International Journal of Biological Macromolecules 108 (2018) 515–522

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Vigilin interacts with CTCF and is involved in the maintenance of imprinting of *IGF2* through a novel RNA–mediated mechanism

Xiaoqin Yu[a,1], Qiuying Liu[a,1], Jinyang He[a], Yuan Huang[a], Lei Jiang[b], Xiaoyan Xie[a], Ji Liu[a], Lihong Chen[a], Ling Wei[a,*], Yang Qin[a,*]

[a] *Department of Biochemistry and Molecular Biology, West China School of Basic Medical Sciences & Forensic Medicine, Sichuan University, Chengdu, 610041, Sichuan Province, China*
[b] *School of Biomedical Science, Hong Kong University, Hong Kong, China*

ARTICLE INFO

Article history:
Received 17 April 2017
Received in revised form
15 November 2017
Accepted 16 November 2017
Available online 20 November 2017

Keywords:
Imprinting
IGF2
CTCF
Vigilin
H19 lncRNA

ABSTRACT

Accumulating evidence has revealed the imprinting of insulin-like growth factor-2 gene (*IGF2*) is maintained by binding of CCCTC binding factor (CTCF) to the unmethylated imprinting control region (ICR) between *IGF2* and *H19* genes. We have previously reported that high-density lipoprotein binding protein (HDLBP/vigilin), a multiKH-domain protein, interacts with CTCF and coexists with it at several CTCF-binding sites on the ICR to regulate general gene expression of *IGF2*. However, the impact of the interaction on imprinting of *IGF2* remains unclear. Here, we demonstrate that cooperation of vigilin and CTCF protects *IGF2* from losing of imprinting. Pull-down experiments show that KH1-7 domains of vigilin interact with zinc-finger domains of CTCF. We also display that some RNAs participate in the vigilin-CTCF interaction, one of which is H19 long noncoding RNA (lncRNA). Furthermore, we confirm that H19 lncRNA−knockdown alters the imprinting of *IGF2*. These data suggest that vigilin interacts with CTCF, mediated by H19 lncRNA, to keep the imprinting of *IGF2*.

1. Introduction

Imprinting is an epigenetic phenomenon in which some certain genes silence from one parental allele due to epigenetic modifications, resulting in monoallelic expression, that is known as maintenance of imprinting (MOL) [1]. Maintenance of normal imprinting is essential for development and growth of human [2]. Aberrant imprinting is a cause of many congenital diseases and various types of cancers, such as Beckwith-Wiedemann and Russell-Silver Syndromes, esophageal squamous cell carcinoma, breast cancer, lung cancer, prostate cancer hepatocellular carcinoma [3–7]. Abnormal imprinting, in which the silenced allele of imprinting gene is reactivated (which leads to expression of both paternal and maternal alleles), is defined as loss of imprinting (LOI) [1].

IGF2 and *H19,* adjacently located at human chromosome 11p15.5, are two most widely studied imprinted genes [8]. Normally, *IGF2*, which is transcribed and translated into a potent growth factor, is expressed exclusively from paternal allele [9], while, *H19*, which transcribes into two lncRNAs, H19 lncRNA and 91H lncRNA, is almost transcribed from maternal allele [10]. LOI of *IGF2* or *H19* commonly occurs in tumors and is involved in tumorigenesis [4–7]. Extensive research on *IGF2/H19* imprinting has revealed a complicated regulating network [11–15]. The best-known mechanism is that the imprinting of *IGF2/H19* almost depends on the binding of CTCF to the differentially methylated region, ICR, located between *IGF2* and *H19* genes [16]. The ICR displays a parental-origin-dependent methylation, that is methylated in paternal allele and unmethylated in maternal allele. CTCF bind to the unmethylated CTCF binding sites on the maternal allele to form an insulator between *IGF2* and downstream enhancer, which prevents enhancer from activating *IGF2* promoter, leading to the silence of *IGF2* and activation of *H19*. On the contrary, the paternal allele keeps a methylated ICR, which prevents CTCF from binding to the ICR and allows the enhancer to activate the *IGF2* promoter, resulting in *IGF2* activation and *H19* silence [17,18].

Functions of CTCF in regulation of genes, especially in the regulation of the *IGF2/H19* imprinted gene, need several cofactors to work together, such as chromodomain helicase protein CHD8 [18], DEAD-box RNA-binding protein p68 [19], PRC2 complex [20] et al. And it is reported that CTCF recruits cohesin complex to its binding sites, and the presence of cohesin is essential for insulator

* Corresponding authors.
E-mail addresses: weiling@scu.edu.cn (L. Wei), qinyang@scu.edu.cn (Y. Qin).
[1] Joint first authors.

https://doi.org/10.1016/j.ijbiomac.2017.11.109
0141-8130/

activity of CTCF [21]. Another report shows that CTCF recruits the PRC2 complex to induce allelic silencing of *IGF2* on the maternal allele through histone K27 methylation by interacting with SUZ12, thereby allowing the exclusive expression of *H19* from the maternal allele [20].

Vigilin, an RNA binding protein, is a ubiquitous and highly conserved protein in almost all eukaryotic organisms [22]. It is supposed to have many functions, such as regulating RNA metabolism, maintaining heterochromatin structure and chromosome segregation, et al. [23–25]. A most striking feature of vigilin is the presence of 14 tandem hnRNP KH domains, which are involved in nucleic acid binding and protein–protein interactions [22]. It is reported that vigilin interacts with SUV39H1 through KH13-14 to take part in vigilin-mediated gene silencing[26].Our previous study has demonstrated that vigilin coexists with CTCF at several CTCF binding sites, and participates in gene expression regulation of *IGF2* [27], however, it is still unclear whether vigilin participates in imprinting regulation of *IGF2* and how vigilin interacts with CTCF.

In this study, we aimed to investigate the impact of the interaction between vigilin and CTCF on imprinting of *IGF2*. So we performed RT PCR-RFLP and Allele-specific RT-qPCR to confirm the alteration of imprinting of *IGF2* after knocking down or overexpressing vigilin and CTCF in MCF-7 and MDA-MB-231 cell lines. We also performed pull-down and immunoprecipitation to determine the details of vigilin-CTCF interaction.

2. Materials and methods

2.1. Cell culture and transient transfections

Human breast cancer cell lines MCF-7 and MDA-MB-231 were cultured in DMEM (GIBCO, USA) and RPMI 1640 medium supplemented with 10% newborn calf serum respectively and maintained in a 5% CO_2 incubator at 37 °C. 1×10^5 cells were seeded in 6-well dishes. Cells were allowed to grow to 80% confluence and then were transfected with specific plasmids by using Lipofectamine2000 (Invitrogen, USA) according to the manufacturer's protocol. Then let the cells grow at 37 °C for an additional 48 h or 72 h before harvesting.

2.2. Antibodies

The antibodies used in this study were: rabbit anti-HDLBP/vigilin antibody (MBL, JPN), mouse anti-CTCF antibody (Abcam, USA), mouse anti-beta actin antibody, goat anti-mouse IgG antibody and goat anti-rabbit IgG antibody (ZSGB-BIO, CHN).

2.3. Construction of recombinant plasmids

For GST-Pull down assay, *vigilin* (GenBankNM_005336.4) cDNA fragments were amplified by PCR, then cloned into the pGEX-5X-3 vector and confirmed by sequencing. The primers were listed in Table S1. *CTCF* (GenBank: NM_006565) cDNA fragments were cloned into the pGEX-4T-2 vector [28]. For RNA interference experiments, two pairs of shRNA sequences were designed and BLAST searched against the human genome to ensure that they were specific for H19 lncRNA. The primers were listed in Table S2. These oligonucleotides were synthesized by Invitrogen. And the specific shRNA duplexes were inserted into AgeI and EcoR I double-digested plk-puro 0.1 vector (BD Bioscience Clontech, USA) to generate plk-H19lncRNA (sh-H19lncRNA1,2). pSIREN-vigilin1, pSIREN-vigilin2, pSIREN-CTCF1, pSIREN-CTCF2 were used for vigilin or CTCF-knockdown [27]. pSIREN-luciferase was used as the control for nonspecific effect.

2.4. DNA extraction and PCR-RFLP experiment for genotype analysis

The Apa I polymorphic site in exon 9 of *IGF2* and the Alu I polymorphic site in exon 5 of *H19* were used to evaluate the genotypes of *IGF2* and *H19* (Fig. S1), respectively [27]. Genomic DNA of MCF-7 and MDA-MB-231 cells were analyzed to identify heterozygosity of *IGF2* and *H19* by using PCR and restriction fragment length polymorphism (RFLP). PCR amplification was performed with 30 cycles under conditions of 30 s at 94 °C, 30 s at optimal annealing temperature (55 °C for *IGF2* and 58 °Cfor *H19*), and 30 s at 72 °C. Primers were listed in Table S3. The purified PCR products of *IGF2* were digested with Apa I, and *H19* were digested with Alu I . Then electrophoresis was performed to analyze the genotype of *IGF2* and *H19*, separately.

2.5. RNA extraction and RT PCR-RFLP to determine imprinting of IGF2

Total RNA was extracted from cells by using Trizol (Invitrogen, USA) according to the protocol from manufacturer. cDNA was prepared as follows: 2 μg of total RNA was treated with 10 units of RNase-free DNase I (Fermentas, LTU) at 37 °C for 30 min to eliminate genomic DNA contamination, and then reverse transcription was performed with M-MLV reverse transcriptase (TaKaRa, JPN) according to the instruction for cDNA synthesis. A 2 μL cDNA was used as the template to amplify the *IGF2* with the same primers and reaction conditions as described above in 2.4. The products of the RT-PCR were digested with Apa I, then electrophoresed for imprinting analysis.

2.6. Western blot

The cells were resuspended and then lysed for 5 min on ice with RIPA buffer (Beyotime, Jiangsu, CHN) supplemented with complete protease inhibitor cocktail tablet (Roche, GER). Total proteins were obtained by centrifugation at 12000 rpm for 10 min at 4 °C. After centrifugation, extracted proteins were separated by SDS–PAGE and transferred to PVDF membranes (Millipore, USA). Membranes were blocked for 2 h using 5% milk powder in TBS-T. Immunoblotting was then performed with the indicated antibodies.

2.7. RT-q PCR

cDNA was prepared as in 2.5. The relative expression of *vigilin, CTCF, IGF2* and *H19* were measured by Quantitative Real-time PCR with SYBR premix 2xTaq (TAKARA, JPN). transcriptional level of target genes was normalized by β-actin mRNA level. The primers required in this part were designed as in the Table S3.

2.8. Allele-specific RT-qPCR to confirm imprinting of IGF2

The RNA specific product (1120 bp) was amplified by an exon-connected primer pair, P1 and P3 (Fig. S1), which allowed a discrimination of the RNA specific product from the genomic DNA specific product (1400 bp). Cycling conditions were an initial denaturation at 94 °C for 1.5 min, followed by 40 cycles of denaturation at 94 °Cfor 30 s, annealing at 55 °C for 30 s, and then extending at 72 °C for 1.5 min, with a final extension at 72 °C for 5 min. For further elimination of genomic DNA contamination, the products were separated by electrophoresis on two percent agarose gel and purified by using DNA purification kit II (OMEGA, USA). Then the purified product was subjected to a Quantitative Real-time PCR with allele-specific primers. The allele-specific primers were designed as their 3′ ends have a particular base for Apa I polymorphism located in exon 9 of *IGF2* as reported [29]. Briefly, the primer ApaT-R, recognizes 'allele A', which contains the Apa I site, while the other,