Biotechnol Lett
DOI 10.1007/s10529-013-1340-1

ORIGINAL RESEARCH PAPER

A novel VHH nanobody against the active site (the CA domain) of tumor-associated, carbonic anhydrase isoform IX and its usefulness for cancer diagnosis

Fatemeh Araste · Walead Ebrahimizadeh · Iraj Rasooli · Masoumeh Rajabibazl · Seyed Latif Mousavi Gargari

Received: 21 May 2013 / Accepted: 5 September 2013

Abstract Expression of carbonic anhydrase IX (CAIX) significantly increases under hypoxic conditions in tumor cells. CAIX activity is executed by the catalytic domain (CA) located on the extracellular part of the enzyme. Neutralization of CAIX enzymatic activity reduces malignancy and survival of tumor cells. To inhibit the enzymatic activity, a VHH nanobody was developed against the CA domain of CAIX using phage display technology. Following immunization of a camel with the recombinant CAIX, VHH fragments were isolated by nested PCR on lymphocyte cDNA. Binding affinity of isolated nanobodies was tested by ELISA. A clone (K24) with the highest binding affinity was expressed in a soluble form. Affinity of K24 nanobody was determined to be approx. 2.3×10^{-5}. K24 nanobody recognized the expressed CAIX in the HeLa cell lines with high selectivity and specificity. These findings thus have usefulness for the diagnosis and treatment of cancers.

Keywords Carbonic anhydrase IX · Cancer diagnosis · Cancer treatment · Phage display · VHH nanobody

Introduction

Among the 16 isozymes in α-carbonic anhydrase family, carbonic anhydrase IX (CAIX) and occasionally carbonic anhydrase XII is expressed in hypoxic tissues (Koukourakis et al. 2001). CAIX is a transmembrane disulfide-linked dimer protein with four domains, of which one has a high homology to the keratin sulfate's binding domain (Nishimori and Onishi 2001). The three other domains are similar in all members of α-carbonic anhydrase family. Carbonic anhydrase activity is executed by the catalytic domain (CA) which is located on the extracellular part of the protein. Transmembrane (TM) and *C*-terminal (IC) domains consist of 20 and 25 amino acids, respectively. CAIX expression significantly increases under hypoxic conditions and accumulation of CO_2 in tumor cells. The carbonic anhydrase enzyme catalyzes the condensation reaction that converts CO_2 to bicarbonate; $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$. The protons and lactic acid produced by

Electronic supplementary material The online version of this article (doi:10.1007/s10529-013-1340-1) contains supplementary material, which is available to authorized users.

F. Araste · W. Ebrahimizadeh · I. Rasooli · S. L. Mousavi Gargari (✉)
Department of Biology, Faculty of Basic Science, Shahed University, Opposite of Imam Khomeini's Shrine, Tehran-Qom, Express Way, 3319118651 Tehran, Iran
e-mail: slmousavi@shahed.ac.ir

M. Rajabibazl
Department of Clinical Biochemistry, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Koodakyar Alley, Daneshjoo Boulevard, Velenjak, Tehran, Iran

Published online: 26 September 2013

anaerobic glycolysis (Warburg effect) lead to acidification of the cell surface; causing drug resistance by decreasing the passive transport of basic drugs and increases the metastatic migration of tumor cells (Fu and Lee 2006). CAIX also contributes to cell-to-cell adhesion through the proteoglycan (PG) domain.

High expression of CAIX reduces the extracellular pH, thus favoring growth of cancerous cells within the tumor microenvironment. Over-expression of CAIX can also result in decreased cell adhesion via competition with E-cadherin to bind β-catenin, as well as affecting cell growth, cell adhesion and cytoskeletal organization. It is therefore not surprising that several studies have connected CAIX activity to cancer and have begun evaluating the diagnostic and/or treatment potentials of this enzyme (Brennan et al. 2006; Atkins et al. 2004). Several antibodies against the PG domain and active site of CAIX have been developed; such as the murine monoclonal, VII/20, which is currently in clinical trial for cancer treatment (Gieling and Williams 2012). Neutralization of CAIX enzymatic activity has been clearly linked to the survival of malignant cells (Zatovicova et al. 2010).

Nanobodies consist of about 120 amino acids and are the smallest natural antigen-binding fragments identified to date. In addition to their high affinity, nanobodies or VHHs, are more soluble and stable than other antibody fragments in extreme conditions such as high temperature and pH. VHHs also have better tissue penetration and are less immunogenic (Ebrahimizadeh et al. 2013). Due to the extended CDR3 section, nanobodies are able to identify epitopes not recognized by other antibodies (Ebrahimizadeh et al. 2013). Importantly, they are also cleared rapidly, even in conjugated forms, from the circulation (Brekke and Loset 2003) making VHHs an ideal tool for imaging and treatment of some tumors. Thus, we have developed and characterized a VHH nanobody against the CA domain of CAIX using phage display technology.

Materials and methods

Production and purification of the antigen

A 615 bp of human CA domain of *CA9* gene was optimized based on *Escherichia coli* codon usage, synthesized by Shinegene Co. (China) and then subcloned into the pET32a expression vector. The recombinant plasmid was transferred into *E. coli* BL21. The transformed bacteria were grown in LB medium (1 % tryptone, 1 % yeast extract, 1 % NaCl) containing 80 mg ampicillin l^{-1}. Expression was induced by 1 mM IPTG and the recombinant CAIX (rCAIX) analyzed on 12 % SDS-PAGE. The rCAIX was purified using Ni-NTA chromatography and confirmed by western blotting.

Immunization of camel

A two-year old male camel (*Camelus dromedarius*) was immunized by four subcutaneous injections at 14-day intervals. In the first injection, 1 mg rCAIX was mixed with an equal volume of complete Freund's adjuvant. Subsequent booster injections were administered with an equal volume of Freund's incomplete adjuvant with the protein content increased by 0.5 mg at each injection step. Blood samples were collected from the jugular vein prior to each injection and the sera were used to monitor the immunization process by indirect ELISA. All animal studies were conducted under a veterinarian supervision according to the university guidelines.

Lymphocyte isolation, RNA extraction, and cDNA synthesis

Blood was collected 1 week after the last injection, and 2 g EDTA l^{-1} was added as an anticoagulant. Lymphocytes were isolated using Ficoll (Sigma-Aldrich) density gradient. Total RNA was extracted from 10^7 lymphocytes using Pure RNA Isolation Kit (Roche, USA) according to the manufacturer's manual, and used as a template for cDNA synthesis via Revert Aid First-Strand cDNA Synthesis Kit, (Fermentase, Litvonia) and oligo-dT primers.

Library construction

Nested PCR was used to isolate VHH fragments from camel heavy-chain antibodies. The fragments beTween CH2 and framework 1 of all conventional and heavy-chain antibodies were amplified in the first PCR. Fragments corresponding to the heavy-chain antibodies were gel purified (Bioneer gel extraction kit) and used as the template in the second PCR where VHH fragments were amplified and *sfi*I restriction site was inserted in both 5′- and 3′-ends of the gene. The

second PCR primers contained degenerated nucleotides to ensure amplification of all types of heavy-chain antibody variable regions. The amplicon was digested with *sfi*I and ligated into similarly digested pComb3X phagemid. The constructs were electroporated into *E. coli* TG1 cells (Strategen, USA). To determine the library size, 10 µl transformed bacteria was cultured on LB agar containing 80 mg ampicillin l^{-1}. Several colonies were randomly selected and analyzed for presence of the VHH gene by PCR and digestion on recombinant phagemids. The PCR thermocycling conditions and primer sequences used throughout were as described in a previous study (Ebrahimizadeh et al. 2012).

Packaging of VHH expressing phage particles

Transformed bacteria were cultured in 30 ml of super broth (SB) medium (3 % tryptone, 2 % yeast extract, 1 % MOPS) containing 80 mg ampicillin l^{-1} and incubated at 37 °C/250 rpm. When the OD_{600} reached 0.5, 10^9 M13K07 helper phages were added and the culture was incubated (stationary/37 °C/30 min) to allow phage infection. The culture was incubated for another 30 min (37 °C/250 rpm) for expression of antibiotic-resistant genes. The culture volume was then increased to 200 ml by adding sterile SB medium with 70 mg kanamycin l^{-1} and 80 mg ampicillin l^{-1} and the culture incubated overnight (37 °C/250 rpm). Released phage particles were isolated by centrifuging at 5,000×*g* for 20 min. The supernatant was mixed with 20 % (v/v) PEG solution (20 % polyethylene glycol 6000 and 2.5 M NaCl) before being incubated on ice for 2 h. VHH-expressing phage particles were sedimented at 12,000×*g* for 20 min and the phage pellet dissolved in 2 ml PBS containing 1 % BSA (w/v). The solution was then centrifuged (12,000×*g*/5 min) to remove any bacterial debris and the supernatant stored at 4 °C.

Screening of phage antibodies by panning

Four wells of a 96 microplate were coated with 10 µg purified rCAIX in 100 µl carbonate/bicarbonate buffer and incubated overnight at 4° C. Unbound antigens were removed by washing with 200 µl PBS-T [PBS with 0.05 % Tween 20] and unbound sites were blocked with 200 µl of 5 % (w/v) BSA in PBS (45 min/37° C). The phage library was added, and the plate incubated for 2 h at 37 °C with mild agitation. The wells were then washed several times [TBS buffer with 0.1 % Tween 20] before bound phage were eluted under acidic condition (0.1 M HCl/glycine, pH 2.2) and neutralized immediately with basic buffer (1 M Tris base, pH 9). The obtained phages were used to inoculate *E. coli* TG1 at mid-growth phase in SB medium. Phages were propagated as described under Packaging of VHH-linked phage particles. To isolate high affinity phages, after each round of panning, Tween 20 (in TBS-T) concentration and washing cycles were increased. After each round of panning, prior to addition of the helper phage, 10 µl bacterial culture was plated on LB agar and the clones were tested for presence of VHH gene by PCR. Panning was monitored by polyclonal phage-ELISA using pure rCAIX and the phage particles obtained from each panning round. ELISA procedure was similar to that of panning except for using anti-M13 HRP-conjugated antibody (Amersham) instead of elution step. After washing, 100 µl TMB (Sigma) was added as substrate, and the reaction was stopped after 15 min by adding 1.5 M H_2SO_4. The absorbance was measured at 450 nm.

Monoclonal phage ELISA and expression of soluble VHH

Seventy random colonies from panning rounds two and three were used for phage propagation as described under Packaging of VHH-linked phage particles. The phages obtained were tested for binding affinity to CAIX by monoclonal phage-ELISA as described under Screening of phage antibodies by panning. The clone with the highest affinity was selected for expression of VHH in the soluble form. For soluble expression of VHH, the phagemid was extracted and transferred into *E. coli* TOP10, which recognizes the amber stop codon in between VHH gene and pIII coat protein gene. Expression was induced by 1 mM IPTG. To increase the nanobody expression, VHH gene was subcloned in pET28a using specific primers containing *Eco*RI and *Hin*dIII restriction sites. Recombinant vector was transferred into *E. coli* BL21 (DE3). The expression was analyzed by SDS-PAGE and western blotting. VHH gene was sequenced, and the result was blasted in NCBI database.

Affinity measurement of the nanobody

After purification of the nanobody by Ni-NTA chromatography, the nanobody was refolded by sequential dialysis in descending concentrations of urea in PBS. Both CAIX and VHH were used at 20, 10, 5, 2.5, 1.25 and 0.625 µg in ELISA and affinity was determined as described previously (Ebrahimi-zadeh et al. 2012).

Anti-CAIX nanobody efficiency in cell-ELISA

HeLa and PC3 cells as positive and negative controls respectively, were cultured at 4×10^4 per well in a 96-well ELISA plate and incubated in a CO_2 incubator at 37 °C for 24 h. Acetone and methanol (1:1 v/v) was prepared and 100 µl was added to each well to fix the cultured cells, and the plate was incubated at −20 °C for 15 min. The wells were blocked with 2 % (v/v) skimmed milk in PBS. The wells were washed with PBS-T and 5, 10 and 20 µg anti-CAIX VHH were added in separate wells. The plate was then incubated at 37 °C for 2 h. The wells were washed and anti-His tag HRP-conjugated antibody (Qiagen) was added to each well. The wells were washed again and 100 µl TMB substrate was added and the plate was incubated for 15 min. The reaction was stopped by addition of 100 µl 1.5 M H_2SO_4 per well, and the absorbance was read at 450 nm.

Statistical analysis

All the experiments were conducted in triplicate. The data is expressed as mean ± standard deviation (SD). *P* values were calculated by Student's *t* test; with *P* values of <0.05 considered significant.

Results

Preparation and purification of antigen

To facilitate cloning, *Eco*RI and *Xho*I restriction sites were inserted at 5′- and 3′-ends of the CAIX sequence respectively (Supplementary Fig. 1). The expressed antigen was purified by Ni-NTA chromatography, confirmed by SDS-PAGE and western blotting (Supplementary Fig. 2).

Camel immunization

Serial dilutions of camel serum showed high titers of anti CAIX antibody after the final booster injection as compared to that of the un-immunized sera by indirect ELISA (Supplementary Fig. 3).

Library construction

The first PCR resulted in 620 and 690 bp bands associated with camel heavy-chain antibodies and a 900 bp band related to conventional camel antibodies. The fragments of heavy-chain antibodies were isolated and purified from the agarose gel. The second PCR resulted in a single 400 bp VHH band. After ligating the VHH fragment to the phagemid, several colonies of the library were randomly isolated and analyzed by PCR. A 400 bp band in the PCR product validated the reaction (Fig. 1). The library size was estimated about 7.8×10^8.

Phage ELISA

Polyclonal phage-ELISA showed maximum absorbance in the second round of panning (Fig. 2). Following PCR confirmation and phage propagation,

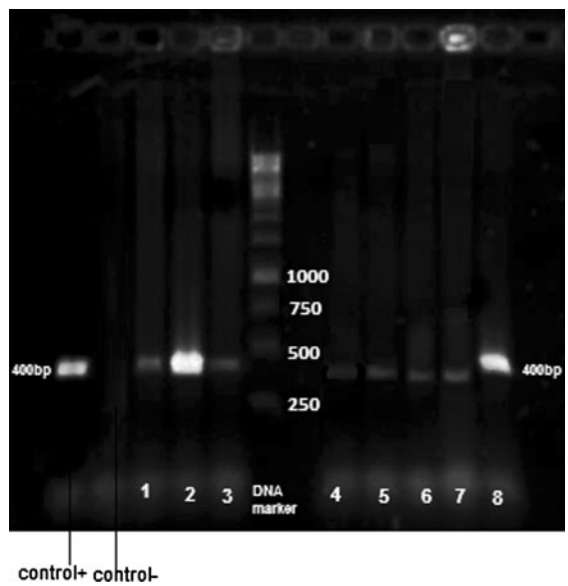

Fig. 1 PCR analysis of random clones selected from the phage-library. To confirm the cloning process and library construction, several colonies were randomly selected and analyzed by colony PCR. The 400 bp bands confirm the cloning of the VHH genes

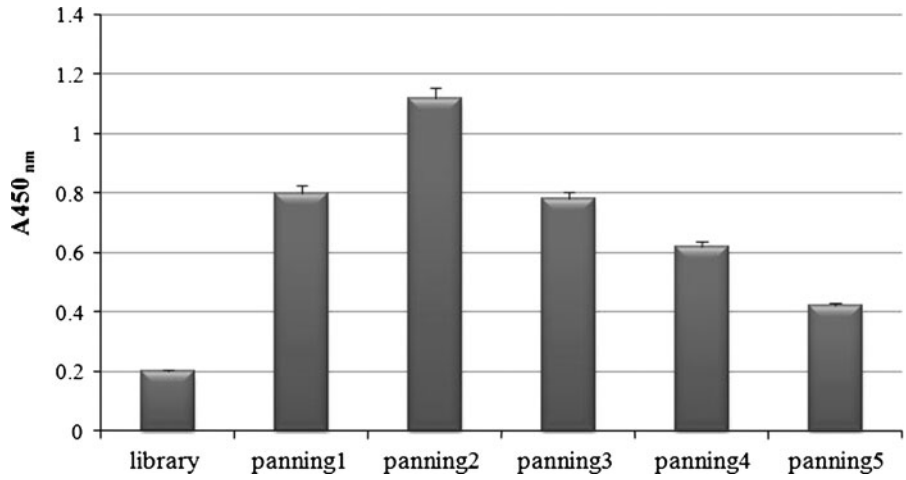

Fig. 2 Polyclonal phage-ELISA of 5 rounds of panning. The round 2 showed the highest immunoreactivity

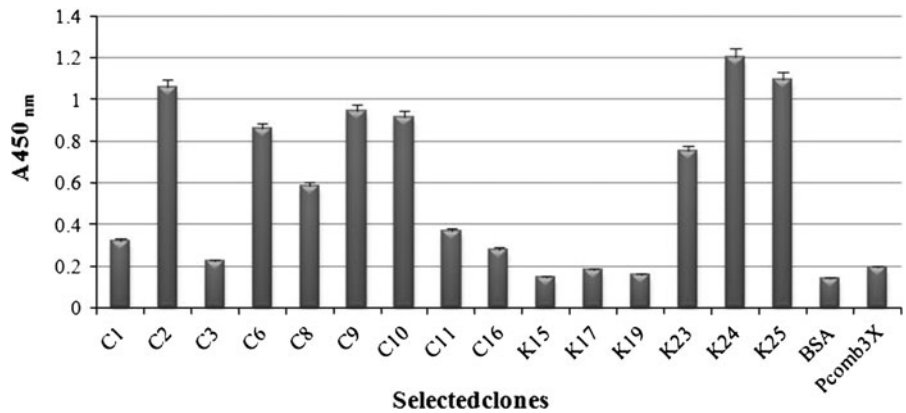

Fig. 3 Monoclonal phage ELISA. To isolate the highest affinity binding nanobody, 15 clones from rounds 2 and 3 of panning were tested by ELISA. Clone K24 exhibited the highest absorption

70 clones from 2nd and 3th rounds of panning were compared in monoclonal phage ELISA; of which 15 showed high absorbance. The colonies were compared again in the second ELISA and the clone exhibiting the maximum absorption (K24) was selected for further analysis (Fig. 3).

K24 evaluation

K24 phagemid confirmed by *Sfi*I digestion led to a 400 bp band representing VHH gene. To confirm isolation of VHH, the PCR product of the K24 clone was sequenced; with a subsequent blast analysis (NCBI nucleotide database) revealing a greater than 90 % similarity with the *Camelus dromedarius* IgVH.

Expression and purification of K24 VHH

Expression of K24 VHH was analyzed under different temperatures and IPTG concentrations. Optimum expression levels were attained at 28 °C and 1 mM IPTG concentration; as confirmed by SDS-PAGE and western blotting (Supplementary Fig. 4).

Affinity analysis

VHH was purified by Ni-NTA chromatography. The nanobody was refolded by sequential dialysis in PBS containing descending urea concentrations. Both refolded and unfolded nanobodies tested against CAIX in ELISA, with no reaction found in unfolded nanobody. Serial dilutions of refolded VHH, however, showed significant ($P < 0.05$) immunoreaction compared to controls (Fig. 4). Affinity was calculated to be approx. 2.3×10^{-5} M. Significant differences ($P < 0.05$) were observed between optical absorbance of HeLa cells and the controls. K24 nanobody could efficiently recognize and attached to the HeLa cells expressing CAIX whereas PC3 cells showed no immunoreaction (Fig. 5).

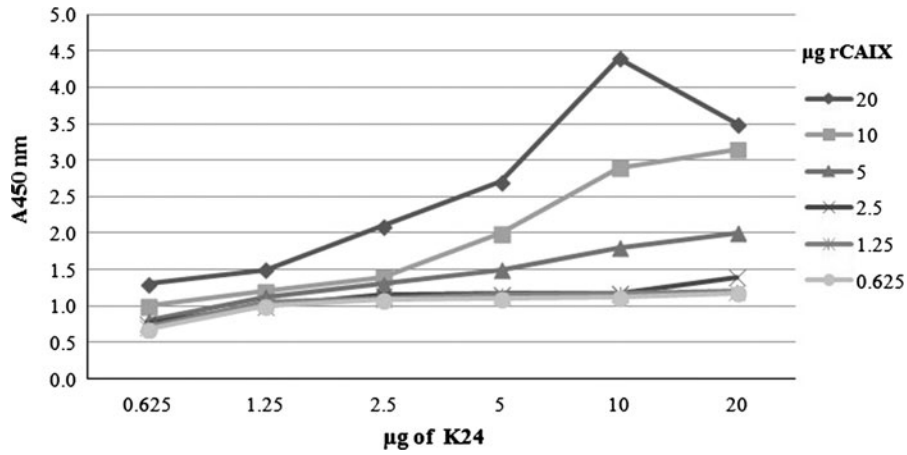

Fig. 4 ELISA immunoreactivity of VHH with CAIX. Different amounts of VHH and CAIX was used in an ELISA to obtain the VHH affinity and optimum reactive amount. The optimum results was obtained with 10 µg K24 nanobody and 10 µg rCAIX

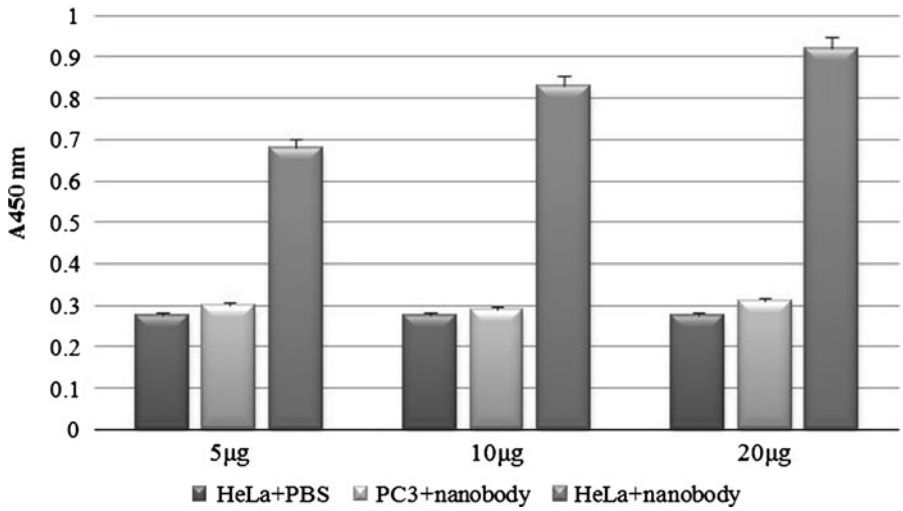

Fig. 5 Efficiency of K24 nanobody in recognition of CAIX tested against PC3 and HeLa cell lines. K24 nanobody could efficiently recognize and attached to the HeLa cells expressing CAIX whereas PC3 cells showed no immunoreactions

Discussion

CAIX has features of a tissue cancer marker such as availability at the cell surface and high expression in common tumors and carcinomas resistant to the conventional treatments: while CAIX expressed in healthy tissue is not easily accessible by circulating antibodies. CAIX, being a metastatic factor involved in pH regulation and cell adhesion of tumor cells, is a suitable candidate for production of diagnostic or therapeutic antibodies.

Attempts have been made to develop imaging, diagnosis or therapeutic tools for cancer by targeting CAIX (Ahlskog et al. 2009; Dubois et al. 2009). Examples of such attempts are sulfonamides, coumarins and their derivatives which inhibit the enzymatic activity of carbonic anhydrase, and various monoclonal antibodies. A well-known antibody is M75, which attaches to the PG domain of CAIX. V10 is a mouse monoclonal antibody against CA domain. V10 and M75 are commonly used in research kits. BAY-79–4620 is a monomethylauristatin E (MMAE) conjugated monoclonal antibody resulted in successful treatment of 10 xenograft tumors, however, significant side effects resulted in termination of human trials (Trail 2013). Nevertheless, antibodies with similar

properties, such as BAY-79–4620 and RENCAREX are still under clinical trials. In this study, a VHH library was constructed from an immunized camel. The highest affinity binding clones were isolated by strengthening the washing conditions after each panning round. This is the first report of a nanobody of this kind against CA domain of CAIX. Although treatment programs using antibodies against the extracellular domains of the enzyme are routinely practiced, the process could be restricted due to protease cleavage of these domains and their shedding into the blood circulation. Though this feature could somehow reduce the therapeutic rate of this approach, however, the diagnostic application by detection of the released CA domains in the sera remains as an advantage.

Under normal conditions, CAIX expression is low but under hypoxia and compact arrangements of cells, the expression is highly increased (Koukourakis et al. 2001). CAIX shedding under hypoxic condition is increased in blood and urine, which could be monitored for diagnosis or treatment of cancerous patients. In this research, the produced K24 nanobody could detect CAIX antigen expressed on HeLa cells. In addition, the cells cultured in minimum compactness of 4×10^4 monolayer cells per well as compared to 5×10^4 (Shvarts et al. 2006) and 10^6 (Dubois et al. 2007; Svastova et al. 2004). CAIX was not detected by western blotting using M75 antibody on normoxic, more intensive cultivated HeLa cells (Dubois et al. 2007), nor a reaction was reported by VII/20 (Zatovicova et al. 2010).

In conclusion, we have isolated a specific VHH nanobody able to recognize the CA domain of the CAIX enzyme. The recognition by K24 nanobody of CAIX on the HeLa cell lines with high selectivity and specificity could be applied in further investigations aimed at diagnosis or treatment of cancerous tissues. The low molecular weight of the nanobody allows better tissue penetration and could be more efficient in the cancer treatment application compared to other antibody fragments. Because the nanobody was generated against the CA domain, and this domain is released in blood, the K24 nanobody can be used for detection of this domain and monitoring the course of treatment in patients suffering from cancer. The similarity of the VHH nanobodies to human VH reduces immunogenicity and enhances the specificity of K24 nanobody in detection of CAIX even in monolayer form leading to increased possibility of therapeutic application of this nanobody.

Acknowledgments The authors wish to thank Shahed University and Biotechnology Development Council of I. R. Iran for their financial support. We also extend our sincere thanks to Prof. Heather MA Cavanagh of Charles Sturt University, Australia for her kind assistance in English editing and polishing of the final version of this paper.

References

Ahlskog J, Schliemann C, Marlind J, Qureshi U, Ammar A, Pedley R, Neri D (2009) Human monoclonal antibodies targeting carbonic anhydrase IX for the molecular imaging of hypoxic regions in solid tumours. Brit J Cancer 101:645–657

Atkins M, mcdermott D, Regan M, Stanbridge E, Upton M, Youmans A, Febbo P, Lechpammer M, Signoretti S (2004) Carbonic anhydrase IX (CAIX) expression predicts for renal cell cancer (RCC) patient response and survival to IL-2 therapy. In: Proceeding American Society of Clinical Oncology, vol 14S. p 4512

Brekke O, Loset G (2003) New technologies in therapeutic antibody development. Curr Opin Pharmacol 3:544–550

Brennan DJ, Jirstrom K, Kronblad A, Millikan RC, Landberg G, Duffy MJ, Ryden L, Gallagher WM, O'Brien SL (2006) CA IX is an independent prognostic marker in premenopausal breast cancer patients with one to three positive lymph nodes and a putative marker of radiation resistance. Clin Cancer Res 12:6421–6431

Dubois L, Douma K, Supuran CT, Chiu RK, van Zandvoort MA, Pastoreková S, Scozzafava A, Wouters BG, Lambin P (2007) Imaging the hypoxia surrogate marker CA IX requires expression and catalytic activity for binding fluorescent sulfonamide inhibitors. Radiother Oncol 83:367–373

Dubois L, Lieuwes NG, Maresca A, Thiry A, Supuran CT, Scozzafava A, Wouters BG, Lambin P (2009) Imaging of CA IX with fluorescent labelled sulfonamides distinguishes hypoxic and (re)-oxygenated cells in a xenograft tumour model. Radiother Oncol 92:423–428

Ebrahimizadeh W, Mousavi Gargari S, Rajabibazl M, Safaee Ardekani L, Zare H, Bakherad H (2013) Isolation and characterization of protective anti-LPS nanobody against V. Cholerae O1 recognizing Inaba and Ogawa serotypes. Appl Microbiol Biotechnol 97:4457–4466

Fu Y, Lee AS (2006) Glucose regulated proteins in cancer progression, drug resistance and immunotherapy. Cancer Biol Ther 5:741–744

Gieling RG, Williams KJ (2012) Carbonic anhydrase IX as a target for metastatic disease. Bioorg Med Chem 21: 1470

Koukourakis MI, Giatromanolaki A, Sivridis E, Simopoulos K, Pastorek J, Wykoff CC, Gatter KC, Harris AL (2001) Hypoxia-regulated carbonic anhydrase-9 (CA9) relates to poor vascularization and resistance of squamous cell head and neck cancer to chemoradiotherapy. Clin Cancer Res 7:3399–3403

Nishimori I, Onishi S (2001) Carbonic anhydrase isozymes in the human pancreas. Dig Liver Dis 33:68–74

Shvarts O, Janzen N, Lam JS, Leppert JT, Caliliw R, Figlin RA, Belldegrun AS, Zeng G (2006) RENCA/carbonic anhydrase-IX: a murine model of a carbonic anhydrase-IX-expressing renal cell carcinoma. Urology 68:1132–1138

Svastova E, Hulíkova A, Rafajova M, Zatovicova M, Gibadulinova A, Casini A, Cecchi A, Scozzafava A, Supuran CT, Pastorek J (2004) Hypoxia activates the capacity of tumor-associated carbonic anhydrase IX to acidify extracellular pH. FEBS Lett 577:439–445

Trail PA (2013) Antibody directed delivery for treatment of cancer: antibody drug conjugates and immunotoxins. In: Antibody-drug conjugates and immunotoxins. Springer, New York, pp 3–22

Zatovicova M, Jelenska L, Hulikova A, Csaderova L, Ditte Z, Ditte P, Goliasova T, Pastorek J, Pastorekova S (2010) Carbonic anhydrase IX as an anticancer therapy target: preclinical evaluation of internalizing monoclonal antibody directed to catalytic domain. Curr Pharm Des 16:3255–3263