VILNIUS UNIVERSITY

Dovilė
STRAVINSKIENĖ

Development of monoclonal and recombinant antibodies against carbonic anhydrases

SUMMARY OF DOCTORAL DISSERTATION

Technological Sciences
Chemical Engineering **T 005**

VILNIUS 2019

This dissertation was prepared between 2012 and 2018 during the studies at Vilnius University Life Sciences Centre Institute of Biotechnology.
The research was supported by the Research Council of Lithuania.

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VILNIAUS UNIVERSITETAS

Dovilė
STRAVINSKIENĖ

Monokloninių ir rekombinantinių antikūnų prieš karboanhidrazes kūrimas

DAKTARO DISERTACIJOS SANTRAUKA

Technologijos mokslai
chemijos inžinerija **T 005**

VILNIUS 2019

Disertacija rengta 2012–2018 metais Vilniaus universiteto Gyvybės mokslų centro Biotechnologijos institute.
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Disertacija ginama viešame Gynimo tarybos posėdyje 2019 m. gruodžio mėn. 4 d. 14 val. Vilniaus universiteto Gyvybės mokslų centro R401 auditorijoje. Adresas: Saulėtekio al. 7, LT- 10257 Vilnius, Lietuva.Tel. +370 (5) 2234374; el. paštas dovile.stravinskiene@gmc.vu.lt

Disertaciją galima peržiūrėti Vilniaus universiteto bibliotekoje ir VU interneto svetainėje adresu:
https://www.vu.lt/naujienos/ivykiu-kalendorius

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LIST OF ABBREVIATIONS

aa – amino acids;

ADCC – antibody-dependent cell-mediated cytotoxicity;

CA IX – carbonic anhydrase IX;

CA IX^{+PG} – recombinant catalytic domain of CA IX with PG domain;

CA IX^{-PG} – recombinant catalytic domain of CA IX without PG domain;

CA XII – carbonic anhydrase XII;

$CA12^{DE3}$ – recombinant catalytic domain of CA XII expressed in *E. coli;*

$CA12^{HEK}$ – recombinant catalytic domain of CA XII expressed in mammalian HEK-293 cells;

CAs – carbonic anhydrases;

CDC – complement-dependent cytotoxicity;

CDR – complementarity-determining regions;

cG250 – chimeric G250 antibody;

ELISA – enzyme-linked immunosorbent assay;

Fab – antigen binding sites (fragment antigen binding);

FC – flow cytometry;

Fc – fragment crystallizable;

FR – framework region;

HAMA – human anti-mouse antibody;

HRP – horseradish peroxidase

IFA – immunofluorescence assay;

Ig – immunoglobulin;

IgG – immunoglobulin G;

IHC – immunohistochemistry;

K_d – apparent dissociation constant;

MAb – monoclonal antibody;

qRT-PCR – real-time (quantitative) reverse transcription PCR;

RAb – recombinant antibody;

scFv – single chain fragment variable;

scFv-Fc – single chain fragment variable fused with human constant fragment;

VH – variable fragment of IgG heavy chain;

VL – variable fragment of IgG light chain;

VU GMC BTI BVTS – Vilnius university, Life Sciences Center, Institute of Biotechnology, department of Biothermodynamics and Drug Design;

VU GMC BTI ILBS – Vilnius university, Life Sciences Center, Institute of Biotechnology, Department of Immunology and Cell Biology;

WB – Western blot.

INTRODUCTION

Cancer is one of the leading causes of death worldwide. The growing demand for the advanced technologies and novel biological tools for screening and treatment of cancer patients promoted intensive developments in cancer immunodiagnostics and immunotherapy. Monoclonal antibodies (MAbs) developed to recognize tumor-specific antigens are considered as core agents in many diagnostic methods to analyse biological specimens. Antibody-based immunotherapy exploits antibodies or their derivatives recombinant antibodies (RAbs) as mediators between the cancer antigen and the toxic substance, such as chemotherapy drugs or radioactive particles, or mediators activating the components of the host's immune system capable of destroying the cancerous cell.

The major challenge of antibody-based cancer diagnostics or therapy is the identification and validation of reliable tumor antigens. The current study was focused on two isozymes carbonic anhydrase IX (CA IX) and XII (CA XII), which are involved in carcinogenesis by contributing to extracellular pH regulation under hypoxia conditions, thus promoting tumor cell growth and survival. The active centers of both transmembrane CAs are directed to the outside of the cell, which is the most important feature to apply antibody-based detection or therapy. CA IX is recognized as cancer biomarker and a potential target for cancer therapy. CA IX-specific MAbs/RAbs have been developed previously and are already at the stage of clinical research in both cancer diagnostics and immunotherapy. Meanwhile, the role of CA XII in carcinogenesis has been less investigated and the results obtained are obscure, thus there is a high demand of new tools for CA XII research. The potential CA XII as a target for cancer therapy is also unclear, as there are no MAbs/RAbs involved in clinical trials.

When developing MAbs, it is important to characterize them comprehensively by various immunochemical methods in order to

investigate their properties and to evaluate the prospects for their application in research, diagnostic or therapy. Only MAbs recognizing native cancer antigens can be used for targeted therapy. The MAbs having antigen-neutralizing properties are even more valuable.

RAbs are genetically engineered MAb derivatives with reduced immunogenicity. Smaller antibody formats, such as single chain fragment variable (scFv), can be used for cancer diagnostics and therapy, because they not only maintain the same antigen binding activities, but also have better tumor penetration. Human constant fragment Fc-fusion proteins, for example scFv-Fc, can provide specificity for target antigen, as well as effector functions through the Fc fragment by activating immune effector cells.

The aim of the dissertation was to develop novel monoclonal and recombinant antibodies against cancer-associated human carbonic anhydrases CA IX and CA XII and evaluate their diagnostic and therapeutic potential.

Tasks of the dissertation:

1. To develop novel hybridoma cell lines secreting monoclonal antibodies (MAbs) against recombinant carbonic anhydrases CA IX and CA XII.

2. To characterise the MAbs by various immunochemical and biochemical assays: to investigate their affinity and neutralising activity, interaction with cellular antigens, cross-reactivity with others carbonic anhydrase (CA) isoforms and to determine MAb epitopes.

3. To evaluate the applicability of the novel MAbs for studying the expression of CA IX and CA XII in tumor cell lines grown under normoxic and hypoxic conditions.

4. To evaluate the diagnostic potential of the novel MAbs by investigating CA IX and CA XII expression in clinical samples.

5. To select the most promising MAb against CA XII and to use genetic material of the MAb-secreting hybridoma to develop recombinant antibodies with potential application in target therapy.

Scientific novelty and practical value of the study

MAbs against cancer-associated antigens have high diagnostic and therapeutic value. In the current study we have developed and characterised novel MAbs against recombinant cancer-associated enzymes - CA IX and CA XII - produced in bacterial and mammalian cells. In previous studies, the MAbs against CA IX and CA XII were developed by immunisation of mice or rats with cancer cells or their lysates and in most cases conformation-dependent MAbs were obtained. We have demonstrated that recombinant protein-derived MAbs were able to recognize native cellular CA IX and CA XII on the surface of viable cancer cells.

The comprehensively characterised MAbs were used to study the expression of CA IX and CA XII in cancer cells grown under normoxic and hypoxic conditions and new results on single-positive or double-positive cancer cell lines were obtained. These results are important for the selection of proper cell lines for studying CA-related cancer. Moreover, the MAbs were employed to investigate CA IX and CA XII expression in tissue specimens of cancer patients. We have demonstrated the diagnostic potential of the generated MAbs, as both enzymes were detected in formalin-fixed and paraffin-embedded tissue sections.

One MAb (clone 14D6) derived against CA XII was reactive with cell-surface CA XII and inhibited the enzymatic activity of recombinant CA XII, thus indicating its therapeutic potential. The genetic material of the MAb-secreting hybridoma cell line was used to determine amino acid sequences of the variable regions of antibody heavy and light chains. For the first time two CA XII-specific RAbs of different formats were generated – scFv and scFv-Fc with human Fc domain. They were able to recognize CA XII by an indirect

enzyme-linked immunosorbent assay (ELISA) and Western blot. Thus, the identified sequences of the MAb 14D6 were correct and allowed development of functional RAbs of potential therapeutic relevance. The MAb sequence and the RAbs are protected by the Lithuanian patent (No. 6331 B).

Defended propositions

1. Recombinant proteins CA IX and CA XII derived from bacterial and mammalian cells are suitable antigens for the generation of hybridoma cell lines secreting high affinity MAbs.
2. MAbs raised against recombinant CA IX and CA XII proteins specifically recognise membrane-exposed CA IX and CA XII, thus allowing investigation of these isoforms in cancer cell lines and clinical specimens.
3. Genetic material of hybridomas producing CA XII-neutralising MAbs can be applied for the generation of RAbs of potential therapeutic relevance.
4. The identified variable regions of CA XII-specific MAb heavy and light chains responsible for antigen binding allows the generation of various forms of functionally active RAbs that retain specificity for the CA XII antigen.

Doctoral thesis contents

The doctoral thesis (in Lithuanian) contains the following parts: Introduction, Literature overview, Materials and Methods, Results, Discussion, Conclusions, List of references (246 citations), List of publications (3 positions), List of patent (1 position), List of book chapters (2 positions), List of conferences attended (10 positions), Figures (61), Tables (29), Total number of page – 167.

1. LITERATURE OVERVIEW

Immunoglobulins (Ig), or antibodies, are humoral components of the adaptive immunity, produced by B lymphocytes (Shishido et al., 2012). They are unique for their versatility and specificity as they are able to recognize almost unlimited number of antigens and their epitopes (Lavande et al., 2013). The molecule of Ig consists of four polypeptide chains (two identical light chains and two identical heavy chains), which form a Y-like shape tertiary structure (Ahmad et al., 2012). There are two antigen binding sites (Fab) formed by variable regions, and one constant part (Fc), which is responsible for mediating Ig effector functions (Byrne et al., 2013). The Fc part is a ligand for receptors on immune effector cells, so the antibody binding to the antigen initiates antibody-dependent cell-mediated cytotoxicity (ADCC) or phagocyte activation, or binding to complement activates complement-dependent cytotoxicity (CDC). Moreover, antibody binding to an antigen, which is determined by three complementarity-determining regions (CDR1, CDR2, and CDR3), or hypervariable sequences, in the variable regions on both heavy and light chains, can directly inactivate the antigen by neutralization (Wang et al., 2007).

Hybridoma technology allows production of standardized antibodies recognizing practically any desired antigen (Ahmad et al., 2012). MAbs are produced by a single cell clone and have the same affinity for the antigen (Kotsovilis and Andreakos, 2014). MAbs are ideal research and diagnostic reagents for many immunochemical assays (Frenzel et al., 2013). However, mouse-derived MAbs are immunogenic and can cause human anti-mouse antibody (HAMA) responses if using for therapy. Antibodies are genetically engineered to reduce immunogenicity (Lonberg, 2005). The chimerization (the variable regions of mouse antibody are attached to human Fc fragment) and humanization (CDRs are of murine origin) of MAbs led

to the emergence of the first therapeutic antibodies for the treatment of cancer (Rituximab, Transtuzumab) (Liu et al., 2008).

Recombinant intact immunoglobulin G (IgG) molecules have long half-life, so they can accumulate at high concentrations in target tumor, despite their weak penetration, and are also capable of mediating ADCC with human effector cells or activating CDC. Small antibody formats, like scFv, which are lacking antibody effector functions, are more rapidly cleared from blood and their tumor uptake is still under discussion, however they can be considered as good immunotoxins when conjugated with drugs or radionuclides. Fc-fusion proteins, for example scFv-Fc, may provide longer half-life of RAbs, as well as effector functions through the interaction of Fc and Fc-gamma receptors on effector cells (Bustamante-Córdova et al., 2018).

The use of MAbs/RAbs for cancer diagnostic and immunotherapy is based on selecting the appropriate tumor-associated antigens - proteins that are necessary for tumor development and survival (Petricevic et al., 2013). Cell surface antigens are easily accessible for antibodies, so they are suitable for antibody-based immunotherapy. Another mandatory characteristics of the selected biomarker is its homogeneous and high expression on cancer cells as well as its absence on normal tissues (Scott et al., 2012).

CA IX and CA XII are considered as new anti-cancer targets for antibody-based diagnostics and immunotherapy as they fulfil the most requirements of a suitable biomarker. They both are transmembrane isozymes of CA family with an extracellular active site that catalyse the reversible hydration of carbon dioxide to bicarbonate (Chien et al., 2012; Nordfors et al., 2010). CA IX and CA XII contribute to the adaptation of malignant cells to hypoxia through regulation of intracellular and extracellular pH. High CA IX expression is found in kidney, lung, colon, breast, cervix, ovaries, brain and few other tumors, CA XII is overexpressed in renal, gastric, colorectal, breast and brain tumors as well (Carradori et al., 2013; Lounnas et al., 2013).

Many attempts are made to produce highly specific diagnostic and therapeutic agents for the detection or targeted therapy of CA IX and CA XII-positive cancer cells. Different classes of CA IX and CA XII inhibitors, for example, small-molecule compounds or MAbs/RAbs have been developed and their pharmacological evaluation is a key priority at the moment (Kazokaitė et al., 2017; Thiry et al., 2006). The selectivity and specificity are the major problems of small-molecule inhibitors. MAbs have a highly specific selectivity for target antigens, therefore they have a great biological potential for cancer therapy (Imai and Takaoka, 2006).

CA IX-specific MAb M75 is broadly used for the detection of CA IX by different immunochemical methods, mainly by immunohistochemistry (IHC) (Lau et al., 2017). MAb G250 targeting the CA IX was used for renal cell carcinoma imaging *in vivo*, however its murine format led to the emergence of HAMA (Divgi et al., 1998; Oosterwijk et al., 1986). A chimeric variant of G250 (cG250) was used in numerous clinical trials, for both cancer imaging and therapeutic applications, however more trials are needed to confirm the usefulness of this antibody. As cG250 does not inhibit the enzymatic activity of CA IX and initiates killing CA IX-positive cells through ADCC, new antibodies were produced using phage-display technology (Oosterwijk-Wakka et al., 2013).

Targeting CA XII is as important as targeting CA IX. It was shown that *CA9* knock-down decreases tumor growth and cancer cell number, however *CA12* mRNA level is up-regulated. The combined silencing of both izoforms gives two times lower volume of the xenograft tumor and increases cell death (Chiche et al., 2009; Doyen et al., 2012). Recently, rat MAbs inhibiting CA XII were developed and are extensively investigated for antitumor activity (Battke et al., 2011; Gondi et al., 2013). Rat MAbs 6A10 interfere with tumor cell growth *in vitro* and *in vivo* animal studies. Genetically engineered Fab of this MAb was labelled with ^{177}Lu or ^{64}Cu with intention to be used as an agent for radioimmunotherapy or positron emission tomography and

showed specific binding to CA XII on tumor cells in mice xenografts (Fiedler et al., 2018a, 2018b). However, more investigations are needed to apply CA XII-specific antibodies for clinical studies.

2. MATERIALS AND METHODS

Materials. Unless otherwise stated, all reagents were purchased from Carl Roth (Germany), Merck (Sigma-Aldrich, Millipore) Group (Germany, USA) or Thermo Fisher Scientific (USA, UK, Lithuania). Primers and probes were purchased from Metabion (Germany).

Cell lines and bacterial strains used for MAb/RAb generation and characterisation were deposited in Vilnius university, Life Sciences Center, Institute of Biotechnology, Department of Immunology and Cell Biology (VU GMC BTI ILBS) (Vilnius, Lithuania).

Recombinant Carbonic Anhydrases. Recombinant proteins, corresponding to the catalytic domain of CA IX with or without the PG domain (CA IX^{+PG} (amino acids (aa) 38 – 414) and CA IX^{-PG} (aa 138 – 390), respectively) and recombinant N-terminal catalytic domain (aa 30 – 291) of CA XII expressed in *E. coli* ($CA12^{DE3}$) or in mammalian HEK-293 cells ($CA12^{HEK}$) were used as antigens for immunization and characterization of MAbs.

Recombinant antigens for MAb cross-reactivity analysis (CA I, II, IV, VB, VI, VII, XIII, and XIV) were described previously (Dekaminaviciute et al., 2014b, 2014a; Dudutienė et al., 2014; Jogaitė et al., 2012; Linkuvienė et al., 2016; Stravinskiene et al., 2019). Proteins were obtained from the VU GMC BTI department of Biothermodynamics and Drug Design (BVTS).

Mice were maintained at the breeding colony of the Centre for Innovative Medicine (Vilnius, Lithuania) or vivarium of VU GMC Institute of Biochemistry, Department of Biological Models under standard animal housing conditions. Animal housing, care, and experimental protocols were performed by certified staff in accordance with FELASA guidelines and conformed to Lithuanian and European legislation (License No. LT-59-902, Permission No. 184 for breeding of mice, and Permission No. 209 for generation of polyclonal and monoclonal antibodies).

Generation of MAbs. MAbs were generated as described previously (Köhler and Milstein, 1975). BALB/c mice were immunized 3 times every 28-30 days with 50 µg of antigen. Hybridoma screening was performed using standard indirect ELISA with immobilized antigen or, additionally, clones were selected with a robotic clone imaging and picking module ClonePix2 (Molecular Devices, USA).

Characterization of MAbs was performed by various types of ELISA. An indirect ELISA was used for the analysis of blood samples of the immunized mice, selection of hybridomas secreting antigen-specific MAbs, and determination of MAb specificity and affinity. Recombinant proteins were used as antigens for coating diluted to a concentration of 5 µg/mL. A direct ELISA was used for testing the activity of MAb-HRP (horseradish peroxidase) conjugates. Competitive ELISA was used to determine if the MAbs compete for a binding site on antigen. Sandwich (two epitope) ELISA was used to detect soluble CA IX by using two MAbs directed to different epitopes of CA IX. Epitope mapping was performed with overlapping biotinylated peptides by an indirect ELISA or recombinant protein fragments by Western blot (WB). WB was also applied to show MAb binding to linear epitopes or to analyse recombinant proteins or cell lysates. For WB analysis, 1 µg of recombinant proteins or 30 µg of cell lysates per lane were separated by electrophoresis using 12% concentrating SDS polyacrylamide gels.

Determination of the inhibitory activity of the MAbs was investigated by a stopped flow CO_2 hydration assay at the Department of Chemistry, University of Florence, Florence, Italy (dr. D. Vullo, prof. C. T. Supuran) or at the VU GMC BTI BVTS (dr. L. Baranauskienė).

Analysis of biological samples. Flow cytometry (FC) was applied to determine the interaction of the MAbs with native CA IX or CA XII located on the surface of live cells (1×10^6 cells per test). Human tumor cell lines representing various cancer types (lung (A549), brain (U-

89), renal (A498), hematopoietic cells (Jurkat, K-562, HUT, H9, CEM), cervical (HeLa, CaSki), skin (A431) breast (MCF-7, MDA-MB-231) colon (COLO, HCT), and liver (Huh 7)) were grown either in normoxic (at 37 °C, 5% CO_2, and 21% O_2) or hypoxic (at 37 °C, 1% O_2, 5% CO_2, 94% N_2) conditions and analysed by FC and optimised real-time (quantitative) reverse transcription PCR (qRT-PCR) to evaluate the applicability of the newly generated MAbs for studying CA IX and CA XII expression. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), beta-actin (*ACTB*) and TATA-binding protein (*TBP*) coding genes were used as a reference genes. In order to optimize the qRT-PCR assay and to quantify *CA9* and *CA12* mRNA expression level, RNA transcripts were generated and used as standards for the calibration curves.

The MAbs were also tested for their ability to isolate native CA IX from cell lysates by immunoprecipitation. All MAbs were tested in immunofluorescence (IFA) microscopy using cancer cell lines.

MAbs against CA IX were examined for their ability to detect CA IX expression in cervical carcinoma *in situ* specimens by immunohistochemical technique. Formalin-fixed and paraffin-embedded tissue sections were immunostained according to the protocol previously developed for the detection of CA IX with anti-CA IX antibodies (clone EP161, Cell Marque, USA) at the National Center of Pathology (Vilnius, Lithuania). IHC staining using CA XII-specific MAbs was performed on formalin-fixed and paraffin-embedded samples of colon adenoma, colon carcinoma, renal carcinoma and normal colon, and kidney tissues at the Institute of Biomedical Technology, University of Tampere (Tampere, Finland) in collaboration with prof. S. Parkkila group. IHC staining of breast, lung and kidney tumours was performed at the National Center of Pathology (Vilnius).

3. RESULTS AND DISCUSSION

3.1. Generation, characterization and application of MAbs against CA IX

Recombinant proteins CA IX^{+PG} and CA IX^{-PG} were used as antigens for immunization. They were produced in HEK-293 cells expecting high similarity to the native CA IX in terms of post-translational modifications (Thomas and Smart, 2005). Antigens were immunogenic, however only a moderate-titered antibody response in the immunized BALB/c mice was induced. Thus, after the fusion of mouse spleen cells with Sp2/0 cells the screening of IgG-producing hybrid clones was performed with a robotic clone imaging and picking module Clone Pix2 (Molecular Devices, USA) to increase screening efficiency. Further steps involved screening of clones by an indirect ELISA to select CA IX-specific clones and generation of stable monoclonal hybridoma cell lines by limiting dilution. Thirteen hybridoma clones secreting CA IX specific-MAbs were selected and the MAbs were subjected to a subsequent characterization by different immunoassays. Eight of them were raised against recombinant CA IX^{-PG} protein, indicating their specificity to the catalytic domain of CA IX and 5 MAbs were generated against recombinant CA IX^{+PG} protein.

An indirect ELISA was used in the first stage of MAb characterization to determine the specificity and affinity of MAbs to their respective antigens as well as investigate their possible cross-reactivities with other CA isoforms. All selected MAbs were of IgG1 subclass, showed high affinity to CA IX (Table 1) and did not react with other CAs (CA I, II, III, IV, VB, VI, VII, XII, XIII, and XIV).

From all our MAb collection only the MAb H7 recognized the denatured CA IX, in particular, CA IX^{+PG} protein, which was determined by WB (Table 1). The linear epitope of the MAb H7 is localized in the PG domain as no reaction was observed with CA IX^{-PG} (Figure 1, A). The exact MAb H7 epitope was determined by an

indirect ELISA using overlapping synthetic biotin-conjugated peptides covering 38–112 aa sequence of the PG domain and was identified as 12 aa-long sequence 55-GEDDPLGEEDLP-66.

In the next step the MAbs were employed for the detection of cellular CA IX by various immunochemical assays. Most of the MAbs (8 out of 13) were reactive with native CA IX on the surface of human lung carcinoma cells (A549) grown under hypoxic conditions as determined by FC (Table 1, Figure 1, D). We have also shown the applicability of the MAbs in IFA after staining human cervical adenocarcinoma cells (HeLa) grown in hypoxia, demonstrating expression of membrane-bound and cytoplasmic CA IX (Figure 1, C). Our study demonstrated that MAb H7 has unique features and exceptionally broad application possibilities. No other clone from the newly generated MAb collection was reactive with CA IX in formalin-fixed and paraffin-embedded cervical carcinoma *in situ* tissue sections by IHC (Figure 1, B).

Table 1. Summarized characteristics of the MAbs generated against CA IX.

MAb clone	**Apparent K_d value, *M***	**MAb reactivity with CA IX variants**					
		CA IX^{-PG}		**CA IX^{+PG}**		**FC**	**IFA**
		ELISA	**WB**	**ELISA**	**WB**		
A3	1.3×10^{-10}	+	–	+	–	+	+
F12	5.1×10^{-10}	+	–	+	–	+	+
F8	3.3×10^{-10}	+	–	+	–	+	+
F7	1.4×10^{-10}	+	–	+	–	+	+
F4	6.5×10^{-10}	+	–	+	–	+	+
D8	6.2×10^{-10}	+	–	+	–	+	+
C9	2.5×10^{-10}	+	–	+	–	+	+
G8	2.1×10^{-10}	+	–	+	–	+	±
H7	8.9×10^{-10}	–	–	+	+	+	+
E3	1.8×10^{-10}	±	–	+	–	–	±
A10	1.8×10^{-10}	±	–	+	–	–	+
H11	1.7×10^{-10}	±	–	+	–	–	±
D3	2.2×10^{-10}	±	–	+	–	–	±

K_d – apparent dissociation constant "+" strong reaction; "±" moderate/weak reaction; "–" no reaction.

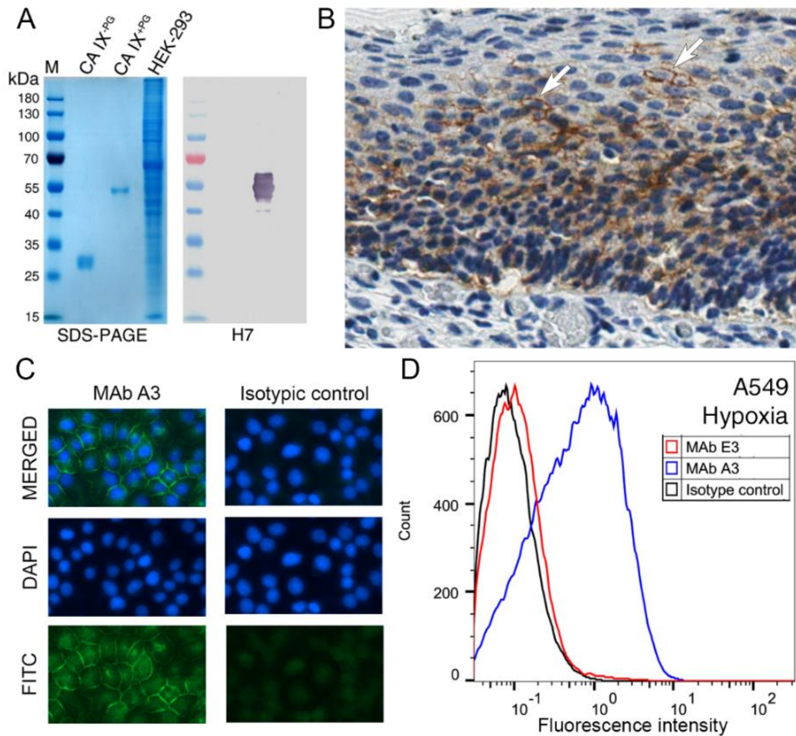

Fig.1. (A) The reactivity of the MAb H7 with denatured recombinant CA IX^{-PG} and CA IX^{+PG} proteins. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (left) and WB pictures are shown. Line M, pre-stained MW marker (Thermo Fisher Scientific); purified recombinant CA IX^{-PG} and CA IX^{+PG} were fractionized; lysate of HEK-293 cells were used as a negative control. (B) IHC detection of CA IX in the tissue section of cervical carcinoma *in situ* using MAb H7. Staining of the cellular membranes is evident in several regions (shown by white arrows). Magnification x 40. (C) Visualization of CA IX expressed in HeLa cells grown under hypoxia by IFA using MAb A3 and secondary Alexa Fluor 488 antibody. Fluorescence microscopy images were taken at x 20 magnification. (D) FC fluorescence histograms of A549 cells grown in hypoxia and stained with anti-CA IX MAbs to determine interaction of MAbs with native CA IX protein. Positive MAb A3 was compared with negative clone E3 and isotype control (irrelevant IgG1 subclass in-house MAb).

The MAb H7 was suitable to immunoprecipitate CA IX protein from cell lysates, which allowed to observe changes in protein expression level due to hypoxia effect. The MAb H7 was also selected as a detection antibody in a pair with the capture MAb A3 to develop sandwich ELISA for the detection of soluble CA IX shedded of the tumor cells by metalloproteases (Zatovicova et al., 2005).

The developed sandwich ELISA was applied to detect CA IX in the blood plasma specimens of 95 individuals with or without cervical pathology. Overall, 40% of specimens were found to be CA IX-positive (data not shown). These results demonstrate the reactivity of the MAbs with native soluble CA IX present in human blood and the potential of the newly developed ELISA to detect CA IX in biological samples.

In conclusion, a new collection of CA IX-specific MAbs was raised against the catalytic domain of CA IX with or without the PG domain. The MAbs were characterized by different immunoassays and their selectivity for CA IX was demonstrated. The MAb of clone H7 that recognizes a linear epitope within the PG domain was extensively investigated for its applicability to detect CA IX by various antibody-based techniques including FC, immunoprecipitation, IHC, IFA, sandwich ELISA, WB. No restrictions in terms of its use to detect the native, denatured, soluble or cell-bound CA IX were identified, which confirms its potential for diagnostics, research and possibly future therapeutic applications.

3.2. Generation, characterization and application of MAbs against CA XII

In order to generate hybridomas, BALB/c mice were immunized with recombinant either non-glycosylated $CA12^{DE3}$ or glycosylated $CA12^{HEK}$ proteins. In addition, synthetic peptide (CA12-KLH) spanning surface-exposed sequence (aa 167-180) located in a close proximity of the catalytic center of CA XII was selected by bioinformatic analysis, conjugated to KLH carrier protein and used as antigen. The immunization with antigens was successful and resulted in 33 stable hybridoma cell lines producing CA XII-specific MAbs that differed in their reactivities with CA XII in various immunochemical methods.

$CA12^{DE3}$-derived MAb 15A4 showed no cross-reactivities with other CA isoforms and was strongly reactive with both $CA12^{DE3}$ and $CA12^{HEK}$ by ELISA and WB. The ability of the MAb 15A4 to recognize cellular CA XII was confirmed by FC as this MAb showed strong specific immunostaining of fixed and permeabilized A-498, U-87, A-549, CaSki and HeLa cells expressing CA XII. Moreover, the MAb 15A4 recognized cellular CA XII protein by IHC on formalin-fixed and paraffin-embedded specimens of renal carcinoma, colon adenoma, colon carcinoma as well as normal kidney and colon tissues. These data demonstrate the potential of the MAb 15A4 as a highly specific reagent for studying CA XII expression in tumor and normal tissues.

One of our purpose was to generate inhibitory MAbs against CA XII, thus a synthetic peptide CA12-KLH was used as an immunogen, afterwards two hybridoma cell lines producing high-affinity CA XII-specific MAbs of IgG1 subtype were generated. The MAbs recognized both $CA12^{DE3}$ and $CA12^{HEK}$ recombinant proteins and did not cross-react with other CA isoforms. Moreover, one of the MAbs (clone 3D8) was capable of recognizing cellular CA XII in formalin-fixed and paraffin-embedded specimens of human tumour tissues, which

demonstrates the potential of this MAb as a specific diagnostic reagent. Inhibitory test by the stopped flow CO_2 hydration assay has revealed that both MAbs 1D8 and 3C8 are highly inhibitory against CA XII. Inhibitory activity of the MAb 3C8 (6.6 nM) is comparable to that previously reported for the MAb 6A10 (3.1 nM), raised against cellular CA XII (Battke et al., 2011). These MAbs are a successful example of immunization strategy with synthetic peptide to direct the humoral immune response towards target epitope of selected protein.

Despite low immunogenicity, the largest collection of 24 CA XII-specific MAbs was obtained by immunizing mice with $CA12^{HEK}$ recombinant protein. MAbs were characterized using ELISA, WB and FC assays. Most of the MAbs (18 out of 24) were reactive with native cellular CA XII on the surface of live A549 and human renal cell carcinoma (A498) cells determined by FC (Figure 2, A). MAb 14D6 distinguished from this group since it was the only MAb exhibiting high inhibition of enzymatic activity of recombinant CA XII protein, which was investigated by a stopped flow CO_2 hydration assay. The calculated inhibition constant (~42 nM) was close to the inhibition constant of the classic CA inhibitor acetazolamide (AZM) (~37.5 nM) (Figure 2, B).

The inhibitory effect of MAb 14D6 can be explained by the epitope localization of this MAb. The WB analysis of lysates of *E. coli* expressing overlapping His-fusion CA XII fragments revealed the 8 aa-long linear sequence 35-FGPDGENS-42 of CA XII recognized by the MAb 14D6. Computer model of CA XII catalytic domain based on PDB entry 1JD0 was developed and the MAb 14D6 epitope was localized in a close proximity of the catalytic center (Figure 2, C). According to Whittington and his colleagues, this surface-exposed sequence is near the active site cleft (Whittington et al., 2001), thus, we assume, antibody binding to the CA XII can block substrate entry to the active site or alter the structure of the protein, making it inactive.

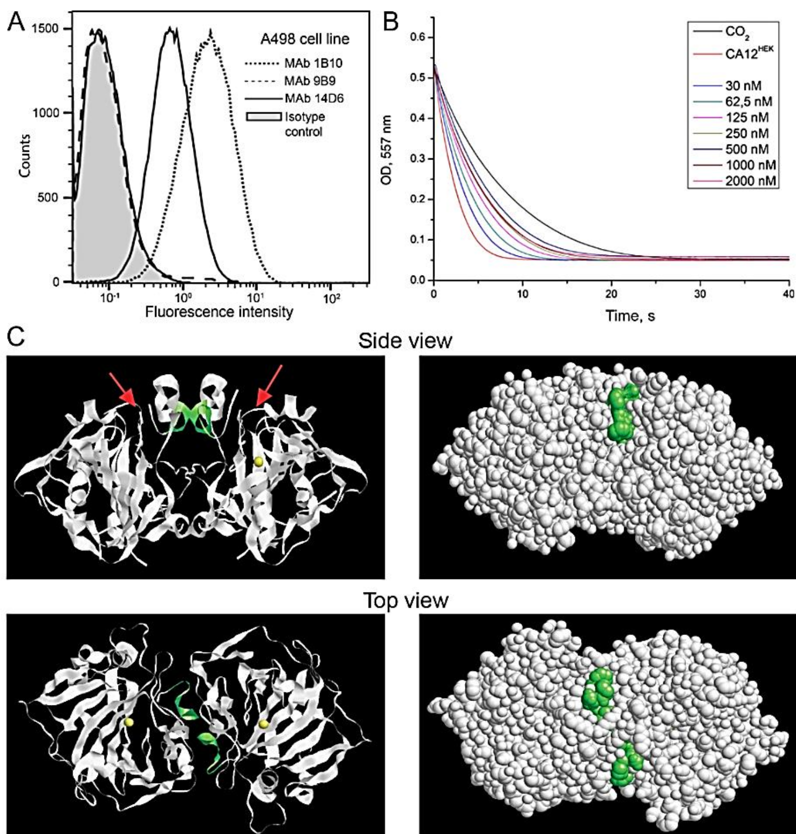

Fig. 2. (A) Live A498 cells were stained by FC to evaluate binding of MAbs against CA XII with native protein on cell surface. Analysis revealed highly reactive MAb 1B10 (dotted line), moderately reactive MAb 14D6 (solid line) and non-reactive MAb 9B9 (dashed line). Grey filed solid line represents isotype control. Secondary Alexa Fluor 488-conjugated Goat anti-Mouse IgG (H+L) (Thermo Fisher Scientific) were used. (B) Absorbance decrease due to enzymatic acidification of the medium by CA XII for various MAb 14D6 concentrations. (C) Localization of linear MAb 14D6 epitope within the CA XII dimmer model (PDB ID: 1JD0, RasMol Version 2.7.5.2). Side and top views of molecule's ribbon display (left panel) and molecular surface (right panel) are shown. Yellow spheres represent zinc atoms, green colour – the sequence of identified epitope, red arrows indicate the active site clefts (accordind to Whittington et al. 2001).

MAb 14D6 is unique, as no other MAb from the collection showed similar properties. MAb 14D6 also differs from the CA XII-specific MAb 6A10 described previously (Battke et al., 2011). Firstly, MAb 6A10 was generated in rats after immunization with A549 cells and was selected by FC. Secondly, although the authors do not specify, but the strategy used for immunization and selection suggests that the MAb 6A10 epitope is conformational (Battke et al., 2011). MAb 6A10 blocks the catalytic activity of CA XII and was administered in a mouse xenograft model of human cancer (Gondi et al., 2013). Radiolabelled recombinant Fab derivative of MAb 6A10 is now considered as a radioimmunotherapy agent targeting carbonic anhydrase XII (Fiedler et al., 2018a).

These findings demonstrate wide application possibilities of MAbs targeting CA XII. Our immunization strategies allowed generation of highly specific MAbs with diagnostic and therapeutic potential. MAb 15A4 can be applied for the diagnostics of CA XII-positive cancer by IHC. CA XII peptide-derived MAbs 1D8 and 3C8 inhibited enzymatic activity of recombinant CA XII, thus the surface-exposed linear CA XII epitope may serve as a target for inhibitory antibodies with a potential immunotherapeutic application. Finally, we have generated MAb 14D6 against recombinant CA XII expressed in mammalian cells. This MAb distinguished by its unique features to recognize cellular CA XII on the surface of live cancer cell and to block the catalytic activity of recombinant CA XII. These two key properties are important if considering antibody application for the cancer therapy. Consequently, we have selected MAb 14D6 for the generation of RAbs expecting to obtain novel specific tools with a potential antitumor activity.

3.3. Generation of RAbs against CA XII

Two RAb formats were selected –scFv and scFv-Fc. To generate CA XII-targeting RAbs, the hybridoma cell line secreting MAb 14D6 was used. First of all, cDNA sequences encoding the variable fragments of IgG light (VL) and heavy (VH) chains were cloned from hybridoma cells and their aa sequences were determined. VH and VL regions were amplified by PCR using primers specific for mouse Ig heavy and light chains framework one region (FR1) and isotype specific region adapted from (Krebber et al., 1997; Orlandi et al., 1989; Wang et al., 2000). After DNA sequencing of PCR products, sequences with occurred frameshifts, stop codons, deletions or atypical aa were eliminated (Figure 3). Cysteine is a conserved aa which always has same position. There are two cysteine residues in the aa sequences of VL and VH prior to CDR1 and CDR3. If cysteines are not in the right position or additional cysteines appears, then the proper structure may not be formed and the functional activity of RAb can be affected (Janeway, 2001).

Analysis with KabatMan data base and NCBI tool IgBlast revealed plausible VH and potentially functional VL sequences (Figure 3), which were further used to produce scFv and scFv-Fc constructs. After all manipulations using different techniques of genetic engineering and molecular biology, two constructs of RAbs, scFv VL-$(G_4S)_4$-VH and VH-$(G_4S)_4$-VL with a polypeptide linker were obtained.

Expression of RAbs in *E. coli* Tuner strain has resulted in the formation of inclusion bodies (Figure 4, A). Recovery of scFvs from inclusion bodies involved step-wise refolding of denatured scFv by dialysis against buffer containing folding additive L-arginine and decreasing concentrations of guanidine hydrochloride with subsequent on-column nickel-affinity purification. Only VL-$(G_4S)_4$-VH scFv was successfully refolded and purified (Figure 4, B) and the CA12^{HEK}-binding activity was verified by an indirect ELISA and WB (Figure 4, D, E).

Light chain	FR1	CDR1	FR2	CDR2
LB12	MVVMTQTTSSLSASLGDRVTINCRAS	QDI---NN---Y	LNWYQQRPDGTVKLLIY	YTS
VK1BACK	DIQLTQSPASLAVSLGQRATISYRAS	KSVSTSGYS--Y	MHWNQQKPGQPPRLLIY	LVS
LB17	MILLTQSPASLAVSLGQRATISYRAS	KSVSTSGYS--Y	MHWNQQKPGQPPRLLIY	LVS
LB11	MIVMTQSPSSMYASLGERVILTCKAS	QDI---KS---Y	FTWYQWKPWRSPKTLIS	CAT
LB6	MIKMTQSPSSMYASLGERVTLTCKAS	QDI---KS---Y	FTWYQWKPWRSPKTLIS	CAT

Light chain	FR3	CDR3	FR4
LB12	KLHSGVPSRFSGSGSGTNYSLTISNLDQEDIATYFC	QQGNTLPFT	FGSGTKLEIK
VK1BACK	NLESGVPARFSGSGSGTDFTLNIHPVEEEDAATYYC	QHIRELTRS	EGGPSWRSNN
LB17	NLESGVPARFSGSGSGTDFTLNIHPVEEEDAATYYC	QHIRELTRS	EGGPSWRSNN
LB11	SLADGVPLRFSGSGSGQDYSLAISCLESDDTTTYYC	LHLGESPHV	RCWDQAGDQI
LB6	SLADGVPLRFSGSGSGQDYSLAISCLESDDTTTYYC	LHLGESPHV	RCWDQAGDQI

Heavy chain	FR1	CDR1	FR2	CDR2
VH-IgG1/MH1	QVKLEQSGGGLVQPGGSRKLSCAAS	GFTFSDYG	MAWVRQAPGKGPEWITF	ISNLAYRI
VH1BACK	-VQLQESGAELARLGASVKMSCKAS	GYTFTSYT	IHWVKQRPGQGLEWIGY	INPSSLYI

Heavy chain	FR3	CDR3	FR4
VH-IgG1/MH1	FYADTVTGRFTVSRENAKNTLYLEMSSLRSEDTATYYC	VR-ATEYA	LDYWGQGTSVTVSSAK
VH1BACK	NLNQKFKDKVTMTAEKSSNTAYMHLSSLTSEDCAVYFC	TRKEVRPW	FAYWGQGTTVTVSSIF

Fig. 3. Sequence alignment of potentially functional (red) and aberrant (black) variable domains of light and heavy chains expressed by the hybridoma cell line 14D6. Green arrow – conserved cysteines, blue box – incorrect cysteine residues.

Later, stable CHO cell line secreting RAb 14D6 scFv-Fc was established by transfecting CHO cells with newly constructed pFUSE-VL-$(G_4S)_4$-VH plasmid, which contains interleukin-2 secretion signal and human IgG1 Fc fragment. CHO growth medium was analysed by an indirect ELISA using anti-human Fc secondary antibodies to select CA XII-specific scFv-Fc expressing cells. RAbs were purified using „rProtein A Sepharose Fast Flow“ (GE Healthcare, JAV) and further analysed by WB under reducing and non-reducing conditions (Figure 4, C). Reduced scFv-Fc migrated at ~56 kDa line, which is near the theoretically calculated protein size. Non-reduced scFv-Fc was ~112 kDa size and this confirmed a homodimeric structure of RAbs. Proper structure of Fc is important for the Fc-mediated antibody effector functions such as ADCC (Yang et al., 2017).

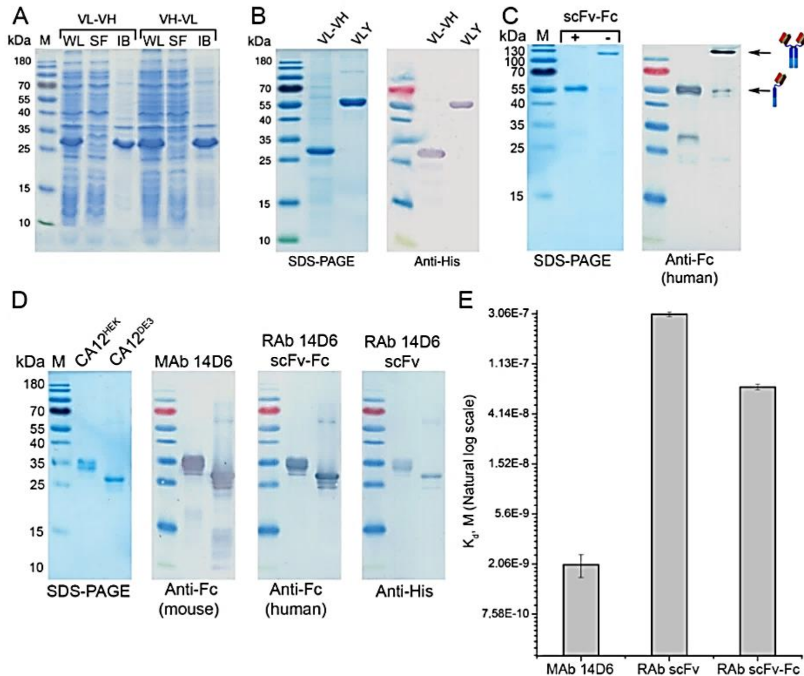

Fig. 4. (A) SDS-PAGE analysis of scFv expressing *E. coli* Tuner whole lysates (WL), soluble protein fraction (SF) and scFv aggregated into insoluble inclusion bodies (IB); (B) – Analysis of refolded and purified RAb scFv VL-VH by SDS-PAGE and WB using Anti-His antibodies showing His-Tag of scFv and in house produced vaginolysin (VLY) protein, previously reported to contain His-Tag (Zvirbliene et al., 2010). (C) Analysis of purified RAb scFv-Fc by SDS-PAGE and Western Blot under reducing (+) and non-reducing (–) conditions. Anti-human Fc secondary antibodies were used. (D) Comparison of MAb 14D6, RAb scFv and RAb scFv-Fc interaction with denatured recombinant CA XII proteins. (E) A comparison of apparent dissociation constants (K_d, M) of MAb 14D6, RAb scFv and RAb scFv-Fc determined by indirect ELISA.

Purified RAbs 14D6 scFv and scFv-Fc were tested for interaction with denatured CA XII recombinant proteins by SDS-PAGE/WB (Figure 4, D) and for apparent dissociation constants K_d by indirect ELISA using anti-His and anti-human Fc secondary antibodies (Figure

4, E). Both scFv and scFv-Fc were able to recognize denatured antigens, indicating the retained specificity of the parental MAb 14D6. The determined K_d of RAb scFv-Fc (7.1×10^{-8} M) showed 4 times higher affinity than scFv (3.0×10^{-7} M) and 35 lower than original MAb 14D6 (2.0×10^{-9} M).

To summarise, in this work we have succeeded to generate specific RAbs targeting cancer-associated CA XII. The potential of laboratory-developed RAbs to be applied in larger-scale studies is limited since it requires additional resources, equipment and experimental optimization to obtain sufficient amounts of proteins and to improve their stability or affinity. Nevertheless, the obtained results confirmed that the determined sequence of MAb variable regions is correct and functional, and developed RAbs are promising therapeutic agents.

3.4. Expression of CA IX and CA XII in cancer cell lines and clinical samples

Expression of CA IX and CA XII is under investigation in normal and malignant tissues to confirm their relevance as cancer biomarkers (Hynninen et al., 2006). MAbs are key components in different immunochemical methods like ELISA, FC, radioimmunoassay, IHC, or IFA for the detection of CA IX and CA XII (Battke et al., 2011; Hynninen et al., 2006; Oosterwijk-Wakka et al., 2013). Clinical specimens like tissue sections or blood are analysed, as well as cancer cell lines, which are considered as reliable models to study the biology of cancer (Gillet et al., 2013; İlie et al., 2010).

In our study cancer cell lines were grown in normoxia and hypoxia and analysed by FC using MAb H7 against CA IX and MAb 1B10 against CA XII to investigate the differences in expression levels of CA IX and CA XII. In addition, the developed qRT-PCR was used for studying *CA9* and *CA12* mRNA expression. Highly correlating results were obtained when comparing protein and mRNA expression of CA IX and CA XII in cell lines (Table 2). FC and qRT-PCR independently verified various expression levels of cancer-related CAs. It was shown that Jurkat and HUT cells do not express CA IX or CA XII regardless of oxygen conditions. This is in line with previous studies that did not find CA IX expression in malignant hematopoietic cells, including Jurkat cell line (Ivanov et al., 2001; Leppilampi et al., 2002). A498 cell line was previously characterized as a poor producer of CA IX (Závada et al., 2003), however these cells express CA XII ((Kallio et al., 2010). Our study showed constitutive CA XII expression in a normoxic environment and an increased CA XII level in hypoxia.

CA XII was more frequently expressed in normoxia than CA IX (A549, MDA-MB-231), however when cells were grown in hypoxia both enzymes were overexpressed. This correlates well with previous studies where cell lines or specimens of the same type cancer were analysed (Ilie et al., 2011; Ivanova et al., 2015; Swinson et al., 2003).

Glioblastoma cell line U-87 was previously characterized by a constitutive CA IX and CA XII expression in a normoxic and hypoxic environment (Said et al., 2006) and these results were confirmed at protein and mRNA level in our study.

Table 2. Comparison of CA IX and CA XII protein and mRNA expression in cancer cell lines, grown under normoxic and hypoxic conditions, determined by FC and qRT-PCR.

Cell lines	**FC**		**qRT-PCR**	
	Normoxia	**Hypoxia**	**Normoxia**	**Hypoxia**
A549	CA XII	CA IX, CA XII	CA XII	CA IX, CA XII
U-87	CA IX, CA XII	CA IX, CA XII	CA IX, CA XII	CA IX, CA XII
A498	CA XII	CA XII	n	n
Jurkat	–	–	–	CA IX
A431	–	CA IX	n	n
HCT	n	n	CA XII	CA IX, CA XII
HUT	n	n	–	–
CEM	n	n	–	CA XI
MDA-MB-231	CA XII	CA IX, CA XII	CA XII	CA IX, CA XII
Huh 7	CA XII	CA XII	CA XII	CA XII

"–" – not detected, "n" – not tested.

It is important to analyse cell lines for the expression of CA IX and CA XII, especially before planning preclinical studies of CA inhibitors. For example, cell viability can be misinterpreted if cell express both isoforms, but inhibition is performed only with one of them. Previous *in vivo* experiments showed that silencing of *CA9* gene alone up-regulates mRNA levels of *CA12*, thus CA IX function is partially compensated (Chiche et al., 2009). Our study showed that majority cell lines when cultivated in hypoxia co-express both CA IX and CA XII (Table 2). Only CA IX was found in A431 cells and only CA XII was expressed in Huh 7 cells.

IHC and MAbs against CA XII (clone 15A4) and CA IX (clone H7) were selected to investigate expression of enzymes in formalin-

fixed and paraffin-embedded tissue sections of various cancer types. For CA XII detection human tumor and normal tissues samples were prospectively collected from patients with different types of breast (n=59), lung (n=34) and kidney (n=13) cancers treated at the Institute of Oncology of Vilnius University. A written informed consent was obtained before study entry. The study was approved by the Lithuanian Bioethics Committee (2013-02-12, No. 158200-13-564-180). IHC staining was done at the National Center of Pathology and the results were analysed by three independent investigators involved in project LIG-09/2012. Samples were evaluated by semi-quantitative scoring, in which the cell percentage stained was taken into consideration as well as the staining intensity in those cells.

The obtained results showed that CA XII was more often overexpressed in cancer tissues, while normal tissues were characterized by low levels of CA XII expression (Figure 5, A). This study confirmed, that CA XII expression was significantly higher in breast cancers. However, we found no significant difference when CA XII expression between cancer and normal tissue specimens of lung and kidney were analysed.

For CA IX detection cervical specimens (n=37) were collected after conizations at the National Cancer institute of Lithuania and stained at the National Center of Pathology by IHC using MAb H7 and analysed by pathologist. Patients were diagnosed with cervical cancer (n=2), cervical carcinoma *in situ* (CIS, n=25), and the rest had cervical intraepithelial neoplasia I, chronic cervicitis or no dysplasia was detected (group “no dysplasia”, n=10). CA IX was detected in 35.1% of all specimens (Figure 5, B). Area of stained tissue ranged from 5 to 70% (mean 20%). No correlation was observed between patient diagnosis and tissue staining area or staining intensity.

Study groups arranged according diagnosis were analysed and the most CA-positive samples were determined in the group of cancer patients (50%), however this group is too small to compare with other groups or to draw any conclusions about the association between CA

IX and malignant changes. In the CIS group, CA IX was detected in 40% of samples and 20% specimens were CA IX-positive for women with no dysplasia. The difference between these groups was not significant, however a tendency of CA IX expression correlation with the degree of cervical alterations can be seen.

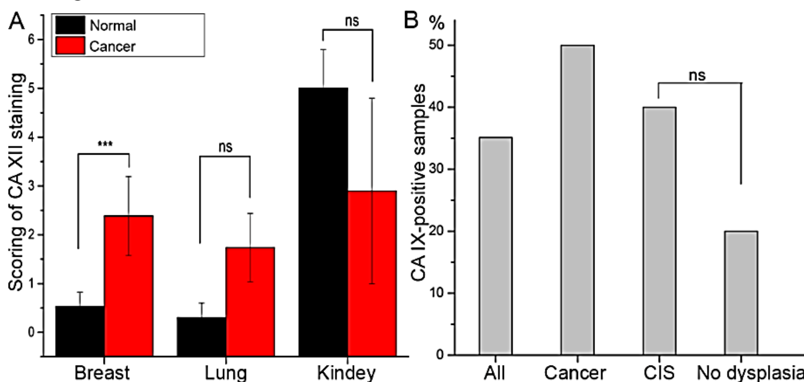

Fig. 5. (A) Expression of CA XII in normal and malignant breast, lung and kidney tissues determined by IHC with MAb 15A4. *** Statistically significant difference, $p<0.05$, in accordance with Wilcoxon test. (B) The prevalence of CA IX-positive specimens among all patients and groups of women with diagnosed cervical cancer, CIS and no dysplasia. ns – statistically non-significant difference, $p>0.05$, in accordance with χ^2 test.

In conclusion, CA IX and CA XII-specific MAbs along with qRT-PCR assay are promising diagnostic tools which can be applied for the diagnostics of various types of cancers. These methods can be applied when describing or selecting CA IX and/or CA XII-positive cancer cell lines for cancer-related studies. Analysis of large-scale groups of cancer patients can provide more accurate medical knowledge and potentially improve the survival and life quality of cancer patients.

CONCLUSIONS

1. Novel stable hybridoma cell lines secreting high affinity MAbs of IgG isotype against CA IX (a collection of 13 MAbs) and CA XII (a collection of 33 MAbs) were generated after immunization with recombinant antigens.

2. After examining the cross-specificity of MAbs, most MAbs were found to be specific only to the CA isoforms against which they were generated. MAbs of both collections interact with linear or conformational epitopes, bind to different protein domains, and are suitable for CA IX and CA XII analysis by various immunochemical methods.

3. MAb 15A4 against CA XII and MAb H7 against CA IX demonstrated their potential diagnostic value by specifically interacting with cellular CA XII and CA IX in human tissue samples. Analysis of clinical specimens by IHC using these MAbs has shown that CA IX and CA XII are more frequently detected in cancerous than in healthy tissues.

4. Expression studies of CA IX and CA XII in cancer cells by flow cytometry revealed that A549, U-87 and MDA-MB-231 cells express both isoforms, whereas A498 cells are CA XII-positive, and A431 cells are CA IX-positive. Under hypoxic conditions, CA IX and CA XII expression increased in several cancer cell lines.

5. MAb 14D6 against CA XII has a potential for anti-tumour targeting as it inhibited enzymatic activity of CA XII and reacted with native CA XII on the surface of live tumor cells.

6. DNA sequences coding the variable regions of MAb 14D6 have been identified and two variants of recombinant antibodies have been constructed, scFv and scFv-Fc, which specifically interacted with recombinant CA XII.

SANTRAUKA

Monokloniniai ir rekombinantiniai antikūnai (MAk ir RAk) yra aukštu specifiškumu antigenui pasižymintys baltymai, kurie plačiai naudojami vėžio tyrimams, diagnostikai ir terapijai. Jų taikiniais gali būti su vėžiu siejami baltymai, tokie kaip karboanhidrazės CA IX ir CA XII – membraniniai fermentai, kurių raiška padidėja esant vėžiniams pakitimams, susidarant hipoksijos sąlygoms, kurios būdingos kietiems navikams. Abu fermentai siejami su vėžio vystymusi, todėl yra potencialūs biožymenys vėžio diagnostikai ar taikinių terapijai.

Šio darbo tikslas: sukurti naujus monokloninius ir rekombinantinius antikūnus, skirtus žmogaus karboanhidrazių CA IX ir CA XII raiškos tyrimams biologiniuose mėginiuose ir turinčius potencialų pritaikymą taikinių terapijai.

Darbo metu panaudojus rekombinantinius antigenus buvo sukurta 13 naujų stabilių hibridomų linijų, sekretuojančių aukšto gimininingumo IgG klasės MAk prieš CA IX ir 33 naujos hibridomos sekretuojančios MAk prieš CA XII. MAk sąveikavo su linijiniais arba konformaciniais epitopais, jungėsi su skirtingomis baltymų sritimis ir buvo tinkami CA IX ir CA XII tyrimams įvairiais imunocheminiais metodais. Dauguma MAk buvo specifiški tik tai CA izoformai, prieš kurią buvo sukurti. MAk prieš CA XII ir CA IX buvo panaudoti šių CA izoformų raiškos tyrimams vėžinėse ląstelėse tėkmės citometrijos metodu. Buvo nustatyta, kad A549, U-87 bei MDA-MB-231 ląstelėse sintetinamos abi izoformos, A498 ląstelėse sintetinama tik CA XII, o A431 ląstelėse – tik CA IX. Hipoksijos sąlygomis tirtose ląstelėse CA IX ir CA XII raiška padidėjo ir tai atitinka ankstesnius tyrimus.

Buvo gauti MAk turintys potencialią diagnostinę vertę (15A4 prieš CA XII ir MAk H7 prieš CA IX), kadangi specifiškai sąveikavo su ląstelėse lokalizuotomis CA XII ir CA IX žmogaus audinių mėginiuose. Šiuos MAk panaudojus klinikinių mėginių tyrimams IHC

metodu buvo nustatyta, kad vėžiniame audinyje CA IX ir CA XII aptinkamos dažniau nei sveikame audinyje.

Buvo sukurtas MAk 14D6 prieš CA XII, kuris gali turėti potencialią vertę taikinių terapijai, kadangi slopino CA XII fermentinį aktyvumą ir reagavo su natyvia CA XII gyvų vėžinių ląstelių paviršiuje. Naudojant hibridomų genetinę medžiagą buvo nustatytos MAk 14D6 variabiliąsias sritis koduojančios DNR sekos ir pirmą kartą sukonstruoti du rekombinantinių antikūnų variantai – viengrandis antikūno fragmentas (scFv) ir scFv prijungtas prie žmogaus pastoviojo fragmento Fc (scFv-Fc), specifiškai sąveikaujantys su rekombinantine CA XII. Sukurti MAk/RAk prieš navikams svarbius fermentus CA IX ir CA XII turi potencialią diagnostinę ir terapinę vertę.

Šis darbas priskiriamas tarpdisciplininių tyrimų kategorijai, nes jo metu buvo panaudoti įvairūs imunologijos, ląstelės biologijos, molekulinės biologijos ir genų inžinerijos metodai.

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PATENT

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1. **Dekaminavičiūtė D**, Lasickienė R, Zilnytė M, et al., Novel monoclonal antibodies against human carbonic anhydrase XII and evaluation of their diagnostic potential, (oral presentation) Lithuanian Biochemical Society (LBS) conference 2012, Tolieja, Lithuania.

2. **Dekaminavičiūtė D**, Lasickienė R, Zilnytė M, et al., Immunodetection of human carbonic anhydrase XII as a new potential biomarker of tumor cells, (poster) ECI 2012, Glasgow, UK.

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5. **Dekaminavičiūtė D**, Kairys V, Petrikaitė V, et al., Novel inhibitory monoclonal antibodies against tumour-associated carbonic anhydrase XII, (poster) FEBS Congress 2014, France, Paris.

6. **Dekaminavičiūtė D**, Kairys V, Petrikaitė V, et al., Novel inhibitory monoclonal antibodies against tumour-associated carbonic anhydrase XII, (oral presentation) international LBS conference, young scientist symposium 2014, Birštonas, Lithuania.

7. **Dekaminavičiūtė D**, Lasickiene R, Vaitiekaitė A, et al., Immunodetection and quantitative evaluation of tumor-associated carbonic anhydrase XII expression in cancer cells, (poster) the International Conference on Carbonic Anhydrase 2015, Maastricht, Netherlands.

8. **Dekaminavičiūtė D**, Lasickiene R, Vaitiekaitė A, et al., Investigation of tumor-associated carbonic anhydrase XII expression in cancer cells, (poster) ECI 2015, Vienna, Austria. ***Best poster in the section of Tumormarkers***.

9. **Dekaminavičiūtė D**, Vaitiekaitė A, Matulis D, Žvirblienė A, Investigation of expression of carbonic anhydrase XII in cancer cell lines by immunochemical and molecular biology methods, (oral presentation) The Lithuanian Academy of Sciences young scientist conference2015, Vilnius, Lithuania. ***1st place and prize for the best presentation in the section***.

10. **Stravinskienė D,** Žvirblienė A. Determination of antibody-binding region of monoclonal antibody targeting carbonic anhydrase XII for the development of recombinant antibodies of potential therapeutic relevance, (poster) Life Sciences Baltics 2018, Vilnius, Lithuania.

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ACKNOWLEDGEMENTS

I am sincerely thankful to my supervisor Aurelija Žvirblienė for her trust and granted opportunity to carry out this work, for her constant encouragement and inspiration and help with writing the thesis. I am grateful to the former and current colleagues of the VU GMC Institute of Biotechnology, Department of Immunology and Cell Biology: Indrė Kučinskaitė-Kodzė, Indrė Dalgėdienė, Martynas Simanavičius, Rita Lasickienė, Vaida Simanavičienė, Milda Zilnytė, Karolina Juškaitė, Vytautas Rudokas and the rest for valuable lessons and help at work, for discussions, for a good and friendly atmosphere. I would like to thank all the students: Aistė Imbrasaitė, Aušra Vaitiekaitė, Jovita Tamulytė, Pijus Andrejauskas, Dmitrij Plotnikov, Enrika Leiputė, Vaiva Žvirblytė for their contribution to this work and the opportunity to improve together.

I am grateful to dr. Milda Plečkaitytė for sharing her knowledge and for her significant help then creating recombinant antibodies. I would like to thank for her comments and corrections while writing the thesis.

I am thankful to dr. Arvydas Kanopka and his group members: Eglė Jakubauskienė, Laurynas Vilys and Inga Pečiulienė for the access to a hypoxia incubator and useful discussions.

I am thankful to dr. Daumantas Matulis and dr. Jurgita Matulienė for the opportunity to participate in their projects and for the initiation of carbonic anhydrase studies in our laboratory. I am grateful to the colleagues of the VU GMC Institute of Biotechnology, Department of Biothermodynamics and Drug Design: Vilma Michailovienė, Vilma Petrikaitė, Lina Baranauskienė, Vaidai Juozapaitienė and the rest for the synthesis and purification of antigens, for determination of neutralizing activity of antibodies, for the help with collection and analysis of clinical samples.

I am thankful to prof. dr. Seppo Parkkila from the University of Tampere for the opportunity to visit and work in his laboratory and for

sharing valuable knowledge of immunohistochemistry methodology and analysis.

I would like to thank to The Research Council of Lithuania for the funding of LIG-16/2010, LIG-09/2012, MIP-37/2012 and SEN-04/2015 projects, for their financial support to participate in international conferences, and for providing doctoral fellowships.

Special thanks to my beloved parents, Aušra and Pranas, for giving me the opportunity to pursue my dreams and for priceless help during writing the thesis. I am thankful to my dear husband Giedrius, wonderful son Tadas and all my loved ones for their support, understanding and being together.

NOTES

Vilnius University Press
Saulėtekio av. 9, LT-10222 Vilnius
Email: info@leidykla.vu.lt,
www.leidykla.vu.lt
Edition 15 copies