partial intragenic miRNA have independent transcription elements, and the accurate transcription of these miRNA requires complicated regulatory system (Chou et al., 2013). The factors influencing the transcription of miRNA mainly include methylation, demethylation, inhibited or activated regulatory factors. It suggested that the ssc-miR-146b expression must be regulated by the above factors since it is an intergenic miRNA.

The present study found one CpG island by analyzing the ssc-miR-146b promoter region. This CpG island can probably affect the normal transcription of the promoter. Studies found that the occurrence of tumor is closely related to the DNA methylation. To some extent, the abnormal transcription of oncogenes and cancer suppressor genes involved the DNA methylation (Hoque et al., 2005). Moreover, the development of tumor is usually associated with inflammation. It was shown that the miR-146b inhibited the inflammation in normal breast cells by forming a feedback loop (Xiang et al., 2014), but its expression was under-regulated in tumor cells due to the methylation of promoter, and this further activated inflammatory reaction (Yang and Yu, 2013). It indicated the over-expression of miR-146b in the small intestine of weaned piglets was possibly an inhibitor mechanism of inflammation for self-protection. However, more experiments should be done to confirm whether this mechanism is associated with the CpG island that was predicted in the present study.

Transcription factors are some DNA-binding proteins that could have special interaction with transcription factor binding sites in the promoter region, and they can activate or inhibit genetic transcription. Various stimuli from external environment and signals of developmental stages can activate the binding of transcription factors and transcription regulatory elements. This activation further affects the transcription of miRNA (Kuang et al., 2014). Studies on the miR-146a promoter in humans showed that the binding sites of NF-κB and PLZF significantly affected the miR-146a expression (Pauley and Chan, 2008; Taganov et al., 2006). Similarly, studies on the miR-146b promoter found the transcriptional activity of the 950 bp site and confirmed the activity of the STAT and NF-κB binding sites (Curtale et al., 2013; Kutty et al., 2013; Shi et al., 2014). The present study found that the ssc-miR-146b expression may be regulated by some transcription factors, such as genes *Sp*1, *AP*-1, *MyoD* and *GATA* etc. Among them, the *Sp*1 and *AP*-1 have been widely studied and they are not only closely related to cell proliferation, differentiation and apoptosis but also involved in the body's inflammation and immune response. This suggested that they probably affect the expression of target genes by regulating the miR-146b expression in the small intestine, and finally participate in the stressful progress of weaned piglets. Certainly this deduction should be further confirmed by performing some molecular experiments. Moreover, the predictions of transcription factor binding sites only included what was known. Those that are new and unknown could not be predicted by existing programs, so it may omit some more important biological information. This depends on improving the data of bioinformatics.

Table 5
Predictions of transcription factor binding sites of the ssc-miR-146b promoter.

Gene names and sequences	Position	Strand	Score	*P*-value
MyoD				
CCTCAGCTGATG	946	+	8.49	0.000575
GATCCGGTGTTG	563	+	8.14	0.000750
*Sp*1				
TAGGCATAAA	1,043	+	7.29	0.000350
TGGGCAGGGT	1,228	+	7.18	0.000475
AGGGCAGAGT	1,488	−	6.96	0.000775
GAGGCTGACT	177	+	6.77	0.000825
AAGGCTCGGA	602	+	6.65	0.000975
CREB				
TGACCTCA	1,208	−	9.79	0.000325
NF-1				
CTCCTGTGGAATATGGAGATTCCCAGGCT	669	−	6.42	0.000675
CREB				
ATGAAGCCTCTTCTC	1,376	−	8.09	0.000325
ATGACTCTTCTCTCC	79	+	7.21	0.000725
AP-1				
AGTGACTTTCT	1,525	−	8.88	0.000775
AATGACACACA	428	+	8.41	0.000975
MyoD				
TGCAGCTGAT	927	+	10.42	0.000275
CCCAGCTGGC	1,089	+	8.14	0.000700
NF-1				
AAATGGCAAAAAGACAAA	707	+	12.20	0.000050
TCCTGGCCCAAGACCCTT	147	+	8.33	0.000750
*Sp*1				
TGTAGGTGGAATT	833	−	8.34	0.000300
AAAGGGCCTATTT	1,050	+	7.44	0.000850
GATA				
GATACCTCCT	891	+	11.79	0.000525
GACAAGGCTC	599	+	11.02	0.000925
HNF-1				
AGGGGAAGAATGGCCAC	18	−	7.90	0.000975
*p*53				
AGGCAAGCC	1,455	−	10.92	0.000575
*HOXA*9				
TGAGAGTTAAACTG	1,511	−	6.75	0.000800
TGACACACAAACGG	430	+	8.50	0.000575
TGAGAGTTAAACTG	1,511	−	7.46	0.000925

ssc-miR-146b = Sus Scrofa microRNA-146b-5p.

5. Conclusion

This study predicted the *CpG* island and transcription factor binding sites in the ssc-miR-146b promoter region using the biological analysis. The predictions provided significant information and scientific basis for further studying the transcriptional regulation mechanism of ssc-miR-146b on the small intestinal injury due to weaning stress. However, the above predicted information must be confirmed by the methods of corresponding molecular experiments in view of limitations of bioinformatics.

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