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Nanobody against the E7 oncoprotein of human papillomavirus 16

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ABSTRACT

The persistent infection of high-risk human papillomavirus (HPV) is one of the most common causes of cervical cancer. It is well documented that expression of two oncogenes (E6/E7) plays a key role in tumor progression. HPV16E7 -targeting via nanobody (Nb) therefore could be beneficial for HPV16-associated cancer diagnosis and therapy. In this work, phage-display approach was employed to select the high affinity HPV16E7-specific Nb. Firstly; a high-quality immune library was constructed. After three round of biopanning, high-affinity HPV16 E7-specific nanobodies were retrieved. By phage ELISA and sequencing, four different sequences of anti- HPV16E7 nanobodies were selected. Then recombinant nanobody Nb2 was cloned and expressed in *E. coli*, and the specificity and thermal stability of purified Nb2 was evaluated. To examine the potential of Nb2 as an inhibitor of E7 function, Nb2 was expressed within HPV16 positive cells. Proliferation assay showed that the intracellular expressed Nb2 as an intrabody can decrease the growth of HPV16-positive cells. The results indicate that Nb2 as an intracellular antibody directed towards HPV oncoprotein E7 has great promise in applications for the therapy of HPV16-associated disease.

1. Introduction

Cervical cancer is the second most common cancer in the world among women. Nearly all cervical cancer are associated with human papillomaviruses (HPV) infection, with two types, HPV16 and HPV18, accounting for 70% of cases, while HPV16 alone cause about 54% of invasive cervical cancers and accounts for > 80% of HPV + anal and head-and-neck cancers (Hsu et al., 2018; Akuzum et al., 2018). HPV is a small double-stranded circular DNA virus with a genome of approximately 8000 base pairs. The two primary oncoproteins of HPV16 are E6 and E7. HPV16 associated cancers express E6 and E7 that can induce and maintain the malignant phenotype through their interaction with the cellular p53 and retinoblastoma proteins (RB1). Inactivation of RB1 induced by high risk HPV16 E7 is critical for cellular transformation and uncontrolled proliferation. E7 disrupts the function of host RB1 protein and competes for RB1 binding and induces the disassembly of the E2F transcription factors from RB1, freeing the E2F to transactivate subsequent target genes transcriptional activity, thus stimulating the progression from G1 to S phase (Mirabello et al., 2017). E7 can also interfere with host histone deacetylation mediated by HDAC1 and HDAC2, leading to transcription activation (Brehm et al., 1999). Since oncogenic HPV16 E7 is the causative agent of cervical cancer, this is an obvious target for diagnosis and therapy.

To date, there are various PCR-based methods or hybridization methods for HPV DNA detection and genotyping. The detection of HPV DNA has been done predominantly by L1 general or consensus primer PCR assay, which enables the detection of a broad spectrum of HPV types. Following the general primer PCR assays, the HPV type determination can be performed by nucleotide sequencing or oligonucleotide probe hybridization (Southern dot blotting) of the PCR products (Clifford et al., 2016). In addition, the type specific PCR assays can be chosen based on the sequence variations present in the E6 and E7 genes of HPV subtypes (Paes et al., 2015). Although PCR method used for HPV detection, is more specific and more sensitive, PCR methods require sophisticated equipment and are very laborious. Recently, ELISA (Enzyme-linked immunosorbent assay) methods to detect the HPV18 L1 have been reported (Zhang et al., 2018). HPV16 E7-binding affibody molecules were also reported to have great potential for molecular imaging and diagnosis of HPV-induced cancers (Xue et al., 2016).

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In addition, prior research suggested that inhibition of E6 and/or E7 function inhibits the growth of HPV-positive cervical cancer cells (Tan et al., 2012; Li et al., 2015b; Nishida et al., 2016). Blocking expression of high risk-HPV E7 and disruption of its downstream pathways have previously been proven a successful approach for inhibition of tumor cell growth in cervical cancers, as demonstrated in several approaches to counteract the E7 oncoprotein at gene or protein level (Accardi et al., 2014; Li et al., 2015a; Phaeton et al., 2015). The most common approach applied in vitro is targeting the E6/E7 transcripts by siRNA or shRNA. E7-targeting shRNA reduced the E7 mRNA levels and inhibited proliferation of SiHa and CaSki cells (Li et al., 2015b). HPV16E7 siRNA could inhibit growth of CaSki cells (Li et al., 2015a). E6 and E7 RNA interference alone inhibited growth of human cervical carcinoma xenografts in mice. Systemic delivery of siRNA for silencing the E6 and E7 was found to significantly suppress the growth of subcutaneous SiHa and HeLa tumors (Nishida et al., 2016).

An alternative approach is the use of antibody or small peptide targeting the E7 oncoproteins, it was reported that targeting E7 with specific radiolabeled monoclonal antibodies inhibited experimental tumors growth (Phaeton et al., 2015). Recently a small peptide targeting HPV16 E7 has been shown to bind and degrade E7, induced G1-phase arrest and suppressed proliferation of SiHa cells in vitro and inhibited SiHa tumor growth in mice (Tan et al., 2012; Guo et al., 2011). Therefore, searching for suitable inhibitors of E6 or E7 oncoprotein is of great value for the treatment of HPV-induced malignancies.

In recent years, the use of intracellular antibodies (intrabodies) to inhibit protein function holds promise for the treatment of human disease (Chen et al., 2019; Bertier et al., 2018; Accardi et al., 2005). HPV E7 function is mediated largely by protein–protein interactions, therefor targeting HPV16E7 via intracellular antibodies to interfere with the function of the targeted E7 could be beneficial for HPV16-associated cancer therapy (Accardi et al., 2011).

Camelids have unique type of antibodies called the heavy chain antibodies (HCAbs) which are naturally devoid of light chains (Hamers-Casterman et al., 1993). Unlike conventional IgGs, the antigen-binding fragment of these heavy chain antibodies consists of only one single domain referred to as nanobody (Nb) or VHH (the heavy chain antibody variable region), with a molecular weight of approximately 15 kD. VHH have interesting physicochemical features, such as solubility and stability, and a high yield produced in E. coli or yeast. Nanobodies have been successfully generated against many antigens for biotechnological or research purposes along with medical applications (Liu et al., 2017; Conrath et al., 2001). It was also reported that neutralization of HPV was achieved by specific nanobodies against HPV16 major capsid protein L1 (Minaeian et al., 2012). The ease of generation and unique features of nanobodies make them promising molecules for the generation of new HPV diagnostics and therapeutics. The main objective of this study was to develop the HPV16 E7-specific nanobody through phage display technique. In this regard, anti-HPV16E7 nanobody from immune VHH phage library was isolated and produced. This nanobody was recombinantly produced in E. coli and recognized HPV16 E7 selectively and can be used in laboratory diagnostic immunoassays for detecting HPV16 E7. The inhibition of HPV16-positive cell proliferation by anti-HPV16 E7 intracellular nanobody was also demonstrated.

2. Material and methods

2.1. Preparation of recombinant HPV16E7 proteins and camel immunization

The plasmid pSUMO-HPVl6E7 for the expression of HPVl6E7 was constructed in our laboratory. Briefly, the synthetic HPVl6E7 cDNA was ligated into a psumo-mut vector and transformed into E. coli cells. Induction with IPTG, the His-tagged fusion protein was expressed in E. coli BL21 (DE3) cells. Soluble recombinant protein was recovered by bacterial sonication and then purified by immobilized metal affinity chromatography (IMAC) by NI-NTA superflow sepharose columns in a gradient of increasing imidazole concentration. Purified HPVl6E7 were analyzed by SDS-PAGE and stained with Coomassie blue. HPVl6E7 preparations were stored at −80 °C until further use. To obtain HPVl6E7 specific binding VHHs, an adult healthy bactrian camel was immunized subcutaneously 7 times at 7-day intervals with HPVl6E7 (1 mg) mixed with equal volume of Freund's complete adjuvant for the first time, and all subsequent boosts were with incomplete Freund's adjuvant according to standard immunization protocols. Two weeks after 7 times immunization, 100 mL of anticoagulated blood from the HPVl6E7 immunized camel was collected. Lymphocytes were isolated from peripheral blood by density gradient centrifugation and used for the library construction. Animal welfare and experimental procedures were performed strictly in accordance with high standard animal welfare and other related ethical regulations approved by Southeast University.

2.2. Library construction

Total RNA was extracted from lymphocyte and cDNA was synthesized by reverse transcription with oligo-dT primers. To avoid contamination of VH genes, two steps nested PCR approach was used to amplify VHH coding regions as we previously described (Liu et al., 2017). The first round PCR was performed with a template of the first strand cDNA using the primers nest-PCR1 up and nest-PCR1 down, which was used for isolation of VHH genes from classical VH genes. The first PCR products were analyzed by agarose gel electrophoresis and re-extracted the 700 bp fragments to use as the template for the secondary PCR reaction. Then, the VHH encoding gene fragments were amplified by second PCR using the nested primers including EcoRI and HindIII restriction sites (nest-PCR2 up and nest-PCR2 down) (Table 1).The amplified 500 bp VHH amplicons were agarose gel purified and digested by restriction enzymes EcoRI and HindIII. Then purified fragments were ligated into EcoRI/HindIII linearized T7Select10- 3b vector arms. The ligation mixtures were packaged in vitro using the T7Select packaging extract according to the manufacturer's instructions. Then gradient dilutions of the packaging reactions were mixed with E. coli BLT5403 and plated on LB plates as described in the T7 Select System Manual, the size of the library was measured by the number of plaques. Seventeen individual plaque were randomly selected to perform PCR and to measure the percentage of library colonies containing correct insertion.

2.3. Phage library biopanning and Nbs selection

The Nbs against HPV16E7 were selected by phage display. Briefly, VHH library was amplified, HPV16E7 protein was immobilized onto agarose beads in phosphate-buffered saline (PBS). After washing the beads by PBST (PBS containing 0.1% Tween20), the beads were incubated with phage libraries for 1 h, and washed unbound phage particles 3 times with PBST. By adding E. coli BLT5403 to the washed beads and incubated at 37 °C until cell lysis occurred, by centrifugation the bound phage particles were rescued and then phage titer was determined. The process represented one round of biopanning and these recovered phage particles were used in the next round of panning. After three rounds of biopanning, the HPV16E7 specific phages were enriched gradually. Seventy individual plaques were randomly selected for further analyses to identify HPV16E7 specific VHH by performing

Table 1
Primers used for PCR of VHH genes in this study.

nest-PCR1 up	5′-GTCCTGGCTGCTCTTCTACAAGG-3′
nest-PCR1 down	5′-GGTACGTGCTGTTGAACTGTTCC-3′
nest-PCR2 up	5' > CCGGAATTCTCAGGTGCAGCTGGTGGAGTCTGG < 3'
nest-PCR2 down	5' > GCCCAAGCTTTGAGGAGACGGTGACCTGGGT < 3'

phage ELISA. Finally 30 positive plaques were performed PCR and sequenced, and these phage-displayed HPV16E7-specific VHH antibodies were isolated and classified according to the diversity of amino acid sequences.

2.4. Cloning of nanobody gene into pET28a expression vector and nanobody expression and purification

After selections, the output T7 phage was used as a template for PCR and PCR-amplified nanobody genes were cloned into pET28a (+) vector and the cloned genes in recombinant plasmid of pET28a (+)-VHH was checked and confirmed by sequencing. For protein expression, the recombinant plasmid of pET28a (+)-VHH was transformed into E. coli strain BL21 (DE3) (Novagene). The selected positive clones were cultured at 37 °C in TB medium.When the optical density (OD) of culture reached 0.6. anti- HPV16E7 Nbs were expressed by inducing with 1 mM IPTG in shaker flasks incubated overnight at 28 °C. Then the culture was centrifuged, the cell pellet was resuspended in lysis buffer and sonicated. Nanobodies were purified by Nickel Sepharose affinity chromatography and dialyzed in PBS buffer. Then the nanobody Nb2 was further purified by gel filtration on a Superdex 75 column (GE Healthcare). The collected fractions containing 99% pure nanobody were pooled and concentrated using an ultrafiltration unit (3000 molecular weight cutoff, Millipore).

The purity of collected proteins was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using Coomassie blue staining.

2.5. ELISA for nanobody specificity

To detect the specificity of Nb2 which we have purified, Nb2 was tested with several kinds of HPVE7 by ELISA. BSA was used as control. Each HPVE7 (5 μg/mL) was coated onto microtiter plates overnight at 4 °C. After blocking with 1% bovine serum albumin (BSA) at room temperature for 2 h, 10 μg/ml of nanobody was added and the plates were incubated at room temperature for 1 h. After washing, anti-HA tag mouse monoclonal antibody was added for 1 h. then washing with PBST (PBS with 0.05% Tween 20), 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG diluted in PBST was added to the wells and incubated for 2 h at room temperature. After washing, TMB solution was added to the wells and incubated for 30 min at room temperature. Finally absorbance at 450 nm was measured by a ELISA reader. Values were the means of three replicates.

2.6. The affinity constant of Nb2 for binding to HPV16E7 was determined by ELISA

The affinity constant of the Nb2 was determined as described previously (Beatty et al., 1987; Zhang et al., 2013). The plate was coated with HPV16E7 of three concentrations 5ug/mL, 2.5ug/mL and 1.25ug/mL respectively. The NB2 of known concentrations (1 mg/mL or serial diluted to 40000ug/L; 10000ug/L; 2500ug/L; 625ug/L; 156.3ug/L; 39.1ug/L; 9.8ug/L; 2.5ug/L) was added into different wells and the plate was incubated at 37 °C for 1 h followed by washing and then incubated with a HRP-conjugated secondary antibody for 1 h and the color was developed by the addition of substrate. The OD values were read at 450 nm wavelength. Three reaction curves were plotted for the OD values versus antibody dilution. The plateau of each curve was designated as OD100 and the Nb2 concentration at OD50 was designated as [Ab]t. According to Beatty, three different concentrations of [Ag], are used, thereby enabling the calculation of three different Kaff values, which generate the average affinity constant. Three [Ab]t values were obtained against three different concentrations of HPV16E7, ([Ab]t at 5 ug/mL,[Ab']t at 2.5 ug/mL and [Ab"]t at 1.25 ug/mL) and were used for the calculation of three K values according to the Beatty formula: Kaff = (n-1)/2(n[Ab']t -[Ab]t), K1 = 1/2(2[Ab']t-[Ab]t), K2 = 1/2(2[Ab"]t-[Ab']t) and K3 = 3/2(4[Ab"]t-[Ab]t). The final affinity constant is the average result of three calculations : Kaff = (K1 + K2 + K3)/3.

2.7. Cloning of anti- HPV16E7 Nb2 sequences in Nb2 expression plasmids pCMV/myc/Nb2

Anti- HPV16E7 Nb2 coding sequence was subcloned into the vector pCMV/myc/cyto (invitrogene) to construct the recombinant plasmid pCMV/myc/Nb2. By cloning in the pCMV/myc/cyto vector, expressed Nb2 acquire a c-myc-tag at the C-terminus for Nb2 detection use.

2.8. Cell lines and transfection

Two HPV16 positive cervical epithelial tumour cell lines, CaSki and SiHa, and the HPV16-negative human cervical squamous carcinoma cell line C33 A, were used in our study. Cells at 50% of confluence were transiently transfected with the recombinant anti-HPV16E7 Nb2 expression plasmids pCMV/myc/Nb2 using Lipofectamine® 2000 (ThermoFisher Scientific) according to the manufacturer's recommendations.

2.9. MTT assay

MTT assay was used for the evaluation of cells proliferation according to the manufacturer's instructions. Briefly, cells transfected with plasmid pCMV/myc/Nb2 or control plasmid were incubated in 96-well-plates with DEME medium supplemented with 10% FBS. 10 μl MTT dye was added into every well for additional incubation for 4 h in a humidified incubator with 5% CO2 at 37 °C. Then the media were drained and cells were treated with 150 μl of DMSO for 15 min to promote the lysis of formazan crystals. The absorbance at 570 nm was measured on a microplate reader.

3. Results

3.1. HPV16E7 antigen preparation

To generate specific anti-HPV16E7 Nbs with high affinity, firstly we prepare the recombinant full-length HPV16E7 because it could provide diverse epitopes to elicit the strongest possible immune response in camels. The recombinant HPV16E7 proteins were expressed in soluble form and purified by Ni2 + -IDA column. The yielded product was about 95% purity as revealed by SDS-PAGE analysis (Fig. 1A). This recombinant HPV16E7 antigen was used to immunize the camel and for subsequent panning, identification and characterization of nanobodies.

3.2. Construction of phage display nanobody library

A 1-year-old healthy bactrian camel was immunized with HPV16E7 for seven times to induce the camel to raise a good immune response. In order to isolate nanobodies against HPV16E7 with high affinity and specificity, phage display nanobody library was constructed according to the procedure shown in Fig. 1B. VHH sequences (The variable region of heavy-CHain antibody) were amplified from lymphocyte cDNA. In the first PCR, a 900 bp gene fragment for VH-CH1-CH2 exons and 700 bp for VHH CH2 exons were amplified with primers annealing at the leader sequence and at the CH2 exon of the heavy chains of all dromedary immunoglobulin. The 700bp PCR product for VHH-CH2 was purified by gel extraction as the nested PCR template (Fig. 1C). The gene for the VHH domain of about 500 bp was then re-amplified with the nest-PCR2 up and nest-PCR2 down primers. For library construction, two restriction enzymes, EcoRI and HindIII were introduced into the 5′ and 3′ ends of the final VHH PCR fragments, respectively (Fig. 1D). Then, VHH fragment was digested and inserted into the T7Select 10-3 vector. After in vitro phage packaging, T7 phage VHH

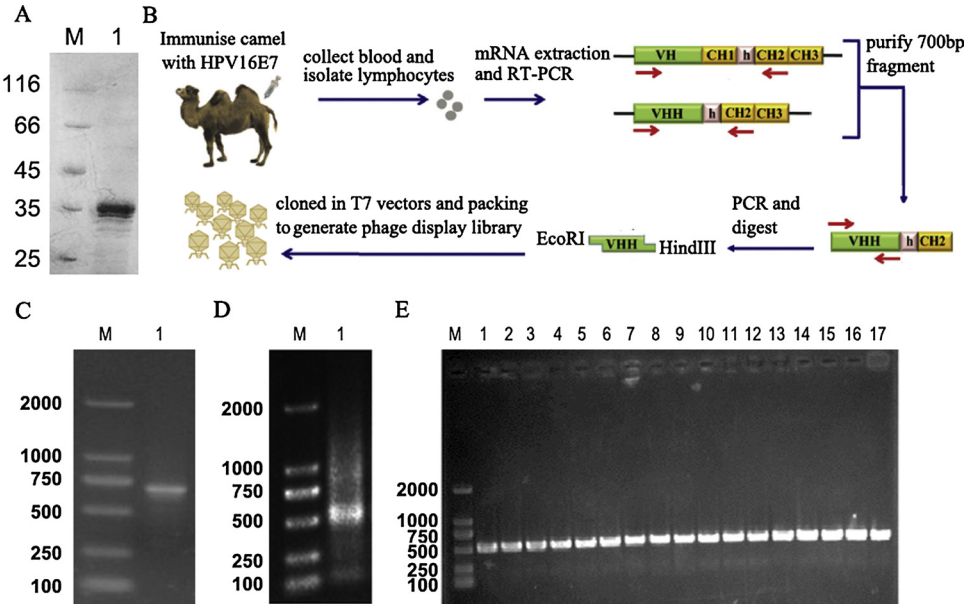

Fig. 1. Construction of phage-displayed library. (A) SDS-PAGE of purified recombinant sumo-tagged HPV16E7 immunogen, reveals molecular mass around 35 kD and high purity, lane M: MW of the marker, lane 1: purified recombinant sumo-tagged HPV16E7 protein. (B) Schematic representation of the strategy that HPV16E7-specific VHH genes were cloned. (C) The first PCR fragments had evident bands around 700 bp. lane M: the MW of the marker, lane 1:1st PCR product. (D) VHH genes were re-amplified by second PCR, lane M: the MW of the marker, lane 1: 2nd PCR product. (E) Clones were randomly selected to detect the percentage of clones containing an insert of a proper size for a VHH. The correct insertion rate detected by PCR of 17 individual clones was nearly 100%. Lane M is a MW size marker, lane 1–17 show the 500 bp PCR product of 17 individual clones, the correct size of the Nb amplicon.

libraries were constructed. Plaque assay was performed to calculate the phage titer. The total number of pfu in the packaging reaction is 5×10^7pfu. Meanwhile, seventeen lysis plaques were chosen to detect the insertion rate of the library by PCR. The result showed that the correct insertion rate was nearly 100% (Fig. 1E). All these results suggested that a high quality immunized phage display library was successfully constructed. Then prior to biopanning, the phage products was used for infection of host BLT5403 to perform a single round of amplification.

3.3. Bio-panning of phage display library against HPV16E7

After acquiring the T7 phage display nanobody library, we performed bio-panning to isolate HPV16E7 specific Nbs. Because coding sequences for the VHH are cloned in T7Select 10-3 vector and translate to form capsid fusion proteins. So the capsid protein fused with nanobody was displayed on the surface of the T7 phage. To obtain anti-HPV16E7 Nbs, phages particles were panned against HPV16E7. To evaluate the enrichment during the process of panning, the ratio of plaques numbers between pannings on HPV16E7 and PBS as negative control was compared. After 3 rounds of panning, the ratios have been gradually increased from 5-fold to 80-fold. (Fig. 2A). After panning, 70 plaques were randomly chosen for ELISA to test for the presence of nanobody which bind HPV16E7. Subsequently, 30 plaques showed a good target recognition with binding ratios relative to a non-coated well of more than 2 and these plaques were selected as positive colonies (Fig. 2B). After ELISA, the DNA of positive plaques were prepared to perform PCR and sequencing across the cloning region of the phage DNA. Based on sequencing data, the VHH fragments from these 30 colonies were analyzed and all 30 colonies contained the correct VHH fragments. Sequence analysis further classified these 30 nanobodies into 4 classes according to the variety of amino acid sequences (Fig. 2C).We define these four classes of anti- HPV16E7 VHH as Nb2, Nb12, Nb4, and Nb27, respectively. CDR and amino acid numbering were according to the methods of Kabat et al. (1991). Similar to previously reported distinct feature of VHHs, beside the strictly conserved intradomain disulphide bridge (cysteine 22 in FR1-cysteine 103 in FR3), which is characteristic for the immunoglobulin fold, the presence of a second interloop disulphide bond is common occurrence in VHHs of the dromedary (Arbabi Ghahroudi et al., 1997). Three of the four clones here contain a cysteine in the CDR3 which could possibly form a disulphide bond with a second cysteine located in the CDR1.

3.4. Expression and purification of HPV16E7 specific VHHs

The plaque 2, showing maximum binding with HPV16E7, was selected for further studies. The isolated HPV16E7 specific VHH2 genes were cloned into pET-28a expression plasmid. Subsequently with 1 mM IPTG for 16 h at 28 °C, induced soluble anti-HPV16E7 VHH2 (Nb2) was expressed in E. coli cells. After pelleting the cells and sonication, soluble Nb2 containing his-tags were purified from the cell lysate by immobilized metal affinity chromatography (IMAC). After washing with PBS, the his-tagged Nb2 proteins were eluted with a gradient increasing concentration of imidazole and subsequent dialysis of the fractions of interest into PBS. Then Nb2 were further purified by gel filtration to remove the remaining contaminants. SDS-PAGE analysis showed that the resultant recombinant nanobody Nb2 had single bands with high purity (Fig. 3A). The molecular weights of soluble Nb2 were nearly 19 kDa (Fig. 3B).

3.5. Heat resistance analysis of Nb2

It was reported that nanobodies were highly stable to extreme pH and temperatures (van der Linden et al., 1999; Omidfar et al., 2007). To determine the thermal stability of anti-HPV16E7 Nb2, Nb2 were incubated at 37 °C for different amounts of time or incubated for 4 h at a range of temperatures (0, 40, 50, 60, 70, 80 and 90 ∘C) before their introduction to an ELISA plate coated with antigen. Then the residual binding capacity of the Nb2 was detected. As shown in Fig. 3C1, even under the treatment for 96 h, Nb2 still keep more than 80% activities. To test the Nb2 stability to thermal denaturation, the residual antigen-binding activity of the purified nanobody Nb2 was determined following incubation at a range of temperatures for 4 h. Nb2 showed considerable resistance to denaturation, they still displayed superior binding to HPV16E7 after incubation at high temperature, retaining more than 40% of maximum binding activity following incubation even at 90 ∘C (Fig. 3C2). These results illustrated that Nb2 were with very good thermo stability and may become promising diagnostic material for clinical application in warm areas lacking low temperature storing condition.

3.6. Determination of affinity constants and binding specificity

The affinity constant of Nb2 for binding to HPV16E7 was determined by ELISA. The method was developed by Beatty et al. (1987).

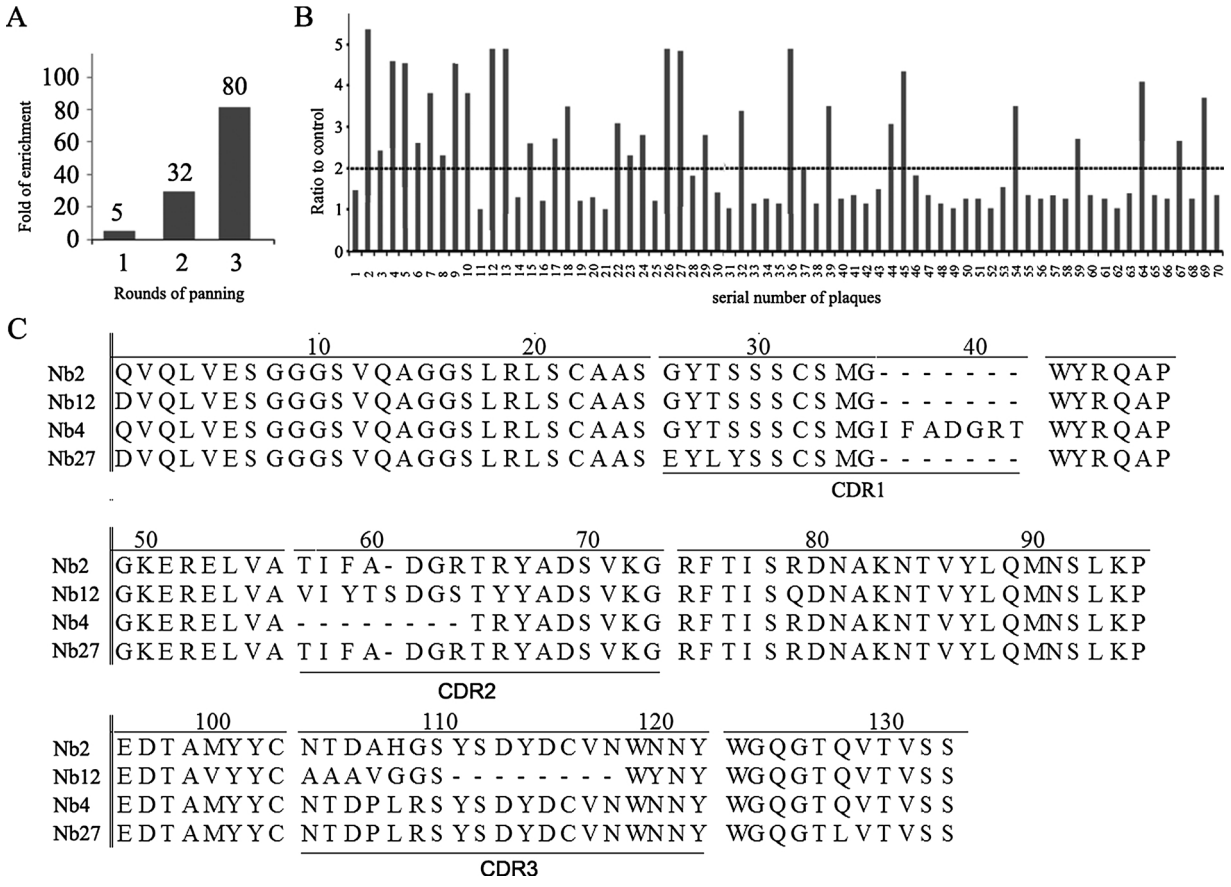

Fig. 2. Selection of nanobodies (Nbs) against HPV16E7 by phage display library. A. After three rounds of panning, HPV16E7-specific VHHs were enriched 80-fold. (B) ELISA for 70 plaques. The plaques, whose absorbance signal (plaques incubated with HPV16E7) is more than two fold higher than that for the negative control, were considered as positive. (C) Four kinds of different amino acid sequences of anti-HPV16E7 VHHs were identified, named Nb2, Nb12, Nb4, Nb27. Sequence alignment of the isolated nanobodies and amino acid numbering were according to the methods of Kabat et al. (1991).

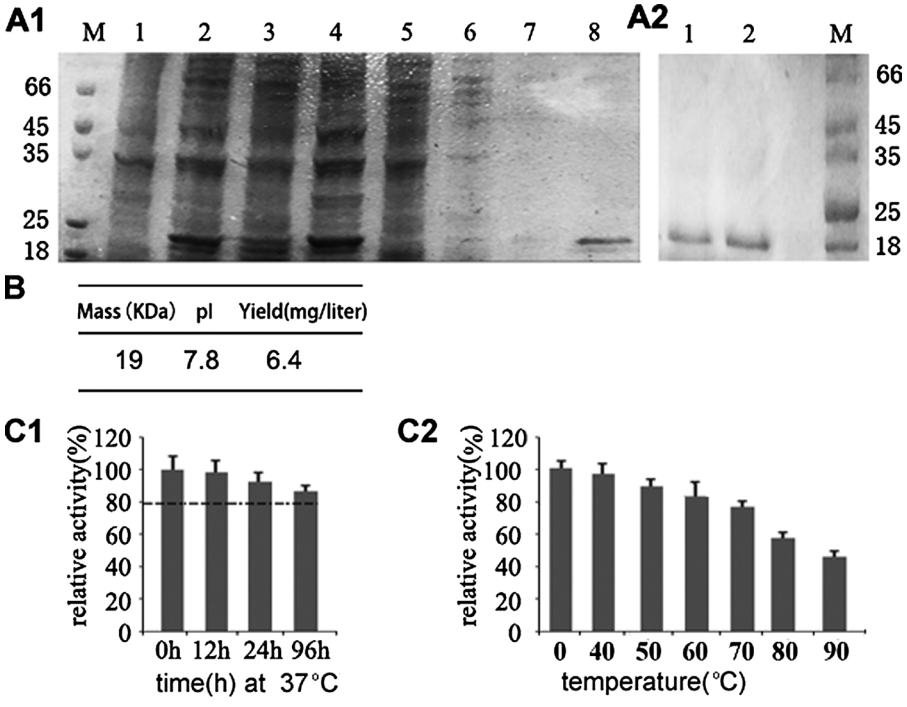

Fig. 3. Purification of anti-HPV16E7 Nb2 and thermo stability analysis of purified Nb2. (A) SDS-PAGE results showing expression and purification of anti-HPV16E7 Nb2.

A1.The expressed protein was purified with Ni2+ affinity column. Lane M, molecular weight markers; lane 1, Total protein before IPTG induction of Nb2-infected BL21 (DE3) cells.; lane 2, Total protein of induced culture of Nb2-infected BL21(DE3) cells, showing the anti- HPV16E7 VHH band at 19 kDa. lane 3, Supernatant after ultrasonication; lane 4, Precipitation after ultrasonication; lane 5, The flow-through fraction ; lane 6,7, The wash fraction at 50 mM imidazole; lane 8, The elution fraction at 500 mM imidazole. A2. SDS-PAGE from gel filtration fractions. Lane M, molecular-mass markers; lane 1 and 2, purified protein Nb2. (B) Properties of anti-HPV16E7 nanobodies Nb2: molecular mass includes HA and His6 tags. Theoretical pI is calculated using the ExPASy ProtParam Tool. (C) Antigen binding after temperature treatment of HPV16E7 specific nanobody Nb2. (C1) The relative activity of Nb2 which were incubated at 37 °C for different amounts of time. Nb2 was treated at 37 C for 0,12, 24 and 96 h, then the activities were tested by ELISA compared to the untreated Nbs. (C2) Nb2 were incubated for 4 h at different temperatures, cooled down to room temperature and subsequently ELISA was performed.

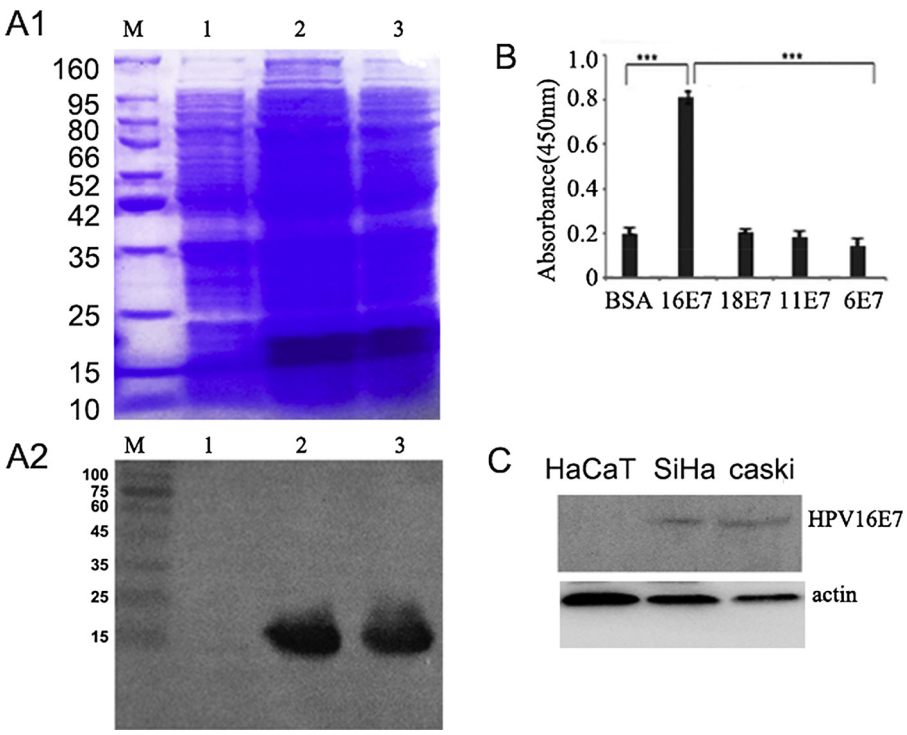

Fig. 4. Specificity of purified Nb2. A1. SDS-PAGE (A1) and Western blot (A2) results showing Nb2 can recognize the pet28a-HPV16E7 expressed recombinant protein. A1. SDS-PAGE. Lane M: molecular weight markers. Lane 1: Total protein before IPTG induction of pet28a-HPV16E7-transformed BL21 (DE3) cells. Lane 2 and lane 3: 2 h and 4 h respectively after induction with IPTG of culture of pet28a-HPV16E7-transformed BL21 (DE3) cells showing pet28a-HPV16E7 expressed recombinant HPV16E7 band at about 15 kDa. The SDS-PAGE was stained with coomassie brilliant blue. A2. Western blot. M. molecular weight markers. Lane 1: uninduced culture of pet28a-HPV16E7-transformed BL21 (DE3) cells. Lane 2 and 3: induced culture of pet28a-HPV16E7-transformed BL21 (DE3) cells. The recombinant HPV16E7 protein was detected by using anti-HPV16E7 nanobody Nb2. B. Cross-reactivity analysis based on ELISA revealed Nb2 was specific against HPV16E7. To test the specificity of Nb2 to four types of different viral proteins by ELISA, one hundred microliters of each protein (5 μg/mL) was coated onto microtiter plates, and 100 μL nanobodies (10 μg/mL) were added. After reaction with mouse anti-HA tag antibody, then anti mouse IgG-HRP and TMB substrate, the absorbance at 450 nm was read. The values were the means of three replicates. ***P < 0.01 versus control. C. Western blot showed that anti-HPV16E7 nanobody Nb2 can recognize the HPV16E7 protein expressed in the HPV16 positive SiHa and caski cells. Actin as control.

This method used serial dilutions of both antigen (coated to the plate) and antibody for measuring affinity constants. The affinity constant is then calculated using the formula: Kaff = (n-1)/ 2(n[Ab']t -[Ab]t). The mean affinity constant of the purified Nb2 was 7.5×10^7 M^- as determined by indirect ELISA, which was calculated from the following Kaff values: 10×10^7 M-, 5.5×10^7 M-, 7×10^7 M-.

To test the immuno-reactivity of Nb2, recombinant pet28a-HPV16E7 plasmid was constructed and transformed into E.coli BL21 cells, HPV16E7 was expressed in E. coli upon induction with IPTG. Protein bands of HPV16E7 with apparent molecular weight of 16 kDa was clearly present in the induced E. coli and were visualized by SDS-PAGE (Fig. 4A1), and can be recognized by Nb2 by the method of western blot (Fig. 4A2).

The nanobody Nb2 specificity to the virus proteins E7 was determined by ELISA method (Fig. 4B). In this assay, Different virus E7 protein (HPV16E7, HPV18E7, HPV11E7 and HPV6E7) were coated onto microtiter plates. Nb2 were added to incubate with them, respectively. Binding of Nb2 were detected by incubation with anti-HA-tag and HRP-conjugated antibody. The absorbance value was measured by a plate reader at 450 nm. The result showed that no cross-reactivity with other immobilized E7 was detected.

To analyze the ability of Nb2 to bind HPV16E7 in HPV16 positive SiHa and caski cells, western blot was performed, our results demonstrated that the Nb2 were able to recognize HPV16E7 expressed in SiHa and caski cells(Fig. 4C).

3.7. Nb2 expression decreases the growth of HPV16-positive cells in vitro

To determine whether the Nb2 expression have impact on proliferation of cancer cells, Nb2 expression plasmids pCMV/myc/Nb2 was transfected to HPV16-positive SiHa and CasKi cells and HPV-negative C33 A cells. Confirmation of the eukaryotic expression of the Nb2 was performed by western blot analysis using anti-myc antibody to detect the c-myc tag within target cells (Fig. 5A). Cell viability after transfection 72 h was measured by MTT assay. As shown in Fig. 5B, Nb2 expression significantly inhibited proliferation of HPV16-positive CasKi cells and SiHa cells, no inhibition of cell growth was observed when the HPV-negative C33 A cells were transfected with the Nb2 expression plasmids, indicating that inhibition of cell growth was specific for the HPV16-positive cells.

4. Discussion

The association of high-risk HPV types with several kinds of human cancer has been

universally recognized since many years; HPV16 is the most frequent, with almost 60% of cervical cancer cases worldwide (Forman et al., 2012). The existing studies have shown that the high-risk HPV E6 and E7 genes are the main oncogenes of high-risk HPVs (Ghittoni et al., 2010). E6 and E7 exert different and concerted pro-tumor actions mainly based on their capacity to target cellular proteins. Overall, the concerted activities of E6 and E7 influence cell cycle control and resistance to apoptosis, with the consequence of immortalizing and transforming human keratinocytes (Chellappan et al., 1992; Thomas et al., 1999). For their well-established roles in the neoplastic progression of the HPV-caused lesions, HPV16 E6 and E7 viral proteins are main target for immunotherapy of the HPV-associated tumors (Manzo-Merino et al., 2013; Accardi et al., 2005).

In the present study, we successfully expressed the HPV16 E7 protein in vitro and applied it in the immunization of a camel. Then an immune phage-displayed nanobody library is constructed. This technology of cloning the repertoire of antigen-binding fragments from an immunized animal into a phage display vector and selection of antigen-specific clones by panning has become a routine method in the past decade to identify antigen-specific molecules. Compared to conventional antibodies, the unique intrinsic properties of nanobodies contained in camel make them attractive alternatives for medical and biotechnological applications. In this study, four nanobodies were successfully isolated from phage-displayed nanobody library. We expressed and purified one of these nanobodies, the Nb2, and verified the specificity of it and developed the immunoassay for HPV16E7 detection. To our knowledge, this is the first report for the identification of nanobodies against HPV16E7.

Previously it was reported that the ScFv intrabody, specific for the

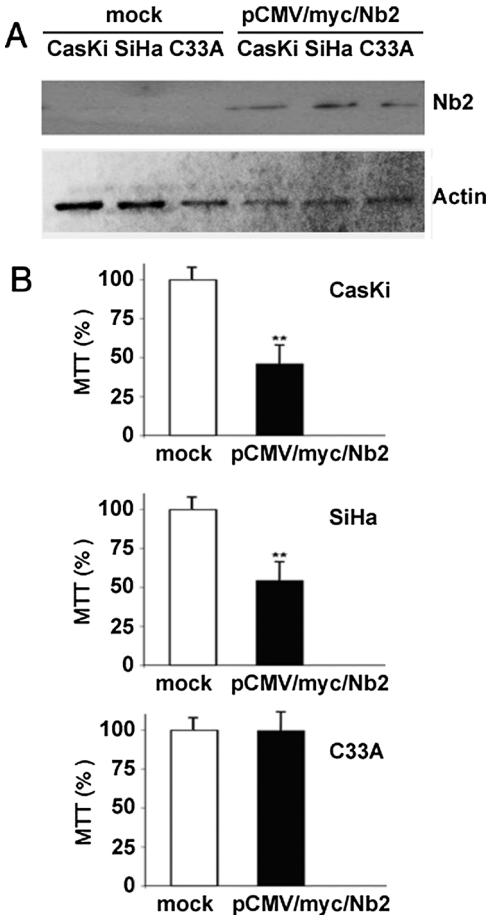

Fig. 5. Effect of anti-HPV16 E7 Nb2 expression on tumor cell proliferation. A. Expression of the anti-HPV16 E7 Nb2 in pCMV/myc/Nb2 transfected cervical carcinoma cells was determined by western blot. The human cervical carcinoma cell lines SiHa (HPVI6 positive), CasKi (HPVI6 positive) and C33 A (HPV16 negative) were transfected with the control plasmid pCMV/myc/cyto (mock) or Nb2 expressing plasmid pCMV/myc/Nb2. At 48 h posttransfection, the cell lysates were submitted to western blot analysis using anti-myc antibody. B. Different human cervical carcinoma cell lines were transfected with the plasmids pCMV/myc/Nb2 to express the anti-HPV16 E7 nanobody Nb2, control plasmid pCMV/myc/cyto was used as mock transfection. 72 h posttransfection, MTT assay was performed to evaluate cell proliferation. **P < 0.01 versus control.

E7 oncoprotein of HPV16, expressed in the HPV16-positive SiHa cells, was able to inhibit cell proliferation (Accardi et al., 2014). Now our anti-E7 nanobody Nb2 also presented the ability to inhibit tumor cell proliferation when expressed in the HPV16 positive cell as intracelluar nanobody. These results suggest that nanobody Nb2 may be employed as therapeutic molecules for HPV16-associated lesions. In deed, recently a growing number of data support that the use of intracellular nanobodies to inhibit protein function holds promise for the treatment of human disease (Boons et al., 2014; Newnham et al., 2015; Tremblay et al., 2010).

In conclusion, we reported for the first time the production and application of a specific HPV16 E7 nanobody. Additionally, nanobodies produced in this study are highly stable and inexpensive. This suggests the possibility that anti-HPV16E7 Nbs may be a good candidate biomolecular agents for diagnosis and therapy of HPV16 associated disease. Nevertheless, further research is still needed to evaluate the clinical utility of the anti-E7 nanobody in the studies of HPV genotyped cervical samples and biopsies.

Conflict of interest statement

The authors have no conflict of interest.

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