RESEARCH ARTICLE

Aptamer TY04 inhibits the growth of multiple myeloma cells via cell cycle arrest

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Abstract The aptamer TY04 is a single-stranded DNA. However, its biological function has not been elucidated. Here, we found that TY04 specifically bound to multiple myeloma cells MM.1S, and some membrane proteins on the surface of MM.1S cells constituted the target molecules of TY04. TY04 inhibited the growth of multiple myeloma cell lines, induced cell cycle arrest in mitosis, and resulted in a significant accumulation of binucleated cells. Following TY04 treatment, a concomitant increase in CDK1 and cyclin B1 expression occurred. In addition, TY04 treatment also resulted in a significant downregulation of γ-tubulin. Considering the unique advantages of aptamers, TY04 shows great potential as a drug candidate to treat multiple myeloma.

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Introduction

Multiple myeloma (MM), also known as plasma cell myeloma, is characterized by the accumulation of clonal plasma cells in bone marrow and overproduction of monoclonal immunoglobulin (Ig) in blood or the urine [1]. MM is the second most common hematological cancer worldwide, accounting for approximately 10 % of all hematologic malignancies [2]. Currently, the 5-year median survival rate for MM patients undergoing conventional chemotherapy is approximately 25 % [3]. Despite recent advances in the understanding and treatment of this disease, MM remains an incurable disease for the vast majority of patients, prompting the continued search for additional therapeutic strategies.

Aptamers are single-stranded RNA or DNA sequences that can fold into unique three-dimensional (3D) structures, allowing them to form stable and specific complexes with different target molecules of complementary shape [4–6]. The molecular recognition properties of aptamers, such as high affinity and specificity, are similar to antibodies. However, compared to antibodies, aptamers have unique advantages, including easy chemical synthesis and modification, low toxicity, lack of immunogenicity, and rapid tissue penetration [7]. In addition to recognition, some aptamers can regulate biological pathways and interfere with disease development through binding to molecular targets involved in pathogenesis [8]. Based on these advantages, aptamers show great potential for therapeutic application. For example, Macugen, an anti-VEGF aptamer, which recognizes the majority of human VEGF-A isoforms, is the first aptamer-based therapeutic approved by the U.S. Food and Drug Administration (FDA) for treatment of age-related macular degeneration (AMD). Other aptamers are currently undergoing clinical evaluation. For example, AS1411, a G-rich aptamer, is able to inhibit cell proliferation in a broad range of cancers through binding to nucleolin and is currently in phase 2 trials for AML [9].

Aptamers are generated from large libraries containing 10^{13} to 10^{16} random nucleic acid sequences through Systematic Evolution of Ligands by Exponential Enrichment (SELEX) [5, 6]. To obtain cell-specific binding aptamers, cell-based SELEX (CELL-SELEX) was specifically developed to select aptamers against live cells [10]. CELL-SELEX does not require identification of molecular targets. Additionally, it is unnecessary to purify targets by processes that may disrupt the native conformation. Therefore, aptamers selected using live cells can bind to the naturally folded conformation of the target on the surface of the cells. Using CELL-SELEX, Cerchia's group generated a panel of aptamers that were able to bind with high affinity to malignant glioma cells rather than non-tumorigenic glioma cells. Interestingly, these aptamers showed functional activity against the proliferation of malignant glioma cells [11].

In previous work, we have generated a series of aptamers for the specific recognition of different cells through CELL-SELEX. We found that the aptamer TY04 could inhibit the growth of MM cell lines, including MM.1S, NCI-H929, KM3, and OPM2. Flow cytometric analysis showed that TY04 bound to MM.1S cells. However, TY04 partly lost its recognition ability when MM.1S cells were briefly treated with trypsin and proteinase K before incubation with TY04, indicating that cell surface membrane proteins must constitute some of the target molecules recognized by TY04. Furthermore, we revealed that TY04 induced significant cell cycle arrest at mitosis and resulted in the accumulation of binucleated cells. Following TY04 treatment, a concomitant increase of CDK1 and cyclin B1 expression occurred. In addition, TY04 treatment also resulted in a downregulation of γ-tubulin. These results provide a framework for further exploration of the mechanism of TY04 anti MM.

Materials and methods

Cell lines and cell culture

All cultures were grown in a humidified incubator maintained at 37 °C with 5 % CO_2. NCI-H929 cells (CRL-9068) and MM.1S cells (CRL-2974) were obtained from the American Type Culture Collection (ATCC, USA). KM3 cells were purchased from Shanghai YRGene Co. of China, and OPM2 cells were purchased from Shanghai Bioleaf Biotech Co. of China. All of the cells were grown in RPMI 1640 medium (Gibco) supplemented with 10 % fetal bovine serum (FBS, Gibco), penicillin (100 units/ml), and streptomycin (100 mg/ml). The TY04 aptamers were obtained from Sangon Biotech Shanghai Co., Ltd. and were synthesized using the following sequences: 5′-ATCGTCTG CTCCGTCCAATATATCAAAGGCGAATTTTGTCAAGG TGTTAAACGATAGTCCCTACCTTTGGTGTGAGGTCG TGC-3′ , and 5′-FITC-TY04.

Cell viability assay

Cells were seeded at a density of 3×10^6 cells/ml in a 96-well plate, at a final volume of 100 μl/well, and then treated with various concentrations of TY04. After incubation for 72, 96, or 120 h, the cells were exposed to 10 μl of Cell Counting Kit-8 (CCK-8, Beyotime, China) for 2 h at 37 °C; next, the absorbance at 450 nm was measured in a microplate reader.

Binding assay

The binding affinity of the aptamer TY04 for MM.1S cells was detected by incubating 1×10^6 MM.1S cells with varying concentrations of FITC-labeled TY04 on ice for 50 min in the dark in 1 ml of binding buffer containing 20 % FBS. The binding buffer used for selection was prepared by adding yeast tRNA (0.1 mg/ml; Sigma) and BSA (1 mg/ml; Sigma) into the wash buffer to diminish background binding. Cells were then washed twice with 0.7 ml of binding buffer with 0.1 % sodium azide, suspended in 0.4 ml of binding buffer with 0.1 % sodium azide, and then subjected to flow cytometric analysis within 30 min. The FITC-labeled unselected ssDNA library was used as a negative control to distinguish nonspecific binding from specific binding. Cell fluorescence was determined using the FACScan cytometer. All of the experiments for the binding assays were repeated three times.

Treatment of cells with trypsin or proteinase K

MM.1S cells (1×10^6) were washed with 1 ml of PBS and then incubated with 1 ml of PBS containing either 0.05 % trypsin or 0.1 mg/ml proteinase K at 37 °C for 2 or 10 min. After treatment, the cells were washed twice with 2 ml of binding buffer. The treated cells were then used for the binding assays, as described in the section of binding assay.

Analysis of cell cycle by flow cytometry

Cells were plated in RPMI 1640 medium supplemented with 10 % (v/v) FBS (Hyclone) at a density of 3×10^6 cells/ml in a 96-well plate. Cells were treated by direct addition of the oligonucleotide to the culture medium at a final concentration of 4 μM. After incubation for 96 h, cells were fixed and stained with propidium iodide with the Cycle Test Plus kit (Beckman), then analyzed using the FACScan cytometer. The percentage of cells in G0/G1, S, and G2/M phases was determined using Modfit software. In a few cases where Modfit was unable to assign these parameters, they were assigned by integrating peak areas with CellQuest software.

Apoptosis assay

Cells were plated at 3×10^6 cells/ml in a 96-well plate in RPMI medium 1640 supplemented with 10 % (*v/v*) FBS (Hyclone). After cells were treated with 4 μM TY04 for 96 h, the cells were collected and stained with Annexin V and propidium iodide (PI; Roche) for 10 min. After washing with PBS, the cells were suspended in 400 μl PBS and analyzed using the FACScan cytometer.

Nuclear staining of MM.1S cells with Hoechst 33258

After cells were treated with TY04 for 96 h, the cells were collected and stained with 0.01 mg/ml Hoechst 33258 for 10 min. After washing with PBS, the cells were suspended in PBS and mounted with coverslips. The nuclei of MM.1S cells were observed using fluorescence microscopy.

Analysis of protein expression by flow cytometry

Cells were plated in RPMI medium 1640 supplemented with 10 % (*v/v*) FBS at a density of 3×10^5 cells per well in a 96-well plate. After cells were treated with 4 μM TY04 for 96 h, the cells were collected and fixed with 4 % paraformaldehyde for 40 min, followed by treatment with 0.2 % Triton X-100 (including 5 % FBS) for 10 min for membrane permeabilization. A total of 10^6 cells were subsequently incubated with appropriate concentrations of antibodies on ice for 1 h. Because the antibodies were directly conjugated with PE or FITC, secondary antibody staining was not needed for CDK1-PE (Bioss, bs-0542R) and cyclin B1-FITC (Bioss, bs-0572R). For γ-tubulin (Santa Cruz Biotechnology, sc-17787), the cells were subsequently washed twice with PBS-BSA buffer (PBS supplemented with 0.1 % BSA) and incubated with AlexaFluor 488-conjugated anti-mouse IgG for 30 min on ice. Samples were analyzed using a FACScan cytometer. Unstained cells (for CDK1 and cyclin B1 staining) or preimmune IgG were used as negative controls. The mean fluorescence intensity was used as a measure of the level of protein expression.

Statistical analysis

All results were analyzed using SPSS 15.0 software. The significant difference between the groups was determined by analysis of variance and Tukey's range test. Differences among groups were considered statistically significant at *P* less than 0.05.

Results

Aptamer TY04 inhibits proliferation of MM cell lines

In our previous work, we generated a series of aptamers for the specific recognition of different cells through CELL-SELEX. However, most of these aptamers have not been evaluated for therapeutic potential. To identify whether these aptamers have the ability to inhibit the growth of MM cell lines, multiple aptamers were selected for testing using the Cell Counting Kit-8 assay. We found that aptamer TY04 could inhibit the growth of a panel of MM cell lines, including MM.1S, NCI-H929, KM3, and OPM2 cells (Fig. 1a showed the relative survival rate of different cell lines after TY04 treatment for 96 h). Because the MM.1S cell line was the most sensitive to TY04 among these cell lines, it was selected as a model to further explore the detailed action of TY04 on MM. We found that TY04 decreased the survival of MM.1S cells in a dose-dependent manner. The concentration of TY04 needed to inhibit 50 % of the growth (IC_{50}) of MM.1S cells was 3.89 μM. In contrast, the unselected ssDNA library (Lib) did not exhibit the significant inhibitory effect on the growth of MM.1S cells (Fig. 1b).

Aptamer TY04 has binding affinity for MM.1S cells

To investigate whether TY04 has binding affinity for MM.1S cells, the unselected ssDNA library and TY04 were incubated with MM.1S cells at 4 °C, respectively. As shown in Fig. 2a, no positive signals were observed in the unstained group (red histogram) and the ssDNA library (blue histogram). However, a large increase of fluorescence intensity (green histogram) was observed after the cells were incubated with TY04. Additionally, as MM.1S cells were incubated with increasing concentrations (0, 25, 125, and 500 nM, respectively) of TY04, the fluorescence intensity correspondingly increased (Fig. 2b). These results suggest that TY04 has binding affinity for MM.1S cells.

To identify the binding ability of TY04 at human physiological temperature (37 °C), MM.1S cells were incubated with TY04 at 4 or 37 °C for 30 min, and binding affinity was measured by flow cytometry. We found that TY04 bound to MM.1S cells with the same efficiency at either 4 or 37 °C (Fig. 2c). Furthermore, it was confirmed using fluorescence microscopy that TY04 bound to the surface of MM.1S cells at both 4 and 37 °C (Fig. 2d).

Some membrane proteins on the surface of MM.1S cells constitute the target molecules of TY04

To demonstrate whether the binding targets of TY04 on MM.1S cells are cell surface membrane proteins, FITC-
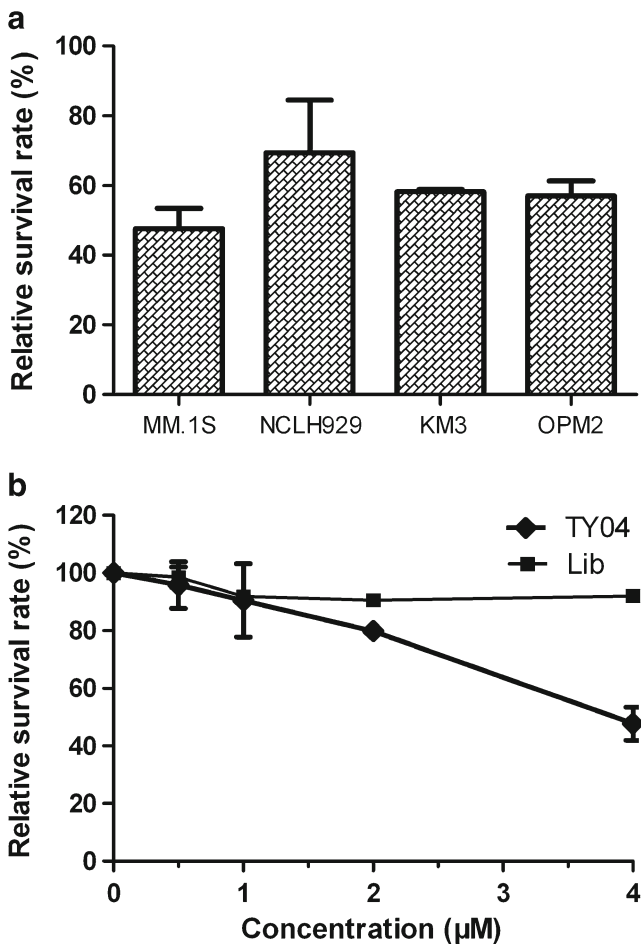
Fig. 1 TY04 inhibited the proliferation of MM (*MM*) cell lines. **a** A panel of MM cell lines was treated for 96 h with 4 μM TY04. **b** MM.1S cells were treated with different concentrations of unselected ssDNA library (Lib) and TY04 for 96 h. The relative survival rate of the above treatments was assessed by the CCK-8 method. Optical density was recorded at 450 nm. The data are presented as the mean±SD for three independent experiments

labeled TY04 was incubated with trypsin- or proteinase K-treated MM.1S cells followed by flow cytometric analysis. As shown in Fig. 3a and b, TY04 showed decreased binding to MM.1S cells after treatment with trypsin or proteinase K for 2 or 10 min (blue line or yellow line) compared to the untreated group (red line). In addition, we also found that the binding ability of TY04 to MM.1S cells could not be completely abolished by trypsin or proteinase K, suggesting that only some membrane proteins on the surface of MM.1S cells were target molecules for TY04 binding.

TY04 induced mitotic arrest in MM.1S cells

To investigate whether TY04 could affect the cell cycle progression of MM.1S cells, synchronized cells treated with 4 μM TY04 for 96 h were subjected to flow cytometric analysis after DNA staining. Representative histograms for cell cycle distribution in MM.1S cells were shown in Fig. 4a. The effects of TY04 on MM.1S cell cycle distribution were summarized in Fig. 4b. We found that exposure of MM.1S to a growth-suppressive concentration of TY04 (4 μM) for 96 h induced a marked accumulation of cells in G2/M phase. The percentage of cells in G2/M phase increased from 30.1 ± 1.56 % in the control to 53.2 ± 6.36 % in populations treated with TY04 ($P<0.05$), which was accompanied by a decrease in G0/G1 phase and S phase cells (Fig. 4a and b). These results revealed that the growth-inhibitory effect of TY04 against MM.1S cells correlated with a cell cycle arrest in G2/M phase.

Furthermore, the nuclei of MM.1S cells were stained with Hoechst 33258 and examined by fluorescence microscopy. We found that most cells treated with 4 μM TY04 morphologically resembled cells in late mitosis, i.e., in anaphase and
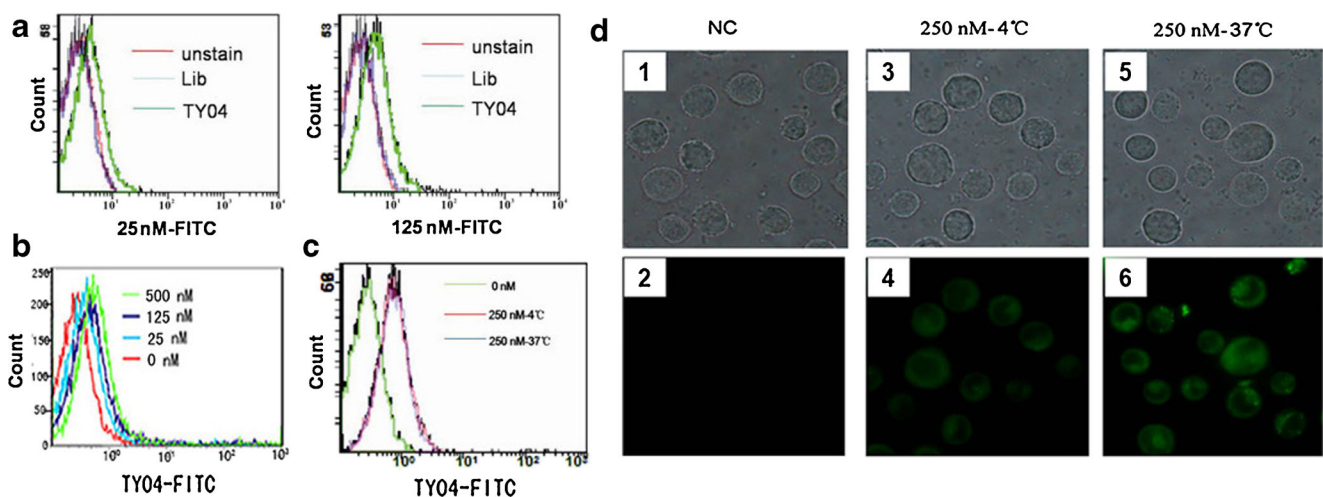

Fig. 2 Binding affinity assays of TY04 with MM.1S cells. The fluorescence intensity of MM.1S cells binding **a** with unselected ssDNA library (*Lib*) and TY04, **b** with different concentrations of TY04, and **c** with 250 nM TY04 at 4 or 37 °C was assayed by flow cytometry. **d** The fluorescence images of MM.1S cells were observed using fluorescence microscopy after incubation with FITC-labeled TY04 at 4 and 37 °C

Fig. 3 Binding affinity of TY04 for trypsin- or proteinase K-treated MM.1S cells. After 2 or 10 min of **a** trypsin or **b** proteinase K treatment of the cells, the fluorescence intensity of MM.1S cells binding with 250 nM TY04 was detected by flow cytometry. (*NC*=negative control)
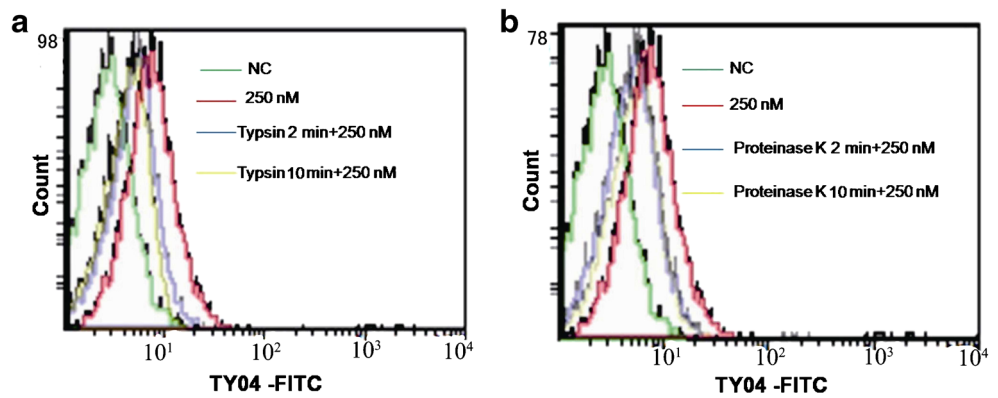
telophase, and that these cells were not frequently observed in the untreated cells, resulting in a significant accumulation of binucleated cells from 5±2 % in the untreated cells to 28±5 % in the TY04-treated cells (Fig. 5).
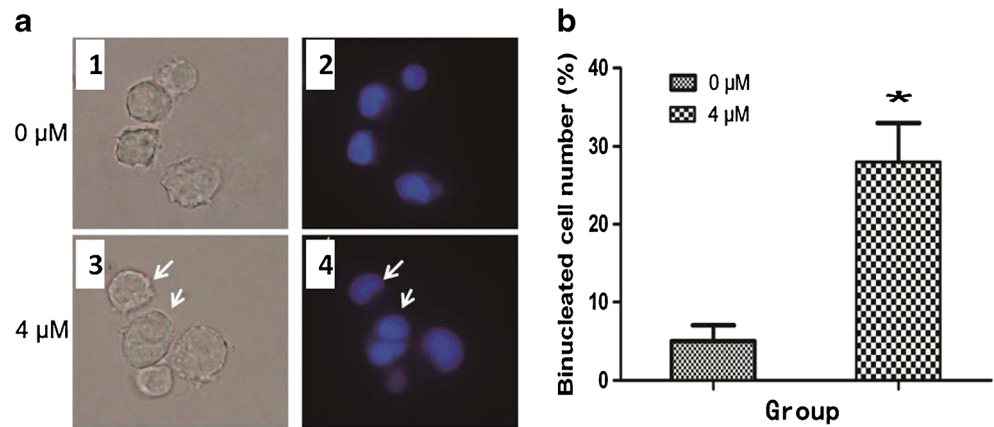
Fig. 4 TY04 induced cell cycle arrest in G2/M phase. After MM.1S cells were grown in medium alone or treated with TY04 at the indicated concentrations, cells were harvested and washed with PBS, fixed with ice-cold 70 % ethanol, stained with PI, and treated with RNase A. **a** Cell cycle distribution was analyzed by flow cytometry. **b** TY04 caused an increase of cells in G2/M phase. The data were presented as the mean±SE from three independent experiments. An *asterisk* (*) indicates a statistically significant difference ($P<0.05$) compared to the control group
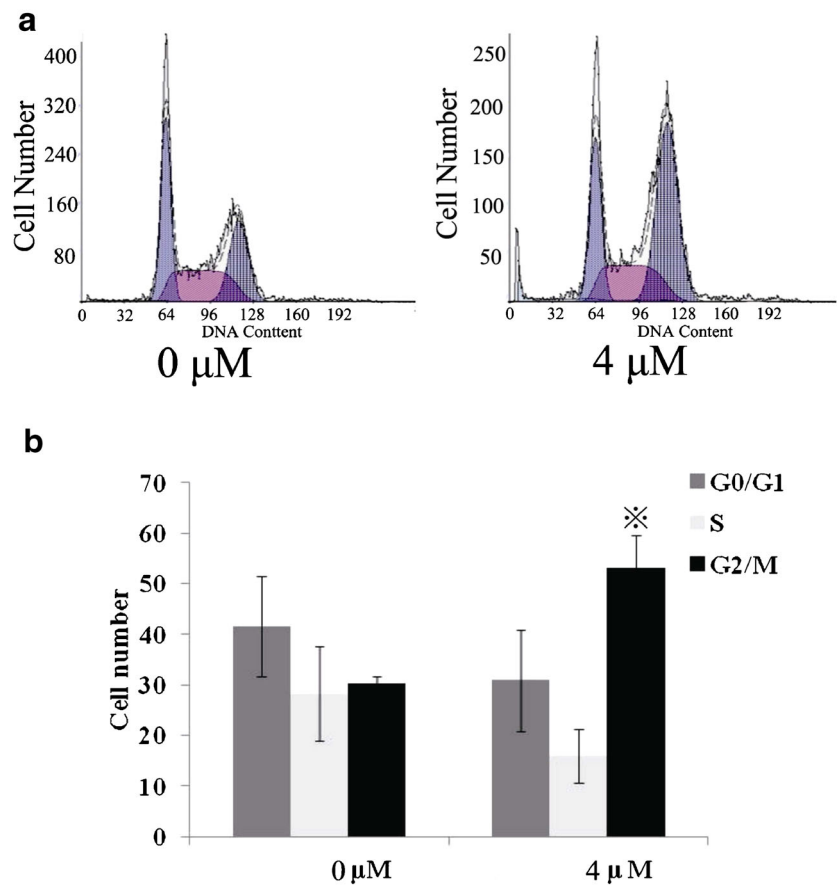
| The concentration of TY04（μM）	G1/G0 $\bar{x} \pm s$	S（%） $\bar{x} \pm s$	G2/M（%） $\bar{x} \pm s$
0	41.6±7.85	28.3±9.04	30.1±1.56
4	30.9±8.91	15.9±5.29	53.2±6.36※

（※ P＜0.05）

Fig. 5 TY04 increased the number of binucleated cells. After MM.1S cells were treated with 4 μM TY04 for 96 h, **a** nuclei were stained with Hoechst 33258 and binucleated cells (*arrow*) were obviously observed under fluorescence microscopy. **b** The number of binucleated cells was calculated from 200 cells. An *asterisk* (*) indicates a statistically significant difference (*P*<0.05) compared to the control group

CDK1 and cyclin B1 were upregulated, while γ-tubulin was decreased upon treatment with TY04 in MM.1S cells

To gain insight into the molecular mechanism of TY04-induced cell cycle arrest, the expressions of several proteins involved in controlling G2/M transition were measured by mean fluorescence intensity (MFI) using flow cytometry. CDK1 interacts with cyclin B1 to form a complex known as the maturation-promoting factor (MPF), which is essential for cell cycle progression from interphase to mitosis. After treating MM.1S cells with TY04, we observed an increase in MFI of CDK1 and cyclin B1 from 12 ± 4 to 149 ± 27 and from 214 ± 13 to 271 ± 22, respectively, indicating that TY04 can upregulate the expression of CDK1 and cyclin B1 in MM.1S cells. In addition, TY04 also caused a decrease in the expression of γ-tubulin compared to untreated MM.1S cells (Fig. 6).

Discussion

Through screening a series of aptamers generated by using the strategy of CELL-SELEX, we found that the aptamer TY04 inhibited the growth of MM cell lines. Comparing across a panel of MM cell lines, our analysis showed the MM.1S cell line was the most sensitive to TY04. The IC_{50} of TY04 for MM.1S cells was 3.89 μM. Currently, TY04 is the first known ssDNA aptamer to be evaluated against MM cell lines in vitro.

Cell surface membrane proteins play key roles in cancer. Their dysregulation activates aberrant cell signaling pathways, which leads to abnormal cell division and tumor metastasis [12]. Many therapeutically useful aptamers have the ability to bind to membrane proteins and interfere with their abnormal function. For example, epidermal growth factor receptor (EGFR) family proteins are cell membrane receptor tyrosine

Fig. 6 Effects of TY04 on the expression of CDK1, cyclin B1, and γ-tubulin in MM.1S cells. After MM.1S cells were treated with 4 μM TY04 for 96 h, the fluorescence intensity of **a** CDK1, **b** cyclin B1, and **c** γ-tubulin was assayed by flow cytometry. **d** Mean fluorescence intensity (MPI) value of CDK1, cyclin B1, and γ-tubulin
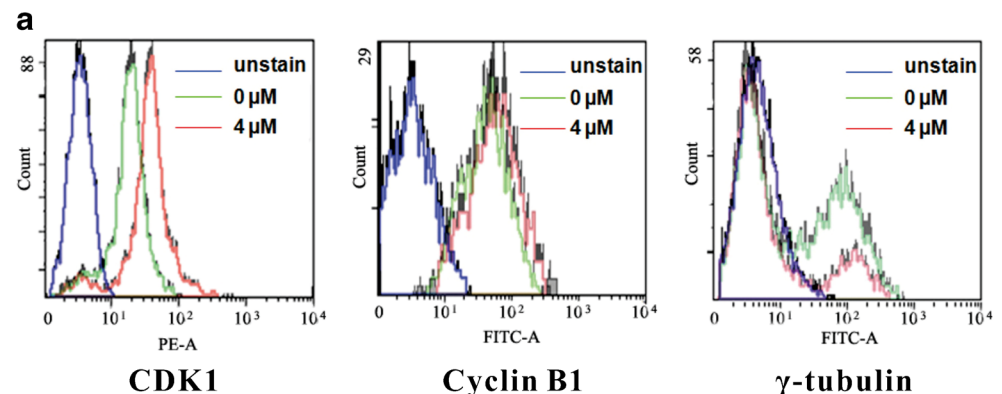
Protein	Mean fluorescence (n＝3)		Changed fold	*p* value
	0 μM	4 μM		
CDK1	12±4	149±27	12	＜0.01
Cyclin B1	214±13	271±22	1.26	＜0.05
γ-tubulin	59±7	37±4	-0.62	＜0.01

kinases often associated with the development of a wide variety of cancers through activation of their kinase activity and downstream signaling pathways. Aptamers targeting EGFR can inhibit activation of the heregulin-dependent pathway by blocking receptor autophosphorylation and preventing proliferation of tumor cells [13–15]. The aptamer TY04 was generated by CELL-SELEX in our group without prior knowledge of the target molecules. To determine whether TY04 targets membrane proteins on the surface of MM.1S cells, the binding affinity of TY04 was detected by treating MM.1S cells with trypsin or proteinase K. Interestingly, the binding ability of TY04 to MM.1S cells could not be completely abolished by trypsin or proteinase K, suggesting that some membrane proteins on the surface of MM.1S cells were involved in the binding of TY04. Therefore, we conclude that TY04 may confer a more complex binding affinity not only for membrane proteins but also for other molecules on the surface of MM.1S cells. Thus, to elucidate the molecular mechanism of TY04 against MM cells, further exploration of the target molecules is required.

In cancer, defects in the regulation of the cell cycle result in uncontrolled cell proliferation. Cell cycle progression is stimulated by sequential activation of CDKs and their association with cyclins [16]. Studies have shown that active CDK1 complexed to cyclin B1 is required for cell cycle progression from G2 to M phase. The Wee1 kinase phosphorylates and inactivates CDK1, whereas dephosphorylation of these sites by the phosphatase Cdc25C increases CDK1 activity, which in turn promotes cell cycle entry into mitosis [17]. Alternatively, Chk1 inactivates Cdc25C through phosphorylation of Cdc25C, which causes the phosphorylation and inactivity of CDK1-cyclin B1 and G2/M arrest [18]. However, it is the sustained activity of the CDK1-cyclin B1 complex that contributes to the ability of cells to remain in mitosis [19, 20]. Our studies indicate that TY04 can increase the expression of CDK1 and cyclin B1 in MM.1S cells. Furthermore, we observed that TY04 induced mitotic arrest in anaphase and telophase, which resulted in a significant accumulation of binucleated cells compared to untreated MM.1S cells. Previous studies revealed that γ-tubulin is a component of the microtubule-organizing center and plays a key role as a nucleator of spindle and cytoplasmic microtubules [21]. Inhibition of γ-tubulin during mitosis leads to disorganization of the mid-body and prevents the completion of cytokinesis [22, 23]. Consistent with previous research, our results revealed that the expression of γ-tubulin is inhibited in TY04-treated MM.1S cells. We conclude that γ-tubulin is involved, at least in part, in the mitotic arrest caused by TY04 in MM.1S cells. In addition, we also performed apoptosis detection in MM.1S cells using the Annexin V and PI assay after treatment with TY04. However, we did not observe big difference between control group and the group treated with 4 μM TY04 (data not shown).

In conclusion, the aptamer TY04 bound to MM cells and inhibited the growth via cell cycle arrest in mitosis. Upregulation of CDK1 and cyclin B1 expression and downregulation of γ-tubulin expression were involved in TY04-induced cell cycle arrest. Considering the unique advantages of aptamers, TY04 shows great potential as drug candidate to treat MM.

Conflicts of interest None.

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