A New Monoclonal Antibody that Blocks KIT Dimerisation and Inhibits Gastrointestinal Stromal Tumour Growth

Chen Guang Bai
Department of Pathology, Changhai Hospital,Second Military Medical University
Yi Xu
Department of Pathology, Yueyang Integrative Medicine Hospital, Shanghai University of Traditional Chinese Medicine
Cen Qiu (qiucendeviant@163.com)
Shanghai Jiao Tong University School of Medicine

Research

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Abstract

Gastrointestinal stromal tumour (GIST) is the most common sarcoma of the gastrointestinal tract, and arises owing to oncogenic mutations in c-kit that result in constitutive auto-phosphorylation of KIT in the absence of ligand binding. Small-molecule tyrosine kinase inhibitors have shown good clinical activity by inhibiting ATP binding to the receptor. Unfortunately, majority of patients eventually develop drug resistance, which limits the long-term benefits of the tyrosine kinase inhibitors and poses a significant challenge in the clinical management of GIST. Here, we demonstrate c-kit mutation-driven KIT auto-dimerisation prior to tyrosine kinase phosphorylation as same as the procedure in ligand-dependent signalling pathway and describe a monoclonal antibody, KITMAb, with strong affinity to the dimerisation domain of KIT that blocks the important step in both the KIT signalling pathways. Treatment of KIT-dimer-expressing cells with the KITMAb slowed down cell growth. Furthermore, KITMAb reduced the proportion of cells in the proliferative phase (S+G2-M). Finally, we also demonstrate that KITMAb treatment accelerated cell apoptosis. These results indicate that KITMAb strongly inhibits KIT receptor dimerisation-mediated signalling pathway and cell growth responses *in vitro.* Further, the results suggest that treatment with KITMAb may be potentially therapeutic in imatinib-resistant GIST.

Introduction

Gastrointestinal stromal tumour (GIST) is the most common mesenchymal tumour of the digestive tract. Most GISTs are caused by gain-of-function mutations in c-kit that trigger intrinsic receptor tyrosine kinase activity and downstream signalling cascades including phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathways even in the absence of the binding of its ligand, stem cell factor (SCF)[1–5]. Although the small-molecule tyrosine kinase inhibitor, imatinib, has shown optimum clinical activity in c-kit-driven GIST patients, the effects are often limited because of intrinsic and acquired resistance resulting in tumour recurrence and progression in most patients[6]. To overcome primary and secondary resistance to imatinib, more effective KIT-targeted therapies are urgently needed.

KIT belongs to the transmembrane growth factor receptor family with an activation mechanism that involves the crosslinking of two receptors following SCF binding[7–8]. Under normal conditions, in the ligand-dependent receptor signalling pathway, the intrinsic tyrosine kinase phosphorylation is followed by receptor dimerisation. However, we detected the expression of KIT-dimers in 293 cells transfected with pcDNA3.1 expressing mutated-type c-kit. These results demonstrated that the c-kit mutation drove auto-dimerisation, and promoted receptor phosphorylation, and ligand-independent receptor signalling pathway. Therefore, dimerisation is the common step in both the activation processes of KIT prior to phosphorylation and therefore blocking receptor dimerisation may be more effective than blocking the phosphorylated receptor.

Based on the design of pertuzumab, a monoclonal antibody that sterically blocks Her-2-dimerisation, we prepared a murine monoclonal antibody, KITMAb, which specifically binds to the dimerisation domain of KIT (the fourth and the fifth extracellular motifs)[9]. The results showed that KITMAb inhibits the expression of KIT-dimers and cell growth responses due to receptor dimerisation. Taken together, our findings confirmed that KITMAb inhibits dimerisation upstream of the phosphorylation in KIT signalling pathway suggesting the potential of dimerisation blocking therapy in imatinib-resistant GIST patients.

Materials And Methods

Cell lines and animals

Human embryonic kidney cells (HEK 293 cells) were obtained from the Laboratory of Thoracic Surgery, Changhai Hospital, Shanghai (China). Cells were cultured in DEME medium with serum (GIBCO BRL, Grand Island, NY, USA) at 37.5°C in a humidified 5% CO_2 atmosphere. The four plasmid vectors used in our study including blank pcDNA3.1, c-kit wild-type pcDNA3.1, c-kit mutated-type pcDNA3.1, and pcDNA6.2 were stored in our laboratory. Imatinib was purchased from Novartis Pharma, Basel, Switzerland. Female BALB/c mice (8-week-old) were obtained from the Experimental Animal Centre of the Second Military Medical University. All animals were treated in accordance with the guidelines of the Committee on Animals of the Chinese Academy of Science.

Design, preparation, and purification of KITMAb

The antigen primer for KIT extracellular domains 4 and 5 (that are involved in receptor dimerisation) were synthesised using polymerase chain reaction (PCR) according to the protocol described in previous studies[9] based on its sequence from the GenBank. The cDNA fragment of interest obtained from the recombination plasmid of c-kit wild-type pcDNA3.1 was ligated to vector PET28 and then transformed into DH5a competent cells. The positive bacterial clones were selected using streptomycin and electroporated into *Escherichia coli* BL21 cells. Expression of the antigenic protein was induced using isopropyl-β-D-thiogalactopyranoside (IPTG), collected by centrifugation, and purified using nickel affinity chromatography. KITMAb was prepared by immunising BALB/c mice with the mixture of the antigenic proteins using hybridoma technique[10]. Ascetic fluid from the mice was analysed for antibody secretion using enzyme linked immunosorbent assay (ELISA). KITMAb was purified using protein G affinity chromatography.

Generation of cells expressing the KIT dimer

PcDNA3.1 containing the c-kit gene was transfected into 293 cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). To observe the transfection efficiency directly under a fluorescence microscope, the cells were cotransfected with pcDNA6.2 harbouring green fluorescent protein. Stably transfected cell lines were isolated through limiting dilution in the presence of 400 μg/mL G418.

Mutational analysis of the c-kit gene

Genomic DNA was extracted from cells using the standard proteinase-K digestion/phenol-chloroform procedure. Exon 11 of the c-kit gene was amplified by PCR using 2 μL DNA solution, 25 μL 2× Taq PCR MasterMix, and 1 μL of each primer (10 μmol/L) in a final volume of 50 μL. The sequences of the primers used for PCR were as follows: forward, 5'-CCAGAGTGCTCTAATGACTG-3' and reverse, 5'-AGCCCCTGTTTCATACTGAC-3'. The PCR conditions included a total of 38 amplification cycles, consisting of denaturation at 90°C for 30 sec, annealing at 60 or 56°C for 30 sec, and extension at 72°C for 1 min, and were performed in a PCR system (Thermo Fisher Scientific, Waltham, MA, USA). The PCR products were directly subjected to sequence analysis.

Western blot analysis

Cells were plated at a density of 2×10^5 cells/well in 6-well plates and serum starved overnight. The following day the cells were either treated in presence or absence of KITMAb or imatinib at varying doses for 72 h at 37°C. The cells were rinsed in PBS and lysed in 1× cell lysis buffer. The expression of KIT dimer was detected by western blotting using native-PAGE, whereas the expression of KIT-monomer was detected by western blotting using SDS-PAGE. The blots were incubated with primary antibodies to KIT (1:2000, DAKO, Glostrup, Denmark) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:3000, DAKO, Glostrup, Denmark).

Analysis of cell cycle and apoptosis

A total of 1×10^6 mutated-type cells were collected, treated with 75% alcohol, washed with PBS, and resuspended in 10 μL propidium iodide solution. The cells were dissociated with RNaseA (Thermo Fisher Scientific, Waltham, MA, USA), stained with Annexin V/FITC, and then analysed by flow cytometry.

Statistical Analysis

Data analysis was performed using SPSS17.0 (IBM, Armonk, NY, USA). $P < 0.05$ was considered to be statistically significant ($\alpha = 0.05$).

Results

Identification of the gene that encodes the dimerisation region of KIT

KIT is characterised by the presence of an extracellular region with five immunoglobulin (Ig)-like motifs, of which the fourth and fifth motifs are involved in dimerization[4–5□9]. Therefore, based on the c-kit gene sequence from the GenBank, primers were designed to span this region as follows: antigen1-1 expressed proteins in the fourth and fifth extracellular motifs, antigen1-2 expressed proteins in the fourth extracellular motif and antigen1-3 expressed proteins in the fifth extracellular motif. The upstream primers were 5'-CGCGGATCCCCCATGATAAACACTACAGT-3', 5'-CGCGGATCCCCCATGATAAACACTACAGT-3' and 5'-CGCGGATCCCCAGAAATCCTGACTTACGA-3'. All the restriction enzyme cutting sites were BamHI. The downstream primers were 5'-CCGCTCGAGTGCAAAGTTAAAATAGGCAG-3', 5'-CCGCTCGAGATTCACATAAACATTAAATG-3' and 5'-CCGCTCGAGCAGGGTGTGGGGATGGATT-3'. All the restriction enzyme cutting sites were XhoI. After amplification of the corresponding cDNA fragments of three antigens from c-kit wild-type pcDNA3.1 using PCR, the gene segments were analysed using restriction mapping and localised between 100–250 bp, consistent with the expected sizes, in agarose gel electrophoresis (Fig. 1). Analysis of sequence homology using BLAST software revealed that the sequence of the amplified c-kit gene was identical to that published in the GenBank.

Induction of expression and purification of the recombinant antigen

Sequence information of antigens generated were as follows: the sequence of antigen 1-1 that localised to 317–507 aa was PMINTTVFVNDGENVDLIVEYEAFPKPEHQQWIYMNRTFTDKWEDYPKSENESNIRYVSELHLTRLKGTEGGTYTFLVSNSDVNAAIAFNVYVNTKPEILTY DRLVNGMLQCVAAGFPEPTIDWYFCPGTEQRCSASVLPVDVQTLNSSGPPFGKLVVQSSIDSSAFKHNGTVECKAYNDVGKTSAYFNFA (molecular weight: 21.44 kDa). The sequence of antigen 1-2 that localised to 317–401 aa was PMINTTVFVNDGENVDLIVEYEAFPKPEHQQWIYMNRTFTDKWEDYPKSENESNIRYVSELHLTRLKGTEGGTYTFLVSNSDVNAAIAFNVYVN (molecular weight: 10.93 kDa). The sequence of antigen 1-3 that localised to 413–517 aa was PEILTYDRLVNGMLQCVAAGFPEPTIDWYFCPGTEQRCSASVLPVDVQTLNSSGPPFGKLVVQSSIDSSAFKHNGTVECKAYNDVGKTSAYFNFAFKEQIHPHTL (molecular weight: 11.53 kDa). The products of the antigenic proteins were mainly in the form of inclusion bodies following IPTG induction of the proteins. The molecular weights of three purified antigenic proteins were 26.01, 16.13, and 16.55 kDa, respectively, by western blotting with SDS-PAGE (Figure 2). These results were consistent with the molecular size of the designed antigenic protein. The concentration of three antigenic

proteins analysed using BCA protein assay (Abcam, Cambridge, MA, USA) was 0.5, 0.3, and 0.4 mg/mL, respectively, and the purity of the antigens was 85, 80, and 90%, respectively.

Identification of KITMAb

After successful generation of a set of 11 murine monoclonal antibodies named KITMAbs that block the KIT receptor dimerisation domain, the effective titres of the KITMAbs were analysed using ELISA (Table 1). The titre marked in green was an effective titre (determination criterion: dilution value of OD450 greater than two times of the negative control and greater than 0.25). The results of the immunohistochemical assay indicated that six of the eleven KITMAbs (1-Ab, 3-Ab, 4-Ab, 6-Ab, 7-Ab, and 8-Ab) bound to KIT proteins in the GIST tumour tissues optimally, and the positive colour rendering was located in the cytoplasm or membrane of the GIST cells (Fig. 3a). Furthermore, KIT protein was extracted from GIST tissues, lysed, and KITMAb was identified by western blotting following SDS-PAGE. The results indicated that the selected KITMAbs bound to KIT protein very well, and the positive bands were of the molecular weight of 145/125 kDa (Fig. 3b).

Table 1
Titer of KITMAb

Number	3.125K	6.25K	12.5K	25K	50K	100K	200K	400K	800K	PC	NC
1	2.638	2.448	2.088	1.36	0.776	0.446	0.252	0.162	0.118	2.82	0.07
2	2.699	2.688	2.677	2.61	2.390	2.044	1.334	0.890	0.507	2.84	0.07
3	2.439	2.388	2.272	1.99	1.496	0.933	0.560	0.364	0.225	2.58	0.09
4	2.541	2.524	2.456	2.42	2.200	1.890	1.505	0.976	0.625	2.69	0.08
5	2.663	2.687	2.671	2.50	2.464	2.007	1.443	0.821	0.487	3.08	0.05
6	2.674	2.517	2.517	2.22	1.856	1.215	0.669	0.345	0.195	2.88	0.06
7	2.624	1.968	1.191	0.53	0.291	0.191	0.124	0.098	0.080	3.08	0.18
8	1.300	0.750	0.426	0.29	0.167	0.163	0.129	0.090	0.077	2.24	0.1
9	2.643	2.688	2.590	2.46	1.893	1.401	0.903	0.548	0.360	2.97	0.1
10	2.688	2.565	2.430	2.09	1.490	0.978	0.564	0.328	0.224	2.96	0.09
11	2.093	1.937	1.814	1.69	1.338	1.229	0.973	0.639	0.372	1.62	0.09
□ PC,Positive Control□NC□Negative Control											

Establishment of KIT-dimer-expressing cell lines

Fluorescence microscopy revealed that the transfection efficiency was approximately 50% 48 h following transfection (Fig. 4a–d). The expression of KIT-monomers and -dimers were investigated using western blot. As shown in Fig. 4e, both KIT-monomers and KIT-dimers were expressed in 293 cells transfected with c-kit mutated-type pcDNA3.1, while only KIT-monomers were expressed in 293 cells transfected c-kit wild-type pcDNA3.1. No KIT expression was detected in blank pcDNA3.1 transfected or untransfected 293 cells. MTT colorimetric assay was also conducted to measure cell proliferation in each cell line. There was no significant difference in the OD at 490 nm between 293 cells transfected with c-kit wild-type pcDNA3.1 and blank pcDNA3.1. Interesting, the OD value of 293 cells transfected with c-kit mutated-type pcDNA3.1 was much higher that of the other two groups of 293 cells ($P < 0.01$, $F = 101.593$, Fig. 4f). These results confirmed that mutated c-kit gene promotes cell proliferation. DNA sequence analysis revealed no c-kit mutation in the 293 cells transfected with c-kit wild-type pcDNA3.1, while exon 11 point mutation was detected in 293 cells transfected with c-kit mutated-type pcDNA3.1 (Fig. 4g).

Inhibition of receptor dimerisation in KIT-expressing cell lines in vitro

Following dimerisation of the receptor, the KIT protein becomes phosphorylated and the downstream signalling pathway is activated. Therefore, to measure the activation of the receptor, detecting the expression of KIT dimer is more informative than detecting the expression of phosphorylated KIT because KIT has several phosphorylation sites. KITMAb was used at the concentration of 0.5 μg/mL (based on the clinically effective therapeutic dose of imatinib) in our study. Four of the previously selected KITMAbs (4-Ab, 6-Ab, 7-Ab, and 8-Ab) were found to significantly decrease the expression of KIT-dimers in 293 cells transfected with c-kit mutated-type pcDNA3.1 after 72 h of treatment as detected by western blot analysis of the corresponding cell lysates using native-PAGE. To demonstrate the specificity of the antibodies, imatinib was used as a positive control and it showed a bright band of KIT dimer protein expression. In addition, there was no significant difference in the expression level of KIT-monomer protein in the different cell samples (Fig. 5a). Our finding suggests that KITMAb strongly binds to the dimerisation domain of KIT and results in inhibition of KIT receptor dimerisation. On the other hand, imatinib was found to have little effect on inhibition of KIT dimerisation.

To examine the ability of KITMAb to inhibit cell growth induced by dimerisation of KIT *in vitro*, cell proliferation, cell cycle, and apoptotic assays were performed. 293 cells expressing KIT dimer were exposed for 72 h to either KITMAb or imatinib as experimental group or positive control, respectively. Untreated 293 cells expressing KIT dimer were used as a negative control. Cell proliferation activity was measured using MTT assay and the results are depicted in Fig. 5b. Cell proliferation activity in all the four experimental groups and positive control group of cells was significantly lower than that of the negative control group of cells. Further observation revealed that the cell growth in the experimental group decreased more compared to that of the positive control group. Neither KITMAb nor imatinib had any effect on cell proliferation of c-kit wild-type pcDNA3.1 transfected and untransfected 293 cells (data not provided here).

The results of cell cycle assay showed that the proportion of cells in the proliferative phases (S + G2-M) in negative control untreated KIT-dimer-expressing 293 cells, positive control cells treated with imatinib, and four experimental groups of cells exposed to KITMAb were 38.82 ± 0.80%, 38.39 ± 0.33%, 37.87 ± 0.19%, 37.69 ± 1.17%, 38.71 ± 0.38%, and 37.99 ± 0.55%, respectively (Fig. 5c). Compared to the negative control group, the proportion of proliferative cells in the positive control and the four experimental groups was decreased although the difference was not statistically significant when tested using ANOVA ($P = 0.246$, $F = 1.552$).

The results of the apoptotic assay showed that the apoptotic rates of the negative control, positive control, and the four experimental groups were 2.84 ± 0.09%, 6.09 ± 0.13%, 8.56 ± 0.45%, 16.70 ± 3.61%, 2.90 ± 0.04%, and 3.92 ± 0.53% (Fig. 5d). Compared to the negative control group, apoptosis was higher in the positive control and the four experimental groups ($P < 0.01$, $F = 35.642$). Further analysis using least significant difference T test (LSD-t) showed that the LSD-t values between 4-Ab and negative control, 6-Ab and negative control, and imatinib and negative control were − 0.539 ($P < 0.01$), -1.353 ($P < 0.01$), and − 0.292 ($P < 0.01$), respectively. Experimental groups treated with the other KITMAbs were not statistically different from the control group (data not shown).

Discussion

Aberrant activation of the ligand-independent KIT signalling axis has been implicated in mutated-type GIST and accounts for 80% of all GIST cases[1–2]. Small-molecule tyrosine kinase inhibitors resemble adenosine triphosphate (ATP) structurally and competitively bind to the ATP-binding domain of KIT thus contributing to good clinical efficacy in GIST patients harbouring constitutively phosphorylated KIT receptors[11]. Unfortunately, the effects are limited based on the mutation site of c-kit. Only patients harbouring exon 11 mutation of c-kit are sensitive to imatinib therapy, while other ligand-independent KIT signalling pathways activated by mutations in exon 9, exon 13, exon 17, and others are not blocked by imatinib. Further, primary drug resistance mainly appears in cases without mutation of c-kit[12–14]. In addition, long-term imatinib application induces secondary mutations to activate new ligand-independent KIT signalling pathways that are insensitive to imatinib and lead to secondary resistance eventually[15–16]. A functional antibody that targets receptor dimerisation is a viable alternative approach to treat imatinib-resistant GISTs, as it would inhibit both ligand-dependent and ligand-independent receptor signalling pathways.

In this study, we applied this strategy of blocking receptor dimerisation toward treatment of GIST and prepared a monoclonal antibody, KITMAb, that targets the receptor dimerisation domain. The properties of the antigen designed for KITMAb were consistent with the result in the GenBank and this guaranteed specific binding of KITMAb to KIT. To characterise the binding properties of KITMAb further, we used liposome-mediated transfection to first obtain KIT-dimer-expressing cell lines. The results demonstrated that KIT auto-dimerisation owing to the c-kit mutation rendered the 293 cells more proliferative, similar to the observation in GISTs. The expression of KIT-dimers was significantly decreased after treatment with KITMAb *in vitro*, which confirmed that KITMAb precisely blocked the dimerisation of KIT receptor. As a consequence of decreased expression of KIT-dimers, treatment with KITMAb reduced cell proliferation and cell cycle progression, and promoted cell apoptosis. Consistent with our expectation, KITMAb blocked the receptor dimerisation-activated KIT signalling pathway.

The development of monoclonal antibody-based therapy effectively addresses the significant challenge associated with imatinib-resistant GIST. However, the activated KIT binding antibody, SR1, lacks extensive *in vivo* characterisation and has agonistic activity owing to its large molecular weight[17]. Another fully human IgG1 monoclonal antibody, CK6, blocks the interaction of KIT with SCF, but only interferes with ligand-stimulated signalling and cell growth responses[18]. Both the KIT antibodies generated in the previous reports have some limitations in application caused by the inadequacies in the antibodies themselves. Unlike SR1 that antagonises the intracellular activated receptor, KITMAb binds to the extracellular region. This implies that KITMAb does not need to enter the tumour cells to reach the effective concentration, which can be affected by molecular weight of the antibody. Furthermore, blocking of both the ligand-dependent and -independent signalling pathways by KITMAb is likely to exert greater clinical effect than blocking of only the ligand-dependent signal pathway as with CK6.

In summary, we describe the anti-tumour growth properties of KITMAb, a monoclonal antibody with affinity for the dimerisation region of KIT. KITMAb represents a potential therapeutic agent that can be effectively used to treat imatinib-resistant GIST patients.

Declarations

Acknowledgements

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Authors' contributions

Cen Qiu and Chenguang Bai conceived and designed the study. Cen Qiu and Yi Xu performed the experiments. Cen Qiu wrote the paper. Cen Qiu and Chenguang Bai reviewed and edited the manuscript. All authors read and approved the manuscript.

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Figures

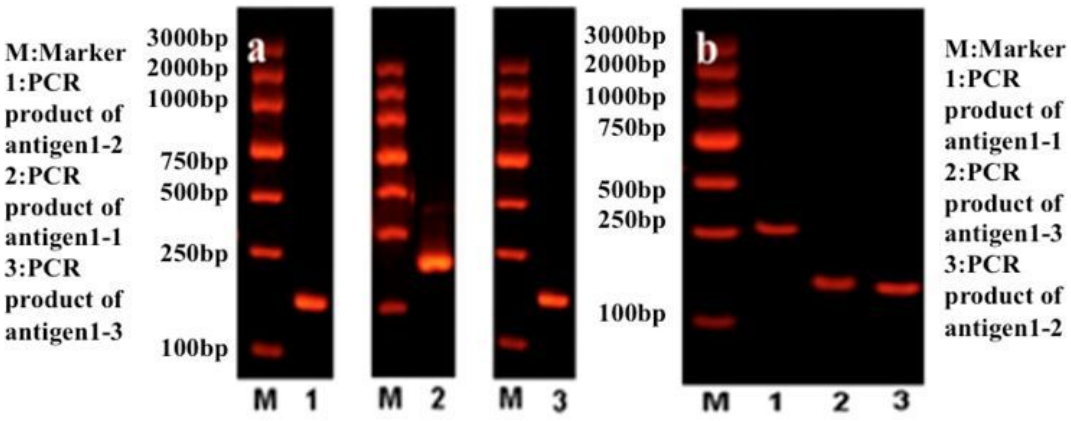

Figure 1

Restriction map of recombinant plasmids containing antigen-related cDNA fragments. (a) PCR products from positive bacterial colonies were identified by agarose gel electrophoresis. (b) The enzymatic digestion product was identified by agarose gel electrophoresis.

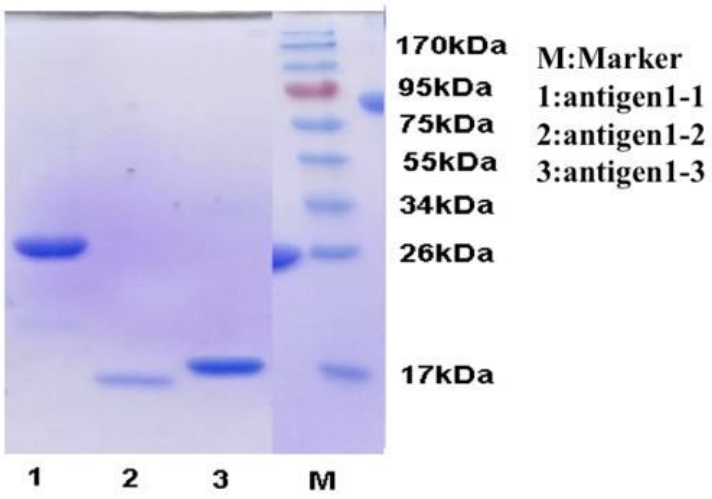

Figure 2

Identification of the purified antigenic proteins by SDS-PAGE

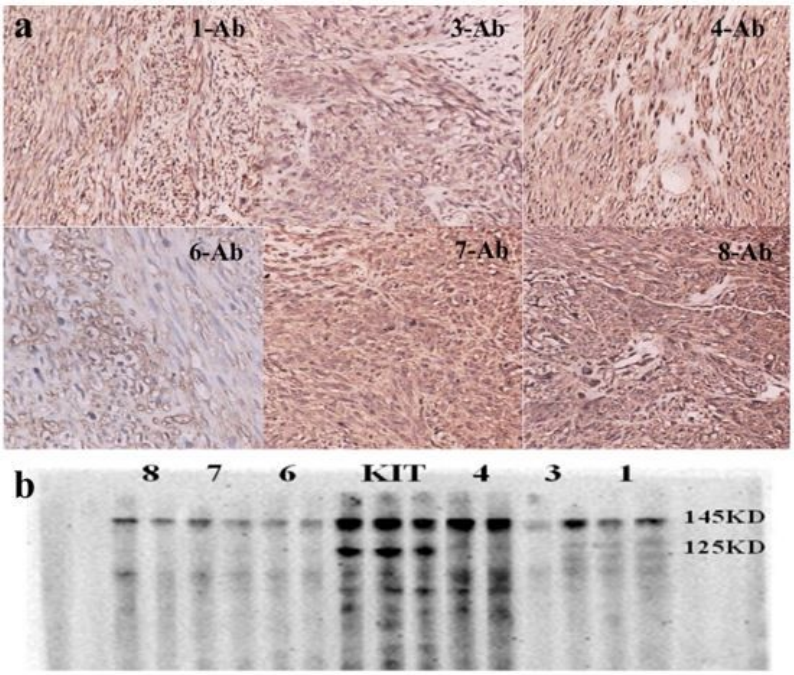

Figure 3

Identification of KITMAb. (a) Identification of KITMAb by immunohistochemical analysis. (b) Identification of KITMAb by SDS-PAGE.

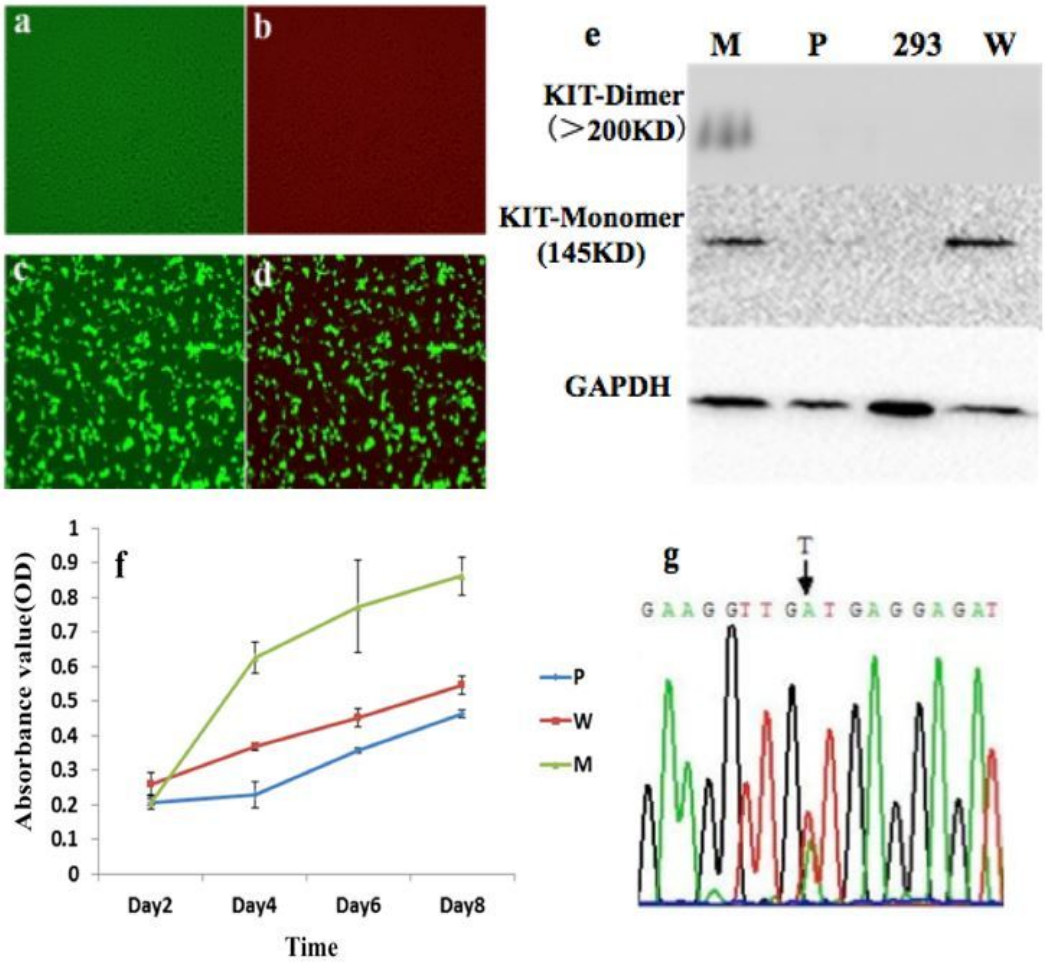

Figure 4

Establishment of KIT-dimer-expressing cell lines. (a) Untransfected 293 cells were observed in green background after 48 h. (b) Untransfected 293 cells were observed in red background after 48 h. (c) Transfected 293 cells were observed in green background after 48 h. (d) Transfected 293 cells were observed in red background after 48 h. (e) KIT expression in untransfected and transfected 293 cells was detected using SDS-PAGE (M meant 293 cells transfected with c-kit mutated-type pcDNA3.1, W meant 293 cells transfected with c-kit wild-type pcDNA3.1, P meant 293 cells transfected with blank pcDNA3.1, and 293 meant untransfected 293 cells). (f) Cell proliferation of untransfected and transfected 293 cells was detected using MTT (M meant 293 cells transfected with c-kit mutated-type pcDNA3.1, W meant 293 cells transfected with c-kit wild-type pcDNA3.1, and P meant 293 cells transfected with blank pcDNA3.1). (g) DNA sequence analysis of exon 11 mutation (point mutation in V560D, GTT→GAT) in c-kit mutated-type pcDNA3.1 transfected 293 cells.

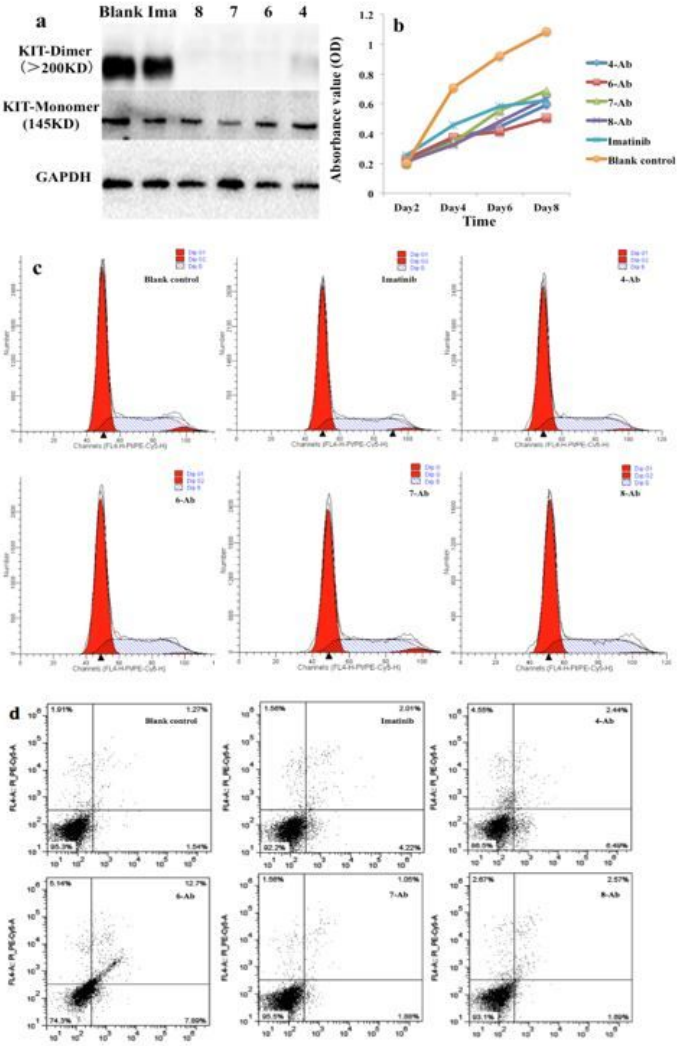

Figure 5

Detection of inhibition of KITMAb in KIT-Dimer-expressing cell lines in vitro. (a) Detection of KIT-Dimers and KIT-Monomers in KIT-Dimer-expressing cells by native-PAGE or SDS-PAGE. (b) Cell proliferation of untreated, imatinib-treated, and KITMAb-treated KIT-Dimer-expressing cells detected using MTT assay. (c) Cell cycle in untreated, imatinib-treated, and KITMAb-treated KIT-Dimer-expressing cells detected using flowcytometry. (d) Cell apoptosis in untreated, imatinib-treated, and KITMAb-treated KIT-Dimer-expressing cells were detected using Annexin V staining.