Host immune defense peptide LL-37 activates caspase-independent apoptosis and suppresses colon cancer

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Short title: LL-37 induces AIF- and EndoG-mediated cell death

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Abbreviations: 5AZA, 5-aza-2'deoxycytidine; AIF, apoptosis-inducing factor; DZNep, 3-deazaneplanocin A; EGFR, epidermal growth factor receptor; EndoG, endonuclease G; FPR2, formyl peptide receptor 2; GPCR, G-protein-coupled receptor; hCAP18, human cationic antimicrobial protein 18; IGF-1R, type I insulin-like growth factor receptor; TLR-9, Toll-like receptor 9; TSA, trichostatin A.

Disclosure of Potential Conflicts of Interest
The authors declare that they have no conflict of interest.
ABSTRACT

Cathelicidins are a family of bacteriocidal polypeptides secreted by macrophages and polymorphonuclear leukocytes (PMNs). LL-37, the only human cathelicidin, has been implicated in tumorigenesis but there has been limited investigation of its expression and function in cancer. Here we report that LL-37 activates a p53-mediated, caspase-independent apoptotic cascade that contributes to suppression of colon cancer. LL-37 was expressed strongly in normal colon mucosa but downregulated in colon cancer tissues, where in both settings its expression correlated with TUNEL-positive apoptotic cells. Exposure of colon cancer cells to LL-37 induced phosphatidylserine externalization and DNA fragmentation in a manner independent of caspase activation. Apoptogenic function was mediated by nuclear translocation of the pro-apoptotic factors AIF and EndoG, through p53-dependent upregulation of Bax and Bak and downregulation of Bcl-2 via a pertussis toxin-sensitive G-protein-coupled receptor pathway. Correspondingly, colonic mucosa of cathelicidin-deficient mice exhibited reduced expression of p53, Bax and Bak and increased expression of Bcl-2 together with a lower basal level of apoptosis. Cathelicidin-deficient mice exhibited an increased susceptibility to azoxymethane-induced colon tumorigenesis, establishing pathophysiological relevance in colon cancer. Collectively, our findings show that LL-37 activates a GPCR-p53-Bax/Bak/Bcl-2 signaling cascade that triggers AIF/EndoG-mediated apoptosis in colon cancer cells.

Keywords: Cathelicidin; colon cancer; p53; caspase-independent apoptosis

PRECIS

Findings suggest that a bacteriocidal factor secreted by macrophages, PMNs and colonocytes contributes to colon cancer suppression by activating a novel pathway of apoptosis in colon cancer cells.
INTRODUCTION

Cathelicidin is a host defense peptide secreted by bone marrow cells, circulating leukocytes, and numerous types of epithelial tissues. It plays an active role in the maintenance of innate immunity. Not only does this peptide eliminate pathogenic microbes directly by serving as a “natural antibiotic”, but it also orchestrates a complex integration of host defense responses, including chemotaxis, cytokine production, and tissue repair (1). In human, cathelicidin is expressed as a 18-kDa preproprotein known as cationic antimicrobial protein 18 (hCAP18). Proteolytic cleavage of hCAP18 is required for the release of the mature peptide LL-37. In the gastrointestinal tract, cathelicidin protects against *Helicobacter pylori* infection, promotes gastric ulcer healing and alleviates inflammation in ulcerative colitis (2-4). LL-37 is highly expressed in colonic epithelial cells of the surface and upper crypts. Deeper crypt regions also have low-to-moderate-level expression of LL-37 (5).

Emerging evidence supports that cathelicidin is implicated in malignant diseases. The expression of LL-37 is dysregulated in gastric, ovarian, lung and breast cancers as well as melanoma and leukemia (6-11). The role of cathelicidin in tumorigenesis is complex and believed to be context-dependent (12). Acting as a tumor-suppressor, the expression of hCAP18/LL-37 is downregulated in gastric adenocarcinomas and acute myeloid and lymphocytic leukemia (6, 11, 13). In this regard, LL-37 induces cell cycle arrest and apoptosis in gastric cancer cells and T lymphocytes, respectively (6, 14). The C-terminal fragment of LL-37 also exerts cytotoxic effects on both drug-resistant and drug-sensitive oral epitheloid carcinoma cells (15). Cathelicidin also enhances the antitumor activity of natural killer cells (16). The expression and function of cathelicidin in colon cancer, however, remain unclear.

A loss of balance between cell proliferation and cell death is central to tumorigenesis. Apoptosis or type I programmed cell death is frequently dysregulated in human cancers. In the colon, reduced rates of apoptosis are associated with an increased risk of colon adenomas (17). In mammalian cells, apoptotic signals are generally mediated through two caspase-dependent molecular pathways, namely, intrinsic and extrinsic cascades. Accumulating evidence supports that the third molecular pathway of apoptosis does exist. This pathway is characterized by the nuclear translocation of apoptosis-inducing factor (AIF) and endonuclease G (EndoG) that trigger chromatin condensation and DNA fragmentation independent of caspase activation (18). In the present study, we demonstrate that LL-37 was substantially downregulated in human colon cancer tissues and induced apoptosis in cultured colon cancer cells through the third molecular pathway of apoptosis.
Materials and Methods

Reagents
All primary antibodies for Western blot were purchased from Cell Signaling Technology (Beverley, MA, USA) except FPR2 antibody (Abcam, Hong Kong). All other chemicals and reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified.

Patient samples and immunohistochemistry
Paraffin-embedded tissue microarrays consisting of a total of 102 formalin-fixed colon adenocarcinoma tissues were used. The corresponding non-cancerous colon mucosal tissues available from 60 matched cases were also included. The median age of the patients was 68 years (32-92 years) and the male to female ratio was 1.12:1. Immunohistochemical staining of mature LL-37 and Ki-67 was performed using polyclonal LL-37 (C14) antibody (Santa Cruz) and Ki-67 antibody (ab15580), respectively, with standard avidin-biotin method. The cytoplasmic expression of LL-37 and Ki-67 was assessed by a proportion score (0, none; 1, <5%; 2, 5-20%; 3, >20% for LL-37; 0, none; 1, <20%; 2, 20-40%; 3, >40% for Ki-67).

Experimental Animals
129/VJ wild-type and cathelicidin-knockout (Cnlp−/−) mice were produced as previously described (19). The mice were maintained on normal diet and under standard laboratory condition. The present study was approved by the institutional Laboratory Animals Ethics Committee.

Cell culture and assays for cell viability, cell cycle and necrotic cell death
The human colon cancer cell lines HT-29, HCT116, SW1116, SW620, SW480 and LoVo and the normal colon fibroblasts CCD-18Co were obtained from the American Type Culture Collection (Manassas, VA, USA) and authenticated with short-tandem repeat profiling by the vendor. Bax-knocked out (Bax−/−) and Bax-hemizygously deleted (Bax+/−) HCT116 cells were generated as previously described (20). Cells were maintained in their respective recommended culture media, supplemented with 10% fetal bovine serum (1% during treatment), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Cell viability, cell cycle distribution and necrotic cell death were determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], flow cytometry and lactate dehydrogenase release assays, respectively, as previously described (6).

Methylated-DNA capture (MethylCap)-qPCR
Genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen) and bisulfite-modified using EZ DNA Methylation Kit (Zymo Research). Methylated DNA was isolated from sonicated DNA
(200-600 base-pair fragments) with affinity purification using MethylMiner™ Methylated DNA Enrichment kit (Invitrogen). Equal amounts of amplified MethylCap and input DNAs were quantified using PCR primers targeting the hypermethylated regions by SYBR Green-base detection (Applied Biosystems) as previously described (21).

**Nuclear protein extraction and Western immunoblotting**

The isolation of nuclear and cytosolic protein was performed using NucBuster Protein Extraction Kit (EMD Biosciences, Darmstadt, Germany). For isolation of whole-cell protein, cells were harvested in radioimmunoprecipitation buffer containing proteinase and phosphatase inhibitors. Equal amount of proteins were resolved by SDS–PAGE followed by a standard immunoblotting procedure.

**Immunofluorescence**

Cells grown on coverslips were fixed with 4% (v/v) paraformaldehyde for 30 min. The cells were then covered with 10% (v/v) goat serum for 60 min at room temperature followed by incubation with diluted primary antibody at 4 °C overnight. Cells were then probed with Alexa Fluor 488 secondary antibodies (Invitrogen). Fluorescent signals were detected using a confocal fluorescence microscope (Nikon EZ-C1, Nikon, Tokyo, Japan).

**RNA interference and Bcl-2 overexpression**

The expression of AIF, EndoG, Bak and p53 were lowered using pre-designed target-specific small interference RNAs (siRNAs) purchased from Qiagen (Valencia, CA, USA). The Flag-Bcl-2 expression vector (18003) was obtained from Addgene, deposited by Dr. Clark W. Distelhorst (Case Western Reserve University, Cleveland, OH). siRNAs or purified plasmids were transfected into cells using Lipofectamine™ 2000 reagent (Invitrogen).

**Quantitation of DNA fragmentation**

DNA fragmentation in human colon cancer cell lines and mouse tissue samples was measured by in situ terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit from Roche Molecular Biochemicals (Mannheim, Germany). The mouse mucosal TUNEL-positive cells were evaluated in five randomized fields at 400 × magnification, and the average was taken and expressed as the number of apoptotic cells of each samples. For human tissue microarrays, apoptotic cells were measured using the TUNEL stain with the Dead-End kit (Promega, Madison, WI, USA). Apoptosis was assessed by a proportion score (0, none; 1, <5%; 2, 5-10%; 3, >10%).

**Quantitation of phosphatidylserine externalization**
Treated cells were re-suspended in staining buffer containing propidium iodide and annexin V–
oflorescein isothiocyanate (Invitrogen). After incubation for 15 min in dark, double-labeled cells were analyzed by the FACSCalibur System and CellQuest program.

**Statistical analysis**
Results were expressed as means ± SD of multiple experiments unless otherwise specified. Statistical analysis was performed with Student’s t-test or an analysis of variance (ANOVA) followed by the Tukey’s t-test where appropriate. *P* values less than 0.05 were considered statistically significant.
Results

Tissue microarray revealed the downregulation of LL-37 and its correlation with apoptosis in human colon cancer tissues

In non-cancerous colonic mucosa, intermediate-to-high levels of cytoplasmic stain of LL-37 was observed (Fig. 1A). In colon cancers, 68 out of 102 (66.7%) cases showed complete loss of LL-37 expression. Weak, intermediate and strong immunoreactivities were noted in 18.6% (19/102), 5.9% (6/102) and 8.8% (9/102) of the cases, respectively (Fig. 1A). In 48 corresponding adjacent non-cancerous tissues where LL-37 immune scores were available, downregulation of LL-37 was seen in 46 out of 48 (95.8%) cases (Fig. 1B; \( p < 0.0001 \)). The overall expression of LL-37 in 102 cases of colon cancer was also significantly lowered than that of non-cancerous colonic tissues (Fig. 1C; \( p < 0.0001 \)). Colonic epithelial cells were the major cell type that expressed LL-37, although immunoreactivity to LL-37 was also detected in submucosal leukocytes. The immune score of LL-37 was also positively correlated with TUNEL- but not Ki-67-positive (Fig. 1D and E) cells. The downregulation of LL-37 was not associated with the differentiation status or stage of the tumors.

DNA demethylation restored LL-37 expression in colon cancer cells

Cultured human colon cancer cells were treated with different epigenetic modifying agents, namely, 5-aza-2’-deoxycytidine (5AZA; a DNA demethylating agent), trichostatin A (TSA; a histone deacetylase inhibitor) and 3-deazaneplanocin A (DZNep; a histone methylation inhibitor) (22). 5AZA but not TSA or DZNep restored LL-37 expression in cultured colon cancer cells (Fig. 2A, B and C). Concordantly, methylated-DNA capture coupled with quantitative PCR revealed that DNA methylation in the promoter of \( CAMP \) (the gene encoding hCAP18/LL-37) was significantly higher in primary colon cancer tissues and human colon cancer cell lines HCT116 and LoVo as compared with normal colonic mucosa (Fig. 2D). By contrast, 5AZA treatment reduced the DNA methylation level (Fig. 2E). These findings would suggest that promoter DNA methylation was responsible for the downregulation of LL-37 in human colon cancer.

LL-37 induced DNA fragmentation and phosphatidylserine externalization without caspase activation in colon cancer cells

A panel of human colon cancer cell lines that consisted of HCT116 (p53 wild-type), LoVo (p53 wild-type), SW1116 (p53 mutant), SW480 (p53 mutant), and SW620 (p53 mutant), were treated with increasing concentrations of synthetic LL-37 peptide (Invitrogen) for 24 h. The effect of LL-37 on normal colon fibroblasts CCD-18Co was also determined. MTT assay showed that LL-37 strongly reduced cell viability at doses ranging from 20 to 60 \( \mu M \) in p53-wild-type HCT1116 and LoVo. LL-37 did not show any effect on the viability of these two cell lines at low doses (500 ng/ml.
to 10 µg/ml; equivalent to 111 nM to 2.2 µM) that have been shown to stimulate cell proliferation in other cancer types (7-9). LL-37 also exerted significant but lesser inhibitory effects on p53-mutant SW1116, SW480 and SW620 but exhibited minimal cytotoxicity in CCD-18Co (Fig. 3A). In addition, mCRAMP (the murine cathelicidin) was used to treat the mouse cancer cell line Colon-26 (p53-wild-type) and the mouse immortalized colonocytes YAMC. Similar to the effect of LL-37, mCRAMP reduced cell viability of Colon-26 but had minimal effect on YAMC (Fig. 3A). These findings suggest that cathelicidin may have differential cytotoxicity towards p53-wild-type colon cancer cells over normal cells and p53-mutant colon cancer cells. Lactate dehydrogenase release assay indicated that LL-37 at the tested concentrations did not induce necrotic cell death (data not shown). Cell cycle analysis showed that LL-37 treatment resulted in the accumulation of the sub-G<sub>1</sub> phase, which was suggestive of DNA fragmentation associated with apoptosis (Fig. 3B and S1A). The apoptogenic effect of LL-37 in colon cancer cells was subsequently confirmed by TUNEL assay (Fig. 3C and S1B) and Annexin V staining (Fig. 3D), which detects DNA fragmentation and phosphatidylserine externalization, respectively. Activation of caspases and the subsequent cleavage of poly (ADP-ribose) polymerase (PARP) are classical molecular markers of apoptosis. Unexpectedly, after 24 h and 48 h of LL-37 treatment, no increase in PARP cleavage nor activation of caspases-3, -7 and -9 could be observed. On the contrary, both activation of caspases and cleavage of PARP were reduced by LL-37 (Fig. 3E), hinting at the possibility that LL-37 induced apoptotic cell death in a caspase-independent manner. This postulation was corroborated by the finding that the pan-caspase inhibitor z-VAD-fmk failed to reverse the loss of cell viability caused by LL-37 in HCT116 and LoVo (Fig. 3F).

**LL-37 triggered nuclear translocation of AIF and EndoG to mediate apoptosis**

AIF and EndoG are known mediators of cell death in caspase-independent apoptosis. These mediators, originally localized in the mitochondria, translocate into the nucleus to initiate DNA degradation upon activation by apoptotic signals (18). As shown in Fig. 4A, LL-37 increased the nuclear protein levels of AIF and EndoG and inversely, reduced their cytosolic levels. The increases in the nuclear expression of AIF and EndoG were peaked at 6 h after LL-37 treatment. The change of subcellular localization of AIF and EndoG in response to LL-37 treatment was confirmed by immunofluorescence. After exposure to LL-37 for 6 h, AIF and EndoG redistributed from the cytosol to the nucleus (Fig. 4B, 4C and S1C). To determine if AIF and EndoG were functionally involved in LL-37-induced apoptosis, RNA interference was used to knock down their expression. Transfection with AIF- or EndoG- small interfering RNA (siRNA) reduced nuclear protein levels (Fig. 4D) and nuclear localization (Fig. 4E) of respective target induced by LL-37. RNA interference targeting AIF and EndoG, alone or in combination, significantly reduced the DNA fragmentation caused by LL-37 as revealed by TUNEL assay (Fig. 4F and S1D).
Altered expression of Bcl-2 family members was required for AIF- and EndoG-mediated apoptosis induced by LL-37

The mitochondrial release of AIF and EndoG is mediated by members of the Bcl-2 family (23). In this connection, LL-37 upregulated the pro-apoptotic Bax and Bak and reduced the anti-apoptotic Bcl-2 (Fig. 5A and S1E). The upregulation of Bax and Bak and the downregulation of Bcl-2 were most prominent at 6 h after LL-37 treatment. To this end, genetic ablation of Bax, knockdown of Bak or restoration of Bcl-2 expression reduced the nuclear protein levels of AIF and EndoG (Fig. 5B) and apoptosis (Fig. 5C) that were induced by LL-37. The reversal effect of genetic ablation of Bax was more prominent than that was detected in the knockdown of Bak. In this regard, knockdown of Bak in Bax^-/- cells or overexpression of Bcl-2 almost completely reversed LL-37-induced AIF/EndoG nuclear expression and apoptosis (Fig. 5B and C). These results would suggest that upregulation of Bax and Bak and downregulation of Bcl-2 were required for caspase-independent apoptosis that was induced by LL-37 in colon cancer cells.

p53 activation was required for LL-37-induced apoptosis

The tumor suppressor protein p53 has been shown to mediate both caspase-dependent and -independent apoptosis in a wide variety of biological contexts (24-26). As shown in Fig. 6A, LL-37-treated HCT116 cells (p53-wild type) showed increased total and nuclear levels of p53. LL-37 also increased p53 expression in LoVo cells (Fig. S1E). The protein expression of PUMA, a direct target gene of p53, was increased upon treatment with LL-37 in HCT116. The increased nuclear levels of p53 were peaked at 3 h after LL-37 treatment, which preceded the altered expression of Bax, Bak and Bcl-2. To confirm if the altered expression of Bcl-2 family members caused by LL-37 was p53-dependent, we used siRNA to silence p53 in HCT116. Knockdown of p53 significantly reversed the induction of Bax and Bak as well as the downregulation of Bcl-2 caused by LL-37 (Fig. 6B). Above all, knockdown of p53 reversed the increase in the nuclear expression of AIF and EndoG (Fig. 6C) and apoptosis (Fig. 6D) in LL-37-treated HCT116 cells. In line with proposed central role of p53, LL-37 failed to induce phosphatidylserine externalization in the p53-mutant SW1116 colon cancer cells (Fig. 6E).

The apoptogenic action of LL-37 was mediated by G protein-coupled receptor (GPCR)

The biological effect of LL-37 has been reported to be mediated through G_i-coupled GPCR in several cell types (27, 28). Here, we demonstrated that pertussis toxin (PTX) as an inhibitor of G_i-coupled GPCR reversed the upregulation of p53, Bax and Bak and the downregulation of Bcl-2 by LL-37. More importantly, PTX was also observed to reduce LL-37-induced phosphatidylserine externalization in HCT116 (Fig. 6F). We found that formyl peptide receptor 2 (FPR2; formerly known as formyl peptide receptor like-1), a G_i-coupled GPCR that has been shown to mediate the biological action of LL-37 in other cell types, was expressed in colon cancer cells (data not
shown). Nevertheless, the specific antagonist WRW4 failed to reverse the action of LL-37 (Fig. 6F), suggesting that a non-FPR2 G_i-coupled GPCR was responsible for the activation of p53-Bcl-2/Bax/Bak axis by LL-37 to induce caspase-independent apoptosis.

Cathelicidin-knockout mice showed reduced basal apoptotic rate in colonic mucosa and increased susceptibility to azoxymethane-induced gross colonic tumors

The protein expression of p53, Bax, Bak and Bcl-2 together with the basal level of apoptotic cells were determined in wild-type and cathelicidin-knockout (Cnlp^{-/-}) mice. As compared with the wild-type mice, Cnlp^{-/-} mice exhibited lower basal expression of p53, Bax, and Bak and higher basal expression of Bcl-2 in the colonic mucosa (Fig. 7A). Phenotypically, Cnlp^{-/-} mice had significantly reduced number of TUNEL-positive cells in the colonic mucosa as compared with the wild-type mice (Fig. 7B). However, genetic ablation of Cnlp in mice did not have any substantial effect on crypt length or cellularity of the colon under normal conditions. To study the in vivo function of cathelicidin in the context of colon tumorigenesis, wild-type and Cnlp^{-/-} mice (n = 7 in each group) were injected intraperitoneally with azoxymethane (AOM) (Fig. 7C), a colon-specific carcinogen capable of inducing only microscopic premalignant lesions (i.e. aberrant crypt foci) but not normally gross tumors in mice. At 16 weeks after the last injection of AOM, all 7 Cnlp^{-/-} mice developed gross colonic tumors. In contrast, only 1 out of 7 wild-type mice developed a minor colonic tumor while the remaining mice were free from macroscopic lesion. The multiplicity and total tumor mass of AOM-injected Cnlp^{-/-} mice were significantly higher than those of wild-type mice (Fig. 7D).
Discussion

LL-37 is the only cathelicidin found in humans. The context-dependent function of LL-37 poses some difficulties to define and understand its exact role in tumorigenesis. For example, while LL-37 has been shown to promote the growth of ovarian (7), lung (8) and breast cancers (9), the same peptide exerts tumor-suppressing effects in gastric cancer (6) and acute myeloid (13) and lymphocytic leukemia (11). Consistent with its role as a tumor suppressor, LL-37 expression is downregulated in colon cancer. The correlation between LL-37 and apoptosis but not cell proliferation has also been shown by clinical sample analysis. Subsequent experiments revealed that promoter DNA hypermethylation might account for the downregulation of LL-37. Cathelicidin-deficient mice also exhibited increased susceptibility to carcinogen-induced colonic tumors. The prominent downregulation of LL-37 and its association with apoptosis in human colon cancer prompt us to investigate if LL-37 has apoptogenic function in colon tumorigenesis. In this study, our data revealed that, in p53 wild-type colon cancer cells, LL-37 induced phosphatidylserine externalization and DNA fragmentation without caspase activation, suggesting the occurrence of caspase-independent apoptosis. Such an apoptogenic role of cathelicidin has been reported in other cell types, such as periodontal ligament cells and infected airway epithelium (29, 30). But for the first time through our investigations it is demonstrated that cathelicidin acts through the caspase-independent pathway to mediate apoptosis in colon cancer cells.

The tumor-suppressor protein p53 accumulates in cells in response to DNA damage, oncogene activation and other stresses. Depending on the cellular context, activation of p53 could lead to cell cycle arrest, apoptosis, cellular senescence, differentiation, and autophagy (31). Restoring p53 activity is an attractive approach for cancer therapy and has led to the identification of a number of potential anticancer therapeutics, such as CP-31398 and Nutlin (32). Here we show that LL-37 increased whole-cell p53 expression and p53 nuclear accumulation accompanied by induction of PUMA, Bax and Bak and reduction of Bcl-2. In agreement, p53 has been shown to transcriptionally activate \textit{BAX} and repress \textit{BCL2} (33, 34). The regulation of p53 and the Bcl-2 family members (i.e. Bax, Bak and Bcl-2) by cathelicidin was also recapitulated in \textit{Cnlp}⁻⁻ mice. Our findings indicated that p53 plays a central role in cathelicidin-induced caspase-independent apoptosis.

AIF/EndoG-mediated DNA fragmentation represents a major mechanism of caspase-independent apoptosis (18). Here we clearly showed that LL-37 induced nuclear translocation of AIF and EndoG to mediate apoptosis. We also showed that knockdown of AIF partially reduced the nuclear expression and localization of EndoG and vice versa (Fig. 4D & E), suggesting a novel reciprocal regulation between these two cell death mediators. Although AIF has been shown to
bind to EndoG (35), it is the first time to demonstrate the existence of such a cooperative interaction. The release of AIF and EndoG is regulated by mitochondrial outer membrane permeability, which is in turn controlled by the relative abundance of the pro- and anti-apoptotic members of the Bcl-2 family. Consistent with its role in apoptosis, LL-37-induced nuclear translocation of AIF/EndoG was paralleled by the upregulation of Bax and Bak and the downregulation of Bcl-2. The BH3-only protein PUMA, which is required for Bax- and Bak-mediated cell death (36), was also upregulated by LL-37. Bax and Bak can form complexes at the mitochondrial outer membrane to facilitate the release of apoptogenic mediators whereas Bcl-2 sequesters BH3-only molecules to prevent the activation of Bax and Bak (37). In particular, Bax and Bak could promote the release of AIF and EndoG in a caspase-independent manner (38). Bcl-2 could also interact with Bax directly to prevent its homo-oligomerization that is required for membrane permeabilization (39). Our study shows that altered expression of these Bcl-2 family members triggers the activation of AIF and EndoG in LL-37-treated colon cancer cells.

In this study, PTX as an inhibitor of G\textsubscript{i}-coupled GPCR blocked the apoptogenic effect and downstream signaling of LL-37. Several receptors, including FPR2, epidermal growth factor receptor (EGFR), P2X\textsubscript{7} purinoceptor, Toll-like receptor 9 (TLR-9) and type I insulin-like growth factor receptor (IGF-1R), have been reported to mediate the biological actions of cathelicidin (27, 40-43). Among these, only FPR2 is a PTX-sensitive GPCR. The others are receptor tyrosine kinases (EGFR and IGF-1R), membrane ion channel (P2X\textsubscript{7}), or MyD88-dependent receptor (TLR-9) that are not coupled to G-protein. Although FPR2 was expressed in colon cancer cells, the specific antagonist WRW4 failed to reverse the action of LL-37, suggesting a FPR2-independent mechanism. However, it is still untested whether LL-37 by itself could bind to FPR2 and to initiate the downstream signaling in colon cancer cells. It is also unclear whether the binding of LL-37 to FPR2 could be blocked by WRW4. Aside from FPR2, Niyonsaba et al. reported that the chemotactic effect of LL-37 on mast cells is mediated by another G\textsubscript{i}-coupled GPCR (28). Further investigations will be needed to identify the receptor for LL-37 in colon cancer cells. Whether the tissue-dependent expression of receptors may alter the oncogenic or tumor-suppressing function of LL-37 also warrants further study.

In cancer therapy, the rapid regrowth of tumor after initial response to chemotherapeutic agents constitute a major clinical challenge. A recent study demonstrated that caspase-3 activation in dying cancer cells triggered the release of prostaglandin E\textsubscript{2}, which in turn stimulated the growth of surviving tumor cells (44). This finding would suggest that novel therapeutic agents that modulate through a caspase-independent mechanism may hold specific promises for cancer drug development. Our data supports that LL-37 could induce caspase-independent apoptosis in human colon cancer cells via the activation of G\textsubscript{i}-coupled GPCR-p53-Bcl-2/Bax/Bak-AIF/EndoG
cascade. Our study not only elucidated the tumor-suppressing mechanism of LL-37 in colon tumorigenesis, but also support the development of synthetic LL-37 peptide as an inducer of caspase-independent apoptosis.

**Acknowledgments**
We thank Prof. Wing-Tai Cheung (CUHK) for his helpful discussion and Dr. Susan Wei for critical editing of this manuscript.

**Grant Support**
This work was supported by research grant from the CUHK Group Research Scheme (3110043) and CUHK Focused Investments Scheme-Scheme C.
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Figure legends

**Fig. 1. Downregulation of LL-37 and its correlation with apoptosis in human colon cancer tissues.** (A) Immunostaining of LL-37 was performed in 102 cases of colon cancer on tissue microarrays. Representative LL-37 immunostaining in (i) the non-cancerous colonic mucosa and (ii) – (iv) colon cancer tissues with LL-37 expression scores from 1 to 3 were shown. (B) The percentage of LL-37-expressing cells was significantly lower in tumor tissues as compared with corresponding adjacent non-cancerous tissues in 48 colon cancer patients ($p < 0.001$; paired t-test). (C) The overall expression of LL-37 in 102 colon cancer tissues was significantly lower as compared with 48 non-cancerous mucosal tissues ($p < 0.001$; unpaired t-test). (D) Representative TUNEL and Ki-67 staining in colon cancer tissues with different levels of LL-37 expression were shown. (E) TUNEL score was significantly higher in colon cancer tissues with high expression of LL-37 than those with no or low-level LL-37 expression. (F) Ki-67 score had no association with LL-37 expression in colon cancer tissues. *, $p < 0.05$; **, $p < 0.01$, significantly different between indicated groups.

**Fig. 2. Restoration of LL-37 expression by DNA demethylation in colon cancer cells.** (A) HCT116 cells were treated with 5-aza-2’dexocytidine (5AZA; 1 µM) for 72 h, trichostatin A (TSA; 300 nM) for 20 h or 3-deazaneplanocin A (DZNep; 1 µM) for 48 h, in combination or alone, followed by RNA extraction and quantitation of hCAP18/LL-37 mRNA by real-time RT-PCR. (B) Treatment with 5AZA (1 µM, 72 h) restored hCAP18/LL-37 mRNA expression in LoVo. (C) Western blot shows upregulation of LL-37 protein expression in HCT116 and LoVo treated with 5AZA (1 µM, 72 h). (D) DNA methylation of CAMP promoter was quantitated by MethylCap-qPCR in normal colonic mucosa, HCT116, LoVo and primary colon cancer tissues ($n = 15$). (E) Demethylation of CAMP promoter was confirmed by reduced enrichment of CAMP promoter (-233 to -132 bp) DNA in HCT116 and LoVo treated with 5AZA (1 µM, 72 h) as measured by MethylCap-qPCR. Results were representative of 3 independent experiments. *, $p < 0.05$; **, $p < 0.01$, significantly different from respective control group.

**Fig. 3. Induction of caspase-independent apoptosis by LL-37 in colon cancer cells.** (A) Effects of 24 h treatment of LL-37 on viability of p53-wild-type (HCT116, LoVo) and mutant (SW480, SW620, SW1116) human colon cancer cells and colon fibroblasts (CCD-18Co) were determined by MTT assay. mCRAMP, the murine cathelicidin, also dose-dependently reduced the viability of mouse colon cancer cells (Colon26) but not the mouse immortalized colonocytes (YAMC). (B) Flow cytometry of propidium iodide-stained HCT116 cells treated with LL-37 (24 h) revealed the accumulation of sub-G$_1$ phase indicative of DNA fragmentation. (C) TUNEL staining confirmed the induction of DNA fragmentation by LL-37 (24 h) in HCT116. (D) Flow cytometry of
annexin V-stained HCT116 and LoVo cells (p53-wild type) revealed the induction of phosphotidylserine externalization by LL-37 (24 h). (E) HCT116 cells treated with or without LL-37 were examined for cleaved caspases 3, 7, and 9 and PARP by Western blot. Protein lysate from HCT116 cells treated with cisplatin (30 μM, 24 h) was used as positive control. (F) Co-treatment with the pan-caspase inhibitor z-VAD-fmk (20 μM) did not reverse the loss of cell viability induced by LL-37 (24 h) in HCT116 and LoVo as measured by MTT assay. Results are averages from 3 independent experiments. **, p < 0.01, significantly different from respective control group.

Fig. 4. AIF and EndoG as mediators of LL-37-induced apoptosis. (A) Cytosolic and nuclear levels of AIF and EndoG by were determined by Western blot of fractionated proteins. GAPDH and Lamin A/C were used as loading controls for cytosolic and nuclear proteins, respectively. (B-C) Nuclear translocation of (B) AIF and (C) EndoG in HCT116 cells treated with or without LL-37 for 6h was determined by immunofluorescence. (D-E) AIF- and EndoG-siRNA were transfected into HCT116 cells followed by LL-37 treatment for another 6 h. (D) Nuclear expression and (E) subcellular localization of AIF and EndoG were determined by Western blot and immunofluorescence, respectively. Reciprocal regulation between AIF and EndoG in LL-37-induced apoptosis was noted. (F) TUNEL staining showed that AIF- and EndoG-siRNA, in combination or alone, significantly reversed the apoptogenic action of LL-37 (40 μM; 24 h) in HCT116. Results were representative of 3 independent experiments.

Fig. 5. Requirement of altered expression of Bcl-2 family members in LL-37-induced caspase-independent apoptosis. (A) LL-37 (40 μM) increased Bax and Bak but decreased Bcl-2 expression. The effect of LL-37 were peaked at 6 h after incubation. (B) The nuclear expression of AIF and EndoG induced by 6 h treatment of LL-37 (40 μM) were reversed by siRNA-mediated knockdown of Bak, genetic ablation of Bax or restoration of Bcl-2 expression in HCT116. The reversal effect was most prominent in Bak-downregulated Bax-ablated cells (siBak + Bax−/−) and Bcl-2-overexpressing cells. (C) TUNEL-positive staining indicated that knockdown of Bak, genetic ablation of Bax or restoration of Bcl-2 reduced LL-37 (40 μM; 24 h)-induced apoptosis in HCT116. Results were representative of 3 independent experiments.

Fig. 6. Central role of G1-coupled GPCR-mediated p53 activation in LL-37-induced caspase-independent apoptosis. (A) Total and nuclear p53 together with PUMA (a p53 target) were upregulated by LL-37 (40 μM) in HCT116. (B) Knockdown of p53 reversed the upregulation of Bax and Bak and downregulation of Bcl-2 by LL-37 (40 μM; 6 h). (C) Nuclear expression of AIF and EndoG induced by LL-37 (40 μM; 24 h) were abolished by p53 knockdown. (D) Annexin V-staining revealed p53 was required for LL-37 (24 h)-induced apoptosis in HCT116. (E) The p53-mutant SW1116 colon cancer cells were resistant to the apoptogenic effect of LL-37 (40 μM; 24 h).
h) as measured by Annexin V-staining. (F) G-coupled GPCR was inactivated by pre-incubation with pertussis toxin (PTX; 40 ng/ml) for 2 h prior to addition of LL-37 for 6 h in HCT116. Inactivation of G-coupled GPCR reversed the upregulation of p53, Bax and Bak and downregulation of Bcl-2 induced by LL-37. The apoptogenic effect of LL-37 (20 μM; 24 h) was also partially blocked by PTX but not by the FPR2 antagonist WRW4 (5 μM; 24 h pretreatment) as measured by Annexin V staining in HCT116. Results were representative of 3 independent experiments.

Fig. 7. Reduced basal apoptosis in colonic mucosa and increased susceptibility to azoxymethane-induced gross colonic tumors in cathelicidin-knockout mice. (A) Colon were isolated from wild-type (WT) and cathelicidin-knockout (Cnlp-/-) mice (n=6 in each group). Colon tissues were randomly selected from the middle and distal part and homogenized for protein extraction. Expression of p53, Bax, Bak and Bcl-2 in individual WT and Cnlp-/- mice and pooled samples were determined by Western blot. (B) Basal level of apoptosis in colonic mucosa was determined by TUNEL labeling in WT and cathelicidin-knockout (Cnlp-/-) mice. (C) Wild-type and Cnlp-/- mice (n = 7 per group) were subject to six consecutive intraperitoneal injections of azoxymethane (AOM) at the dose of 6 mg/kg at one-week intervals followed by maintenance for another 16 weeks for the development of neoplastic lesions before euthanasia. (D) Control wild-type mice showed no observable macroscopic lesions whereas Cnlp-/- mice developed multiple gross colonic tumors (blue arrows) after AOM injections. The number of tumors and total tumor mass per mice were significantly higher in Cnlp-/- mice than the wild-type mice.
Fig. 2
Fig. 7 A

**p53**

β-actin

![Graphs showing protein expression levels for p53 and β-actin in WT and KO samples.](image)

**B**

Wild-type  Cnlp<sup>−/−</sup>

Nucleus

TUNEL

Merged

![Immunofluorescence images showing nuclear and TUNEL staining in WT and KO samples.](image)

**C**

WT KO

AOM AOM AOM AOM AOM

1 wk 1 wk 1 wk 1 wk 16 wk

**D**

WT KO1 KO2 KO3

Number of Tumors

![Graph showing number of tumors in WT and KO samples.](image)

Tunnel-positive cells

![Bar graph showing tunnel-positive cell counts in WT and Cnlp<sup>−/−</sup> samples.](image)

Total Tumor Volume (mm<sup>3</sup>)

![Graph showing total tumor volume in WT and KO samples.](image)
Host immune defense peptide LL-37 activates caspase-independent apoptosis and suppresses colon cancer

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Cancer Res  Published OnlineFirst October 24, 2012.

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