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14. ABSTRACT
We planned to employ phage-display methodology to unveil novel breast cancer epitopes for the development of multivalent breast cancer vaccines. Some of the patients on our study were receiving Herceptin, a monoclonal antibody, that could complicate our analysis. We demonstrated that, in fact, our screening strategy would identify Herceptin. So, it was necessary to remove Herceptin from the sera before screening. We developed high affinity Herceptin mimotopes that could be employed to remove Herceptin from the serum samples, however we failed to successfully employ the Herceptin mimotope in a manner that would remove Herceptin from our test serum. Future studies will require samples from patients who were not receiving monoclonal antibody therapy. Over the same period, we successfully refined our algorithms to enable epitope definition based on results from polyclonal antiserum by using a collection of 5 monoclonal antibodies with defined specificity as a test case.

15. SUBJECT TERMS
Phage Display, Epitopes, Antibody, Herceptin

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1.0 Introduction

Immunization against breast cancer antigens is a promising method to enhance current therapies since the immune system has the unique capacity to "seek-out-and-destroy" small deposits of tumor cells. Although clinical trials investigating breast cancer vaccines have generated promising initial data, it is clear that further refinement is required. One key factor is the limitation of vaccines that only target individual antigens since antigen-loss variants can develop quickly. We would like to develop multivalent vaccines that employ epitope libraries composed of shared antigens. Therefore, we propose to use a high-throughput method to screen and identify shared epitopes within breast cancer patients. Filamentous bacteriophage have been used as a universal scaffold for the expression of random antigenic peptides (termed "phage display")[1,2]. The advantage of the random peptides displayed by phage is that they can represent peptidomimetics of conformational and discontinuous antibody epitopes ("mimotopes"). Thus, this method is highly amenable to rapid identification of previously unknown epitopes that can be used directly as immunogens. Specifically, we will use patient serum to screen for breast cancer-specific mimotopes within a bacteriophage library where random cysteine-looped 12-mer peptides were incorporated in the pVIII protein of phage f88. The constrained nature of these cysteine-looped peptides promotes the peptides to adopt a tertiary structure which can mimic natural antibody epitopes, even though the mimotope may only share limited sequence similarity with the actual residues in the protein. In this way, we hope to identify mimotopes specific for antibodies in breast cancer patient serum that could be used as the basis of a vaccine.

Any group of breast cancer patients will have two classes antibody circulating in their bloodstream: 1) individual and 2) shared. The individual antibodies will reflect unique exposures that are patient-specific. However, the shared antibodies will reflect common exposures among the patients which will include: specific immunizations (ex. Measles virus), common environmental pathogens (ex. Influenza), and ***common tumor-specific antigens***. By pooling serum from multiple patients, the individual antibodies will be diluted but the shared antibodies will not be. Therefore, by screening random phage display libraries using pooled serum from breast cancer patients, we hypothesize that we will be able to identify common breast cancer-specific epitopes/antigens that can be used as the basis for an immunization strategy. We will test our hypothesis in a pilot study with 30 serum specimens. To determine whether pooling of patient sera truly offers an advantage with regard to identification of shared epitopes, we will compare the results of screening with serum from individual patients to the results obtained using small pools (10 patients per pool) and large pools (30 patients per pool). We predict that the larger pools will yield the greatest number of shared epitopes, since the results from less complex pools may be unduly affected by individual patients with high-titer antibodies.

2.0 Body

2.1 The presence of Herceptin in patient blood confounds the screening strategy.

As a first step in our evaluation of this methodology, we screened 2 individual patients for "breast cancer" specific antibodies. To avoid phages specific for environmental pathogens and vaccine antigens, we first prepared a "depleted" phage library where the library was adsorbed to a pool of serum from 10 healthy male volunteers. We then screened the depleted phage library (method described in legend for Table 1) with serum from patient MTL-003 and found few specific phages (sequences are listed in Table I). We carried out the same procedure with serum from a separate patient (HH-002) and again only recovered few phages. Strikingly, all 7 phages recovered with the serum from HH-002 carried the same insert (Table I). These results revealed that when employing a phage library depleted of mimotopes for common antigens, the number of recovered phage was very low. This observation prompted a shift in strategy. Since the number of phage that we are recovering is not very high, we will screen the "depleted" phage library with individual sera first. Subsequently, we will pool all the recovered phages and screen them again with individual sera to determine which phage present mimotopes that are commonly recognized among a large fraction of patients.

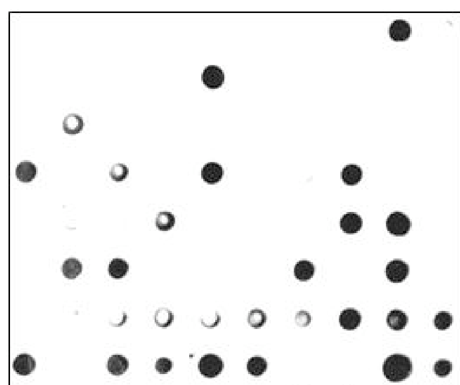
While conducting these pilot experiments, we became aware that MTL-003 had received treatment with Herceptin, a monoclonal antibody. To determine whether the presence of Herceptin had influenced our results, we tested all of the recovered phages with Herceptin. Two of the 6 six unique phage that were recovered with serum from MTL-003 bound to Herceptin indicating that, indeed, the presence of Herceptin can confound our results.

Patient ID	Phage sequence	Reactivity to Herceptin
MTL-003	CRRVLVSANVNERC	No
	CSERRVAEKEQGC	No
	CGEATHHEGAKERS	No
	CGGMTTEKVAEQPC	No
	CAMGWLSGDAKGSC	Yes
	CAMTRPTQKEPQWC	Yes
HH-002	SCESSQHVAPGDCL	No

Table 1. Plasic plate was coated with anti-human antibody, blocked with 1% gelatin and incubated with individual breast cancer patient sera. Then the immobilized serum antibody were incubated with 12-mer cystein-looped phage display library. Unbound phages were washed out and bound phages were eluted by glycine-HCl buffer (pH 2.5). The eluted phages were used to infect E.coli DH5alfa F+ for amplification. The procedure was repeated two times where the sequentially amplified eluates were used instead of the original library. Bacteria infected with the last eluate (#3) were plated onto selective agar plates and individual clones were grown in 96-well plate, the phages were purified from the growth media of these clones and transferred onto nitrocellulose membrane which in turn was reacted with the original serum (1:1000) and probed with anti-human IgG conjugated with HRP. The reaction was detected by ECL. DNA's of positive phage clones were purified and their inserts were sequenced. Amino acid sequences were deduced. All phages were subsequently tested for reactivity to Herceptin and presented on the figure B. Partial homology motifs are presented in bold.

2.2 Development of a method for depleting Herceptin from patient sera.
Given the widespread use of Herceptin (trastuzumab) and possible confounding effects on our screening strategy, we decided to develop a method for removing Herceptin from the patient sera prior to analysis. Given the observation that Herceptin can bind to specific phage, we decided to screen our phage library with Herceptin as a means of identifying specific, high-affinity phage. Our strategy for depleting Herceptin from the serum was to link the Herceptin-specific phage to a solid matrix, pass the serum over the matrix such that the Herceptin antibodies would remain bound and employ the recovered, unbound sera for phage screening. Herceptin-specific phages were isolated by two rounds of biopannings using immobilized Herceptin. An example of these results are shown in Figure 2.

A: Screening of the phage display library with Herceptin

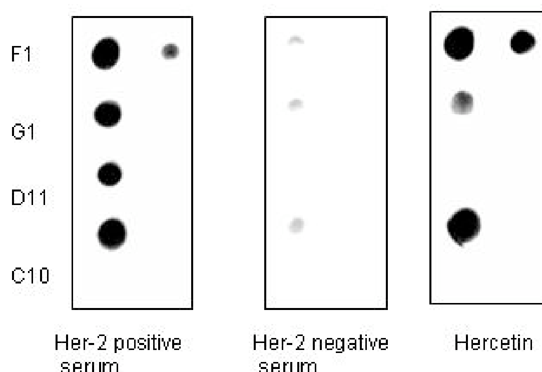


1 2 3 4

1 – wild type phage
2 - eluate after first biopanning
3 – eluate after second biopanning
4 – human HER-2

Sequence:
CAMGWLSGDAKGSC

B: Herceptin-binding phages found by screening phage display library with human serum



	Sequence	Reaction with Herceptin
F1	CAMGWLSGDAKGSC	++
G1	CAMTRPTQKEPQWC	+
C10	CEARITMGGAHTAC	++

Figure 2. Identification of Herceptin mimotope. ***A.*** Immunoaffinity purified phage were blotted onto a nitrocellulose filter and screened with Herceptin using Western blot. Dark spots represent positive phage. A single sequence was found in all the positive phage. ***B***. Selected phage were tested for reactivity to human serum. ***Left panel***, dot blot of selected phage using serum from a patient on Herceptin (Her-2 positive); ***Middle panel***; dot blot of selected phage using serum from a patient who was not receiving Herceptin (Her-2 negative); ***Right panel***, dot plot of selected phage using Herceptin.

We ultimately identified 8 independent mimotopes that bound Herceptin to varying degrees (Table 2). We attempted to bind the phage carrying the CAMGWLSGDAKGSC insert to a variety of solid matrices to permit a simple, column-based method for removing Herceptin from serum. Unfortunately, we were unsuccessful. We subsequently attempted to introduce the

CAMGWLSGDAKGSC mimotope into the mannose-binding protein (MBP) based on a previous report demonstrating that insertion of the mimotope into MBP can recapitulate its antibody-binding properties [3]. The MBP-mimotope reagent could be bound to a mannose matrix permiting the development of a column-based removal strategy. However, we were not able to complete the cloning within the time-frame of the current grant.

Sequence	Binding of Herceptin
CAMGWLSGDAKGSC	++++
CAEFTEGRADGSSC	++++
CEARITMGGAHTAC	++++
CAPHESTKDVSPSC	+++
CGLRTVSPSC	+++
CMIYGSGTGERVVC	+++
FKHRGSDHQETEAC	+++
CAMTRPTQKEPQWC	+++

Table 2. List of Herceptin mimotope sequences identified in our study. The relative binding to Herceptin (+++ or ++++) is based on the intensity of signal obtained using dot blot analysis. The intensity was scored relative to individual phages and negative control phages.

2.3 Refinement of epitope prediction algorithm. The true power of this technology stems from the potential to identify common elements within the selected mimotope sequences that would permit identification of the target protein. Our original algorithm for achieving this goal has been described previously [4]. However, this algorithm is dependent upon the identification of amino acid “pairs” that are positionally-defined based the structure of the folded protein. While this method has proven to be highly efficient for mapping of monoclonal antibody specificities where it is reasonably easy to prepare a large selection of unique peptide sequences from the phage libraries, our original method does not work well with small selections of sequences, such as the scale that we are generating from the patient sera. Additionally, many protein structures have not yet been solved, so our original algorithm is also limited by the requirement for this information. To improve the selectivity and predictive capacity of our algorithm, we have incorporated an additional parameter where we score the frequency of amino acid pairs that are present within our phage peptides and the putative target protein, regardless of the structure of the folded protein. To develop this new algorithm, we required a test set of sequences. To this end, we employed a set of sequences that we had obtained previously for a 5 monoclonal antibodies specific for the West Nile virus E protein. While we had sufficient sequences from our previous work to define the specific epitope using our standard method, we have also made use of these sequences to develop and validte our novel algorithm. The increased power of our new algorithm is shown in Figure 2. While our novel algorithm correctly identifies residues that

were identified using the previous algorithm (An example of our results for a specific monoclonal antibody, E24, is shown in Figure 2A; amino acids shown in blue), the new method also greatly increases the number of predicted residues (Figure 2A; amino acids shown in cyan). Pairwise analysis of the sequences revealed a broader range of pairs resulting a more definitive prediction of the target epitope (Figure 2B; prediction with original algorithm shown in blue, additional residues defined by the new algorithm are shown in cyan). Thus, our novel algorithm defines a clearer picture of the target epitope and it also independent of solved structures, so we can use it for a broader range of protein targets.

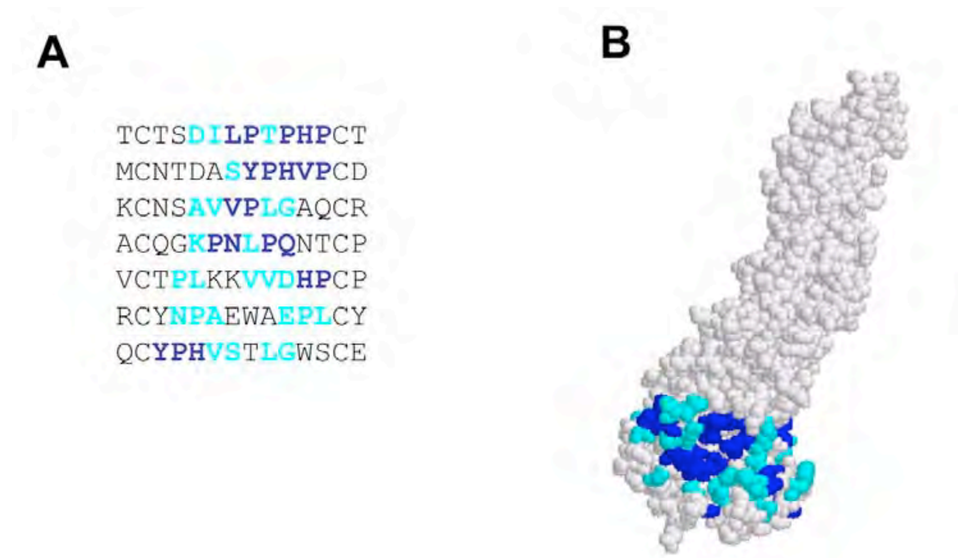


Figure 2 A. List of peptide sequences found by phage library screening with mAb E24. Putative epitope residues defined by original algorithm are shown in blue and additional residues defined by the novel algorithm are shown in cyan. **B.** The position of the putative epitope residues are shown on a 3-D rendering of the E protein using the same color code as ***A.***

3.0 Key Research Accomplishments

Identified that monoclonal antibody therapy is a potential confounding factor which must be considered for future experimental design.

Development of a novel algorithm for predicting epitopes based on the use of phage-display libraries.

4.0 Reportable outcomes

Isolation of high-affinity Herceptin-specific mimotopes for removal of Herceptin from patient sera.

5.0 Conclusion

It is too early in this project to make definite conclusions about the suitability of this method to identify novel mimotopes for vaccine development. While we have shown that this method can be used to identify antibody reactivities that are specific for individual patients, further analysis must be carried out to determine how broadly applicable these results will be. The novel algorithm that we have developed for epitope prediction should be helpful in validating novel mimotope targets.

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