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ORIGINAL ARTICLE

Gene expression profiling of hybridoma cells after bursal-derived bioactive factor BP5 treatment

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Abstract Bursa of Fabricius is the acknowledged vital humoral immune system for B cell differentiation and antibody production. To study the molecular mechanism underlying the effect of bursal-derived BP5, we used gene microarray to analyze the genomic expression profiling of BP5-treated hybridoma cells. BP5 exhibited an immunomodulatory effect on antibody production in hybridoma cells and induced alterations in the gene expression profiles related to the immune-related biological processes, such as T cell activation and proliferation, B cell activation, B cell-mediated immunity, and cytokines cytokine production involved in immune response. In addition, 26 biological pathways associated with immunomodulatory functions were regulated in BP5-treated hybridoma cells, in which p53 signal pathway played an important role in antitumor. Among these regulated genes, 12 differentially expressed genes were verified by qRT-PCR. The activation of p53 activity by BP5 was further confirmed by p53 luciferase reporter assay and p53 expression. Our data revealed that bursal-derived BP5 could regulate various immune-related cellular processes, including antitumor factor p53 signal pathway, perhaps partially accounting for the reported immunomodulatory roles and novel antiproliferation on tumor cells functions of bursal-derived bioactive factor BP5.

Keywords Bursal-derived BP5 · Gene microarray · Go analysis · p53 pathway · p53 expression

Introduction

Bursa of Fabricius (BF) is the central humoral immune organ unique to birds, absent in humans, which is the primary site of chicken B cell development (Davison et al. 2008). It has also become clear that the mechanism by which rearrangement of immunoglobulin gene segments in chickens generates antibody diversity differs fundamentally from that seen in mouse and human (Reynaud et al. 1987, 1989; Thompson and Neiman 1987). The chicken therefore represents a species in which there has been a co-evolution of lymphoid structure and function away from that seen in the more commonly studied models of rodents and primate bone marrow (Reynaud et al. 1987, 1989; Thompson and Neiman 1987).

It was reported that BF contained various bioactive factor. For example, BF extracts from 2- to 3-month-old Peking ducks could increase E-rosette formation of swine thymus cells (Zheng and Gao 1991). In addition, chicken BF extract at high concentration enhanced blastogenic responses to T cell mitogens (Murthy and Ragland 1992). It has been proved that Bursal-derived BPP-I and BPP-II

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could induce antibody production and cell-mediated immune responses (Feng et al. 2011, 2012). These observations suggest that there are some immunomodulatory elements present in BF to enhance or modulate the immune response.

Recently, we isolated a new biological active factor BP5 from BF, avian humoral immune system, which was proved to be a multi-functional modulator in immune response (Li et al. 2010). In this paper, the effects of BP5 and its underlying molecular mechanism were examined in mouse-derived hybridoma cell (H5F9 strain) through microarray analysis. Results from these analyses showed that p53 is an important modulator of gene expression in BP5-treated hybridoma cells. Furthermore, BP5-induced p53 expression was confirmed by a luciferase reporter assay in Vero/p53 transgenic cells and by Western blotting analysis in Vero cells.

Method and materials

Cell lines and BP5

Hybridoma cell, H5F9 strain (Feng et al. 2011), was fused with the spleen cell from immunized BALB/c mouse by the inactivated JEV (SA14 strain) and SP2/0 cell (NO. TCM18) and the monoclonal antibody was IgG1 κ subtype antibody. Hybridoma cells were cultured with RPMI 1640 supplemented with 20 % heat-inactivated fetal bovine serum (Invitrogen) at 37 °C with 5 % CO_2. Vero cell (NO.GNO10) and Hela cell (TCHu 19) were purchased from Cell Bank of Chinese Academy, and MCF-7 cell was a gift from Ming Yao (Shanghai Cancer Institute, China). These three cells were maintained with DMEM (Invitrogen) supplemented with 10 % heat-inactivated fetal bovine serum (Invitrogen) at 37 °C with 5 % CO_2.

BP5, a synthesis peptide with purity over 97 % (Huachen, Xi'an, China) was dissolved in double-distilled BPS buffer.

Cell culture and antibody detection

Hybridoma cells were prepared in 96-well plates and incubated for 24 h before the BP5 treatment, respectively. Various concentrations of BP5 (20, 2, 0.2, or 0.02 μg/mL) were added to hybridoma cells for 48 h. The control cells were treated with equal amounts of PBS buffer only. Antibody production from the hybridoma supernatant was determined by ELISA as described previously (Feng et al. 2010) with inactivated JEV coated plates.

3-[4,5-Dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann 1983)

After the BP5 treatment for 48 h, MTT 20 μL/well (5 mg/mL) was added to the cells for 4 h. The purple formazan crystals were solubilized in 150 μL dimethyl sulfoxide (DMSO), and the absorbance was measured at a test wavelength of 570 nm and a reference wavelength of 650 nm using a microplate reader. The relative survival rate was calculated using the following equation: survival rate (%) = (absorbance of BP5-treated cells/absorbance of untreated control cells) × 100 %.

Total RNA extraction, microarray and data analysis

The experiment program was tested by KangChen Bio-tech (China, Shanghai). Simply, hybridoma cell was treated with BP5 at 2 μg/mL for 4 h, and total RNAs are harvested using TRIzol (Invitrogen) and the RNeasy kit (Qiagen) according to the manufacturer's instructions. Hybridoma cells (1×10^5 cells/mL) were stimulated by BP5 treatment. PBS was used as negative control. Total RNA was extracted from hybridoma cells grown in 75 cm^2 flasks following treatment with BP5 (2 μg/mL) ($n = 3$) using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). The total RNA samples with A_{260}/A_{280} ratios greater than 1.8 were further evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA samples with an RNA integrity number greater than 8.0 were used for the subsequent microarray analysis.

After having passed RNA measurement on the Nanodrop ND-1000 and denaturing gel electrophoresis, the samples were amplified and labeled with Cy3 using the Agilent Quick Amp labeling kit and hybridized with Agilent whole genome oligo microarray in Agilent's SureHyb Hybridization Chambers. After hybridization and washing, the processed slides were scanned with the Agilent DNA microarray scanner (G2565BA) using settings recommended by Agilent Technologies.

The microarray analysis was performed as previously described (Chen et al. 2010a, b). Briefly, the resulting text files extracted from Agilent Feature Extraction Software (version 10.5.1.1) were imported into the Agilent GeneSpring GX software (version 11.0) for further analysis. The microarray data sets were normalized in GeneSpring GX using the Agilent FE one-color scenario (mainly median normalization). Differentially expressed genes were identified through fold-change screening (fold change $\geq$ 1.5).

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

According to genomic expression profiling of BP5-treated hybridoma cell, ten genes were randomly selected to further validate by qRT-PCR. All primers were designed to amplify cDNA across exon–intron junctions. And expressed genes were estimated using One Step SYBR® PrimeScript® RT-PCR Kit (Takara, DRR066A). Each assay was

run on an Applied Biosystems 7300 Real-time PCR system in triplicate. Fold changes were calculated using the comparative CT method. The primer sets for selected genes are shown in Table S1.

P53 luciferase activity and expression

Vero cell, wild-type p53 (Shen et al. 2009), were prepared in 24-well plates (5×10^4 cells/well). After 16–20 h, Vero cells were transfected with the p53-LUC and pRL-TK (reference) construct in a LipofectAMINE 2000/DNA conjugate (Invitrogen). After 24 h, transfected Vero cells were treated with 20, 2, 0.2 and 0.02 μg/mL of BP5 for ~24 h and assaying for luciferase activity as previously described (Promega). Also, transfected Vero cells were treated with 20 μM α-pifithrin for 2 h (Walton et al. 2005), and then stimulated with BP5 and assayed for luciferase activity 22 h later. All assays were performed in triplicate.

To estimate the probable role on p53 protein expression of bursal peptide, Vero cells were treated with BP5 for 24 h. A positive control cells was treated with Dox (Sigma). Vero cell protein samples were prepared by the cell culture lysis reagent (Promega) according to the manufacturer's instructions. Western blotting was performed as previously described (Qiu et al. 2008) using antip53 monoclonal antibody (DO-1, Santa Cruz, USA), anti-Bax polyclonal antibody (N-20, Santa Cruz), anti-b-actin monoclonal antibody (AC-15, Sigma, USA).

Tumor cell proliferation

MCF-7 and Hela cells were prepared (1×10^5 cells/mL) and stimulated with BP5 at different concentrations for 48 h. PBS was used as negative control. Cell viabilities were determined with the MTT (Sigma) (Mosmann 1983).

Statistical analysis

Data are presented as the mean ± SD Student's *t* test was used for comparisons between the control and BP5-treated groups. A value of $P < 0.05$ was determined to be statistically significant.

Results

Antibody production and cell proliferation in BP5-treated hybridoma cells

It was reported that BP5 induced immune responses, and directly enhanced B lymphocyte proliferation (Li et al. 2010). In this paper, we examined the potential roles of BP5 on hybridoma cell which shares the dual characteristics of unlimited proliferating and secreting antibody. As shown in Fig. 1a, BP5 stimulated antibody production of hybridoma cell. The antibody levels were increased by 18.32, 33.5, 15.18, and 2.1 % at 20, 2, 0.2, and 0.02 μg/mL BP5, respectively.

The cell viability (using the MTT assay) was measured after exposing hybridoma cells to various concentrations of BP5 (from 20 to 0.02 μg/mL) (Fig. 1b). The results showed that hybridoma cell proliferations were increased with BP5 treatment (range from 2 to 0.02 μg/mL) by 9.76, 7, and 2.1 %. However, at 20 μg/mL, BP5 could decrease hybridoma cell proliferation by 4.79 %. There was no significantly hybridoma proliferation changes observed. Compared with the results of BP5-treated hybridoma cell, we speculated that BP5 could induce the hybridoma secreting antibody, which might not be related to the hybridoma cell proliferation.

According to these results, we chose the 2 μg/mL as a dose to further study the gene regulation in hybridoma cell exposed to BP5.

Overview of gene expression patterns

cDNA microarray systems are broadly used to investigate gene expression patterns and functional classification in stem cell (Pan et al. 2002). In this paper, to elucidate the mechanisms underlying the inducing effect of BP5 on immune cells, we extracted RNA from hybridoma cells that were in extro subjected to BP5 stimulation for 4 h, and hybridized in mouse cDNA microarrays. The resulting data were analyzed by the GABRIEL (Genetic Analysis by Rules Incorporating Expert Logic) system, a knowledge-based system of computer algorithms (Pan et al. 2002). We found 2,671 genes were up-regulated and 952 genes that were down-regulated at least 1.5-fold by BP5 (Table S2). That so many genes are differentially transcribed in the BP5 versus control comparisons suggests the biological active peptide BP5 might be involved various biological functions.

Biological processes analysis of the gene expression profiles in BP5-treated hybridoma cells

GO analysis is a functional analysis associating differentially expressed genes with GO categories. The GO categories are derived from Gene Ontology (http://www.geneontology.org), which comprises three structured networks of defined terms to describe gene product attributes. This functional analysis was used to predict significant differences among the gene expression levels in hybridoma cells in response to 2 μg/mL BP5 treatment. As shown in Table S3, cellular process, metabolic process, biological regulation, regulation of biological process, cellular

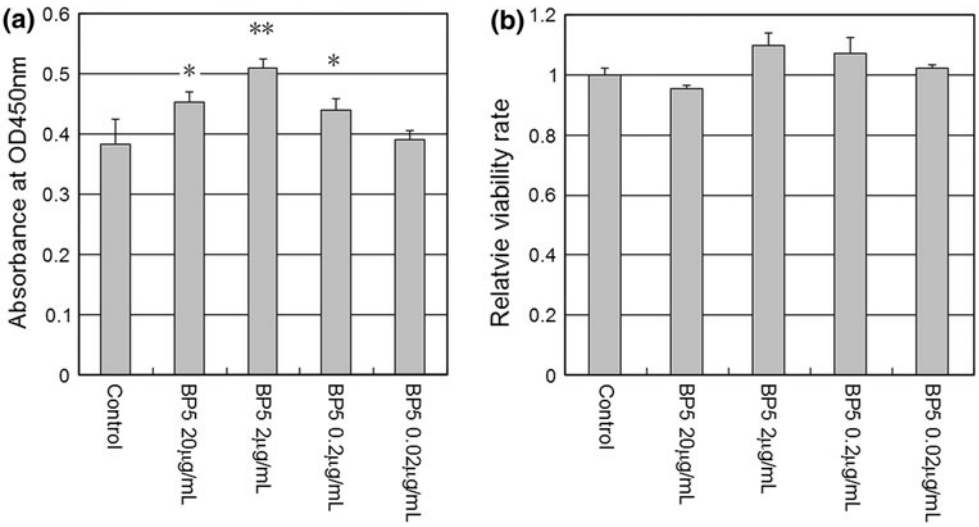

Fig. 1 Roles of BP5 on mouse-derived hybridoma cell. Hybridoma cells were treated with various concentration range from 20 to 0.02 μg/mL of BP5. **a** Antibody production detection. After 48 h, antibody level of hybridoma cell supernatants was determined by ELISA. **b** Hybridoma cell proliferation determination. Dispatch the culture medium, MTT was added to hybridoma cell to measure the cell viability. Values are mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared to control cells without BP5 treatment

metabolic process, regulation of cellular process, primary metabolic process, macromolecule metabolic process, cellular macromolecule metabolic process, signaling, nitrogen compound metabolic process, cellular nitrogen compound metabolic process were shown to be significantly regulated with more than 500 genes differential expression in hybridoma cells in BP5 treatment group (P value < 0.01).

Biological processes B cell involved by BP5 treatment

BF is critical for normal development of the B lymphocytes responsible for antibody production (Davison et al. 2008). It was reported that bursal-derived BP5 enhanced anti-hemagglutinin antibody (IgG, the isotypes IgG1 and IgG2a) production in BALB/c mice immunized with inactivated AIV (Li et al. 2010). In this paper, the results showed that genes related to various B cell-related biological processes were regulated with BP5 treatment (Tables 1, S4). The down-regulated gene Exonuclease 1 (Exo1) was involved in B cell activation (GO:0042113), humoral immune response (GO:0006959) and humoral immune response mediated by circulating immunoglobulin (GO:0002455). Also, Fc receptor, IgE, high affinity I, gamma polypeptide (Fcer1g) and alpha polypeptide (Fcer1a), TNF, PTPRC, LTA, and GIMAP5 were involved in various humoral immune response and regulation, in which Fcer1g was down-regulated, other genes were up-regulated in hybridoma exposed to BP5 (Tables 1, S4). Also, up-regulated genes STAT5B, STAT5A, and BAD were involved in B cell activation (GO:0042113), regulation of B cell activation (GO:0050864), regulation of B cell differentiation (GO:0045577), and positive regulation of B cell differentiation (GO:0045579). These results provide some cellular level insight on humoral immunity mechanism of BP5, a bioactive peptide from avian humoral immune system.

Biological processes T cell involved by BP5 treatment

Our recent report proved that BP5 increased proliferations of splenic lymphocyte subsets CD4+ T cells (CD3+CD4+), CD8+ T cells (CD3+CD8+) and B cells, and enhanced cytotoxic T-lymphocyte activity of the activated splenocytes against NIH3T3 cells in immunization mice (Li et al. 2010). T cell is key immune cell for cellular mediated immunity, and regulation play vital roles on various immune responses (Jonathan 1994). There were 17 terms of T cell-related biological processes, as shown in Table S5. Except for GO:0046640 (regulation of alpha–beta T cell proliferation) and GO:0002710 (negative regulation of T cell-mediated immunity), activated STAT5B and STAT5A genes were involved in all T cell-related biological processes (Tables 2, S5). The up-regulated PTPRC and GIMAP5 genes were involved in gamma–delta T cell differentiation, activation and regulation biological processes. Although not involved in gamma–delta T cell-related biological processes and T cell differentiation in the thymus (GO:0033077), CASP3 was involved in all T cell activation, proliferation, regulation and homeostasis. Also, TNFRSF14, CDKN2A, IL20RB, and ICOSL were involved in various T cell biological processes. Among these regulated genes, only cyclin-dependent kinase inhibitor 2A (Cdkn2a) was down-regulated by BP5 in hybridoma cell (Tables 2, S5).

Cytokine-related biological processes involved by BP5 treatment

The cytokines play an important role in cell-mediated immune response (Kourilsky and Truffa-Bachi 2001). It was reported that BP5 remarkably increased both Th1-type (IL-2 and IFN-γ) and Th2-type (IL-4 and IL-10) cytokine productions, which suggested that BP5 is capable of

Table 1 Genes involved in biological processes B cell involved by BP5 treatment

Gene symbol	Regulated change	Genbank accession	UniGeneID	Description
Exo1	−1.63543	NM_012012	Mm.283046	Exonuclease 1
Fcer1g	−1.64746	NM_010185	Mm.22673	Fc receptor, IgE, high affinity I, gamma polypeptide
Fcer1a	+1.916035	NM_010184	Mm.5266	Fc receptor, IgE, high affinity I, alpha polypeptide
Tnf	+1.533966	NM_013693	Mm.1293	Tumor necrosis factor
Ptprc	+2.123588	NM_011210	Mm.391573	Protein tyrosine phosphatase, receptor type, C
Lta	+1.707351	NM_010735	Mm.87787	Lymphotoxin A
Gimap5	+2.16353	AK087160	Mm.455917	Hypothetical protein
Stat5b	+1.520192	NM_011489	Mm.34064	Signal transducer and activator of transcription 5B
Stat5a	+1.956247	NM_011488	Mm.277403	Signal transducer and activator of transcription 5A
Bad	+1.787754	AK029400	Mm.4387	Bcl-associated death promoter

To identify the genes that are differentially expressed, we performed a fold-change screening between the two groups obtained from the experiment. The threshold we used to screen up (+) or down (−) regulated genes is fold change ≥ 1.5

Table 2 Genes involved in biological processes T cell involved by BP5 treatment

Gene symbol	Regulated change	Genbank accession	UniGeneID	Description
Stat5b	+1.5201918	NM_011489	Mm.34064	Signal transducer and activator of transcription 5B
Stat5a	+1.9562465	NM_011488	Mm.277403	Signal transducer and activator of transcription 5A
Ptprc	+2.123588	NM_011210	Mm.391573	Protein tyrosine phosphatase, receptor type, C
Gimap5	+2.1635299	AK087160	Mm.455917	Hypothetical protein
Casp3	+1.7084149	U49929	Mm.34405	ICE-like cysteine protease (Lice)
Tnfrsf14	+1.8990159	BC022125	Mm.215147	Tumor necrosis factor receptor superfamily, member 14
Cdkn2a	−1.5040325	NM_009877	Mm.4733	Cyclin-dependent kinase inhibitor 2A
Il20rb	+1.7414869	AK076401	Mm.242896	Unclassifiable
Icosl	+1.7000047	NM_015790	Mm.17819	Icos ligand

To identify the genes that are differentially expressed, we performed a fold-change screening between the two groups obtained from the experiment. The threshold we used to screen up (+) or down (−) regulated genes is fold change ≥ 1.5

enhancing T cells and T cell-mediated immune responses, and balancing Th1 and Th2 responses (Li et al. 2010). Also, we determined the effect of BP5 on endogenous cytokine production involved in immune response and regulation in BP5-treated hybridoma cell. There were four differential expression genes (Table 3), in which heme oxygenase (decycling) 1 (HMOX1), an anti-oxidative stress protein (Chen et al. 2010a, b), and GIMAP5 were up-regulated while Fc receptor, IgE, high affinity I, gamma polypeptide (FCER1G) and CD36 were down-regulated, which were involved in cytokine production involved in immune response and regulation.

Molecular function and cellular component by BP5 treatment

Genes differentially expressed between groups were categorized according to Gene Ontology (GO) (Ashburner et al. 2000). In the cellular component ontology, genes associated with alpha–beta T cell receptor complex (GO:0042105) generally demonstrated activated expression in BP5-treated mouse-derived hybridoma genes samples (Table 4). However, Ms4a2 and Fcer1g genes were involved in Fc receptor complex (GO:0032997), and Fc-epsilon receptor I complex (GO:0032998) generally demonstrated reduced expression exposed to BP5 (Table 4).

In the molecular function ontology, except for Ms4a2 and Fcer1g genes, Fcer1a gene related to immunoglobulin receptor activity (GO:0019763), IgE receptor activity (GO:0019767), and IgE binding (GO:0019863) were up-regulated expressed in BP5-treated hybridoma cell (Table 4). BP5-treated hybridoma also showed up-regulated expression of T cell receptor binding (GO:0042608), which include Fyn(T) [L-fyn(T)] and Adducin 1 (alpha) (Add1) (Table 4).

Pathway analysis of gene expression profiles in BP5-treated hybridoma cells

Given the fact that BP5 has a broad range of effects on many cell types, it is conceivable that there are multiple transcriptional changes caused by BP5 involved in various

Table 3 Genes involved in cytokines production involved in immune response by BP5 treatment

Ontology	*P* value	Gene	Gene symbol	Regulation change	Genbank accession	Description
Cytokine production involved in immune response						
Biological process	0.01636	+2/−2	Hmox1	+2.447585	NM_010442	Heme oxygenase (decycling) 1
			Gimap5	+2.16353	AK087160	Hypothetical protein
			Fcer1g	−1.64746	NM_010185	Fc receptor, IgE, high affinity I, gamma polypeptide
			Cd36	−1.52109	NM_007643	CD36 antigen

To identify the genes that are differentially expressed, we performed a fold-change screening between the two groups obtained from the experiment. The threshold we used to screen up (+) or down (−) regulated genes is fold change ≥ 1.5

Table 4 Molecular function and cellular component of TCR, Fc receptor and IgE receptor by BP5 treatment

Term	Ontology	*P* value	Gene symbol	Regulated change	Genbank accession	Description
alpha–beta T cell receptor complex	Cellular component	0.0373	Ptpn6	+1.7611192	NM_001077705	Protein tyrosine phosphatase, non-receptor type 6
			Cd3d	+1.6991402	NM_013487	CD3 antigen, delta polypeptide
			Cd247	+1.7576624	NM_001113391	CD247 antigen (Cd247)
Fc receptor complex	Cellular component	0.01324	Ms4a2	−7.1796646	NM_013516	Membrane-spanning 4-domains, subfamily A, member 2
			Fcer1g	−1.6474578	NM_010185	Fc receptor, IgE, high affinity I, gamma polypeptide
Immunoglobulin receptor activity	Molecular function	0.0128	Ms4a2	−7.1796646	NM_013516	Membrane-spanning 4-domains, subfamily A, member 2
			Fcer1g	−1.6474578	NM_010185	Fc receptor, IgE, high affinity I, gamma polypeptide
			Fcer1a	+1.9160347	NM_010184	Fc receptor, IgE, high affinity I, alpha polypeptide
T cell receptor binding	Molecular function	0.0133	Fyn	+2.0808187	U70324	Fyn(T) (L-fyn(T)
			Add1	+1.5538745	NM_013457	Adducin 1 (alpha)

The biological pathways affected by BP5 in hybridoma cells were examined by GO categories functional analysis. This function computes a *P* value to test the hypothesis that the selected genes in a pathway tend to be differentially expressed (*P* value < 0.05). To identify the genes that are differentially expressed, we performed a fold-change screening between the two groups obtained from the experiment. The threshold we used to screen up (+) or down (−) regulated genes is fold change ≥ 1.5

signal pathways. The GeneSetTest function was used to investigate which biological pathways could be regulated in hybridoma cells in response to treatment with 2 μg/mL of BP5. In this study, pathways with *P* values <0.05 in all BP5 treatment groups were considered differentially regulated. The pathway analysis revealed that 26 pathways were regulated following all BP5 treatments (Table S6). Most of the BP5-regulated pathways were associated with immunomodulatory functions, indicating that BP5 might participate in the regulation of immune genes.

Evaluation of gene expression changes by qRT-PCR

Twelve regulated genes (down-regulated: CCR3, FGF8, MS4A2, FCER1G, and CDKN2A; up-regulated: CD3D, FGF21, CD80, PTPRC, Fas, CBLC and Fyn) were chosen for qRT-PCR verification. The expression levels of regulated genes that were validated by qRTPCR showed correlations in the general trends for the microarray analysis (Fig. 2).

Genes altered in the p53 signaling pathway

Activation of p53 can induce several functional responses in cells, including cell cycle arrest and apoptotic cell death (Bates and Vousden 1999). These two responses allow p53 to inhibit the growth of stressed cells either by a cycle arrest, which may be irreversible or transient to allow repair and recovery before further rounds of replication, or by permanent removal of these cells from the organism by apoptosis. Either response would prevent replication of cells undergoing oncogenic changes and so would inhibit tumor development.

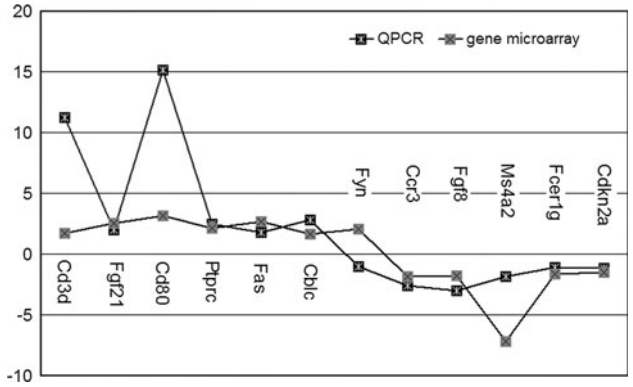

Fig. 2 Evaluation of gene expression changes by qRT-PCR. Eight regulated genes (down-regulated: CCR3, FGF8, MS4A2, FCER1G, and CDKN2A; up-regulated: CD3D, FGF21, CD80, PTPRC, Fas, CBLC and Fyn) were chosen for qRT-PCR verification

In this paper, we found that genes involved in cell cycle, cell arrest and cell apoptotic were regulated in p53 signaling pathway of BP5-treated hybridoma (Table 5). We found that BP5 exposure repressed cyclin B1 (Ccnb1), cyclin B2 (Ccnb2), cyclin D2 (Ccnd2), and cyclin E1 (Ccne1), whereas BP5 treatment induced cyclin G1 (Ccng1) and cyclin G2 (Ccng2) gene expression. Also, we found that BP5 treatment up-regulated cyclin-dependent kinase inhibitor 1A (P21) (Cdkn1a) gene, whereas down-regulated cyclin-dependent kinase inhibitor 2A (Cdkn2a). Also, there were three growth arrest and DNA-damage-inducible genes up-regulation, such as 45 alpha (Gadd45a), 45 beta (Gadd45b), and 45 gamma (Gadd45g). However, leucine-rich and death domain containing (Lrdd) was down-regulated. Another family of effector of apoptotic signaling genes, such as apoptotic peptidase activating factor 1 (Apaf1) and PERP, TP53 apoptosis effector (Perp), which activate signaling through various feedback mechanisms, was markedly induced in BP5-treated hybridoma (Table 5).

Potential functions on p53 expression

In order to analyze the transcriptional function of p53, we transfected a p53-Luc reporter plasmid (p53-Luc) that contains 14 tandem repeats of the p53 consensus binding sites in Vero cells that were BP5-treated 24 h after transfection, and the results clearly revealed that luciferase activation folds were observed apparently higher in BP5-treated transfected Vero cell than in non-treated transfected Vero cell in dose-dependent manner (Fig. 3a), indicating that BP5 is able to induce the transcriptional activation of p53. In contrast, luciferase levels were no clearly increasing in non-transfected Vero cells. BP5 at 2 μg/mL resulted in a significant enhancement of the luciferase activity compared with the non-treatment, whereas the enhanced luciferase activity was significantly reduced by 20 μM α-pifithrin, especial inhibitor of p53 pathway (Fig. 3b). Remarkable change of p53 in protein level was observed in all BP5-treated Vero cells (Fig. 3a). These results suggested that BP5 activated the transcriptional activity of p53.

As previously mentioned, regulation of the apoptotic function of p53 is associated with selective activation of apoptotic target genes. p53 functions primarily as a transcriptional factor that triggers cell cycle arrest or apoptosis by inducing an increasing number of proteins such as Bax (Miyashita and Reed 1995). To further explore the significance of p53 activation, p53-inducing proapoptotic gene, bax protein expression was analyzed. Western blotting analysis revealed dose-dependent up-regulation of p53 and Bax proteins in BP5-treated Vero cells (Fig. 3c). Taken together, these data confirm that BP5 treatment causes p53 activation and Bax induction in Vero cell.

P53 protein is one of the most important tumor suppressor proteins, as its gene is mutated or pathway inactivated, in nearly all human cancers (Boehme and Blattner 2009). We investigated the antiproliferation on tumor cell, which tested using a MTT assay. In this paper, we chose the MCF-7 and Hela cell as tumor cell model. The results illustrated that tumor cells cultured with different concentrations of BP5 displayed a decreased cell proliferation rate in dose-dependent manner. It was clear that BP5 of 20, 2, and 0.2 μg/mL inhibited DNA synthesis at less incorporation of 24.79, 12.40 and 1.795 % for MCF-7, respectively (Fig. 3d). In Hela cell, the values were significantly less than the control groups ($P < 0.001$) (Fig. 3). The values with BP5 were 31.79, 18.40 and 9.90 % for 20, 2, and 0.2 μg/mL, respectively. Cells with BP5 at 0.02 and 0.002 μg/mL have identical viability value to non-treated tumor cell with no significant difference ($P \geq 0.05$) in both MCF-7 and Hela cell.

Discussion

Regulatory peptides are components of a complex system responsible for biological regulation. Microarray analysis has become a popular and useful tool to study the effects of agents on gene expression in cells, tissues, and organs; however, few studies have applied DNA microarray analysis to investigate the immunostimulative function of bursal-derived bioactive peptide. In this in vitro study on the gene expression profiles of BP5-treated (hybridoma) cells, the mouse-derived hybridoma cell line was used, which shares characteristics of B lymphocyte secreting antibody (Galfre and Milstein 1981).

Here, we reported characterization of the hybridoma cell transcriptional profile of genes influenced by BP5

Table 5 Genes altered in the p53 signaling pathway by BP5 treatment

Gene symbol	Regulated change	Genbank accession	Description
Apaf1	+1.673021	NM_001042558	Apoptotic peptidase activating factor 1
Atr	+1.840748	NM_019864	Ataxia telangiectasia and Rad3 related
Bbc3	+2.251081	NM_133234	BCL2 binding component 3
Casp3	+1.708415	U49929	ICE-like cysteine protease (Lice)
Casp9	+1.659161	NM_015733	Caspase 9
Ccnb1	−1.78114	NM_172301	Cyclin B1
Ccnb2	−1.66763	NM_007630	Cyclin B2
Ccnd2	−1.51729	NM_009829	Cyclin D2
Ccne1	−1.54143	NM_007633	Cyclin E1
Ccng1	+1.560522	NM_009831	Cyclin G1
Ccng2	+1.545857	NM_007635	Cyclin G2
Cdkn1a	+2.405733	NM_007669	Cyclin-dependent kinase inhibitor 1A (P21)
Cdkn2a	−1.50403	NM_009877	Cyclin-dependent kinase inhibitor 2A
Ei24	+1.619767	NM_007915	Etoposide induced 2.4
Fas	+2.66967	NM_007987	Fas (TNF receptor superfamily member 6)
Gadd45a	+1.685429	NM_007836	Growth arrest and DNA-damage-inducible 45 alpha
Gadd45b	+1.615533	NM_008655	Growth arrest and DNA-damage-inducible 45 beta
Gadd45 g	+1.575556	NM_011817	Growth arrest and DNA-damage-inducible 45 gamma
Lrdd	−2.29311	NM_022654	Leucine-rich and death domain containing
Mdm4	+1.921434	NM_008575	Transformed mouse 3T3 cell double minute 4
Perp	+2.293525	NM_022032	PERP, TP53 apoptosis effector
Rrm2	−1.75346	AK088907	Ribonucleotide reductase M2
Sesn2	+1.966706	AK170547	Sestrin 2
Sesn3	+1.831066	NM_030261	Sestrin 3
Siah1a	+1.793212	NM_009172	Seven in absentia 1A
Trp73	−1.52286	NM_011642	Transformation related protein 73

To identify the genes that are differentially expressed, we performed a fold-change screening between the two groups obtained from the experiment. The threshold we used to screen up (+) or down (−) regulated genes is fold change ≥ 1.5

exposure. It was recently reported that BP5 was a multi-functional biological active factor, which could induce antibody production and Th1 and Th2 type immune responses, and enhanced T cell subsets, and lymphocyte proliferation (Li et al. 2010). Our results showed that hybridoma cells were sensitive to BP5-induced antibody production and cell proliferation in reachable concentration of BP5-treated hybridoma cells, compared with that of control without BP5 (Fig. 1). The microarray analysis showed that there were significantly altered genes and biological processes in the hybridoma cell after the BP5 treatment. These will only be selectively discussed here.

Due to a bioactive peptide from avian central humoral immune system, we first discussed the cellular level gene expression of hybridoma cell in B cell and immunity after BP5 treatment. It was shown that transcripts of genes related to biological processes involving B cell activation, humoral immune and regulation function were generally abundant in BP5-treated hybridoma cell (Tables 1, S4).

Signal transducer and activator of transcription 5 (STAT5) plays an important role in a variety of cellular processes, including proliferation, differentiation, and apoptosis (Buitenhuis et al. 2004). Expression of STAT5B and STAT5A involved in B cell activation and differentiation was elevated in BP5-treated hybridoma cell, which are two highly homologous STAT5 isoforms, and are predominantly observed in immature myeloid cells (Buitenhuis et al. 2004), suggesting a potential role of BP5 on regulation of myeloid differentiation. It was reported that STAT5 prevents premature Igk recombination in pro-B cells (Malin et al. 2010), which suggested that BP5 might play role on B cell development and in maturation.

B cells receive various signals in the germinal center, provided by different receptors, such as the B cell receptor (BCR), Fc and complement receptors, or the receptors binding T cell derived, cell bound or soluble products (CD40L, IL4) (Bernasconi et al. 2002; Crotty and Ahmed 2004). The generation of highly diverse antibodies with

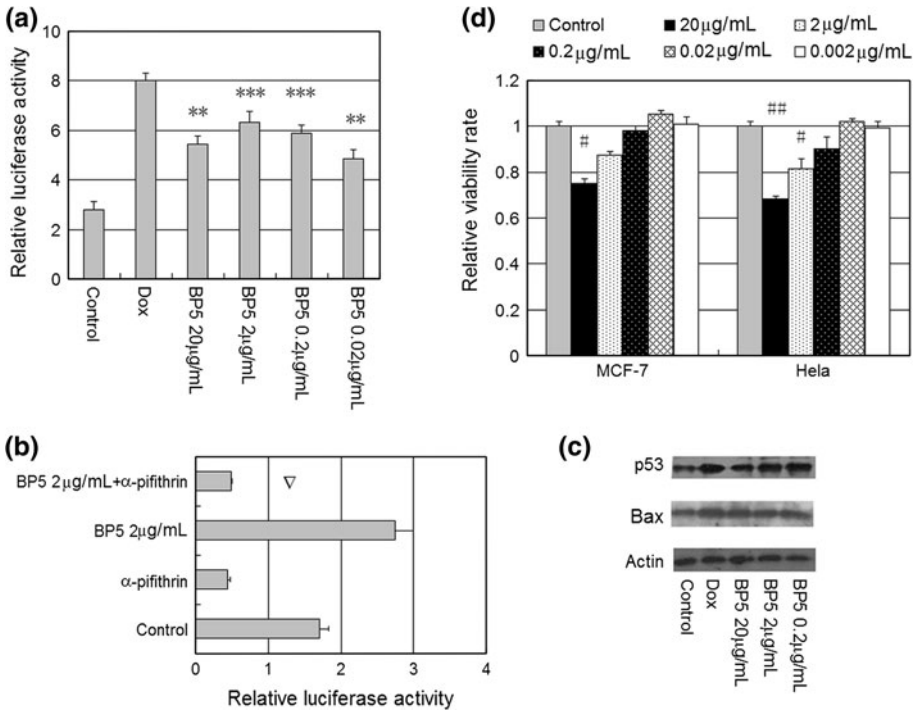

Fig. 3 Potential functions on p53 expression and antiproliferation on tumor cell. **a** Inducing effect of BP5 on p53 transcriptional activity. Vero cells were transiently transfected with p53-Luc and incubated for 24 h. The luciferase activity of lysates prepared from the transfectants was analyzed. **b** Inhibition on BP5-stimulated p53 transcriptional activity by α-pifithrin. Transfected Vero cells were treated with α-pifithrin 20 μM for 2 h before BP5 treatment. After 22 h, p53 luciferase activity was measured. **c** Induction of p53 and bax protein expression by BP5. Vero cells were treated with BP5 at different concentrations for 24 h. Equal amounts of total cell lysates were collected and protein expression was detected by Western blot analysis using the indicated antibodies. Actin was included as an internal loading control. Dosages of BP5 treatment are indicated in the below *lanes*. Vero cells untreated with BP5 were used as negative control, and Dox used as positive control. **d** Antiproliferation of BP5 on tumor cells. Target tumor cell MCF-7 and Hela were treated with BP5 at different concentrations for 48 h, and cell relative viability activity was determined in presence of BP5 treatment or absent. Results are presented as the mean ± SD from three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$, compared to transfected cells without BP5 treatment. $\nabla P < 0.001$, compared to transfected cells with 2 μg/mL BP5. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared to control cells without BP5 treatment

high affinity is a key element of acquired immunity. Fcer1g, which present equal to complement as components of the humoral arm of innate immunity (Moalli et al. 2010; Blink and Fu 2010), were down-regulated in BP5-treated hybridoma, whereas alpha polypeptide (Fcer1a) found on the surface of mast cells and basophiles, which mediates allergic diseases, anaphylaxis and asthma through binding of IgE (Sandomenico et al. 2009), was up-regulated. We found that various affinity IgE receptors were involved in Fc receptor complex (cellular component), and immunoglobulin receptor activity (molecular function), including of down-regulated MS4A2 and FCER1G gene, and up-regulated FCER1A gene. Therefore, we thought that IgE receptor-related genes might be the down-stream after BP5 stimulus.

It has been reported that tumor necrosis factor (TNF) and lymphotoxin-alpha (LT-alpha) are members of a family of secreted and cell surface cytokines that participate in the regulation of immune and inflammatory responses (Crowe et al. 1994). One member of TNF superfamily, BAFF, the B cell-activating factor, could stimulate human B cell proliferation and immunoglobulin production in vitro upon BCR cross-linking (Schneider et al. 1999). In this paper, we found that TNF and LTA were up-regulated by BP5 treatment. Signal transduction events depend on the subtle balance between protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPase) which, in concert, control phosphorylation and dephosphorylation events (Sasaki et al. 2001; Hermiston et al. 2003). Protein-tyrosine-phosphatase receptor type C (PTPRC), also designated as the transmembrane glycoprotein CD45, is expressed by all leukocytes, whose intracellular domain exhibits phosphatase activity that takes part in different kinds of signal transduction pathways of the immune system (Sasaki et al. 2001; Hermiston et al. 2003). In this paper, we found PTPRC up-regulation by BP5 treatment. CD45 is a critical positive regulator of T cell receptor (TCR) and B cell receptor (BCR)-mediated signaling required for the activation and development of lymphocytes (Kishihara et al. 1993). It was reported that

lymphotoxin might play physiological role for IgE in the regulation of T helper cell differentiation (Byth et al. 1996). Fcer1g activation is important for the induction of a T cell dependent, systemic autoimmune condition (HgIA) by inorganic mercury (Martinsson et al. 2010). Also, it has been reported that CD45 function as a hematopoietic JAK phosphatase which negatively regulates cytokine receptor signaling (Irie-Sasaki et al. 2001). In immune system, humoral immune, cellular immune and cytokines responses are mutually correlated. These indicated that BP5 could induce various B cell-mediated immune-related cellular processes, which correlate with cellular immunity and cytokines responses.

The mechanisms whereby BP5 effects converge to regulate T cell signaling are not clear. Based on our gene expression profiling, it appears that BP5 has affected hundreds of genes that involved in various biological processes of T cell proliferation, differentiation, activation and related immunity. To consider how bursal-derived peptide signaling converges on T cell processes, we will briefly consider the roles of various genes and their possible roles in regulation of T cell-related biological processes.

T cell development and differentiation is carefully orchestrated by a series of cytokines (Wei et al. 2009). The balance of Treg and Th17 cells is thought to be critical in maintaining immune tolerance, while preserving effective host defense. Stat5a/b are emerging to be key players in T cell differentiation and homeostasis, which are indispensable in T regulatory (Treg) cell development and maintenance, and negatively regulate T helper 17 (Th17) cell differentiation (Wei et al. 2009). In hematopoietic cells, Stat5 has been demonstrated to play a critical role in regulating apoptosis (Socolovsky et al. 1999), and in promoting proliferation and cell cycle progression (Nosaka et al. 1999). The role of STATs in novel aspects of T cell function and T cell subsets continues to accumulate.

The TNF ligand and receptor families represent a paradigm of cytokine and signaling network, and their biological function covers the regulation of various immune system, such as innate and adaptive immunity, the induction of apoptosis, and the reversible formation of secondary lymph organs (Eissner et al. 2004). TNFRSF14, a receptor expressed by T lymphocytes, is naturally expressed on immature dendritic cells (DCs) and activated T cells (Mauri et al. 1998; Tamada et al. 2000). Our observed up-regulation of TNFRSF13B and TNFRSF14 was involved in various biological processes, including T cell activation, proliferation and regulation, and up-regulated TNF or TNFRSF13B was involved in many B cell activation, B cell-mediated immunity, and regulation, suggests that BP5 treatment changed the expression of TNF superfamily, which, in turn, plays a up-regulatory role in T cell and B cell signaling.

Cytokines are highly regulated for their functions during normal physiological conditions. We found that up-regulation of IL-20RB was involved in negative regulation of T cell-mediated immunity. IL20RB is one of receptor subunits for IL-20 which was identified as a new class II cytokine with high structural homology to IL-10 (Kotenko 2002; Wegenka 2010). CD8+ T cells, not CD4+ T cells, could serve as target cells for IL-20, which up-regulated the transcripts of KGF in these cells (Wei et al. 2005). In this paper, it was observed that after BP5 treatment, up-regulated genes HMOX1, also as haem oxygenase-1 (HO-1), which encode an anti-oxidative stress protein (Chen et al. 2010a, b) and down-regulated FCER1G and CD36 were involved in cytokine production involved in immune response (GO:0002367) and regulation (GO:0002718), suggesting a potential mechanism of BP5 on cytokine and T cell responses.

Key steps during cellular immune response are T cell activation and proliferation. It has been reported that ICOS ligand (ICOSL) plays an important role in controlling specific aspects of T cell activation, differentiation, and function, including immunoregulation and protective immunity (Kadkhoda et al. 2010). Except for broadly distributed on various cell types, ICOS-L is the ligand of the TNF receptor-related co-stimulators (Khayyamian et al. 2002), and costimulates Th1 and Th2 cytokine secretion by memory CD4+ T cells. Also, we found that there were a variety of genes such as FYN, ADD1, PTPN6, CD3D, CD247, to take part in T cell-related process, T cell receptor complex and binding activity, T cell activation, differentiation and proliferation. It was reported that TCR stimulation (with or without CD4 or CD8 co-receptors) results in the activation of Fyn. In the thymus, Fyn acts as a tyrosine kinase that transduces the leptin signal independently of JAK2 activation, and mediates some of the immunomodulatory effects of leptin in this tissue (Girasol et al. 2009; Hermiston et al. 2002). Current models for signal transduction in lymphocytes envision a dynamic equilibrium between membrane-proximal PTPase and PTKs that serve to maintain cells in a resting state (Roose et al. 2003). These suggests that BP5 treatment changed the expression of genes involved alpha–beta T cell receptor complex and T cell receptor binding, which, in turn, plays a regulatory role in TCR stimulation signaling (Tables 2, S5).

In the immune system, signal transduction pathways are functionally important for the appropriate development of properly selected T and B lymphocytes as well as in controlling responses to antigen by more mature cells (Crotty and Ahmed 2004). The pathway analysis showed that most of the BP5-regulated pathways were associated with

immunomodulatory functions, indicating that BP5 might participate in the regulation of immune genes.

Pathway analysis is a functional analysis mapping genes to KEGG pathways. The *P* value (Fisher *P* value) denotes the significance of the pathway correlated with the conditions. Less the *P* value, more significant is the pathway. Among these pathways of hybridoma cell after BP5 treatment, p53 is a sequence-specific nuclear transcription factor that can mediate many of its downstream effects by the activation or repression of target genes (Laptenko and Prives 2006; Bai and Zhu 2006). Activation of p53 could induce various cellular responses, such as differentiation, senescence, DNA repair and the inhibition of angiogenesis, and well-known cell cycle arrest and apoptotic cell death (Bates and Vousden 1999). To prevent replication of cells undergoing oncogenic changes and so would inhibit tumor development, cell cycle arrest was irreversible or transient to allow repair and recovery before further rounds of replication, which allow p53 to inhibit the growth of stressed cells. Also, some stressed cells were permanently removed from the organism by apoptotic cell death (Bates and Vousden 1999).

Among these differentially expressed genes after 2 μg/mL BP5 treatment (Table 5), six gene-related cell cycle was regulated, including the down-regulation genes of cyclin B1 (Ccnb1), cyclin B2 (Ccnb2), cyclin D2 (Ccnd2), and cyclin E1 (Ccne1), and up-regulation genes of cyclin G1 (Ccng1) and cyclin G2 (Ccng2). Also, we found that BP5 treatment up-regulated cyclin-dependent kinase inhibitor 1A (P21) (Cdkn1a) gene, whereas down-regulated cyclin-dependent kinase inhibitor 2A (Cdkn2a). Cell cycle inhibitors (CKIs) are subject to precise topological control, and escape from this regulation may be a critical feature of neoplastic transformation (el-Deiry et al. 1995), which suggested that CKIs are therefore potential mediators of developmental control of cell proliferation. It is reported that the p53-regulated gene product p21WAF1/CIP1 is the prototype of a family of small proteins that negatively regulate the cell cycle (Owen et al. 1998), which is transcriptionally regulated by p53 and can induce growth arrest (Parker et al. 1995). Furthermore, three growth arrest and DNA-damage-inducible genes, such as 45 alpha (Gadd45a), 45 beta (Gadd45b), and 45 gamma (Gadd45g) which were involved in cell cycle that is related to the regulation of cell growth, were also shown to be up-regulated following BP5 treatment in this study. Therefore, we thought that the cell cycle and growth arrest responses might be BP5-stimulated target processes in p53 signal pathway.

Apoptosis is one of the downstream cellular responses of p53 pathway (Bates and Vousden 1999). In this paper, we found differential expressions of various gene involved in apoptotic were regulated by BP5 (Table 5). A down-regulated gene identified in this study was leucine-rich and death domain containing (Lrdd). The p53-inducible and death domain-containing PIDD/LRDD protein could activate the initiator caspase-2, and p53-mediated apoptosis by forming large protein complexes with RAIDD (Pick et al. 2006). Another family of effector of apoptotic signaling genes, such as apoptotic peptidase activating factor 1 (Apaf1) and PERP, TP53 apoptosis effector (Perp), which activate signaling through various mechanisms, were markedly activated in BP5-treated hybridoma. It was reported that Apaf1, an important component of the intrinsic apoptotic pathway, played a central role in the common events of mitochondria-dependent apoptosis in most death pathways which is critical for normal development (Yoshida et al. 1998). PERP, TP53 apoptosis effector, is a direct p53 target gene, and selectively mediates the p53 apoptotic response (Ihrie et al. 2003). We speculated that the regulation of p53 regulated genes by BP5 may partly explain the antiproliferation of bursal-derived BP5 on tumor cell.

To further understand the role of BP5 on p53, we investigated the p53 transcriptional function during the BP5 treatment. To achieve this, we transfected a plasmid, containing the luciferase reporter gene cloned under the control of 14 p53-binding DNA sequences, into Vero cells. Luciferase activity increase during the BP5 treatment indicated that BP5 is able to induce the transcriptional activation of p53, which was significantly reduced by 20 μM α-pifithrin, an especial inhibitor of p53 pathway. As previously mentioned, regulation of the apoptotic function of p53 is associated with selective activation of apoptotic target genes. P53 functions primarily as a transcriptional factor that triggers cell cycle arrest or apoptosis by inducing an increasing number of proteins such as Bax (Miyashita and Reed 1995). For this reason, WB analysis of the presence of this protein in BP5-treated and non-treated cell extracts was performed. Using this approach, we detected specific increase of the expression of the proteins Bax after BP5 treatment (Fig. 3). Concurrently, our study showed that BP5 significantly restrained tumor cell proliferation in MCF-7 and Hela cells, compared with the control cells. Therefore, we thought that BP5 might play antiproliferation on tumor cell by activating p53 expression, leading to cell cycle arrest, or apoptotic activation, resulting in tumor cell proliferation activity decrease.

The tumor-immune system competitive interaction is very complex, and the cellular components of the innate immune system integrate into an antitumor effector response (Dònofrio 2008). Immunotherapeutic strategies that mobilize the innate and adaptive immune responses may well prove to be a powerful integrative approach toward antitumor therapy (Soloski 2001). In this paper, we

also found that various genes involved in the regulation of G-protein-coupled receptor protein signaling pathway and activation of protein kinase C activity by G-protein-coupled receptor protein signaling pathway were regulated in hybridoma cell after BP5 treatment (the results not shown). Chemokines are small secreted proteins which bind to chemokine receptors that are coupled to G proteins of the Gi type. In the immune system, chemokines exert vital homeostatic functions, thus influencing inflammation and adaptive immune responses (Butcher and Picker 1996; Muller and Lipp 2003). It has also been realized that tumor cell migration and growth is dependent on direct chemokine signals to tumor cells (Krieg and Boyman 2009). It has been proved that B cells play role on antitumor cellular responses against a lung metastatic tumor, which provide evidences of the importance of B cell responses in tumor defenses (Jones et al. 2008). BF is the central humoral immune organ responsible for B cell difference (Davison et al. 2008). In this paper, we found that bursal-derived bioactive factor BP5 could regulate various genes involved in antitumor p53 signal pathway. Therefore, we thought that these results are important in the context that various cellular responses against tumor cell proliferation could be enhanced after BP5 treatment either directly by initiating an antitumor effector response or indirectly through the instructive role that the innate immune system has on the adaptive response.

Data from multiple epidemiological and clinical studies on the immune effects suggest that BF is the central humoral immune organ unique to birds, which has a critical role in immune system (Davison et al. 2008). In the present work, we demonstrated that treatment with bursal-derived bioactive peptide BP5 resulted in an increase of both the antibody production and the hybridoma cell proliferation activities. It was proved that BP5 could regulate gene expression profiles related to various immune effector cell biological processes, such as T cell activation and proliferation, B cell activation, B cell-mediated immunity, and cytokines related response. In addition, various signal pathways associated with immunomodulatory functions were regulated in BP5-treated hybridoma cells, and p53 pathway played an important role in antitumor. The activation of p53 activity by BP5 was further confirmed by p53 luciferase reporter assay, p53 expression, and antiproliferation on tumor cell. Information generated from these studies may be the basis at transcriptional level for future experiments and may be highly relevant for the development of novel therapies for the treatment of immune and antitumor diseases.

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Conflict of interest The authors declared no conflict of interest.

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