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A new ligand of CD105 screened out by phage display technology provides a reliable identification of recurrent or metastasizing pleomorphic adenoma from pleomorphic adenoma

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ABSTRACT

Objectives: To assess CD105 expression in pleomorphic adenoma (PA), recurrent pleomorphic adenoma (RPA) and metastasizing pleomorphic adenoma (MPA), to identify new epitopes and screen a ligand with high affinity to CD105 by phage display technology, to evaluate the reliability of the new ligand for identifying RPA/MPA from PA.

Methods: Phage display technology was used to screen ligands with high affinity to recombinant human CD105. The ligand with strongest affinity to CD105 was synthesized by FMOC Chemistry according to the sequencing results. The archived formalin fixed paraffin-embedded (FFPE) tissues of 35 PA cases, 12 RPA cases and 2 MPA cases were sliced and immunofluorescent stained. CD105 expression were detected by Confocal laser scanning microscopy (CLSM). The relative fluorescence intensity was calculated with the image processing software Image J. Statistical analyses were performed by the software Graph Pad Prism (Version 7.0a). Using PROC logistic, receiver operating characteristic (ROC) curves, area under ROC curves (AUCs) were generated to assess the sensitivity and specificity of the new ligand for identifying RPA/MPA from PA cases.

Results: A ligand with specialty and high affinity to CD105 i.e. ligand nABPK296 were developed. FITC-labeled ligand nABPK296 confirmed the difference of CD105 expression in RPA/MPA and PA. The AUC of nABPK296 was 0.9418.

Conclusions: CD105 is a promising biomarker for identification of RPA/MPA from PA cases. Ligand nABPK296 provides a promising approach to CD105 detection. This study also validated the reliability of phage display technology in finding new episodes and ligands with high affinity for antigens.

1. Introduction

PA is the most common salivary neoplasm. RPA and MPA are the two histological sub-type of PA according to WHO International Histological Classification of Tumors [1,2], which may be associated with malignant transformation and often cast a shadow over prognosis [3]. Besides the malignant tendency, RPA is also a challenge for oral and maxillofacial surgeons due to the difficulty to avoiding local functional and cosmetic sequelae [4,5]. As for MPA, it is a rare tumor with a high mortality rate [6], histologically indistinguishable from PA yet produces secondary tumors in distant sites. Metastasizing sites included bone, lung, neck lymph nodes, liver, kidney, sphenoid, scapular, etc. [7–14]. Therefore, it is of great significance to find a reliable biomarker for identification of RPA/MPA from PA.

CD105 is a membrane glycoprotein forming the TGF-β receptor complex on cell membranes. CD105 consists of a large extracellular domain, a hydrophobic transmembrane domain, and a short intracellular domain. It plays an important role in regulating cellular

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proliferation, differentiation, migration, adhesion and angiogenesis, which are vital for tumor growth, survival and metastasis [15–17]. The expression of CD105 in a variety of malignant tumors is increased (including breast cancer [18], renal cell carcinoma [19,20], pancreatic cancer [21], pediatric rhabdomyosarcoma [22], acute leukemia [23], Hepatocellular Carcinoma [24], Colorectal Cancer [25], laryngeal squamous cell carcinoma [26], oral squamous cell carcinoma [27], etc.). Those with higher CD105 levels had significantly poorer 5-year disease-specific survival rate (DDS) and overall survival rate (OS) [28]. Tadbir and his colleagues reported that the increased expression of CD105 is related to the cellular proliferation, angiogenesis, malignant transformation of Salivary gland tumor [29]. As for RPA and MPA, there is no final conclusion about the role of CD105 in tumor recurrent or metastasis [30].

Phage display technology has become increasingly mature. According to different application purposes, a variety of peptide libraries have been constructed. This technique has become a tool for finding high affinity bioactive ligand molecules, exploring the interaction sites between receptors and ligands, and detecting the unknown protein spatial structure epitopes [31–33].

Based on the abovementioned theories and findings, we speculated that the expression of CD105 in RPA/MPA and PA is different. To compare the expression of CD105 in RPA/MPA and PA with a reliable and sensitive biomarker, we designed this experiment. First, we plan to screen ligands with high affinity for CD105 by phage display technology. Then, using the fluorescence labeled ligand with the strongest affinity for CD105, we try to provide a reliable and sensitive biomarker for identification of RPA/MPA from PA.

2. Materials and methods

2.1. Cases inclusion criteria and grouping

This study was approved by the ethics committee of Stomatology Hospital, Sun Yat-sen University. The approval number is ERC-2017-11. A retrospective study was conducted on 49 cases which were previously histopathological diagnosed as PA between 2000 and 2010 (i.e. 35 cases without recurrence, metastasis or malignant transformation were included in PA group, 12 cases with subsequent recurrence were included in the RPA group, 2 cases with subsequent metastasis were included in the MPA group) (Fig. 1). The mean follow-up period was 10 years. To exclude interference factors affecting tumor recurrence and metastasis such as tumor location, surgical methods, cases inclusion criteria was set as follows: the primary tumors were located in the superficial lobe of parotid gland, excision of tumor and parotid lobectomy were performed by the same surgeon.

Sections made of archived FFPE tissues of PA, RPA and MPA groups were prepared for IF staining.

2.2. Phage display and synthesis of ligand nABPK296

A peptide phage display library was commercially constructed (Ph.D.-12 phage display library, NEB, Beverly, MA, USA). The recombinant human CD105 (catalog# 1097-EN-025, R&D Systems, Minnesota, USA) at 100 μg/ml in sterile PBS was added to individual sterilized MaxiSorp plate (Thermo Fisher Scientific, USA) and incubated the plate at overnight 4 °C. The plate was washed with TBST (0.1% tween) for 6 times. And then the plate was blocked for 1 h at 4 °C using 1% BSA in PBS. M13 phage display libraries were allowed to bind for 1 h at room temperature and the unbound phages were washed away by TBST (0.1% tween) for 10 times. The binding phages were eluted by elution buffer for another round biopanning. After three rounds biopanning, target M13 phages were enriched. 16 monoclonal phages were selected randomly for ELISA binding essay. After the MaxiSorp ELISA plate was repeatedly washed with 0.1% TBST, bound phages were detected by incubation with Rabbit anti-M13 phage antibody and HRP-conjugated goat anti-rabbit IgG. ABTS/H2O2 substrate was used to measure the amount of HRP bound and monitor the absorbance at 405 nm. The inserted DNA sequence of the monoclonal phages with the strongest affinity to CD105 was determined using the primer: 5′-CCCT CATAGTTAGCGTAACG-3′ (New England Biolabs). Then, the ligand nABPK296 which composes 12 amino acids(Trp-Ile-Tyr-Asp-Thr-Thr-Arg-Val-Ile-Val-Pro-Gly) (Fig. 2A) was synthesized by Fmoc Chemistry (China Peptide Company, Jiangsu, China), confirmed and purity analyzed by reversed phase high performance liquid chromatography-electrospray ionization mass spectrometry (RP-HPLC/ESI MS). An extra FITC was linked at the amino (N) terminus of nABPK296 for labeling.

2.3. Specificity verification of nABPK296 binding to CD105

ELISA binding assay of nABP296 to CD105 were performed. The unrelated CD106 protein was used as control group. The experimental steps are as follows: (1) The recombinant human CD105 and CD106 (100 μg/ml) (catalog# 809-VR-050, R&D Systems, Minnesota, USA) were incubated in 6 individual sterilized MaxiSorp plates with sterile PBS overnight, at 4 °C. (2) The plate was washed with 0.1% TBST for 6 times and blocked with 1% BSA for 1 h in PBS, at 4 °C. (3) FITC labeled nABP296 (2 μM) was incubated for 1 h at room temperature. (4) The unbound peptide was removed by PBS for 10 times. (5) The absorbance was measured using Victor X5 (PerkinElmer, Singapore) at 490 nm and results reported as optical density (OD).

By flow cytometry, CD105 positive cell line MNNG and CD105 negative cell line Cal27 [34] were used to confirm the specific affinity of nABPK296 to CD105. MNNG and Cal27 cell lines were obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. MNNG and Cal27 cells (5×10^5 cells/100 μl for each group) were collected in a 0.25% (w/v) trypsin solution with 0.025% (w/v) ethylene diamine tetraacetic acid (EDTA), incubated respectively in FITC-labeled nABPK296 (2 μM) at 4 °C for 30 min and in CD105 mAb (Product code: ab11414, 1:1000, Abcam, Cambridge, UK) at 4 °C for

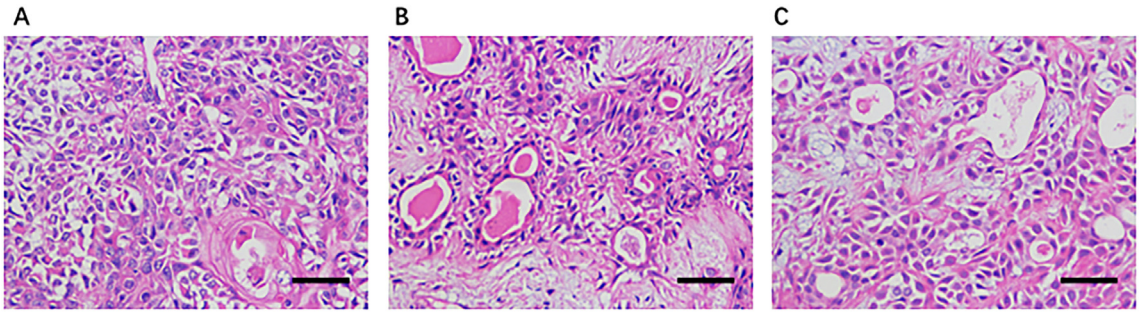

Fig. 1. RPA and MPA are histologically indistinguishable from PA. Hematoxylin and eosin (HE) stain of PA(A),RPA(B)and MPA (C) cases. The lesions showed an admixture of epithelial, myoepithelial and stromal components without atypia cell. Scale bar, 100 μm.