# Construction of a natural phage antibody library of human anaplastic thyroid carcinoma

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**ABSTRACT**. The objective of this study was to identify and construct a human natural phage single-chain antibody (scFv) library of human anaplastic thyroid carcinoma (ATC) using phage display technology. Total RNA was extracted from lymphatic tissue near an ATC and used to amplify variable heavy chain ($V_H$) and variable light chain ($V_L$) fragments with added linker sequences using reverse transcription-polymerase chain reaction (RT-PCR). After purification, the $V_H$ and $V_L$ amplicons were used to produce scFv fragments with added *Sfi*I and *Not*I restriction enzyme recognition sites using splicing-overlap-extension PCR. Following digestion, the scFv gene was cloned in the pCANTAB-5E plasmid, and the recombinant phagemids were transformed into the susceptible *Escherichia coli* TG1 strain. After infection by the helper phage M13K07, a human ATC phage antibody library was successfully constructed. Clear 28 S and 18 S bands could be seen in the total RNA from the library, and the sizes of the $V_H$, $V_L$, and scFv genes contained therein were approximately 370, 350, and 750 bp, respectively. In addition, the conversion efficiency as measured by the pUC19

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standard plasmid was $10^8$ CFU/μg, and the positive insert ratio was 86.4% (19/22). These results demonstrated the successful construction of a human ATC scFv antibody gene library, and might provide the experimental basis for the further screening and identification of a phage single-chain antibody with ATC cell-specificity.

**Key words:** Phage display; Single-chain antibody; Construction; Anaplastic thyroid carcinoma

## INTRODUCTION

Anaplastic thyroid carcinoma (ATC) accounts for 7-8% of all thyroid cancers and is one of the most aggressive malignant tumors in humans. ATC is characterized by aggressive, local invasion and common distant metastases (Amodeo et al., 2003). Only 20% of affected patients survive for one year after diagnosis; the median survival duration is 3-9 months (Giuffrida and Gharib, 2000). The 5-year survival rate for those with ATC is only from 5-15%; most patients usually die within 12 months of being diagnosed (Kihara et al., 2004). In addition, patients with ATC are typically elderly (most >60 years old). ATC is responsible for 14-40% of all thyroid carcinoma-related deaths (Xia et al., 2006), and the cancer manifests as a rapidly enlarging anterior neck mass that is accompanied by dyspnea, dysphagia, and vocal cord paralysis. Furthermore, 20-50% of patients present with distant metastases, most often pulmonary (Van Glabbeke et al., 1999; Derbel et al., 2011; Sherman et al., 2011; Wein and Weber, 2011; Ito et al., 2012). ATC progresses quickly and readily invades adjacent structures, and in the early stages, can metastasize distally through the blood and lymphatic vessels (Giuffrida and Gharib, 2000; Xia et al., 2006). The treatments for this disease are less effective than are those for other types of thyroid cancer. ATC cells cannot take up polyiodides because of their lack of differentiation; therefore, radioactive iodine therapy has no effect on this type of cancer. In addition, surgical management, radiotherapy, and chemotherapy can only partially control the tumors. Regardless of the treatment methods used, it is very difficult to permanently cure ATC, and although ATC is, overall, one of the most aggressive malignancies in humans, there remains a lack of effective treatments; therefore, it is critical that new therapeutic strategies be evaluated to improve survival.

The use of radioimmunoassay drugs, i.e., radioimmunotherapy (RIT), which combines specific antibodies with radionuclides, has shown efficacy in the treatment of many human cancers, and it appears to provide a new idea for the diagnosis and treatment of ATC (Li, 1998; Vezzosi et al., 2007).

Many investigators have utilized murine monoclonal antibodies in their RIT research protocols and have made some progress; however, certain problems remain including the side reaction of the human anti-mouse antibody (HAMA), which, along with murine antibodies in general as well as large molecular mass monoclonal antibodies, have poor penetration, a low ratio of target to non-target specificity, and a low serum clearance rate.

To resolve these problems, ATC human single-chain antibodies selected as the specific antibody by using phage antibody library technology were used for RIT research in ATC. In this paper, a human ATC single chain variable fragment (scFv) phage display library was constructed using phage display technology, and was used to screen out the phage single-chain

antibodies that could identify ATC-specific antigens. In addition, the scFv phage display library might be used for RIT *in vivo*, which aims to improve the survival rate and the prognosis of patients with ATC.

# MATERIAL AND METHODS

## Phagemids and reagents

The tissue RNA Kit, RNA PCR Kit, Gel Extraction Kit, and Plasmid Kit were purchased from Omega Bio-Tek, Inc. (Norcross, GA, USA). The cDNA reverse transcription kit, cDNA reverse transcriptase, 2X Power pfu PCR MasterMix, and 2X hot PFU PCR mix with PCR primers were purchased from BioTeke Company (Beijing, China). The Taq DNA polymerase, T4 DNA ligase, and the restriction enzymes *Sfi*I and *Not*I were purchased from TaKaRa Bio, Inc. (Shiga, Japan). The phagemid vectors pCANTAB-5E, *Escherichia coli* TG1, and helper phage M13KO7 were purchased from Bio-View Shine Company (China).

## Extraction of total mRNA from lymphatic tissue originating near the ATC

Lymphatic tissue was separated from near the ATC in a patient who was undergoing a second surgery in the Department of Endocrine and Breast Surgery in the First Affiliated Hospital of Chongqing Medical University, Chongqing, China, and who had been diagnosed with a clear case of ATC.

The peritumoral lymph nodes were wrapped with sterile gauze and quickly placed in liquid nitrogen. A portion of the peritumoral lymph nodes was retrieved and the total RNA was immediately extracted. The rest of the tissue was stored in liquid nitrogen. The total RNA was extracted according to kit instructions. Agarose gel electrophoresis was used to estimate the integrity of the RNA, and the A260/A280 ratio was measured by spectrophotometer to estimate its purity. Finally, the extracted RNA was stored at -80°C.

## Reverse transcription-polymerase chain reaction (RT-PCR)

The RT process (synthesis of first strand cDNA) was performed with reference to the kit instructions, added 1µL 50 µM Oligo-dT primer, 1µL RNA and 1 µL 10 mM of each dNTP Mixture into the EP tube to amplify the cDNA.

## PCR primer design

According to the literature (Marks et al., 1991; Osbourn et al., 2003), all PCR primers were designed by the mesh filter method. The primers were synthesized by the Beijing Sunbiotech Company, Ltd. (Beijing, China) and purified using polyacrylamide gel electrophoresis. Primer sequences were as follows: variable heavy chain ($V_H$): Hu$J_H$3FOR: 5'-TGA AGA GAC GGT GAC CAT TGT CCC -3', Hu$V_H$5aBACK: 5'-GAG GTG CAG CTG TTG CAG TCT GC -3'; variable light chain ($V_L$): Hu$J_k$4FOR: 5'-ACG TTT GAT CTC CAC CTT GGT CCC-3', Hu$V_k$5aBACK: 5'-GAA ACG ACA CTC ACG CAG TCT CC-3'; $V_H$-Linker: Hu$J_H$3FOR: 5'-AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC TGA AGA GAC GGT GAC CAT

TGT CCC-3', HuV$_H$5aBACK: 5'-GAG GTG CAG CTG TTG CAG TCTGG-3'; V$_L$-Linker: HuJ$_k$4FOR: 5'-ACG TTT GAT CTC CAC CTT GGT CCC-3', LinkerHuV$_k$5aBACK: 5'-GTT CAG GCG GAG GTG GCT CTG GCG GTG GCG GAT CGG AAA GAC ACT CAC GCA GTC TCC-3'; restriction enzymes: HuV$_H$5aBACKSfi: 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG TTG CAG TCT GC-3', HuJ$_k$4FORNot: 5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT CTC CAC CTT GGT CCC-3'.

## Amplification of antibody $V_H$ and $V_L$ genes

A set of nested PCR primers for the antibody $V_H$ and $V_L$ genes were used in this paper. cDNA was obtained by RT using total RNA as a template. PCR was performed using the outer primers under the following conditions: $V_H$: one cycle of 94°C for 5 min; 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s; and 1 cycle at 72°C for 10 min. $V_L$: one cycle at 94°C for 5 min; 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and one cycle at 72°C for 10 min. The resulting $V_H$ and $V_L$ DNA fragments were detected by agarose gel electrophoresis (1.0%) and harvested using the purification kit.

## Construction of scFv by splicing-overlap-extension (SOE) PCR

### *Preparation of the $V_H$- and $V_L$-linkers*

The V$_H$-linker was amplified with the corresponding HuJ$_H$3FORLinker and hHu-V$_H$5aBACK primers, whereas the V$_L$-linker was amplified with the corresponding Linker-HuV$_k$5aBACK and HuJ$_k$4FOR primers. The purified $V_H$ and $V_L$ DNA fragments were used as templates.

### *SOE-PCR*

The PCR products of the amplified V$_H$- and V$_L$-linkers were connected by SOE-PCR without primers. The reaction conditions were as follows: equal amounts of $V_H$ and $V_L$ DNA fragments were mixed as a template for SOE. In the first five cycles, primers were absent and the fragments were mixed as follows: one cycle of 94°C for 5 min; and five cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min. The primers HuV$_H$5aBACK *Sfi* and HuJ$_κ$4FOR *Not* were then added for the next 30 cycles as follows: 94°C for 30 s, 60°C for 1 min, and 72°C for 1.5 min. scFv fragments were detected by agarose gel electrophoresis (1.0%) and harvested using the purification kit.

## Construction of the scFv library

### *Bacterial transformation*

First, the resulting scFv fragment was digested by the restriction enzyme *Sfi*I and incubated overnight at 37°C in a swing bed. The restriction enzyme *Not*I was then used to digest the single-digested scFv fragment with incubation overnight at 50°C in a swing bed. After recovery and purification, the double-digested fragment was ligated into the plasmid

pCANTAB-5E. The recombinants were chemically transformed into competent *E. coli* TG1, incubated for 1.0 h at 37°C in a swing bed, and seeded on a SOB medium plate containing 100 mg/L ampicillin and 20 g/L glucose (SOBAG) at 37°C for 24 h. In parallel, the conversion efficiency was measured using the standard plasmid pUC19.

### *Titration of the scFv library*

From the above solution, 10 μL aliquots were diluted with 2X YT culture medium at the ratios of 1:10, 1:100, 1:1000, and 1:10,000. Then, 1.0 μL antibody library from the respective gradients were used to infect 100 μL *E. coli* TG1 ($OD_{600}$ = 1), incubated for 1.0 h at 37°C in a swing bed, seeded onto SOBAG medium plates, and incubated at 37°C overnight. The next day, the colony forming units (CFUs) were calculated to estimate the titration of the antibody library.

## PCR determination of the antibody gene insertion rate

Twenty-two isolated colonies were randomly chosen and 5.0 mL 2X YT culture medium plus 100 mg/mL ampicillin was added. The solution was shaken overnight at 37°C. A small amount of plasmid was extracted according to the instructions in the plasmid extraction kit. Then, 1.0 μL extracted plasmid was used as a template for PCR with the primers HuVH5a-BACK *sfi* and HuJκ4FOR *Not*, with the conditions as described previously. Finally, the positive insertion rate was detected by gel electrophoresis (1.0%).

## Enzyme digestion of the positive clones

The PCR positive clones were digested with the restriction enzymes *Sfi*I and *Not*I. The digestion products were detected by gel electrophoresis (1.0%) to discover whether gene fragments of approximately 750 bp could be released from the clones. Sequencing of the positive plasmids was performed by Invitrogen (Shanghai, China).

## Phage antibody expression

To express the phage antibodies, the positive clones confirmed by PCR and restriction digestion were diluted with 10 mL 2X YT culture medium to $OD_{600}$ = 0.3. The ampicillin was then added at a concentration of 100 μg/mL and the glucose at a concentration of 2.0%. And then, shaken the solution for 1.0 h at 220 rpm at 37°C, after which l x $10^{10}$ PFU Ml3K07 helper phage was added and the solution was shaken again for 1.0 h at 220 rpm at 37°C. The bacteria were precipitated by low-speed centrifugation, and then re-suspended with 100 mL 2X YT-AK culture medium and shaken overnight at 37°C. The next day, the broth was centrifuged and polyethylene glycol/sodium chloride was added to a final concentration of one-fifth the volume of the supernatant. The solution was taken an ice bath for 45 min, and then centrifuged for 10 min. After that, the precipitate was re-suspended with 1.0 mL 2X YT broth and centrifuged again. Then, the recombinant phage supernatants were filtered. Finally, sodium azide was added to a final concentration of 0.02% and the solution was stored at 4°C.

## RESULTS

### Extraction of total lymph node RNA

The integrity of the total ATC lymph node RNA was estimated by 1.0% agarose gel electrophoresis as shown in Figure 1. In the figure, the two bands of 18 S and 28 S RNA can be clearly visualized. The brightness of the 28 S band appears to be about twice that of the 18 S band. The purity of the RNA was >1.8, which was estimated by measuring the absorbance $A_{260}/A_{280}$ ratio with a spectrophotometer.

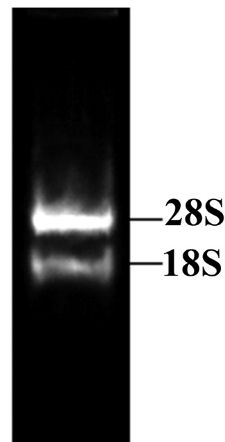


**Figure1.** Total RNA of lymph nodes around anaplastic thyroid cancer.

### Amplification of $V_H$ and $V_L$ genes

As measured by 1.0% agarose gel electrophoresis, the size of the $V_H$ gene was approximately 370 bp and the $V_L$ gene was approximately 350 bp (Figures 2 and 3).

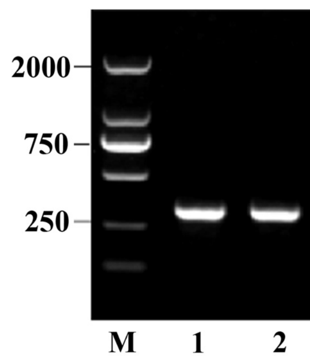


**Figure 2.** Agarose gel electrophoresis identification of antibody variable range $V_H$ . *Lane M* = DL2000 marker; *lanes 1* and *2* = $V_H$ fragment.

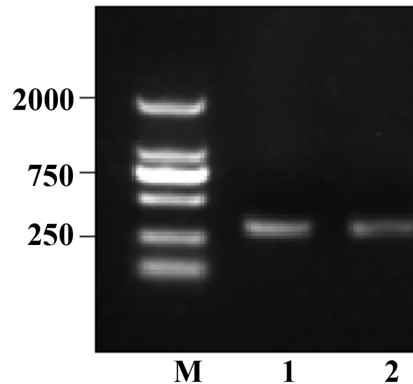


**Figure 3.** Agarose gel electrophoresis identification of antibody variable range $V_L$. *Lane M* = DL2000 marker; *lanes 1* and *2* = $V_L$ fragment.

## Construction of scFv by SOE

The $V_H$ and $V_L$ DNA fragments were successfully connected by SOE. Agarose gel electrophoresis demonstrated that scFv was approximately 750 bp, as shown in Figure 4.

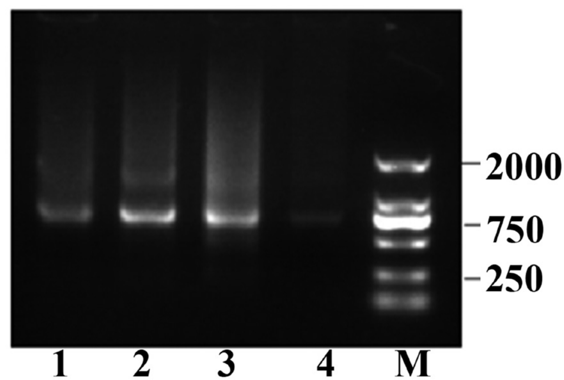


**Figure 4.** Agarose gel electrophoresis identification of single antibody gene after link. *Lane M* = DL2000 marker; *lanes 1*, *2*, *3*, and *4* = scFv fragment.

## Conversion efficiency of the PCR ligation product

The conversion efficiency of *E. coli* TG1 as measured by the standard plasmid pUC19 was $10^8$ CFU/μg, and the conversion efficiency of the PCR ligation product was 3.1 x $10^7$ CFU/μg.

## Identification of the antibody gene insertion rate by PCR

Twenty-two monoclonal plasmids were randomly chosen and analyzed by PCR. The


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results showed that scFv could be amplified from 19 monoclonal plasmids, and that the insertion rate of the antibody gene was therefore 86.4%, as shown in Figure 5.

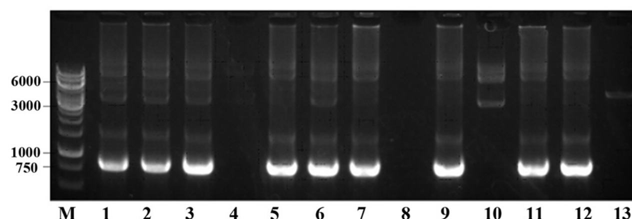


**Figure 5.** PCR identification of randomly chosen plasmid clones. *Lane M* = 1-kb DNA ladder; *lanes 1-3*, *5-7*, *9*, *11*, and *12* = PCR positive; *lanes 4*, *8*, and *10* = PCR negative; *lane 13* = phagemid vectors pCANTAB-5E.

## Identification of the antibody gene insertion rate by double restriction enzyme digestion

Eight monoclonal plasmids were randomly chosen from the positive clones and digested with the restriction enzymes *Sfi*I and *Not*I. The subsequent release of the 750 bp scFv fragment from the plasmid can be seen in Figure 6.

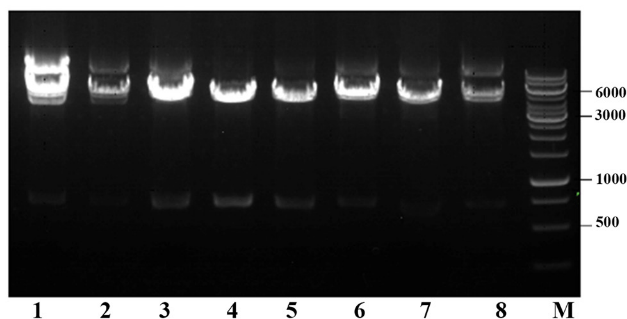


**Figure 6.** *Sfi*I/*Not*I double digestion results of positive insert clones. *Lane M* = 1-kb DNA ladder; *lanes 1-8* = scFv fragments (down) and phages (up).

## Sequencing results of the positive plasmid digestion

Sequencing of the positive plasmids was performed by Invitrogen (Shanghai, China), and the results showed that compared with the entire coding sequence, the positive scFv plasmid sequence was 98.3% identical to the native gene.

## DISCUSSION

ATC is the most aggressive of all malignant thyroid tumors. Histopathologically, ATC is characterized by an elevated mitotic rate and lymphovascular invasion. Although its incidence is not high, its insidious onset, lack of specific clinical manifestations, and rapid growth

usually results in a diagnosis later than is considered optimal for the possibility of effective interventions. However, even if diagnosed early, patients have often lost any chance of useful treatment because of ATC's rapid progression and early metastasis through a variety of channels (Are and Shaha, 2006). In recent years, RIT for cancer patients has been considered an important therapeutic tool and has been widely applied and researched in the clinic. This strategy provided a new direction for the diagnosis and treatment of ATC.

Human scFv is a genetically engineered antibody with a small molecular weight that is considered to be the smallest unit with antigen binding activity (Zhao et al., 2004). It is concatenated with a short peptide sequence between antibody $V_H$ and $V_L$ variable regions. Because scFv not only overcomes HAMA detection limitations but also has a small molecular weight, it exhibits good penetration, has a high target to non-target ratio, is quickly cleared from the serum, and is easily prepared and transformed for the purposes of genetic engineering. Accordingly, scFv plays an important role in the study of genetically engineered antibodies. In turn, phage display technology can join together the features of specific antigen recognition with the power of phage amplification through use of scFv, and thus can obtain the rapid generation of a wide variety of antibodies with a high degree of specific antigen binding activity. This strategy is predicted to have a broad range of application prospects in the future (Gu et al., 2012).

To take advantage of these benefits, phage display library technology was used in this study. RNA was extracted from the peripheral lymph nodes of patients with ATC. The $V_H$ and $V_L$ genes of the antibody variable region were amplified by RT-PCR, and the resultant scFv fragment, which was spliced together using SOE-PCR technology, was transferred into a pCANTAB-5E vector and used to infect *E. coli* along with the helper phage M13K07. Thus, a fully human ATC scFv phage display library was successfully constructed.

The crucial features of this technology are a sufficiently large storage capacity. Compared with other technologies, the greater capacity of the phage to display library technology, the greater possibility to select the specific antibodies (Sidhu et al., 2000). The capacity of the library depends on the transfer efficiency of the bacterial gene, which can satisfy the requirements of screening when its value is $10^7$-$10^8$ CFU/μg, as is customarily achieved.

In this study, the conversion efficiency of the PCR ligation products was 3.1 x $10^7$ CFU/μg, and the positive insertion rate of the antibody gene identified by PCR was 86.4%. This indicated that constructing human ATC single-chain antibody phage libraries meets the basic requirements for library capacity and lays the foundation for the further screening, identification, and preparation of a high-affinity and high-specificity human monoclonal antibody phage for the treatment of ATC.

## Conflict of interest

The authors declare no conflict of interest.

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## REFERENCES

Amodeo C, Caglià P, Gandolfo L, Veroux M, et al. (2003). Undifferentiated carcinoma of the thyroid. *Tumori* 89: 205-206.

Are C and Shaha AR (2006). Anaplastic thyroid carcinoma: biology, pathogenesis, prognostic factors, and treatment approaches. *Ann. Surg. Oncol.* 13: 453-464.

Derbel O, Limem S, Ségura-Ferlay C, Lifante JC, et al. (2011). Results of combined treatment of anaplastic thyroid carcinoma (ATC). *BMC Cancer* 11: 469.

Giuffrida D and Gharib H (2000). Anaplastic thyroid carcinoma: current diagnosis and treatment. *Ann. Oncol.* 11: 1083-1089.

Gu YJ, Yang XY and Zhang XK (2012). Constructing of human lung cancer phage antibody library. *Guide Chin. Med.* 19: 22-24.

Ito K, Hanamura T, Murayama K, Okada T, et al. (2012). Multimodality therapeutic outcomes in anaplastic thyroid carcinoma: improved survival in subgroups of patients with localized primary tumors. *Head Neck* 34: 230-237.

Kihara M, Miyauchi A, Yamauchi A and Yokomise H (2004). Prognostic factors of anaplastic thyroid carcinoma. *Surg. Today* 34: 394-398.

Li GP (1998). Technology of pretargeted in imaging and treatment of tumor radioimmun. *For. Med. Sci. Cancer Sect.* 25: 26-29.

Marks JD, Hoogenboom HR, Bonnert TP, McCafferty J, et al. (1991). By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J. Mol. Biol.* 222: 581-597.

Osbourn J, Jermutus L and Duncan A (2003). Current methods for the generation of human antibodies for the treatment of autoimmune diseases. *Drug Discov. Today* 8: 845-851.

Sherman EJ, Lim SH, Ho AL, Ghossein RA, et al. (2011). Concurrent doxorubicin and radiotherapy for anaplastic thyroid cancer: a critical re-evaluation including uniform pathologic review. *Radiother. Oncol.* 101: 425-430.

Sidhu SS, Lowman HB, Cunningham BC and Wells JA (2000). Phage display for selection of novel binding peptides. *Meth. Enzymol.* 328: 333-363.

Van Glabbeke M, van Oosterom AT, Oosterhuis JW, Mouridsen H, et al. (1999). Prognostic factors for the outcome of chemotherapy in advanced soft tissue sarcoma: an analysis of 2,185 patients treated with anthracycline-containing first-line regimens - a European Organization for Research and Treatment of Cancer Soft Tissue and Bone Sarcoma Group Study. *J. Clin. Oncol.* 17: 150-157.

Vezzosi D, Bennet A and Caron P (2007). Recent advances in treatment of medullary thyroid carcinoma. *Ann. Endocrinol.* 68: 147-153.

Wein RO and Weber RS (2011). Anaplastic thyroid carcinoma: palliation or treatment? *Curr. Opin. Otolaryngol. Head Neck Surg.* 19: 113-118.

Xia J, Bi H, Yao Q, Qu S, et al. (2006). Construction of human ScFv phage display library against ovarian tumor. *J. Huazhong Univ. Sci. Technol. Med. Sci.* 26: 497-499.

Zhao Y, Wang QM, Fu XQ, Chen JZ, et al. (2004). Construction of phage display antibody library to MCF-7 cells and screening of single-chain antibodies against breast cancer cells. *Sheng Wu Gong Cheng Xue Ba* 20: 667-672.

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