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Original Article

A novel tumor-specific broad-spectral monoclonal antibody to PL2L60 is highly effective for the treatment of various types of cancers from human and mouse

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Abstract: There are numerous antibodies used for cancer therapy in clinic, but they are essentially less efficacy than expected. None of them has tumor-specific and broad-spectral properties. PIWIL2-like (PL2L) protein 60 (PL2L60) is a product of alienated activation of *PIWIL2* gene, and has been found to be specifically and widely expressed in various types of cancers, including hematopoietic and solid ones. Current study aims to investigate whether a monoclonal antibody (mAb) to PL2L60 has both tumor-specific and broad-spectral properties, which can be used universally to treat various types of cancers. The expression of PL2L60 protein in the cell surface and cytoplasm were determined in a panel of human and mouse tumor cell lines by flow cytometry, immunofluorescent microscopy and Western Blotting. The apoptosis and the cell cycle arrest of the tumor cells treated with mAb KA03 were evaluated by flow cytometry. The tumorigenesis of the mAb KA03-pretreated tumor cells was determined by tumor incidence and tumor size, and the efficacy of mAb KA03 treatment on tumor growth in tumors-bearing mice were kinetically evaluated. Complement-dependent cytotoxicity (CDC) assay was used to determine the capacity of mAb KA03 to kill tumor cells. Treatment of human or mouse tumor cells from hematopoietic or solid tumors with mAb KA03 at the time of inoculation efficiently inhibited tumorigenesis in the severe combined immunodeficient (SCID) mice. Moreover, injection of mAb KA03 into established tumors significantly inhibited their growth, and prolonged survival of the tumor-bearing mice, including lymphoma, breast cancer, lung cancer and cervical cancer. The efficacy of mAb KA03 treatment is likely associated with its binding to PL2L60 expressed on tumor cell surface, which may lead to cancer cell death through blocking cell cycling and/or activation of complement. In conclusion, we have identified a tumor-specific mAb to PL2L60 (KA03), which may be used potentially to treat all the types of human cancers including from both hematopoietic and solid ones.

Keywords: PIWIL2, PL2L60, mAb KA03, immunotherapy, cancer

Introduction

The attention has been increasingly garnered to “cancer immunotherapy” because of its efficacy in several cancer types [1-5]. However, the target for cancer immunotherapy is largely individualized or personalized at present and most of the targets are only distributed in a few of cancer types rather than all cancer types [1]. Moreover, these molecular targets are not cancer-specific rather required for normal cell functions. Therefore, the bottleneck for current immunotherapy is lack of a specific but broad spectral target or antigen which is widely expressed on both hematopoietic and solid cancers. Recently, we have found that the PIWIL2-like (PL2L) protein 60, a product of alienation-activated PIWIL2 gene by intragenic promoter [6, 7], is widely expressed in various hematopoietic and solid tumors, mediating tumorigenesis through promoting tumor cell proliferation and inhibiting apoptosis as well [8].

The PIWIL2 is usually expressed in testis [9-11], but can be activated in somatic cells upon DNA

damages to promote DNA repair through remodeling chromatin [12]. It plays crucial roles in self-renewal and maintenance of germline stem cells [13, 14]. Recent studies have indicated that the ectopic expression of PIWIL2 has been observed in various types of primary tumors and tumor cell lines [8, 15], including breast cancer [16], cervical cancer [17], gastric cancer [18], acute myeloid leukemia [19], colorectal cancer [20], colon cancer [21], ovarian cancer [22] and testicular germ cell tumors [23].

It has been reported that PIWIL2 can promote tumorigenesis through regulating several signal transduction pathways [8, 24-31] and inhibiting apoptotic death of tumor cells via activation of Stat3/Bcl-XL pathway [15, 24]. However, the function of PIWIL2 in tumor development remains controversial, because most of the commercial available antibodies specific for PIWIL2 could not distinguish the full length PIWIL2 from its variants [8]. In the primary breast and cervical cancers, full length PIWIL2 proteins were detected mainly in apoptotic tumor cells but little in living tumor cells [8]. In contrast, PIWIL2 variants PL2L proteins, such as PL2L60, are abundantly detected in various types of tumor tissues and tumor cell lines [8, 23], suggesting that the tumorigenic function of PIWIL2 might be mediated mainly by PIWIL2 variants [31].

We and others have found that PIWIL2 has multiple variants including PL2L80, PL2L80A, PL2L60, PL2L60A, PL2L50 and PL2L40 [8, 23, 31]. Some of the variants appear to be transcribed by intragenic promoters rather than a canonical promoter [6, 7]. While full length PIWIL2 can mediate DNA repair acting as a barrier gene to the initiation of tumorigenesis and promote apoptotic cell death in tumor tissues [8, 32], its variants such as PL2L60 [6, 8] and PL2L60A [23] can promote tumorigenesis. Among the variants mentioned above, PL2L60 is predominantly expressed in precancerous stem cells (pCSCs) as well as in various types of human and murine tumor cell lines with a level much higher than full length of PIWIL2 [8, 33-35]. PL2L60 can promote tumor cell survival and proliferation *in vitro* through up-regulation of STAT3 and BCL2 genes. It can also coordinate with NF-κB to promote tumorigenesis, probably representing a common pathway for the development of tumors in various types of tissues [8, 36, 37]. Importantly, peptides derived from PL2L60 can serve as strong immunogens targeting various types of cancers [8, 38]. In addition, PL2L60 is also detected in the testicular cells of mice, suggesting its roles in gametogenesis or development [6, 23].

In this study, we investigated the efficacy of mAb KAO3 to PL2L60 in immunotherapy of cancers, which was generated in our laboratory [8]. It demonstrated a unique capability to directly induce apoptosis of cancer cells and to inhibit cell proliferation through arresting cell cycle. It effectively inhibited tumorigenesis of pCSCs in the severe-combined immunodeficient (SCID) mice and furthermore suppressed established tumor growth when injected intratumorally into lymphoma, breast cancer, lung cancer and cervical cancer. Collectively, the anti-PL2L60 mAb (clone KAO3) is a potentially useful drug for immunotherapy of various types of cancers from either hematopoietic or solid tissues.

Materials and methods

Mice, cell lines, medium and mAb KAO3

SCID mice were obtained from Shanghai SLAC Laboratory Animal Co., Shanghai, China, bred in the pathogen-free animal facility at Ren Ji Hospital, School of Medicine, Shanghai Jiao Tong University, and maintained under standard conditions according to the institutional guidelines of Renji Hospital animal care and ethics review committee. Mice were used at the age of 6-8 weeks old.

The following human and mouse cell lines were used: human colon cancer cell line HCT116 (ATCC® Number: CCL-247™), human hepatoblastoma cell line HepG2 (ATCC® Number: HB-8065™), human breast cancer cell line MDA-MB-231 (ATCC® Number: HTB-26™), human lung cancer cell line A549 (ATCC® Number: CCL-185™), human cervical cancer cell line HeLa (ATCC® Number: CCL-2™), mouse lymphoma cell line EL4 (ATCC® Number: TIB-39™), mouse melanoma cell line B16-F10 (ATCC® Number: CRL-6475™), and mouse Lewis lung carcinoma cell line LL/2 (LLC1) (ATCC® Number: CRL-1642™) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in D10F [DMEM (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 0.1 mg/ml Penicillin-Streptomycin (Gibco)]. Other mouse lymphoma cell lines 2C4

and 326T-4 were prepared and maintained in our laboratory [8]. Murine precancerous stem cells (pCSCs) clone 2C4 was cloned from a mouse with dendritic cell-like leukemia, as previously described [33]. Murine cancer stem cell (CSC) clone 326T-4 was a subclone of 326T clone, which was generated from a thymomas [8]. The cells were maintained in R10F (RPMI 1640 plus 10% fetal calf serum supplemented with 5 mM glutamine, 50 mM 2-mecaptoethonal, 100 U/ml penicillin, and 100 ug/ml streptomycin) at 37°C in a humidified incubator containing 5% CO_2 (v/v).

Monoclonal antibody (mAb) to PL2L60 (clone KAO3, isotype IgM) was generated and maintained in our laboratory. The culture supernatants was used as described except for where indicated [8]. The filtered culture supernatants of KAO3 hybridoma usually contained about 40-60 ug/ml IgM, as determined by ELISA. The content is dependent on each batch of cultivation.

Flow cytometric analysis of cell surface expression of PL2L60 and cell apoptosis

Cancer cells were dissociated with 0.25% trypsin-EDTA (1 mM; Invitrogen) for 1-3 min. Cells (1×10^6/well) in U-bottomed 96-well plates were washed twice with 200 µl washing buffer (PBS containing 1% fetal calf serum), resuspended with 50 µl serum-free culture supernatant of mAb KAO3 (1:25 dilution), and incubated for 1 hour at 4°C. Following incubation, the cells were washed twice and then incubated with 50 µl APC-conjugated goat anti-mouse IgM (1:250 dilution; BioLegend) for 30 min at 4°C. After two washes with PBS, the cells were resuspended with 1% paraformaldehyde in PBS and analyzed by flow cytometry (BD, San Jose, CA, USA).

For cell apoptosis assay, the Annexin V-APC Apoptosis Detection kit (Biolegend) was used. Tumor cells were harvested and washed twice with pre-cooled PBS after they were treated with or without various concentrations of mAb KAO3 for 48 h. Then, the cells were incubated with a mixture containing annexin V-APC (BioLegend) and propidium iodide (Sigma) in the binding buffer for 15 min in darkness. Apoptotic cells were detected using Annexin V-APC and propidium iodide (PI), and were analyzed using a flow cytometer (BD, C6). Three independent experiments were performed.

Immunofluorescent microscopic analysis of PL2L60 expression in the cell membrane and cytoplasm

For cell surface staining, tumor cells were harvested, resuspended (5×10^6/ml) in PBS, and then plated 0.2 ml cells (1×10^6 cells/well) in 96 well plates in triplicates. The cells were spun (1,000 rpm for 5 min) and the supernatants were discarded. The cells were resuspended in 100 µl PBS containing 5 µl supernatants of anti-PL2L60 mAbs (1:20 dilution) and gently vortexed for 5 s. The samples were incubated at 4°C for 30 min and washed twice with PBS. Then, the samples were incuated with 50 µl FITC-conjugated goat-anti-mouse IgM antibody (1:200 dilution; BioLend) at 4°C for 30 min, and washed twice with PBS. Finally, the cells were resuspended in 100 µl PBS containing 1% paraformaldehyde to each well to fix the cells.

For cytoplasmic staining, tumor cells cultured on cover slips were fixed in 1% paraformaldehyde for 20 min before being washed, and subsequently blocked with 1% bovine serum albumin in PBS for 30 min. Cells were incubated at room temperature with anti-PL2L60 mAbs (1:20 dilution) in 1% bovine serum albumin. One hour after incubation, cells were washed and incubated with FITC-conjugated goat anti-mouse antibody (1:200 dilution, BioLegend).

Nuclei were counterstained with 4', 6-Diamidino-2-phenylindole (DAPI, 1:500) for all cells before microscopic analysis.

Western blot analysis of PL2L60 expression in tumor cells

Tumor cells were washed in cold PBS twice after they were harvested with trypsin. Then cells were lysed with protein extraction reagent. The total protein concentrations of whole-cell lysates were determined using a BCA Protein Assay Kit (Beyotime, Shanghai, China). Then the protein was separated using 12% polyacrylamide gel, and transferred to polyvinyl difluoride membranes. After being blocked with 5% bovine serum albumin (BSA) in TBS/Tween 20 (TBST) and incubated with PL2L60-specific antibody (mAb KAO3 supernatants) at 4°C overnight, the membranes were washed three time with TBS-T for 5 min each. Then, the membranes were incubated with horseradish peroxidase conjugated anti-mouse IgM antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h, followed by a

5 min wash with TBST, which was repeated three times. Then the membranes were analyzed with the ECL chemiluminescent detection system (Bio-Rad), and the images were acquired by a Kodak Imaging Station 2000R (Eastman Kodak, USA).

CCK-8 assay for cell proliferation

The effect of anti-PL2L60 mAb (KA03) on tumor cell proliferation was assessed using CCK-8 assays (Dojindo, Japan) according to the manufacturer's protocol. To perform the CCK-8 assay, the cells (1×10^4/well) were cultured in the 100 μl R10F (for hematopoietic cells) or D10F medium (for epithelia) in the presence of various concentrations of mAb KA03 supernatants (0, 1, 2, 4, and 8 μl/well) in flat-bottomed 96-well plates for 48 hours. Then, the cells were washed, resuspended in fresh medium containing 20 μl of CCK-8 reagent, and incubated for 1 hours at 37°C in a humidified atmosphere with 5% CO_2 before measured for O.D. value. The absorbance (A) at 490 nm was then recorded using a Spectra® Max M5 Series (Molecular Devices). Cellular viability was calculated as (A sample-A blank)/(A control-A blank) ×100%. All experiments were repeated at least three times, with triplicate samples for each experiment.

Cell cycle assay

The cells were fixed in chilled 75% ethanol and stained with propidium iodine (PI) solution containing 100 μg/ml RNase (Tiangen Biotech) and 50 μg/ml PI (Biolegend) in PBS for cell cycle analysis. The percentage of cells at each phase of the cell cycle was measured by C6 flow cytometer (BD).

Tumorigenesis assay and tumor immunotherapy

For tumorigenesis assay, the tumor cells [5×10^6 in 200 μl PBS with or without mAb KA03 supernatants (1:2 dilution)] were injected at groins of the SCID (male, 6-8 weeks old) except for where indicated. The tumor incidence and size were measured every two days. For tumor immunotherapy, the tumors were treated intratumorally with 100 μl KA03 mAb supernatants or equal volume of vehicles every two day, when they become palpable. The tumor bearing mice were randomly separated into appropriate groups (group 1: control → control; group 2: control → KA03; group 3: KA03 → control; and group 4: KA03 → KA03). The length and width of tumors were measured with a caliper every 2 days and the area of tumor was calculated by the formula length × width. Mice were euthanized when the tumor diameter reached 2.0 cm.

Human complement-dependent cytotoxicity (CDC) assay

For complement-dependent cytotoxicity (CDC) assay, tumor cells were harvested, washed twice with PBS, and resuspended in the supernatants of mAb KA03 (3×10^6/ml). Then 100 μl cells were plated in 96-well plates in triplicates (3×10^5/well), incubated at 37°C for 30 min followed by addition of 100 μl freshly isolated human plasma (1:2 dilution with PBS) and incubation at 37°C for 1 h. After the incubation, 15 μl PI (50 μg/ml) were added into each well of samples, which were immediately put in the ice bucket for 15 min. The samples were then analyzed for PI-positive cells by flow cytometry. Three groups of controls were set up: (a) tumor cells alone; (b) tumor cells with mAb KA03 without complements; (c) tumor cells with complement without mAb KA03.

Statistical analysis

All data were derived from at least three independent experiments. Values are expressed as the mean ± SD. Experimental groups were compared with the respective controls by Student's t-test. Three or more groups were compared by the one-way analysis of variance (ANOVA). Differences were considered significant when *P* values were less than 0.05. *indicated that *P* value <0.05; **indicated that *P* value <0.01; and ***indicated that *P* value <0.001. Survival analyses were performed using Kaplan-Meier survival curves, and significant differences between groups were tested using the log-rank test. Correlation coefficients were assayed by Spearman's analysis.

Results

PL2L60 protein is expressed on the surface and cytoplasm of cancer cells

In the human primary breast and cervical cancers, full length PIWIL2 proteins were detected mainly in apoptotic tumor cells but little in living tumor cells by immunohistochemistry. In con-

trast, PIWIL2 variants PL2L proteins, such as PL2L60, are abundantly detected in various types of tumor tissues and tumor cell lines [8], suggesting that the tumorigenic functions of PIWIL2 might be mainly mediated by PIWIL2 variants [6, 7]. Therefore, the wide expression of PL2L proteins in various type of cancers renders them an ideal tumor-specific broad spectral target for immunotherapy of solid and hematopoietic caners. To test the hypothesis, we first investigated whether the PL2L proteins were expressed on the cell surface of various types of tumor cell lines, using a monoclonal antibody (mAb KAO3) to a PIWIL2 peptide homologous for both human and mouse [8]. In addition to cytoplasm [8], PL2L proteins were also detected by flow cytometry (**Figure 1A**, **1B**) and fluorescent microscopy (**Figure 1C**) on the surface of tumor cell lines, including mouse hematopoietic precancerous stem cell (pCSCs) line 2C4 [33] and cancer stem cell (CSC) line 326T-4 [8], and human breast cancer cell line MDA-MB-231, lung cancer cell line A549 and cervical cancer cell line HeLa (**Figure 1A-C**). Intracellular immunofluorescent analysis (**Figure 1C**, right panel) revealed that PL2L60 protein was expressed predominantly in cytoplasm of various types of human and murine tumor cells. Western blotting data also revealed that PL2L60 protein was highly expressed in various types of murine and human tumor cell lines, including murine melanoma (B16-10F), lung cancer (LLC1), lymphoma (EL-4), pCSCs (2C4) and CSCs (326T-4) (**Figure 1D**, **1F**), and human colon cancer (HCT116), cervical cancer (HeLa), hepatoblastoma (HepG2), lung cancer (A549) and breast cancer (MDA-MB-231) (**Figure 1E**, **1G**). The expression of PL2L60 protein is the highest in cytoplasm of pCSC 2C4 (**Figure 1D**, **1F**), consistently with its expression on cell surface (**Figure 1B**), suggesting that surface expression of PL2L60 is correlated with its intracellular expression in quantity.

The PL2L60-specific mAb KAO3 effectively inhibits tumor cell proliferation through induction of apoptosis

PL2L60 can promote tumor cell survival and proliferation *in vitro* through up-regulation of STAT3 and BCL2 genes. It may also coordinate with NF-κB to promote tumorigenesis [8]. Therefore, we used antibodies to PL2L60, such as mAb KAO3, to examine whether it could inhibit cancer cell proliferation by blocking the function of PL2L60 [8]. Cancer cells were cultured for 48 hours in the presence of various concentrations of mAb KAO3 (0, 1, 2, 4, and 8 μl per well) and examined for their number and viability. As shown in **Figure 2**, KAO3 mAb was able to inhibit proliferation of various types of cancer cells in a dose-dependent manner, including murine hematopoietic pCSC line (2C4) and CSC line (326T-4) and human epithelial cancer cell lines such as MDA-MB-231, A549 and HeLa (**Figure 2A**). This was associated with cancer cell apoptosis induced by mAb KAO3 (**Figure 2B**, **2C**). Some epithelial cancer cells became round, karyopyknosis was observed under high power microscope and semi-detached cells were observed in the microwells (**Figure 2A**). Flow cytometry analysis revealed that apoptotic cells in the KAO3 mAb-treated wells were increased significantly (**Figure 2B**, **2C**). Accordingly, the number of viable cells was greatly reduced in a dose-dependent manner, too, as revealed by CCK-8 assay (**Figure 2D**). These results suggest that anti-PL2L60 mAb (KAO3) may block the function of surface PL2L60, leading to cancer cell apoptosis.

The KAO3 mAb induces cell cycle arrest at the G_2/M phase in cancer cells

To determine the mechanisms underlying cell apoptosis induced by mAb KAO3, the cell cycle distributions of 2C4, 326T-4, MDA-MB-231, A549 and HeLa after anti-PL2L60 mAb (KAO3) treatment were examined. As shown in **Figure 3A**, compared with control cells, cancer cells that were treated with anti-PL2L60 mAb (KAO3) for 48 h had slightly decreased or increased proportion of G_0/G_1 phase and S phase, except for 2C4, which was increased at G_0/G_1 phase and almost suppressed at S phase (**Figure 3B**, **3C**). The number of cells that were in G_2/M phase increased in all lines to various degrees. 2C4, 326T-4 and A549 cells were arrested at G_2/M phase with higher percentages than MDA-MB-231 and HeLa cells (**Figure 3D**), consistently with the observation for their cell viability (**Figure 2D**) and their surface expression level of PL2L60 (**Figure 1B**). In short, the cell cycling analysis demonstrated that anti-PL2L60 mAb (KAO3) caused significant G_2/M-phase arrest in five cancer cell lines, which was associated with surface expression level of PL2L60.

The mAb KAO3 effectively inhibits tumorigenesis and tumor growth of various types of cancers from human and mouse

Since anti-PL2L60 mAb (KAO3) treatment inhibited cancer cell proliferation and induced

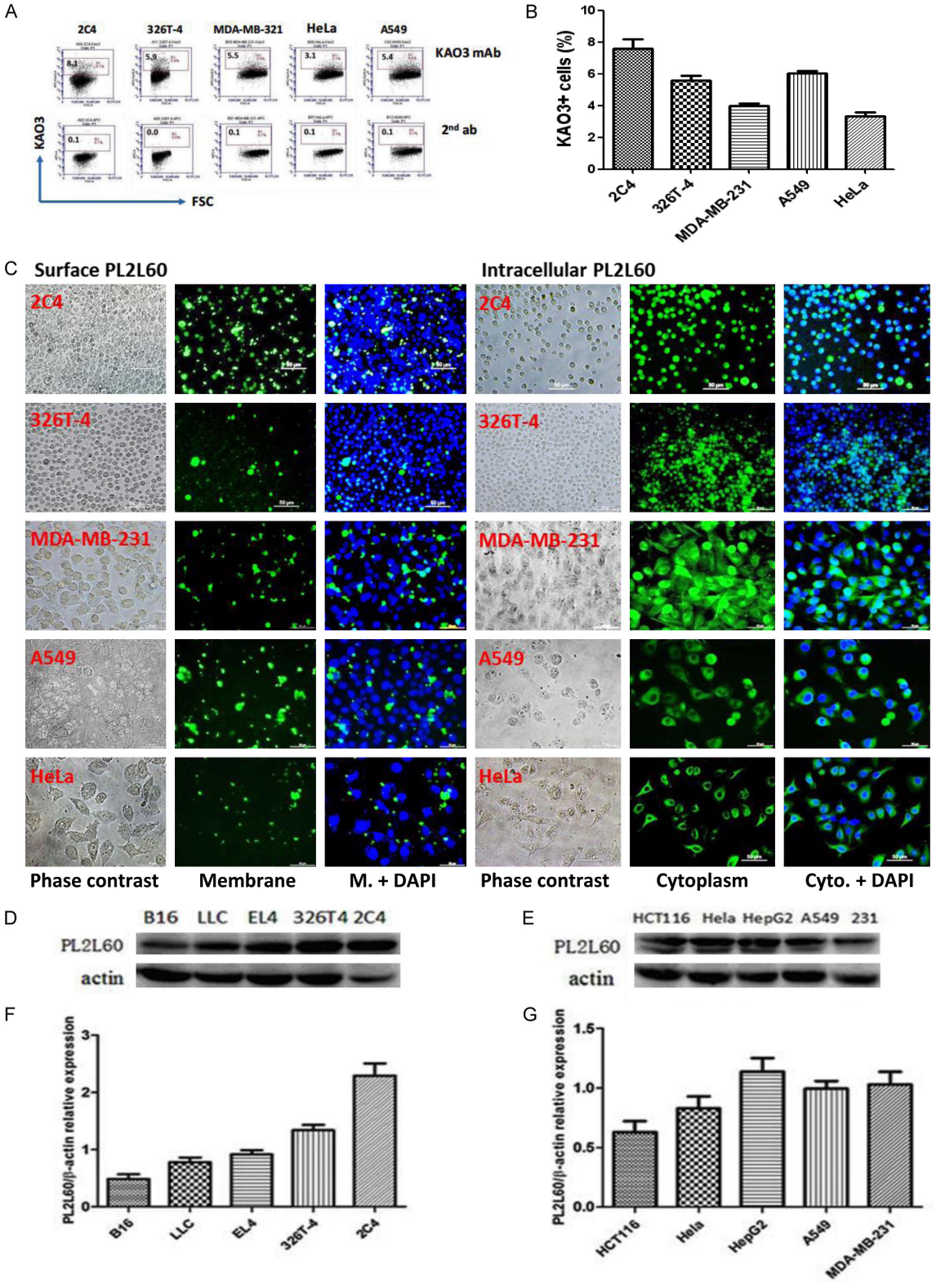


Figure 1. Expression of PL2L60 protein in cancer cells. Binding activities of anti-PL2L60 mAb were measured by flow cytometry (A, B), immunofluorescent microscopy (C), and Western blotting (D-G). (A) Flow cytometric analysis of expression of PL2L60 on cell surface of a panel of human (MDA-MB-231, A549 and HeLa) and mouse (2C4 and 326T-4) cancer cell lines. Cells were harvested using cells stripper, incubated with mAb KAO3 followed with APC-conjugated goat anti-mouse IgM for 30 min each at 4°C, and then analyzed by flow cytometry with BD C6 software. (B) Summary of three independent experiments from (A). (C) Micrographs of the immunofluorescent phase-contrast

microscopy show surface and intracellular expressions of PL2L60 in both human and mouse cancer cell lines. M.: membrane; Cyto.: cytoplasm. Bars: 50 μm. (D-G) Western blotting analysis of protein expression of PL2L60 in a panel of murine and human cancer cell lines. Mouse cell lines include B16, LLC1, EL4, 326T-4 and 2C4 (D, E). Human cell lines include HCT116, HeLa, HepG2, A549 and MDA-MB-231 (F, G). Quantitative analysis of protein expression of PL2L60 in mouse and human cancer cell lines shown in (E) and (G), respectively.

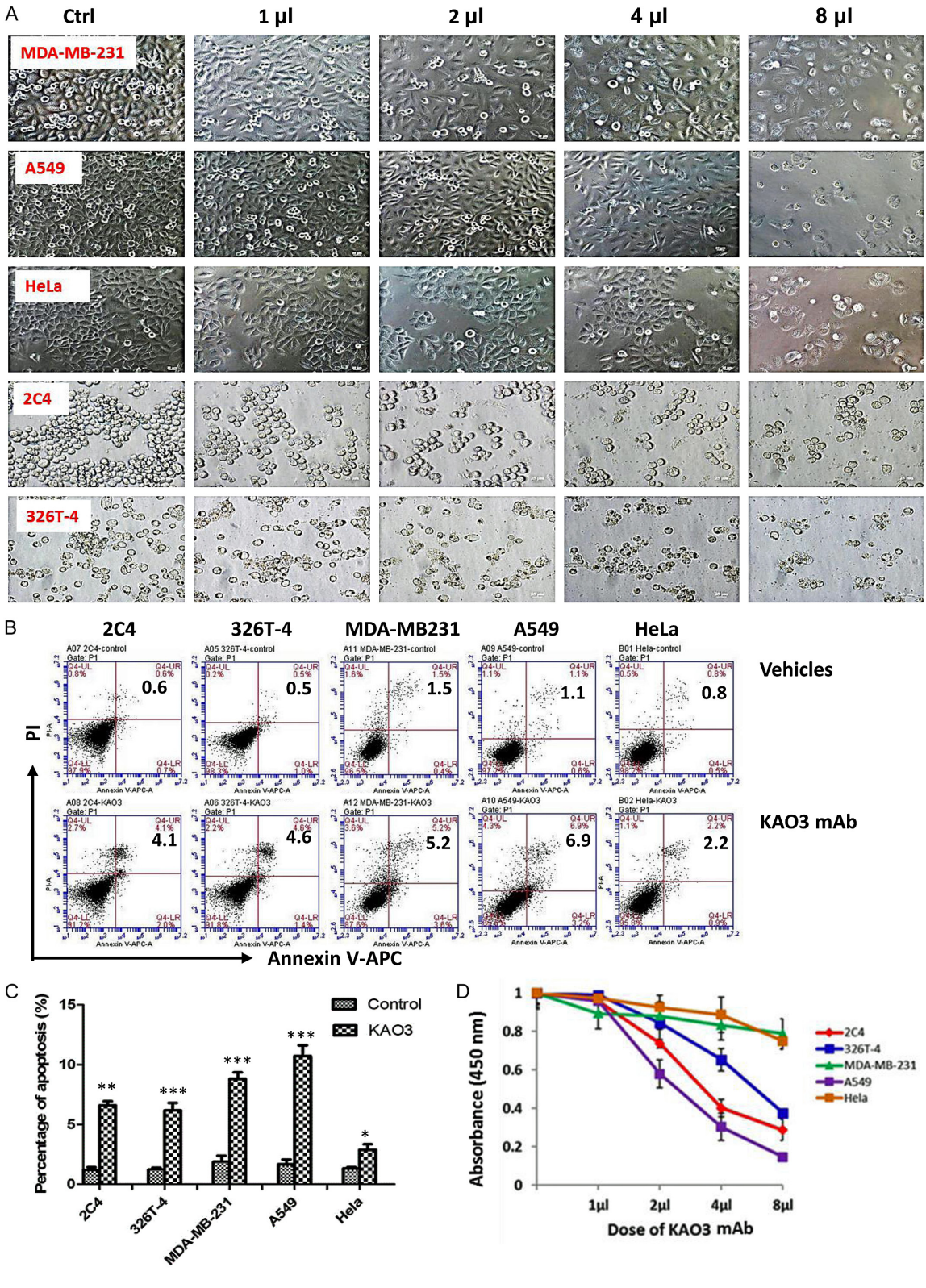


Figure 2. The anti-PL2L60 mAb KAO3 inhibited proliferation and induced apoptosis of cancer cells *in vitro*. A. Phase contrast micrographs of the mAb KAO3-treated cancer cells at 48 h of cultivation in 96-well plates. The reduction of cell in number and cell apoptosis were remarkably dose-dependent. Bars represent 25 mm. B, C. Increased apoptotic cells (Annexin V^+PI^+) from 2C4, 326T-4, MDA-MB-231, A549 and HeLa cells treated, respectively, with anti-PL2L60 mAb for 48 h. B. Representative dot plots; C. Summary of three independent experiments. Vehicles: controls. D. Dose-dependent decreases of viable cells after the cells were treated with KAO3 mAb for 48 h, as revealed by CCK8 assay. $^*P<0.05$; $^{**}P<0.01$; and $^{***}P<0.001$.

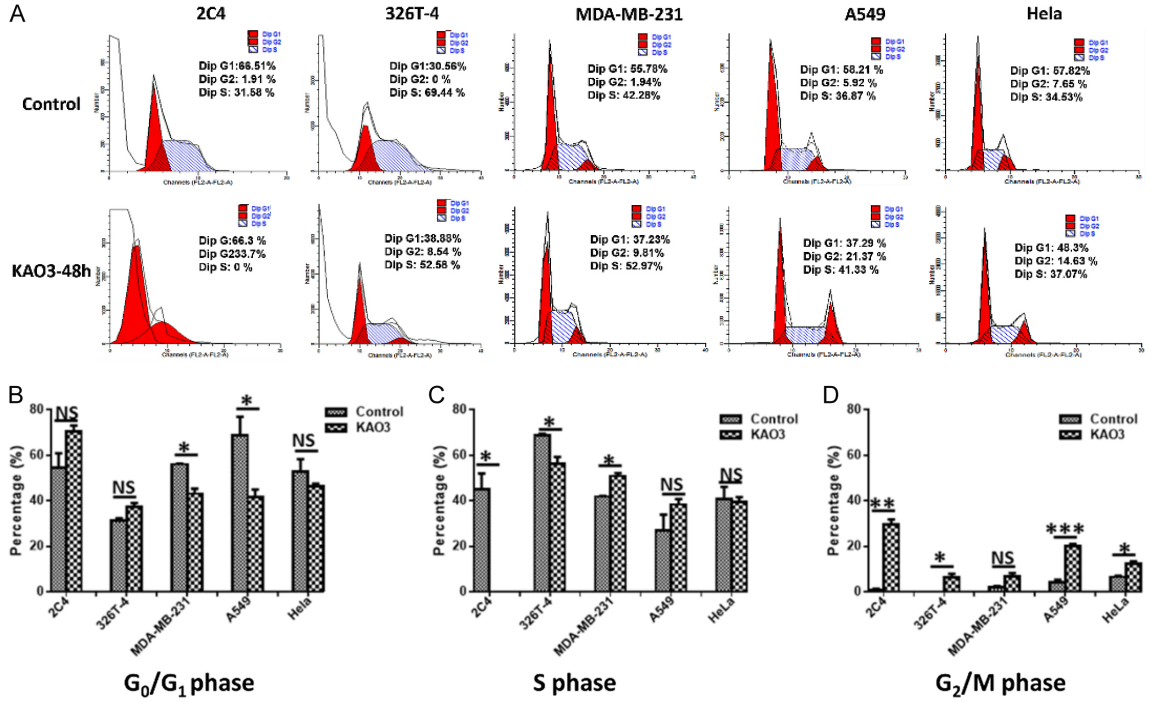


Figure 3. KAO3 mAb induced the cell cycle arrest at G_2/M phase of human and mouse cancer cells. A. The distribution of cell cycle in 2C4, 326T-4, MDA-MB-231, A549 and HeLa cell lines after KAO3 mAb treatment. Shown is a representative of cell cycle analysis from three independent experiments. B-D. The proportions of cell cycles at G_0/G_1, S and G_2/M phases were compared between the control and treatment groups. The results shown are from three independent experiments. NS, not significant; $^*P<0.05$; $^{**}P<0.01$; and $^{***}P<0.001$.

cancer cell apoptosis *in vitro* (**Figures 1-3**), we investigated whether KAO3 mAb could suppress tumorigenesis and inhibit tumor growth *in vivo*. In order to determine the effects of mAb KAO3 on tumorigenicity of tumor cells, tumor cells were pretreated with mAb KAO3 immediately before inoculation. Then, growing tumors were treated once they became palpable after inoculation to determine the effectiveness of the mAb KAO3 on inhibiting tumor growth.

Human and mouse cancer cells were suspended in the culture supernatants of KAO3 hybridoma (treatment group) or R10F medium (control group) and then inoculated into severe-combined immunodeficient (SCID) mice. After tumor formation, tumor-bearing mice were treated intratumorally with (a) mAb KAO3 supernatants or (b) vehicles (control groups) every two days, and tumor growth rate was measured every two days.

At the initial stages of tumorigenesis, tumor incidence in different groups were monitored until they became palpable. We found that the tumorigenesis of pCSCs (2C4) in the mice was almost completely inhibited by KAO3 mAb. Within 3 weeks of observation, only 60% of the mice (8/14) developed tumors from day 13 to day 21 after inoculation. In contrast, all the mice (100%, 12/12) in the control group developed tumor within 15 days after inoculation. There was a very significant statistical difference between the two groups (**Figure 4A**; P=0.001). The tumor incidence in mice inoculated with KAO3-pretreated human breast can-

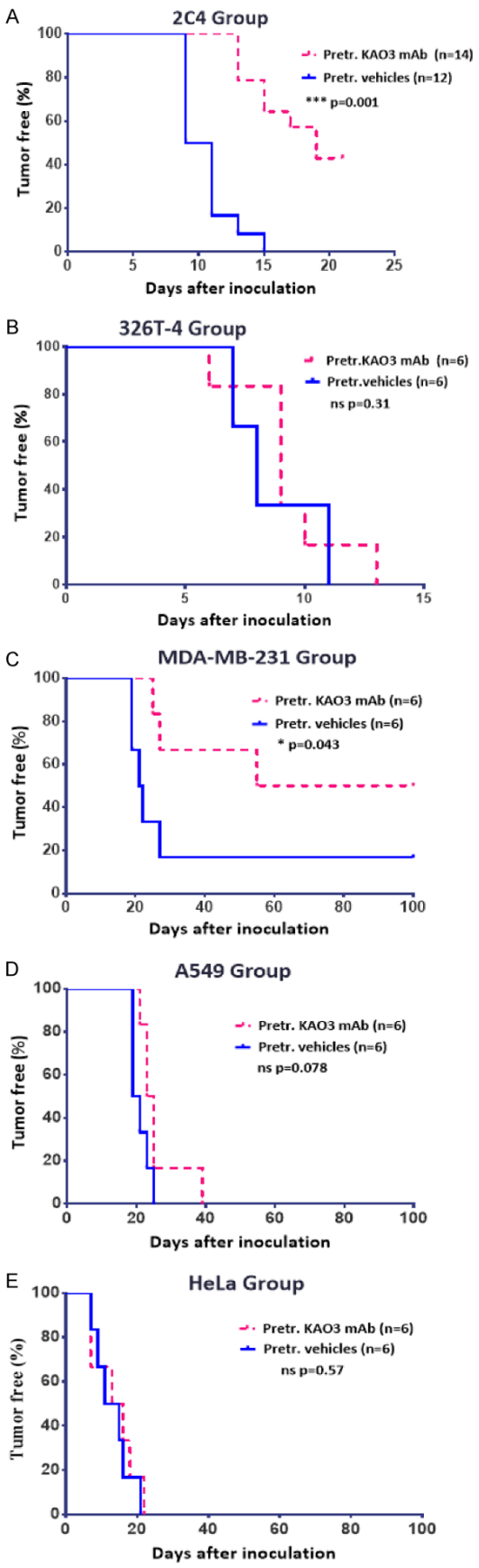


Figure 4. The effects of KAO3 mAb on tumorigenesis of mouse and human cancer cells. The mouse (2C4 and 326T-4) and human (MDA-MB-231, A549 and HeLa) tumor cells (5×10^6 cells in a volume of 200 μl) were injected into the groin of SCID mice with or without KAO3 mAb supernatants (1:2 dilution). Tumorigenesis was monitored every two days until they were palpable. (A) 2C4; (B) 326T-4; (C) MDA-MB-231; (D) A549 and (E) HeLa. *P<0.05, and ***P<0.001.

cer cells (MDA-MB-231) was 50%, whereas it was 83% in control group (**Figure 4C**; P=0.043). The tumor incidence of other three cell lines were 100%, and there were no differences between KAO3 pretreatment groups and the control groups (**Figure 4B**, **4D** and **4E**).

Further analysis of the tumor growth kinetics showed that tumors derived from KAO3-pretreated pCSCs grew significantly slower than the tumors derived from control (medium-treated) pCSCs (2C4) (**Figure 5A**). Intra-tumoral treatment of tumors from control group with 100 μl of KAO3 mAb supernatants (Ctrl → KAO3) significantly inhibited tumor growth (**Figure 5B**, 5P/2C4). The tumors which derived from the pretreated pCSCs were further treated with KAO3 mAb (KAO3 → KAO3). Tumor size was further reduced and smaller than those in the control treatment group (KAO3 → ctrl). But there was no statistical difference between these two groups, because the tumor volume was both restrained after they were pretreated with the KAO3 mAb (**Figure 5C**, 5P/2C4: KAO3 → ctrl vs. KAO3 → KAO3). The results indicate that the mAb KAO3 is effective in preventing tumorigenesis of pCSCs and suppressing the growth of established tumors. The same treatments of murine hematopoietic cancer stem cells (CSCs; clone 326T-4, unpublished data) also led to various degrees of tumorigenic suppression or inhibition of tumor growth in the SCID mice (**Figure 5D-F**, 5P/326T-4).

Since mAb KAO3 also recognizes human PL2L60 proteins, we treated human breast cancer cells (MDA-MB-231), lung cancer cells (A549) and cervical cancer cells (HeLa) with the culture supernatants of hybridoma KAO3 as described for pCSC (2C4). Similar results were observed that the KAO3 mAb could also effectively inhibit the tumorigenesis of human cancer cells and the growth of established tumors in SCID mice (**Figure 5G-P**). The results suggest that mAb KAO3 can also effectively kill or sup-

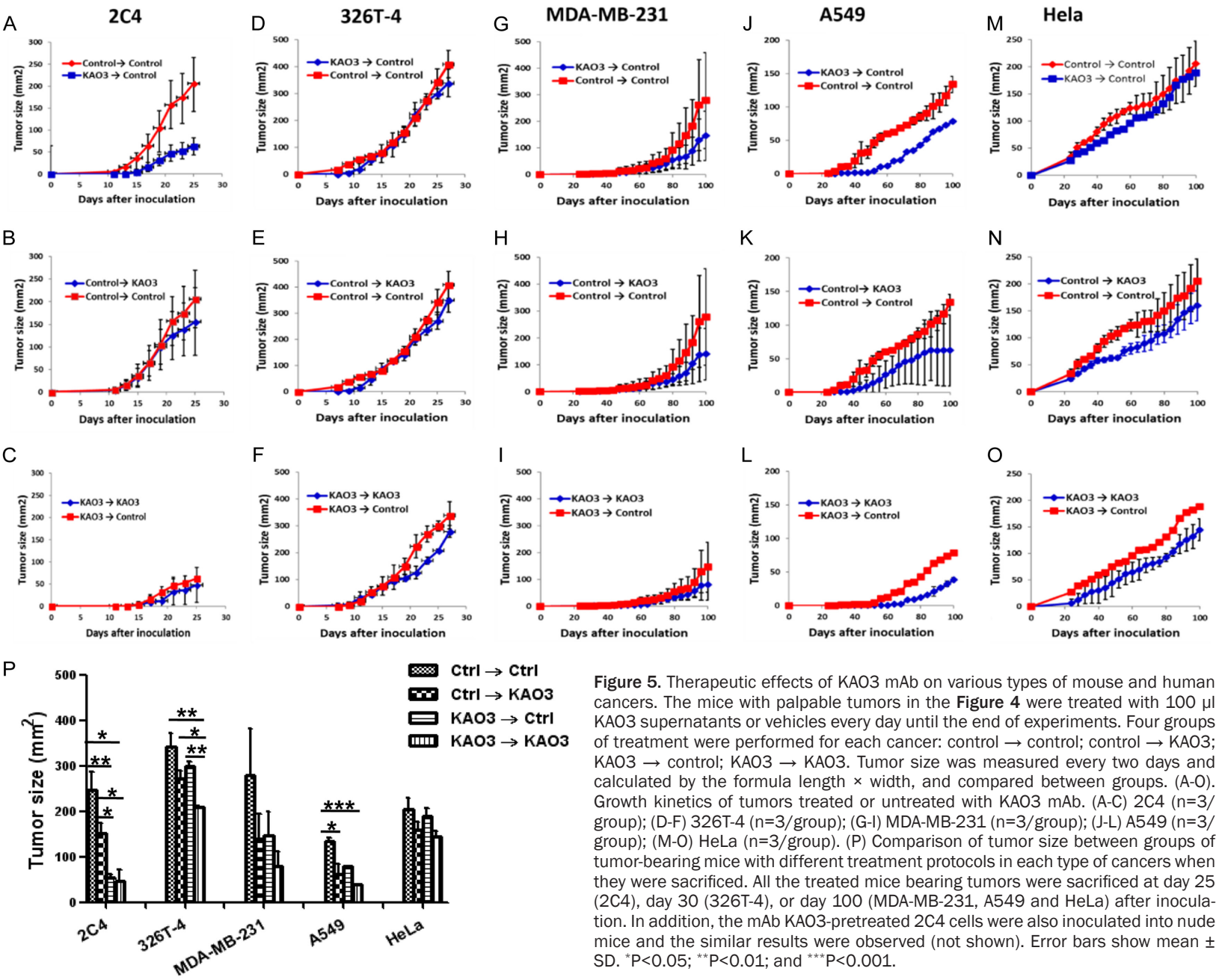
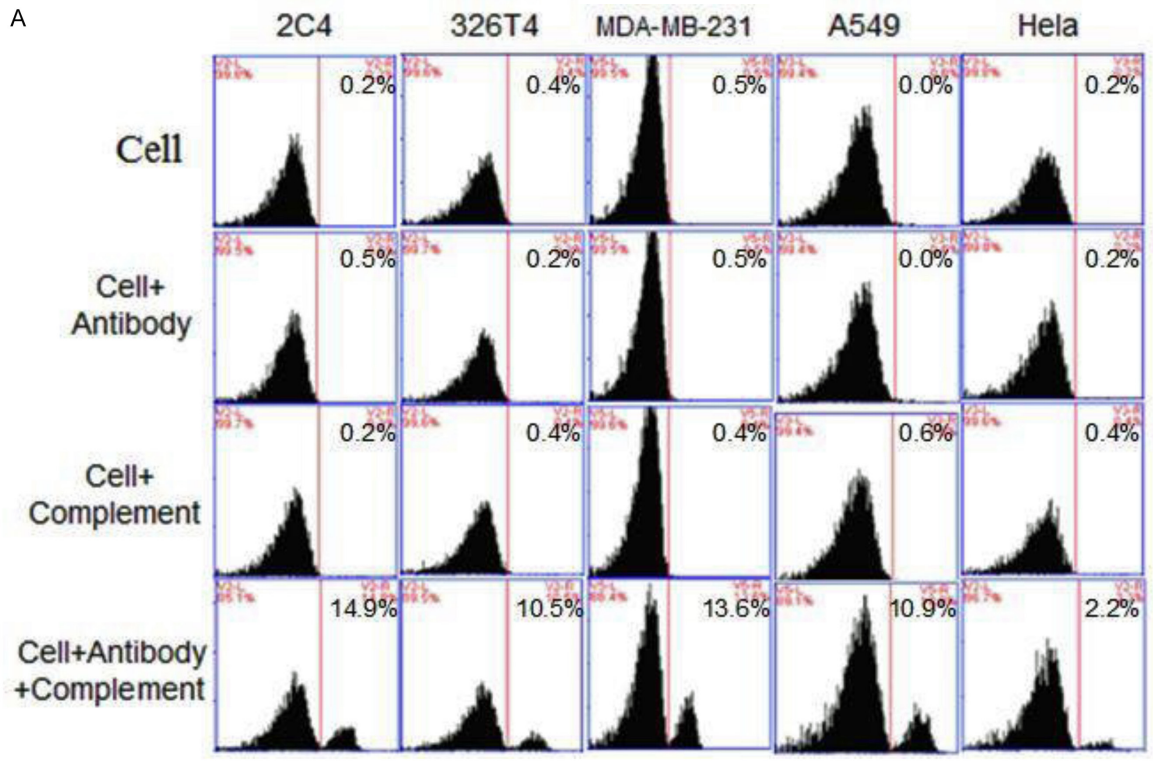


Figure 5. Therapeutic effects of KAO3 mAb on various types of mouse and human cancers. The mice with palpable tumors in the **Figure 4** were treated with 100 μl KAO3 supernatants or vehicles every day until the end of experiments. Four groups of treatment were performed for each cancer: control → control; control → KAO3; KAO3 → control; KAO3 → KAO3. Tumor size was measured every two days and calculated by the formula length × width, and compared between groups. (A-O). Growth kinetics of tumors treated or untreated with KAO3 mAb. (A-C) 2C4 (n=3/group); (D-F) 326T-4 (n=3/group); (G-I) MDA-MB-231 (n=3/group); (J-L) A549 (n=3/group); (M-O) HeLa (n=3/group). (P) Comparison of tumor size between groups of tumor-bearing mice with different treatment protocols in each type of cancers when they were sacrificed. All the treated mice bearing tumors were sacrificed at day 25 (2C4), day 30 (326T-4), or day 100 (MDA-MB-231, A549 and HeLa) after inoculation. In addition, the mAb KAO3-pretreated 2C4 cells were also inoculated into nude mice and the similar results were observed (not shown). Error bars show mean ± SD. $^{*}P<0.05$; $^{**}P<0.01$; and $^{***}P<0.001$.

A



B

PI positive cells（%）	cells	cells + antibody	cells + complement	cells + antibody + complement
2C4	0.3±0.1	0.5±0.2	0.3±0.2	15.8±2.7
326T-4	0.4±0.2	0.4±0.3	0.5±0.2	10.8±2.6
MDA-MB-231	0.4±0.1	0.5±0.2	0.4±0.3	13.9±3.9
A549	0.4±0.2	0.6±0.2	0.6±0.3	11.5±3.4
Hela	0.2±0.2	0.5±0.3	0.4±0.2	3.5±1.8

Figure 6. The KAO3 mAb effectively activated human complement and induced complement-dependent cytotoxicity (CDC). CDC experiments were carried out in 5 types of cancer cells (2C4, 326T-4, MDA-MB-231, A549, and HeLa) with human complements. (A) Flow cytometric analysis of CDC induced by KAO3 mAb. Shown are histograms from a representative experiment, demonstrating a complement-dependent cytolysis. (B) Summary of three experiments performed in (A). All the groups of the cells + mAb + human complement were significantly higher than other control groups in CDC activity ($P<0.001$).

press human cancer cells in the SCID mice same as observed *in vitro*. These results verify the *in vitro* finding, and indicated that KAO3 mAb had preventive effects on tumorigenesis and therapeutic effects on tumor growth either from human or mice.

The mAb KAO3 mediates complement-dependent cytotoxicity (CDC) to cancer cells of human and mice

To further determine the mechanisms underlying therapeutic effects of mAb KAO3 on the cancers of human and mice, we treated cancer cell lines 2C4, 326T-4, MDA-MB-231, A549 and HeLa cells with mAb KAO3 supernatants plus human complement (**Figure 6**). The results showed that mouse pCSC line 2C4 and human breast cancer cell line MDA-MB-231 displayed the strongest oncolytic effect in the CDC experiment, followed by mouse lymphoma cell line 326T-4 and human lung cancer cell line A549. The CDC effect in human cervical cancer cell line HeLa was the weakest (**Figure 6A**, **6B**). These results were consistent with the expression level of PL2L60 protein on cancer cell surface, suggesting that the mAb KAO3 targeting PL2L60 protein on the tumor cell surface effec-

tively suppressed tumor growth at least through the CDC-mediated mechanisms.

Discussion

We and others have found that PIWIL2 has multiple variants including PL2L80, PL2L80A, PL2L60, PL2L60A, PL2L50 and PL2L40 [8, 23]. Some of the variants appear to be transcribed by intragenic promoters rather than a canonical promoter [6, 7]. While full length PIWIL2 can mediate DNA repair acting as a barrier gene to the initiation of tumorigenesis and promote apoptotic cell death in tumor tissues [7, 8, 31]. Its variants such as PL2L60 [8] and PL2L60A [23] can promote tumorigenesis. Among the variants mentioned above, PL2L60 is predominantly expressed in precancerous stem cells (pCSCs) as well as in various types of cancer cell lines including hematopoietic and solid cancers of human and mice with a level much higher than full length PIWIL2 [8]. PL2L60 can promote tumor cell survival and proliferation *in vitro* through up-regulation of STAT3 and BCL-2 genes. It can also coordinate with NF-κB to promote tumorigenesis, probably representing a common pathway for the development of tumors in various types of tissues [7]. Therefore, the development of therapeutic antibodies, which target PL2L60, has great potential for eradicating tumors. Importantly, peptides derived from PL2L60 may serve as strong immunogens targeting various types of cancer. A PIWIL2 peptide which is located in the sequence of all the PL2L proteins and shared between human and mice is a strong immunogen successfully used for generation of rabbit polyclonal antibody and murine monoclonal antibody (mAb) [8]. The mAb clones KA02 and KA03 have stronger affinity for PL2L60 than the clone KA01 in the immunohistochemical staining assay and Western blot analysis [8]. The findings implicate that PL2L proteins, especially PL2L60, might be a common target for cancer immunotherapy.

In this study, we used the mAb KA03 developed in our laboratory to examine whether PL2L60 proteins are a common target for cancer immunotherapy, because PL2L60 proteins are widely expressed in various types of cancer cells including hematopoietic and solid tumors [8]. As expected, mAb KA03 has strong capacity to inhibit tumorigenesis and tumor growth of various cancer cell types. The differential capability of mAb KA03 to inhibit tumorigenesis and tumor growth appeared to be associated with the expression level of surface PL2L60 proteins between the cancer cell lines (**Figure 1A**, **1B**), but not with intracellular expression levels (**Figure 1C-G**). The efficiency of KA03 mAb to inhibit tumorigenesis was associated with surface KA03$^+$ cells in percentage. HeLa cell lines contained less KA03$^+$ cells than other lines and had less sensitivity to mAb KA03 treatments (**Figure 5**). Therefore, the therapeutic efficacy of mAb KA03 may be determined by the amount of surface PL2L proteins expression.

Based on the *in vitro* results, two pathways may be involved in the mechanisms by which the mAb KA03 inhibits tumorigenesis and tumor growth. First, mAb KA03 may block PL2L60-meidated cancer proliferation through inducing apoptosis or G_2/M arrest. Treatment with KA03 mAb inhibited the proliferation of human and mouse cancer cells and this was associated with significantly increased percentage of cells in the G_2/M phase of the cell cycle and reduced number of viable cells in a dose-dependent manner. Especially the percentage of 2C4 cells in the S phases were almost completely blocked. Secondly, the mAb KA03 can directly kill tumor cells expressing surface PL2L60 through activation of complements (CDC), as revealed by CDC assay. In this case, human plasma containing fresh/active complement is required. The mAb KA03 bound to PL2L60 on the tumor cell surface triggered activation of complement and lysis of cell membranes. The effects were confirmed in a SCID mouse model. Pretreatment of cancer cells with mAb KA03 effectively inhibited tumorigenesis of cancer cells from both human and mice, and treatment of the established tumors with mAb KA03 also effectively inhibited tumor growth (**Figures 4** and **5**). In addition to these mechanisms, antibody-dependent cytotoxicity (ADCC) might also be involved. However, the ADCC, if any, should be much weaker than CDC, because mAb KA03 is IgM isotype. Our findings provide direct evidence that PL2L60 is a potential novel target for cancer immunotherapy.

As a novel target for cancer immunotherapy, active immunotherapy such as vaccination should be of choice. However, current cancer immunotherapy is largely predominant with passive antibody therapy, because there are many technical bottlenecks for active immuno-

therapy at present. For passive immunotherapy, immune cell transplantation might be possible. It has been reported that PL2L60 bears at least one cytotoxic T lymphocyte (CTL) epitope that may be enhanced by 4-chlorophenylalanine substitution at position 1 [38]. However, HLA-restriction rendered the CTL-mediated cancer therapy very expensive and time-consuming. Since there is no HLA-restriction for antibody-mediated cancer therapy, the antibody-mediated immunotherapy currently is much more cost-effective and convenient compared to CTL therapy. Therefore, invention of PL2L60-specfic antibody provides a new opportunity for promoting efficacy of cancer immunotherapy.

Previously, the PL2L60 proteins is mainly detected in the cytoplasm and nuclei [8]. In this study, we have detected the PL2L proteins on the surface of some tumor cells. Importantly, KA03$^+$ cells were detected on all the types of tumor cells used either from mouse or human. The preliminary findings raise a number of interesting issues that are worthwhile to be elucidated further, such as whether PL2L60 contains a transmembrane region, how PL2L60 promotes tumorigenesis, and what is the ligand for PL2L60? In addition, is it possible that mAb KA03 recognize other protein(s) on the cell surface?

We have for the first time demonstrated that the mAb KA03 may recognize the surface PL2L proteins expressed on various types of human and mouse tumor cell lines, including the lymphoma and cancers of breast, lung and cervix despite unknowing of its ligand. Since PL2L60 has been shown expressed in almost all hematopoietic and solid tumor cell lines [8, 23], it is likely that the mAb KA03 may be universally used for treatment of cancers without limitation for their origin and developmental stages. In addition, PL2L60 is specifically expressed in tumor cells but not in normal somatic tissue cells. Thus, the mAb KA03 may have little side-effects when used as a drug for treatment of cancer patients. If the hypothesis is verified, mAb KA03 will lead to next generation of cancer immunotherapy. In addition, the mAb KA03 is one of three mAb clones to PL2L60 proteins [8]. Another two clones mAb KA01 and mAb KA02 had the same therapeutic efficacy as the mAb KA03 (unpublished data), despite the mAb KA01 used in an immunohistochemical staining (HIS) experiment showed an affinity for PL2L60 in the apoptotic cancer cells higher than in the living cells [8].

Conclusions

We have for the first time demonstrated that PL2L60 is a universal tumor-specific biomarker for cancer therapy. The mAb KA03 can effectively inhibit tumorigenesis and tumor growth either through blocking PL2L60-mediated cancer cell proliferation pathway, inducting cell apoptosis or activating complements to kill and lyse cancer cells. Potentially, the mAb KA03 can be used universally for immunotherapy of both hematopoietic and solid cancers in clinic.

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Disclosure of conflict of interest

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Abbreviations

mAb, monoclonal antibody; mAb KA03, monoclonal antibody clone KA03; PL2L60, Piwil2-like protein (PL2L) 60; pCSC, precancerous stem cells; CSC, cancer stem cells; APC, allophycocyanin; SCID, severe-combined immunodefi-

ciency; STAT3, signal transducers and activators of transcription 3; CDC, complement-dependent cytotoxicity; DAPI, 4', 6-Diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; PI, propidium iodide; PBS, phosphate buffered saline; CCK=8, cell counting Kit-8.

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