Pathol. Oncol. Res. (2013) 19:259–266
DOI 10.1007/s12253-012-9577-x

RESEARCH

Clinical Significance of Mannose-Binding Lectin Expression in Thyroid Carcinoma Tissues

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Received: 16 July 2012 / Accepted: 24 September 2012 / Published online: 19 December 2012

Abstract Mannose-binding lectin (MBL) plays an important role in the host defence against pathogens and carcinogenesis. This study aimed to analyze differential expression of MBL protein in thyroid cancer tissues and then to investigate the effects of rhMBL in thyroid cancer cells. Tissue specimens from 45 thyroid carcinoma patients and 45 adenoma patients were recruited for immunohistochemical analysis of MBL expression. Cell viability, apoptosis, RT-PCR and Western blot assays were used to detect changes in tumor cell viability, apoptosis, and gene expression, respectively, after treatment of thyroid cancer cells with rhMBL. MBL was differentially expressed in papillary thyroid carcinoma, adenoma, and the distant normal tissues (0.322±0.008, 0.227±0.003, and 0.113 ±0.003, respectively, $P<0.05$). MBL expression was associated with the advanced disease stage, histological grade, or lymph node metastasis in cancer patients ($P<0.05$). Moreover, rhMBL treatment of thyroid cancer cells reduced tumor cell viability but induced apoptosis in a dose- and time-dependent manner. rhMBL treatment also downregulated Bcl2 protein expression in thyroid cancer cells ($P<0.05$). In addition, expression p53 protein was increased in thyroid cancer cells after rhMBL treatment ($P<0.05$). The data from the current study demonstrate that MBL overexpression is associated with advanced thyroid carcinomas, and rhMBL treatment significantly reduced viability but induced apoptosis of thyroid cancer cell lines. Further studies will clarify whether overexpressed MBL in thyroid cancer tissues is functional.

Keywords Thyroid carcinoma · Apoptosis · Mannose-binding lectin

Introduction

Thyroid carcinoma is the most common endocrine malignancy in human. The incidence of thyroid carcinoma is continuously rising worldwide. Approximately 80 % of thyroid cancer is papillary carcinoma and 15 % of thyroid cancer is follicular carcinoma, both of which are derived from thyroid follicular epithelial cells. These two types of thyroid carcinoma can give rise to poorly differentiated thyroid carcinoma, and if they are not properly treated, fatalities will occur [1, 2]. Although significant progression has been made in both basic and clinical research in thyroid cancer, the molecular mechanism responsible for development of thyroid carcinoma, like most other cancers, remains to be determined, and a number of gene alterations may contribute to thyroid gland carcinogenesis [3, 4]. Thus, further studies will clarify the pathogenesis of thyroid cancer and provide the molecular targets for effective treatment of thyroid cancer.

To this end, mannose-binding lectin (MBL) is an acute phase protein synthesized and secreted by the liver. It belongs to the class of collectins in the calcium-dependent lectin superfamily and plays an important role in the first line of host defence [5]. MBL can specifically recognize and bind to glycoproteins with terminal mannose, fucose, N-acetylglucosamine, or N-acetylmannosamine on the surface of many pathogens where it can activate the lectin pathway of the complement system [5]. More recently, it has been reported that MBL could bind to tumor cells through

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collectin receptor and induce phagocytosis or lysis of tumor cells that MBL bound to [6]. Our previous study revealed differentially expressed MBL in the resected human papillary and anaplastic thyroid carcinoma tissues vs. the distant normal thyroid tissues using the mRNA differential display PCR technique. However, little is known about the role of MBL in thyroid carcinoma. Moreover, other studies showed that polymorphism of the MBL gene was associated with the risk of different cancer development, glioma and gastric, colorectal, and breast cancers, and that MBL has antitumor activity in vivo [7–10].

In the current study, we first analyzed MBL expression in normal, adenoma, and cancerous thyroid tissues using immunohistochemistry for association with clinicopathological data from thyroid cancer patients. After that, we then investigated the effects of rhMBL on the regulation of thyroid cancer cell viability, apoptosis, and gene expression. The data could provide more insightful information regarding the functions of MBL in thyroid carcinoma, which may further verify whether expression of MBL saves as a biomarker for early detection or prediction of the progression of thyroid cancer.

Materials and Methods

Tissue Specimens

A total of 90 chemotherapy-, radiotherapy-, and immunotherapy-naive patients received surgery at Tianjin Cancer Hospital, Tangshan Workers Hospital, and Tangshan People's Hospital from 2008 to 2009. There were 45 histologically confirmed papillary thyroid carcinoma and 45 thyroid adenoma tissue samples. A fragment of fresh tumor and distant normal thyroid tissues 5 cm away from the cancers was obtained from the surgery and immediately snap-frozen in liquid nitrogen for RT-PCR analysis. Most of the remaining tissues were fixed in formalin and embedded in paraffin for immunohistochemical staining. Clinicopathological characteristics of the patients were listed in Table 2. Our institutional review board has approved this study and all patients or their guidance have agreed to participate in this study.

Immunohistochemistry

Tissue sections (4 μm-thick) were deparaffinized in xylene and rehydrated in a series of ethanol, and then the endogenous peroxidise activity was quenched with 3 % hydrogen peroxide in methanol for 10 min. Next, antigen retrieval was achieved by heating the tissue sections in 10 mM of citrate buffer at pH 6 for 20 min in a microwave. To block the potential binding of secondary antibody, the sections were incubated with 20 % of normal goat serum for 30 min and then with a polyclonal rabbit anti-MBL-C antibody (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) at a dilution of 1:100 overnight at 4 °C. The next day, the sections were washed with PBS trice and then incubated with a horseradish peroxidase-conjugated secondary antibody (Zhongshan Jinqiao Biotech, Beijing, China) at room temperature for 30 min. After being washed with PBS three times, the sections were incubated with diaminobenzidine solution as the chromogen (Zhongshan Jinqiao Biotech) to visualize the positive signal, and then counterstained with Mayer's haematoxylin for 30 s and dehydrated in upgraded ethanol solutions, clarified with xylene and sealed with cover slips. The negative control sections were processed as the same as the above but using PBS instead of the primary antibody. After that, two pathologists double-blindly reviewed and scored each tissue section without knowing the patients' information. Cells with brown or yellow staining in the cytosol or cell membrane were considered as MBL positive. Cells within five randomly selected fields from three different parts of each section were counted under a microscope (10×20) and quantified by using the average optical density (AOD).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was isolated from the cancerous and normal tissues samples using the Trizol reagent (Biyuntian Biotech Institute, Shanghai, China) according to the manufacturer's instructions. cDNA was then synthesized from 1 μg of the freshly isolated RNA using BeyoRT cDNA synthesis kit (Biyuntian Biotech Institute), i.e., 20 μl reaction mixture was denatured at 42 °C for 10 min and reversely transcribed at 50 °C for 50 min. After that, PCR analysis of MBL mRNA was performed using MBL primers. The MBL primer sequences were 5'- CACAAACTGGAACGAGGGTGA-3' and 5'-CAATACTGGATATGAGGAAATTG-3', which produces a 232 bp band in size. GAPDH gene primers were 5'-CCGACC TGCCCTACGACTA-3' and 5'-CTGGGCTGTAA CATCTCCCTT-3', which produced 226 bp of the amplified fragment. A total of 25 μl PCR reaction contained 2 μl of 10 × PCR buffer, 1.2 μl of 2.5 mM dNTP, 0.1 μl of TaqDNA polymerase, 0.6 μl of each primer, 1 μl of cDNA, and water. Negative control was processed the same but without template cDNA. PCR program was 94 °C for 3 min and then 30 cycles of 94 °C for 30 s, 58 °C 30 s, and 72 °C for 1 min and followed by a final extension at 72 °C for 5 min. PCR products were then loaded onto a 1 % agarose gel for electrophoresis and stained with ethidium bromide. The data were summarized and expressed as a ratio to GAPDH.

Enzyme-Linked Immunosorbent Assay (ELISA)

Serum samples from 45 cases each of papillary thyroid carcinoma, thyroid adenoma, and healthy volunteers were collected and used for ELISA analysis of serum levels of MBL protein expression with an ELISA kit from Hufeng Biotech (Shanghai, China) according to the kit instructions. The data were summarized as mean ± SD and compared to each tissue types.

Cell Lines and Culture

Human PTC K1 cells were obtained from the European Collection of Cell Cultures (CAMR, Salisbury, UK) and maintained in DMEM: Ham's F12: MCDB105 (2:1:1) (Sigma, St Louis, MO, USA) supplemented with 10 % fetal calf serum (FCS) (Gibco Co., Grand Island, NY, USA) at 37 °C in a humidified atmosphere containing 5 % CO_2 and 95 % air. Human FTC-133 cells were from the European Collection of Cell Cultures and cultured in DMEM: Ham's F12 (1:1) with 10 % FCS.

Protein Extraction and Western Blotting

Cancer cells in exponential phase of growth were treated with different amounts of rhMBL (R&D systems, Minneapolis, MN, USA) for 48 h, and were lysed with a lysis buffer (Suolaibao Biotech, Beijing, China) on ice for 15 min followed by spinning down for 15 min at top speed. Concentration of the total cellular protein was measured using a BCA kit (Suolaibao Biotech, Beijing, China). Cell lysates containing approximately 40 μg protein were separated by using SDS/polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Sigma). After that, the membranes were blocked with 5 % milk in Tris buffered saline-0.1 % Tween-20 (TBST) for 2 h at room temperature and then incubated with different monoclonal primary antibodies against MBL (at a dilution of 1:500), Bcl-2 (1:500), p53 (1:200), or GAPDH (1:500) overnight at 4 °C. In the following days, the membranes were washed with TBST trice for 5 min each and then incubated with the secondary antibody (1:1000) for 2 h at room temperature. The positive protein target bands were detected by using the Odyssey Infrared Imaging System (LI-COR Bioscience, Lincoln, Nebraska, USA). Expression of GAPDH protein served as a loading control.

Cell Viability MTT Assay

Thyroid cancer cells were seeded in each well (10^3 to 10^4) of 96-well plates and treated with different doses of rhMBL (0.01, 0.1, 1, or 10 μg/ml) for 12, 24, or 48 h and then 20 μl of MTT solution (5 mg/ml; from Sigma) was added and the cells were incubated for 4 h. After that, the cell culture medium was removed and 100 μl of DMSO was added to dissolve MTT. Absorbance of each well was then measured at OD_{490} using a spectrophotometer. The cell culture medium without rhMBL treatment served as the negative control. Suppression of K1 and FTC-133 cell proliferation by rhMBL was calculated using the following equation: survival rate (SI) = OD of the experimental cells/OD of the negative control x 100 %; suppression rate = 1 – survival rate.

Flow Cytometry Analysis After Cells Stained with Annexin V-FITC and Propidium Iodide (PI)

Thyroid cancer cells were seeded in six-well plates with 10^6 per well overnight and then treated with 1 μg/ml rhMBL for 48 h. The control cells were treated with PBS only. At the end of experiments, the cells were trypsinized and collected by centrifugation at 1,000 r/min for 5 min, and then incubated with 0.5 ml of the binding buffer and 1 μl Annexin V-FITC from a Annexin V-FITC apoptosis detection kit (Merck, Darmstadt, Germany) at 18 to 24 °C for 15 min. After that, the cells were resuspended in fresh 0.5 ml of the binding buffer containing 5 μl PI and filtered through a 40-micron filter. Then, thyroid cancer cell apoptosis was measured by flow cytometry.

Statistical Analyses

All statistical analyses were performed using SPSS17.0 software (SPSS, Chicago, IL, USA). For association with the clinicopathological features, P values were calculated using the χ^2 test. MBL mRNA expression was shown in mean ± SD. Student's t-test was used to analyze the difference between groups. A P-value less than 0.05 was considered statistically significant.

Results

Overexpression of MBL Protein in Thyroid Carcinoma Tissues

In this study, we first assessed differential expression of MBL protein in 45 papillary thyroid carcinomas and the corresponding distant normal tissues, as well as 45 thyroid adenoma tissue samples using immunohistochemistry. Immunohistochemistry data showed that MBL protein was localized in the cytosol of thyroid follicular epithelial cells as brown or yellow particles (Fig. 1b and c). Quantitative expression of MBL protein in papillary thyroid carcinomas, distant normal tissues, and thyroid adenoma tissue samples were significantly different (0.322±0.048, 0.113±0.004, and 0.227±0.037, respectively; $P<0.05$). Similarly, expression of MBL mRNA in papillary thyroid carcinomas, distant normal, and thyroid adenoma tissue samples were dramatically different (0.514±

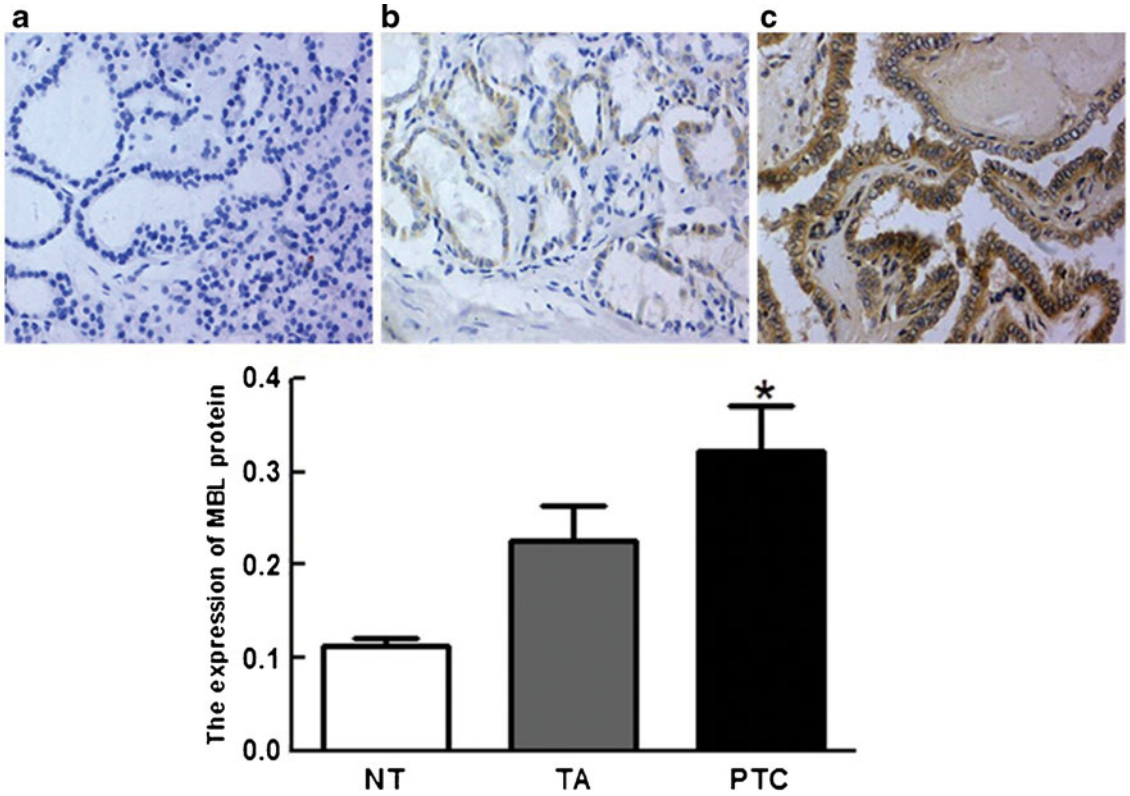

Fig. 1 Differential expression of MBL protein in thyroid carcinoma tissue specimens. Immunohistochemical staining of MBL protein was conducted in normal thyroid tissue (**a**), thyroid adenoma (**b**), and papillary thyroid carcinoma (**c**) tissue samples. Quantification of the staining data is shown in the bottom figure (*P<0.05). Note: NT, normal thyroid glands; TA, thyroid adenoma; PTC, papillary thyroid carcinoma

0.052, 0.171±0.022, and 0.397±0.025, respectively, P<0.05; Fig. 2). Furthermore, we also analysed serum levels of MBL protein in these patients and found an increase in MBL protein expression in thyroid carcinoma (Table 1). These data suggested that expression of both MBL mRNA and protein was dramatically enhanced in thyroid carcinoma tissue specimens.

Association of MBL Protein Expression with Clinicopathological Features from Thyroid Cancer Patients

After that, we associated MBL protein expression with clinicopathological data from thyroid cancer patients and found that expression levels of MBL protein were associated with histological grade, advanced disease stage, or lymph node metastasis of papillary thyroid carcinoma (P<0.05, Table 1). In contrast, other clinicopathological features, such as age, sex, or tumor size, were not associated with MBL protein expression (P>0.05, Table 2).

Expression of MBL Protein in Thyroid Cancer K1 Cell and FTC-133 Cells

To determine the role of MBL protein in thyroid carcinoma, we performed an in vitro study to assess levels of MBL protein in two thyroid carcinoma cell lines using Western blotting. We found that MBL protein was expressed in K1 cells and FTC-133 cells with similar levels (0.754±0.078 vs. 0.863±0.121, respectively; Fig. 3).

Effect of rhMBL Protein on Regulation of Thyroid Carcinoma Cell Viability In Vitro

Next, we assessed the effect of rhMBL protein on regulation of thyroid carcinoma cell viability *in vitro* using an MTT assay. Our data showed that rhMBL significantly reduced viability of both K1 and FTC-133 cell lines in a dose-dependent manner (P<0.05, Fig. 4). Moreover, the inhibitory effect of rhMBL protein was observed after a 12-h treatment and reached its maximum at 72-h of treatment (P<0.05, Fig. 4).

Induction of Thyroid Carcinoma Cells to Apoptosis After rhMBL Treatment

After that, we performed flow cytometry analysis to detect apoptosis levels induced by hrMBL protein and found that 48-h treatment with 1 μg/ml rhMBL significantly enhanced apoptosis of K1 cells vs. the control cells (9.36±0.35 % vs. 3.24±0.71 %, respectively; P<0.05, Fig. 5). Similarly, 1 μg/ml

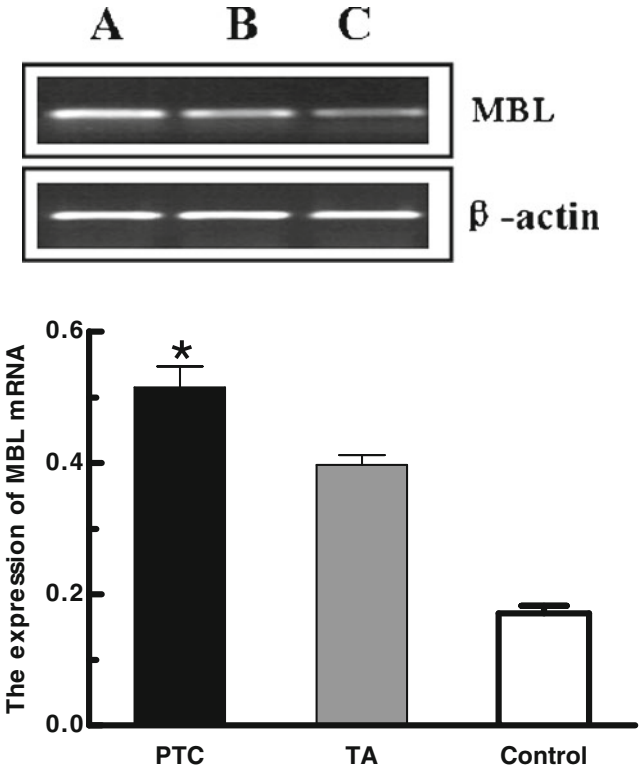

Fig. 2 Differential expression of MBL mRNA in thyroid carcinoma tissues. RT-PCR was performed to assess expression of MBL mRNA in papillary thyroid carcinoma (**a**), thyroid adenoma (**b**), and normal thyroid tissues (**c**). Quantification of RT-PCR data is shown in the bottom panel (*P<0.05)

of rhMBL dramatically enhanced apoptosis of FTC-133 cells compared to control cells (12.87±0.47 % vs. 5.15±0.52 %, respectively; P<0.05).

Effects of rhMBL on Gene Expression in Thyroid Cancer Cells

We then explored the effect of rhMBL protein on the regulation of apoptosis-related gene expressions. Our data showed that rhMBL significantly reduced expression of Bcl-2 protein in K1 cell compared to the controls (0.26± 0.05 vs. 0.49±0.09, respectively; P<0.05, Fig. 6), while p53 protein was significantly induced in K1 cell compared to the controls (0.74±0.11 vs. 0.56±0.08, respectively; P<0.05,

Table 1 Serum levels of MBL concentration in thyroid carcinoma and adenoma patients and healthy control

Group	No.	MBL concentration		
		mean±SD	F	P
Control	45	152.417±16.952		
TA Patients	45	324.413±25.405	48.289	0.000
PTC Patients	45	563.817±82.840		

Table 2 Association of MBL protein expression with clinicopathological features from thyroid cancer patients

Characteristics	No.	MBL protein expression		
		Mean ± SD	t	P
Gender				
Male	25	0.316±0.021	0.384	0.720
Female	20	0.310±0.019		
Age (yrs)				
≤40	28	0.317±0.032	0.627	0.565
>40	17	0.302±0.026		
Tumor size (cm)				
≤4	22	0.324±0.032	0.736	0.502
>4	23	0.343±0.030		
Disease stage				
I~II	14	0.296±0.024	2.980	0.041
III~IV	31	0.355±0.025		
Histological grade				
I	19	0.291±0.029	5.113	0.000
II~III	26	0.335±0.028		
Lymph node metastasis				
N0	21	0.290±0.022	3.570	0.023
N+	24	0.361±0.027		

Fig. 6). Similarly, expression of Bcl-2 was significantly reduced in rhMBL-treated FTC-133 cells compared to the control cells (0.38±0.06 vs. 0.55±0.10, respectively; P< 0.05, Fig. 7). Meanwhile, Western blot data showed the levels of p53 protein in rhMBL-treated FTC-133 cells were also induced to 0.85±0.12 compared to the control cells (0.65±0.09, P<0.05, Fig. 7).

Discussion

In this study, we detected MBL protein expression in normal, adenoma, and cancerous thyroid tissues and serum samples from the normal and cancerous thyroid patients. We found that overexpression of MBL protein in thyroid carcinomas was associated with histological grade, advanced disease stage, or lymph node metastasis of papillary

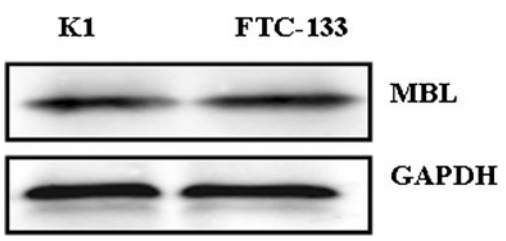

Fig. 3 Detection of MBL protein in thyroid cancer K1 and FTC-133 cell lines. A western blot was performed to detect levels of MBL protein in K1 and FTC-133 cells (0.754±0.078 and 0.863±0.121, respectively)

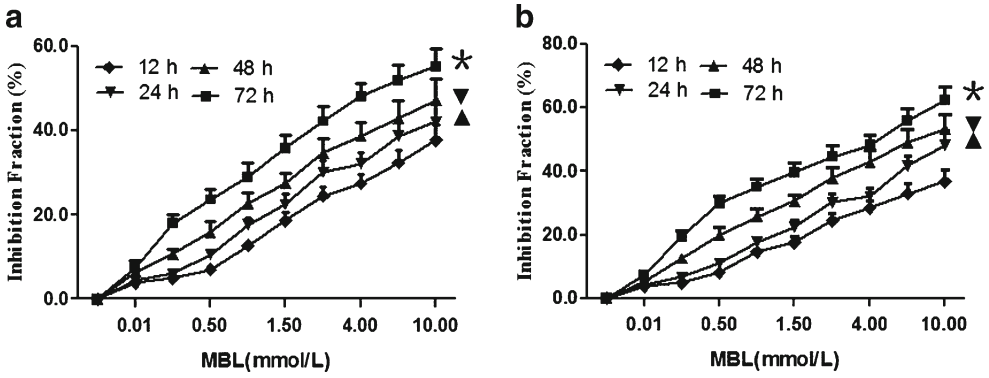

Fig. 4 Effects of rhMBL treatment on regulation of thyroid carcinoma K1 and FTC-133 cell viability. Cell viability MTT assay revealed the inhibitory effect of MBL on viability of K1 (**a**) and FTC-133 (**b**) cells

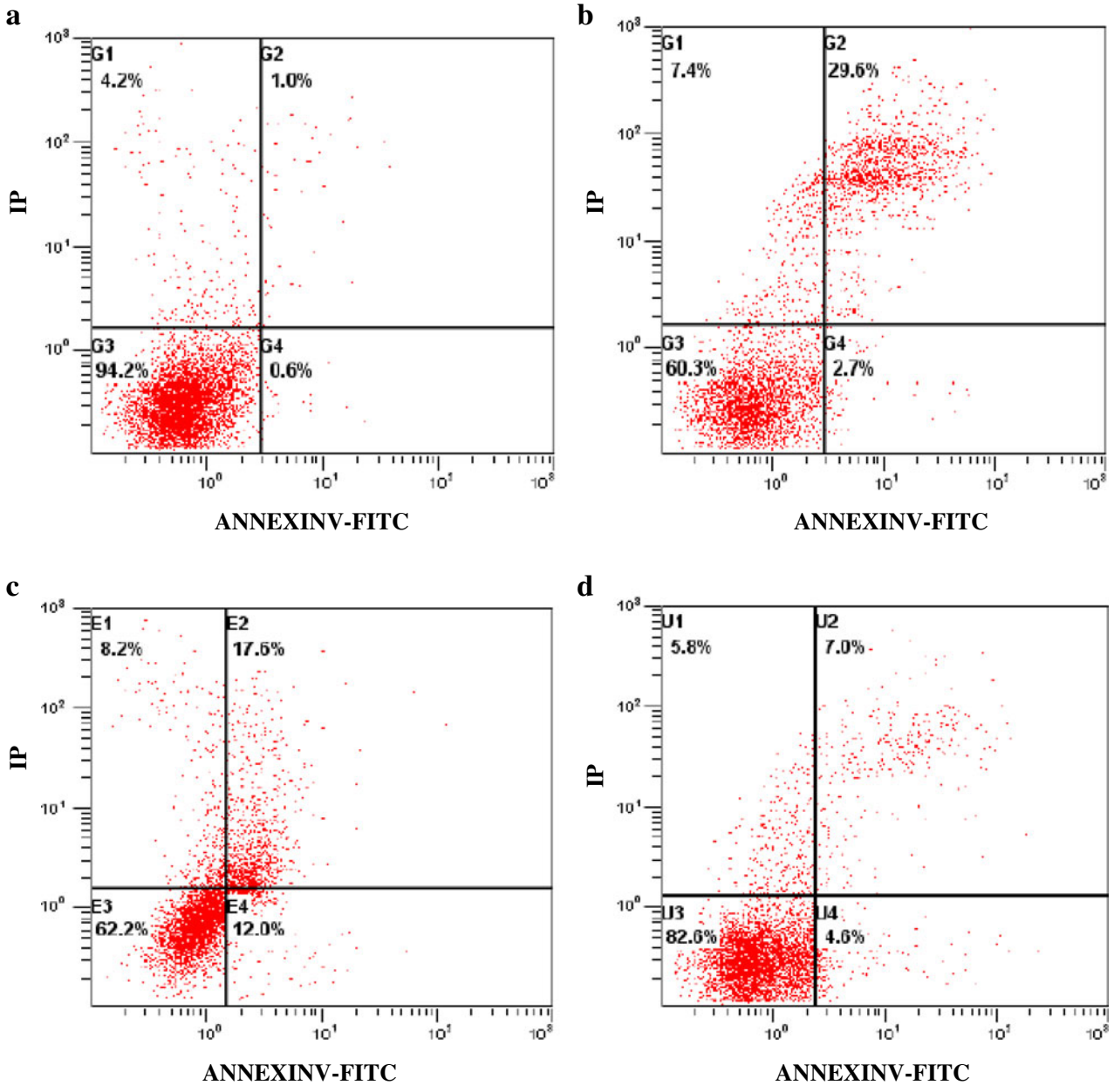

Fig. 5 Effects of rhMBL on induction of thyroid cancer cell apoptosis. Flow cytometric analysis revealed induction of apoptosis in K1 cells treated with rhMBL (**a**) compared to the untreated cells (**b**). Similar effects were shown in FTC-133 cells treated with rhMBL (**c**) compared to the untreated cells (**d**)

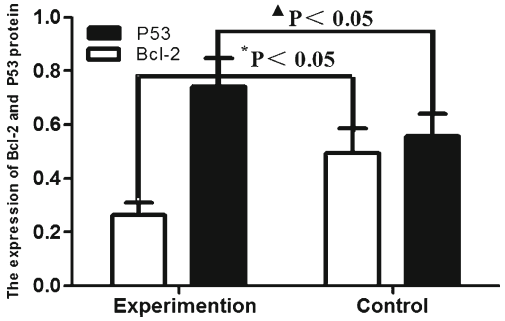

Fig. 6 Effects of rhMBL on Bcl-2 and p53 protein on regulation of apoptosis-related gene expression in thyroid carcinoma cells. Western blot analysis showed a reduction of Bcl-2 or p53 protein expression in rhMBL treated in K1 cells (**a**) relative to the untreated K1cells (**b**)

thyroid carcinoma. However, treatment of thyroid cancer cell lines with hrMBL significantly reduced tumor cell viability in a dose-dependent manner, but induced tumor cells to undergo apoptosis, which was associated with reduction of Bcl2 protein levels. These data demonstrate that overexpression of MBL protein was associated with advanced or aggressive thyroid carcinomas, whereas rhMBL treatment significantly reduced viability but induced apoptosis of thyroid cancer cell lines, indicating that either the overexpression of MBL protein in thyroid carcinomas may be nonfunctional or treatment with hrMBL protein could stress thyroid carcinoma cells and therefore cause apoptosis. Further studies will clarify the role of MBL protein in thyroid cancer.

At the protein level, several MBL identical peptide chains together form the oligomeric structure and the dimers and trimers are not biologically active but at least a tetramer is needed for activation of MBL protein [11]. Moreover, MBL protein is subjected to significant posttranslational modifications, such as proteolytic cleavage of the N-terminal signal peptide, hydroxylation and glycosylation and any aberrations could result in a non-functional protein [6]. Our current study demonstrated that MBL protein was

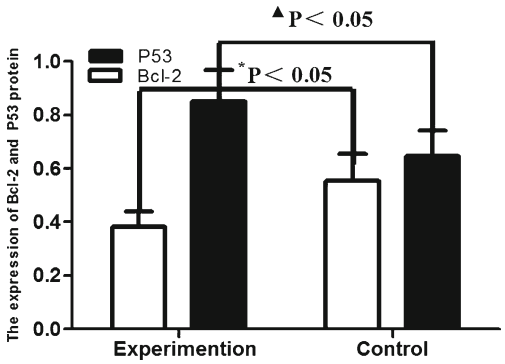

Fig. 7 Effects of rhMBL on Bcl-2 and p53 protein on regulation of apoptosis-related gene expression in thyroid carcinoma cells. Western blot analysis showed a reduction of Bcl-2 or p53 protein expression in rhMBL treated in FTC-133 cells (**a**) relative to the untreated FTC-133 cells (**b**)

overexpressed in thyroid carcinoma tissues compared to the distant normal and adenoma tissues, MBL overexpression was associated with the advanced and aggressive disease, but treatment of thyroid cancer cells with rhMBL protein induced tumor cell apoptosis and reduced Bcl2 expression. We therefore predict that the immnuohistochemically positive MBL protein in thyroid cancer tissues may carry a non-functional MBL protein, which allowed treatment of thyroid carcinoma cells with rhMBL protein the ability to induce tumor cell apoptosis. However, this assumption needs further study to verify.

Previous studies have shown that MBL protein is an important component of the innate immune system and functions as the first line of defense in the pre-immune host through its recognition of carbohydrate patterns that are found on the surface of pathogenic microorganisms [6]. To date, there are four known functions of the MBL protein, i.e., activation of the complement system, promotion of opsonophagocytosis, regulation of inflammation, and promotion of cell apoptosis [11–16]. An increasing number of studies have demonstrated that abnormal MBL levels are not only associated with infectious or inflammatory diseases but also with cancers because both the adaptive and innate immunity can regulate tumor cell growth and migration and protect normal tissues against tumor transformation [14]. In addition, activation of the complement system is a central mechanism in the innate immune response and induces activation of inflammatory cells, which could also contribute to tumorigenesis [14]. Due to the change in glycosylation on the surface of tumor cells, MBL might recognize and target these tumor cells for subsequent phagocytosis or lysis [17–19]. To date, most studies focused on MBL protein in certain types of cancer, such as glioma and gastric, colorectal, and breast cancers [7–10]. Our previous study has identified MBL as a differentially expressed gene in resected human papillary and anaplastic thyroid carcinoma, indicating that MBL protein may be critical in thyroid cancer progression [20]. In the current study, we confirmed the overexpression of MBL protein, which was associated with the tumor stage, histological grade, and lymph node metastasis. However, this needs further study to verify whether the overexpressed MBL protein in thyroid cancer tissues is functional. Normally, MBL can immune-protect thyroid cells from tumor development. However, due to the chronic inflammation in the thyroid, highly expressed MBL on cell membrane is able to recognize and bind to the tumor cells and then to activate C3 and in turn to phagocytise abnormal cells [6].

Indeed, our in vitro experiments revealed that rhMBL possessed the ability to reduce viability of thyroid cancer K1 and FTC-133 cells and to induce them to undergo apoptosis. These data provide experimental evidence showing the effects of MBL protein on thyroid cancer cells,

although it needs further study as to whether the overexpressed MBL protein in thyroid cancer is functional or nonfunctional.

Conflicts of interest All authors have no conflicts of interest.

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