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Long non-coding RNA PVT1 as a novel candidate for targeted therapy in hematologic malignancies

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ABSTRACT

Cancerous cells show resistance to various forms of therapy, so applying up to the minute targeted therapy is crucial. For this purpose, long non-coding RNA PVT1 as shown by recent studies is an important oncogene that interacts with vital cellular signaling pathways and different proteins such as c-Myc, NOP2 and LATS2. Due to the enormous role of long non-coding RNAs in development of leukemias, we aimed to show the role of PVT1 knock-down on fate of different hematologic cell lines. owing to this matter, various experiments such as Real-time PCR, cell cycle analysis and apoptosis assay were performed. Meanwhile, proliferation rate by CFSE, protein expression of c-Myc and hTERT by western blot and flow cytometry analysis were investigated. Our results demonstrated that PVT1 knock-down results in c-Myc degradation, proliferation down-regulation, induction of apoptosis and G0/G1 arrest. Simultaneously, for the first time, we posited the relation between this oncogene with hTERT that reduced after PVT1 knock-down. Considering these results, long non-coding RNA PVT1 may be a potential option for targeted therapy in hematologic malignancies.

1. Introduction

Hematologic malignancies are heterogeneous diseases which account for about 9% of all cancers in developed countries (Smith et al., 2011). Conventional treatments of such malignancies are widely varied from common chemotherapy drugs to biological therapy and bone marrow transplantation (Lichtman, 2008). Meanwhile, by the emergence of new agents for targeted therapies such as monoclonal antibodies like rituximab, or signal transduction inhibitors like Imatinib new era of treatments has initiated (Younes, 2011; Cilloni and Saglio, 2009). However different studies have revealed that, as cancerous cells find their way to circumvent targeted therapy and proceed through activation of compensatory pathways (von Manstein et al., 2013; Trusolino and Bertotti, 2012), the advent of new forms of treatment is of the essence. (Masui et al., 2013).

c-Myc, an oncogenic transcription factor, has a hand in the development of leukemia and lymphoma and inhibition of these molecules is considered as a suitable therapeutic target (Delgado and Leon, 2010). c-Myc enhances tumor metastasis and regulate cell cycle, apoptosis and above all transcripts from diverse genes like human telomerase reverse transcriptase (hTERT) (Tansey, 2014). Higher expression of hTERT is a prominent feature of most cancer cells which allow them to divide infinitely (Wu et al., 1999). Meanwhile, the interrelationship of this oncogene with long non-coding RNA (lncRNA) PVT1 draws researcher's attention because of their leading role in tumorigenesis (Iaccarino, 2017).

Plasmacytoma variant translocation 1 (PVT1) locates in the chromosomal region of 8q24 in downstream of c-Myc, and produces 6 microRNAs and about 20 lncRNAs variants. It has elucidated that PVT1 and c-Myc have reciprocal interactions by which c-Myc regulate PVT1 expression and lncRNA PVT1 stabilize c-Myc and prevent it from degradation (Colombo et al., 2015). The oncogenic potential of lncRNA PVT1 is not limited to c-Myc interaction and acts as a competing endogenous RNA (ceRNA) by sponging different tumor suppressor microRNAs such as mir-186 and mir-195 (Lan et al., 2017; Zhou et al., 2016a,b). Moreover, it can induce drug resistance and also engage in

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autophagy related pathways (Zhang et al., 2015; Xu et al., 2017a,b).

Due to the importance of new forms of targeted therapy and prominence of lncRNA PVT1 partake in cancer development, we determined to shed light on the role of PVT1 knock-down in some hematologic malignancies cell lines.

2. Materials and methods

2.1. Cell culture

For results generalization, we decided to work on cells from distinct cell lines. K562, TF-1, HL-60 and JJN3 were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 100 U/ml Penicillin, 100ug/ml Streptoc-Mycin at 37 °C in a humidified 5% CO2 atmosphere.

2.2. RNA interference

To determine the effect of PVT1 knock-down, two siRNAs against lncRNA PVT1 (Hs-PVT1-5 flexitube siRNA and Hs-PVT1-6 flexitube siRNA) that interact with two different parts of lncRNA PVT1, purchased from flexitube siRNA (Qiagen, Germany). Meanwhile, AllStars siRNA (Qiagen, Germany) served as negative control. 10^5 cells of each cell lines were seeded in 24 well plates. SiRNA transfection was performed after 24 h of cell seeding by exposure of the cells to lipofectamine 3000 according to the manufacturer's instruction (Invitrogen, USA). All the experiments were performed 48 h after transfection.

2.3. Real-time PCR

To analyze the efficacy of PVT1-siRNA, the level of lncRNA PVT1 was determined before and after treatment by real-time PCR. Also, to clarify the role of PVT1 in some target mRNAs, the expression of c-Myc, hTERT, Bcl2, Caspase-3, p15 and p16 mRNA were investigated. Total RNA was extracted from each cell line, using Qiazol (Qiagen, Germany). The concentration and purity of RNA were confirmed by Nanodrop spectrophotometer. Using cDNA synthesis kit (Takara, Japan) RNAs were reverse transcribed into cDNA. Real-time PCR performed by applying SYBER Green master mix (Takara, Japan) and the results were normalized with endogenous β-actin mRNA as an internal control. The relative expression of target mRNAs and lncRNA were subtracted by 2–ΔΔCt formula. The designed primers were listed in Table 1.

2.4. Flowcytometry detection of target proteins

To verify the protein expression of c-Myc and h-TERT on treated cells, we used c-Myc, and h-TERT rabbit anti human primary antibodies and FITC conjugated anti rabbit antibody (all from Abcam,USA) according to manufacturer's instruction. Briefly, 10 microliters of primary antibodies were added to each test tubes contain 10^5 cells of treated and control groups which were fixed for intracellular staining. In parallel, for all samples isotype controls were used to put aside non-specific reactions. After 20 min incubation in 4 °c and washing the cells, FITC conjugated secondary antibody was added to cells and incubated in 4 °c for 20 min. During incubation with antibodies, the cells were exposed to permeabilization reagent. Finally, a total of 2×10^4 cells were analyzed by Partec CYflow space (Germany) and positive reactions were screened by Flomax software.

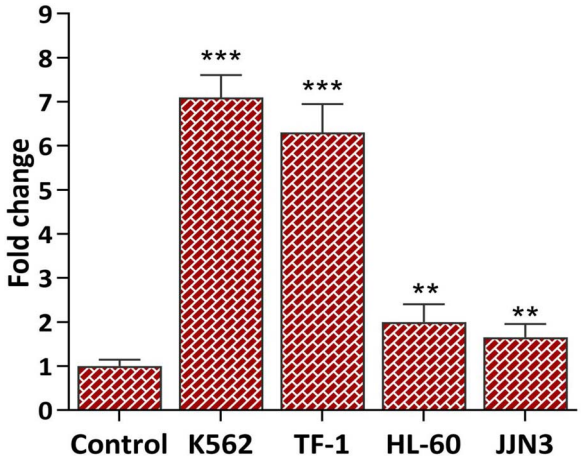

Fig. 1. Fold change of PVT1 in different cell lines *: $P < 0.05$, **: $P < 0.01$ and ***: $P < 0.001$.

2.5. Western blot analysis of c-Myc

Treated and non-treated experimental groups were homogenized in ice cold PBS and lysed with ice cold lysis buffer and after centrifugation, total proteins were extracted. The concentration of proteins was measured using BCA protein assay method (Pierce™ BCA Protein Assay Kit, Thermo Scientific) according to the manufacturer protocol. The protein content was incubated with sample buffer including 2-mercaptoethanol and subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then proteins were transferred at 150 V onto Polyvinylidene difluoride (PVDF) membrane. After that, it blocked with TBS-Tween buffer (0.2%) containing 5% skim milk at room temperature. Afterwards, membranes were incubated with c-Myc antibody (abcam. USA) for 24 h at 4 °C (1:10000). Following this, they were washed with TBS-Tween buffer (0.2%). Secondary rabbit HRP conjugated antibody was used for detection of related antigen. Afterwards, membranes were washed with TBS-Tween buffer (0.2%) and after chemiluminescence reaction, they become visualized on autoradiography film. Finally, the band of c-Myc proteins was semi-quantified by densitometry analysis by applying Total LAB V1.10 software, then the gained results were normalized to β-actin protein expression.

2.6. Apoptosis assay

Cell apoptosis assay was carried out to investigate any alteration in cell viability during PVT1 knocking down. In this manner, the cells were washed twice with PBS, and 10^5 cells were suspended in 1 mL binding buffer which contains Ca ions to facilitate Phosphatidylserine and Annexin V binding. 5 μL of FITC conjugated Annexin V, and 5 μL of

Table 1
Primer pairs sequences for qRT-PCR amplification.

Primer	Forward	Reverse
PVT1	5′ GTGCTCTGTGTTCACCTGGTTCATC 3′	5′ GCCCGTTATTCTGTCCTTCTCATG 3′
c-Myc	5′ AGCGACTCTGAGGAGGAAC 3′	5′ CTGCGTAGTTGTGCTGATG 3′
hTERT	5′ TGTACTTTGTCAAGGTGGATGTG 3′	5′ ATGTACGGCTGGAGGTCTG 3′
p15	5′CTGGACCTGGTGGCTACG 3′	5′ACATTGGAGTGAACGCATCG 3′
p16	5′ AAGGTCCCTCAGACATCC 3′	5′ TCGGTGACTGATGATCTAAG 3′
Caspase 3	5′ATTGATGCGTGATGTTTCTAAAG 3′	5′ CAATGCCACAGTCCAGTTC 3′
Bcl2	5′ CAACATCACAGAGGAAGTAG 3′	5′ GGAACACTTGATTCTGGTG 3′