Radio sensitizing effect of aloe polysaccharide on pancreatic cancer bxpc-3 cells

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Abstract: The roles of autophagy in pancreatic cancers are complex and may differ depending on tumor type or context. This study aimed to investigate if aloe polysaccharide can be used as a radio-sensitizing chemical to induce autophagy in pancreatic cancer cells. Pancreatic carcinoma BxPC-3 cells were divided into four groups: A control group, an aloe polysaccharide only group, a radiation only group and a radiation combined with aloe polysaccharide group (combination group). Transmission electron microscopy was used to observe ultra structural changes of autophagosomes in each cell group. The mRNA expressions of *ULK1*, *GABARAPL1*, *BECN1,* and *BCL-2* were detected by real-time PCR. Autophagosomes or autophagolysosomes were observed in all experimental groups except the control group. Compared with the control group, *ULK1* mRNA expression was up-regulated and *BECN1* and *BCL-2* mRNA expression were down-regulated in all groups ($P<0.05$); changes in expression were most obvious in the combination group ($P<0.05$). *GABARAPL1* mRNA expression was up-regulated in the combination group ($P<0.05$), but had no significant changes among other groups. In brief, aloe polysaccharide induces autophagy in pancreatic carcinoma BxPC-3 cells both alone and in concert with radiation. Autophagic cell death may be one of the mechanisms producing a radiosensitizing effect.

Keywords: Aloe polysaccharide; pancreatic carcinoma; radio sensitizing; autophagy.

INTRODUCTION

Cell autophagy, a mechanism of programmed cell death in which eukaryocytes degrade and recycle materials in the cytoplasm, allows an organism to adapt to differing internal environments. Autophagy is necessary for survival; it is involved in metabolism, cellular repair, and protecting against infections (Levine and Kroemer, 2008). Recent evidence indicates that autophagy plays a role in the treatment of cancer: indeed, adding radio sensitizing chemicals such as berberine and emodin to radiation therapy can induce autophagy of tumor cells (Peng *et al.*, 2008; Liu *et al.*, 2009).

Aloe, a member of the lily family, produces compounds that can enhance immune function, promote wound healing, and act as anti-peptic ulcer, anti-viral, and anti-fungal agents (Kong and Wang, 2003). In particular, one compound, aloe polysaccharide, can delay cell division of eukaryocytes, resulting in G1 or G2 phase arrest, S phase accumulation, and formation of giant cells; G2 and G1 phase arrest are the most common. Some of the arrested cells can be repaired and resume the cell cycle, while other arrested cells stay irreversibly in the G1 or G2 phase, with some entering into apoptosis (Ma *et al.*, 2006; Wang *et al.*, 2004). The ability of aloe polysaccharide to arrest cell division makes it of particular interest for treating tumors, which often progress through uncontrolled cell division (Wang *et al.*, 2005).

To further explore the relationship between radio sensitization and the development of autophagy in tumors, cultured pancreatic cancer cells were treated with aloe polysaccharide prior to radiation. Electron microscopy and real-time PCR were used to investigate the formation of autophagosomes and to detect expression of autophagy-related genes.

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MATERIALS AND METHODS

Materials

Main reagents

Aloe polysaccharide, a light, brownish-yellow, granular powder, extracted and separated from Chinese aloe, *A. vera L.var. Chinensis* (Haw.) Berger, and then the molecular weight was determined for 14000 by gel permeation chromatography (GPC), and the content of total sugar was 91.2% by Phenol-sulfuric acid method. Before use, the extract was dissolved in saline and sterilized. The pancreatic cancer cell line BxPC-3 was purchased from Shanghai Cell Bank, Chinese Academy of Sciences. Other reagents used were: TRIzol REAGENT (Invitrogen Company, USA); isopropyl alcohol and chloroform were domestic analytical reagents; DEPC-treated water; Revert Aid^{TM} First Strand cDNA Synthesis Kit (Fermentas Company, USA); Fast Start Universal SYBR Green Master (Rox) (Roche); sterile water for injection; and RPMI 1640 (Gibco).

Main instruments

The main instruments utilized in this study included: ultra-low-temperature high-speed centrifuge 5810R (Germany Eppendorf Company); ABI 7300 Real-time

PCR thermocycler; Nano Drop 2000 (Thermo Company); gel imager (BIO-RAD); inverted phase contrast microscope Axiovert 200 (Carl Zeiss, United Kingdom). ^{60}Co ray (product of Canada Theratronics) was used as the radiation source, with a dose rate of 94.41cGy/min; irradiation was performed for the appropriate dose according to the experimental requirements.

Primer design

Specific PCR primers were designed according to human *ULK1, GABARAPL1, BECN1,* and *BCL-2* nucleotide sequences provided by NCBI database, with β-actin (*ACTB*) as an internal reference. Primer synthesis was completed by Sangon Bioengineering (Shanghai). The primer sequences and product sizes are shown in table 1.

Experimental methods

Cell culture

Cells were subcultured in RPMI 1640 culture medium containing 10% fetal calf serum in a 5% CO_2 incubator at 37°C; cells in logarithmic growth phase were harvested and utilized as described below.

Treatment groups

Cells were divided into four treatment groups. Blank control group (C group): cells were cultured in 1640 complete medium. Aloe polysaccharide group (AP group): cells were cultured for 24 hours in 1640 complete medium supplemented with aloe polysaccharide at a final concentration of 50μg/mL; subsequently, cells were rinsed and incubated for 16 hours in fresh medium not containing aloe polysaccharide. Radiation group (R): cells were cultured for 16 hours after ^{60}Co2Gy irradiation. Aloe polysaccharide combined with radiation (AP + R group): cells were cultured for 24 hours in complete media containing aloe polysaccharide with a final concentration of 5mg/L units must be same throughout the manuscript; subsequently, cells were irradiated with ^{60}Co2Gy, rinsed, and cultured in fresh medium for 16 hours. Cells were washed three times with PBS, harvested using trypsin, and centrifuged into pellets. Pre-cooled glutaraldehyde with a mass fraction of 3% was added, and cells were stored overnight at 4°C before being analyzed.

Transmission electron microscopy

After the above processings, the cells were washed with PBS for 3 times, then trypsinized and centrifuged into ball-like masses. Add the pre-cooled glutaraldehyde with a mass fraction of 3%, then placed overnight at 4□ and send for test.

Real-time PCR

Total cell RNA was extracted using RNA isolator (TaKaRa Biotech, Code D312), and the concentration was measured using the Nano Drop 2000, with an A260/A280 reading between 1.9 and 2.1; agarose gel (10g/L) electrophoresis was used to determine RNA integrity. 2 μg total RNA were used for cDNA synthesis in accordance with the instructions of the reverse transcription kit; PCR conditions are shown in table 2. The standard curve was established according to methods in the literature correct the reference style (8). The following formula was used to calculate the relative expression index of the target genes: $F = 10^{\triangle C_{T.T}/A_T \triangle C_{T.R}/A_R}$ (Zhang *et al.*, 2005). In this formula, $\Delta C_{T.T}$ represents the difference of the target gene cross threshold (Ct) value of the experimental group minus that of the blank control group; $\Delta C_{T.R}$ represents the difference of the internal reference gene Ct value of the experimental group minus that of the blank control group; AT represents the slope of the curve of the target genes; AR represents the slope of the curve of the internal reference genes; F represents the expression level of the target gene of the experimental group relative to that of the blank control group.

STATISTICAL ANALYSIS

SPSS17.0 statistical software was used for statistical analysis and experimental results are expressed as mean ± standard deviation ($\overline{x} \pm sd$). One-way analysis of variance (ANOVA) and one-way ANOVA-based inter-group multiple comparison (SNK method) were used to compare morphology parameters between different groups. All tests were two-sided, with a test level α of 0.05; $P < 0.05$ was considered statistically significant.

RESULTS

Changes in cellular ultrastructure

Transmission electron microscopy was used to detect changes within cells that would be related to autophagy. The AP group, R group, and AP+R group all displayed the following features: increased vacuoles in the cytoplasm; swollen and denatured mitochondria; and vacuole-like structures appeared around denatured organelles. Some vacuole-like structures had double membranes (fig. 1). These features, typical of autophagy for autophagosomes and autolysosomes, were particularly evident in the AP+R combination group.

Expression of autophagy-related genes

To detect changes in expression of autophagy-related genes, real-time PCR was used to measure differences in mRNA levels (fig. 2). In all experimental groups, *ULK1* mRNA expression was increased, while *BECN1* and *BCL-2* mRNA expression were decreased ($P<0.05$) compared to the control group; changes in expression in the AP+R combination group were most dramatic ($P<0.05$). *GABARAPL1* mRNA levels were only significantly different (increased) in the AP+R combination group ($P<0.05$).

DISCUSSION

This study investigated the utility of aloe polysaccharide as a radio sensitizing chemical to induce autophagy in

Table 1: Primer sequences and product lengths

Gene		Primer sequence	Product length (bp)
ULK1	Sense	5'-CAGTCACCCACCCAGTTCCAAA-3'	152
	Antisense	5'-CCTCCACCCAGAGACATCTTCC-3'	
GABARAPL1	Sense	5'-TATCGGAAAAAGGAAGGAGAAA-3'	136
	Antisense	5'-CAACAGTAAGGTCAGAGGGCAC-3'	
BECN1	Sense	5'-GCTGAGGGATGGAAGGGTCTAA-3'	132
	Antisense	5'-GTTCCTGGATGGTGACACGGT-3'	
BCL-2	Sense	5'-TGTGGCCTTCTTTGAGTTCG-3'	148
	Antisense	5'-ATCCCAGCCTCCGTTATCC-3'	
ACTB	Sense	5'-AACTCCATCATGAAGTGTGA-3'	247
	Antisense	5'-ACTCCTGCTTGCTGATCCAC-3'	

Table 2: PCR amplification reaction conditions

Gene	Denaturation	Annealing	Extension	Cycles
ULK1	95ºC 45 s	58ºC 40 s	72ºC 45 s	40
GABARAPL1	95ºC 45 s	56ºC 40 s	72ºC 45 s	40
BECN1	95ºC 45 s	61ºC 40 s	72ºC 45 s	40
BCL-2	95ºC 45 s	60ºC 40 s	72ºC 45 s	40
ACTB	95ºC 45 s	59ºC 40 s	72ºC 45 s	40

pancreatic cancer cells. Two methods were used to explore this question, transmission electron microscopy and real-time PCR.

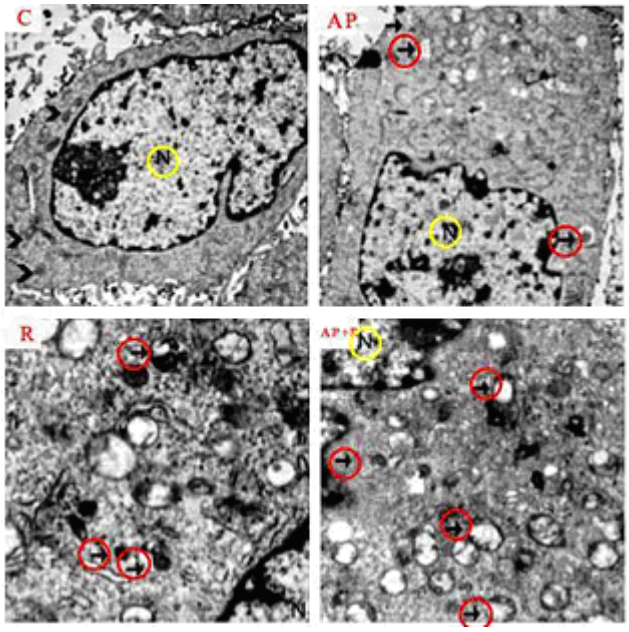

Fig. 1: Ultra structural changes among cells of groups: Nucleus (N) and autophagosomes (→). Note: C, Control group; AP, Aloe polysaccharide group; R, Radiation group; AP+R, Aloe polysaccharide + Radiation group. Edit the arrows and N labeling in fig 1 to make prominent

Electron microscopy

Specialized structural and morphological changes occurring during the process of autophagy can be observed by transmission electron microscopy. Cells are induced to form small and flat autophagic vacuoles in the cytoplasm with double membranes; the autophagic vacuoles extend to wrap around damaged organelles, denatured proteins, and nucleic acids to form closed spherical autophagosomes. Autophagosomes merge together with structures absorbed by cells via endocytosis such as phagosomes and pinocytotic vesicles to form an autolysosome (Ma *et al.*, 2012).

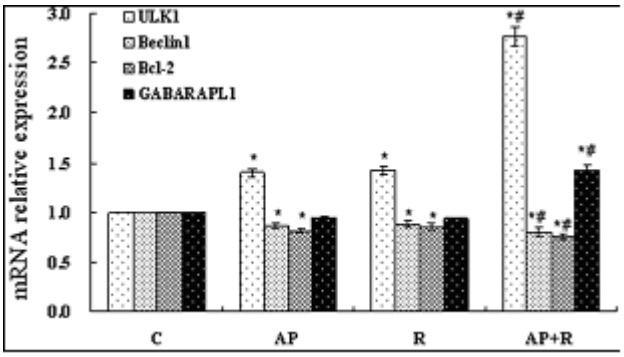

Fig 2: Autophagy-related gene mRNA expression among cells of groups. Note: C, Control group; AP, Aloe polysaccharide group; R, Radiation group; AP+R, Aloe polysaccharide + Radiation group. $P<0.05$: **vs.* C group; $^{\#}$ *vs.* AP and R group.

Our study demonstrates that autophagic vacuoles or autophagosomes formed in cells treated with aloe polysaccharide, radiation, or both. However, treatment with both aloe polysaccharide and radiation led to

formation of the largest number of autophagosomes and autolysosomes. Thus, pre-treatment with aloe polysaccharide appeared to induce greater autophagy in pancreatic cancer cells.

Real-time PCR

Autophagy involves multiple genes/proteins and a complex program. ULK1 is a serine/threonine kinase that can be activated via dephosphorylation by upstream signals to form a protein complex critical for regulating autophagy (Jung *et al.*, 2009; Kim *et al.*, 2011). Beclin1 and Bcl-2 expression levels are closely related to autophagic activity. Beclin1 is an important positive regulatory factor that affects the positioning of autophagy-related proteins in precursor structures, mainly through forming a complex with PI3K3C (Scarlatti *et al.*, 2008). Up-regulation of the expression of Beclin1 in mammalian cells can stimulate the occurrence of autophagy. Bcl-2 is located in the mitochondria, endoplasmic reticulum, and continuous nuclear membrane, decreased *BCL-2* expression can cause increased autophagy (Rikiishi H, 2012). At intracellular homeostasis, Beclin1 and Bcl-2 can interact to inhibit the formation of autophagosomes; when the internal environment is unbalanced, Beclin1 and Bcl-2 will separate from each other, causing an increase in Beclin-dependent autophagy (Sadasivan *et al.*, 2008). GABARAPL1 is mainly bound on the membrane of autophagosomes or lysosomes; Up-regulation of GABARAPL1 enables the formation of unique membranous structures that extend until they completely wrap the cytoplasmic fraction that is to be degraded. GABARAPL1 expression levels will decrease when the autophagosome and lysosome merge together (Chakrama *et al.*, 2010). Regulation of these genes was altered in cells treated with AP, radiation, or both; however, the most significant expression changes occurred in cells treated with the combination.

Taken together, these results indicate that aloe polysaccharide, both alone and in concert with radiation, can induce autophagy in pancreatic cancer cells. However, further study is needed to investigate the mechanism of action of aloe polysaccharide on the autophagy process.

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