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A novel beta-galactose-specific lectin of the tubeworm, *Ridgeia piscesae*, from the hydrothermal vent

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Abstract

Lectins are sugar-specific binding proteins or glycoproteins that play important physiological roles in cellular recognition and regulation. And they are also valuable in medicine and pharmacy. Tubeworm is the representative species around the hydrothermal vent in the deep sea. They have developed unique mechanisms to adapt to the harsh environment. In this study, a 1 092 bp cDNA, designed as *rpgal*, was first cloned and characterized from the tubeworm *Ridgeia piscesae*. Sequence analysis showed that RPGAL had low homology with the known galectin. And it had two homologous carbohydrate-recognition domains, which is the characteristic of the tandem-repeat type galectins. The RPGAL was successfully recombinant expressed in *Escherichia coli* and purified. Analysis of biological activity revealed that RPGAL was metal ion independent and it could agglutinate all the vertebrate erythrocytes tested. It was stable at 10–50°C and pH 5–10. And the hemagglutinating activity of RPGAL was strongly inhibited by D-Lactose and lipopolysaccharide. Although RPGAL had no effect on the microorganisms tested, it showed anti-tumor activity towards HeLa cells and HT1080 cells, which was accomplished by apoptosis. The study demonstrated that RPGAL was a novel galectin and provided a potential candidate for therapy of anti-tumor.

Key words: galectin, antitumor, apoptosis, tubeworm, *Ridgeia piscesae*

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1 Introduction

Lectins are sugar-specific binding proteins or glycoproteins that play important physiological roles in cellular recognition and regulation through protein-carbohydrate interactions, and they are widely distributed in many organisms. Since the beginning of the last century, a large amount of lectins from plants and various marine invertebrates, including tunicates (Nair et al., 2000), sponges (Gamulin et al., 1994), crustaceans (Takahashi et al., 1995), echinoderms (Giga et al., 1987), actinia (Gaphurov et al., 1999) and clams (Renwrantz and Stahmer, 1983; Suh-Chae et al., 1988; Dam et al., 1992) have been isolated and characterized. Based on their source, lectins can be divided into three classes, including phytolectin, animal lectins and microbial lectins. Additionally, according to the features of sequences and structures, animal lectins can be further categorized into C-type lectin, S-type lectin, P-type lectin, I-type lectin and Pentraxins (Barondes et al., 1994a). They always have some important biological activities, such as promotion of phagocytosis (Mercy and Ravindranath, 1994), antibacterial activity (Tunkijjanukij and Olafsen, 1998), regulation of cell adhesion, cell growth (Cooper and Barondes, 1999; Kasai and Hirabayashi, 1996), apoptotic events (Bernerd et al., 1999) and so on.

Galectin, a member of S-type lectin, has ability to recognize β-galactoside through evolutionary conserved carbohydrate-recognition domain (CRD) (Barondes et al., 1994b). So far, 15 members of galectin family have been identified. They were found in sponges, fungi, nematodes, insects, vertebrates and even virus (Cooper, 2002). Based on the number and the unique structure of CRDs, members of galectin family have been classified into three subtypes, prototype, chimera and tandem repeat group. (Hirabayashi and Kasai, 1993). The prototype group (Galectin-1, -2, -5, -7, -10, -11, -13, -14 and -15) contains one CRD, and the chimera group (Galectin-3) has one CRD and glycine-rich domain, while the proteins that have two distinct but homologous CRDs belong to the tandem repeat group (Galectin-4, -6, -8, -9, and -12). These members of the galectin family play different roles in various biological processes (Barondes et al., 1994a; Sacchettini et al., 2001; Nakahara et al., 2005). Galectin-3 can modulate cell growth, cell cycle and is involved in regulation of apoptosis (Nakahara et al., 2005), while Galectin-1 and Galectin-9 were found to be involved in apoptotic events in T cell immunity (Fajka-Boja et al., 2002; Hadari et al., 2000). Due to its multiplicity and diversity of functions, galectin attract much research, not only on glycobiology, but also on medicine and pharmacy. For example, in cancer re-

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search, Galectin-1 was a possible biomarker or therapeutic target (Kim et al., 2013). And Galectin-3 can protect cells against pro-apoptotic anticancer drugs (Fukumori et al., 2007). Earlier, a GlcNAc-specific lectin from the sea tubeworm *Serpula vermicularis* was found to have anti-HIV-l activity (Molchanova et al., 2007). Thus, isolation and identification of galectins will have both academic and commercial rewards.

Currently, more and more studies are focusing on deep-sea creatures, especially tubeworms. *Ridgeia piscesae*, a tubeworm living around the hydrothermal vent of Juan de Fuca Ridge, is one of the most representative species (Urcuyo et al., 2003). And they develop the unique adaptive mechanisms to extreme environment. Therefore, the study on the functional genes will help to understand the characteristics of the adaptation, as well as to develop the potential genetic resources. In this study, a novel β-galactose-specific lectin, designed as RPGAL, was first cloned and characterized from *R. piscesae*. Further study revealed that it had cytotoxicity activity against tumor cells.

2 Materials and methods

2.1 *Materials*

R. piscesae was collected from the hydrothermal vent of Juan de Fuca Ridge (47°56′N, 129°05′W, 2 181 m depth). The samples were immediately dissected on ice after collection and kept in RNAlater buffer (QIAGEN).

Human erythrocytes of type A, B and O were donated by the Blood Bank in Xiamen. Rabbit and mouse were purchased from Xiamen University Laboratory Animal Center, Xiamen, China. And chicken were purchased from a supermarket in Xiamen, China. 2% (v/v) suspensions of a variety of animal erythrocytes were prepared in Tris-buffered saline (TBS) (50 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl) and stored at 4°C until use.

Escherichia coli BL21, *Staphylococcus aureus*, *Vibrio parahaemolyticus* and *Saccharomyces cerevisiae* (SMD1168) were stored in our laboratory. HeLa cells, HT1080cells, A549 cells, HL60 cells were the kind gifts from Dr. Tang Xixiang (Third Institute of Oceanography, SOA, China). The cells were cultured in RPMI 1640 medium (Hyclone) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and 1 μg/mL penicillin/streptomycin, and maintained at 37°C with 5% CO_2.

2.2 *Total RNA extraction and cDNA synthesis*

Total RNA from *R. piscesae* was extracted according to the instructions of Trizol Reagent (MRC). After treatment with DNase I (TaKaRa), total RNA was primed with Oligo$(dT)_{18}$ primer (Fermentas) and reverse-transcribed with SuperScript™ III reverse transcriptase (Invitrogen).

2.3 *Sequence analysis*

The cDNA and amino acid sequences of RPGAL were analyzed by using the BLAST program at the National Center for Biotechnology Information (NCBI) and the Expert Protein Analysis System (ExPASy). Domain search was performed with the Pfam database and the conserved domain search program of NCBI. The molecular mass and theoretical isoelectric point (pI) were predicted using ProtParam tool in ExPASy. Prediction of signal peptide was accomplished by using Signal P (version 4.1). Phylogenetic tree was constructed with the MEGA 5.0 program.

2.4 *Recombinant expression and purification of RPGAL*

The cDNA sequence encoding RPGAL was amplified by PCR. The primers were as follows: CGCGGATCCATGATGAACGCACCAGGAATGGCTG (forward) and CTAGCTAGCTCATTGGACGCGGACATGTGTAAGG (reverse). *Bam*H I and *Nhe* I sites were incorporated in the primers, respectively (as shown by italic letters). The thermal cycling protocols were 30 cycles of 98°C for 10 s, 60°C for 15 s and 72°C for 70 s. The amplified sequence was inserted into the expression vector pET-His (Gene Power Lab). The recombinant plasmid was transformed into *E. coli* BL21 (DE3) and the recombinant RPGAL was purified by affinity chromatography with TALON®Metal Affinity Resins (Clontech) under the native conditions as described by the manufacturer. The purified protein was dialyzed with the TBS and the concentration of protein was determined according to Bradford's method using Coomassie (Bradford) Protein Assay Kit (Thermo). Crystalline bovine serum albumin (BSA) was used as the standard protein (Bradford, 1976).

2.5 *Hemagglutination and microorganism agglutination assay*

To assay the hemagglutinating activity, the protein solution of RPGAL was serially diluted by 2-fold with TBS in microtiter V-plates. Equal volume of 2% suspension of erythrocytes were added into the protein solutions and mixed. After incubation for 1 h at room temperature, the hemagglutination was observed under a microscope (Olympus). TBS was used as blank control, and 1 mg/mL of BSA solution (prepared with TBS) was used as negative control. To analyze whether the hemagglutination requires calcium, TBS with calcium (TBS-Ca) (50 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl and 10 mmol/L $CaCl_2$) was used instead of TBS. The experiment was performed as described above.

For microorganism agglutination assay, tested microorganisms (OD_{600}=0.6) were suspended in TBS and TBS-Ca at 2.5×10^9 cells/mL, respectively. The suspensions of tested microorganisms were used instead of erythrocytes, and agglutinating activities were assessed by using the same method described above for the hemagglutination assay.

2.6 *Hemagglutination inhibition assay*

For the hemagglutination inhibition assay, all carbohydrates used were dissolved in TBS at an initial concentration of 100 mmol/L for the following mono- and disaccharides: D-galactose, D-xylose, D-glucose, D-mannose, D-fructose, D-lactose, Maltose, D-cellobiose and Sucrose. Lipopolysaccharide (LPS) from *E. coli*, 055:B5, Peptidoglycan and Zymosan were also dissolved in TBS at an initial concentration of 1 mg/mL. All carbohydrate solutions were diluted by 2-fold serially with TBS in microtiter V-plates. Equal volume (25 μL) of the protein solution of RPGAL were added and mixed. After incubation for 30 min at room temperature, 50 μL of suspension of mouse erythrocytes were added. The mixture obtained was continued to be kept for 1 h at room temperature. Carbohydrate solutions were used as blank controls, and the protein solution of RPGAL without treatment was used as positive control.

2.7 *Effect of EGTA/EDTA, temperature and pH on hemagglutinating activity*

To analyze whether the hemagglutination required other metal ions, the protein solution of RPGAL was serially diluted by 2-fold with TBS containing 50 mmol/L EGTA/EDTA (pH 7.5) and incubated at room temperature for 30 min. Then equal volumes of mouse erythrocyte suspension were added. After incubation for 1 h at room temperature, the hemagglutination was evaluated again.

To study the effects of temperature on hemagglutinating activity, the protein solution of RPGAL was serially diluted by 2-fold and incubated at 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100°C

for 1 h. Then the mouse erythrocytes were used and the hemagglutinating activity was assayed as the method described above.

The effect of pH was evaluated as follows. The protein solutions of RPGAL were titrated in the following buffers, respectively (25 mmol/L CH_3COONa (pH 4.0 and 5.0), 25 mmol/L NaH_2PO_4 (pH 6.0 and 7.0), 25 mmol/L Tris-HCl (pH 8.0 and 9.0) and 25 mmol/L Na_2CO_3 (pH 10.0 and 11.0)). Then the mouse erythrocytes were used and the hemagglutinating activity was assayed as the method described above.

2.8 *Cytotoxicity test*

The cytotoxic activity of RPGAL was measured by using Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories). The cells were cultured in the 96-well flat bottom plate. When the cells grew at 1×10^4 cells per well, the protein solutions of RPGAL, which were serially diluted by 2-fold with TBS, were added into the wells accordingly and the cells were incubated for another 12 h at 37°C in CO_2 incubator, respectively. Then the aliquot was removed and 100 μL of fresh medium and 10 μL of CCK-8 solution were added into each well. The cells were continued to be incubated again at 37°C for 2 h. Subsequently, the values of absorbance were read at 450 nm. Cells treated with BSA were used as control and the blank well was used as blank control. The percentage of cell viability was calculated as follows: Cell viability (%)=[OD450 (experiment)–OD450 (blank)]/[OD450 (control)–OD450 (blank)]×100.

2.9 *Caspase-3 fluorescent assay*

To further characterize the effect of RPGAL on tumor cells was accomplished by apoptosis or proliferation inhibition, the tumor cells were analyzed by ApoAlert®Caspase Fluorescent Assay Kit (Clontech). Tumor cells cultured with or without RPGAL at 37°C for 0, 3, 6, 12 and 24 h were harvested and suspended with 1 mL medium. Then, fluorometric detections for caspase-3 were performed using a 400-nm excitation filter and 505-nm emission filter according to the manufacturer's instruction. TBS was used as blank control, Caspase-3 inhibitor from the kit was used as negative control, and apoptosis inducer (Apoptosis A and Apoptosis B from Beyotime) was used as positive control.

3 Results

3.1 *Molecular characteristics of RPGAL*

The complete cDNA sequence of RPGAL (GenBank accession No. KJ742830) from *R. piscesae* contained a 1 092 bp open reading frame (ORF) encoding a 363 amino acid peptide with a predicted molecular mass of 39.42 kDa and a pI of 7.67. According to silico analysis, RPGAL did not contain a putative signal peptide. BLAST searching against NCBI database showed that the deduced amino acid sequence of RPGAL had low homology with other galectins, and it shared the highest similarity of 32% with a galectin-8-like gene from *Saccoglossus kowalevskii*. In addition, two Gal-binding lectin domains (GLECT) were found in the RPGAL. Furthermore, phylogenetic trees constructed by Maximum-Likelihood method, also showed that it had the closest relationship with *Saccoglossus kowalevskii* galectin-8 (GenBank™ accession no.XP_002731585) (Fig. 1). The results indicated that RPGAL may be a novel Galectin/galactoside-binding lectin.

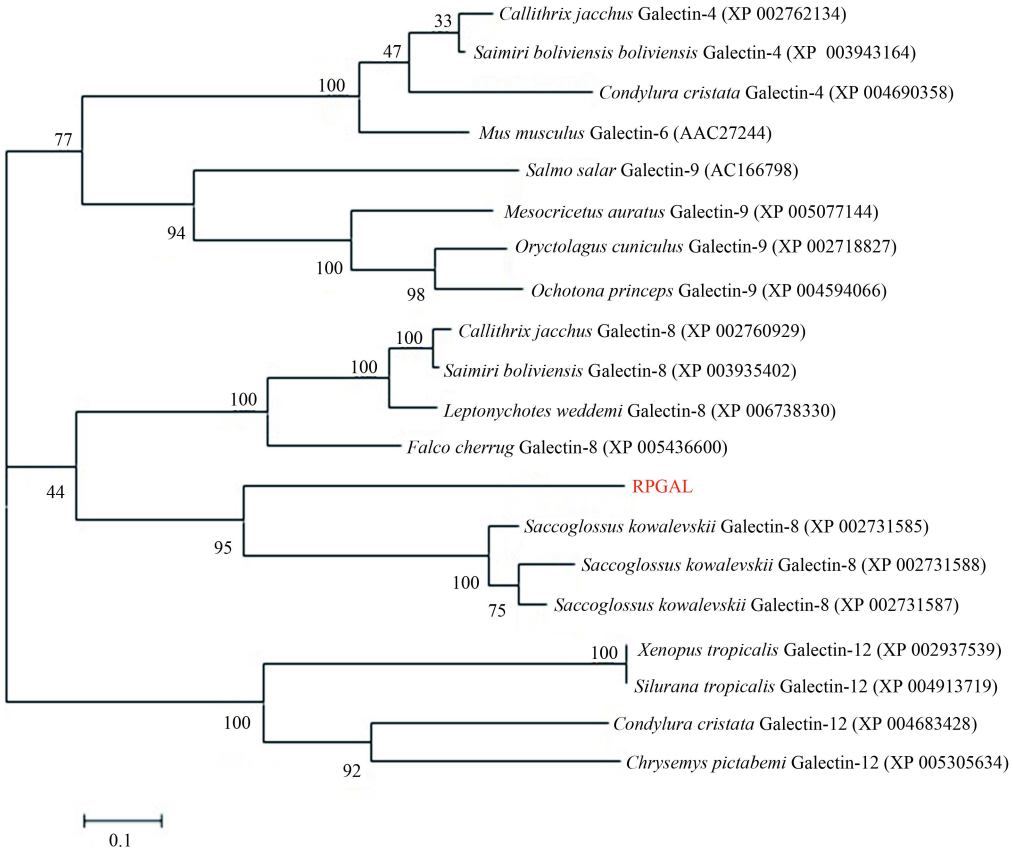

Fig. 1. Phylogenetic analysis by maximum-likelihood method based on amino acid sequences of RPGAL and other known galectins. Bootstrap percentage values based on 1 000 replicates are indicated at the nodes. The accession no of other known galectins was provided in the brackets.

3.2 *Expression and purification of recombinant RPGAL*

The RPGAL gene was inserted into the vector pET-His and highly expressed in *E. coli*. The recombinant RPGAL protein was purified under native conditions. SDS-PAGE analysis showed that the recombinant RPGAL was about 40 kDa, which was identical to the theoretical molecular mass. And the purity was estimated to be higher than 90% (Fig. 2).

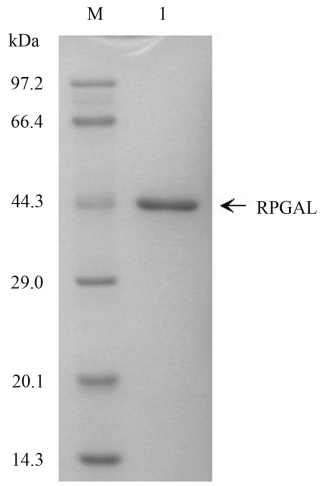

Fig. 2. Recombinant expression of the RPGAL protein. After 12% SDS-PAGE, the protein was stained in Coomasse blue. Lane M represents low molecular weight marker (TaKaRa, Dalian), and Lane 1 purified RPGAL protein.

3.3 *Biological activity of RPGAL*

RPGAL was found to have a strong activity of hemagglutination, including Human, rabbit, chicken and mouse erythrocytes. Among them, RPGAL agglutinated rabbit erythrocytes most effectively (Table 1). The minimum agglutinating concentration of RPGAL for rabbit erythrocytes was estimated to be 1.6 μg/mL.

Table 1. Hemagglutinating activity of RPGAL towards vertebrate erythrocytes

Erythrocytes RPGAL	Minimum agglutinating concentration/μg·mL^{-1}
Mouse(Ca^{2+}+)	6.4
Mouse (Ca^{2+}–)	6.4
Rabbit (Ca^{2+}+)	1.6
Rabbit (Ca^{2+}–)	1.6
Chicken (Ca^{2+}+)	6.4
Chicken (Ca^{2+}–)	6.4
Human group A (Ca^{2+}+)	6.4
Human group A (Ca^{2+}–)	6.4
Human group B (Ca^{2+}+)	6.4
Human group B (Ca^{2+}–)	6.4
Human group O (Ca^{2+}+)	12.8
Human group O (Ca^{2+}–)	12.8

Note: Ca^{2+}+ represents addition of 10 mmol/L Ca^{2+} in agglutination, and Ca^{2+}– no addition of Ca^{2+} in agglutination.

Further study showed that the hemagglutinating activity of RPGAL could not be enhanced by the addition of calcium. In addition, the hemagglutinating activity of RPGAL was not interfered with the addition of reagents (EDTA and EGTA), indicating that the activity of hemagglutination was independent of metal ions, such as Ca^{2+}, Mn^{2+} and Mg^{2+} (data no shown). The results were identical to the report that GLECT does not require metal ions for activity (Barondes et al., 1994b). Thus, the following assays were all carried out under calcium-free conditions.

To test whether RPGAL can interact with the microorganisms, an agglutinating assay using microorganisms was performed. Neither bacteria nor fungus listed in the materials were agglutinated by RPGAL with or without Ca^{2+} (data no shown).

3.4 *Properties of RPGAL*

In the hemagglutination inhibition assay, hemagglutinating activity of the RPGAL towards mouse erythrocytes was inhibited by D-lactose and LPS from *E. coli*, 055:B5 (Table 2). The minimum concentrations of inhibitors that could completely inhibit hemagglutination were 12.5 mmol/L for D-lactose and 0.004 mg/mL for LPS. These data strongly suggested that RPGAL protein was a β-galactose-specific lectin.

Table 2. Effect of carbonhydrates on the hemagglutination activity of RPGAL

Carbonhydrates	Minimal inhibitory concentration
D-xylose	no inhibition
D-galactose	no inhibition
D-glucose	no inhibition
D-mannose	no inhibition
D-frutose	no inhibition
D-lactose	12.5 mmol/L
Maltose	no inhibition
D-cellobise	no inhibition
Sucralose	no inhibition
LPS from *E. coli* 055:B5	0.004 mg/mL
Peptidoglycan	no inhibition
Zymosan	no inhibition

The effects of pH and temperature on RPGAL were also evaluated based on hemagglutination towards mouse erythrocytes. The hemagglutinating activity of RPGAL was relatively heat- and pH-stable. The RPGAL protein was fully active at the temperatures between 10°C and 40°C, and the activity was decreased dramatically above 50°C (Fig. 3a). The activity was completely lost and irreversible after incubation for 1 h at above 80°C. For pH stability, the RPGAL was fully active at pH 5, and the activity was still relatively stable between pH 6 and 10. When the condition was below pH 5 or above pH 11, the activity was totally lost (Fig. 3b).

3.5 *Cytotoxic activity*

When the tumor cells were treated with RPGAL, the cellular morphology was first observed under phase contrast microscopy. It was found that HeLa cells and HT1080 cells were agglutinated and the cellular morphology became abnormal, while the A549 cells and HL60 cells were not affected (Fig. 4). The results indicated that RPGAL had potent toxic effect on HeLa cells and HT1080 cells. Furthermore, HeLa cells, HT1080 cells and A549 cells were used for assay of RPGAL cytotoxic activity. The results showed that RPGAL could inhibit the proliferation of HeLa cells and HT1080 cells in dose-dependent and time-dependent manner. Especially, the effect of RPGAL on HT1080 was more marked. When the HT1080 cells were incubated with 51.5 μg/mL of RPGAL after 12 h, the viability of cell was reduced to about 54%. However, A549 cells were not affected (Fig. 5).

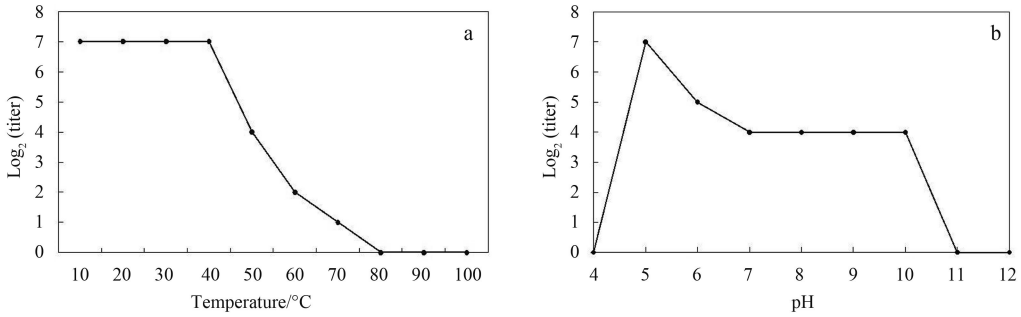

Fig. 3. Biological properties of RPGAL. a. The effect of temperature on the haemagglutination activity of RPGAL and b. the effect of pH on the haemagglutination activity of RPGAL.

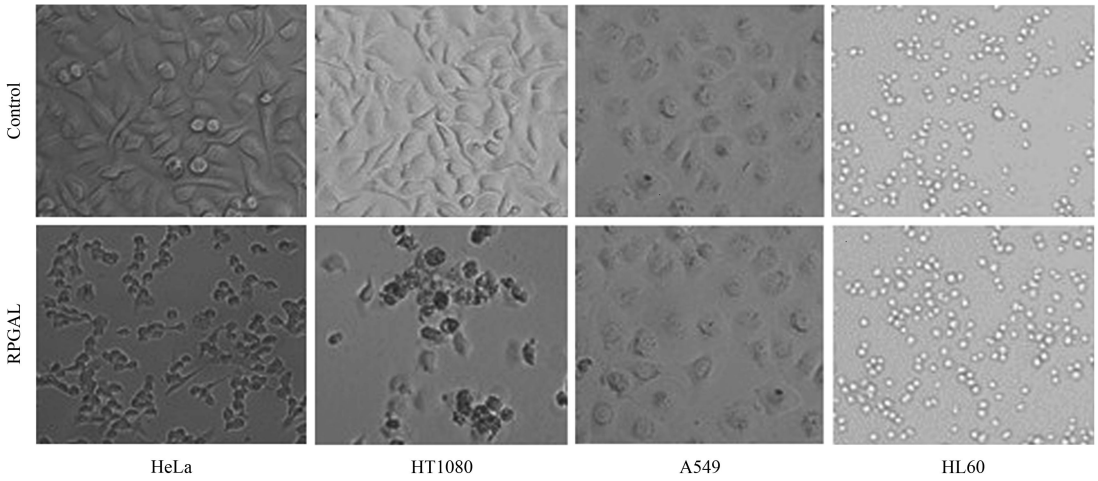

Fig. 4. The cellular morphology of tumor cells cultured in the absence (control) and presence of RPGAL (51.5 μg/mL) for 12 h (original magnification of 100×).

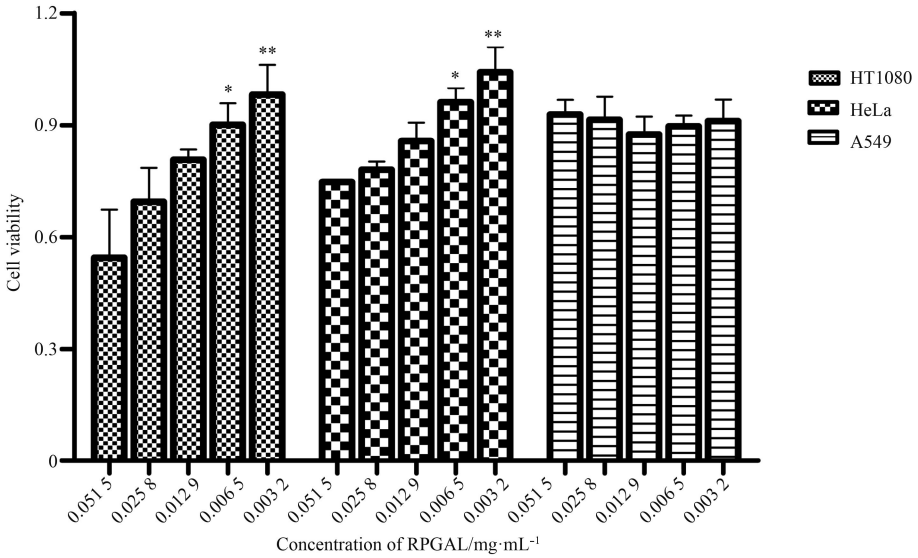

Fig. 5. Cytotoxic activity of RPGAL to the tumor cells. The tumor cells were treated with various doses of RPGAL for 12 h. The viability ratios of cell were measured by using Cell Counting Kit-8 (n=3, mean±SD). Asterisks indicate the level of statistical significance (one asterisk, P<0.05; two asterisks, P<0.01).

3.6 *Assay of apoptosis in tumor cells*

HT1080 cells were chosen for further study, because they were more sensitive to the RPGAL protein. Caspase-3 is crucial mediator of programmed cell apoptosis and it was used for detection of cell apoptosis. Compared with the control groups, RPGAL-induced apoptosis was obvious. When the cells were treated with RPGAL, the activity of caspase-3 was increased markedly at 3 h (Fig. 6). The data suggested that the effect of RPGAL on

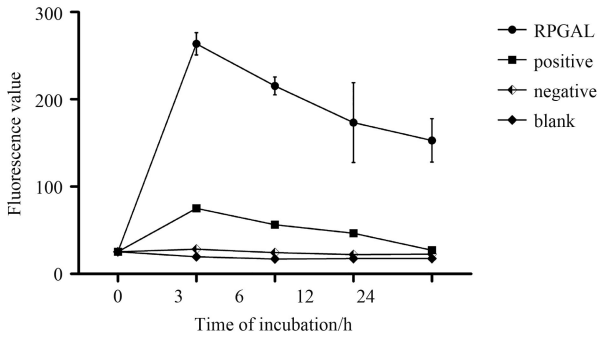

Fig. 6. Apoptotic analysis of HT1080 cells induced by RPGAL. Cell treated with RPGAL at different time points was used for Caspase-3 activity-based assay by ApoAlert® Caspase Fluorescent Assay Kit (Clontech) (n=3, mean±SD).

HT1080 cells was accomplished by apoptosis.

4 Discussion

Lectins are proteins involved in the innate immune in marine invertebrates, and their abilities affect the antibiosis of tumor cells and pathogenic organisms have also been reported. Tubeworm is the representative species around the hydrothermal vent in the deep sea. They have developed unique mechanisms to adapt to the extreme environment. However, knowledge about their molecular characteristics remains limited.

In this study, a novel lectin from *R. piscesae*, namely RPGAL, was cloned and characterized. Sequence analysis revealed that RPGAL had low homology with the known galectins, but it had the characteristic of tandem-repeat type galectins. It had two homologous CRDs, which are responsible for β-galactoside sugar binding. In addition, RPGAL did not have a signal peptide like other galectins (Cooper, 2002). The data suggested that RPGAL was a novel lectin.

The RPGAL was cloned and recombinant expressed. It was found that the recombinant RPGAL could strongly agglutinate all the tested vertebrate erythrocytes in vitro, and the activity could be inhibited by D-lactose. The results further confirmed that RPGAL was a galectin.

It has been reported that many lectins from marine invertebrates showed antibacterial activity (Tunkijjanukij and Olafsen, 1998). Differently, RPGAL did not exhibit the antibacterial activity against all microbes tested. However, it was interesting that the activity of RPGAL could be inhibited by LPS. It is well known that LPS on different Gram-negative bacteria strains are various, and lectin may have selective binding activity for different types of LPS. Similar LPS-lectin interaction pattern was reported on the lectin PPL of shrimp *Litopenaeus schmitti*. PPL could recognize the LPS of *E. coli* (0128:B12) and *E. coli* (0111:B4) rather than that of *E. coli* (055:B5) (Cominetti et al., 2002). Our result showed that RPGAL could not agglutinate *E. coli* (BL21), but could interact with LPS from *E. coli* (055:B5). Whether it has the same effect on other gram-negative bacteria needs to be further studied.

In the assay of biological activity, the RPGAL was found to be metal ion independent. It was relatively pH-stable and could keep active up to the temperature of 70°C. In some invertebrates, such as *Serpula vermicularis*, a GlcNAc-specific lectin from which was stable at pH 6–9, but lost activity once the temperature was above 40°C (Molchanova et al., 2007; Wang et al., 2006; Liu et al., 2013). The characteristics of RPGAL might be associated with the harsh living condition of *R. piscesae*, and help them to adapt to the extreme environment.

Galectins, which are consisted of a large family of β-galactosides-binding proteins, have received much attention for their remarkable anti-tumor activities in potentially cancer therapeutical applications (Boronkai et al., 2009). For example, a α-D-galactose-binding lectin isolated from breadfruit seeds could rapidly target to the HeLa cell's nucleus and induce cell apoptosis (Oliveira et al., 2011). And a GlcNAc-specific lectin from *Serpula vermicularis* could inhibit cytopathic effect induced by HIV-1 (Molchanova et al., 2007). In the study, RPGAL was used to test their effect on tumor cells. And it was found that RPGAL was toxic to HeLa cells and HT1080 cells, while had no effect on A549 cells and HL60 cells. Further study revealed that the effect was accomplished by apoptosis, which was induced by RPGAL. RPGAL resembled some lectins in possession of anti-tumor activity toward different tumor cells due to its specificity to β-galactosides (Dhuna et al., 2007; Yan et al., 2009). Thus, RPGAL has a great potential application in the therapy of anti-tumor.

In summary, this is the first report on cDNA cloning, expression and characterization of a new β-galactose-specific lectin from *R. piscesae*. Moreover, RPGAL was shown to be toxic to some tumor cells. And the mechanism of anti-tumor is interesting to be further investigated.

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