The cloning and activity of human Hes1 gene promoter

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Received June 20, 2017; Accepted September 15, 2017

DOI: 10.3892/mmr.2017.8240

Abstract. The aim of the current study was to obtain and analyze the activity of the human Hes1 gene promoter. The genomic DNA of human HeLa cell was used as template, polymerase chain reaction (PCR) was used to amplify the 5' end sequence of Hes1 gene and then the amplified segment was connected to pMD18-T vector. Subsequently, double enzyme digestion was used for identification and the sequence was detected; the promoter with the correct sequence was inserted into pGL3-Basic, and the sequence was identified by double enzyme digestion. The recombinant DNA with correct sequence was transiently transfected into cervical cancer cells, and the dual luciferase reporter gene assay system was used to detect the activity of the promoter. The results demonstrated that the human Hes1 gene promoter amplified by PCR was the same as that of the sequence in the gene bank, and the dual luciferase reporter gene assay system demonstrated that there was promoter activity in cervical cancer cells. In conclusion, the Hes1 luciferase reporter recombinant vector was successfully established and transfected into HeLa cells to verify that it has promoter activity, and the core area of the promoter has several tumor-promoting and tumor suppressor genes. This provides a basis for understanding the regulatory mechanism of Hes1 transcription and translation.

Introduction

During the development of normal cells, the proliferation and differentiation remain a dynamic balance, which is important to the normal physical function of the body (1,2). Among the pathways that regulate the balance of proliferation and differentiation, Notch-Hes is one of the key pathways (3-5). Previous studies have identified that Hes1 protein is extensively expressed in various cell types in adult mice (6-8). The Hes1 gene is an important member of the basic Helix-Loop-Helix gene family, which is abundantly expressed during embryonic development (9-11). It is located at 3q28-q29 on human chromosome 3, the mRNA contains 1,471 base pairs and 4 exons. The Hes1 protein is encoded by the Hes1 gene and contains 280 amino acids and the relative molecular weight is 29,400 (12,13). As an important downstream effector molecule of the Notch signaling pathway, Hes1 serves an important role in the proliferation and particularly the differentiation of the mammalian cells (14,15). Previously, in the research of cervical caner, it has been identified that Hes1 protein is particularly expressed in the nucleus and cytoplasm of the cervical epithelial cells (16,17). The Hes1 expression levels in the epithelial cells of cervical intraepithelial neoplasia and cervical cancer are significantly increased compared with normal cervical epithelial cells. As the severity of cervical epithelial neoplasia increases, the Hes1 expression is increased (18-20). It was hypothesized that Hes1 protein overexpression may be involved in the carcinogenesis of the cervical epithelium. In the present study, the Hes1 promoter was cloned and the activity was analyzed in order to provide a basis for the research on transcription and regulation of Hes1 during the occurrence and development of cervical cancer.

Materials and methods

Materials. The human cervical cancer HeLa cell line (Institute for Regenerative Medicine, ZhuJiang Hospital of Southern Medical University, Guangzhou, China), GenBank (National Center for Biotechnology Information, Bethesda, MD USA), Dulbecco's modified Eagle's medium (DMEM; GE Healthcare Life Sciences, Logan, UT, USA), fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA), Ex Taq, T4 DNA ligase, pMD18-T vector, restriction enzymes *Sac*I and *Hind*III (all from Takara Bio, Inc., Otsu, Japan), primer synthesis (Sangon Biotech Co., Ltd., Shanghai, China), pGL3-Basic vector, PGL3-control vector, PRL-TK vector, dual-luciferase reporter assay kit (all Promega Corporation, Madison, WI, USA), *Escherichia coli* DH5α (Institute for Regenerative

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Key words: Hes1 gene, promoter, luciferase reporter gene

Table I. Hes1 promoter sequences and their associated transcription factors.

Start point	Sequence (5'-3')
-889	gggattcaagaactaccttgctccgaaaaacctgcatttgtgaggtagaaggcaattttt
-829	ccttttctgcatggaaacaggaaaatttttttggcccttttcctttaccatctactttc
-769	accctcctga atgtaaagtctg*agcgggaactttagatgtgtcggtaactcacattctta
-709	cacccgtcccccctcccccgccccctttaaccactgctgtttttttctttattgttt
	MZF1 SP1 SPY
-649	ataccttaaaaaaaatatgtttcaaatgaacttactacagtcaaagcagctctgttaca
-589	tatgagagagggcataaagagcaaagaccctggctccaaaagaaatagacaagatcaaga
	CdxA SRY Evi1
-529	ccaaagcggaaagaaaaaaaaaatctctaaaccaaagcccagagggagagagcaaagg
	SRY
-469	ttaaaatcctttgattgacgttgtagcctccggtgccctgggctcaggcgcgcgccatt
	SRY
-409	ggccgccagaccttgtgcctggcggccaatgggggggcgcggtccacgagcggtgccgcg
	SP1
-349	tgtctcctcctcccattggctgaaagttactgtggga*aagaaagtttgggaagtttcaca
	SPY Lyf1
-289	cgagccgttcgcgtgcagtcccagatatatatagaggccgccagggcctagggatcacac
-229	aggatccggagctggtgctgataacagcggaatccccgtctacctctctccttggtcct
	GATA-1 C-REL
-169	ggaacagcgctactgatcaccaagtagccacaaaatataataaaccctcagcacttgctc
	CdxA
-109	agtagttttgtgaaagtctcaagtaaaagagacacaaacaaaaaattctttttcgtgaag
-49	aactccaaaaataaaattctctagagataaaaaaaaaaaaaaaaggaaaatgccagctga
	GATA-1
12	tataatggagaaaaattcctcgtccccggtggctgctaccccagccagtgtcaacacgac
72	accggataaaccaaagacagcatctgagcacagaaaggtaagggcggtacctgtatctct
132	ttgcagcccctcaaaattaag

*The area between the stars represents the core sequence. MZF1, myeloid zinc finger 1; Sp1, transcription factor Sp1; CdxA, Caudal-type homeodomain protein A; SRY, sex-determining region Y; Lyf1, lymphoid transcription factor LyF-1; GATA-1, erythroid transcription factor; C-REL, Proto-oncogene c-Rel.

Medicine, ZhuJiang Hospital of Southern Medical University), genomic DNA extraction kit, agarose gel extraction kit, plasmid minipreparation medium kit (DP304; Tiangen Biotech Co., Ltd., Beijing, China), Lipofectamine™ 2000 (Thermo Fisher Scientific Inc.), and electrophoresis-grade agarose (12% separation gel and 5% spacer gel) were used.

Cell culture and the extraction of genome. HeLa cells were cultured in high glucose DMEM containing 10% fetal bovine serum. The cells were maintained at 37˚C, in a saturated humidity and 5% CO_2 incubator, and were passaged every 2-3 days. The cells at the logarithmic phase were used in all the experiments. The TIANamp Genomic DNA kit (DP304) was used to extract the genomic DNA of HeLa cells, and all procedures were conducted according to the manufacturer's protocol (Tiangen Biotech Co., Ltd.).

Amplification of human Hes1 gene promoter and the purification of the product. The primer was designed according to the 5'end of Hes1 gene from GenBanksp16 (ncbi.nlm.nih.gov/nuccore/NT_005612.17?from=100428715&to=100434890&report=genbank). The Hes1 gene sequence was analyzed using BIO-XM™ (Biomax Informatics AG, Planegg, Germany) and the -747-+66 segment at the 5' end was amplified. The following primers were used: Upstream, 5'-CGAGCTCAGCGGGAACTTTAGATGTG-3' and downstream, 5'-CCCAAGCTTGTTGACACTGGCTGGGGTA-3'. The underlined parts indicate the enzyme sites-*Sac*I and *Hind*III. The genomic DNA of HeLa cells was used as a template, and

Table II. Analysis of Hes1 promoter activity following HeLa cell transfection.

Group	n	Relative luciferase activity	F-value	P-value
pGL3-Basic	3	1.076±0.214	84.434	<0.001
pGL3-Hes1-promoter	3	34.44±13.76		
pGL3-Control	3	100.47±9.13		

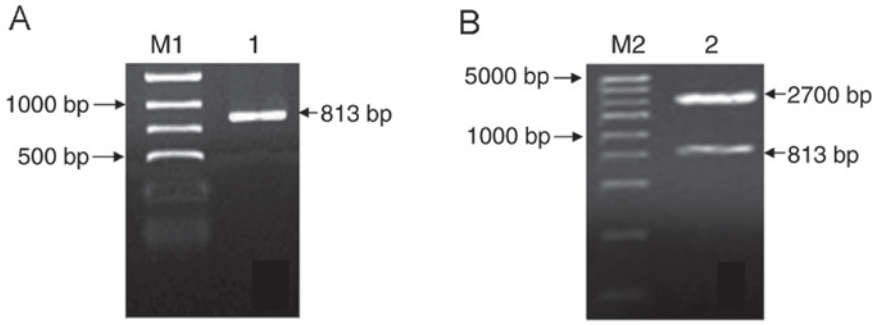

Figure 1. (A) The PCR amplification product and (B) dual-enzyme digestion product of the Hes1 promoter. M1, DNA Marker 2000; M2, DNA Marker 5000; 1, PCR product; 2, the product of recombinant plasmid pMD18-T-Hes1-promoter after *Sac*I and *Hind*III dual enzyme digestion. PCR, polymerase chain reaction; bp, base pairs.

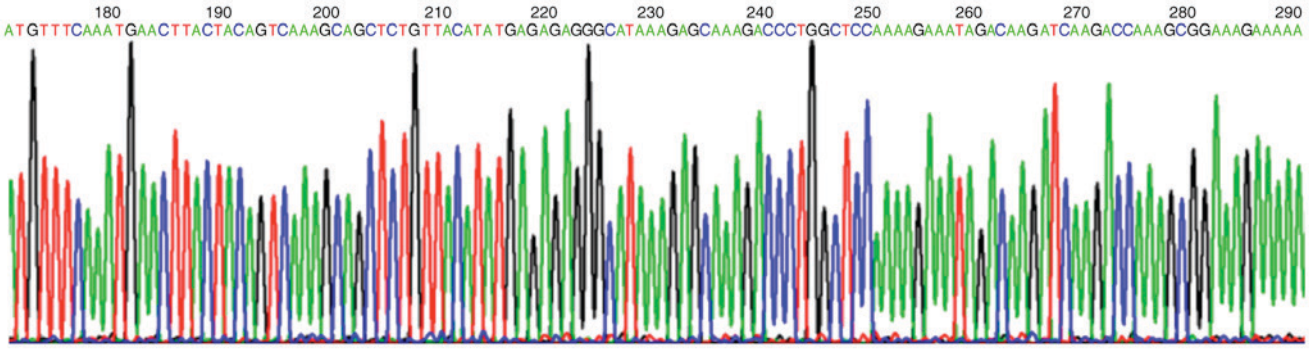

Figure 2. The sequence image of recombinant T cloning vector pMD18-T-Hes1-promoter.

polymerase chain reaction (PCR) was used to amplify the Hes1 promoter segment. The Hes1 promoter sequences are listed in Table I.

The amplification conditions were pre-denaturation at 95˚C for 5 min, denaturation at 95˚C for 30 sec, annealing at 56.2˚C for 30 sec, elongation at 72˚C for 45 sec, there were 35 cycles in total, followed by elongation at 72˚C for 10 min. Following PCR, 5 μl product was added in 1.5% agarose gel for electrophoresis. Subsequently, the gel extraction kit was used for the purification of the PCR product. The procedures were conducted according to the manufacturer's protocol [DNA amplification and extraction kit (cat. no. ER103; Tiangen Biotech Co., Ltd.)].

The connection and identification of purified target segment and cloning vector. The collected PCR product (Hes1 gene) was transfected by Lipofectamine™ 2000 into the pMD18-T vector, which was transformed and amplified to extract the plasmids. Subsequently, restriction enzymes for *Sac*I and *Hind*III dual-enzyme digestion were used and the sequences were detected, which was termed the pMD18-T-Hes1-promoter.

The establishment and identification of recombinant expression vectors. The recombinant T vector with the correct sequence and pGL3-Basic plasmid received *Sac*I and *Hind*III dual-enzyme digestion and separated by agarose electrophoresis, the gel extraction kit was used to collect the target segment, and then T4 DNA ligase was used to transform, amplify and extract the plasmids. The product received

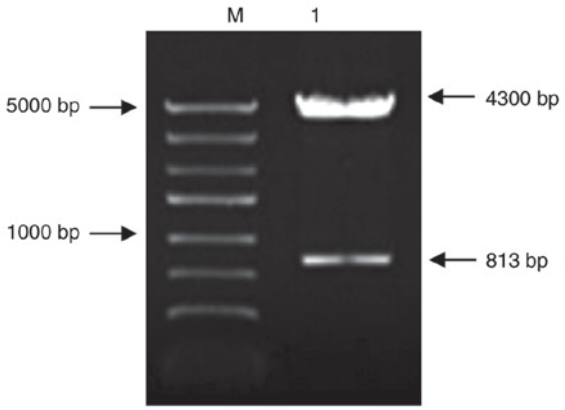

Figure 3. Electrophoresis image of recombinant expression vector after *Sac*I and *Hind*III dual-enzyme digestion. M, DNA Marker 5000; 1, the dual-enzyme digestion product of recombinant expression vector pGL3-Hes1-promoter. bp, base pairs.

dual-enzyme digestion and sequence detection, which was termed the pGL3-Hes1-promoter.

Transfection of recombinant expression vector into the cells and activity detection. The HeLa cells were inoculated in 24-well plate at 4 h before transfection, 0.5 ml complete medium and $0.5x10^5$ cells were added in each well and the transfection was conducted when the cells reached 70-80% confluency. The procedures were done according to the manufacturer's instructions for Lipofectamine 2000. The transfected plasmids included negative control plasmid pGL3-Basic, positive control

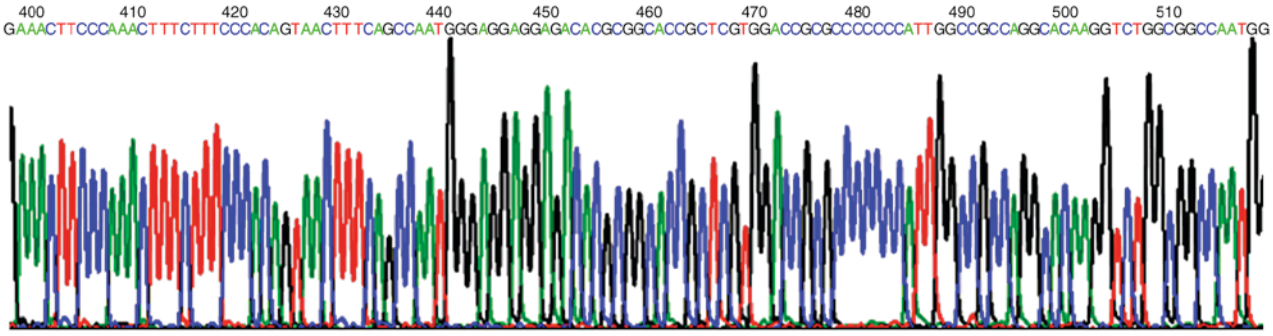

Figure 4. The sequencing image of recombinant expression vector of pGL3-Hes1-promoter.

plasmid pGL3-Control containing the SV40 enhancer/promoter, recombinant plasmid pGL3-Hes1-promoter and internal control PRL-TK plasmid. A total of 48 h after transfection, the Dual-luciferase reporter assay kit from Promega was used to detect the activity of the transfected plasmid, and there were 3 repeated wells for every group and the experiments were repeated 3 times.

Statistical analysis. The data were analyzed by SPSS software, version 18.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance and the Q test were used to analyze the data. The data were presented as mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Target segment after PCR product electrophoresis. The genomic DNA of HeLa cell was used as a template and the promoter segment of the Hes1 gene was amplified by PCR, and the electrophoresis results indicated that there was a specific band at 813 base pairs (bp), as presented in Fig. 1A.

Enzyme cutting identification of recombinant T cloning vector. The PCR production of the Hes1 promoter was purified and connected to the pMD18-T vector to establish the recombinant vector pMD18-T-Hes1-promoter. Subsequent to transformation the plasmids were extracted for *Sac*I and *Hind*III dual enzyme digestion. The electrophoresis results demonstrated that there were two specific bands at 813 and 2,700 bp, as presented in Fig. 1B.

Sequence detection of recombinant T cloning vector. DNA sequence analysis demonstrated that compared with the Hes1 gene promoter sequence from GenBank, the 5' and 3' ends of the cloned target segment of recombinant T cloning vector were located upstream -747 bp and downstream +66 bp of ATG, the size was 813 bp as presented in Fig. 2.

Electrophoresis results of recombinant expression vector pGL3-Hes1-promoter after dual enzyme digestion. The electrophoresis of recombinant expression vector pGL3-Hes1-promoter after *Sac*I and *Hind*III dual-enzyme digestion demonstrated that there were specific bands at 813 and 4,300 bp, as presented in Fig. 3.

Sequencing results of recombinant expression vector. DNA sequencing results demonstrated that compared with the Hes1 promoter sequence from GenBank, the 5' and 3' end of the target segment sequence of cloned expression vector pGL3-Hes1-promoter located at upstream -747 bp and downstream +66 bp of ATM, the size was 813 bp, as presented in Fig. 4.

Activity analysis of recombinant expression vector following HeLa cell transfection. At 48 h after HeLa cell transfection by recombinant expression plasmid pGL3-Hes1-promoter, pGL3-Basic and pGL3-Control, the luciferase expression was detected. The ratio of first and second fluorescence was defined as the relative luciferase activity. The relative luciferase of pGL3-Basic was 1.076, of pGL3-Control was 100.47 and of pGL3-Hes1-promoter was 34.44, there was significant difference ($P<0.05$; Table II).

Discussion

During the normal development of cells, the proliferation and differentiation remains in dynamic balance, which is important for normal physical function (21-23). The pathways to regulate the balance between cell proliferation and differentiation include the leukemia inhibitory factor-bone morphogenetic protein pathway, octamer-binding transcription factor 3/4 pathway, Wnt pathway and Notch-Hes pathway (24,25). These pathways are composed of various cytokines and transcription factors and among these pathways, the Notch-Hes signaling transduction pathway is the most important pathway. If there is abnormity of Notch signaling pathway, the cells cannot differentiate normally, thus there is possibility of oncogenesis, and it has been reported that the Notch receptor is abnormally expressed in numerous human solid tumor types including cervical, head and neck, endometrial, kidney, lung and breast cancer, pleural mesothelioma and salivary gland tumors) and hematological malignancies (26-28).

Cervical cancer is the second most common type of female malignant cancer, following breast cancer in developing countries and the most common type of genital cancer in Asia (29). The morbidity of cervical cancer is increasing and the mortality is the second highest cause of cancer-associated mortality in women in Asia (29-31). In the early stages of cervical cancer, there is usually no symptoms and the physical signs are not obvious, however early diagnosis and early

treatment are the key methods to increase the survival rate. Previous studies have demonstrated that the low degree of cell differentiation is associated with poor prognosis (32-34). As an important downstream target gene of Notch signaling pathway, Hes1 genes can directly combine with specific DNAs to block the pathway activation and inhibit cell differentiation (35,36). Thus, exploring the regulatory mechanism of its transcription is significant in the prevention and treatment of cervical cancer.

The regulation of gene expression in eukaryotic cells include transcriptional-level regulation and post-transcriptional regulation according to the sequencing, and transcriptional-level regulation is the predominant method (37). Transcriptional-level regulation is the most important step in the regulation of gene expression in eukaryote, which mainly depends on the effects of certain regulatory sequences (including the promoter and enhancer) and certain protein factors (such as transcription factors) on the initiation of transcription. The key regulation of gene expression is on a transcriptional level, and the promoter is the most important domain in transcriptional regulation. Thus, the study on Hes1 is important. The luciferase reporter gene vector doesn't contain the promoter and enhancer, which can clone the target gene into the expression plasmid vector containing the reporter gene. The expression of luciferase is associated with the promoter sequence in the recombinant plasmid. Subsequent to transfection, the expression of luciferase activity can directly reflect the activity of the promoter. At present, this method is widely applied (12,13). The novelty of the current study is that the promoter sequence was newly designed.

In the current study, the Hes1 luciferase reporter recombinant vector was successfully established and transfected into HeLa cells to verify that it had promoter activity, and the core area of the promoter had several tumor-promoting and tumor suppressor genes. This provides a basis for the further study of the regulatory mechanism of Hes1 transcription and translation. In addition, it can provide novel strategy, target and experimental evidence for the prevention and treatment of cervical cancer.

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