Peer Review File

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Reviewer A

This manuscript describes about the identification of CD44v6 binding peptides. Authors used CD44v3-v10-expressing HEK 293 cells for screening of phage library and selected ELT peptide. There are some comments and concerns about the manuscript.

1. Authors claimed that they identified a CD44v6 specific peptide. However, the screening was done using CD44v3-v10-expressing cells. Also, the binding activity was measured using the same protein-coated plates. In this regard, the peptides in this study should be described as CD44v3-v10 specific peptides.

Reply: In this study, we aimed to screen a peptide using the target protein in the natural form. In this way, the original advanced structure of CD44 protein can be maintained. Encoding region of v6 exon is an important cancer biomarker, but it is only a part in natural CD44 variants and cannot exsist in isolation. We searched *homo sapien* CD44 variants in Genebank and found that CD44v3-v10 (NM 0010011389.1) was the smallest one with v6 exon. CD44v3-v10 was also available for isolation in a *homo sapien* cDNA library (Shanghai Genechem Co.,Ltd.). Thus, CD44v3-v10 was chosen as the target protein.

To obtain peptides specific to CD44v6, we applied selective elution in screening. Differ from traditional elution using acids, candidate phages were competitively eluted with a monoclone anti-CD44v6 antibody. In this way, phages targeted the same antigenic determinant on CD44v6 can be enriched.

Change in the text: We revised unclear statement in discussion part (Page 18 line 378-379, 386).

2. In page 4, line 94, authors mentioned that peptide specific to CD44v6 has not been reported yet. However, CD44v6 specific peptides have already been reported (Matzke-Ogi A et al. Gastroenterology. 2016;150:513-25 e10; Orian-Rousseau V, Ponta H. Arch Toxicol. 2015;89:3-14; Tremmel M et al. Blood. 2009;114:5236-44). Authors should acknowledge these peptides in the Introduction or Discussion section and describe about the novel or significant of their peptide I comparison.

Reply: We carefully read the papers mentioned above and studied some other work related to the peptide. We learned the CD44v6 peptide before, and had cited a related reference in the manuscript (Page 22, line 487-488). However, we did not mention these peptides in manuscript. CD44v6 was assumed as a co-receptor of Met. To block activation of Met, researchers studied the whole amino acid sequence of CD44v6, and designed a tripeptide and a pentapeptide. These CD44v6 peptides were not ligands for

CD44v6, but a minimal active motif in CD44v6 sequence (Matzke A et al. Cancer Research. 2005; 65: 6105-10). Researches focused on these peptides demonstrated the activity as an inhibitor to block CD44v6 related pathway (Matzke-Ogi A et al. Gastroenterology. 2016; 150:513-25 e10; Tremmel M et al. Blood. 2009; 114:5236-44). However, we did not found evidence of binding activity of these peptides to CD44v6.
Change in the text: We deleted statement may lead to ambiguity and add the work mentioned above in the discussion part (Page 4 line 94-95; Page 18 line 371).

3. In Fig. 2, the binding activity of the ELT peptide to a control protein (e.g., BSA) should be included.

Reply: We added the binding activity study of the ELT peptide to BSA protein.
Change in the text: Data was added (Fig.2; Page 7 line 178; Page 11 line 281; Page 13 line 286).

4. In Fig. 3, the binding activity of FITC-labeled control peptide should be included.
Reply: We added the binding study of the control peptide to cells.
Change in the text: Data was added (Fig.3; Page 13 line 302; Page 14 line 306, 308-309).

5. In Fig. 5, was the in vivo fluorescence imaging done only in one mouse? Otherwise, provide a graph showing the average and S.E. of fluorescence intensity.
Reply: We performed *in vivo* imaging detection with five mice in each group. We add statistical graphs showing the average and S.E. of fluorescence intensity in Fig.5.
Change in the text: Data was added (Fig.5; Page 16 line 340, 344; Page 17 line 352).

6. In Fig. 5, immunofluorescence microscopic analysis of FITC-peptide homing to tumor tissue and control organs (such as liver) should be included.
Reply: We really appreciated the constructive proposal from the reviewer. When we performed the *in vivo* examinations of the peptide probes, we referred to published work those identified peptide probes for tumor targeting (Zhu L et al. J Control Release. 2011; 150(3): 248-255; Kim S et al. Angew. Chem. Int. Ed. 2012, 51, 1890-1894). In these works, *in vivo* examinations focused on the tumor specificity, pharmacokinetic and bio-distribution of peptide probes, and did not contain an immunofluorescence microscopic analysis. Immunofluorescence can really help observing FITC-peptide homing to tumor tissue and control organs at the micro level, but it is not a decisive test. In other ways, bio-distribution examination showed statistic difference in fluorescent intensity between tumor tissue and control organs. Immunohistochemistry presented peptide homing to tumor tissue and corresponding pericarcinous tissues at the micro level. We considered the existing data could exhibit bio-distribution, binding specificity to tumor and binding location of peptide.

We are so sorry that we cannot meet the request of reviewers. Due to the epidemic in China, laboratory in our university is still unavailable for animal experiments. It is impossible to finish the immunofluorescence analysis in a period.

Reviewer B

This manuscript deals with the discovery of peptides with binding capacity for CD44v6, in an attempt to develop moieties to serve as diagnostics in gastric cancers. Despite the high relevance of this topic, the manuscript lacks proper rational and discussion.

1. The results are depicted as a sequence of experiments, but with poor interlink. The discussion section is, indeed, a repetition of the results section. No single reference is used to justify/compare the results.

Reply: We carefully revised the discussion section based on the reviewers' valuable comments and suggestions to make the section full and accurate. The specific changes were listed at response for comments point by point. We also added related work and reference in this part to compare the work we did. (Page 18 line 364, 371).
Other points need clarification/discussion:

2. The authors mentioned that compared to antibodies, peptides own lower toxicity and immunogenicity. This statement needs support and references, as it cannot be generalized. There are also peptides with significant toxic effects.

Reply: The statements were appeared in page 4, line 93-94, page 20, line 419-420. We referred to several review articles (Klenske E et al. Dig Liver Dis. 2018; 50(9):878-885; Muguruma N et al. Clin Endosc. 2013; 46(6): 603–610.). We add these references in the manuscript and rewrite the sentence to make it more rigorous.
Change in the text: We added references and revised the statement (Page 4 line 93-94; Page 20 line 419-421).

3. The potential route of administration is a clinical setting is not elaborated. Being the target gastric cancer, is it expected to be an oral delivery? If so, how to assure that peptides are resistant to degradation and mucus penetrating?

Reply: As the reviewer concerned, route of administration is a critical part in clinical practice. Systemic intravenous administration is a common application of contrast agent for imaging examination recently. In this way, small metastases can be found out. For gastroenterology, some new type endoscopy can detect contrast agent administrated topically. However, due to degradation and mucus, oral delivery is not the first choice. After the mucus was washed away with mucus eliminating agent and physiological saline, contrast agent could be sprayed on mucosa directly using endoscopy. Similar work with this application has been reported (Sturm MB et al.

Science translational medicine. 2013; 5:184ra161.).
Change in the text: We added related discussion in manuscript (Page 18, line 360).

4. In vivo proof of concept used subcutaneous xenograph, and then, intravenous administration. Why not consider intratumor deposition?

Reply: We performed the *in vivo* examinations of the peptide probes using intravenous administration. It is a classic way to identified peptide probes in pharmacokinetic and bio-distribution, as well as binding difference between tumor and normal organs. Compared to topical administration, systemic intravenous administration can provide comparable dose to different organs. The protocol was designed according to related published work (Zhu L et al. J Control Release. 2011; 150(3): 248-255; Kim S et al. Angew. Chem. Int. Ed. 2012, 51, 1890-1894).
Is it expected that peptides (ELT) could be used as ligand for targeted delivery of nanosystems, for instance? How do modify the peptide for grafting application?
Reply: We really appreciate the reviewer's constructive suggestion. Nanosystems can provide higher signal intensity and enable multi-valent binding to increase the affinity. We will carefully consider this proposal in the former work.
As after 4 hours no signal of peptide was detected in animals, how to justify the strong binding affinity in vitro and this short half-life?
Reply: After 4 hours, peptide probes were eliminated from animals. In this study, we consider the short half-life *in vivo* was affected by multiple factors, including peptide characteristic, individual metabolism characteristic, administration route. Binding condition *in vivo* was complicated and could influence the metabolization and elimination. In the former work, we will consider the reason for this short half-life.

5. May Immunofluorescence modification alter the biding affinity of the native peptides?

Reply: When we modified peptide for fluorescence imaging, we linked fluorophore at one end of the peptide chain to avoid big change of advanced structure of native peptide. The result of immunofluorescence showed that the modified peptide also exhibited specific binding activity to target cell and tissues.

Reviewer C

The manuscript “Screening and identification of a CD44v6 specific peptide using improved phage display for gastric cancer targeting” identified a CD44v6 specific peptide by using the phage display technology. The study is interesting and significant, and the authors identified new peptides that have the comparable targeting effect on CD44v6. However, I would like to see the following points addressed before publication.

Concerns/suggestions:

1. The author should provide more information of the phage display peptide library used in this study, such as the peptide number in the library.

Reply: We used a commercial Phage display peptide library in this research (New England BioLabs, Beverly, MA, Catalog # E8111L). The Ph.D.-12 Phage Display Peptide Library is based on a combinatorial library of random dodecapeptides fused to a minor coat protein (pIII) of M13 phage. The displayed peptide (12-mer) is expressed at the N-terminus of pIII. The peptide is followed by a short spacer (Gly-Gly-Gly-Ser) and then the wild-type pIII sequence. The library consists of approximately 10^9 electroporated sequences amplified once to yield approximately 100 copies of each sequence in 10 μl of the supplied phage.

Change in the text: We added catalogue and information of the phage display peptide library (Page 5 line 131).

2. The author should provide detailed information of a, b, c and d in Figure 4A.

Reply: We added the detailed information of a, b, c and d in Figure 4A.

Change in the text: Data was added (Page 16 line 330).

3. As shown in Figure 3B, the transfection efficiency in the empty control group is low compared with the overexpression group. I think the result is not very convincing. The author should repeat the experiment and comparing the binding capacity after the transfection efficiency is comparable.

Reply: We performed experiment with more than five slides in each group, we are sorry for choosing a view with low transfection efficiency. We carefully overviewed all the slides and chose other ones with comparable transfection efficiency.

Change in the text: Photos was replaced (Figure 3B).

4. As shown in Figure 5A, the distribution of the ELT peptide and control peptide was not consistent, the author should discuss about this.

Reply: The distribution of peptide could be affected by multiple factors, including peptide characters, peptide binding affinity to plasma and tissues, blood flow and so on. Considering we observed the fluorescence imaging at different time point, different metabolism and elimination in different individuals also influence fluorescent intensity in organs during the whole examination. Although different peptide and different mouse caused distribution not consistent, statistic analyze showed that tumor fluorescent intensity of one peptide had similar tendency at different time point after peptide administration (Figure 5C).

Change in the text: We added discussion in the manuscript (Page 19, line 413-415).