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Evolutionary selection of personalized melanoma cell/tissue dual-homing peptides for guiding bionanofibers to malignant tumors†

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Abstract

Both melanoma cells and tissues were allowed to interact with an identical pool of billions of human-safe phage nanofiber clones with each genetically displaying a unique 12-mer peptide at the tips, respectively, resulting in the discovery of bionanofibers displaying a melanoma cell/tissue dual-homing peptide for personalized targeted melanoma therapy.

Melanoma, as one of the most deadly cancers, accounts for 75% of all skin cancer deaths worldwide.¹ The incidence of malignant melanoma is still increasing and the median overall survival rate of treated patients is still low for the available therapeutic methods such as radiation therapy, surgery and chemotherapy.² The low survival rate is due to the lack of a drug that can selectively target melanoma cells and tissues of a specific patient to avoid off-target side effects.^{3,4} Hence, there is a pressing need in the search for a patient-specific melanomatargeting molecule and the development of novel personalized tumor-homing therapeutic agents for targeted melanoma therapy in precision medicine. Filamentous phages are human-safe nanofiber-like viruses (~900 nm long and 7 nm wide), which are made of DNA (as a core) and proteins (as a shell, coded by the DNA core) and can be cost-efficiently produced by specifically infecting bacteria (Scheme 1c and d).^{5–7} Compared with other

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Conflicts of interest

There are no conflicts to declare.

targeting moieties such as antibodies, short peptides have advantages, such as flexibility in chemical conjugation and minimized immunogenicity.⁸ A library of phage nanofibers, where billions of phage clones are present with each displaying a unique peptide sequence,^{8,9} can be used to identify target-specific binding peptides. Both the cancer cells and vasculatures are the unique zip codes for the solid tumors such as melanoma.^{10,11} However, it remains unknown whether a peptide can be identified from an identical library of phage nanofibers to not only target melanoma cells *in vitro* but also circulate in the body to home to melanoma tumor tissue through vasculatures *in vivo*.^{8,9,12–16}

To answer this question, here we first used melanoma cancer cells as a target and then select peptides that can target the cells *in vitro* from a phage library by *in vitro* biopanning (Scheme 1a). Then we used the same melanoma cancer cells to form a melanoma tumor model, injected the same phage library into the tumor-bearing mice, allowed the phage library to circulate in the blood vasculatures, excised the tumor tissues to find out the associated phages, and eventually identified a peptide that made the associated phages home to tumor tissue through blood circulation *in vivo* by *in vivo* biopanning (Scheme 1a). Finally, we compared and evaluated the peptides from *in vitro* and *in vivo* biopanning to identify the best cell/tissue dual-homing peptides. By this strategy, we discovered a cell/tissue dual-homing peptide that enabled the phage nanofibers, conjugated with a photosensitizer (PS), to home to the melanoma tumor tissue for effectively destructing malignant melanoma by photodynamic therapy (PDT, Scheme 1b).

During *in vitro* biopanning, the phage library was allowed to interact with Petri dish first and then with normal skin cells (human epidermal melanocytes, HEM) to remove the phages that recognized the Petri dish and the normal skin cells. The depleted phages (input) were then allowed to interact with melanoma cells. The non-binding or weakly binding phages were washed away from the cells using a washing buffer. Then the cell-binding phages (output) were eluted from the cell surface using an elution buffer and amplified by infecting bacteria. The output was then used as an input to interact with the target cells again to repeat the biopanning. During the *in vivo* biopanning, the phage library (input) was injected into a tumor-bearing nude mouse (generated by injecting the same melanoma cells used for *in vitro* biopanning) through tail vein. After the phages were circulated in the blood for 24 h, the tumor was excised and the tumor-associated phages were amplified to form an output, which was used as a new input for the next round of biopanning. After three rounds of biopanning with the phage library *in vitro* or *in vivo* (Scheme 1a), random phage clones were picked and sequenced. Several sequences were enriched with a higher frequency. 40 melanoma cell-targeting phage clones were randomly picked and sequenced after *in vitro* biopanning, allowing us to find that 7 melanoma cell-targeting peptide sequences had an occurrence frequency higher than 1 out of 40 (Fig. 1a). 36 melanoma tissue-homing phage clones were randomly chosen and sequenced after *in vivo* biopanning, which showed that 3 melanoma tissue-homing peptide sequences had an occurrence frequency higher than 1 out of 36 (Fig. 1b). A comparison between Fig. 1a and b shows that the melanoma cell-targeting peptides C2 and C3 discovered by *in vitro* biopanning are exactly same as the melanoma tissue-homing peptides T2 and T3 discovered by *in vivo* biopanning, respectively. In addition, C1 is the cell-targeting peptide with the highest occurrence frequency and is only discovered by *in vitro* biopanning (Fig. 1a).

The melanoma cell binding affinity of the peptides displayed on the selected cell-targeting phages was determined by phage output/input assay and ELISA. In the phage output/input assay, the binding affinity of a specific peptide was estimated by the output (the nuput/input assay and ELISA). In the phage output/input assay, the binding affinity of a specific peptide was estimated by the output (the number of the same phage originally added to the cells). In the phage ELISA assay, the binding affinity of a peptide was transformed to the optical density (OD) at 405 nm, which reflects the number of the phages bound to the cells after washing. Both assays showed that C1 peptide has the best binding affinity against melanoma cells A375 (Fig. 1). C2 and C3 peptide had a better frequency in both *in vitro* and *in vivo* biopanning (Fig. 1a and b), but C2 peptide showed a much better binding affinity than C3 peptide in both assays (Fig. 1c).

T1 is only discovered by *in vivo* biopanning and is the peptide with the highest frequency after the *in vivo* biopanning (Fig. 1b). To find out which peptide out of C1, C2, C3, and T1 has the best tumor-homing capability when displayed at the tip of phage nanofibers, we injected the peptide-displaying phage nanofibers into the tumor-bearing nude mice. One day after the injection, we found that the number of phage nanofibers associated with each milligram of tumor tissues is decreasing in the order of C2, C1, T1, C3, and wildtype phage according to phage titering assay (Fig. 2). Among these groups, C1, C2 and T1 have the higher tumor-homing capability than the other groups. Moreover, C2 shows the best tumor homing capability because it not only homes to tumor tissue through blood circulation but also binds to cancer cells. Indeed, it is surprising that T1 did not accumulate in the tumor as much as C1 and C2.

Phage immunofluorescence was used to estimate the recognition between the selected peptides (displayed on the phages) and A375 cells. HEM cells and wild type phage (M13) were used as a control cell and a control phage, respectively. The results showed that the phages displaying peptide C1 and C2 could bind to the melanoma cells significantly with the former showing better binding capability (Fig. 3). However, the wild type phage (*i.e.*, without displaying any peptide) and the T1 (images similar to wildtype phage, not shown) could not (Fig. 3). Moreover, we found that the cell-targeting peptide C1 (Fig. 3) bound to the melanoma cells but not the HEM cells, suggesting that it could specifically recognize A375 cells.

In order to further understand the cell-targeting capability of the best two cell-targeting peptides (C1 and C2) identified by *in vitro* biopanning, we employed National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) to search for the protein that shares a similar motif with them. Surprisingly, we found that the two peptides share the similarity with a tumor-targeting cell-adhesion molecule, termed carcinoembryonic antigen-related cell adhesion molecule 3 (CEACAM3)-like isoform X1 (NCBI reference sequence: XP_021010770.1), which is predicted by a computational method (Fig. S1, ESI[†]). Specifically, C1 and the CEACAM3-like protein share a motif of SNTXLYLH (X = unshared amino acid) while C2 and the same protein share a motif of SGLY. Namely, BLAST shows that the C1 peptide is more similar to the CEACAM3-like

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protein, which is consistent with the result that C1 has a higher melanoma cell targeting affinity than C2 (Fig. 3).

A comparison between Fig. 2 and 3 confirms that C2, as a melanoma cell/tissue dual-homing peptide, has the best tumor-homing capability for *in vivo* drug delivery. To demonstrate that this peptide can be used to guide drug delivery to melanoma tumors, we followed our previous method¹⁷ to conjugate a photosensitizer (PS), pyropheophorbide-a (PPa), to each of the ~3000 copies of the p8 protein on the side wall of phage nanofibers by ethyl(dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS) chemistry. The resultant phage nanofibers are bifunctional in that their tip displays five copies of the dual-homing peptide and their side wall bears many copies of PS, the drug for PDT. Then we injected the bifunctional phage nanofibers into the melanoma bearing nude mice by tail vein. After the phage nanofibers were circulated in blood for 1 day, the tumor site was irradiated by the 658 nm laser for 5 min under a power density of 1.25 W cm⁻² to trigger PDT. We found that the tumor size was decreasing over time only in the cell/ tumor dual-homing peptide-modified groups (C2). In the C1, T1 and C3-modified group, the tumor is still growing but in a rate much slower than the control groups including wild type phage and phosphate buffered saline (PBS) group (Fig. 4). The cell/tissue dual-homing peptide (C2) is more efficient in guiding bifunctional phages to tumors than the other peptide group (C1, T1 and C3) because C2 can guide PS-carrying phages to tumors most efficiently (Fig. 2). Apparently, the tumor-homing capability of each peptide (Fig. 2) is consistent with the tumor therapy efficacy (Fig. 4) as expected. These results clearly indicate that the cell/tissue dual-homing peptides are ideal agents for guiding the nanofibers. In addition, nanomaterials were found to induce endothelial leakiness so that they could be delivered to tumors.^{18,19} However, since phages displaying different peptides showed different tumor homing capabilities, we believe that the nanomaterials-induced endothelial leakiness effect might not be the dominant factor for the phage-based tumor homing. These *in vivo* data show that the dual-homing peptides can indeed guide the nanodrugs to tumors, resulting in effective inhibition of the malignant melanoma growth.

It should also be emphasized that phage display is powerful for discovering cancer cell- and tissue- dual-homing peptides when we do not have any prior knowledge on the unique receptors on the target cancer cells and tumors, as is the case for the melanoma cells and tissues in this work. This advantage will speed up our development of therapeutics for targeted cancer therapy and compromise the disadvantage of this technique that the receptors targeted by the peptides are unknown when the peptides are discovered. Moreover, the peptides targeting the same cancer cells used to generate tumor models are used to guide the therapeutics to the same tumor model for targeted therapy. Namely, the peptides are patient-specific in guiding the drugs. In reality, patients with the same type of cancer present different status in the tumor microenvironments. Hence, integrating *in vitro* and *in vivo* biopanning can be used to discover patient-specific peptides that are customized to the cancer cells and tumor tissues of a specific patient. Therefore, our strategy is promising in advancing the search for patient-specific cancer therapeutics.

In summary, by a combination of *in vitro* and *in vivo* biopanning, we discovered a peptide that can guide nanofibers to home to both melanoma tumor tissues and cancer cells. By

conjugating the peptide-displaying phage nanofibers and a photosensitizer, we demonstrated that such cell/tumor dual-homing peptides could be used to achieve targeted photodynamic melanoma therapy. Our work suggests that a combination of *in vitro* and *in vivo* biopanning is powerful in searching for peptides that can enable their modified nanodrugs to not only home to tumor tissues but also target tumor cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Notes and references

1. DeSantis CE, Lin CC, Mariotto AB, Siegel RL, Stein KD, Kramer JL, Alteri R, Robbins AS and Jemal A, *Ca-Cancer J. Clin*, 2014, 64, 252–271. [PubMed: 24890451]
2. Eggermont AM, Spatz A and Robert C, *Lancet*, 2014, 383, 816–827. [PubMed: 24054424]
3. Felcht M and Thomas M, *J. Dtsch. Dermatol. Ges*, 2015, 13, 125–136. [PubMed: 25631130]
4. Lo JA and Fisher DE, *Science*, 2014, 346, 945–949. [PubMed: 25414302]
5. Zhao SJ, Zhao WX and Ma L, *Peptides*, 2010, 31, 2027–2034. [PubMed: 20713104]
6. Hamzeh-Mivehroud M, Alizadeh AA, Morris MB, Church WB and Dastmalchi S, *Drug Discovery Today*, 2013, 18, 1144–1157. [PubMed: 24051398]
7. Molek P, Strukelj B and Bratkovic T, *Molecules*, 2011, 16, 857–887. [PubMed: 21258295]
8. Yang M, Sunderland K and Mao C, *Chem. Rev*, 2017, 117, 10377–10402. [PubMed: 28723101]
9. Willats WG, *Plant Mol. Biol*, 2002, 50, 837–854. [PubMed: 12516857]
10. Narasimhan K, *Nat. Med*, 2002, 8, 116. [PubMed: 11821892]
11. Ruoslahti E and Rajotte D, *Annu. Rev. Immunol*, 2000, 18, 813–827. [PubMed: 10837076]
12. Karimi M, Mirshekari H, Moosavi Basri SM, Bahrami S, Moghoofei M and Hamblin MR, *Adv. Drug Delivery Rev*, 2016, 106, 45–62.
13. Cheung CS, Lui JC and Baron J, *J. Orthop. Res*, 2013, 31, 1053–1058. [PubMed: 23440926]
14. Cao B, Yang M and Mao C, *Acc. Chem. Res*, 2016, 49, 1111–1120. [PubMed: 27153341]
15. Sunderland KS, Yang M and Mao C, *Angew. Chem., Int. Ed. Engl*, 2017, 56, 1964–1992. [PubMed: 27491926]
16. Rajotte D, Arap W, Hagedorn M, Koivunen E, Pasqualini R and Ruoslahti E, *J. Clin. Invest*, 1998, 102, 430–437. [PubMed: 9664085]
17. Gandra N, Abbineni G, Qu X, Huai Y, Wang L and Mao C, *Small*, 2013, 9, 215–221. [PubMed: 23047655]
18. Setyawati MI, Tay CY, Docter D, Stauber RH and Leong DT, *Chem. Soc. Rev*, 2015, 44, 8174–8199. [PubMed: 26239875]
19. Setyawati MI, Tay CY, Chia SL, Goh SL, Fang W, Neo MJ, Chong HC, Tan SM, Loo SC, Ng KW, Xie JP, Ong CN, Tan NS and Leong DT, *Nat. Commun*, 2013, 4, 1673. [PubMed: 23575677]

Scheme 1.

Schematic of evolutionary selection of melanoma cell/tissue dual-homing phage nanofibers from a phage library made of billions of phage nanofiber clones by *in vitro* and *in vivo* biopanning against melanoma cells and tumor tissues, respectively, for guiding photosensitizer (PS) to tumors for targeted PDT. (a) A phage library was intravenously (*i.v.*) injected into mice and biopanning was conducted *in vivo* to select melanoma tissue-homing peptides while it was allowed to interact with melanoma cells *in vitro* to select melanoma cell-targeting peptides. In both *in vitro* and *in vivo* biopanning, after the input-output process was repeated for three rounds, the phage DNA was sequenced to know the targeting peptide sequences displayed on the targeting phages. One best peptide, displayed at the tip of phage nanofibers, was found in both *in vitro* and *in vivo* biopanning and thus was melanoma cell/tissue dual-homing. (b) A PS (PPa) was conjugated to the side wall of the dual-homing phage nanofibers by NHS/EDC chemistry. The resultant nanofibers were *i.v.* injected into the tumor-bearing mice model (same as used for *in vivo* biopanning). After 1 day, a laser light was irradiated on the tumor site to trigger PDT. The tumor disappears on Day 20 due to targeted PDT. (c) Structure of the phage nanofibers constituting the phage library used for *in vitro* and *in vivo* biopanning. The dual-homing peptide was displayed as fusion to the N-terminal end of the five copies of pIII (p3) of the phage nanofibers. (d) The atomic force microscopy (AFM) image of the dual-homing phage nanofibers. The dashed rectangle highlights an individual phage nanofiber illustrated in (c).

Fig. 1.

Characterization of the binding affinity of the selected peptides (displayed on phages) with melanoma cells. (a) Melanoma cell-targeting peptides (with an occurrence higher than 1 out of 40 randomly chosen clones) discovered after the third round of *in vitro* biopanning. (b) Melanoma tumor-homing peptides (with an occurrence frequency higher than 1 out of 36 randomly chosen clones) discovered after the third round of *in vivo* biopanning. (c) Phage output/input assay. The presence of more phage clones on the plates (*i.e.*, higher output under the same input) indicates the better binding affinity of the peptide displayed on the phage clones. (d) Phage ELISA. A higher OD value represents a stronger binding affinity against the melanoma cells for this particular peptide. M13 denotes wildtype phages.

Fig. 2.

Number of phage nanofibers (determined by a titering assay) accumulated in each unit mass of the tumors one day after the same amount of phages displaying a specific peptide is separately injected into tumor-bearing mice through tail vein. Wildtype is the phages that do not display any peptides. *, $p < 0.05$ when compared to wild type phages.

Fig. 3. Immunofluorescence to estimate the affinity and specificity of different phage nanofibers against melanoma cells. A375 melanoma cells or HEM cells were incubated with C1-displaying and C2-displaying phage nanofibers or wild type (termed M13) phage nanofibers. After washing, the phages bound with the cells were allowed to interact first with anti-M13 antibody (1 : 200) and then with the secondary antibody conjugated with a red dye TRITC (1 : 2000). Finally, the cell nuclei were stained with DAPI. In this assay phages were stained with red fluorescence and nuclei were stained with blue. Scale bars: 50 μ m.

Fig. 4.

Tumor size change after PDT for treatment groups. After PS-conjugated phages (engineered, displaying C1, C2, C3, and T1, and wild type) as well as PBS (no PS) were injected into the melanoma-bearing mice for 1 day, PDT was triggered by applying a 658 nm laser to the tumor for 5 min. Then the tumor size was monitored every 5 days for a period of 25 days.