

A novel transcript variant of proteasome activator 28 γ : Identification and function in oral cancer cells

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Received January 27, 2015; Accepted March 4, 2015

DOI: 10.3892/ijo.2015.2980

Abstract. Proteasome activator 28 γ (PA28 γ) binds to and activates the proteasome in an ATP-independent manner to promote mainly ubiquitin-independent protein degradation in cells. Previously, four transcript variants of PA28 γ have been identified, which have been closely correlated with the progression of cancers. In the present study, we predicted the alternative splicing of PA28 γ via the bioinformatics tool ASPicDB and 49 splices were predicted. Then, we cloned some new segment according to predication in oral cancer cells using reverse transcription PCR and a novel variant of PA28 γ was found. The novel transcript encodes a truncated form compared with other isoforms of PA28 γ . However, it contains most of the conserved residues and the 'activation loop' of the PA28 γ family. In order to explore its function, we overexpressed the variant in HEK293 cells and demonstrated that this variant is likely to further regulate cell cycle and apoptosis via regulating p53 and the mouse double minute2 homolog (Mdm2).

Introduction

Proteasome activator 28 γ (PA28 γ) is a component of the proteasome system, which is one of the most important proteolytic systems in eukaryotes. It binds to the 20S core and mainly promotes proteins degradation in a ubiquitin- and ATP-independent manner (1-4). Strikingly, PA28 γ not only plays a role as a proteasome activator, but also has been shown to be involved in cancer progression and virus infection (Fig. 1B). Loss of PA28 γ expression in PA28 γ -deficient mice results in

reduced body size and cell-specific mitotic defects (5-7). It directs degradation of the steroid receptor coactivator SRC-3, which is an oncogene frequently amplified in breast cancer (8). It is also a novel serum marker for human colorectal cancer (CRC) because it can be detected in sera and is significantly elevated in CRC patients compared with healthy donors and patients with benign bowel disease (9). Moreover, overexpression of PA28 γ occurs in many different cancer types, including thyroid, colon, liver, lung, ovary and gastric cancer (10-14). PA28 γ binds to and regulates the stability and nuclear retention of hepatitis C core protein, contributing to hepatitis C core protein-induced insulin resistance and hepatocarcinoma (15-18). In addition, PA28 γ promotes coxsackievirus B3 (CVB3) replication (19-21) and interacts with human T-lymphotropic virus type 1 (HTLV-1) p30 to increase viral spread (22,23). A series of cell cycle, apoptosis, and cancer progression-related PA28 γ target proteins have been identified, including p21, p16, p19, p53, Mdm2 (24-26). In recent years, more targets of PA28 γ have been identified using antibody array analysis. These proteins include protein kinase A catalytic subunit- α (PKA α), SirT1, and casein kinase (CK)1 δ , which play important roles in angiogenesis, hepatic lipid metabolism and premature aging, respectively (27-29).

Alternative splicing of mRNA allows many gene products with different functions to be produced from a single coding sequence according to the cell type, developmental stage, or in response to acute stimuli. It explains how enormous mammalian proteomic diversity can be achieved with the limited number of genes found in higher eukaryotes. Based on deep sequencing of alternative splicing complexity in the human transcriptome, it is estimated that transcripts from ~95% of multiexon genes undergo alternative splicing (30,31). A study involving probabilistic analyses indicated that >60% of human disease-causing mutations affect splicing rather than directly affecting coding sequences. Another study concluded that one-third of all hereditary diseases are likely to have a splicing component (32,33). Abnormally spliced mRNAs are also found in a high proportion of cancerous cells. Combined RNA-Seq and proteomics analyses technology revealed striking divergent expression profile of splice isoforms of key proteins in important cancer pathways. For example, several abnormally spliced DNMT3B mRNAs are found in tumors and cancer cell lines. In two separate studies, expression of

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Key words: proteasome activator, alternative splicing, PA28 γ , PA28 γ isoform 5, p53, Mdm2

two of these abnormally spliced mRNAs in mammalian cells caused changes in the DNA methylation patterns in those cells. Cells with one of the abnormal mRNAs also grew twice as fast as control cells, indicating a direct contribution to tumor development by this product (34-37).

In a previous study, we identified 85 differentially and constantly expressed proteins (>2-fold change, $P < 0.05$) in six pairs of oral leukoplakia tissues with dysplasia and oral squamous cancer tissues via two dimensional electrophoresis (2-DE) followed by ESI-Q-TOF-LC-MS/MS. Among them, three homologs of proteasome activator PA28 α , PA28 β , and PA28 γ were shown to have upregulated mRNA levels in oral squamous cell carcinoma (OSCC) cells relative to oral keratinocytes (38). In this study, we analyzed alternative splicing of PA28 γ using the alternative splicing prediction data base ASPicDB (39,40), and found that there are theoretically nearly 50 alternative transcripts. We therefore tried cloning these predicted splice variants. We successfully cloned a novel (the fifth) transcript variant of PA28 γ in oral cancer cells. This variant encodes a truncated isoform that retains the most conserved residues of the PA28 family. Furthermore, it is involved in regulation of p53 and Mdm2.

Materials and methods

Bioinformatics prediction. The alternative splicing prediction data base (ASPicDB) is a program designed to provide access to reliable annotations of the alternative splicing pattern of human genes, and to the functional annotation of predicted isoforms. Alternative splicing prediction of PA28 γ (PSME3) was performed in ASPicDB.

Cell type and cell culture. Six oral squamous cell carcinoma-derived cell lines (HSC-3, HOK, UM1, UM2, Cal27 and HN31) and human embryonic kidney 293 (HEK293) cells were cultured in DMEM with 10% fetal bovine serum.

Gene cloning and DNA sequencing. Total RNA of each cell line was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The RNA concentration was determined by absorbance at 260 nm with a Nanovue™ spectrophotometer (GE Healthcare). Equal amounts of RNA (1.0 μ g) were used as template in each reverse transcription reaction (total volume, 30 μ l) with the PrimeScript® RT Reagent kit with gDNA Eraser (Takara, Shiga, Japan). The reaction conditions were 42°C for 2 min to remove genomic DNA, 37°C for 15 min, followed by 85°C for 5 sec to obtain total cDNA. The primers designed for PA28 γ amplification were: 5'-TTGTATTTCCAGGGCATGGCCTCGTTGCTG-3' (forward primer) and 5'-CAAGCTTCGTCATCATCAGTACAGAGTCTC-3' (reverse primer). PCR was performed for 30 cycles with PrimeSTAR® HS DNA Polymerase and 2 μ l total cDNA was used as template in 50 μ l reaction volume. The reaction conditions were a denaturation step at 98°C for 10 sec, an annealing step at 55°C for 15 sec, and an elongation step at 72°C for 1 min. PCR product (10 μ l) was separated on a 1% agarose gel, and observed under ultraviolet light (Bio-Rad, Hercules, CA, USA). DNA bands were eluted from the agarose gel using a gel extraction kit (Doupson) and cloned into the p15TV-L vector for sequencing.

Plasmid transfection. Plasmids for overexpressing human influenza hemagglutinin A epitope (HA)-tagged PA28 γ isoforms were constructed by Chengdu Bio-atom Biotechnology. HEK293 cells were used as experimental cells. The control and positive group cells were transiently transfected with empty vector and HA-PA28 γ isoform 5-expressing plasmids, respectively.

Western blotting. Seventy-two hours after treatment, cells were harvested and protein was extracted with RIPA lysis buffer (Beyotime, P0013B). Antibodies to GAPDH (XP® Rabbit mAb, Cell Signaling Technology), p53 (mouse mAb, Cell Signaling Technology), Mdm2 (Phospho-Mdm2 (Ser166) Antibody, Cell Signaling Technology), PA28 γ isoform1 (Purified mouse anti-PA28 γ , BD Transduction Laboratories), PA28 γ isoform 5 (HA-tag antibody, ZSGB-BIO) were used to test the amount of corresponding protein by western blotting (Bio-Rad electrophoresis).

Quantitative PCR. The amount of mRNA of PA28 γ transcript variant 1 was measured by the ABI 7500 Real-time PCR (RT-PCR) System with One Step SYBR® PrimeScript™ Plus RT-PCR kit (Takara, RR096A). Gene-specific primers were designed as follows: PA28 γ transcript variant 1 forward primer, 5'-ATGGACTGGATGGTCCCACT-3'; PA28 γ transcript variant 1 reverse primer, 5'-ACAGCCGGATCTCAGGTTTC-3'; 18S rRNA forward primer, 5'-CTACCACATCCAAGGAA GGCA-3'; 18S rRNA reverse primer, 5'-TTTTTCGTCCTA CCTCCCCG-3'. Gene-specific primers for PA28 γ transcript variant 1 were designed crossing the lost sequences which lack in PA28 γ transcript variant 5. Mean Ct values for target genes were normalized to mean Ct values for the endogenous control 18S [$-\Delta C_t = C_t (18S) - C_t (\text{target gene})$]. The ratio of mRNA expression of target gene versus 18S was defined as $2^{-\Delta C_t}$. All experiments were repeated at least three times.

Results

Alternative splicing prediction of PA28 γ via bioinformatics. The genomic location of human PA28 γ is at chromosome 17: 40985423-40995777, with a size of 10,354 bp. Expression sequence tags (EST) (1,180) exist in this cluster. Forty-nine alternative transcripts are predicted and 45 of them are protein-coding forms. To date, only four alternative transcripts are reported in GenBank. PA28 γ transcript variant 1 (Nucleotide accession: NM_005789.3) encodes the predominant protein with 254 amino acids. Variant 2 (Nucleotide accession: NM_176863.2) uses an alternative in-frame splice site; variant 2 is 13 amino acids longer than variant 1. Variant 3 (Nucleotide accession: NM_001267045.1) is distinguished in the 5'-untranslated region and 5'-coding region, and initiates translation at an alternative start codon. Variant 3 has a distinct N-terminus and is 12 amino acids longer than variant 1. Variant 4 (Nucleotide accession: NR_049772.1) is a long non-coding RNA.

Cloning and identification of a novel PA28 γ transcript variant in oral cancer cells. As shown in Fig. 2A, three DNA bands were observed on the agarose gel. According to the DNA markers, from top to bottom, the first band likely corresponds to PA28 γ transcript variant 1, and the third band

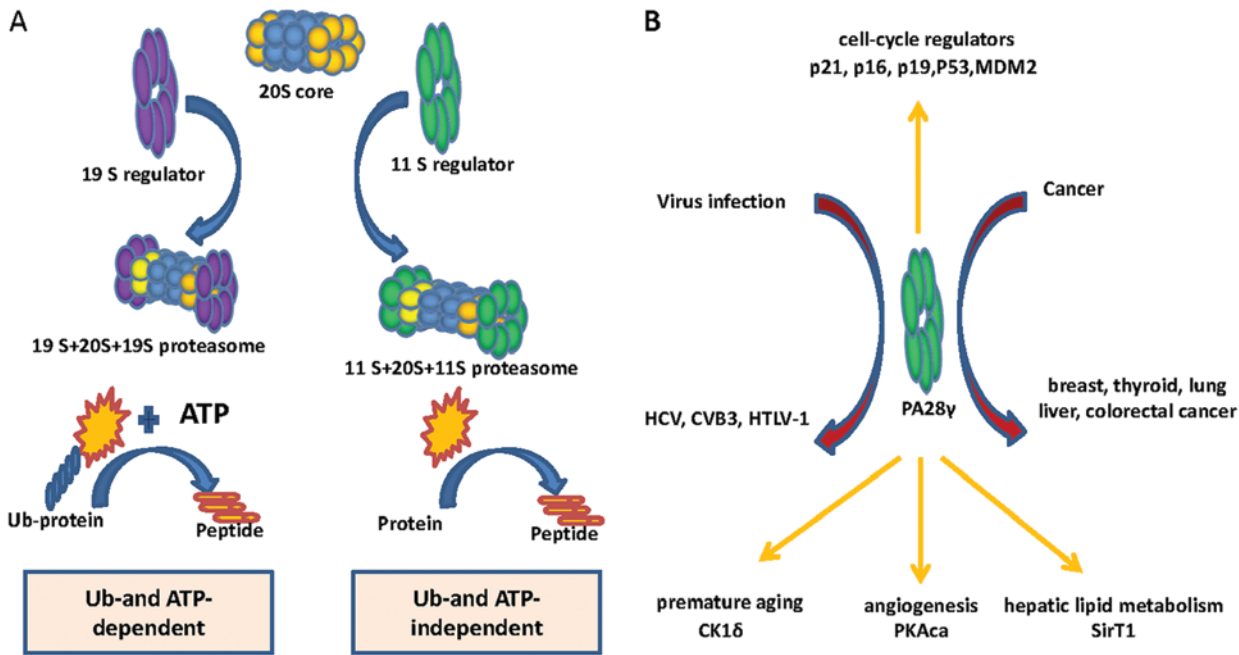


Figure 1. Proteasome degradation system and biological functions of PA28 γ . (A) The composition of proteasome degradation system and diagram of protein degradation mediated by PA700 (19S) and PA28 γ (11S) in this system via ubiquitin/ATP-dependent and ubiquitin/ATP-independent manner, respectively. (B) All the diseases and target proteins involved in PA28 γ are collected.

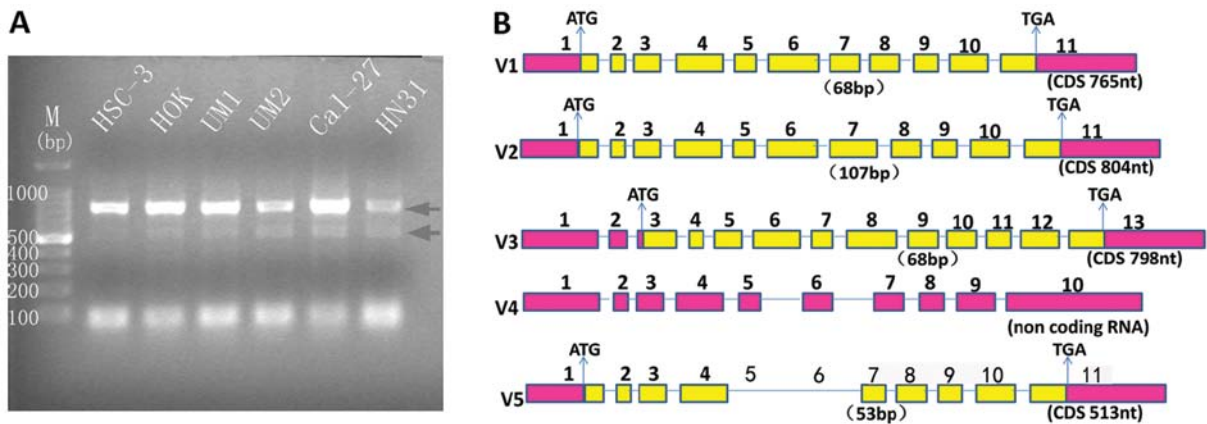


Figure 2. Identification of novel transcript variant of PA28 γ in oral cancer cells. (A) The nucleic acid electrophoresis result of RT-PCR products and the PA28 γ -related bands are labeled with arrows. (B) Schematic open read frame (ORF) comparison of 5 transcript variants of PA28 γ . V1, V2, V3, V4, V5 represent the transcript variant 1, variant 2, variant 3, variant 4 and variant 5 of PA28 γ , respectively. The coding sequences (CDS) of exons are labeled in yellow frame, while the untranslated regions in dark red. ATG is start codon and TGA is the stop codon.

to oligonucleotide primers. In addition, we observed a weak band between the first and third bands. The sequencing data were analyzed by the BLAST tool in NCBI. As expected, the first band was confirmed as PA28 γ transcript variant 1. We translated the nucleotide acid sequence of the second band into amino acids and a structure-based sequence alignment was performed. As shown in Fig. 3, the new isoform lacks the ‘homolog-specific insert’ region (41), but it belongs to the PA28 γ subfamily according to the high identity in the C-terminal sequence. Therefore, we termed the new alternative splicing as transcript variant 5 and submitted it to GenBank (Nucleotide accession: JX156303.1). Compared with PA28 γ variant 1, PA28 γ variant 5 lacks the nucleotide acids regions in coding sequence among 185-483 in PA28 γ variant 1, which corresponds to the sequence from exon 4 to exon 7 in variant 1

(Fig. 2B). Taken together, these findings indicate that PA28 γ transcript variant 5 is a novel transcript variant in the PA28 γ subfamily.

PA28 γ isoform 5 involved in p53 and Mdm2 regulation. We predicted that PA28 γ transcript variant 5 encodes a truncated protein of 170 residues, which is 84 residues shorter than PA28 γ isoform1. However, structure-based sequence alignment showed that isoform 5 retains most of the conserved residues and the ‘activation loop’ of PA28 family (Fig. 3). As mentioned in the introduction, PA28 γ has a series of target proteins and plays an important role in cell cycle regulation. It was reported that PA28 γ regulates p53 by enhancing Mdm2-mediated degradation (25). This prompted us to explore whether PA28 γ isoform 5 could play a similar role in this process. As shown

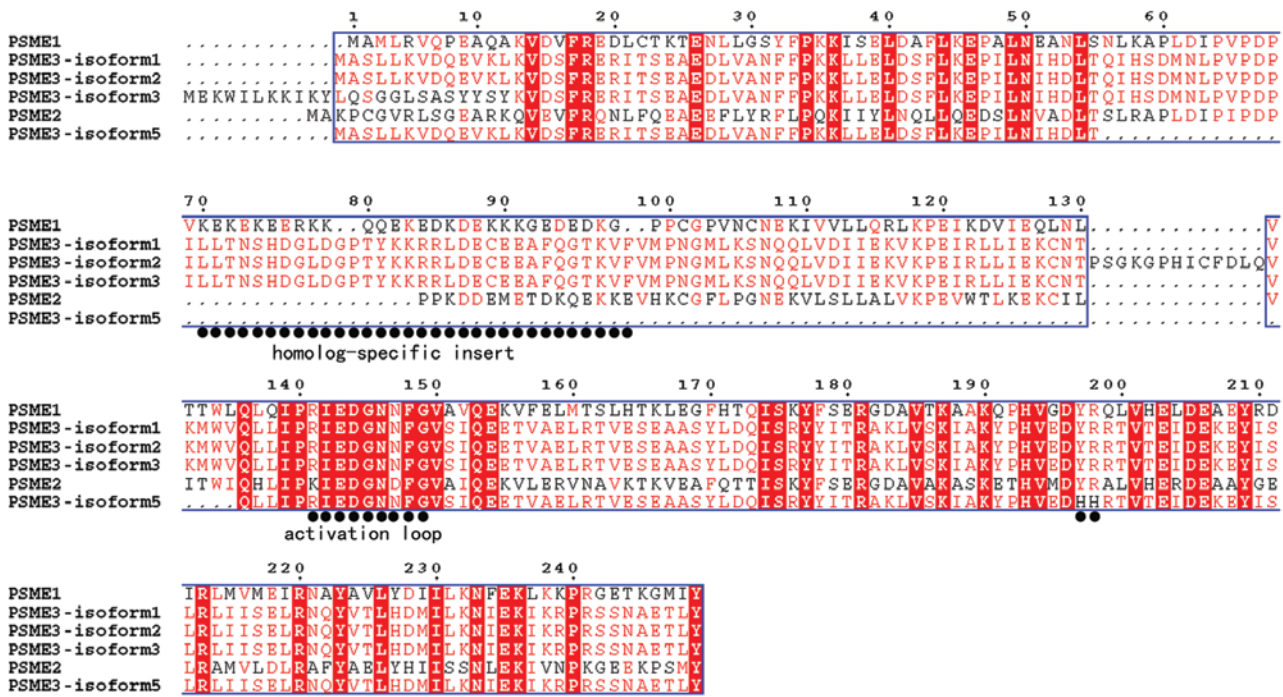


Figure 3. Structure-based sequence alignment of PA28 family members. Since α , β , γ cannot be indicated in the alignment software, the PA28 α , PA28 β , PA28 γ were substituted by PSME1, PSME2, PSME3 (the official symbol) respectively. White characters on a red background show strictly conserved residues. Residues that are well conserved are drawn in red and framed in blue. The remaining residues are black. The special residues and regions are marked in filled circles. Sequence alignment was performed with the programs of MultAlin and ESPript.

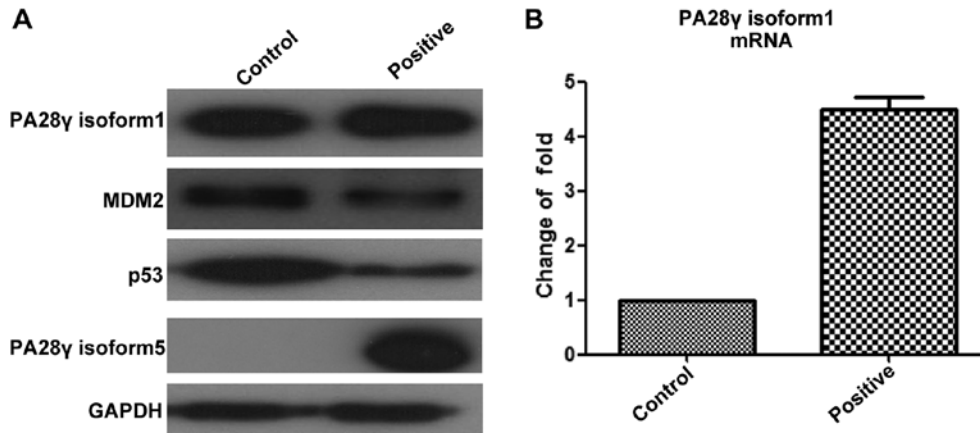


Figure 4. Effect of PA28 γ isoform 5 after overexpression. (A) Protein level change of PA28 γ isoform 5, PA28 γ isoform1, p53 and MDM2 via transient expression after plasmids transfection treatment. (B) Transcription level change of PA28 γ transcript variant 1 after plasmids transfection treatment. The mRNA expression amount of 18S rRNA was used as the normalized gene and set as 1.

in Fig. 4, the positive group overexpressed PA28 γ isoform 5, which resulted in significant decrease of p53 and Mdm2 at protein level. Furthermore, we tested the amount of PA28 γ isoform1 at both mRNA and protein levels. Strikingly, the mRNA level of PA28 γ isoform1 in the positive group was 4.5-fold higher than in the control group. The change in protein levels of PA28 γ isoform1 between positive and negative groups was not as great as for mRNA levels, but still increased. To explain these changes, we propose two possibilities: i) increased PA28 γ isoform 5 triggers the transcription of PA28 γ alternative transcript1, and the increased PA28 γ isoform1 regulates the degradation of p53 and Mdm2, as reported before; or ii) overexpressed

PA28 γ isoform 5 maintains a pool of functional PA28 γ , and represses the translation of PA28 γ alternative transcript1 as feedback regulation. In either case, the downregulation of p53 and Mdm2 at the protein level resulted from overexpression of PA28 γ isoform 5, which indicates that PA28 γ isoform 5 is a functional isoform that may play a complementary role in regulating p53 and Mdm2.

Discussion

Based on the results of bioinformatics analysis via ASPicDB, 30 alternative transcripts of PA28 α , one alternative transcripts of PA28 β , and 49 alternative transcripts of PA28 γ

were predicted (39,40). We checked the alternative transcripts of these PA28 members deposited in GenBank, and found that four transcript variants of PA28 α , one transcript variant of PA28 β and four transcript variants of PA28 γ have been reported. Therefore, the number of reported transcript variants of PA28 β is consistent with the bioinformatics prediction. However, there are many more alternative transcripts of PA28 α and PA28 γ that are not yet defined. Typically, alternatively spliced transcripts have been found by comparing expression sequence tags (ESTs), but this requires sequencing of very large numbers of ESTs. Most EST libraries come from a very limited number of tissues, so tissue-specific splice variants are likely to be missed in any case. However, high-throughput approaches to examine splicing have been developed, such as DNA microarray-based analyses, RNA-binding assays, and deep sequencing. When combined with splicing assays, including *in vivo* reporter gene assays, the functional effects of polymorphisms or mutations on the splicing of pre-mRNA transcripts can be analyzed (33,42-44). Herein, utilizing bioinformatics, RT-PCR technology and gene overexpression in model cells, we successfully predicted, cloned, and confirmed a novel transcript variant of PA28 γ . Moreover, we found PA28 γ isoform 5 lacks long regions compared with other reported isoforms of PA28 γ . Therefore, the siRNA target to this region of PA28 γ will affect other reported isoforms of PA28 γ but not on PA28 γ transcript variant 5, which may result in a significant off-target effect of siRNA interference.

Generally speaking, the diversity of transcript variant corresponds to the functional significance of the gene regardless of whether the transcript variant has the same function or not. The variants with the same function will compensate the loss-of-function mutation to maintain homeostasis, whereas patterns of divergent functional variants are likely to be indicators for some disease. In a retrospective analysis of 80 individuals with gastrointestinal sarcoma, predominant expression of the immunosuppressive NKp30c isoform (over the immunostimulatory NKp30a and NKp30b isoforms) was associated with reduced survival of the subjects (45). Previously, we identified three alternative transcripts of oral cancer overexpressed 1 gene (ORAOV1), and an inverse correlation was found between the expression frequency of ORAOV1-A and the degree of differentiation in OSCC (46). In future studies, we will assess the clinical value of PA28 γ isoform 5 in tissue samples from normal, precancerous to infiltrative OSCC. Moreover, the relationships of immunostaining with survival rate and recurrence will be analyzed.

Several studies have evaluated the interaction of PA28 γ variant 1, p53 and Mdm2. P53 encodes a tumor suppressor protein and responds to diverse cellular stresses to regulate expression of target genes, thereby inducing cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism. Mutations in this gene are associated with a variety of human cancers. Thus, it is a focus of numerous investigations for reversing tumor progression. MDM2 encodes a nuclear-localized E3 ubiquitin ligase. The protein can promote tumor formation by targeting tumor suppressor proteins, such as p53, for proteasomal degradation. Amplification of this locus is detected in a variety of different cancers. Noteworthy, the

polymer form of PA28 γ variant 1 interacts with both p53 and Mdm2, which facilitates ubiquitination and Mdm2-dependent proteasomal degradation of p53. The decreased p53 attenuated apoptosis stimulation after DNA damage (47,48). In our study, we confirmed that PA28 γ isoform 5 also mediates the downregulation of p53 and Mdm2, which may serve as a complementary mechanism for PA28 γ isoform1 in regulating p53 and Mdm2. Given that PA28 γ is involved in cancer progression, it suggests to examine the PA28 γ isoform 5 before we determine PA28 γ isoform1 as a therapy target via regulating p53 and Mdm2 pathway.

The proteasome is a primary proteolytic system in eukaryotes. This system is a multi-subunit protease complex composed of 20S catalytic core and proteasome activators (Fig. 1A). The 20S core is a cylindrical stack of four heptameric rings with two outer α rings and two inner β rings. The PA700 (19S) activator binds to the 20S core and primarily mediates degradation of ubiquitinated proteins in ATP-dependent manner. In contrast, the PA28 (11S) activator binds to the 20S core and mainly promotes protein degradation in Ub- and ATP-independent manner (1-4). To date, three classes of PA28 have been identified: PA28 α , PA28 β , and PA28 γ . PA28 α and β form a heteroheptamer, which is mainly localized in the cytosol. PA28 γ exists as a homoheptamer and is primarily found in the nucleus. PA28 α and β mediate proteolytic cleavage after basic, acidic, and hydrophobic residues. PA28 γ stimulates proteasomal hydrolysis of peptides with basic residues (41,49-56). Thus, the subcellular location of PA28 γ isoform 5 and its effect on proteolytic activity in the proteasome system need to be further studied.

In this study, we made an attempt to transiently transfect HA-tagged PA28 γ isoform 5 overexpressing plasmids into oral cancer cells. However, we failed because oral cancer cells are highly keratinized. Although we determined the primary function of PA28 γ isoform 5 in HEK293 cells, further investigation of its effects on cell cycle, apoptosis and its correlation with progression of oral squamous cell carcinoma will be performed in oral cancer cells using lentivirus transfection.

Acknowledgements

We acknowledge Min Zhou, at State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University, for preparing some experimental materials. This project was supported by grants from National Natural Science Foundations of China (nos. 81321002, 81472533, 81302371, and 81072218) and 111 Project of MOE, ISTCPC (2012DFA31370).

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