

# A Novel Monoclonal Antibody to PL2L60 is Effective For Therapy of Various Types of Cancers in Human and Mice

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## Research

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# Abstract

**Background:** PL2L60 is a PIWIL2-like (PL2L) protein that is specifically and widely expressed in various types of hematopoietic and solid tumors. However, it is unknown whether PL2L60 is an effective target for cancer immunotherapy. The current study aimed to investigate the efficacy of an monoclonal antibody (mAb) to PL2L60 (clone KAO3 IgM isotype) in treatment of the cancers either from human or mouse.

**Methods:** 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 staining and Western Blotting. The apoptosis and the cell cycle arrest of the tumor cells treated with mAb KAO3 were evaluated by flow cytometry. The tumorigenesis of the mAb KAO3-pretreated tumor cells was determined by tumor incidence and tumor size, and efficacy of mAb KAO3 treatment on tumor growth in tumors-bearing mice were kinetically evaluated. Complement-dependent cytotoxicity tests were utilized to determine the mechanism of mAb KAO3 killing tumor cells.

**Results:** Treatment of human or mouse tumor cells with mAb KAO3 at the time of inoculation efficiently inhibited their tumorigenesis in the immunodeficient mice. Moreover, injection of mAb KAO3 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 inhibitory effects of mAb KAO3 were likely associated with its binding to the PL2L60 expressed on tumor cell surface, which may induce cell apoptosis through either activation of complement or blocking cell cycling. The cell cycling was arrested at G<sub>2</sub>/M phase and DNA synthesis may be inhibited.

**Conclusion:** We have identified the PL2L60 as a novel tumor-specific and broad-spectral biomarker, which is recognized by the mAb KAO3 as an efficient target for immunotherapy of both solid and hematopoietic cancers.

## Background

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 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- $\kappa$ B 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 KAO3 is a potentially useful drug for immunotherapy of various types of cancers.

## Materials And Methods

### Mice, cell lines, medium and mAb KAO3

Severe-combined immunodeficiency (SCID) mice, nude mice and C57BL/6 mice were obtained from SLACCAS (Shanghai Laboratory Animal Center), 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 Ren Ji Hospital animal care and ethics review committee. Mice were used at the age of 8–12 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 0.1mg/ml Penicillin-Streptomycin (Gibco)]. Other mouse lymphoma cell lines 2C4 and 326T-4 were prepared in our laboratory. 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 mg/ml streptomycin) at 37°C in a humidified incubator containing 5% CO<sub>2</sub> (v/v).

Monoclonal antibody (mAb) to PL2L60 (clone KAO3, isotype IgM) was generated in our laboratory, and the culture supernatants was used as described except for where indicated (8).

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

Cancer cells were dissociated with 0.25% trypsin-EDTA (1 mM; Invitrogen) for 1–3 min. Cells were washed with cell sorting buffer (PBS containing 1% fetal calf serum), and then incubated for 1 hour at 4°C with anti-PL2L60 mAbs. Cells were then incubated with phycoerythrin-conjugated goat anti-mouse IgM (1:250 dilution; BioLegend) for 30 min at 4°C. After a final wash, the cells were re-suspended with 1% paraformaldehyde in PBS containing 1% FBS 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 mAb KAO3 for 24 h. 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 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 tumor cells ( $5 \times 10^6$ /ml) in PBS, and then plated 0.2 ml cells ( $1 \times 10^6$  cells/well) in 96 well plates. The cells were spun (1000 rpm for 5min) and the supernatants were discarded. The cells were resuspended in 100  $\mu$ l PBS containing 5  $\mu$ l supernatants of anti-PL2L60 mAbs KAO3 and gently vortexed for 5 s. The samples were incubated at 4°C for 30 min, washed twice with PBS and resuspended in 100  $\mu$ l 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 (KAO3, 1:100 dilution) in 1% bovine serum albumin. One hour after incubation, cells were washed and incubated with FITC-conjugated goat anti-mouse antibody (IgM, 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

Cell samples were washed in cold PBS twice before 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 with TBS-T for 5 min. The wash was repeated three times. After this, 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 (KAO3) 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 ( $1 \times 10^4$ /well) were cultured in the 200  $\mu$ l R10F (for hematopoietic cells) or D10F medium (for epithelia) in the presence of various concentrations of mAb KAO3 (0, 1, 2, 4, and 8  $\mu$ l/ml) in flat-bottomed 96-well plates for 48 hours. Then, the cells were washed, resuspended in fresh medium containing 20  $\mu$ l of CCK-8 reagent, and cultured for 1–4 hours at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> 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  $(A \text{ sample} - A \text{ blank}) / (A \text{ control} - A \text{ blank}) \times 100\%$ . 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 iodide (PI) solution containing 100  $\mu\text{g/ml}$  RNase (Tiangen Biotech) and 50  $\mu\text{g/ml}$  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 [ $5 \times 10^6$  in 200  $\mu\text{l}$  PBS with or without mAb KA03 supernatants (1:2 dilution)] were injected at groins of the SCID or nude mice (Shanghai slack laboratory animal Co., Shanghai, China). The tumor incidence and size were measured every two days. For tumor immunotherapy, the tumors were treated intratumorally with 100  $\mu\text{l}$  KA03 mAb supernatants or equal volume of vehicles every 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  $\times$  width. Mice were humanely euthanized when the tumor diameter reached 2.0 cm.

## Complement-dependent cytotoxicity assay

For complement-dependent cytotoxicity (CDC) assay, tumor cells were plated out at  $5 \times 10^4$  cells per well. Test antibodies were added at the indicated final concentrations together with either active or heat inactivated (60°C for 30 minutes) human serum (25% final serum concentration; Pathway Diagnostics, Dorking, UK). Plates were incubated for 3 hours at 37°C, following which the cell viability reagent was added. Triton1 X-100 was added to the control cell-only wells to establish the maximum lysis control. After incubation for 1 hour at 37°C, fluorescence was measured by fluorescence microscopy (Olympus, Japan).

## Statistical analysis

All data were derived from at least three independent experiments. Values are expressed as the mean  $\pm$  SD. Experimental groups were compared with the respective control 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

### Expression of PL2L60 protein in 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 contrast, 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 cancer types renders them an ideal broad spectral target for immunotherapy of solid and hematopoietic cancers. 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 (Fig. 1A & B) and fluorescent microscopy (Fig. 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 (Fig. 1A-C). Intracellular immunofluorescent analysis (Fig. 1C right panel) revealed that PL2L60 protein was expressed predominantly in cytoplasm of various types of human and murine tumor cells. Western blotting data 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) (Fig. 1D & F), and human colon cancer (HCT116), cervical cancer (HeLa), hepatoblastoma (HepG2), lung cancer (A549) and breast cancer (MDA-MB-231) (Fig. 1E & G). The expression of PL2L60 protein is the highest in cytoplasm of pCSCs 2C4 (Fig. 1D-F), consistently with its expression on cell surface (Fig. 1B).

## The mAb KAO3 effectively inhibited tumor cell proliferation

PL2L60 can promote tumor cell survival and proliferation *in vitro* through up-regulation of STAT3 and BCL2 genes. It can also coordinate with NF- $\kappa$ B to promote tumorigenesis (8). Therefore, we have developed antibodies to PL2L60, such as mAb KAO3, to examine whether it may 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  $\mu$ l per well) and examined for their number and viability. As shown in Fig. 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 (Fig. 1A). This was associated with cancer cell apoptosis induced by mAb KAO3. Some epithelial cancer cells became round, karyopyknosis was observed under high power microscope and semi-detached cells were observed in the microwells (Fig. 1A). Flow cytometry analysis revealed that apoptotic cells in the KAO3 mAb-treated wells were increased significantly (Fig. 2B & C). Accordingly, the number of viable cells was greatly reduced in a dose-dependent manner, too, as revealed by CCK-8 assay (Fig. 2D). These results suggest that anti-PL2L60 mAb(KAO3) may block the function of surface PL2L60 and induce cancer cell apoptosis.

## The KAO3 mAb induced cell cycle arrest at the G<sub>2</sub>/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 Fig. 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 (Fig. 3B & C). 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 (Fig. 3D), consistently with the observation for their cell viability shown in Fig. 2D). In short, the cell cycling analysis demonstrated that anti-PL2L60 mAb (KAO3) caused significant  $G_2/M$ -phase arrest in five cancer cell lines, 2C4 was most sensitive to mAb KAO3 treatment.

The mAb KAO3 could effectively inhibit various types of tumorigenesis and tumor growth of both human and mice in the SCID mice

Since anti-PL2L60 mAb (KAO3) treatment disrupted cancer cell growth and induced cancer cell apoptosis *in vitro*, we investigated whether KAO3 mAb could be used to directly inhibit tumor growth *in vivo*. In order to observe the effects of mAb KAO3 on tumorigenicity of tumor cells, tumor treatment program was divided into two stages: one is the initial stages of tumorigenesis, and another is the tumor growth stage.

Human and mouse cancer cells were suspended in the culture supernatant 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 in different treatment groups were then divided into two groups, respectively. A group of tumor-bearing mice were injected with KAO3 supernatants intratumorally every two days for treatment, and the other group was injected with culture medium intratumorally as a control. Tumor growth rate was measured every two days.

At the initial stages of tumorigenesis, tumor incidence in different groups were counted when the tumors become 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 (Fig. 4A;  $p = 0.001$ ). The tumor incidence in mice inoculated with KAO3-pretreated human breast cancer cells (MDA-MB-231) was 50%, whereas it was 83% in control group (Fig. 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 (Fig. 4B, D & E).

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 (Fig. 5A). Intratumoral treatment of tumors from control group with 100  $\mu$ l of KAO3 mAb supernatants (Ctrl→KAO3) significantly inhibited tumor growth (Fig. 5B & P/2C4). The tumors which derived from the pretreated pCSCs were further treated with KAO3 mAb (KAO3→ KAO3). Tumor size was smaller than those in the

control treatment group (KAO → ctrl). But there was no statistical difference in the two groups because the tumor volume was both restrained after the KAO3 mAb was pretreated (Fig. 5C & P/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 treatment of murine hematopoietic cancer stem cells (CSCs; clone 326T-4), and 326T-4 also led to various degree of tumorigenic or tumor growth inhibition in the immunodeficient mice (Fig. 5D-F & P).

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 KAO3 mAb could also effectively inhibit the tumorigenesis of human cancer cells and the growth of established tumors in SCID mice (Fig. 5G-P). The results suggest that mAb KAO3 can also effectively kill or suppress human cancer cells in the SCID mice. These results verify the *in vitro* finding that KAO3 mAb has therapeutic effects on tumorigenesis and tumor growth either tumors from human or mice, as compared to controls.

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

To determine the mechanisms underlying therapeutic effects of mAb KAO3 on the cancers of human and mice, we treated with cancer cell lines 2C4, 326T-4, MDA-MB-231, A549 and HeLa cells with mAb KAO3 plus human complement (Fig. 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 (Fig. 6A-B). These results are consistent with the expression level of PL2L60 protein on cancer cell surface. It is further suggest that the target of the anti-PL2L60 mAb KAO3 was the PL2L60 protein on the tumor cell surface, and KAO3 may exert its anti-tumor effect through the CDC-dependent 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 KAO2 and KAO3 have stronger affinity for PL2L60 than the clone KAO1 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 KAO3 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 KAO3 has strong capacity to inhibit various types of tumorigenesis and tumor growth. The differential capability of mAb KAO3 to inhibit tumorigenesis and tumor growth appeared to be associated with the expression level of surface PL2L60 proteins between the cancer cell lines (Fig. 1A-B), but not with intracellular expressions (Fig. 1C-G), as the efficiency of KAO3 mAb to inhibit tumorigenesis was associated with surface KAO3<sup>+</sup> cells in percentage. HeLa contained less KAO3<sup>+</sup> cells than other lines and had less sensitivity to mAb KAO3 treatments (Fig. 5). Therefore, the therapeutic efficacy of mAb KAO3 may be determined by the amount of surface PL2L proteins expression.

Two pathways may be involved in the mechanisms by which the mAb KAO3 inhibits tumorigenesis and tumor growth. First, mAb KAO3 may block PL2L60-mediated cancer proliferation through inducing apoptosis or G2/M arrest. Treatment with KAO3 mAb inhibited the proliferation of human and mouse cancer cells and this was associated with significantly increased percentage of cells in the G2/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, mAb KAO3 can directly kill tumor cells expressing surface PL2L60 through activation of complement (CDC). The effects were confirmed in a SCID mouse model. Pretreatment of cancer cells with mAb KAO3 may effectively inhibit tumorigenesis of cancer cells from both human and mice, and treatment of the established tumors with mAb KAO3 also effectively inhibited tumor growth. Our findings provide direct evidence that PL2L60 is a novel target for cancer immunotherapy.

We have for the first time demonstrated that the mAb KAO3 may recognize the surface PL2L proteins expressed on various types of tumor cell lines, including the lymphoma, breast cancer, lung cancer and cervical cancers. Since PL2L60 has been shown expressed in almost all hematopoietic and solid tumor cell lines (8, 23), it is highly likely that the mAb KAO3 may be broadly used for treatment of cancers without limitation for their origin and developmental stages. In addition, PL2L60 is specifically expressed in tumor cells, mAb KAO3 may have little side-effects when used as a drug for treatment of cancer patients. If the hypothesis is verified, mAb KAO3 will lead to next generation of cancer immunotherapy.

## Conclusions

We have for the first time demonstrated that PL2L60 is an 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 or activating complements to lyse cancer cells. Potentially, the mAb KA03 can be used for immunotherapy of both hematopoietic and solid cancers.

## Abbreviations

mAb: monoclonal antibody

mAb KA03: monoclonal antibody clonal KA03

PL2L60: Piwil2-like protein (PL2L) 60

pCSC: precancerous stem cells

CSC: cancer stem cells

APC: allophycocyanin

SCID: severe-combined immunodeficiency

STAT3: signal transducers and activators of transcription 3

BCL-2: B-cell lymphoma-2

CDC: complement-dependent cytotoxicity

DAPI: 4', 6-Diamidino-2-phenylindole

FITC: fluorescein isothiocyanate

PI: fluorescein isothiocyanate

PBS: phosphate buffer saline

CCK=8: cell counting Kit-8

## Declarations

**Ethics approval and consent to participate:** Studies involving animals are approved by the Institutional Guidelines of Ren Ji Hospital Animal Care and Ethics Review Committee.

**Consent for publication:** not applicable.

**Availability of data and materials:** All data generated or analysed during this study are included in this published article.

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**Authors' contributions:** GJX designed experiments, interpreted the data of the manuscript and wrote the manuscript; LFL designed and performed experiments and analyzed the data; LHM designed and performed experiments, analyzed the data of experiments and wrote manuscript. YJF, NL, WYX, HYC and MYL performed some experiments and analyzed relevant data. All authors read and approved the final manuscript.

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## References

1. Drake CG, Lipson EJ, Brahmer JR. Breathing new life into immunotherapy: review of melanoma, lung and kidney cancer. *Nat Rev Clin Oncol*. 2014;11:24–37.
2. Sabado RL, Bhardwaj N. Cancer immunotherapy: Dendritic-cell vaccines on the move. *Nature*. 2015;519:300–1.
3. Shin DS, Ribas A. The evolution of checkpoint blockade as a cancer therapy: what's here, what's next? *Curr Opin Immunol*. 2015;33:23–35.
4. Geldres C, Savoldo B, Dotti G. Chimeric antigen receptor-redirected T cells return to the bench. *Seminars in Immunology*; 2015.
5. Priceman SJ, Forman SJ, Brown CE. Smart CARs engineered for cancer immunotherapy. *Curr Opin Oncol*. 2015;27:466–74.
6. Liu SS, Liu N, Liu MY, Sun L, Xia WY, Lu HM, Fu YJ, Yang GL, Bo JJ, Liu XX, Feng H, Wu H, Li LF, Gao JX. An unusual intragenic promoter of PIWIL2 contributes to aberrant activation of oncogenic PL2L60. *Oncotarget*. 2017;8:46104–20.
7. Li L-F, Liu N, Liu M-Y, Gao J-X. The Functions of Piwil2 and Its Prospects in Tumorigenesis. *Am J Transl Med*. 2017;1:75–98.

8. Ye Y, Yin DT, chen L, zhou Q, Shen R, He G, Yan Q, Tong Z, Issekutz AC, Shapiro CL, Barsky SH, Lin H, Li JJ, Gao JX. Identification of Piwil2-like (PL2L) proteins that promote tumorigenesis. *PLoS ONE*. 2010;5:e13406.
9. Kuramochi-Miyagawa S, Kimura T, Yomogida K, Kuroiwa A, Tadokoro Y, Fujita Y, Sato M, Matsuda Y, Nakano T. Two mouse piwi-related genes: miwi and mili. *Mech Dev*. 2001;108:121–33.
10. Kuramochi-Miyagawa S, Kimura T, Ijiri TW, Isobe T, Asada N, Fujita Y, Ikawa M, Iwai N, Okabe M, Deng W, Lin H, Matsuda Y, Nakano T. Mili, a mammalian member of piwi family gene, is essential for spermatogenesis. *Development*. 2004;131:839–49.
11. Sasaki T, Shiohama A, Minoshima S, Shimizu N. Identification of eight members of the Argonaute family in the human genome small star, filled. *Genomics*. 2003;82:323–30.
12. Yin DT, Wang Q, Chen L, Liu MY, Han C, Yan Q, Shen R, He G, Duan W, Li JJ, Wani A, Gao JX. Germline stem cell gene PIWIL2 mediates DNA repair through relaxation of chromatin. *PLoS One*. 2011;6:e27154.
13. Lin H, Spradling AC. A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the *Drosophila* ovary. *Development*. 1997;124:2463–76.
14. Unhavaithaya Y, Hao Y, Beyret E, Yin H, Kuramochi-Miyagawa S, Nakano T, Lin H. MILI, a PIWI-interacting RNA-binding Protein, Is Required for Germ Line Stem Cell Self-renewal and Appears to Positively Regulate Translation. *J Biol Chem*. 2009;284:6507–19.
15. Lee JH, Schutte D, Wulf G, Fuzesi L, Radzun HJ, Schweyer S, Engel W, Nayernia K. Stem-cell protein Piwil2 is widely expressed in tumors and inhibits apoptosis through activation of Stat3/Bcl-XL pathway. *Hum Mol Genet*. 2006;15:201–11.
16. Liu JJ, Shen R, Chen L, Ye Y, He G, Hua K, Jarjoura D, Nakano T, Ramesh GK, Shapiro CL, Barsky SH, Gao JX. Piwil2 is expressed in various stages of breast cancers and has the potential to be used as a novel biomarker. *Int J Clin Exp Pathol*. 2010;3:328–37.
17. He G, Chen L, Ye Y, Xiao Y, Hua K, Jarjoura D, Nakano T, Barsky SH, Shen R, Gao JX. Piwil2 expressed in various stages of cervical neoplasia is a potential complementary marker for p16. *Am J Transl Res*. 2010;2:156–69.
18. Wang Y, Liu Y, Shen X, Zhang X, Chen X, Yang C, Gao H. The PIWI protein acts as a predictive marker for human gastric cancer. *Int J Clin Exp Pathol*. 2012;5:315–25.
19. Yazarloo F, Shirkoohi R, Mobasher MB, Emami A, Modarressi MH. Expression analysis of four testis-specific genes AURKC, OIP5, PIWIL2 and TAF7L in acute myeloid leukemia: a gender-dependent expression pattern. *Med Oncol*. 2013;30:368.
20. Oh S-J, Kim S-M, Kim Y-O, Chang H-K. Clinicopathologic Implications of PIWIL2 Expression in Colorectal Cancer. *Korean Journal of Pathology*. 2012;46:318.
21. Li L, Yu C, Gao H, Li Y. Argonaute proteins: potential biomarkers for human colon cancer. *BMC Cancer*. 2010;10:38.
22. Wang QE, Han C, Milum K, Wani AA. Stem cell protein Piwil2 modulates chromatin modifications upon cisplatin treatment. *Mutat Res*. 2011;708:59–68.

23. Gainetdinov IV, Skvortsova YV, Stukacheva EA, Bychenko OS, Kondratieva SA, Zinovieva MV, Azhikina TL. Expression Profiles of PIWIL2 Short Isoforms Differ in Testicular Germ Cell Tumors of Various Differentiation Subtypes. *PLoS ONE*. 2014;9:e112528.
24. Lee JH, Jung C, Javadian-Elyaderani P, Schweyer S, SchÄ¼tte D, Shoukier M, Karimi-Busheri F, Weinfeld M, Rasouli-Nia A, Hengstler JG, Mantilla A, Soleimanpour-Lichaei HR, Engel W, Robson CN, Nayernia K. Pathways of Proliferation and Antiapoptosis Driven in Breast Cancer Stem Cells by Stem Cell Protein Piwil2. *Can Res*. 2010;70:4569–79.
25. Chen Y, Hu W, Lu Y, Jiang S, Li C, Chen J, Tao D, Liu Y, Yang Y, Ma Y. A TALEN-based specific transcript knock-down of PIWIL2 suppresses cell growth in HepG2 tumor cell. *Cell Prolif*. 2014;47:448–56.
26. Jiang S, Zhao L, Lu Y, Wang M, Chen Y, Tao D, Liu Y, Sun H, Zhang S, Ma Y. Piwil2 Inhibits Keratin 8 Degradation through Promoting p38-Induced Phosphorylation To Resist Fas-Mediated Apoptosis. *Mol Cell Biol*. 2014;34:3928–38.
27. Yao Y, Li C, Zhou X, Zhang Y, Lu Y, Chen J, Zheng X, Tao D, Liu Y, Ma Y. PIWIL2 induces c-Myc expression by interacting with NME2 and regulates c-Myc-mediated tumor cell proliferation. *Oncotarget*. 2014;5:8466–77.
28. Lu Y, Zhang K, Li C, Yao Y, Tao D, Liu Y, Zhang S, Ma Y. Piwil2 Suppresses P53 by Inducing Phosphorylation of Signal Transducer and Activator of Transcription 3 in Tumor Cells. *PLoS ONE*. 2012;7:e30999.
29. Zhang K, Lu Y, Yang P, Li C, Sun H, Tao D, Liu Y, Zhang S, Ma Y. HILI inhibits TGF-beta signaling by interacting with Hsp90 and promoting TbetaR degradation. *PLoS One*. 2012;7:e41973.
30. Li D, Sun X, Yan D, Huang J, Luo Q, Tang H, Peng Z. Piwil2 modulates the proliferation and metastasis of colon cancer via regulation of matrix metalloproteinase 9 transcriptional activity. *Exp Biol Med (Maywood)*. 2012;237:1231–40.
31. Gao JXL, Wu N, HL. PIWIL2 (piwi-like RNA-mediated gene silencing 2). *Atlas Genet Cytogenet Oncol Haematol*. 2014;18:919–27.
32. Yin D-T, Wang Q, Chen L, Liu M-Y, Han C, Yan Q, Shen R, He G, Duan W, Li J-J, Wani A, Gao J-X. 2011. Germline Stem Cell Gene *PIWIL2* Mediates DNA Repair through Relaxation of Chromatin. *PLoS ONE* 6: e27154
33. Chen L, Shen R, Ye Y, Pu XA, Liu X, Duan W, Wen J, Zimmerer J, Wang Y, Liu Y, Lasky LC, Heerema NA, Perrotti D, Ozato K, Kuramochi-Miyagawa S, Nakano T, Yates AJ, Carson lii WE, Lin H, Barsky SH, Gao JX. Precancerous Stem Cells Have the Potential for both Benign and Malignant Differentiation. *PLoS ONE*. 2007;2:e293.
34. Gao JX. Cancer stem cells: the lessons from precancerous stem cells. *J Cell Mol Med*. 2008;12:67–96.
35. Shen R, Ye Y, Chen L, Yan Q, Barsky SH, Gao JX. Precancerous stem cells can serve as tumor vasculogenic progenitors. *PLoS ONE*. 2008;3:e1652.

36. Gao J-X, Zhou Q. Epigenetic progenitors in tumor initiation and development. *Drug Discovery Today: Disease Models*. 2009;6:5–12.
37. Gao J-X. Development of Tumor Stem Cells: Implication in Field Cancerization. In: Dakubo G, editor. *Field Cancerization: Basic Science and Clinical Applications*. New York: Nova Science Publishers, Inc; 2011. pp. 26–69.
38. Shi RR, Liu J, Zou Z, Qi YM, Zhai MX, Zhai WJ, Gao YF. The immunogenicity of a novel cytotoxic T lymphocyte epitope from tumor antigen PL2L60 could be enhanced by 4-chlorophenylalanine substitution at position 1. *Cancer Immunol Immunother*. 2013;62:1723–32.

## Figures

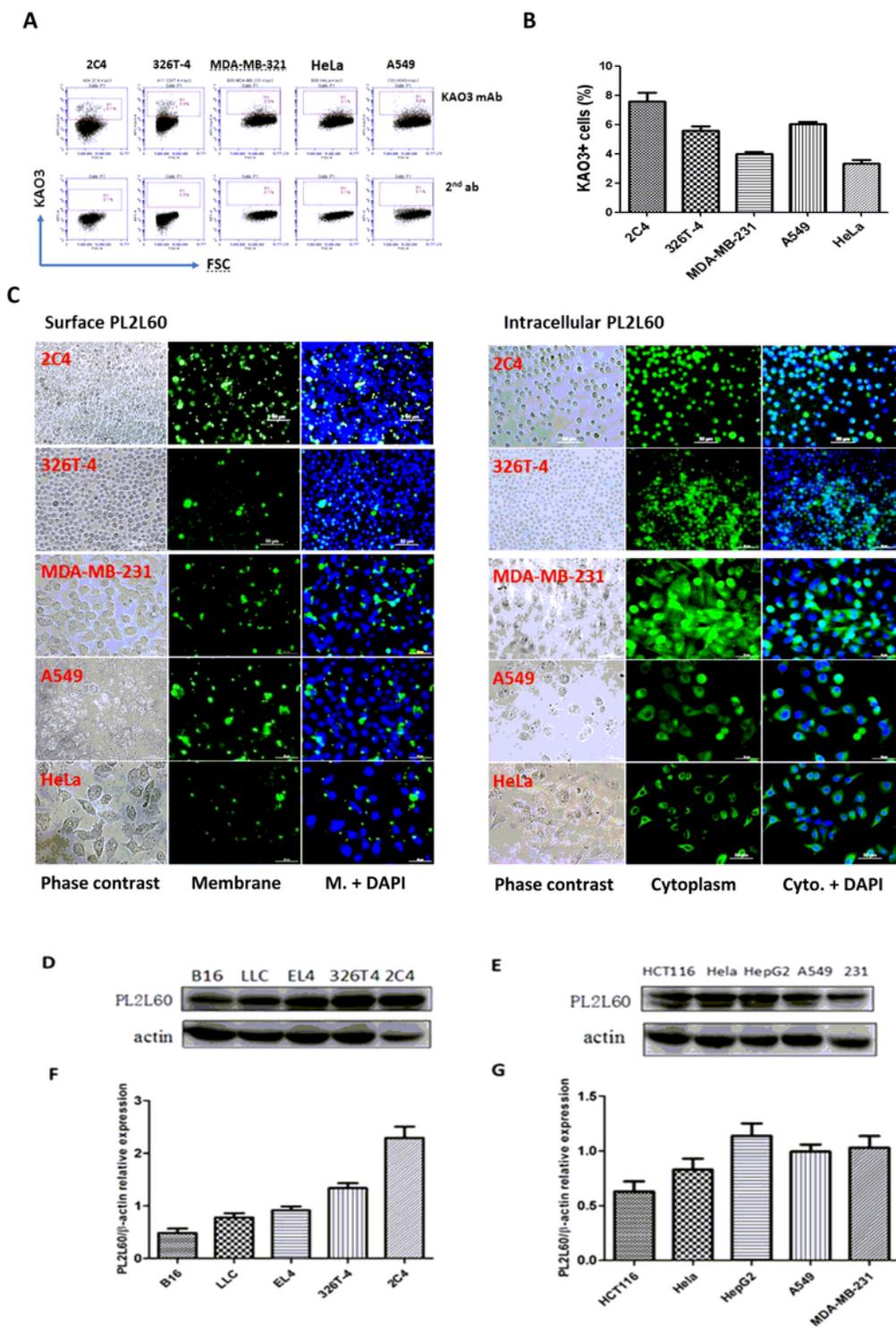


Fig. 1

## 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 cytometry analysis of expression of PL2L60 on cell surface of a panel of cancer cell lines (2C4, 326T-4, MDA-MB-231, A549 and HeLa). Cells were harvested using cells stripper, stained with PL2L60 mAb for 1 h at 4 °C followed by APC-conjugated goat anti mouse IgM, and then analyzed by flow cytometry using BD

C6 software. (B) Summary of three independent experiments from A. (C) Micrographs from immunofluorescent microscopy of surface and intracellular expressions of PL2L60 in cancer cell lines. M.: membrane; Cyto.: cytoplasm. Bars: 50  $\mu$ M. (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 PI2L60 in mouse and human cancer cell lines shown in E and G, respectively.

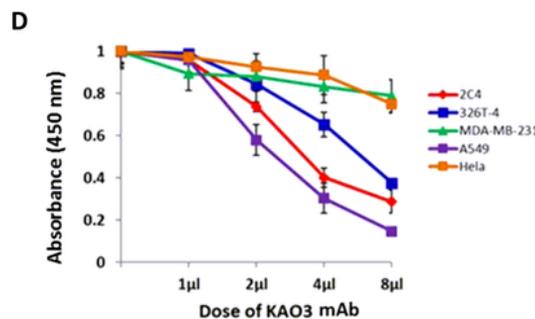
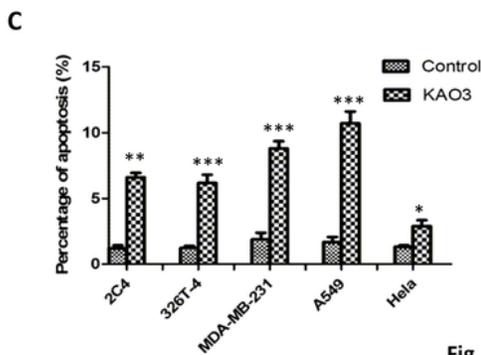
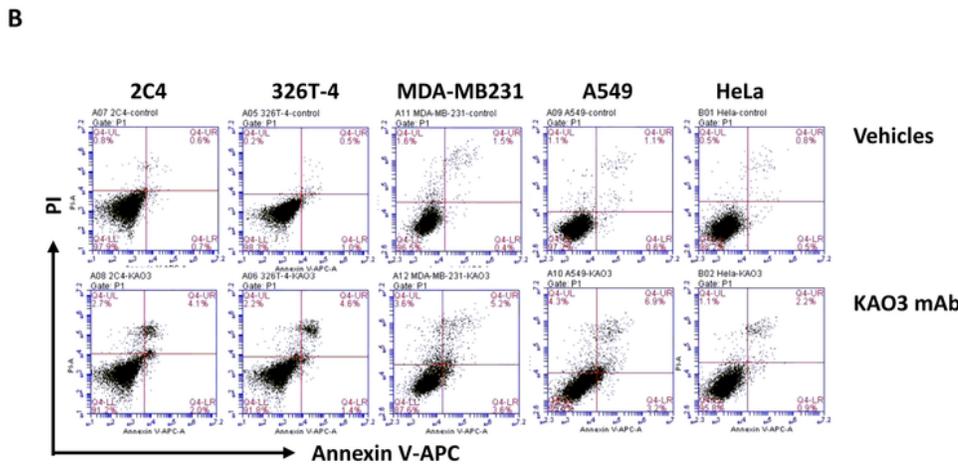
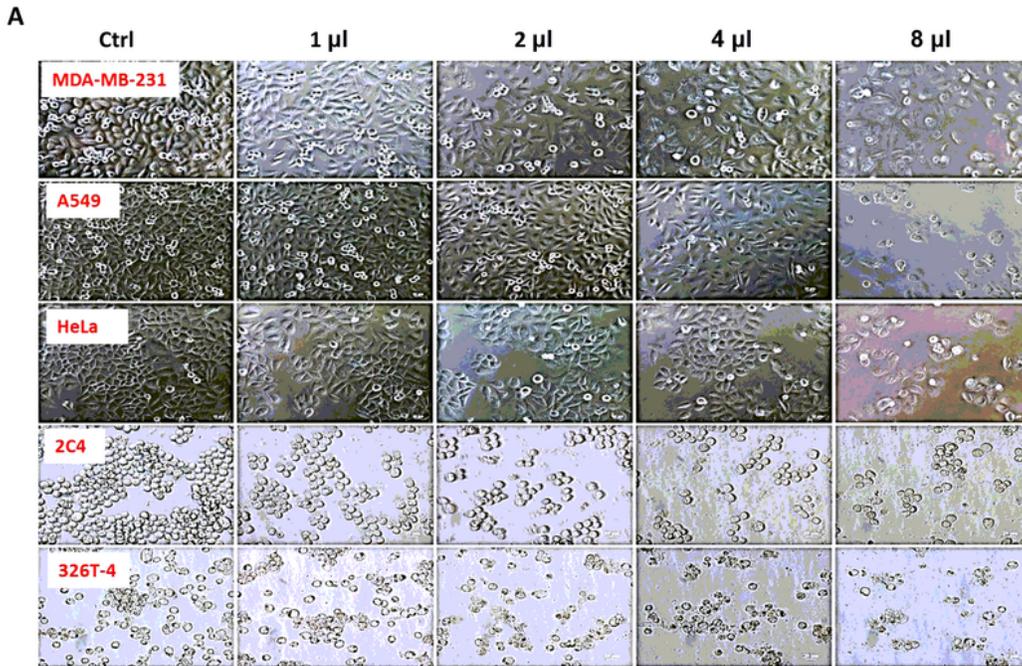
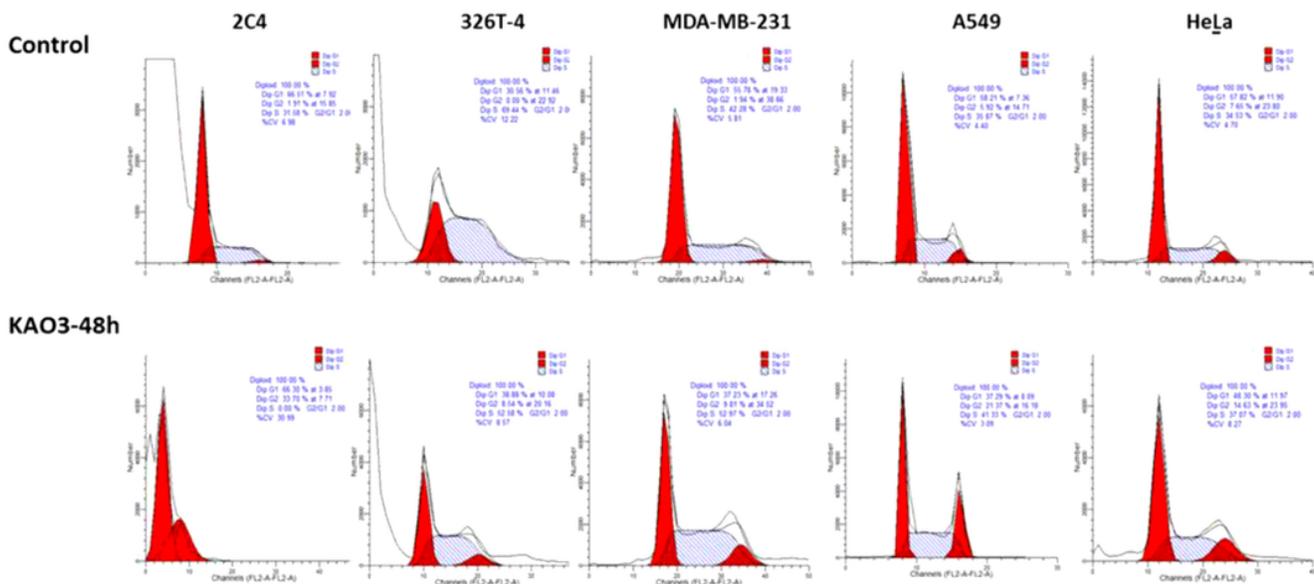


Fig. 2

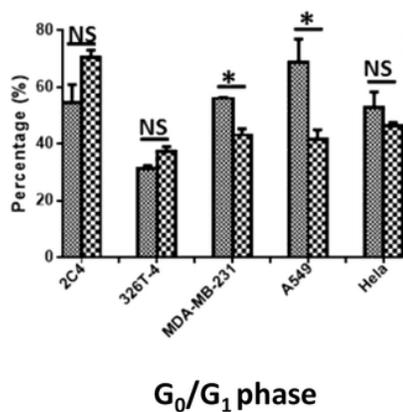
**Figure 2**

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. A dose-dependent cell reduction and apoptotic cells exhibited remarkably. Bars represent 25  $\mu$ m. (B & C) Representative dot plots (B) and summary of three independent experiments (C) revealed by flow cytometry of apoptotic cells from 2C4, 326T-4, MDA-MB-231, A549 and HeLa cells treated by anti-PL2L60 mAb for 48h. Vehicles: controls. (D) Dose-dependent reduction of viable cells after being treated with KAO3 mAb for 48 h, as revealed by CCK8 assay. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; and \*\*\*,  $p < 0.001$ .

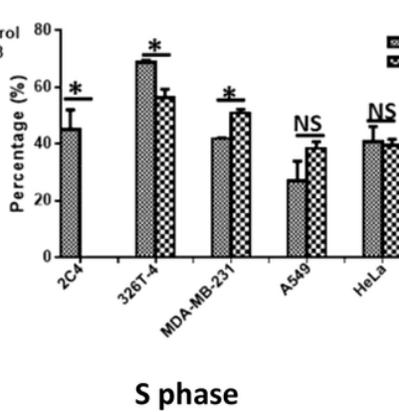
**A**



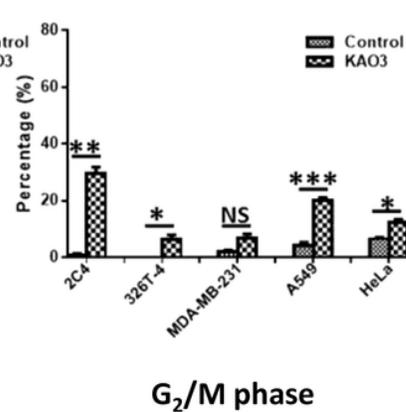
**B**



**C**



**D**



**Fig. 3**

**Figure 3**

KAO3 mAb induced the cell cycle arrest at G2/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 in G0/G1, S and G2/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 \*\*\*,  $p < 0.001$ .

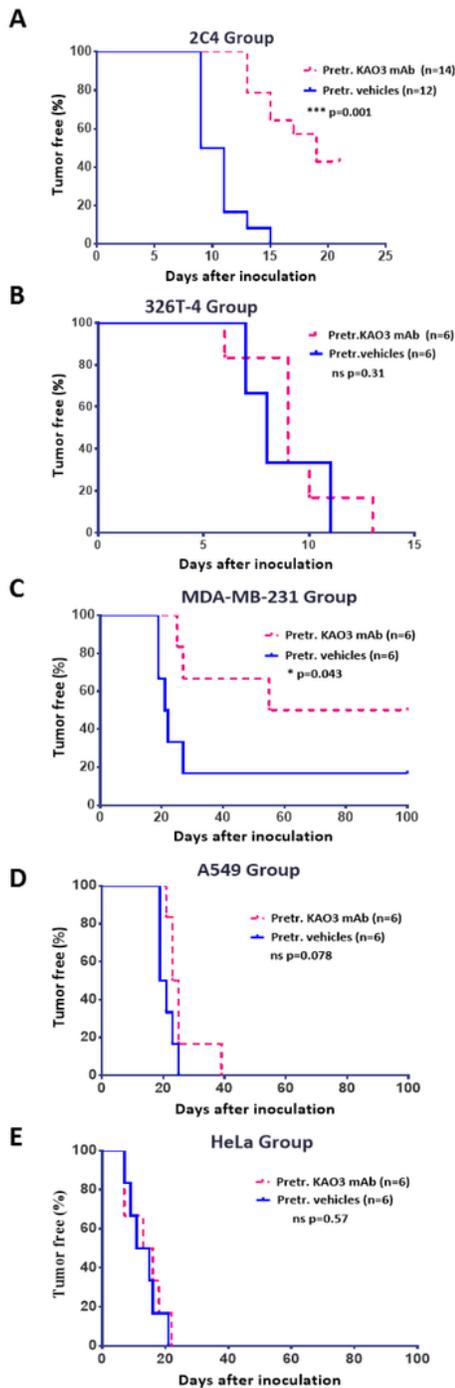


Fig. 4

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 ( $5 \times 10^6$  cells in a volume of 200  $\mu$ l) were injected into the groin of SCID mice or nude mice (2C4) with or without KAO3 mAb supernatants (1:2 dilution). Tumorigenesis was monitored every day until they were palpable. (A) 2C4; (B) 326T-4; (C) MDA-MB-231; (D) A549 and (E) HeLa. \*,  $p < 0.05$ , and \*\*\*,  $p < 0.001$ .

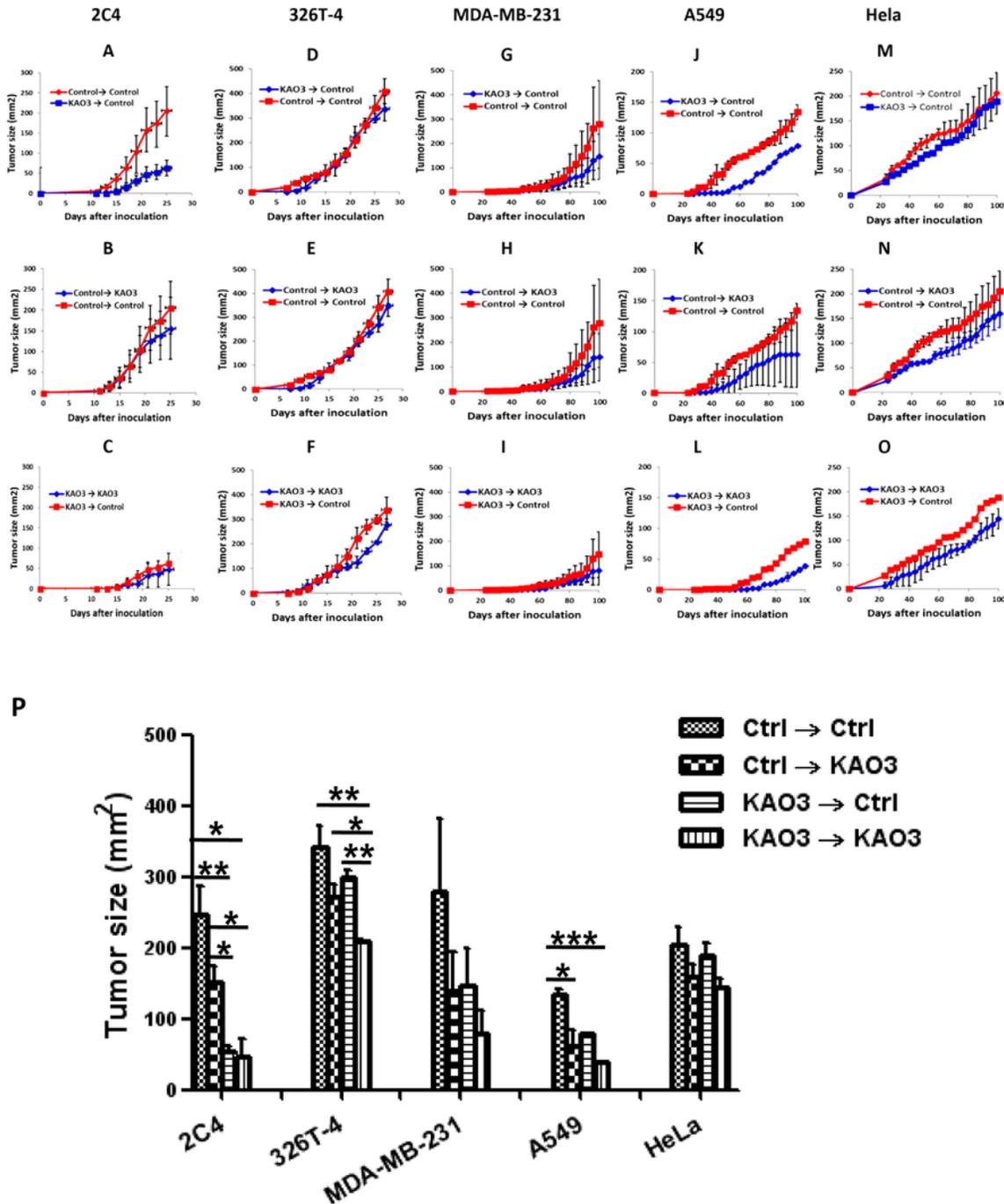
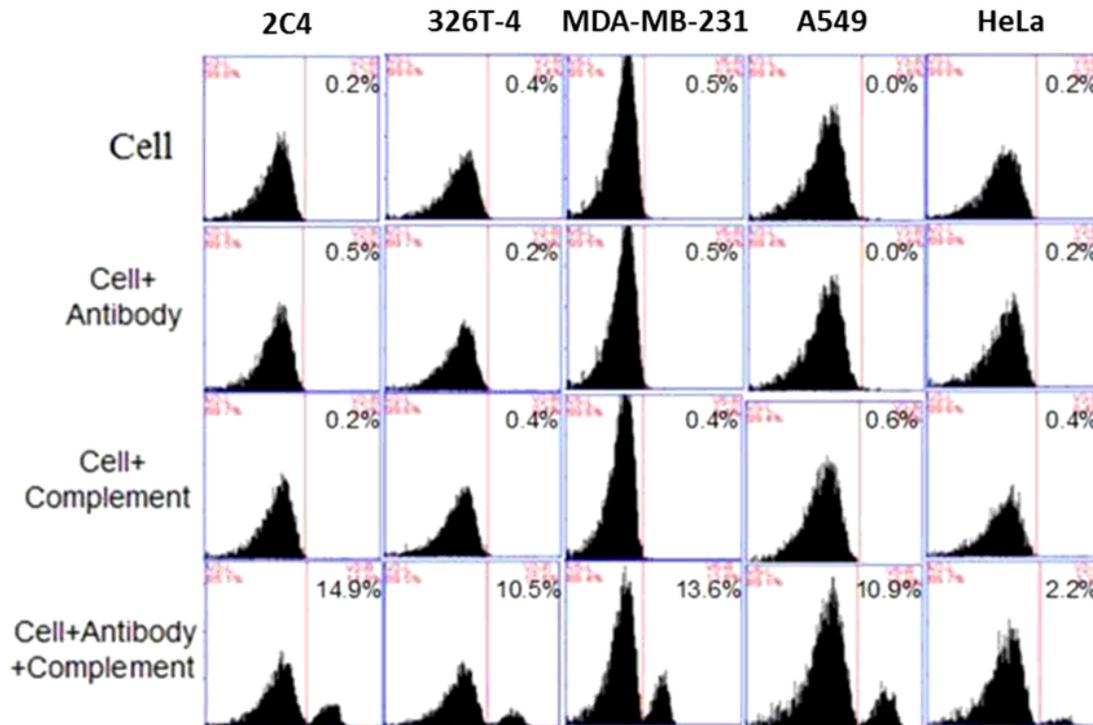


Fig. 5

Figure 5

Therapeutic effects of KA03 mAb on various types of mouse and human cancers. The tumor-bearing mice in the figure 4 were treated with 100  $\mu$ l KA03 supernatants or vehicles every day until the end of experiments. Four groups of treatment were performed for each cancer: control  $\rightarrow$  control (n=3; 2C4: n=6); control  $\rightarrow$  KA03 (n=3; 2C4: n=7); KA03  $\rightarrow$  control (n=3; 2C4: n=6); KA03  $\rightarrow$  KA03 (n=3; 2C4: n=7). Tumor size was measured every two days and calculated by the formula length  $\times$  width, and compared between groups. (A-O): growth kinetics of tumors treated or untreated with KA03 mAb. (A-C) 2C4; (D-F) 326T-4; (G-I) MDA-MB-231; (J-L) A549; (M-O) HeLa. (P) Comparison of tumor size between groups with different treatments in one type of cancers. The mice bearing 2C4 tumors were sacrificed at day 35 of inoculation; 326T-4 at day 30; and MDA-MB-231, A549 and HeLa at day 100days. Error bars show mean  $\pm$  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

**Fig. 6****Figure 6**

The KA03 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 KA03 mAb. Shown are histograms from a representative experiment, indicating a complement-dependent

cytolysis. (B) Summary of three experiments performed in (A). All the groups of the cells + mAb + human complement are significantly higher than other control groups in CDC activity ( $p < 0.001$ ).