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## Original Investigation

# Combination of Curcumin with an Anti-Transferrin Receptor Antibody Suppressed the Growth of Malignant Gliomas In vitro

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### ABSTRACT

**AIM:** Transferrin receptor (TfR) has been used as a target for the molecular cancer therapy due to its higher expression in a variety of tumors. Anti-TfR antibodies combined with chemotherapeutic drugs has showed great potential as a possible cancer therapeutic strategy. In our study, we investigated the anti-tumor effects of anti-TfR monoclonal antibody (mAb) alone or in combination with curcumin in vitro.

**MATERIAL and METHODS:** We detected the apoptosis, proliferation and cell cycle of glioma cells after treated with anti-TfR mAb and curcumin alone or the combinations by flow cytometer.

**RESULTS:** Anti-TfR mAb or curcumin could inhibit proliferation of tumor cells. Anti-TfR mAb marked S phase arrest and curcumin induced G2/M arrest in tumor cells. When anti-TfR mAb and curcumin were used simultaneously, a synergistic effect was detected in relation to tumor growth inhibition and the induction of cells necrosis.

**CONCLUSION:** These results provided a potential role of anti-TfR mAb-containing curcumin in the treatment for gliomas.

**KEYWORDS:** Chemotherapeutic, Curcumin, Glioma, Transferrin receptor

### ■ INTRODUCTION

Malignant glioma is the most common primary malignant tumor in the brain. Although the introduction of temozolomide treatment in addition to radiotherapy after surgical resection has improved survival in patients with glioblastoma (GBM), tumor recurrence is unavoidable (10). After tumor recurrence, chemotherapy still has certain effect, but the patients' survival rates remain poor. Thus, how to improve the effectiveness of the chemotherapy drugs and found new drugs for GBM patients need to solve.

A growing body of research suggests that curcumin, the major active constituent of the dietary spice turmeric, has potential for the prevention and therapy of cancer (3). Being the main ingredient of curries and thus part of the everyday diet of millions of people, curcumin is considered a safe agent in

humans (10). Preclinical data have shown that curcumin can both inhibit the formation of tumors in animal models of carcinogenesis and act on a variety of molecular targets involved in cancer development (3). Accumulating evidence shows that curcumin has a potent anticancer effect both in vitro and in vivo on a variety of cancer cell types, such as leukemia, breast cancer, prostate cancer, and pancreatic cancer (17).

Research on targeted therapy of malignancies has attracted a great deal of interest during the past several years based on its well-justified rationale, the efficient and specific delivery of cytotoxic agents to the tumor tissue (8). The immediate benefit of this tumor-specific delivery is two-fold: Increasing the effective dose of the anti-tumor agent, and decreasing the extent of its related side effects and toxicity (8). Transferrin receptor (TfR) is more abundantly expressed

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in rapidly dividing cells than quiescent cells, and high levels of TfR expression have been identified on many tumors (7). Studies have demonstrated that the TfR is expressed more abundantly in human breast cancer tissue and a potential marker for identifying dividing cells (13). Anti-TfR antibody was supposed to inhibit cell growth and proliferation especially in malignant cancer cells by blocking the engagement of Tf with its receptor and interfere the intake of iron into cell (7).

Combinations of two or more anti-transferrin receptor monoclonal antibodies can interact synergistically to inhibit cell growth in vitro and tumor growth in vivo (15).

In this study, we aimed to assess the potential combined effects of treatment with curcumin and anti-TfR mAbs (7579mAb, a murine monoclonal anti-human TfR IgG antibody) on glioma cell proliferation, necrosis, and cell cycle and to investigate the potential mechanisms of action.

## ■ MATERIAL and METHODS

### *Cells, antibodies and drug*

The glioma cell lines A172 and U87-MG were purchased from the Shanghai Institute for Biological Science of the Chinese Academy of Science. Cells were planted in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (16). Anti-TfR mAb 7579 was kindly provided by Dr. Guanxin Shen (Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China) as described previously (7). Mouse anti-CD71 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Curcumin was purchased from Sigma Chemical (Sigma, St. Louis, MO, USA).

### *Cell viability assay*

Cell viability was determined using the methyl-thiazolyl tetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) quantitative colorimetric assay. After A172 and U87-MG cells were planted in 96-well-plate for 24 hours, cells were co-cultured with curcumin at concentrations of 0, 10, 20, 40, 60 and 80  $\mu$ M for 72 hours. Thereafter, cells were incubated with MTT (0.5 mg/ml) for 3 hours and determined by using a multi-well scanning reader (Tecan GmbH, Crailsheim, Germany) (10). For each experiment, 18 wells were allocated to one treatment or control group. Data are from 4 independent experiments. The viability of untreated cells was considered to be 100%.

### *Cell apoptosis analysis*

A172 and U87-MG cells were treated with 7579 mAb (100 $\mu$ g/mL) alone, curcumin (40 $\mu$ g/mL) alone or the combinations for 72 hours respectively. The same concentrations of non-specific mouse IgG were used as isotype control (16). Apoptosis of tumor cells were detected by labeling FITC-Annexin V and PI (Roche Diagnostics, Mannheim, Germany) using flow cytometry (FCM, Becton-Dickinson LSR II, New York, USA).

### *Cell proliferation analysis*

A172 and U87-MG cells were treated with 7579 mAb alone,

curcumin alone or the combinations for 72 hours after labeling with carboxyfluorescein diacetate (CFSE) (Sigma, St. Louis, MO, U.S.A.) respectively and detected by FCM. The same concentrations of non-specific mouse IgG were used as isotype control (16).

### *Cell cycle analysis*

A172 and U87-MG cells were treated with 7579 mAb alone, Curcumin alone or the combinations for 72 hours respectively. Tumor cells were stained with 50 $\mu$ g/mL propidium iodide (Sigma, St. Louis, MO, USA) containing 0.25 mg/mL RNase A at room temperature for 30 minutes after fixed with 70% ethanol at 4°C overnight and detected by FCM (11).

### *Statistical analysis*

Statistical significance of the data was examined by ANOVA or Student's t-test using SPSS 10.0 statistical software (SPSS, Chicago, USA). A significance level of  $p < 0.05$  was chosen.

## ■ RESULTS

### *Curcumin is a potent inhibitor of GBM viability*

We incubated A172 and U87-MG cells with different concentrations of curcumin and measured cell viability using the MTT assay to detect whether curcumin can effect on cell survival viability. Results showed that cell viability decreased in a dose-dependent manner in both cell types (Figure 1A, B) (\* $P < 0.05$ ). The 50% inhibitory concentration was approximately 40  $\mu$ M for both cell types.

### *Combined effects of 7579 mAb and curcumin on the apoptosis of A172 and U87-MG cells*

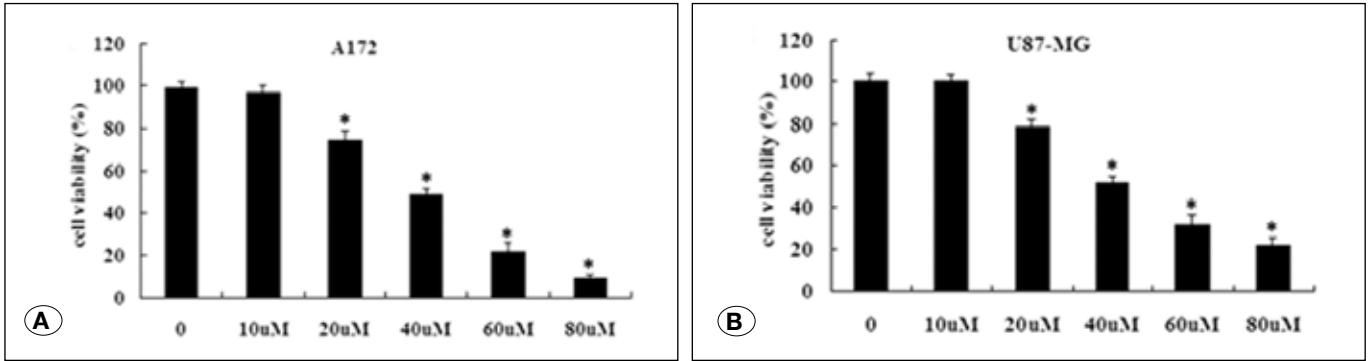
The apoptosis of A172 and U87-MG cells after combined treatment with 7579 mAb and curcumin for 72 hours were examined by FCM with PI-Annexin V staining. As shown in Figure 2A-F, 7579 mAb induced apoptosis in tumor cells. However, curcumin induces necrosis but not apoptosis in A172 and U87-MG cells (\* $p < 0.05$ ).

### *Proliferation inhibitory effects of 7579 mAb in combination with curcumin on A172 and U87-MG cells*

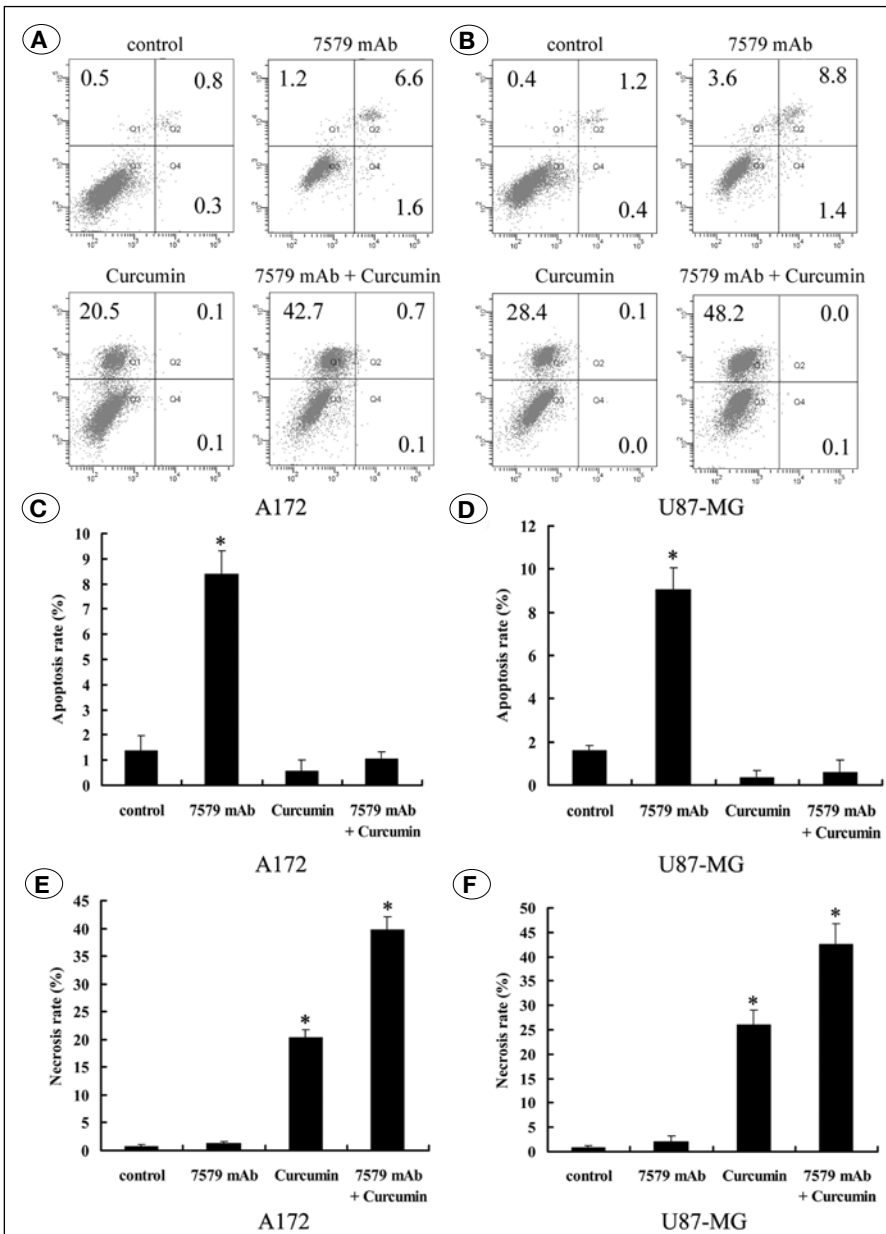
The proliferation of A172 and U87-MG cells after combined treatment with 7579 mAb and curcumin for 72 hours were detected by FCM with CFSE staining. As shown in Figure 3A-D, either 7579 mAb/curcumin combinations or each agent alone inhibited proliferation of tumor cells. Furthermore, compared with curcumin or 7579 mAb used alone, combined treatment with 7579 mAb and curcumin significantly inhibited proliferation of tumor cells (\* $p < 0.05$ ).

### *Combined effects of 7579 mAb and curcumin on the cell cycle of A172 and U87-MG cells*

Figure 4A-D showed the cell cycle distribution of A172 and U87-MG cells after combined treatment with 7579 mAb and curcumin for 72 hours was detected by FCM with PI staining. 7579 mAb marked S phase arrest and curcumin induced G2/M arrest in tumor cells compared to that of isotype control groups (\* $p < 0.05$ ).



**Figure 1:** Curcumin inhibits glioma cell viability. **A)** Dose-dependency of cell viability when A172 cells were treated with curcumin after 72 hours. **B)** Dose-dependency of cell viability when U87-MG cells were treated with curcumin after 72 hours. The viability of untreated cells was considered 100%.



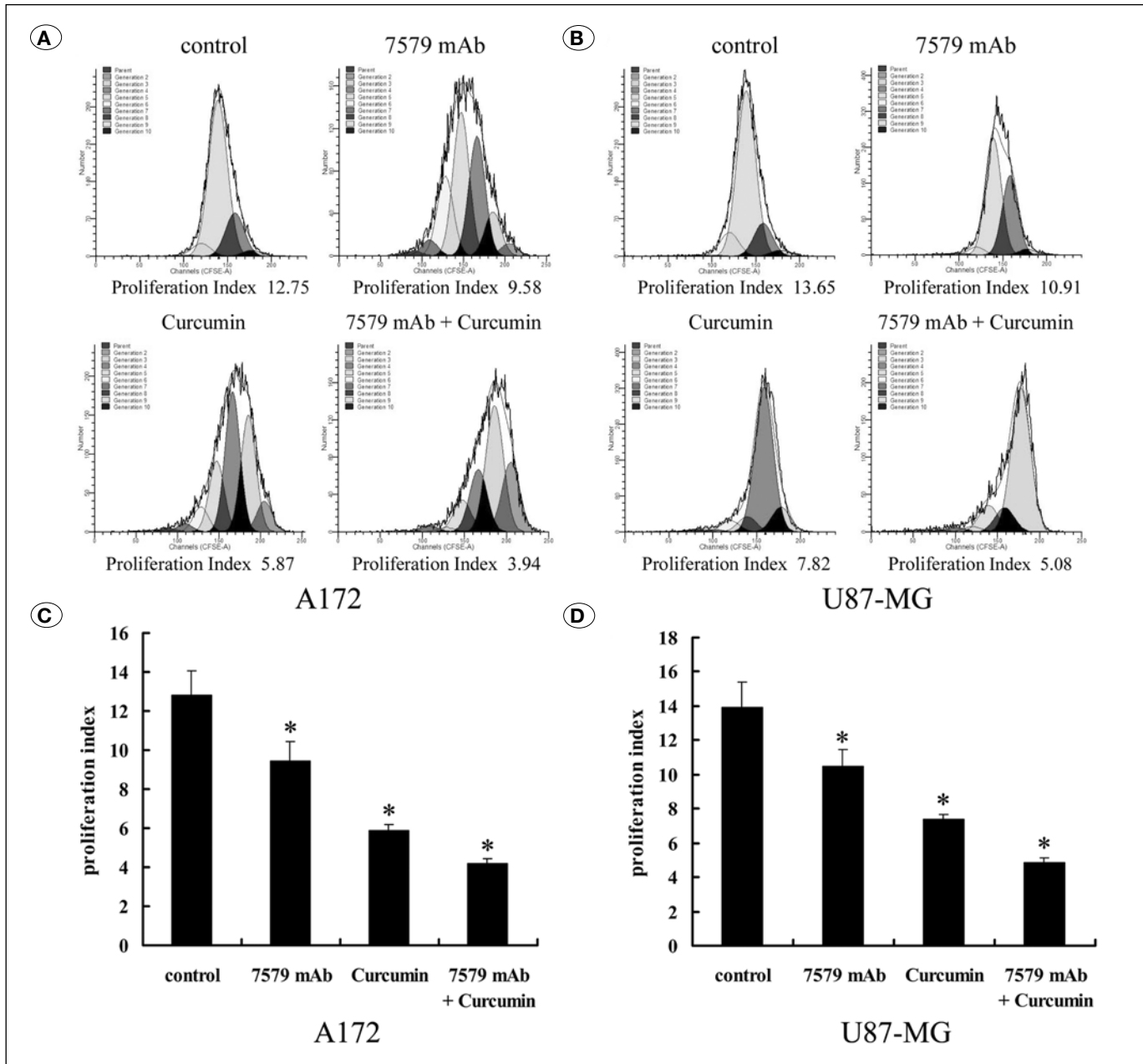
**Figure 2:** Combined effects of mAb and curcumin on apoptosis and necrosis of glioma cells. **A)** Apoptosis and necrosis of A172 cells detected with FCM after treated with mAb/ curcumin combinations or each agent alone. **B)** Apoptosis and necrosis of U87-MG cells detected with FCM after treated with mAb/curcumin combinations or each agent alone. **C)** The statistical analysis of apoptosis of A172 cells. **D)** The statistical analysis of apoptosis of U87-MG cells. **E)** The statistical analysis of necrosis of A172 cells. **F)** The statistical analysis of necrosis of U87-MG cells. Data represent the means and standard deviation of 4 independent experiments.

**DISCUSSION**

Till now, GBMs are incurable malignant tumors. Although the addition of chemotherapy to radiotherapy significantly prolongs survival among patients with newly diagnosed GBM, with a median increase in survival of 2.5 months, nevertheless, the challenge remains to improve clinical outcomes further (14). Ideally, a chemotherapeutic drug would prove efficacious selectively against tumor cells without inducing unwanted side effects (10). Studies showed that the ability of curcumin

to kill tumor cells and not normal cells made it an attractive candidate for drug development (9).

Several studies have shown that TfR is expressed more abundantly in malignant tissues than in their healthy counterparts due to tumor cells in a highly proliferative state (6). This suggests that targeting mediated by TfR might provide a targeting marker in tumor therapeutic strategy. About the mechanisms of anti-TfR antibody mediated growth inhibition, different antibodies had shown different modes of action in



**Figure 3:** Combined effects of mAb and curcumin on proliferation of glioma cells. **A)** Proliferation of A172 cells detected with FCM after treated with mAb/curcumin combinations or each agent alone. **B)** Proliferation of U87-MG cells detected with FCM after treated with mAb/curcumin combinations or each agent alone. **C)** The statistical analysis of proliferation of A172 cells. **D)** The statistical analysis of proliferation of U87-MG cells. Data represent the means and standard deviation of 4 independent experiments.

different models, including blocking Tf binding to the receptor, blocking the internalization of TfR/Tf (transferrin) complex, down-regulating cell-surface TfR or causing TfR intracellular degradation (4).

In previous studies, our have showed that anti-TfR antibody together with chemotherapeutic drugs has potential for cancer therapy and TfR as a surface marker for tumor targeting was a plausible strategy (16). In this study, we have demonstrated that TfR was a suitable targeting molecule for therapeutic strategy in gliomas.

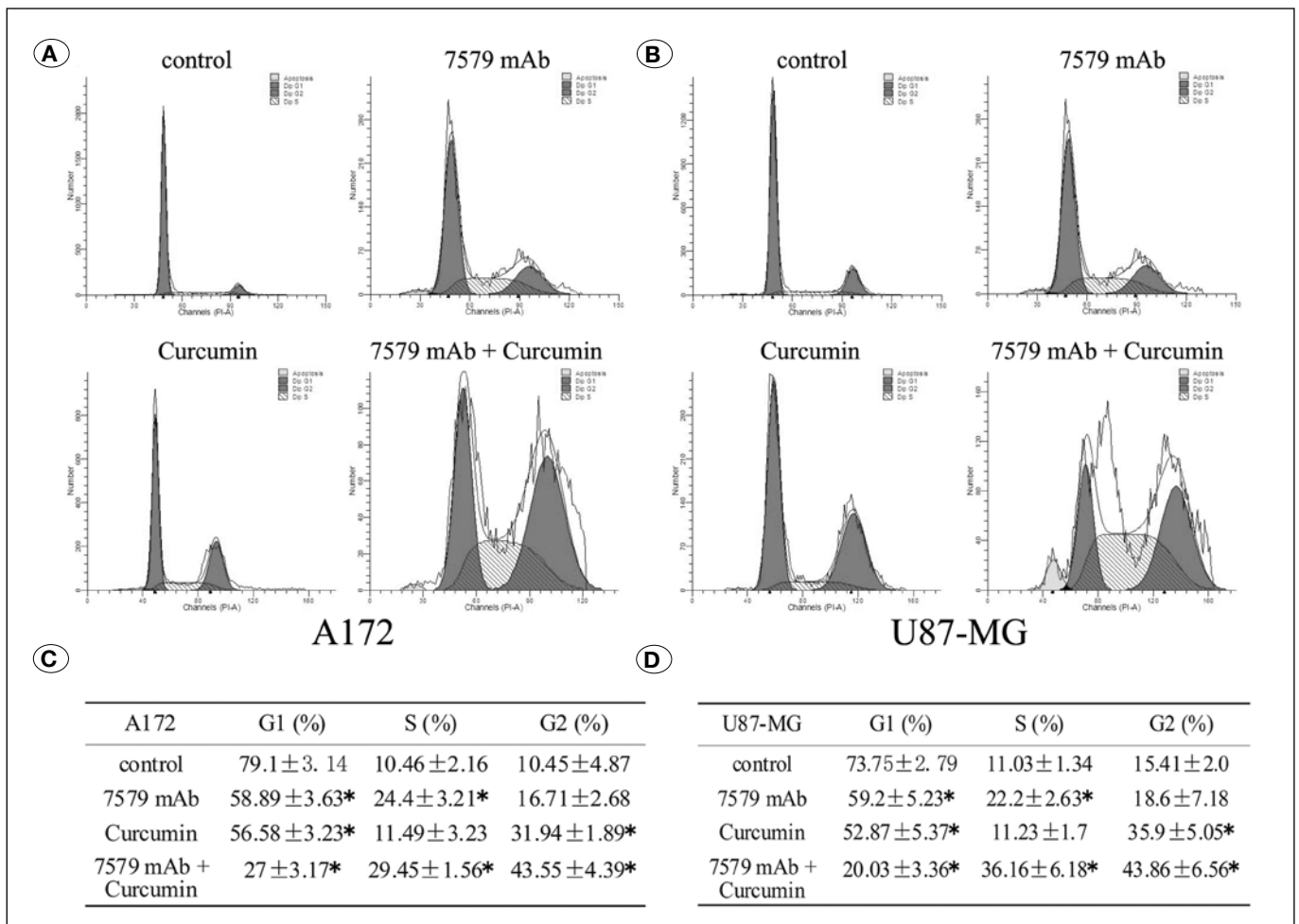
We observed that cell growth was reduced in all cell lines in dose-manner when treated with curcumin and we did not detected significant increase of apoptosis in our apoptosis assays. The suppression proliferation effects of curcumin as detected with MTT and FCM assays indeed reflect an inhibition of tumor cells growth and were caused by an overall cell loss due to necrosis in the assays.

7579 mAb could significantly marked S phase arrest and curcumin induced G2/M arrest in tumor cells. As was shown

in this study, 7579 mAb enhanced the proliferation inhibition of curcumin on tumor cells. We detected the combined effects of 7579 mAb and curcumin on tumor cells viability to illustrate the decreased proliferation of tumor cells after combined treatment and we found that curcumin did not induce tumor cell apoptosis but necrosis. When curcumin was co-cultured with 7579mAb on tumor cells it exhibited synergistic cytotoxic effects.

As showed in the current data that the changes in cell cycle distribution caused by 7579 mAb showed greater therapeutic effect than that expected by the simple addition of the effects of the component drug. 7579 mAb increased the percentage of cells in S phase as well as decreased the percentage of cells undergoing G1 phase, suggesting that an increased sensitivity to curcumin may due to an increase in the percentage of tumor cells in S phase.

Monoclonal antibodies (MAb) are potential large molecule drugs for the brain. However, MAb's do not cross the blood-brain barrier (BBB) (1). A molecular Trojan horse is an



**Figure 4:** 7579 mAb induces S arrest and curcumin induces G2/M arrest. **A)** Cell cycle of A172 cells detected with FCM after treated with mAb/curcumin combinations or each agent alone. **B)** Cell cycle of A172 cells detected with FCM after treated with mAb/curcumin combinations or each agent alone. **C)** The statistical analysis of cell cycle phase distribution of A172 cells. **D)** The statistical analysis of cell cycle phase distribution of U87-MG cells. Data represent the means and standard deviation of 4 independent experiments.

endogenous peptide, or peptidomimetic monoclonal antibody (mAb) (such as the insulin receptor or the transferrin receptor), which enters brain from blood via receptor-mediated transport on endogenous BBB transporters (5). The BBB TfR is a bi-directional transport system and enables the receptor-mediated transcytosis of either transferrin (Tf) or a TfR mAb from the blood to the brain (16). The transferrin receptor (TfR) is expressed at both the BBB and the brain cell membrane (BCM) (6), and data showed that an antisense imaging agent comprised of an iodinated peptide nucleic acid (PNA) conjugated to a monoclonal antibody to the rat transferrin receptor was transported through the blood-brain barrier (12). The present studies have shown that 8D3 rat mAbs to the mouse TfR are effectively transported into brain in vivo and may be used for brain drug-targeting studies in mice such as transgenic mouse models (2). Thus, 7579 mAb can permeate through blood brain barrier and deliver curcumin to brain tumors when combining with curcumin and develop synergistic anti-tumor effects in vivo. The 7579 mAb is a murine monoclonal anti-human TfR IgG antibody and it can permeate through BBB of human exclusively, but it cannot be detected in a mouse model.

In summary, we have analyzed the combined effects of anti-TfR antibody and curcumin on glioma cells in vitro. These studies suggest that combination therapy was more effective than the use of either agent alone and 7579 mAb would have more efficient anti-tumor activity in future clinical trials. Our research results would lead to a greater foundation of its great potential in molecular-targeted application for tumor clinical treatment.

## ■ ACKNOWLEDGEMENTS

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