Synthesis of the mutant sequences for HER2 and survivin mimetic peptides of bladder cancer

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Abstract: Immunotherapy for bladder cancer could not only reduce tumor recurrence, but also more importantly prevent tumor progression of invasive bladder cancer. Studies have found that the tumor markers, HER2 and survivin, are related to the occurrence and prognosis of human bladder cancer. In order to conduct advanced research on immune therapy for bladder cancer, herein, we report the synthetic mutant sequences of HER2 and survivin mimetic peptides for bladder cancer by using phage peptide library technology. This will provide a good foundation, as well as the conditions for constructing a high-affinity epitope mimetic peptide library, in order to develop a polypeptide vaccine.

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Key words bladder cancer; Her2; mutant sequence**;** Ph.D-7 phage display peptide library; Survivin

1. Introduction

In China, urinary bladder cancer is the most common malignant tumor of the urinary system, of which more than 90% comprises transitional cell carcinoma. At the initial treatment, 70% to 80% of patients have a nonmuscle invasive bladder cancer (NMIBC). Although a transurethral resection could be utilized to remove a primary tumor, the recurrence rate of NMIBC is as high as 30% to 85% (Lamm, 1992). For bladder cancer, immunotherapy is better than chemotherapy, such as the internationally recognized Bacillus Calmette-Guerin (BCG). However, because of the likelihood of greater side effects, the application of BCG is limited. Recently, tumor-associated surface markers, HER2 and survivin, have received the attention of researchers. Many clinical trials regarding peptide vaccines have been carried out since the first clinical trial of a melanoma antigen gene-1-derived peptide-based vaccine was reported in 1996 (Hu et al, 1996). Compared with a traditional vaccine, it is considered to be one of the safest vaccines, can induce a specific immune response, and can be synthesized and purified quickly. It has been shown that tolerance to self-antigens could be overcome by certain protein parts that can selectively activate the immune system without activation of suppressor T-helper cells (Disis et al, 1996). HER2 is a member of the human epidermal growth factor receptor (EGFR) family. It is not expressed in normal bladder epithelium but is over-expressed by 40% to 80% in bladder cancer cells (Coogan et al, 2004; Røtterud et al 2005). Survivin is the strongest inhibitor of apoptosis ever discovered and also serves as a new tumor marker. Wang (Wang et al, 2004) found that survivin is not expressed in normal bladder tissue but is expressed in 80% of bladder cancers, and its expression correlated with the degree of malignancy. Phage peptide library technology has recently been established and developed, and it can be used to filter and enrich epitope peptides of interest.

In this study, in order to provide a basis for future potential immunotherapy of bladder cancer, i.e., joining mutant HER2 and survivin peptides onto a carrier to produce polypeptide vaccines, we used the polyclonal antibodies of the human bladder cancer-associated surface markers, HER2 and survivin, to identify each polypeptide from the phage peptide library and synthesize both mutant sequences successfully.

2. Materials and Methods

2.1 Materials

The following materials were used in this study: a Ph.D.-7 phage display library kit (including a 7-peptide library; storage capacity, 2×10^{13} pfu/mL; recipient strain Escherichia coli ER2738; sequencing primer, 5'-CCC TCA TAG TTA GGG TAA CG-3', NEB, USA); polyclonal antibodies of HER2 and survivin (Santa Crus, USA); horseradish peroxidase

(HRP)-labeled anti-M13 monoclonal antibody (Pharmacia Company); HER2 and survivin proteins (Abnova Corporation); and other reagents that were purchased domestically .

2.2 Bio-panning and phage titer determination of a Ph.D-7 phage display peptide library

After coating 96-well plates with HER2 and survivin polyclonal antibodies (control group was coated with streptavidin-biotin), the plates were incubated overnight at 4 ℃ by using gentle agitation in a humidified container. Each plate was filled with a blocking buffer and remained in place at least 1 h at 4℃. Then, each plate was washed rapidly 6 times with Tris Buffered Saline +0.1% Tween-20 (TBST), coated with a diluted phage peptide library by TBST, and rocked gently for 40 min at room temperature. After washing 10 times with TBST, 100 μL of a 0.2M glycine-HCl buffer (pH, 2.2) was used as an elution buffer and rocked gently for 10–20 min at room temperature. This solution was then neutralized with 15 μL of 1M Tris-HCl (pH, 9.1) (the elution buffer was biotin, and Tris-HCl was not used for the control group). A small amount was titrated with the eluate. The rest of the eluate was amplified, and the amplified phage solution was titrated in order to calculate the amount of investment for the next round.

The panning procedure should be performed 3 times. An antibody concentration of 100 μg/mL was added to a phage (4×10^{11} pfu) during the first round. The antibody concentration was adjusted to be 10 μg/mL and was added to an amplified phage (2×10^{11} pfu) during the second and third rounds.

2.3 Amplification of the blue plaque

Twenty blue plaques were randomly picked from the third round of screening titers and were added to a 1-mL culture medium of E. coli ER2738. They were incubated while shaking for 4.5-5 h at 37 ℃. Then, they were transferred to an Eppendorf tube and centrifuged at 14000 rpm for 30 s. The upper 80% of the supernatant was transferred to another tube. This was the amplified phage stock.

2.4 ELISA analysis of phage clones

Twenty holes were coated with HER2 and survivin antibodies in 96-well plates; shook in a humidified container overnight at 4 ℃, by using a blocking buffer for at least 1 h; washed 6 times with TBST (Tween concentration of 0.5%); added to an amplified phage solution (concentration of about 5×10^{11} pfu/mL) of 100 μL; and gently shook for 1 h at room temperature. Each plate was washed with TBST 6 times. A diluted HRP-labeled anti-M13 monoclonal antibody of 200 μL was added and rocked at room temperature for 1 h. Each plate was washed with TBST 6 times. An HRP chromogenic substrate solution of 200 μL was added and shook for 30-60 min at room temperature. The plates were read by using a microplate reader at 405 nm, and a ratio of $antibody_{405}$ to $blocking\ buffer_{405}$ of >2.5 was used to select a positive clone.

2.5 Competitive inhibition experiments

After adding HER2 and survivin antibodies to a 100-μL phage solution, a 50-μL phage solution with differing concentrations was added to 50 μL of HER2 and survivin proteins (0.1 μg/mL, 1 μg/mL, and 10 μg/mL). Then, 200 μL of an HRP-labeled anti-M13 monoclonal antibody was added. Furthermore, an ABST substrate chromogenic solution of 200 μL was added. The plates were read by using a microplate reader at 405 nm and were observed for competitive binding trends, and the protein inhibition rate for the phage was calculated. The formula that was utilized was as follows: inhibition rate = [(did not inhibit A_{405}-inhibited A_{405})/(did not inhibit the A_{405}) × 100%].

2.6 Monoclonal phage DNA extraction, identification, and sequencing

With an NEB kit, the DNA was extracted from the positive phage, and agarose gel electrophoresis was performed. The sequencing primers that were used were provided by the NEB Company for sequencing. The amino acid sequences for the peptides were compared and researched in order to see a conservative core sequence.

2.7 Peptide synthesis and verification of the binding force by ELISA

According to the sequencing results, the polypeptide was synthesized to a purity of >95%. The plates were coated with a synthetic peptide for ELISA. The coated plates with a synthetic peptide were used for competitive inhibition experiments, and by using an HRP-conjugated secondary antibody and ABTS (2,2'-amino-di [2-ethyl-benzothiazoline sulphonic acid-6] ammonium salt), HER2 and survivin protein competitive binding antibodies were used for coloration.

2.8 Construct mutant sequence

The 6 random peptide sequences and Kpn Ⅰ and Eag Ⅰ restriction sites were inserted into both sides of the HER2 and survivin sore sequences.

The first primer design and synthesis was as follows: HER2 sample sequence, 5’cTTTCTATTCTCACTCTNNKNNKNNKNNKNNKNNKACTACGGCTGAGAATAATGAGNNKNNKNNKNNKNNKTc3’; and survivin sample sequence, 5’cTTTCTATTCTCACTCTNNKNNKNNKNNKNNKTCTCCTCCTCATCTTTCGCAGNNKNNKNNKNNKNNKTc 3’.

The second primer design and synthesis was as follows: HER2 sample sequence, 5’ggccgAMNNMNNMNNMNNMNNMNNCTCAT

TATTCTCAGCCGTAGTMNNMNNMNNMNNM NNMNNAGAGTGAGAATAGAAAggtac3'; and survivin sample sequence, 5'ggccgAMNNMNNMNNMNNMNNMNNCTGCG AAAGATGAGGAGGAGAMNNMNNMNNMNN MNNMNNAGAGTGAGAATAGAAAggtac3'.

Fifteen μL of an upstream primer and a downstream primer was mixed and cooked for 5 min in boiling water. Then, it was cooled to room temperature. The primer dimers were used as a purpose fragment.

3. Results

3.1 Phage beneficiation by phage display

We used HER2 and survivin antibodies as solid-phase molecules (streptavidin-biotin as a control) and screened for 3 rounds. In the second and third rounds, we reduced the antibody concentration and increased the concentration of elution fluids (Table 1).

Table 1. Results of the screening phage display peptide library with HER2 and survivin antibodies

Antibody	Panning times	Concentration of the antibody (μg/mL)	Input quantity for the phage (pfu)	Output quantity of the phage (pfu)	Recovery rate
HER2	1	100	4×10^{10}	1.17×10^{6}	2.93×10^{-5}
	2	10	2×10^{10}	5.60×10^{4}	2.80×10^{-6}
	3	10	2×10^{10}	1.17×10^{5}	5.85×10^{-6}
Survivin	1	100	4×10^{10}	4.80×10^{3}	1.20×10^{-7}
	2	10	2×10^{10}	1.70×10^{3}	8.50×10^{-8}
	3	10	2×10^{10}	8.00×10^{3}	4.00×10^{-7}
Streptavidin -biotin	1	100	4×10^{10}	1.50×10^{4}	3.75×10^{-7}
	2	10	2×10^{10}	8.00×10^{3}	4.00×10^{-7}
	3	10	2×10^{10}	6.00×10^{5}	3.00×10^{-5}

3.2 ELISA analysis and competitive inhibition experiments

ELISA was used in order to identify the binding of the selected 20-phage plaques with the antibodies. The results showed that the absorbance value was higher than that of the blocking buffer, which had some affinity and could have diminished their effect. We selected a ratio of antibody$_{405}$/blocking buffer$_{405}$ of >2.5 as the standard. The results showed that the selected 20-phage plaques all met the above-mentioned standard (Figure 1, 2).

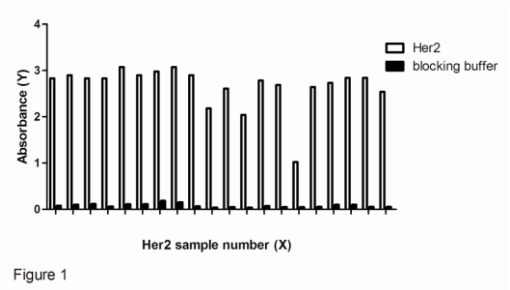


Figure 1 HER2 group: contrast of the absorbance value for the positive clones and blocking buffer

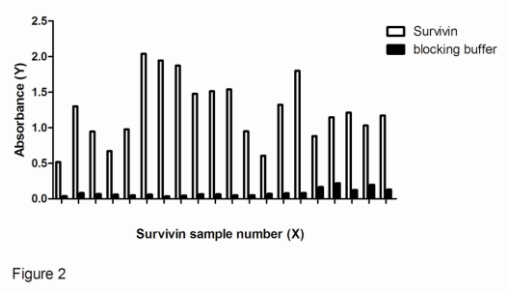


Figure 2 Survivin group: contrast of the absorbance value for the positive clones and blocking buffer

The results showed that as the input of the HER2 and survivin protein concentrations decreased, the absorbance value increased, while the competitive inhibition of the phage decreased. (Figure 3)

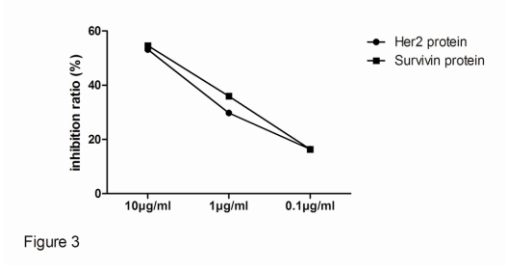


Figure 3 The competitive inhibition ratio for the HER2 and Survivin proteins with the combination of their antibodies and phage

Table 2 Phage DNA sequencing in the HER2 and survivin groups

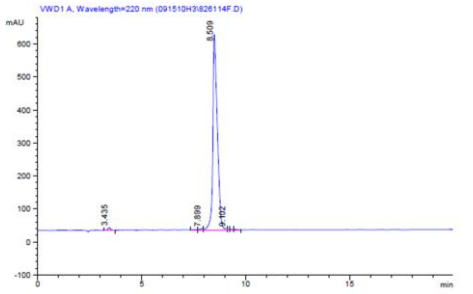
Phage number	HER2 absorbance	HER2 base sequence	Survivin absorbance	Survivin base sequence
1	2.829	GCG ACG GCT GAG AAT ATG GAG	0.516	CAT CCG CCG ATG AGG CCT GAG
2	2.896	ACT GTT GCG GAG AAT ACG TAT	1.301	TGG AAT CAT AAT CAT GTT CCT
3	2.829	GCG ACG GCT GAG AAT ATG GAG	0.946	TCG TAT GTT AAT AAT TGG AAG
4	2.829	ACT GTT GCG GAG AAT ACG TAT	0.670	TGG AAT CAT AAT CAT GTT CCT
5	3.072	TTG ACG ATT GCG TTG GAT GAG	0.976	CAT TTT CCG CAT ATT CTT CAG
6	2.896	ACT CAT CCG GAG AAT ACG TAT	2.041	TCG TCT ATT CAT TCG GTT CTG
7	2.976	TTG ACG ATT GCG TTG GAT GAG	1.944	ACT CTT CTT TCG GTT TAT CAT
8	3.072	TCT CAT CCT GAT CTT CCT CTG	1.872	TAT CCG CAT GTT CCT AAG TAT
9	2.896	TTG ACG ATT GCG TTG GAT GAG	1.476	TCT TTG CAT CCT CGT ATG CGG
10	2.183	GTT AAT CTT GCT GAG AAT GAG	1.513	AAT TCT GCT CAT GTT TCG ATG
11	2.605	TTG ACG ATT GCG TTG GAT GAG	1.538	TCT CCG ACT TCT AAT TAT AAT
12	2.041	GGT ACG CTT GCG GAG AAT AAT	0.950	CTT CAG AAG GCT CCG TCT ACT
13	2.782	GCT ACG ACG GAG AAT CCT GAG	0.606	AAT TCT GCT CAT GTT TCG ATG
14	2.685	GCG ACG GCT GAG AAT ATG GAG	1.322	TTG CCG ACT TTT TTG CCG CGG
15	1.022	ACT GTT GCG GAG AAT ACG TAT	1.799	TCT TCG ATT ATG AGT ACT GCT
16	2.643	ACT CTG GCT GAG AAT TTT TCG	0.882	GCG CCG CCT TCT GTT AGG ACG
17	2.731	ATT ACT GCG GAG AAT CAG GAG	1.146	ATG AAT TTG TAT CCG ACG CCG
18	2.840	TTG ACG ATT GCG TTG GAT GAG	1.212	TCG CAT TGG CTG CAT CAG AAG
19	2.840	ATT ACT GCG GAG AAT CAG GAG	1.030	ACT CCT GCG CTT AAT CCG GAG
20	2.539	ACT CCT GCG CTT AAT CCG GAG	1.172	GCT GT**A** ATT GAT CCT CGT CTG

3.3 Extraction, identification, and analysis of phage DNA

After extracting the phage DNA, agarose gel electrophoresis was used in order to identify its size, and gene sequencing was used to analyze the core sequence. The core sequences of HER2 and survivin mimetic peptides for bladder cancer were: ACT ACG GCT GAG AAT AAT GAG and TCT CCT CCT CAT CTT TCG CAG, respectively (Table 2).

3.4 Peptide synthesis and binding force identification

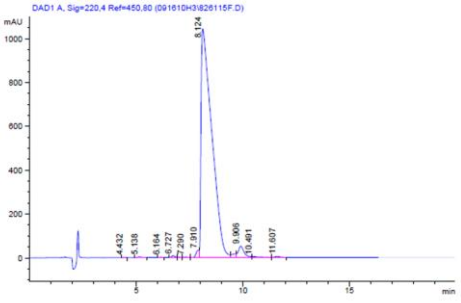
The synthetic HER2 polypeptide was TTAENNE, with high performance liquid chromatography (HPLC), and the purity was 97.5% (Figure 4); survivin polypeptide was SPPHLSQ, by using HPLC, and the purity was 95% (Figure 5).



Signal 1:VWD1 A, Wavelength=220 nm

Peak #	RT [min]	Type	Height	Width [min]	Area	Area %
1	3.435	BP	8.123	0.148	78.887	0.869
2	7.605	VV	1.477	0.197	19.066	0.210
3	7.899	VV F	4.339	0.162	47.074	0.518
4	8.509	VV	565.117	0.229	8857.872	97.552
5	9.102	VV B	3.542	0.106	22.540	0.248
6	9.297	VV B	2.625	0.152	28.866	0.318
7	9.491	VV B	2.120	0.177	25.820	0.284

Figure 4 HER2 peptide synthesis



Signal 1:DAD1 A, Sig=220,4 Ref=450,80

Peak #	RT [min]	Type	Height	Width [min]	Area	Area %
1	4.432	PV	2.082	0.097	12.671	0.033
2	5.138	BP	3.430	0.178	39.968	0.104
3	6.164	VP	1.695	0.108	11.444	0.030
4	6.727	BV	8.333	0.173	95.914	0.251
5	6.996	VV	2.212	0.128	18.867	0.049
6	7.290	VV	2.519	0.196	31.325	0.082
7	7.910	VV	33.035	0.155	329.000	0.860
8	8.124	VV	1041.161	0.491	36365.492	95.009
9	9.686	VV	16.942	0.232	235.367	0.615
10	9.906	VV	51.468	0.280	982.201	2.566
11	10.491	VV	3.563	0.320	83.067	0.217
12	11.607	VBA	4.564	0.228	70.530	0.184

Figure 5 Survivin peptide synthesis

We identified the polypeptide by using ELISA. The results showed that the polypeptides have a certain affinity, and with the reduced HER2 and survivin protein concentrations, the inhibition rate decreased (Figure 6).

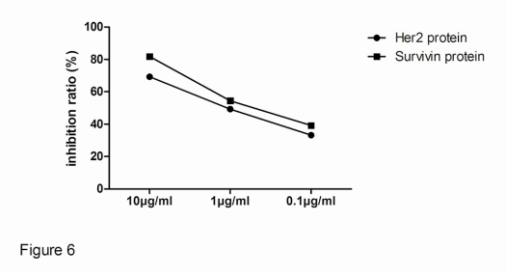


Figure 6 Assessment of antibody-peptide affinity for the HER2 and survivin synthetic polypeptides with a competitive inhibition assay

3.5 Electrophoresis of a mutant primer sequence polymerization fragment

HER2 and survivin mutant primer sequence polymerization fragments could be seen in lane 1 and lane 2, respectively. The fragment size should be synthesized to be 74 bp, in theory. Their electrophoresis positions in the figure appeared to be about 100 base pairs (bp) because of the manufacturer‘s sensitivity. Thus, it demonstrated that they could be synthesized by using routine technology (Figure 7).

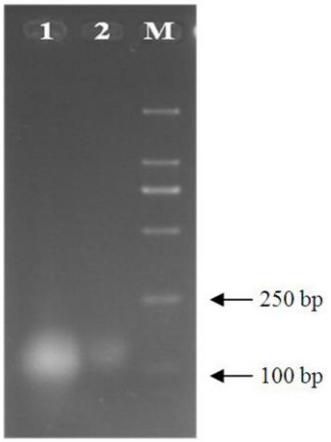


Figure 7 Primer polymerization product with 2% agarose gel electrophoresis

Lane 1: HER2 fragment; Lane 2: Survivin fragment; M: 2000 Marker.

4. Discussion

There are many immunotherapy methods for bladder cancer, including exogenous immune stimulation, cytokines, tumor vaccines, monoclonal antibodies, and gene therapy. BCG is the only recognized drug that has a certain antigen characteristic, sensitization, and residual toxicity. However, about 90% of patients have local side effects, which are exacerbated during administration. Many patients cannot sustain this therapy, and thus, the application is limited.

In recent years, with the proposed concept of biological epitopes, many tumor antigen epitopes have been identified. These small molecules have a higher specificity and can help avoid the shortcomings of immune complexes that are large, antigen molecules. Therefore, they are better candidates for cancer immunotherapy.

A tumor peptide vaccine is synthesized by connecting a small peptide, which is from tumor-specific antigens, viral antigens, mutated oncogenes, or tumor suppressor gene protein peptides, in order for a carrier molecule to form an

antigen-carrier chimera. After injecting into a human body, it can activate an immune response to a specific antigen so it can kill the "target." Its advantages over traditional vaccines are as follows: it can induce a specific and effective immune response and can be synthesized and purified rapidly. Pouyanfard et al. (Pouyanfard et al, 2012) used multivalent T7 phage nanoparticles in order to confer a high immunogenicity to the HER-2-derived minimal CTL epitope in breast cancer research. A survivin-based peptide vaccination was also being researched for several cancers including melanoma (Becker et al, 2012) and pancreatic cancer (Kameshima et al, 2013).

A phage peptide library has a large number of phages with different peptides. Phage peptides can be screened by interacting with a target receptor. After elution and amplification, the specificity of a recombinant phage is enriched, and the structure and sequence can be analyzed. In this experiment, we used a Ph.D-7 phage display peptide in order to screen and pan three times. The recovery rate of the HER2 group increased from the second round, 2.80×10^{-6}, to the third round, 5.85×10^{-6}. The recovery rate of the survivin group increased from the second round, 8.50×10^{-8}, to the third round, 4.00×10^{-7}. The peptides of interest were enriched to some degree. We used an ELISA method to further screen and identify the affinity of the phage.

There was a circular, single-stranded DNA molecule, which was 6407 nucleotides long in the M13 phage. It contained the necessary genetic information for phage DNA replication and proliferation. In this study, we used 0.8% agarose gel electrophoresis in order to identify the correct phage DNA that was extracted. In addition, the initiation sites for M13 DNA replication were located in the gene spacer region, so even if there was a mutation, a deletion, or an insertion of foreign DNA fragments in the nucleotide sequence of the spacer region, the replication of M13 DNA would not be affected. These provided a theoretical basis for synthesizing the mutation sequence, inserting the Kpn Ⅰ and Eag Ⅰ restriction sites, connecting the M13 DNA carrier, and building a mutant phage peptide library.

HER2 has type Ⅰ tyrosine kinase activity. HER2 is overexpressed in many epithelial tumors, such as breast cancer (25-30%) (Seshadri et al, 1989), ovarian cancer (25-32%) (Berchuck et al, 1990), and bladder cancer (40-80%) (Chow et al, 2001; Ravery et al, 1997). Overexpression of HER2 significantly increases the rate of tumor cell proliferation, promotes tumor angiogenesis, and reduces adhesion between cells. A HER2 overexpression of about 62% to 69% was shown to be in the cell membrane of metastatic bladder cancer. Through the follow-up of 90 patients who had muscle-invaded bladder cancer after surgery, Kolla et al. (Kolla et al, 2008) found that the HER2 level and survival of patients were closely related. In this study, through screening and DNA sequencing analysis, we found that the core nucleotide sequence of HER2 was ACT ACG GCT GAG AAT AAT GAG, and the amino acid sequence that was successfully synthesized was Thr-Thr-Ala-Glu-Asn-Asn-Glu (TTAENNE), with a purity of 97.5%.

Survivin is one of the inhibitors of the apoptosis protein (IAP) family that was first found by Ambrosini (Ambrosini et al, 1997) from the human genome library by hybridization screening. Studies have shown (Reed, 2001) that apart from endometrial tissue, other than the placenta and thymus tissue that have small amounts of survivin, no other normal adult tissues express survivin. Kitamura et al. (Kitamura et al, 2006) found that the rate of survivin expression in tumor tissue was 87.5% among 88 patients with bladder cancer. Sun et al. (Sun, et al, 2013) found that the positive expression rates of survivin in superficial bladder transitional cell carcinoma (BTCC) were 66.7% (52/78) and that the tumor recurrence rate in survivin (+) patients was 53.8% (28/52). Gonzalez et al. (Gonzalez et al, 2008) found that the risk of local neoplasm metastasis with bladder cancer significantly increased with survivin overexpression (≥20%) in bladder cancer. Karam et al. (Karam et al, 2007) also found that overexpression of survivin was associated with a greater tumor grade recurrence and progression of non-muscle-invasive bladder tumor. Thus, survivin is a prominent and widespread tumor-associated antigen, which has important value in tumor immunotherapy. In this study, through screening and a DNA sequencing analysis, we found that the core nucleotide sequence of survivin was TCT CCT CCT CAT CTT TCG CAG and that the amino acid sequence, which was successfully synthesized, was Ser-Pro-Pro-His-Leu-Ser-Gln (SPPHLSQ), with a purity of 95%. Moreover, sample number 20 was sequenced twice in the survivin group. It was confirmed that the third base of the second codon in the heptapeptides was A, which does not match the third base that should be G/T in the normal peptide library. A possible explanation might be because of a genetic mutation, and the search for core sequence comparisons did not include this sample.

Because the immunogenicity of these small molecules from the phage peptide library was weaker than that of natural ligands, it was particularly important to increase the affinity of the peptide. Beenhouwer et al. (Beenhouwer et al, 2002) confirmed that the affinity of the capsular polysaccharide peptide could be increased 80 times

by insertion of a random hexapeptide into both ends of the core sequence of the peptide in a study of anti-cryptococcal capsule polysaccharide vaccines. In this study, we inserted the Kpn Ⅰ and Eag Ⅰ restriction sites into both ends of the 6 random peptides in order to join with the M13 vector and successfully synthesize HER2 and survivin mutant epitope mimic peptide sequences. The HER2 sample sequence was: 5'cTTTCTATTCTCACTCTNNKNNKNNKNNKN NKNNKACTACGGCTGAGAATAATGAGNNKN NKNNKNNKNNKNNKTc3'. The survivin sample sequence was: 5'cTTTCTATTCTCACTCTNNKNNKNNKNNKN NKNNKTCTCCTCCTCATCTTTCGCAGNNKNN KNNKNNKNNKNNKTc 3'. Therefore, our experiment helped to improve the screening affinity of HER2 and survivin epitope mimic peptides and to create a platform for further research regarding the construction of mutant peptide libraries and peptide vaccines.

While researching a tumor peptide vaccine, Tsai et al. (Tsai et al, 2009) showed that intramuscular electrogene transfer of an expression vector that encoded a fusion protein, anti-HER2scFv-IL12, which consists of an anti-HER2 single-chain variable fragment (scFv) and a single-chain of IL-12, that can retard tumor growth and prolong survival in a syngeneic bladder tumor model. However, the effect was decided by the amount of IL-12 and the technology of intramuscular plasmid electrogene transfer, which is not simply and commonly used. A more simple and easy method for tumor peptide vaccine preparation was used for the purpose of this experiment.

This study mainly involved the preparation of a tumor peptide vaccine for bladder cancer and provided a good foundation and platform for future study.

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