

## Original Article

# Effect of anti-human IgM antibody on the proliferation, apoptosis and cell cycle of Hep-2 laryngeal squamous cell carcinoma cells and potential mechanisms underlying its antitumor activity

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**Abstract:** To investigate the expression of IgM in laryngeal cancer and the effect of anti-human IgM antibody on the proliferation, apoptosis and cell cycle of Hep-2 human LSCC cells, explore the potential mechanisms underlying its antitumor activity. The expression of IgM protein in Hep-2 cells was examined. The effects of anti-human IgM antibody on cell proliferation, apoptosis and cell cycle were explored. Nude mouse model of Hep-2 tumor xenografts were established, the expression levels of IgM and gp96 and the apoptosis rate of Hep-2 cells in xenograft tumors were examined. IgM protein was expressed in Hep-2 cells. The anti-human IgM antibody group produced a significantly decreased number of colonies than the control groups. The inhibitory effect of anti-human IgM antibody on cell proliferation was concentration- and time-dependent. The anti-human IgM antibody group had a higher apoptotic rate than the control groups *in vitro* and *in vivo*. The G1-phase and S-phase cells of anti-human IgM antibody group accounted for  $51.37 \pm 2.49\%$  and  $38.20 \pm 1.74\%$ , respectively, was different from the control groups. Tumor volume was smaller in anti-human IgM antibody group than the control groups. The IgM and gp96 expression of anti-human IgM antibody group were statistically significantly reduced from the control groups *in vivo*. IgM expressed in Hep-2 human LSCC cells and anti-human IgM antibody treatment can inhibit Hep-2 cells and xenograft tumors proliferation, promote apoptosis and arrest cell cycle to S phase. Anti-human IgM antibodies are likely to become a part of the comprehensive treatment for LSCC in the future.

**Keywords:** Laryngeal squamous cell carcinoma, immunoglobulin M, proliferation, apoptosis, cell cycle

## Introduction

Worldwide, there are approximately 600,000 new cases of head and neck squamous cell carcinoma (HNSCC) diagnosed each year [1], with a high incidence of these cancers occurring in Asia [2]. Laryngeal cancers, the vast majority of which are squamous cell carcinomas (SCC), account for a proportion of head and neck cancers. In clinical practice, a comprehensive multidisciplinary approach is used to treat laryngeal cancer. Patients with laryngeal cancer generally receive surgery as the primary treatment, which is supplemented with radiochemotherapy [3, 4]. However, the efficacy of this comprehensive treatment is far from satisfactory. In particular, the 5-year survival rate in patients who suffer advanced laryngeal cancer with distant metastasis is only 40-

60% [5-7]. Therefore, a comprehensive analysis of the molecular mechanisms of laryngeal cancer and the identification of novel antitumor targets are of great clinical significance for effectively controlling the development of laryngeal cancer and improving its survival rate.

Immunoglobulins (Igs) are important molecules of the immune system. Classical immunological theory states that Igs are only expressed during B lymphocyte development and that other types of cells generally do not express Igs. Contradictory to this theory, recent studies have found that Igs (including IgA, IgG and IgM) are abnormally expressed in various malignant tumors of epithelial origin. Zheng et al. found that the IgA heavy chain is expressed in tumors of epithelial origin and that IgA promotes tumor growth [8]. Liang et al. found

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that IgG is expressed in bladder cancer cell lines and that anti-IgG therapy promotes apoptosis of bladder cancer cells [9]. In addition, IgG promotes tumor growth by inhibiting apoptosis of pancreatic cancer cells [10]. Similarly, Hu et al. have found that IgM is expressed in various tumors of epithelial origin and may be involved in tumor immunity and biological changes in the tumors [11]. Furthermore, IgM complex levels are significantly elevated in liver [12], prostate [13] and ovarian [14] cancers. We previously showed that IgM is expressed in laryngeal squamous cell carcinoma (LSCC) tissues and that IgM expression is correlated with the prognosis of LSCC patients [15]. However, the effects of anti-human IgM antibody on the proliferation, apoptosis and cell cycle of LSCC cells and the antitumor mechanisms of anti-human IgM antibodies have not been reported.

The present study examines the expression of IgM protein in Hep-2 LSCC cells, investigates the effects of anti-human IgM antibody on the proliferation, apoptosis and cell cycle of Hep-2 cells *in vitro* and on the tumorigenesis of these cells in nude mice, and preliminarily explores the antitumor mechanisms of an anti-human IgM antibody, thereby providing the experimental and theoretical basis for applying anti-human IgM antibodies in the prevention and treatment of LSCC.

### Materials and methods

#### Cell lines

The human LSCC cell line Hep-2 and the human Burkitt's lymphoma cell line Raji were provided by the Laboratory of Tumor Biotherapy, State Key Laboratory of Biotherapy, West China Hospital of Sichuan University.

#### Experimental materials

Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS) and trypsin were purchased from Gibco (USA). Goat anti-human IgM polyclonal antibody and purified goat IgG were purchased from Sigma-Aldrich Corporation (USA). The ready-to-use anti-goat IgG StreptAvidin-Biotin Complex (SABC) Kit was obtained from Wuhan Boster Biological Engineering Co., Ltd. Fluorescein isothiocyanate (FITC)-labeled swine anti-goat secondary antibody was purchased from Dako (USA). Protein standards were purchased from MBI Fermentas (USA). Polyvinylidene difluoride (PV-

DF) membrane and Coomassie Brilliant Blue G250 were purchased from Bio-Rad Laboratories, Inc. (USA). The polyclonal rabbit anti-human gp96 antibody was obtained from Beijing Biosynthesis Biotechnology Co., Ltd. The streptavidin-peroxidase (SP) immunohistochemistry kit was purchased from Santa Cruz Biotechnology, Inc. (USA). The TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) Apoptosis Detection Kit was purchased from Roche (USA).

#### Cell culture

Hep-2 cells were routinely cultured and passaged in RPMI-1640 medium containing 10% FBS at 37°C in an environment with 5% CO<sub>2</sub> and saturated humidity. Logarithmically growing cells were used in all experiments.

#### Examination of the expression of IgM protein in Hep-2 cells using the SABC method and western blot analysis

Single-cell suspensions of Hep-2 cells were dropped evenly onto culture plates containing glass coverslips to seed cells on the coverslips. The IgM protein level was analyzed by SABC methods according to the kit instructions. Negative controls were established by replacing the primary antibody with phosphate-buffered saline (PBS). In addition, logarithmically growing Hep-2 cells were harvested. Cellular proteins were extracted from the cells, and the protein concentration was determined. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane. The membrane was blocked with 5% nonfat milk and incubated with primary antibody overnight. After being washed with PBS-Tween 20 (PBST) solution, the membrane was incubated with secondary antibody. The membrane was washed again, and the target protein was visualized.

#### Examination of the effect of anti-human IgM antibody on Hep-2 cell proliferation using the soft agar colony formation assay

RPMI-1640 medium containing 0.6% agar was prepared by mixing 1.2% agar with 2 × medium at a ratio of 1:1 and then added to 6-well culture plates (1 ml/well). The plates were cooled to room temperature (RT), allowing the agar to solidify and form the base layer. The following groups were prepared: the PBS group (20 µl/

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ml), the goat IgG group (20 µg/ml), and 3 goat anti-human IgM antibody groups with different concentrations of the goat antibody (5, 10, and 20 µg/ml) and the blank control group. Each group of cells was suspended in RPMI-1640 medium containing 0.3% agar and 20% FBS at a density of  $1 \times 10^3$  cells/ml. One milliliter of the cell solution was seeded on top of the base agar layer in each well. After the agar solidified, the cells were cultured for 2 weeks, and colony growth was monitored daily. Three replica wells were prepared for each group. At the end of the 2-week culture period, the number of colonies containing more than 50 cells was counted under a microscope.

### *Examination of the effect of anti-human IgM antibody on Hep-2 cell proliferation using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)-based assay*

Logarithmically growing Hep-2 cells were collected, seeded into 96-well plates at a density of  $2 \times 10^3$  cells/well and cultured under conventional culture conditions for 24 h. The experimental groups were treated with culture media (100 µl) containing 12.5, 25, 50, 100 or 200 µg/ml goat anti-human IgM antibody. The control groups were treated with culture media (100 µl) containing corresponding concentrations of goat IgG or corresponding volumes of PBS, while the blank control group was treated with 100 µl of culture medium. At 72 h post-treatment, 10 µl of MTS was added to each well, and the absorbance (A) values were measured at 490 nm using an enzyme immunoassay analyzer. Three replica wells were established for each group and the experiment was repeated 3 times. The mean optical density (OD) values were determined, and the inhibitory rates of cell growth were calculated (cell proliferation inhibitory rate % =  $(1 - \text{mean OD value of a treatment group} / \text{mean OD value of the blank control group}) \times 100\%$ ). In addition, concentration-inhibitory rate curves were constructed. Cells were seeded, cultured and grouped as described above. Three replica wells were prepared for each group. The cells were treated with 100 µl of RPMI-1640 medium supplemented with 2.5% FBS, 100 µl of the above FBS/RPMI-1640 medium containing a corresponding volume of PBS (100 µl/ml), 100 µl of the FBS/RPMI-1640 medium containing a corresponding concentration of goat IgG (100

µg/ml), or 100 µl of the FBS/RPMI-1640 medium containing goat anti-human IgM antibody (100 µg/ml). Subsequently, 10 µl of MTS was added to each well, and the A values at 490 nm were measured at days 1, 2, 3, 4 and 5 using an enzyme immunoassay analyzer. The experiment was repeated 3 times, and the mean A values were determined. In addition, cell proliferation inhibitory rates were calculated, and the concentration-inhibitory rate curves were plotted.

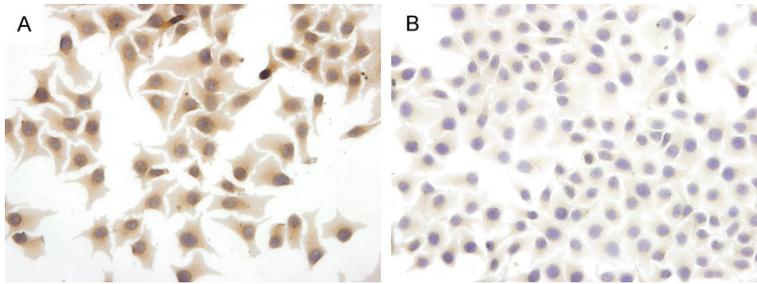
### *Analysis of Hep-2 cell apoptosis by Hoechst staining*

Logarithmically growing Hep-2 cells were collected, seeded onto 96-well plates at a density of  $2 \times 10^3$  cells/well and cultured under conventional culture conditions for 24 h. Culture media containing goat anti-human IgM antibody (final concentration: 100 µg/ml), goat IgG (final concentration: 100 µg/ml), or PBS (100 µl/ml) were added to the cells. The cells were then cultured for an additional 72 h. Subsequently, the cells were fixed in 0.5 ml of fixative solution for 10 min, washed twice with PBS and stained with 0.2 ml of Hoechst staining solution for 5 min. After washing the cells twice with PBS, a drop of antifade mounting medium was added to each well. The apoptosis of Hep-2 cells was examined under a fluorescence microscope, and the rates of apoptosis were calculated (apoptotic rate (%) =  $\frac{\text{the number of apoptotic cells}}{\text{total number of cells}} \times 100\%$ ).

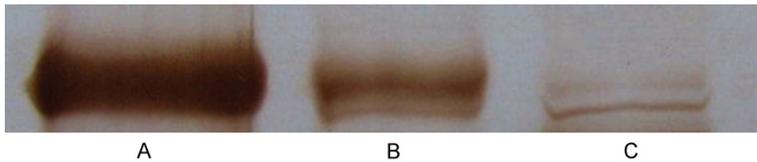
### *Cell cycle analysis*

Logarithmically growing Hep-2 cells were collected, seeded onto 6-well plates at a density of  $2 \times 10^5$  cells/ml and cultured under conventional culture conditions for 24 h. Culture media containing goat anti-human IgM antibody (final concentration: 200 µg/ml), goat IgG (final concentration: 200 µg/ml) or PBS (200 µl/ml) were added to the cells. Three replica wells were established for each group. After being cultured for an additional 72 h, the cells were dispersed into single cell suspensions ( $1-5 \times 10^6$  cells/ml) and centrifuged at 1500 r/min for 3 min. The culture media were discarded, and the cells were resuspended in 3 ml of PBS and fixed in pre-cooled 70% ethanol at 4°C overnight. Subsequently, 100 µl of RNase A was added to the cells. After incubation in a 37°C water bath for 30 min, the cells were mixed thorough-

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**Figure 1.** Immunocytochemical staining of IgM protein in Hep-2 cells ( $\times 200$ ). A: Negative control (replacement of the primary antibody with PBS); B: Cytoplasmic IgM-positive cells.



**Figure 2.** Examination of IgM expression in Hep-2 cells by western blot. A: Purified human IgM; B: Raji cells; C: Hep-2 cells.

ly with 400  $\mu$ l of propidium iodide (PI) staining solution and incubated for 30 min at 4°C in the dark. The cell concentration was then adjusted to  $5 \times 10^5$  cells/ml. Cell cycle analysis was performed using Multicycle software.

### *Tumor inoculation and treatment*

Logarithmically growing Hep-2 cells were collected, and single-cell suspensions ( $1 \times 10^8$  cells/ml) were prepared. The cell suspensions were inoculated subcutaneously into the right back of nude mice. Once the mean diameter of the subcutaneous tumors exceeded 5 mm (approximately 2 weeks after the inoculation), the nude mice were randomly divided into the IgM experimental group, the IgG control group and the PBS control group. Each group contained 5 mice. The IgM group, IgG group and PBS group were given intratumoral injections of anti-human IgM antibody, goat IgG and PBS, respectively. Each mouse received 5 injections (once every 3 days) at a total dose of 1.5 mg. During the injection period, the general conditions of the mice were monitored daily, including their vitality, behavior, food and water intake, and response to external stimuli. Tumor growth in the nude mice was also monitored. The long axis (L) and short axis (W) of each subcutaneous tumor were measured every 3 days using a Vernier caliper. Tumor vol-

umes were calculated (tumor volume (V) =  $(LW^2)/2$ ), and tumor growth curves were established. All experiments were performed in accordance with the appropriate institution or the National Research Council Guide for the care and use of laboratory animals.

### *Examination of the expression of IgM and gp96 proteins in tumor tissues using the immunohistochemical SP method*

The immunohistochemical assay was conducted according to the manufacturer's instructions. Positive protein expression was indicated by the presence of brownish-yellow precipitates in the cytoplasm.

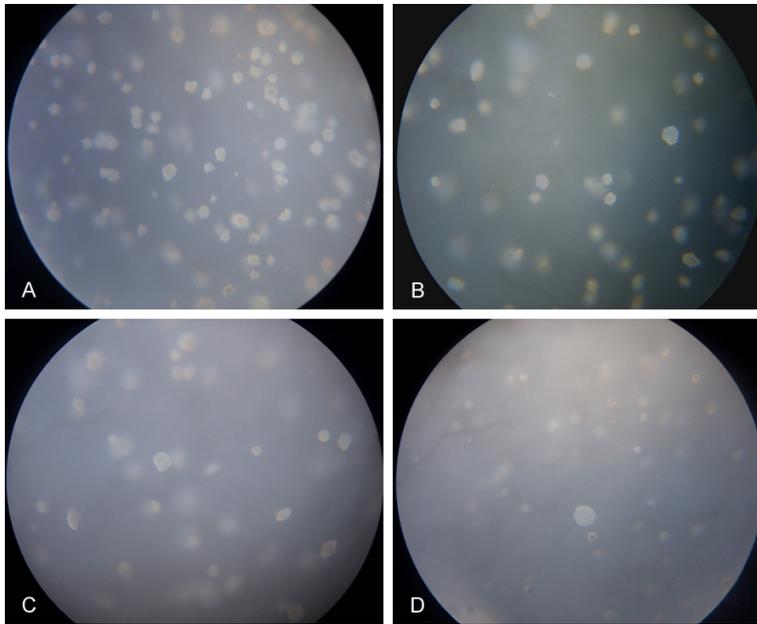
Five microscopic fields were randomly selected under 400  $\times$  magnification, and the cells positive for target protein expression were detected. The results were analyzed using Image Pro-Plus 6.0 software, and the mean OD values were calculated.

### *Examination of apoptosis in tumor tissues using TUNEL-alkaline phosphatase (AP)*

Tissue sections were first subjected to conventional deparaffinization procedures and then incubated with proteinase K at room temperature for 30 min. Subsequently, the sections were washed and counterstained with hematoxylin. TUNEL-positive cells were represented by bright-red or red nuclei. Using a double-blind procedure, a minimum of 1000 tumor cells were examined at 400  $\times$  magnification, and the number of TUNEL-positive cells were counted. The results were analyzed using Image Pro-Plus 6.0 software, and the apoptotic rates were calculated.

### *Statistical methods*

Experimental data were analyzed using SPSS18.0 software. All measurement data are expressed as the mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ). The results of the MTS assays were subjected to pairwise comparisons using two-way analysis of variance (ANOVA) and the Least



**Figure 3.** Colony formation of Hep-2 cells following 2-week treatment with anti-human IgM antibody or control treatment ( $\times 40$ ). A: The blank control group (Hep-2 cells that were cultured in RPMI-1640 medium containing 0.3% agar); B: The PBS control group (20  $\mu\text{l/ml}$ ); C: The goat IgG control group (20  $\mu\text{g/ml}$ ); D: The goat anti-human IgM antibody group (20  $\mu\text{g/ml}$ ).

Significant Difference (LSD) test. Differences between the groups were compared using univariate ANOVA.  $P$  values less than 0.05 indicate statistically significant differences.

## Results

### *Expression of IgM protein in Hep-2 human LSCC cells*

Immunocytochemical assays showed that IgM protein was expressed in Hep-2 cells, mainly in the cytoplasm (**Figure 1**). Western blot analysis of protein extracts from Raji cells and Hep-2 cells revealed a protein band with a molecular weight of 75 kD, indicating that the cells were positive for IgM expression (**Figure 2**).

### *The inhibitory effect of anti-human IgM antibody on Hep-2 cell proliferation is concentration- and time-dependent*

Single-cell suspensions of Hep-2 cells were seeded in RPMI-1640 medium containing 0.3% agar. Two weeks after seeding, a large number of visible colonies (cell clusters containing more than 50 cells) were formed. The experimental group was subjected to a 2-week intervention with goat anti-human IgM antibody. Compared

with the goat IgG group, the PBS group and the blank control group, the experimental group produced a significantly decreased number of colonies. In addition, the colonies derived from the experimental group appeared significantly smaller in size (**Figure 3**).

The results of the MTS assays were subjected to pairwise comparison using two-way ANOVA and the LSD test. Significant differences were detected between the goat anti-human IgM antibody group, the goat IgG control group and the PBS control group ( $P < 0.05$ ). In addition, different concentrations of anti-human IgM antibody produced statistically significant differences ( $P < 0.05$ ) (**Table 1**). Treatment of Hep-2 cells

with increasing concentrations of anti-human IgM antibody resulted in decreased OD values and increased inhibitory rates. These results demonstrate that the inhibitory effect of anti-human IgM antibody on Hep-2 cell proliferation is concentration-dependent (**Figure 4A**).

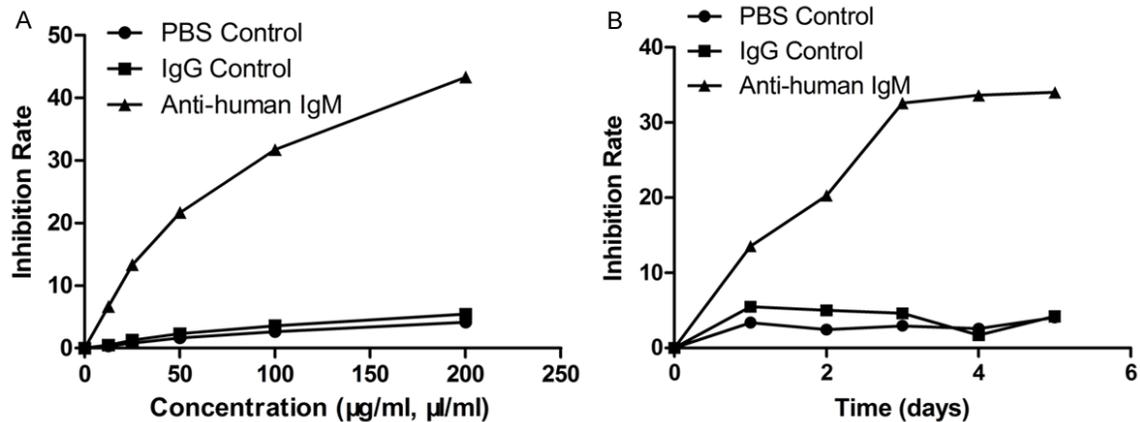
Because 100  $\mu\text{g/ml}$  of the goat anti-human IgM antibody significantly inhibited proliferation, this antibody concentration was subsequently used in the time gradient-based cell proliferation experiments and Hoechst staining assays. The experimental results were subjected to pairwise comparison using two-way ANOVA and the LSD test. Statistically significant differences were detected between the groups that were exposed to anti-human IgM antibody for various lengths of time ( $P < 0.05$ ) and between the groups that received different treatments ( $P < 0.05$ ) (**Table 2**). As the treatment duration of Hep-2 cells with 100  $\mu\text{g/ml}$  goat anti-human IgM antibody increased, both the OD value and the inhibitory rate also increased. These results demonstrate that the inhibitory effect of anti-human IgM antibody on Hep-2 cell proliferation is time-dependent (**Figure 4B**).

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**Table 1.** The inhibitory effects of 72-h treatments with various concentrations of anti-human IgM antibody or control treatment on Hep-2 cell proliferation

Concentration ( $\mu\text{g/ml}$ )	PBS control group		IgG control group		Anti-human IgM antibody group	
	OD values ( $\bar{x}\pm s$ )	Inhibitory rate (%)	OD values ( $\bar{x}\pm s$ )	Inhibitory rate (%)	OD values ( $\bar{x}\pm s$ )	Inhibitory rate (%)
0	0.6081 $\pm$ 0.0027	0.00	0.6051 $\pm$ 0.0236	0.00	0.6010 $\pm$ 0.0043	0.00
12.5	0.6060 $\pm$ 0.0027	0.35	0.6021 $\pm$ 0.0106	0.50	0.5609 $\pm$ 0.0042	6.67
25.0	0.6031 $\pm$ 0.0028	0.83	0.5972 $\pm$ 0.0107	1.31	0.5207 $\pm$ 0.0044	13.36
50.0	0.5981 $\pm$ 0.0027	1.64	0.5911 $\pm$ 0.0108	2.31	0.4707 $\pm$ 0.0043	21.68
100.0	0.5921 $\pm$ 0.0029	2.63	0.5832 $\pm$ 0.0107	3.62	0.4104 $\pm$ 0.0042	31.71
200.0	0.5828 $\pm$ 0.0027	4.16	0.5723 $\pm$ 0.0107	5.42	0.3407 $\pm$ 0.0041	43.31

Note: The PBS concentration unit is  $\mu\text{l/ml}$ .



**Figure 4.** The inhibitory effect of anti-human IgM antibody on Hep-2 cell proliferation. A: *Inhibition-rate curves* depicting the effects of 72-h exposure to various concentrations of anti-human IgM antibody or control treatments on Hep-2 cells; B: *Inhibition-rate curves* depicting the effects of various durations of anti-human IgM antibody treatment or control treatments on Hep-2 cells.

### Effect of anti-human IgM antibody on Hep-2 cell apoptosis

Hep-2 cells treated with anti-human IgM antibody had an apoptotic rate of  $20.45\pm 3.45\%$ . The apoptotic rates in the goat IgG control group and the PBS control group were  $7.30\pm 3.56\%$  and  $6.45\pm 2.98\%$ , respectively. The differences in the apoptotic rate between the anti-human IgM antibody group and the goat IgG group and between the anti-human IgM antibody group and the PBS control group were statistically significant ( $P < 0.05$ ). In contrast, there was no statistically significant difference between the goat IgG group and the PBS group ( $P = 0.574$ ). Typical apoptosis-related morphological changes were detected under a fluorescence microscope after treatment of Hep-2 cells with 100  $\mu\text{g/ml}$  anti-human IgM antibody for 72 h. Specifically, strongly stained dense granules were present in the nuclei of

Hep-2 cells, the cell and nuclear membranes remained intact, a single or multiple spherical or crescent-shaped blank areas were clearly visible at the edge of the nuclei lining the nuclear membrane, and some nuclei displayed a thick, dense stain. In addition, the cytoplasm of Hep-2 cells became condensed, and cell volume was significantly reduced (Figure 5). These results demonstrate that anti-human IgM antibody promotes the apoptosis of Hep-2 cells.

### Effect of anti-human IgM antibody on the cell cycle of Hep-2 cells

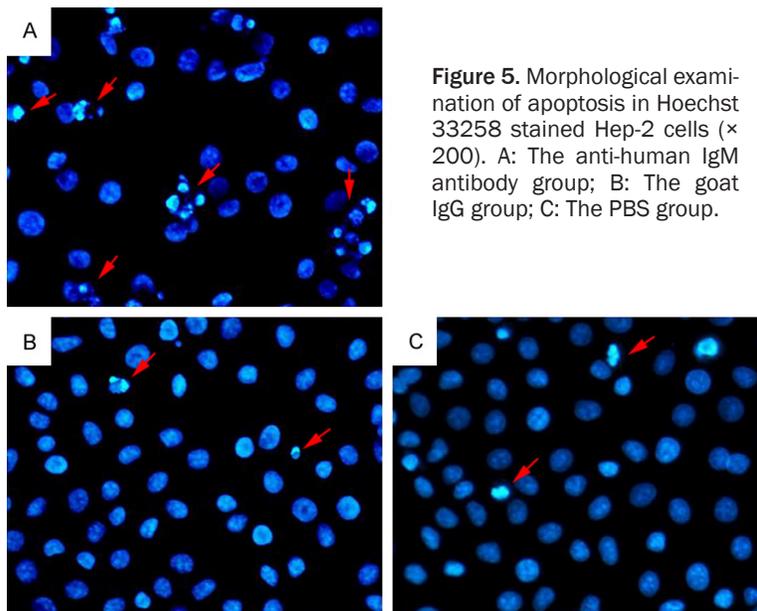
Flow cytometric analysis of PI-stained Hep-2 cells showed that G1-phase cells and S-phase cells accounted for  $51.37\pm 2.49\%$  and  $38.20\pm 1.74\%$ , respectively, of all cells in the anti-human IgM antibody group. In the goat IgG control group,  $74.63\pm 0.67\%$  of the cells were in the G1 phase and  $16.43\pm 0.55\%$  were in the S

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**Table 2.** The inhibitory effects of various durations of anti-human IgM antibody or control treatments on the proliferation of Hep-2 cells

Time (d)	PBS control group		IgG control group		Anti-human IgM antibody group	
	OD values ( $\bar{x} \pm s$ )	Inhibitory rate (%)	OD values ( $\bar{x} \pm s$ )	Inhibitory rate (%)	OD values ( $\bar{x} \pm s$ )	Inhibitory rate (%)
1	0.4021±0.0038	3.39	0.3934±0.0058	5.48	0.3598±0.0018	13.55
2	0.4446±0.0058	2.45	0.4334±0.0056	5.00	0.3637±0.0020	20.28
3	0.6100±0.0017	2.94	0.5995±0.0046	4.61	0.4238±0.0035	32.57
4	0.7097±0.0010	2.59	0.7161±0.0077	1.72	0.4836±0.0023	33.63
5	0.8176±0.0041	4.05	0.8162±0.0077	4.21	0.5624±0.0056	34.00

Note: The PBS concentration unit is  $\mu\text{l/ml}$ .



**Figure 5.** Morphological examination of apoptosis in Hoechst 33258 stained Hep-2 cells ( $\times 200$ ). A: The anti-human IgM antibody group; B: The goat IgG group; C: The PBS group.

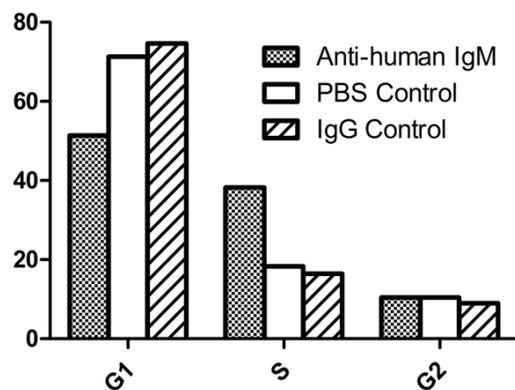
goat IgG and PBS control groups, the anti-human IgM antibody group had a decreased number of cells in the G1 phase and an increased number of S-phase cells, and these differences were statistically significant ( $P < 0.05$ ) (Figure 6). These findings indicate that anti-human IgM antibody affects the cell cycle of Hep-2 cells, resulting in cell cycle arrest mainly during S phase.

### Effect of anti-human IgM antibody on the formation of subcutaneous LSCC (Hep-2) xenografts in nude mice

During the experimental period, all 3 groups of tumor-bearing

nude mice appeared to be in good condition. No deaths occurred among the mice, and the tumor-formation rate reached 100%. ANOVA results found no statistically significant differences in body weight and tumor volume between the 3 groups of tumor-bearing nude mice prior to the treatments (body weight:  $P = 0.157$ ; tumor volume:  $P = 0.468$ ). All mice were sacrificed 3 d after the last injection. Tumor tissues were collected and subjected to routine pathological sectioning and hematoxylin and eosin (HE) staining. The tumors were confirmed as LSCC.

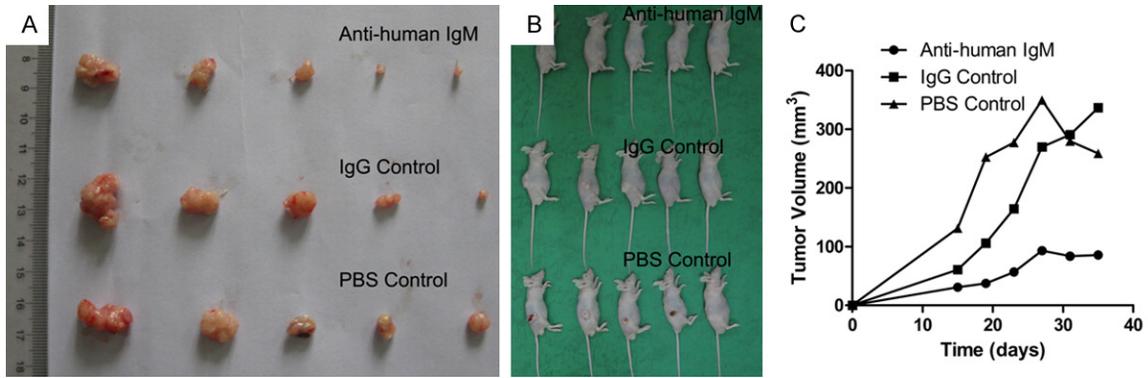
After a 2-week administration of the anti-human IgM antibody, the experimental group exhibited delayed tumor growth. The rate of tumor growth was significantly slower in the experimental group compared with the IgG control group and the PBS control group ( $P < 0.05$ ). Tumor inhibitory rate 1 (compared with the IgG



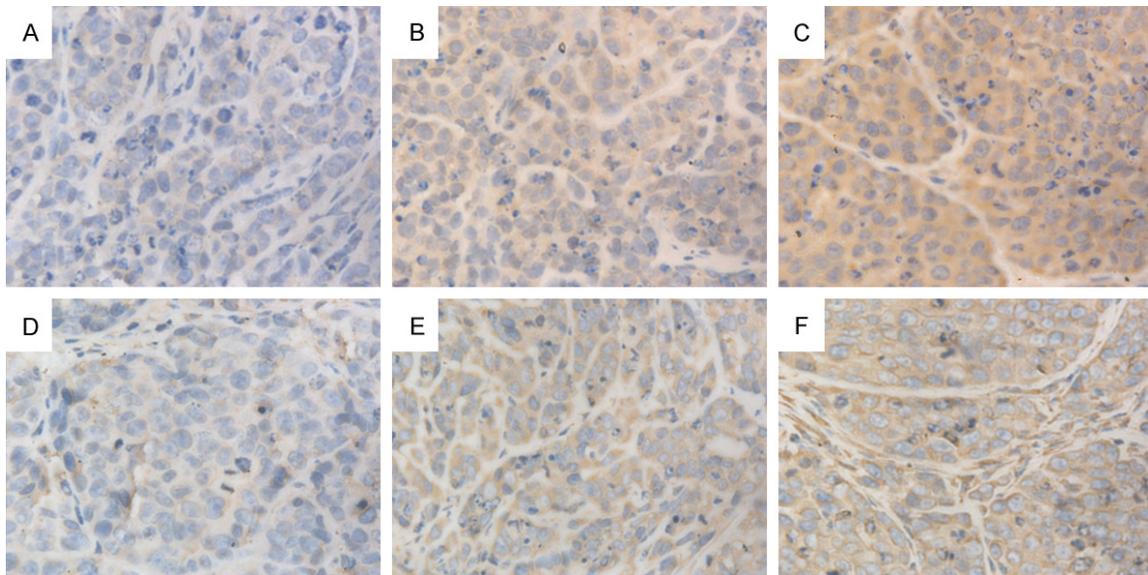
**Figure 6.** Comparison of cell cycle distribution between anti-human IgM antibody-treated Hep-2 cells and the control groups.

phase. In the PBS control group,  $71.30 \pm 1.28\%$  of the cells were in the G1 phase and  $18.27 \pm 0.83\%$  were in the S phase. Compared with the

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**Figure 7.** The growth of Hep-2 LSCC cells in nude mice and tumor growth curves. A: Comparison of the size of tumor nodules; B: Growth status of the nude mice and xenograft tumors; C: Growth curves of the *Hep-2* xenograft tumors.



**Figure 8.** Expression of IgM and gp96 protein in xenograft tumor tissues collected from the nude mice ( $\times 400$ ). A: IgM expression in the anti-human IgM antibody group; B: IgM expression in the IgG control group; C: IgM expression in the PBS control group; D: gp96 expression in the anti-human IgM antibody group; E: gp96 expression in the IgG control group; F: gp96 expression in the PBS control group.

control group) was 73.63%, while tumor inhibitory rate 2 (compared with the PBS control group) was 73.28% (**Figure 7**).

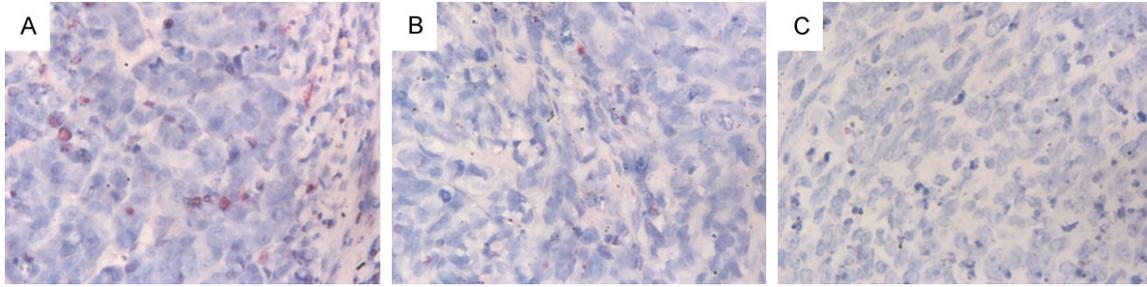
### *Examination of the expression of IgM and gp96 protein and apoptosis in xenograft tumor tissues collected from the nude mice*

In Hep-2 cell-derived xenograft tumor tissues, IgM and gp96 staining (brownish-yellow color) were mainly observed in the cytoplasm of the tumor cells. Anti-human IgM antibody significantly affected the expression of IgM and gp96. Protein expression was quantified by measuring the OD value. For IgM protein expression, the experimental group had a mean OD

value of  $0.04 \pm 0.03$ , which was significantly reduced compared with the IgG control group ( $0.10 \pm 0.03$ ) and the PBS control group ( $0.11 \pm 0.04$ ) ( $P < 0.05$ ). In terms of gp96 expression, the experimental group had a mean OD value of  $0.04 \pm 0.01$ , which was statistically significantly reduced from the IgG control group ( $0.08 \pm 0.02$ ) and the PBS control group ( $0.10 \pm 0.03$ ) ( $P < 0.05$ ). These results are shown in **Figure 8**.

Xenograft tumor tissues derived from Hep-2 cells were collected from the nude mice and subjected to TUNEL staining. TUNEL-positive cells were represented by bright-red or red nuclei, whereas the nuclei of TUNEL-negative

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**Figure 9.** Examination of apoptotic cells in tumor xenografts ( $\times 400$ ). A: The anti-human IgM antibody group; B: The IgG control group; C: The PBS control group.

cells appeared blue. The ratio of TUNEL-positive cells was significantly affected by treatment with anti-human IgM antibody. The percentage of apoptotic cells in the experimental group was  $2.32 \pm 1.03\%$ , compared with  $0.47 \pm 0.23\%$  in the IgG control group and  $0.38 \pm 0.23\%$  in the PBS control group. These differences were statistically significant ( $P < 0.05$ , **Figure 9**).

### Discussion

As molecular biology techniques advance and our understanding of tumor pathogenesis deepens, the relationship between tumors and immunity has become a hot topic in cancer research. Recent studies have found that Igs are expressed in various tumor tissues. However, certain differences in the rearrangement mechanisms and biological activities exist between the Igs expressed by tumor tissues and the classical Igs derived from B cells. A study conducted by Qiu et al. showed that non-B cell-derived, tumor-secreted IgG not only has Ig activities but also promotes the survival and growth of tumor cells. Inhibition of the expression of the tumor-secreted IgG suppresses tumor cell growth [16]. Another type of Ig, IgM, is also expressed in tumor tissues and epithelial tissues, in contradiction to the classical immunological theory [11, 15].

Currently, studies focusing on the biological activities of Igs in malignant tumors of epithelial origin suggest that Igs promote tumor growth and possess growth factor-like activities [16]. Ma et al. found that Igs are highly expressed in breast cancer cells and can enhance the invasive and migratory capabilities of these cells. Knockout of Igs decreases the expression levels of invasion- and migration-related proteins and reduces the invasive and migratory capabilities of breast cancer cells [17]. In the pres-

ent study, *in vitro* experiments showed that anti-human IgM antibody effectively inhibited the proliferation and growth of Hep-2 cells in a concentration- and time-dependent manner. *In vivo* experiments showed that anti-IgM treatment significantly suppressed tumor growth. It is likely that anti-IgM treatment suppresses the growth factor-like activity of IgM, resulting in the inhibition of tumor growth and proliferation. This hypothesis is supported by findings from Devarapu et al. that anti-IgM antibody exerts a cytotoxic effect on melanoma and neuroblastoma cell lines [18].

As an important signaling molecule in mature B cells, IgM plays critical roles in a variety of signal transduction processes related to B cell activation and survival. Blocking B cell antigen receptors with anti-IgM antibody induces apoptosis of B cells [19]. Qiu et al. introduced antisense oligonucleotides into cell lines to inhibit the expression of IgG in cancer cells of epithelial origin and found that tumor cell apoptosis was enhanced [16]. In addition, Qiu et al. employed the antigen-antibody binding strategy to block IgG in cancer cells with a goat anti-human IgG antibody. Treatment with goat anti-human IgG antibody induced apoptosis of tumor cells and inhibited the growth of xenograft tumors in nude mice. The findings of Qiu et al. indirectly suggest that tumor-derived IgG promotes tumor growth. Graves et al. demonstrated that anti-human IgM antibodies may inhibit tumor growth by promoting apoptosis of the tumor cells [19]. The present study showed that anti-human IgM antibody significantly increased the apoptosis of Hep-2 cells in xenograft tumors, supporting the above theory.

The regulatory machinery of the cell cycle is closely related to the development and progression of tumors. Deregulation of the cell

cycle leads to tumorigenesis [20, 21]. A category of antitumor drugs exert their effects through blocking cell cycle progression [22]. In the present study, we found that anti-human IgM antibody affected the cell cycle of Hep-2 LSCC cells, resulting in a decreased number of G1-phase cells and an increased number of S-phase cells. We concluded that the anti-human IgM antibody arrested the cell cycle at the S phase. This hypothesis is consistent with results obtained by other researchers in their studies of other tumor types [8].

gp96 is a major member of the heat shock protein 90 (HSP90) family and an important molecular chaperone. Under physiological conditions, gp96 binds to and hydrolyzes adenosine triphosphate (ATP) in the endoplasmic reticulum, thereby assisting the correct folding and assembly of newly synthesized proteins and promoting the degradation of misfolded proteins [23]. In addition, gp96 contributes to the expression of approximately 20 proteins, such as Ig heavy chain and integrins. Recent studies have found that gp96 is highly expressed in tumor cells and is closely related to the development and progression of tumors and to a poor prognosis. Wu et al. have shown that gp96 is highly expressed in liver cancer and is related to tumor type and stage [24]. It is likely that gp96 participates in tumor development and progression by promoting the degradation of p53. Liu et al. found that gp96 is highly expressed in oral tumors and is correlated with radiotherapy resistance [25]. Moreover, gp96 participates in tumor immune response by activating macrophages and T cells. Activated macrophages and T cells secrete tumor necrosis factor and destroy tumor cells [26]. However, the gp96-mediated immune response has a dichotomous nature; gp96 fails to elicit protective immune responses when the dose of gp96 exceeds or is below optimum levels [27]. Igs are also involved in tumor immunity and are likely to be related to gp96 activity. In the present study, a nude mouse model of Hep-2 tumor xenografts was established, and anti-human IgM antibody was injected intratumorally as an interventional drug. The expression levels of IgM and gp96 in the xenograft tumors were significantly lower in the experimental group compared with the 2 control groups, indicating that anti-IgM treatment inhibits the expression of gp96 in laryngeal carcinoma.

In summary, the expression of Igs in malignant tumor cells of epithelial origin is a novel frontier in cancer immunology research. The present study investigated the expression of IgM in laryngeal cancer and the effect of anti-human IgM antibody on the proliferation, apoptosis and cell cycle of Hep-2 human LSCC cells, thereby providing new pathways for the biological study of laryngeal cancers. An in-depth study of IgM expression may provide novel perspectives for the prevention and treatment of LSCC. Anti-human IgM antibodies are likely to become a part of the comprehensive treatment for LSCC in the future.

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### Disclosure of conflict of interest

None.

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