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AP-57/C10orf99 is a new type of mutifunctional antimicrobial peptide

Meijia Yang [a, 1], Mei Tang [a, 1], Xianjun Ma [a], Lijia Yang [a], Jiangpeng He [a], Xirui Peng [a], Gang Guo [a], Liangxue Zhou [b], Na Luo [c], Zhu Yuan [a, *], Aiping Tong [a, *]

[a] The State Key Laboratory of Biotherapy and Cancer Center/Collaborative Innovation Center of Biotherapy, West China Hospital, West China Medical School, Sichuan University, Chengdu 610041, China
[b] Department of Neurosurgery, West China Hospital, West China Medical School, Sichuan University, Chengdu 610041, China
[c] Nankai University School of Medicine/Collaborative Innovation Center of Biotherapy, Tianjin 300071, China

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ABSTRACT

Antimicrobial peptides (AMPs) are an evolutionarily conserved component of the innate immune response that provides host defence at skin and mucosal surfaces. Here, we report the identification and characterization of a new type human AMPs, termed AP-57 (Antimicrobial Peptide with 57 amino acid residues), which is also known as C10orf99 (chromosome 10 open reading frame 99). AP-57 is a short basic amphiphilic peptide with four cysteines and a net charge +14 (MW = 6.52, PI = 11.28). The highest expression of AP-57 were detected in the mucosa of stomach and colon through immunohistochemical assay. Epithelium of skin and esophagus show obvious positive staining and strong positive staining were also observed in some tumor and/or their adjacent tissues, such as esophagus cancer, hepatocellular carcinoma, squamous cell carcinoma and invasive ductal carcinoma. AP-57 exhibited broad-spectrum antimicrobial activities against Gram-positive *Staphylococcus aureus*, *Actinomyce*, and Fungi *Aspergillus niger* as well as mycoplasma and lentivirus. AP-57 also exhibited DNA binding capacity and specific cytotoxic effects against human B-cell lymphoma Raji. Compared with other human AMPs, AP-57 has its distinct characteristics, including longer sequence length, four cysteines, highly cationic character, cell-specific toxicity, DNA binding and tissue-specific expressing patterns. Together, AP-57 is a new type of multifunctional AMPs worthy further investigation.

1. Introduction

Skin and mucosal surface are continuously exposed to a large number of microorganisms. To manage the substantial microbial exposure, epithelial surfaces produce a diverse arsenal of antimicrobial proteins (AMPs) that directly kill or inhibit the growth of microorganisms [1].

AMPs indentified in human mainly include defensins, LL-37, histatins and hepcidin [2]. The small (29–42 amino acids) cationic defensins represent an important mammalian antimicrobial peptides family that possess potent broad-spectrum microbicidal activities. Human defensins can be divided into two categories: α-defensins and β-defensins. α-Defensins (29–35 residues) are expressed by granulocytes and in epithelial cells of the small intestine, while the β-defensins (38–42 residues) can be found in several epithelial and nonepithelial cell types. Both defensin subclass contain six disulfide-linked cysteines but differ in the position and linkage of the cysteines [3]. A cyclic θ-defensin were found to be present in macaque leukocytes [4]. Another major family of antimicrobial peptides in vertebrates are cathelicidins, which are often devoid of cysteines. The only cathelicidin peptide in human is LL-37. Other AMPs found in human are histatins (present in saliva) and LEAP-1/hepcidin (present in liver) [2].

Overwhelming evidence now exists that the AMPs have many distinct and complementary functions. For example, hepcidin is a key peptide hormone made in the liver that is involved in the control of iron homeostasis in the body [5]. Defensins and LL-37 are recognized not only as direct antimicrobial agents but multifunctional mediators of innate immune system, including increasing or decreasing chemokine production, activating or suppressing

Abbreviations: AMPs, antimicrobial peptides; C10orf99, chromosome 10 open reading frame 99; HD5, human defensin α-5; AP-57, antimicrobial peptide-57; HCC, hepatocellular carcinoma; SCC, squamous cell carcinoma; BCC, basal cell carcinoma; IDC, invasive ductal carcinoma; TAT, tumor adjacent tissues.

* Corresponding authors. Fax: +86 28 85502796.
E-mail addresses: yuanzhu@scu.edu.cn (Z. Yuan), aipingtong@scu.edu.cn (A. Tong).

[1] Both authors have contributed equally.

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different immune cell types, binding microbial- or self-nucleic acid, neutralizing endotoxin, and they also play a role in coagulation, angiogenesis and wound healing [1–3].

Despite the importance of AMPs, other than the four classes identified previously (defensins, LL-37, histatins and hepcidin), rare novel or new type human AMPs was reported in the recent ten years. In the present study, we report the identification and characterization of a new type human AMPs, termed AP-57 (Antimicrobial Peptide with 57 amino acid residues).

2. Materials and methods

2.1. Cell culture

Cell lines were originally purchased from ATCC and have been maintained in our lab. Cells were maintained in DMEM or 1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Proteomic profiling study of secretary proteins in extracellular tissue space of clinical colorectal cancer samples

After carefully washed in PBS, fresh colorectal cancer tissues were cut into small pieces (1–3 mm^3) and placed in a 24-well culture plate containing 0.5–1 ml of PBS. Samples were incubated for 1 h at 37 °C in a humidified CO_2 incubator. Thereafter, the samples were centrifuged at 5000 rpm for 15 min and the supernatant was collected and concentrated by freeze-drying. Protein samples were subjected to SDS-PAGE, in-gel digestion and MS/MS analysis as described previously [6].

2.3. Purification of recombinant AP-57 and antibody generation

AP-57 nucleic acid sequence were synthesized by GenScript Corporation (Nanjing, China). Recombinant proteins expression, purification and antibody generation were performed generally according to a previously reported method with some modifications [7]. AP-57 were purified sequentially by Ni-NTA affinity colomn, EK digestion, SP exchange chromatography and size exclusion chromatography. Two rabbits were immunized by subcutaneous injection of 400 μg of recombinant AP-57 protein with equal amount of complete Freund's adjuvant (Sigma). For subsequent immunizations, 200 μg of AP-57 was mixed with incomplete Freund's adjuvant and injected for 6 times with a 3-week interval.

2.4. Immunohistochemical (IHC) analysis

Tumor/tumor adjacent/normal tissue microarrays were purchased from Shanghai Xinchao Biotechnology (Shanghai, China). IHC were performed as described previously [8]. IHC staining results were examined and scored by two experience pathologists according to a previously reported method [8,9].

2.5. Plasmid transfection, immunofluorescence (IF) and Western Blot (WB) analysis

Transfections were performed by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The images were captured 48 h after transfection. IF and WB were performed according to our previously reported study [10]. Total cellular proteins were extracted in modified RIPA buffer supplemented with protease inhibitor cocktail (Sigma). Equal amounts of protein were resolved on 15% SDS-PAGE gel. Equal volume of culture supernatants from pCDNA3.1-AP-57- or pCDNA3.1 vehicle control-transfected cells were firstly concentrated by ultra-filtration and then subjected to SDS-PAGE and immunoblotting analysis. Golgi were stained with Golgi-tracker from Beyotime Institute of Biotechnology (Jiangsu, China). Cell nucleus were counterstained with DAPI (Roche).

2.6. Microorganisms and treatment

Microorganisms were either purchased from ATCC or isolated from clinical specimens (Table S1). Microorganisms were seeded in a 24-well plate in serum free 1640 medium. Microbe were treated with AP-57 or same amount of BSA in 37 °C for 24 h in 1640 medium. EGFP-expressing lentiviral particles were packaged in HEK293T cells (from Cyagen Biosciences, Guangzhou, China). Lentiviral particles solution were pretreated with AP-57 or BSA in 37 °C for 3 h, and then used for transfection of HEK293A cells. Mycoplasma were stained with hoechst. For all the treatments, PBS treatment were also used as another negative control. Since no obvious difference were observed between PBS- and BSA-treated control groups, these data in PBS control groups were not shown. All experiments were repeated for at least three times.

2.7. Antibodies and other reagents

Primary antibody against AP-57 used in the IHC, IF and WB analysis in this study was generated in rabbit as mentioned above. We also compared its specificity with a commercial antibody purchased from Abcam (ab151109), which was also produced by immunizing with full-length mature Ap-57 protein. As a result, they have very similar staining patterns in IHC, IF and WB assays (Data not shown). Monoclonal antibodies against GAPDH (AG019-1), HRP-conjugated secondary antibodies, and BeyoECL Plus were from Beyotime Institute of Biotechnology (Jiangsu, China).

2.8. Statistical analysis

The significance of the results were calculated by two-tailed unpaired Student's t test utilizing Excel and presented as means ± SD. P values <0.05 were considered significant.

3. Results

3.1. Identification of AP-57 by ESI-Q-TOF analysis

In a proteomic study profiling secretary proteins in extracellular tissue space of clinical colorectal cancer samples, we identified a peptide VPSPNSTNLKGHHVR, which is corresponding to a putative uncharacterized small protein C10orf99 (chromosome 10 open reading frame 99) (Fig. S1).

3.2. Amino acid sequence features of AP-57

Mature human C10orf99 is a short basic peptide of 57 amino acid residues with a net charge +14 (MW = 6.52, PI = 11.28, Fig. 1A). Homology analysis shows that its basic amino acid residues, four cysteines and the hydrophobic C terminal are highly conserved among some mammals, including common model animal Gorilla, Machin, Rat and Mouse (Fig. 1B). When subjected C10orf99 to 3-D structure analysis with Phyre2 Server, the highest homologous protein was human defensin α-5 (HD5), although they only share a 9-amino-acid sequence alignment (HD5 has only 32 amino acid residues) (Fig. S2A). Hydrophobic analysis revealed that it is a

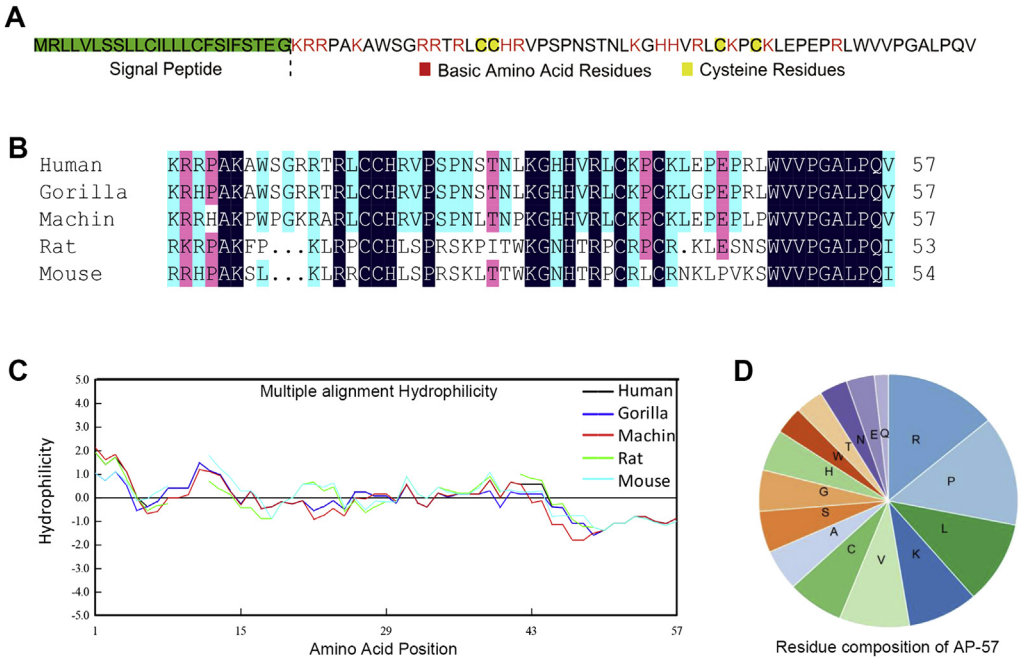

Fig. 1. Features of AP-57 amino acid sequence. (A) Amino acid sequence of AP-57. (B) Sequence conservation analysis. (C) Hydrophilicity analysis. (D) Amino acid composition of AP-57.

typical amphiphilic peptide with a hydrophilic N terminal and a hydrophobic C terminal (Fig. 1C). In consideration of its highly basic and amphiphilic character, composition characteristic of amicno acid residues (Fig. 1D) and structural homology with HD5, we hypothesized that C10orf99 maybe a new type AMPs (all human defensins are characterized by six conserved cysteine residues). To test this hypothesis, we further examined whether C10orf99 possesses antimicrobial activity. For convenience, in the following

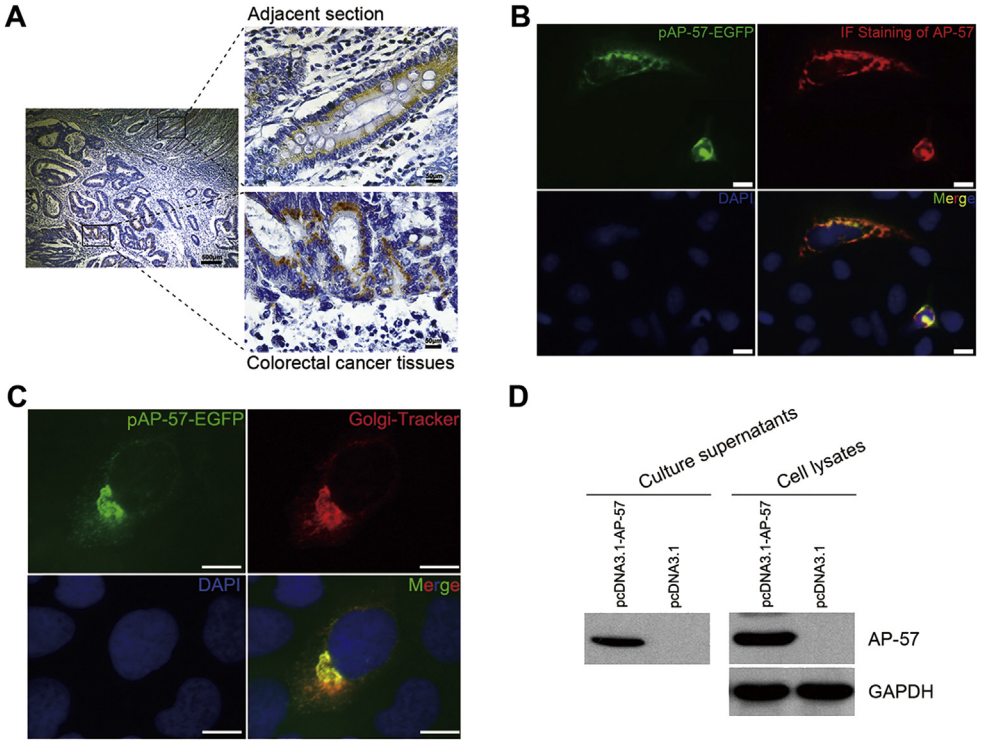

Fig. 2. Identification of secretary protein C10orf99. (A) Positive IHC staining of AP-57 can be observed both in colorectal cancer adjacent tissues and in colorectal cancer tissues in some cases. Scale bar, 50 μm. (B) Specificity of the primary antibody against AP-57. As shown, the primary antibody specifically recognized AP-57-EGFP fusion protein in HEK293A cells transfected with AP-57-EGFP expression vector. Scale bar: 10 μm. (C) Co-localization (yellow) of AP-57-EGFP (green) with Golgi-Tracker (red). Scale bar: 10 μm. (D) Western blot analysis of culture supernatants and cell lysates from transiently transfected HEK293A cells with pcDNA3.1-AP-57. GAPDH was used as loading control for cell lysates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

description, we termed C10orf99 as AP-57 (Antimicrobial Peptide-57, 57 amino acid residues).

3.3. Expression and purification of recombinant AP-57

Firstly, recombinant AP-57 in soluble form were expressed and purified in E. coli system. Disulfide bond formation were examined with reducing and non-reducing SDS-PAGE assayes. As shown in Fig. S2B, there is an obvious difference between the running position of AP-57 under reducing and non-reducing conditions, suggesting that intermolecular disulfide bonds were probably formed in AP-57 protein. We also performed a prediction of the disulfide bond bonding pattern with PredictProtein server. As shown in Fig. S2C, the disulphide linkages of cysteine residues in AP-57 are probably between the first and the fourth cysteine residues (Cys1–Cys4) and Cys2–Cys3.

3.4. Secretory expression of AP-57

Through IHC staining, C10orf99 can be detected both in colorectal cancer tissue and in adjacent normal columnar epithelial cells (Fig. 2A). Fig. 2B analyzed the specificity of the primary antibody against AP-57. As shown, the primary antibody specifically recognized AP-57-EGFP fusion protein in HEK293A cells transfected with AP-57-EGFP expression plasmids. Subcellular location and immnoblotting analysis suggested that C10orf99 is a classical secretary protein (Fig. 2C and D).

3.5. Antimicrobial activity of AP-57

Using recombinant AP-57 protein, we examined its inhibition effects on bacteria, fungi and virus (Fig. 3A–E & Table S1). As shown, for bacteria, only gram-positive bacterium (*Staphylococcus aureus* and *Actinomyce*) exhibited susceptibility to AP-57 treatment (Fig. 3A and B). Nevertheless, we also observed that AP-57 treatment can slow down the movement of some kinds of gram-negative bacterium, such as *E. coli*, *Salmonella typhi* and *pseudomonas aeruginosa*, and hence caused an obvious bacterial aggregation (Fig. 3C). As to fungi, AP-57 shows obviously inhibitory effects on *Aspergillus niger*, while no inhibition of the growth of budding yeast and *Candida albicans* were observed (Fig. 3D & Table S1). We further tested the influence of AP-57 on the infection efficiency of Ad5 adenoviral and lentiviral particles (expressing EGFP) in HEK293A cells. As shown, AP-57 treatment dramatically reduced the infection efficiency of lentivirus, while no apparent changes were observed in adenovirus infection (Fig. 3E & Table S1). Moreover, we found it exerted strikingly membrane-damaging activities against mycoplasma. Fig. 3F shows membrane-damaged mycoplasma aggregated together following treatment with AP-57.

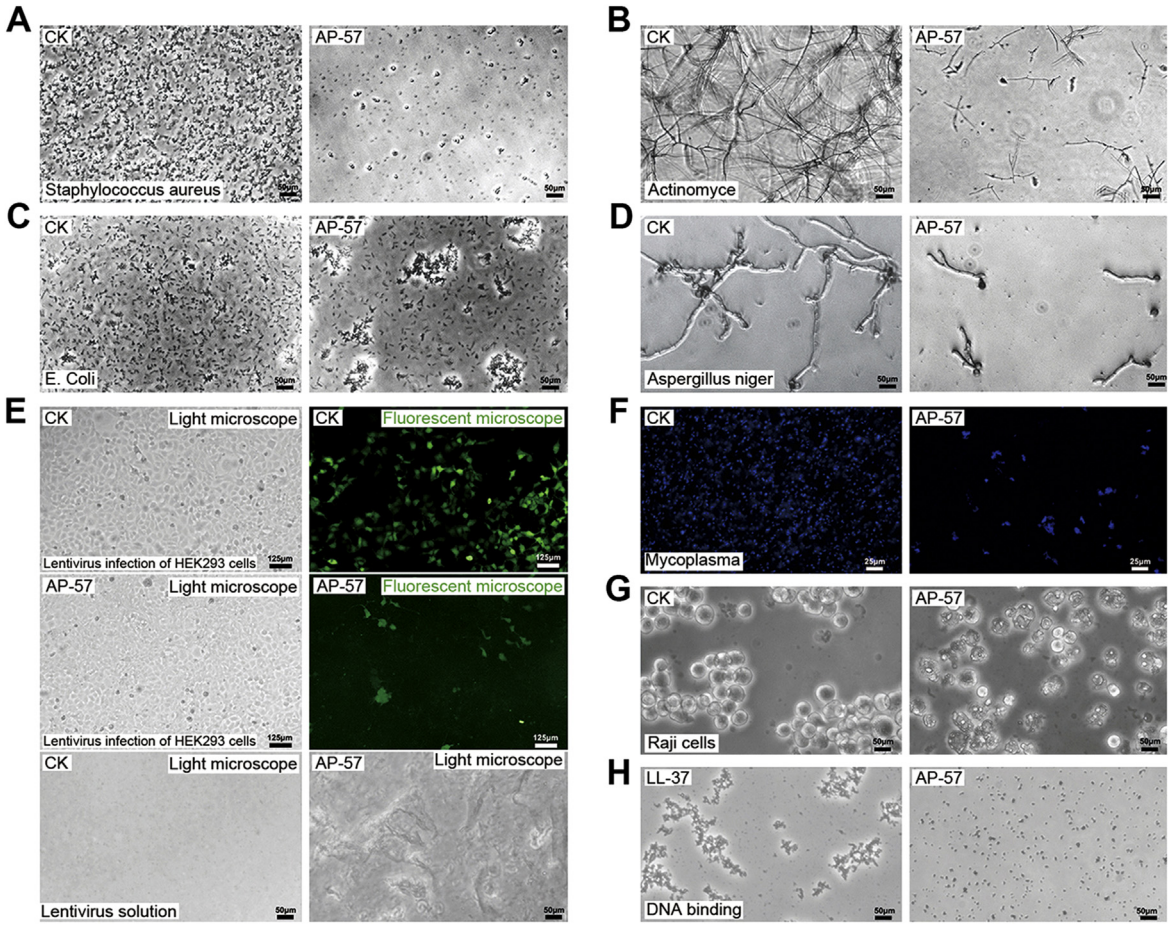

Fig. 3. Representative images of the antimicrobial, antitumor and DNA-binding effects of AP-57. (A–F) Microbe were cultivated and treated in serum-free 1640 medium with 40 μg/ml AP-57 (6 μM, the same order of magnitude as known for other AMPs) or same amount of BSA (CK) in 37 °C for 24 h. EGFP-expressing Lentiviral particles were packaged in HEK293T cells and were pretreated with AP-57 for 3 h before transfection. Mycoplasma hominis were stained with hoechst. (G) Raji Cells were treated in 1640 medium supplemented with 5% FBS as described in supplementary materials. (H) Aggregates formed immediately by mixing genome DNA solution (0.1 μg/μl) with equal volume solution of LL-37 or AP-57 peptides (0.5 μg/μl). All experiments were repeated for at least three times, and PBS treatment was also used as another negative control. Since no obvious difference were observed between PBS- and BSA-treated control groups, these data in PBS control groups were not shown.

3.6. Antitumor and DNA-binding capacity of AP-57

We also tested the potential antitumor effects of AP-57 with sixteen commonly used human cell lines, including Jurcat, Raji, K562, U937, A549, HT-29, LoVo, U87, MCF-7, SKOV3, PC-3, HepG2, L02, HaCaT, HEK293 and HUVEC. As a result, it is very interesting that only human B-cell lymphoma Raji was highly sensitive to AP-57 treatment (Fig. 3G & Fig. S3). As shown, the cell membrane of Raji were almost totally damaged following treatment with 6 μM AP-57 for 24 h. Moreover, it has been reported that LL37 play important roles in immune regulation by sensing self-DNA [11], then we asked whether AP-57 has a similar function. As a result, AP-57 and DNA formed obviously complexes immediately after mixing, suggesting that, just like LL-37, AP-57 may play a role in immune recognition of self-DNA too (Fig. 1H).

3.7. Tissue expression pattern of AP-57

Furthermore, in order to analyze whether the expression pattern of AP-57 were similar to that of other human AMPs, we performed IHC assays using human tissue microarray (Fig. 4 & Fig. S4). The highest level of AP-57 protein expression were detected in stomach and colon. Epithelium of skin and esophagus show obvious positive staining. Strong positive staining were also observed in some tumor and/or tumor adjacent tissues (TAT), including TAT of esophagus cancer and HCC (Hepatocellular Carcinoma), as well as SCC (Squamous Cell Carcinoma), BCC (Basal cell carcinoma) and IDC (Invasive Ductal Carcinoma) tissues. In particular, while weak or negative staining in normal liver and HCC, some cases show strong positive staining in HCC adjacent tissues (TAT), indicating the expression of AP-57 in liver maybe inducible.

4. Discussion

In the present study, we identified a new type human AMPs, AP-57. Compared with other human AMPs (defensins, LL-37, histatins and hepcidin), AP-57 shows some distinct features. Firstly, the sequence length of AP-57 is longer than other human AMPs (normally less than 45 residues). For the second, AP-57 has four cysteine residues, while hepcidin contain eight cysteines, LL-37 and histatins have no cysteine, and all human defensins possess 6 cysteines. Thirdly, AP-57 is widely expressed in mucosa of digestive tract, epidermis of skin, as well as some tumor and/or tumor adjacent tissues, and the expression patterns are obviously different from other human AMPs.

As to the antimicrobial activity, although AP-57 exhibited broad-spectrum antimicrobial effects, it lacks appreciable bactericidal activity against gram-negative bacterium. Nevertheless, AP-57 treatment can slow down the movement and hence caused an obvious bacterial aggregation. Recently, it was reported that human α-defensin 6 (HD6) lacks bactericidal activity, but affords protection against invasion by enteric bacterial pathogens in vitro and in vivo by undergoing ordered self-assembly to form fibrils and nanonets that surround and entangle bacteria [12]. Thus the promotion of bacterial aggregation by AP-57 resembles the surrounding and entangling capacities of HD6 in some way.

Particularly, mycoplasma are the smallest free-living organisms able to self-replicate. Without a cell wall, they are unaffected by many common antibiotics. They can cause a variety of infectious human diseases, and mycoplasma contamination are the long-standing widespread problems in cell culture. The powerful anti-mycoplasma capacity of AP-57 may provide new approach for mycoplasma therapy and prevention. On the other hand, it is really interesting that for the sixteen cell types, only B-cell lymphoma Raji

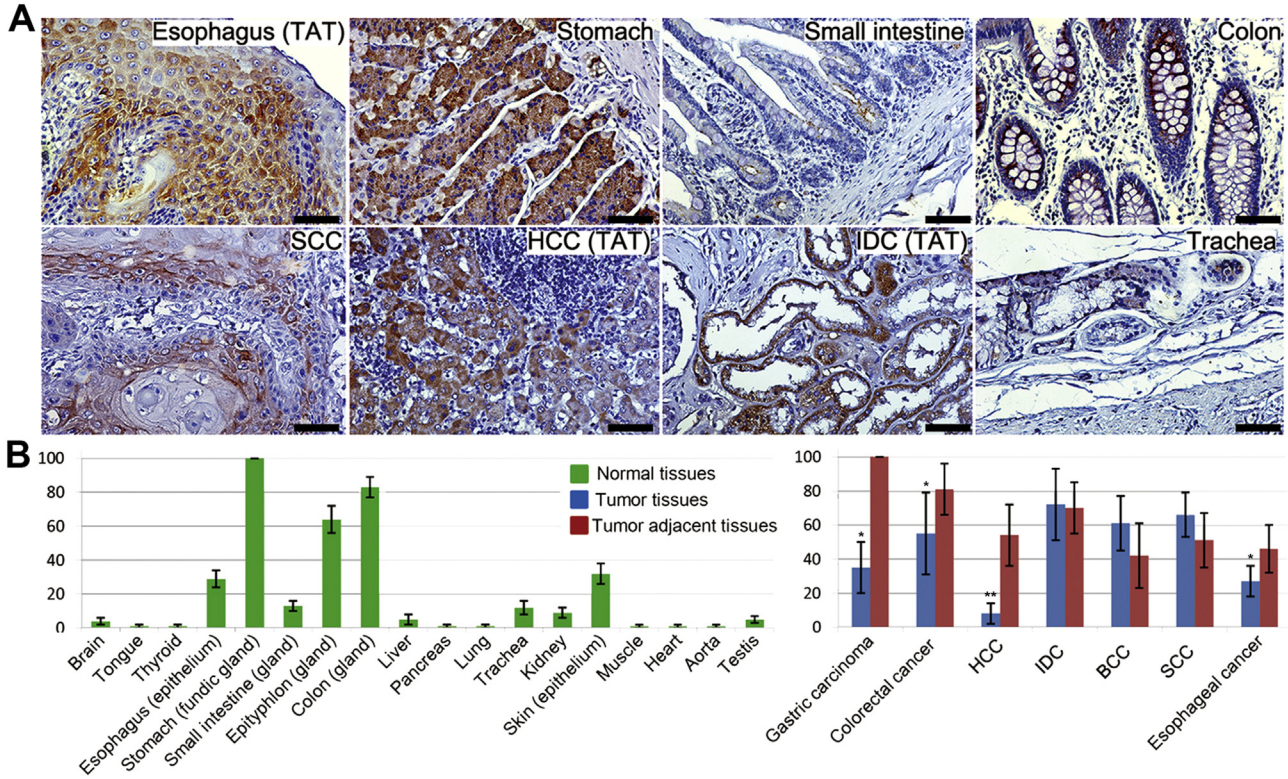

Fig. 4. Positive IHC staining of AP-57 in different human tissue samples. TAT, Tumor adjacent tissue; HCC, Hepatocellular Carcinoma; IDC, Invasive Ductal Carcinoma; BCC, skin Basal Cell Carcinoma; SCC, skin Squamous Cell Carcinoma. The highest expression of AP-57 in fundic gland was set to 100 and all individual expression levels were scored by compared to its expression in fundic gland. Enlarged images were supplemented in Fig. S4. Scale bar: 200 μm *, $P < 0.05$; **, $P < 0.01$.

was highly sensitive to AP-57 treatment. It is worth carrying out further study to explore mechanisms underlying the cell specific effects of AP-57.

During our preparation of this manuscript, Pan et al. reported that C10orf99 maybe a potential cytokine highly expressed in colon tissue [13]. In that study, they reported that C10orf99 can interact with Sushi Domain Containing 2 (SUSD2) and was able to inhibit colon cancer cell growth through inducing G1 arrest. We also examined the effects of AP-57 on the growth of several cell line, including colorectal cancer cell HT-29 and LoVo. As a result, the most sensitive cell line was Raji (Fig. S3). These different results may caused by the different recombinant proteins we used. Pan et al. used C10orf99-Fc fusion protein, while we used untagged protein (just 57 amino acid residue). It is probably that fuse a large tag, like Fc, at C terminal of C10orf99 may influence its function, because the ten aminao acid of C terminal are very conservative and hydrophobic. Previously, in a large-scale mouse mutant study, Tang et al. reported that C10orf99 knockout exhibited an increased ratio of CD4+/CD8+ and a decreased serum IgM level, suggesting that C10orf99 may play a role in immune regulation [14]. Other large scale gene expression analysis indicated that C10orf99 was significantly up-regulated in psoriasis patients [15,16]. This results were consistent with the expression characters of some antimicrobial peptides, such as LL-37 and beta-defensin 2, which was significantly induced in skin infection, wounds and psoriatic lesions [17].

In summary, here we for the first time demonstrate that AP-57 is a new type AMPs. Compared with other human AMPs (Defensins, LL-37, Histatins and Hepcidin), AP-57 has its distinct characters, including longer sequence length, four cysteines, highly cationic character, cell-specific toxicity, DNA binding and tissue-specific expressing patterns. More studies are required to explore the molecular mechanisms underlying the multi-effects of AP-57 on inhibition of microbes, cell-specific toxicity and immune regulation. Nonetheless, we hope that the identification of AP-57 as a distinct antimicrobial peptide will open up fruitful avenues of research.

Declaration of financial disclosure

A patent application (201410669781.3) has been filed related to this work by Aiping Tong & Meijia Yang.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2014.12.115.

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