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Original Article

NUDT21 regulates 3′-UTR length and microRNA-mediated gene silencing in hepatocellular carcinoma

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ABSTRACT

Recent studies have shown that several microRNAs (miRNAs) are involved in hepatocellular carcinoma (HCC) tumorigenesis and metastasis; however, the mechanisms responsible for the differences in the functions of these miRNAs in liver cancer remain a mystery. In our previous study, we identified NUDT21 as an interaction partner of argonaute 2 (AGO2). NUDT21 has been reported to be involved in alternative polyadenylation (APA); thus, the interaction between NUDT21 and AGO2 may be a key component of the crosstalk between APA and miRNA-mediated gene silencing in HCC. Our data showed that NUDT21 expression was decreased in HCC. Moreover, our results showed that NUDT21 co-localized with AGO2 in P/GW bodies in normal liver cells; however, this co-localization was diminished in cancer cells. Functional studies showed that NUDT21 elongated the 3′-UTR of mRNA and enhanced the efficiency of miRNA-mediated gene silencing by increasing the efficiency of AGO2-mRNA binding, which played an important role in cell proliferation. In summary, loss of NUDT21 shortened the 3′-UTR of various oncogenes in HCC cells. The shorter 3′-UTR contained less miRNA binding sites, which enabled the oncogenes to evade miRNA regulation and become overexpressed in HCC, leading to unregulated cancer cell proliferation.

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Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related mortality worldwide. Many risk factors, such as cirrhosis, viral infections, alcohol abuse and toxic chemical exposures, may play a role in the development of HCC [1]. However, dysregulated gene expression and accumulated gene mutations in hepatocytes are the main causes of HCC. Numerous genes are regulated by microRNAs (miRNAs) in HCC; thus, miRNA may be involved in HCC tumorigenesis and metastasis. Recent studies of HCC pathology have revealed that individual miRNAs play crucial biological roles in many cellular processes [2]; however, the mechanisms responsible for the differences in the functions of these miRNAs in liver cancer remain a mystery.

A previous work showed that post-transcriptional regulatory processes, such as alternative splicing or alternative polyadenylation (APA), can generate several different isoforms of a primary transcript. This study also determined the efficiency of miRNA-mediated gene silencing [3]. In cancer cells, substantial amounts of mRNA isoforms with shorter 3′ untranslated regions (3′-UTRs) are expressed as a result of APA [4]. mRNAs with shorter 3′-UTRs exhibit increased stability, as these mRNAs contain less miRNA binding sites and are thus able to evade miRNA-mediated repression [5]. APA is prevalent in at least 50% of genes in mammalian genomes [6]. The following three sequence elements precisely determine the 3′-end cleavage and polyadenylation sites in pre-mRNAs: 1. the poly(A) signal (AAUAAA or its variants); 2. the actual polyadenylation site (PAS), which is 11–30 nt downstream of the poly(A) signal; and 3. the G-U rich sequence, which is 14–70 nt downstream of the PAS [7]. At least 5 proteins, including cleavage stimulatory factor (CstF), poly(A) polymerase (PAP), cleavage factors I and II (CFI and CFII), cleavage and polyadenylation specificity factor (CPSF), and poly(A) binding protein (PABP), participate in polyadenylation [8]. These proteins bind to the precursor RNA to

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List of abbreviations

Ab	antibody
AGO2	argonaute 2
APA	alternative polyadenylation
BRCA	breast invasive carcinoma
CFI	cleavage factor I
CFII	cleavage factor II
CPSF	cleavage and polyadenylation specificity factor
CstF	cleavage stimulatory factor
FBS	fetal bovine serum
HCC	hepatocellular carcinoma
H&E	hematoxylin-eosin
IHC	immunohistochemistry
IP	immunoprecipitate
KD	NUDT21-knockdown
miRNA	microRNA
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide
NAT	normal adjacent tissue
OE	NUDT21-overexpressing
PAP	poly(A) polymerase
PAS	polyadenylation sites
PABP	poly(A)-binding protein
PFA	paraformaldehyde
PRAD	prostate adenocarcinoma
RIP	RNA immunoprecipitation
RISC	RNA-induced silencing complex
THCA	thyroid carcinoma
WB	western blot

form a complex prior to the cleavage and polyadenylation reactions and determine length of the mRNA 3′-UTRs.

With the help of RNA-induced silencing complexes (RISCs), miRNAs match with their complementary sequences within the 3′-UTRs of mRNAs and induce gene silencing by facilitating either mRNA degradation or translation inhibition [9,10]. Thus, 3′-UTR length is essential for efficient miRNA-mediated gene silencing. In our previous study, our co-immunoprecipitation and mass spectrometry results showed that argonaute 2 (AGO2), the catalytic engine of the RISC, interacted with both PABPC1 and NUDT21 (also known as CPSF5, CFIM25) in liver tumors [11]. As PABPC1 and NUDT21 both participate in APA, it is possible that APA plays a mechanistic role in miRNA regulation. In our previous study, we found that PABPC1 increased the recruitment of mRNA to the RISC and enhanced the efficiency of miRNA-mediated inhibitory processes in HCC [11]. In this paper, we will discuss the biological role of NUDT21 in miRNA-induced gene silencing and HCC development.

NUDT21 is a highly conserved component of CFIM that participates in an early step in the assembly of eukaryotic pre-mRNA [12]. A previous work showed that NUDT21 and CFIM68 bound specifically with two UGUA elements upstream of the poly(A) site as a dimer and formed a loop, which prevented CPSF-mediated pre-mRNA cleavage [12,13]. Further study demonstrated that NUDT21 was a key regulator of 3′-UTR length, as NUDT21 knockdown induced proximal poly(A) site selection in several genes with APA and thus reduced the length of their 3′-UTRs [8,14]. As NUDT21 levels were decreased in HCC, and NUDT21 interacted with AGO2 in liver tumors, we hypothesized that loss of NUDT21 in HCC decreased the stability of 3′-UTRs with a distal poly(A) tail, resulting in the loss of several miRNA binding sites. Shortening of the 3′-UTRs of various oncogenes enabled the oncogenes to evade miRNA regulation and thus induced HCC tumorigenesis and metastasis.

Materials and methods

Clinical cancer samples and cell lines

Four cancer samples were obtained from the Third Xiangya Hospital of Central South University and stored in liquid nitrogen until analyzed. The demographic features of all the patients whose tissue samples were used in the study are listed in supplementary file 1. The study was approved by the Ethics Committee of the Third Xiangya Hospital of Central South University, and all experiments were conducted in accordance with the provisions of the Declaration of Helsinki and good clinical practice guidelines.

HepG2 and Bel7402 cells were obtained from the Xiangya Experiment Center (Changsha, China), Female human plateable hepatocytes were purchased from the Research Institute for Liver Disease Co., Ltd (Shanghai, China). All the cells were authenticated before being used. The HepG2 cells were cultured in high-glucose DMEM (HyClone, Logan, UT, USA), and the Bel7402 cells were cultured in RPMI 1640 medium (Gibco, Carlsbad, California, USA), Both types of media were supplemented with 10% fetal bovine serum (FBS) (HyClone) and 1% penicillin and streptomycin sulfate (Solarbio, Beijing, China). The female human plateable hepatocytes were cultured in hepatocyte culture medium using routine culture methods. All the cells were maintained at 37 °C with 5% CO_2, and the medium was changed every 2 or 3 days.

Bioinformatics analysis

The Cancer Genome Atlas (TCGA) database was analyzed by maplab (http://maplab.imppc.org/wanderer/) to identify the genes that are up-regulated in HCC. To select the pattern genes, we screened 57905 genes encoding a total of 196502 transcripts from the Ensembl Human Genome Database. The following genes were selected: 1. genes containing two or more 3′-UTR transcripts with the same starting point but different ending points, 2. genes containing a poly A tail signal sequence (PAS: AAUAAA or a similar sequence) 10–60 nt upstream of their 3′-ends and 3. genes featuring proximal PASs within their long 3′-UTR transcripts that were surrounded by 2 UGUA sequences located within 200 nt of the PASs.

Western blotting

The cells were lysed in RIPA buffer (Beyotime, Haimen, China) containing protease inhibitor cocktails (Sigma, St. Louis, USA), and the nuclear and cytoplasmic proteins were separated by a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime). All proteins were assessed using BCA Protein Assay Kit (Beyotime) and then separated by 10% SDS-polyacrylamide gel electrophoresis. After being transferred to PVDF membranes, which were blocked with 5% milk, the proteins were detected by anti-AGO2 (Abcam, Cambridge, UK), anti-NUDT21 (Abnova, Taipei, Taiwan), anti-GAPDH (Sigma), anti-PCNA (Cell Signaling, Danvers, France), and anti-β-tubulin (Cell Signaling) antibodies.

Immunostaining and immunohistochemistry (IHC)

To image the cells, we seeded them on gelatin-pre-treated slides and then fixed them with 3.7% paraformaldehyde (PFA) in PBS for 15 min. After being blocked with NHS (3‰ Triton-100 and 10% horse serum in PBS) for 1 h, the slides were incubated with anti-AGO2, anti-GW182 (Bioss, Beijing, China) or anti-NUDT21 primary antibodies, followed by Alexa Fluor 488- or 594-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, USA). DAPI (Invitrogen, San Diego, CA, USA) was applied to detect cell nuclei. Coverslips were mounted with Microscopy Aquatex® mounting medium (MERCK, Darmstadt, Germany) and then detected under a Leica Tcs-sp5-II confocal microscope (Leica, Wetzlar, Germany).

To image the tumor tissue samples, liver tissues were fixed with 4% PFA, dehydrated, and embedded in paraffin. Seven μm sections were prepared with a microtome (Leica RM2245). Slides were subsequently deparaffinized and rehydrated, and the antigen was retrieved by microwaving in sodium citrate buffer (10 mM sodium citrate acid, 0.05% Tween 20, pH 6.0) for 2 min. Endogenous catalases were blocked with 3% H_2O_2, and then the slides were blocked with NHS for 1 h. The sections were subsequently incubated with anti-NUDT21 primary antibodies and HRP-conjugated secondary antibodies. The signals were detected with DAB (ZSGB-BIO, Beijing, China), and the sections were counterstained with hematoxylin. The adjacent tissues were stained with hematoxylin-eosin (H&E) for morphological analysis.

Plasmids and transfection

NUDT21 shRNAs were inserted into a GV248 vector to construct NUDT21-knockdown (KD) plasmids. The target sequences of the shRNAs were as follows:

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