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Study on the relationship between the structure and functions of anti-human cervical cancer single-chain antibody and the lengths of linkers

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Summary

Background: This study aimed to find the linker with minimal impact among chains to fight against the structure and function of cervical cancer (CC) single-chain antibody. *Materials and Methods:* The original variable region of heavy chain (VH) and variable region of light chain (VL), and the single-chain antibody with linkers of different lengths (n = 1~8) were modeling by homologous modeling, while the peptide chain structure of $(Gly_4Ser)_n$ was utilized by the linkers. Comparison of the similarity of original VH/VL and VHn/VLn was carried out by applying the algorithm of spatial hierarchical alignment based on the spherical coordinates. The fore and aft distance and diffusion radius of alpha (α) were also calculated. The stability of antibody with different linker length was then compared. ELISA method was adopted to evaluate the immunological activity of single-chain antibody with different linkers. MTT assay was used to analyze the inhibition effect of ScFv-n on CC cells. *Results:* When n = 4, the structures were the most similar between ScFv and the original VH/VL. When n = 3, the influence of adding connecting peptide on the stability of single-chain antibody was the least. The result of ELISA and MTT methods indicated that when n = 3, single-chain antibody gained the highest activity. *Conclusion:* The optimum length of linker of anti-human CC single-chain antibody was n = 3 from the point of mathematical modeling and biology experiments. This study provided new ideas for the design and constructions of single-chain antibody, and theoretical basis for the treatment of CC.

Key words: Cervical Cancer; Single-chain Antibody; Linker; Homologous Modeling; Similarity Algorithm.

Introduction

Cervical cancer (CC) is a malignant tumor occurred in the uterus vagina and cervical canals. It is one of the most common gynecological malignancies [1] and the incidence takes the second place among gynecologic oncology. The age with high incidence of carcinoma in situ (CIS) in 30 to 35 years and that of infiltrating carcinoma is 45 to 55 years. In recent years, patients with CC become younger in age. Due to the universal application of cervical cytology screening in recent decades, early detection and treatment of CC and precancerous lesions have been realized. The incidence and mortality rates of CC have decreased significantly. However, in developing countries, where CC screening is not completely become popular, hence the morbidity of CC remains high. There are about 530,000 new cases around the world every year, while 85% of them are in developing countries. The high morbidity of CC is one of the main reasons of women death in developing countries [2, 3]. The first therapeutic regimen in the early period of CC is operative treatment, and assists with radiotherapy in accordance with the high risk factors after the treatment [4]. Comprehensive treatment methods of later period are mainly by radiotherapy and chemotherapy. In developed countries, most CC can be detected in the early period; while in developing countries, over 80% patients are in the later period when they diagnosed as CC [5]. Moreover, the effective rate of chemotherapy is only range within 20% - 30%. As a result, an insight into the molecular mechanism of genesis and development of CC, and a search for new target spots of diagnosis are imminent.

With constantly thorough study of molecular biology, genetically engineered antibody has been widely used in the diagnoses and treatments of diseases. It has great potential in the diagnosis and treatment of malignancy, which bring a new direction to the treatment of CC. There are many reports regarding targeted therapy of CC that adopt monoclonal antibody (mAb) as the vector. However, mAb shows some limitations in clinic for some defects, as production of human anti-mouse antibody reaction (HAMA) that aimed at mAb, large amount of antibody molecules, low penetrating ability, low clearance rate of blood, and low ratio of tumor blood, etc. [6]. Single-chain variable fragment (ScFv) is a small molecule antibody that and has many advantages, as intact antigen-binding site, small amount of antibody molecules, high penetrating ability, low immunogenicity, and simple to mass produce. It can also neutralize

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virus and toxin to process immune in cells and be a guided carrier. Although this kind of antibody has been applied to the diagnoses and treatments of the clinic, there are few reports of the application to CC. Therefore, it is a great significance to research the single-chain antibody of CC.

ScFv is recombinant protein linked by a length of linker through VH and VL. Different lengths of linkers among chains have different influences on the activity of single-chain antibody; hence an insight into the impact of different lengths of linkers among chains on the biological activity of single-chain antibody has great significance to establish single-chain antibody and screening of linkers among chains. This study design constructed a universal expression vector with different linkers among chains. Through the comparison of the impact of different linkers among chains on the structures and functions of single-chain antibody of CC, this paper provides new ideas of the screening of different linkers among chains of CC and the establishment of single-chain antibody, and also provides some theoretical basis of targeted treatment of CC.

Materials and Methods

Experimental materials

The amino acid sequences of single-chain antibody VH and VL of CC were from the articles related to researches of the anti-human cervical cancer composed by Wang *et al.* [7]. HeLa human cervical epithelial cell line was preserved in the present lab. The authors themselves constructed the general expression vector of single-chain antibody of connecting peptide with different length and purified its expression.

Experimental methods

Modeling

The present authors modeled a three-level structure of VH and VL using homologous modeling. The amino acid sequences of VH and VL obtained from literatures were uploaded to swiss-model server for modeling. Thus the pdb structured data file (pdb code: VH:2gki; VL:3hzm) and three-dimensional (3-D) structured diagrammatic figure corresponding to original VH and VL could be obtained as the data of comparisons and references.

Selection of linker

The amino acid sequences of single-chain antibody could be obtained when appropriate linker was added between VH and VL. The linkers that applied most extensively were proposed by Huston *et al.* [8]. They were the variable region structure of antibody in accordance with analysis of x-ray crystallography, and $(Gly_4Ser)_n$ with rigid structure designed by computer-aided analysis results, which means n times repeated emergences of four glycine, one serine, and five amino acid residues [9, 10]. These linkers had better flexibility, which was equal to the ability of spatial bending, and small spatial steric hindrance, which advanced in the interaction and forming the correct conformation of fragments of VL and VH, and improving the stability of antibody molecules. Also, these structures would not be recognized and degraded by protease easily, which was good for the internal stability of antibody molecules.

$(Gly_4Ser)_n$ (n = 1, 2, 3…) was adopted as the linker of the single-chain antibody in this study, and the structured data file and 3-D structured diagrammatic figure of corresponding VHn and VLn were obtained by homologous modeling of ScFv-n.

Structure comparison and stability analysis

Methods of inference to the functional similarity of biomolecules mainly included two categories. One was to determine the function of undetermined proteins by comparison of sequences of new measurement and the known database, another was structural and functional analysis based on the structure. For the complex mechanism of biological evolution, the function of protein could not be obtained only by the analysis of sequences. By the viewpoint of bioinformatics, the characters and functions were decided by the structures of molecules, and the biological functions of proteins were decided by the spatial structures of proteins. Therefore, the method of comparison of spatial structures was adopted for the comparison of similarity of protein molecules [11].

There were four basic methods of space division, 3-D grid method (GRID), fan type method (ICO), spherical mapping method (SPH), and sphere method or spherical shell method (BALL). The space spherical shell hierarchical matching algorithm based on spherical coordinates, which is put forward by Zhang *et al.* [12] is used for calculation of the similarity. First, coordinate utilized formula (1, 2, 3) of 3-D structure model that provided by pdb file was converted into spherical polar coordinates.

$$r = \sqrt{x^2 + y^2 + z^2} \qquad r \in [0, +\infty) \tag{1}$$

$$\varphi = \arctan\left(\frac{y}{x}\right) \qquad \varphi \in [0, 2\pi) \tag{2}$$

$$\theta = \arccos\left(\frac{z}{r}\right) \qquad \theta \in [0, \pi] \tag{3}$$

Then the largest space of molecules was separated to spherical shell unit with particular radial thickness. The number of the same kind of atoms in every spatial block was calculated to build vector quantity a, b. The amount of the same kind of atoms (mainly C, N, and O) in different space shell was counted and summed up in accordance with predetermined weight. The last number of atoms in that shell was obtained. Multi-dimensional vector a_i and b_i was constituted by the number of atoms in every shell according to the divided order. At last, the commonly used similarity included angle cosine of approximate function was conducted as the approximate function of similarity. It is the following:

$$\cos\langle a, b\rangle = \frac{\sum_{i=1}^{n} a_i b_i}{\sqrt{\sum_{i=1}^{n} a_i^2 \sum_{i=1}^{n} a_i^2}} \tag{4}$$

The stability of ScFv could be expressed by the fore to aft distances or the diffuse radiuses, which means the stabilities of single-chain antibody with different lengths of linkers were compared by the distances from first alpha (α) carbon atom to the last α carbon atom and diffuse radiuses. The fore to aft distances of VH and VL original structure and that of VHn and VLn when added different length of linkers (n = 1~8) were figured out. Then their maximum diffuse radiuses were calculated, respectively.

Gene identification of single-chain antibody

Single-chain antibody expression vectors constructed by connecting peptide containing different length were identified using PCR. PCR products were analyzed using 1.0% agarose gel electrophoresis.

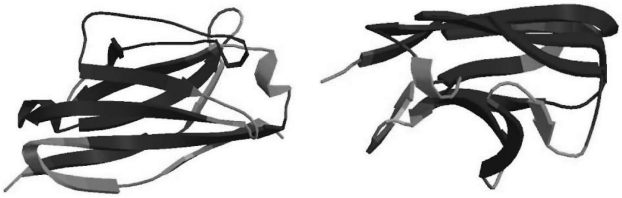

Figure 1. — 3-D structures gained by the independent modeling of VH and VL.

ELISA detection of ScFv-n immunological activity

Human HeLa cells in the logarithmic phase were collected. They were inoculated in 96-well plates of 5×10^4 cell / ml, 100 μL / well. It was cultured at 37°C for 24 hours, washed three times with PBS, and fixed by 2.5% glutaraldehyde at 37°C for five minutes. After washing, equimolar single-chain antibody of connecting peptide with different length was added to each well as the primary antibody. PBS was added in negative control well. Goat-anti-mouse HRP antibody was treated as the second antibody. 100 μl antibody was added to each well, and incubated at 37°C for two hours. Substrate TMB was added after washed with PBS, coloration at 37°C away from light for five minutes. Then 100 μl stop buffer (one mol / L H_2SO_4) was added to each well. Microplate reader was used to detect the OD value at a wavelength under 450 nm.

Detection on single-chain antibody inhibition rates for cervical cancer cells using MTT assay

Human HeLa cells in the logarithmic phase were selected, which were then transferred to $(2 \sim 4) \times 10^5$ cells / ml, inoculated in 96-well plates of 100 μl / well, and cultured in 5% CO_2 at 37°C. Each well in the experimental group was added 100 μl single-chain antibody containing a different length of the connecting peptide. Each length of the linker was made in triplicate. One group of cancer cell suspension without antibody was set up as control group, and a group added culture medium was regarded as zero hole, cultured in 5% CO_2 cell incubator at 37°C. Each well was added 100 μl of MTT (0.2 mg / ml), incubated for four hours at 37°C, and removed the supernatant; 200 μl of it was then added to alkalize DMSO (10% glycine -NaOH buffer), incubated for one hour, vibrated, and determined the *A* value at 57 0nm using microplate reader. Each well was repeated more than three times, and the results were basically the same, with the median test values as the criterion. The inhibition rate of cervical cancer cells represented the activity levels of a single-chain antibody. The formula is as follows:

Inhibition rate = 1 - *A* value in experimental group / *A* value in control group × 100%

Results

Original VH and VL three dimensional structures of ScFv

The original VH and VL were modeled by homology modeling. The result is shown as Figure 1.

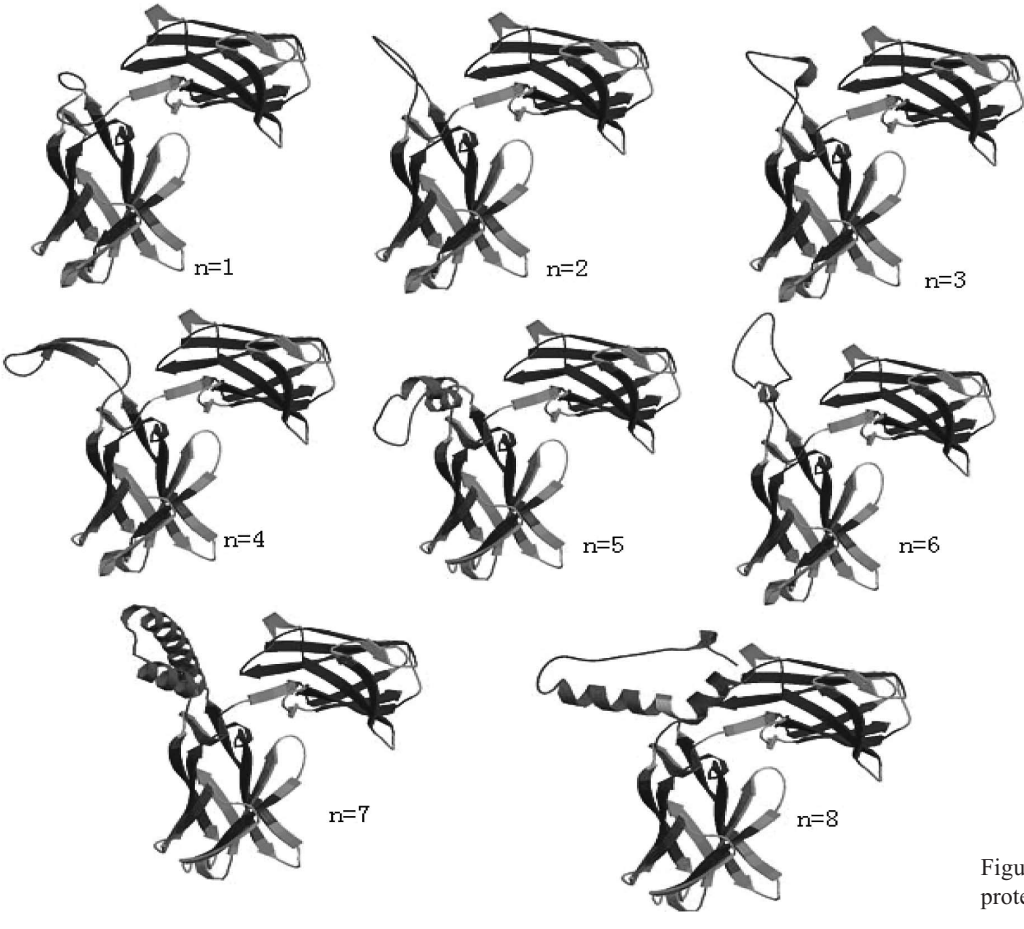

Figure 2. — 3-D structures of protein molecules when n = 1-8.

Table 1. — *Comparative results of similarity.*

Number of layers	2	3	4	Mean value	Number of layers	2	3	4	Mean value	Total mean value
VH1	0.9203	0.8419	0.6324	0.7982	VL1	0.9943	0.9709	0.8867	0.9506	0.8744
VH2	0.9143	0.8419	0.6042	0.7868	VL2	0.9932	0.9709	0.8953	0.9531	0.8700
VH3	0.9150	0.8412	0.6175	0.7912	VL3	0.9932	0.9709	0.8953	0.9531	0.8722
VH4	0.9117	0.8423	0.6463	0.8001	VL4	0.9932	0.9709	0.8953	0.9531	0.8766
VH5	0.9171	0.8416	0.6365	0.7984	VL5	0.9932	0.9697	0.8849	0.9493	0.8738
VH6	0.9154	0.8419	0.6157	0.7910	VL6	0.9932	0.9709	0.8953	0.9531	0.8721
VH7	0.9142	0.8386	0.6474	0.8001	VL7	0.9938	0.9697	0.8942	0.9526	0.8763
VH8	0.9151	0.8391	0.6521	0.8021	VL8	0.9932	0.9697	0.886	0.9496	0.8759

Table 2. — *Distance between head and tail and radius of VH, VL, and VHL before and after adding linkers.*

	Distance between head and tail	Radius		Distance between head and tail	Radius		Distance between head and tail	Radius	Distance between head and tail of linkers
VH1	53.6011	34.7554	VL1	46.7338	30.0575	VHL1	45.6794	216.5253	10.1888
VH2	56.2322	38.7563	VL2	43.6335	27.6651	VHL2	45.6783	216.5592	14.0992
VH3	55.6829	39.5128	VL3	43.6429	27.6653	VHL3	45.6783	216.8067	11.7021
VH4	50.1406	37.4041	VL4	43.6302	27.6654	VHL4	45.6783	215.9425	27.8454
VH5	45.9529	28.5646	VL5	43.6084	27.6612	VHL5	45.6782	216.3861	14.2882
VH6	56.1668	37.7859	VL6	43.6321	27.6654	VHL6	45.6777	217.4843	12.2691
VH7	48.3040	31.8512	VL7	43.6263	27.6624	VHL7	45.6782	217.0619	12.7475
VH8	45.4060	30.8712	VL8	43.6034	27.6596	VHL8	45.6803	216.3476	26.4597
VH	39.9954	41.8673	VL	43.9975	29.1371				

Three dimensional structures of ScFv-n (n = 1~8)

In general, the linkers of ScFv were ranged within 4 to 44 amino acids [12]. The longer the linker was, the more instable structure of ScFv was. In the process of modeling, when n = 9 (the linker was 45 amino acids in length), the searched template had lower homology, so the modeling declared a failure. Therefore, the authors finally obtained the pdb data file and 3-D model of ScFv-n when n = 1~8, which meant the lengths of linkers were ranged within 5-40 amino acids. The 3-D structures are presented in Figure 2.

Figures 1 and 2 had shown that the original structural model of VH and VL had some differences to the structure after adding the linkers. In addition, the structure of the linker was changed following the length of it. It was visible that the lengths of these adding linkers had direct influence on the spatial structure of single-chain antibody.

Similarity and stability analysis of VH and VL of ScFv-n

Then the similarities among the original VH/VL and VHn/VLn with adding linkers were compared using protein similarity algorithm based on spherical polar coordinates hierarchy. The similarities are shown in Table 1.

During the counting process, it was indicated that the sequence of single-chain antibody was fairly short. If the number of molecules' shells was too large, the results would not be stable and correct enough. In this way, spherical molecules were separated into two, three, and four layers. Then the similarities were calculated and the mean value was obtained. The higher similarity indicated that the minimal impact of adding linkers on the structure of protein, and the minimal impact on the activity of single-chain antibody. From Table 1, it is shown that when n = 4, the similarity between that VH/VL and original VH/VL was the highest, which indicated that the influence of the adding linkers on the structures of single-chain antibody in variable region was the least.

The influences of adding linkers on the stability of single-chain antibody were analyzed through the calculation of the fore and aft distances and diffuse radiuses. The results are shown in Table 2. The results in Table 2 show that:

- When n = 8, the fore and aft distance of VH was the closest to that of original VH. While n = 3, the diffuse radius of VL was the closest to that of original VL. When n = 1, the diffuse radius of VL was the closest to that of original VL. Moreover, when n = 2~8, the diffuse radiuses of VL were almost the same. As a result, when n = 3, the influence of adding linker on the stability of single-chain antibody was the least.
- The fore and aft distances and radiuses changed little after added linkers.
- When $n \leq 4$, the fore and aft distances of linkers were increased with the increases of n value. However, when $n > 4$, the fore and aft distances of linkers were decreased for the spatial folding and bending.

The structure of the path of α carbon atom of original VH, VL, and ScFv-n that built by Matlab 2008 was compared with positions in Figures 3 and 4. The two figures

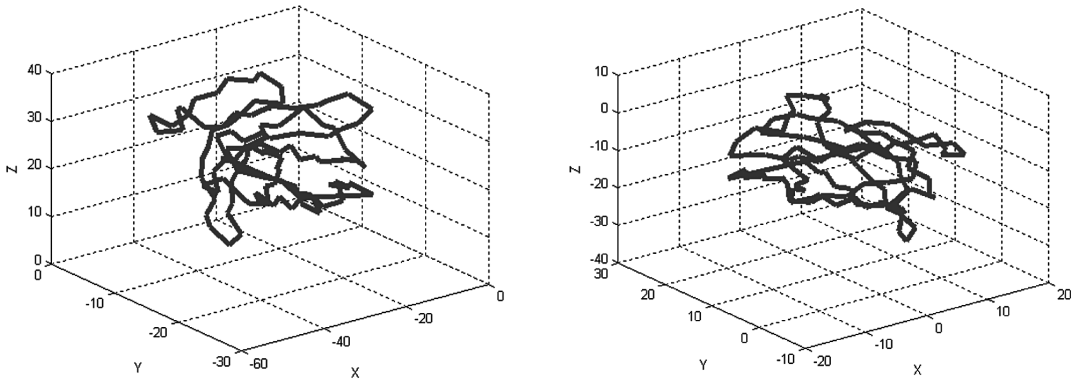

Figure 3. — α carbon atom path of original VH and VL.

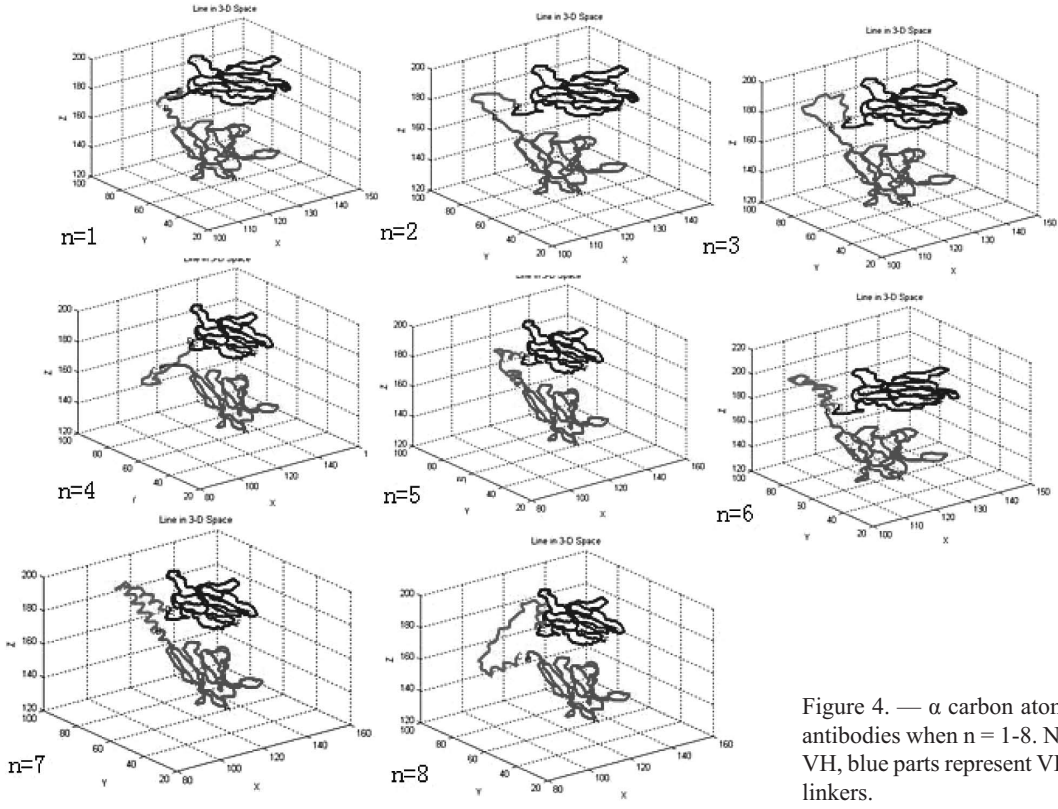

Figure 4. — α carbon atom paths of single-chain antibodies when n = 1-8. Note: red parts represent VH, blue parts represent VH, green parts represent linkers.

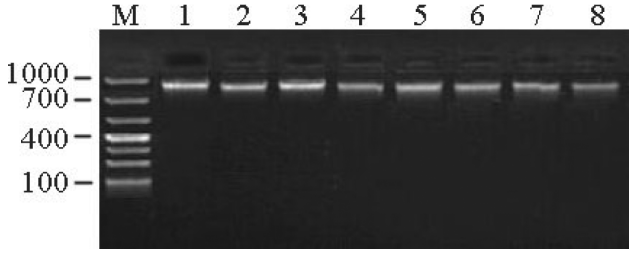

Figure 5. — PCR identification of single-chain antibodies. Note: 1~8 represent electrophoresis results of single-chain antibodies when n = 1~8.

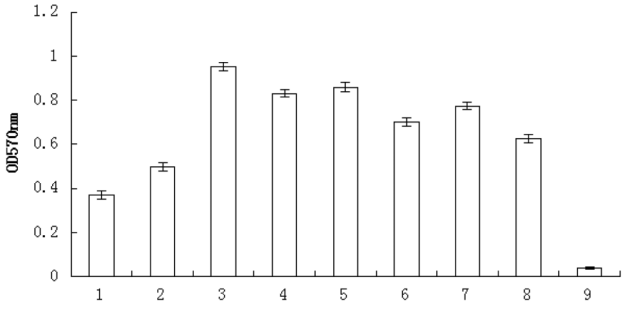

Figure 6. — ELISA detection of single-chain antibody immunological activity.

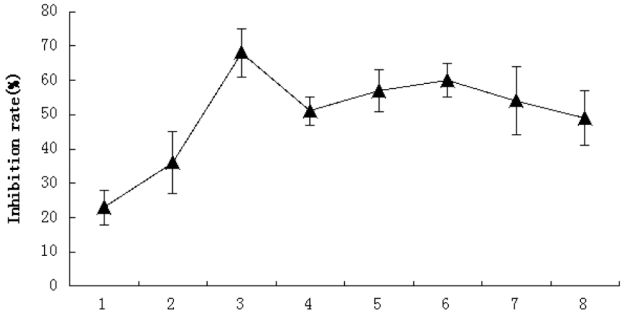

Figure 7. — MTT method detection of inhibition rate on cancer cells of single-chain antibody.

present the fore and aft distance changes of VHn and VLn more directly, indicating small changes in overall radial of molecules after adding linker with different lengths.

Gene identification of single-chain antibody

The established expression vectors of single-chain antibodies with different lengths of linkers were identified. After analyzing PCR products by agarose gel electrophoresis, a DNA fragment at about 830 ~ 950 bp was obtained (Figure 5). The sequencing results were entirely consistent with the expected sequence.

ELISA detection of single-chain antibody immunological activity

ELISA quantitative test results showed that when n = 1 ~ 8, all single-chain antibody had specific binding activity to cervical cancer cells. When n = 3, their immune activity was higher. The results are shown in Figure 6.

MTT method detection of inhibition rate on cancer cells of single-chain antibody

MTT method was used to detect the inhibiting effect of single chain antibody with different of linkers on HeLa cell line. The results are shown in Figure 7. When n = 1 ~ 8, the inhibition rate was more than 20%. When n = 3, the inhibition rate of ScFv against cervical cancer cell was the highest, reaching 68%. When n > 3, the inhibition rate decreased slightly.

Discussion

Single-chain antibody (ScFv) was a new reorganized protein, which belong to a micro molecule antibody of genetic engineering antibody. It has characteristics like small number of molecules (around 27 kD, only 1/6 of the complete antibody), no Fc fragment, low immunogenicity, good appetency, strong penetrability, short half-life period in body circulation, and easily reforming genetic engineering, etc. It has an extensive application prospect, because it has become the oriented isotopic carrier of diagnoses and treatments of tumors. Zitzmann *et al.* [13] took advantages of phage antibody library technology to screen the ScFv (DUP21) of prostate specificity, and the imaging showed that the radioactivity of cancerous area of prostate was three times higher than the normal tissue when marked by I^{131} which indicated that ScFv possessed favorable targeting. DeNardo reported that the combined application of ScFv that marked by IFN-C and I could increase up to two to four times than part of the mark concentration of tumors, which can remarkably strengthen the imaging results [14].

The linkers of VH and VL should have exact length. The over-short linkers will affect the freedom of spatial folding of protein, the structures and functions of protein will be affected at the same time, while the over-long linkers will influence the stability of ScFv, the immunogenicity will be affected and cause heterogenic protein reflection as well [15, 16]. According to the conformation of monomer Fv, the distance of a linker that crosses from C-terminal of a variable region to N-terminal of another variable region was about 35 to 40 lengths of amino acids [17]. So the selection of linker is very important that the linker should possess limpness, not form steric hindrance to interference the folding of heavy chains, and not have bad influence on the solubility of products. $(Gly_4Ser)_n$ link peptide in this study, proposed by Huston *et al.* [8], cannot only link the N-terminal to C-terminal in V region, but can tighten the VH and VL without affecting the interaction between them. Most of the applications of single-chain antibody directed medicine were still at stage of in vitro and animal experiments, while they had many difficulties in application to human body [18], of which the most important was that the appetency of ScFv was lower than the parent McAb. As to this problem, we can change the link peptide among chains to increase the appetency of ScFv.

So far, there have been many studies on the influence of lengths of linkers on the activity of ScFv. Gustavsson *et al.* [19] proposed that the range of the length of linkers to build single-chain bispecific antibody was within four to 44 amino acids. Le Gall *et al.* [20] proposed that the range of the length of link peptides was within six to 27 amino acids. Weisser *et al.* found that when the number of amino acids of linkers was 15, which was the same as n = 3 in $(Gly_4Ser)_n$, the appetencies, activities, and the other rounds could reach a satisfying degree [21]. All these were in an agreement with the results of the present study.

In this study, the changes of the lengths of linkers and the structures of variable regions were analyzed and compared by theoretical 3-D modeling at first. The study found that when n value was 4, the similarity between ScFv and original structure was the highest. By calculation of the distances from the first α carbon atom to the last α carbon atom and diffuse radiuses, the influence of linker on the stability of single-chain antibody in the variable region was the least when n value was 3. Through in-

direct ELISA and MTT methods, it was found that the biological activity of single-chain antibody was much higher when n value was 3. To sum up, the results of this study indicated that when n value of linker was 3, the structure and biological activity of anti-CC single-chain antibody were ideal, and $(Gly_4Ser)_3$ could be regarded as the linker of single chain antibody against human cervical cancer, which provided a basis for further study on anti-human cervical cancer single-chain antibody. It was found that the three-dimensional structure of the protein is more stable than its primary structure. The present study compared the stability of the protein in terms of protein structure. Through the three-dimensional structure model of the protein and the use of spherical polar coordinates, the present authors calculated the similarity of the protein and analyzed the stability of the protein structure, thus providing a new idea on protein function analysis and laid the foundation for the design and construction of single-chain antibody.

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