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Development of Human Recombinant Antibodies Against ROR1 Tumor Antigen

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Abstract

Background: Receptor tyrosine kinase-like orphan receptor 1 (ROR1) is an oncofetal antigen expressed on many types of cancer cells, but not normal adult cells. ROR1 antigen contributes to cancer development and progression by several signaling pathways. ROR1 expression has been associated with tumor growth, survival, and metastasis. In this study specific human recombinant antibodies were selected against ROR1 antigen for their use in cancer immunotherapy.
Methods: Phage display technology was used to produce phage antibody from a human scFv library. Phage concentration was determined to confirm the phage rescue process. Panning procedure was performed to isolate specific scFv clones against ROR1 epitope. Phage ELISA was done to evaluate the reactivity of the selected scFvs.
Results: Two specific human scFvs with frequencies of 20% and 25% were selected against ROR1 peptide. The antibodies showed specific reaction to the corresponding epitopes in phage ELISA.
Conclusions: Cancer targeted therapy using human specific antibodies is a new strategy, which is used in cancer therapy. The selected specific scFvs that target ROR1 epitope are human antibodies that originated from a human library and have the potential to be used in clinic in cancer immunotherapy of ROR1 positive tumors without induction of human anti mouse antibody (HAMA) response.

Keywords: ROR1, Phage display, scFV library, Cancer.

Introduction

Cancer is a major health problem worldwide and imposes a great economic and psychological burden in addition to loss of life and fertility (1). Today, several therapeutic methods are used for cancer therapy such as chemotherapy and radiation therapy, but these therapeutic methods are limited by a narrow therapeutic index, significant toxicities, and high rate of acquired resistance. More recently, an improved understanding of cancer pathogenesis has given rise to new treatment options including targeted therapy (2, 3). Targeted therapy refers to a new generation of cancer drugs designed to interfere with a specific target protein that is believed to have a critical role in tumor growth or progression (4).

One of the most popular approaches for targeted therapy is antibody based-targeted therapy.

Over the past decade, the effectiveness of antibodies in treating patients with cancer has been increasingly recognized in treating patients with haematological malignancies and solid tumors (5, 6). The definition of cell surface antigens that are expressed by human cancers has revealed a broad array of targets that are overexpressed, mutated or selectively expressed compared with normal tissues. Receptor tyrosine kinase-like orphan receptor 1, RoR1, is a single-pass type I membrane glycoprotein belong to the receptor tyrosine kinase (RTK) family which is known to be a

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key regulator of normal cellular processes such as differentiation, migration, proliferation and survival (7) and is evolutionarily conserved in invertebrate and vertebrate (7, 8). ROR1 protein is consist of 937 amino acid residues and composed of three extracellular domains, one transmembrane domain and one intracellular domain. The extracellular domain consists of three parts including an immunoglobulin (Ig)-like domain, a cysteine rich (CRD) frizzled domain and a kringle (KRD) domain (9)**.** ROR1 protein is expressed during embryogenesis but absent from normal adult tissues, apart from a subset of immature B-cell precursors, and low level expression on adipocytes (10). In addition to ROR1expresion during normal embryonic and fetal development, this protein is also expressed on many cancers. ROR1 was first shown to be expressed in B-cell chronic lymphocytic leukemia (B-CLL) and was subsequently identified on the surface of many cancers including mantle cell lymphoma (MCL), acute lymphoblastic leukemia (ALL) and a subset of lung, ovarian, colon, breast pancreas, and renal cancers (11). Their unique expression profiles provided this molecule as a novel class of therapeutic targets for small molecules inhibitors against the kinase or for antibody-based therapies against this receptor (12). Beside its unique expression on the tumor cell, several studies have been shown direct correlation between the expression of ROR1 and aggressive behavior of tumor cells including tumor growth, survival, metastasis (12, 13) and poor clinical outcomes (14).

Single chain fragment variable (scFv) is the smallest part of immunoglobulin molecule with function in antigen-binding activities. ScFv antibody consists of variable regions of heavy (VH) and light (VL) chains, which are joined together by a flexible peptide linker (15). These fragments retain the binding specificity of the whole antibody and offer several advantages compared to full-length mAbs. It can be easily expressed in functional form in *E. coli* and display improved pharmacokinetic properties, such as better tissue penetration (15-18).

In this study specific human scFvs against ROR1 epitope were selected using phage display technology The epitope was obtained from CRD domain of extracellular part of ROR1 (19).

Materials and Methods

Phage rescue of scFv human library

A phage antibody display library of human scFv was produced (20). *Escherichia coli* (*E. coli*)

bacteria containing phagemid vector were cultured on 2TYG agar/ampicillin plates overnight at 30 °C. The bacteria were incubated in 2TYG broth at 37 °C for 1 hour. Helper phage (M13 KO7) was added and incubated at 37 °C for 30 minutes. The culture was shaken for 30 minutes and centrifuged at 0.5 g for 20 minutes. The pellet was transferred to 2TY broth containing ampicillin (100 g/mL) and kanamycin (50 g/mL) and incubated with shaking at 30 °C overnight. Following centrifugation, the supernatant was passed through 0.2 filter and stored at 4 °C.

Panning and selection of anti-ROR1 scFv

Panning process was performed on a phage antibody library of scFv (21, 22). Briefly, the specific phage scFv antibodies were isolated against an immunodominant peptides from extracellular domain of ROR1 (aa 191–205; YMESLHMQGEIENQI). This epitope was used for anti-ROR1 monoclonal antibody production (17). Ten µg/ml of the peptide was coated on immunotubes for **16** hours at 4 °C (Nunc, Roskilde, Denmark). The phage-rescued supernatant (10^{11} pfu/ml) diluted with skimmed milk, as blocking solution, was added to the tubes, and incubated for 1 h at room temperature and then immunotubes were washed 10 times with PBST and 10 times with PBS. Afterward, log phase TG1 *Escherichia coli* bacteria was added and incubated for 2 h at 37 °C. After centrifugation, the bacterial pellet was grown on 2TY-ampicillin agar plates. Four rounds of panning were performed to isolate specific antibodies against the ROR1 epitope.

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DNA fingerprinting of the selected anti-ROR1 clones

Colony PCR was performed to investigate the presence of the desired band corresponding to the scFv gene and DNA fingerprinting with *Mva-I* restriction enzyme was used to reveal the common dominant patterns. An individual clone with the most frequent pattern was selected.

Measuring of the phage concentration of phage rescue supernatant

Ten µl of Phage antibody supernatant was added to 1 ml logarithmic phase TG1 *E. Coli* and incubated at 37 °C for 1 h with shaking. It was diluted serially and plated onto 2TY ampicillin plates. Number of colonies per dilution was counted and phage concentration titer/ml was determined.

ELISA screening of the selected clones

In order to detect antigen recognition, 96 well polystyrene plates were coated with 10 µg/mL of ROR1 peptide and incubated overnight at 4 °C. The experiment was set in triplicate. Equal amounts of an unrelated peptide, which was anti-HER2 scFv were also coated as negative control. Plates were blocked with 2% (w/v) skimmed milk in PBS for 2 h at 37 °C. Wells were washed with PBS/Tween-20 and PBS. Phage-rescued supernatants, at equal concentration of phage particle, 10^{12} pfu/mL, were diluted with blocking buffer and added to the plate and incubated at room temperature (RT) for 2 h. Following washing, the plate was incubated with rabbit anti-fd antibody. After washing anti-rabbit horseradish peroxidase (HRP) conjugated antibody was added. Following 1 h of incubation at RT, the plate was washed, and bound phages were detected with TMB (3,3,5,5-tetramethylbenzidine) peroxidase substrate, the absorbances was measured by ELISA reader. Absorbances were read at 450 nm after 10 min using an ELISA reader (BP-800, Biohit, USA).

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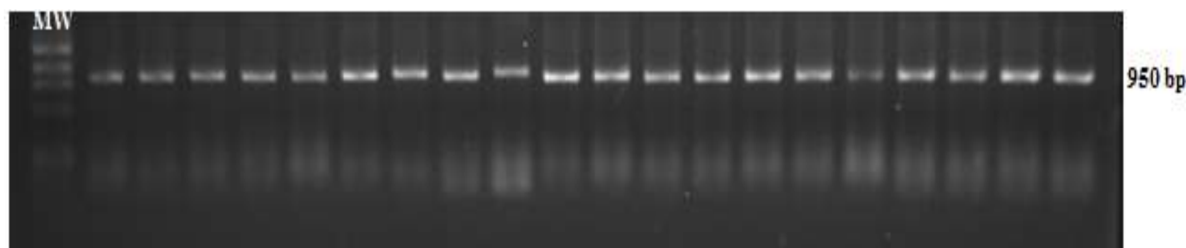
Results

Selection of anti-ROR1 scFv antibodies

After four rounds of panning against ROR1 peptide, PCR of randomly selected clones demonstrated 950 bp bond corresponding to scFv gene (Fig. 1A). *Mva 1* DNA fingerprinting demonstrated two specific clones with frequencies with frequencies 25% (5/20) and 20% (4/20) (Fig. 1B). Fingerprinting of un-panned library, which showed heterogeneous pattern of digestion, is shown in (Fig. 1C).

A



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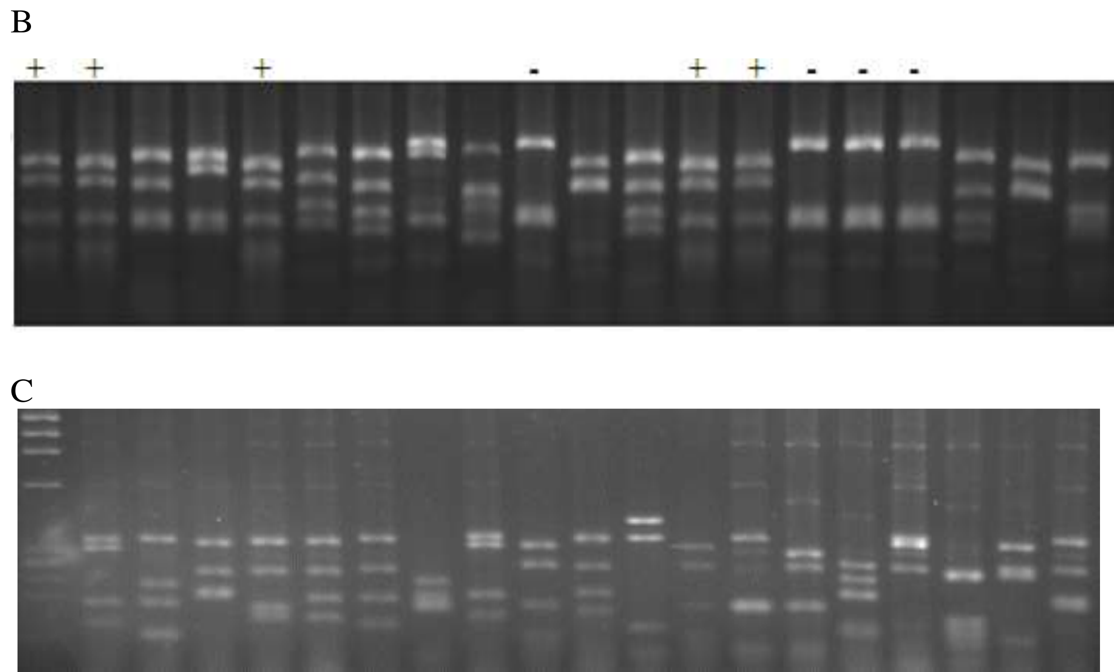

Fig. 1. PCR and *Mva-I* DNA fingerprinting of scFv clones. (A) PCR of 20 clones after panning showed 950 bp bond of scFv insets. (B) Common patterns with frequency of 25% and 20% were obtained against ROR1 peptide. (C) A heterogeneous pattern of fingerprinting in un-panned library.

Assessment of specific phage antibodies in phage ELISA

Phage-rescued supernatant from a clone, which showed dominant pattern in DNA fingerprinting was evaluated by phage ELISA to assess specific binding to ROR1 peptides. Figure 2 demonstrates that the selected anti-ROR1 scFv phage antibodies reacted with the corresponding epitope significantly. The ODs for scFv I and II were 7 and 5 times higher than the OD detected for no peptide well (ODs: 0.15 and 0.16, respectively). In addition, reactivities of M13 KO7 helper phage and unrelated scFvs to ROR1 epitope were negative.

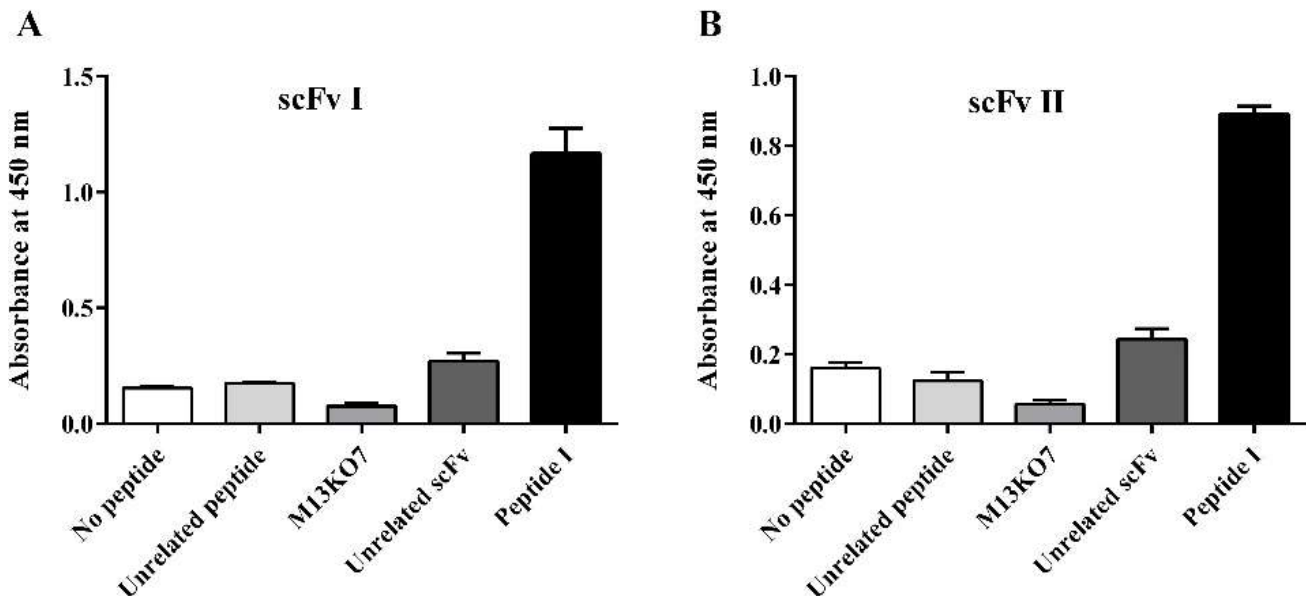

Fig. 2. Phage ELISA results of selected scFvs (I and II) against ROR1 peptide. The antibodies bond to the corresponding peptide significantly higher than reaction obtained for no peptide well controls. Unrelated scFv and M13KO7 did not show reactivity with the ROR1 epitope.

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Discussion

Recombinant antibody (rAb) fragments are becoming popular therapeutic alternatives to full length monoclonal Abs due to their remarkable specificity and unique binding properties. ScFv Abs are one of the most popular rAb format as they have been engineered into larger, multivalent, bi-specific and conjugated forms for many clinical application (23, 24).

The expression of ROR1 as an oncofetal antigen on cancer cells but not normal cells has been an advantage for targeted therapy against this antigen (25). In addition, RoR1 roles in cell signaling, proliferation, migration and metastasis had made this molecule an interesting candidate target for cancer immunotherapy of ROR1 positive tumor (26). Studies on extracellular parts of ROR1 demonstrated that targeting extracellular Ig-like, CRD or KNG domains of ROR1 molecule by mAbs can block ROR1 function (17). To select specific scFvs against ROR1, the epitope was derived from CRD domain of ROR1. This epitope was applied for generation of monoclonal antibodies against ROR1. Two specific mAbs selected against this peptide could significantly induce apoptosis in chronic lymphocytic leukemia cells (19).

Panning process resulted in the isolation of two specific scFv antibodies against the epitope.

Random colony selection following panning showed positive PCR, demonstrating the presence of scFv genes within the selected clones. Fingerprinting results demonstrated two enriched patterns with enriched patterns with 20 % and 25 % frequencies. Panning process has been extensively used as a reliable technique for isolation of a specific binding moiety from a phage display library (27, 28). This strategy has several superiorities than other methods. This strategy is more rapid, simple and cost effective than other laborious methods like hybridoma technology (29, 30). Phage displaying library of scFv antibodies can confer higher diversity over using hybridoma derived from mouse injected by an immunogen (28). Moreover, using a human scFv library provides specific human scFv clones that will not produce human anti mouse antibody, HAMA, response and would be applicable for clinical use.

The results of ELISA demonstrated specific binding of the selected clones to the ROR1 peptide. The selected scFvs did not react with unrelated peptide and very low reactivity was detected for no peptide well. According to previous phage ELISA experiments when average of OD value is at least two fold higher than negative controls, positive phage ELISA is detected (31). In the current study the detected ODs were 5-11 times greater than ODs of no peptide negative control wells.

The anti ROR1 human scFvs isolated in this study that bind to the ROR1 epitopes specifically have the potential to be used in cancer immunotherapy for ROR1 positive tumors. As ROR1 is highly expressed in B-cell chronic lymphocytic leukemia (B-CLL), but not on normal tissues, it would be a good target for immunotherapy using anti-ROR1 human scFvs, which are not only high specific antibodies but also have human origin and will not be rejected in human body. It has been demonstrated that the Naïve immune library can match the best immune-sourced antibodies (32). Therefore, scFvs originated from synthetic or semi-synthetic libraries cannot match high quality sourced antibodies. The anti-ROR1 scFvs selected in this study are originated from a human Naïve library that has the advantage of capturing the natural repertoire of antibodies, which is a great superiority over other forms of antibody selection.

The small fragments of scFvs also facilitates the genetic manipulation of antibody fragments that provides a unique opportunity to produce new more effective immunoconjugates with additional effective function against cancer development. By examining the intracellular signals, we get the idea of how to equip scFv antibodies so that it can better perform anti-cancer action. ROR1 is a receptor that binds to WNt5a that may complex with TCL1 that is a coactivator of

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AKT pathway, which can promote the development of CLL (33). Manipulating of specific anti-ROR1 scFvs and conjugating the antibodies with anti TCL1 scFvs will result in a high potency product that would stop cancer progression dramatically. Therefore, fusion peptides produced by scFv genes can contribute to future cancer therapy for blocking cancer in different ways.

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