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RESEARCH ARTICLE

Preliminary study of thyroid and colon cancers-associated antigens and their cognate autoantibodies as potential cancer biomarkers

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Abstract

Background: Autoantibodies, which are produced against tumor-associated antigens, are potential tumor markers and attract a growing interest for cancer detection, differential diagnostics and prognosis.

Objective: To evaluate the diagnostic significance of 40 antigens identified by immunoscreening of cDNA libraries from thyroid and colon cancers by allogenic screening with different tumor types patients' sera.

Method: Plaque-spot serological assay.

Results: Increased frequency of antibody response in sera of cancer patients compared with that of healthy donors was shown toward 14 antigens, 8 of which (CG016, BTN3A3, FKBP4, XRCC4, TSGA2, ACTR1A, FXYD3 and CTSH) have revealed exclusively cancer-related serological profile.

Conclusion: Allogenic screening of 40 SEREX-antigens with sera from cancer patients and healthy donors allowed us to reveal 14 antigens with potential diagnostic significance. These antigens and their cognate autoantibodies could be considered as valuable targets for further analysis as potential cancer biomarkers.

Keywords: Autoantibody, immunodiagnostics, antigen

Introduction

Malignant transformation, resulting from changes in gene structure and regulation in combination with epigenetic deregulations of critical genes and proteins pathways, causes the host immune response against products of these genes called tumor-associated antigens (TAAs). Mutated, aberrantly regulated or misfolded proteins break down the tolerance of host immune system and serve as targets for immune cells. Dendritic cells, macrophages and mast cells represent the first line of defense and continuously monitor their microenvironment for signs of distress. When tissue homeostasis is perturbed, sentinel macrophages and mast cells immediately release soluble mediators, such as cytokines, chemokins, matrix remodeling proteases, reactive oxygen species and bioactive mediators including histamine, which induce mobilization and infiltration of cells of adaptive immune system – T- and B-lymphocytes into damaged tissue (de Visser et al. 2006). Leading role in destruction of cancer cells belongs to cytotoxic T lymphocytes that recognize peptides of TAAs in the complex with major histocompatibility complex I on the surface of cancer cells. Surface and membrane antigens of cancer cells can be targeted by antibodies produced by B-lymphocytes that can mediate antibody-dependent cytotoxic T-cell response or activate the innate immune system to attack cancer cells. However, most of the TAAs are intracellular proteins and the production of autoantibodies against these antigens is not completely understood. Nevertheless, these autoantibodies might be regarded

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as reporters from immune system, identifying cellular participants in tumorogenesis (Tan 2001). Molecular fingerprint of autoantibodies, which are produced against TAAs called as autoantibody signature, attracts a growing interest for early cancer detection, differential diagnostics and prognosis (Zhang et al. 2003; Anderson et al. 2005; Casiano et al. 2006; Caron et al. 2007; Desmetz et al. 2009c; Desmetz et al. 2009b; Gnjatic et al. 2010). One of the first attempts of studying serological immunogenicity of colon cancer-associated antigens has been made in the pioneering work before 10 years (Scanlan et al. 2002). To date, the autoantibody signatures of lung (Boyle et al. 2011), prostate (Wang et al. 2005), liver (Zhang et al. 2007), pancreas (Gnjatic et al. 2010), ovarian (Li et al. 2008; Gnjatic et al. 2010) and breast (Desmetz et al. 2009a) cancers have been already revealed using different approaches including ELISA (Boyle et al. 2011), different formats of micro-arrays including nucleic acid programmable protein micro-array (NAPPA) (Anderson et al. 2008) based on self-assembling protein micro-array (Ramachandran et al. 2004), "reverse capture" antibody micro-array (Ehrlich et al. 2006; Qin et al. 2006) and phage display micro-array (Zhang et al. 2007).

Thus, identification and characterization of novel TAAs and their cognate antibodies opens new perspectives for cancer immunodiagnostics. Intensive search for TAAs have been performed in the last decade using various methodologies, including SEREX (serological identification of recombinant expressed clones), SEPRA (serological proteome analysis), micro-arrays and multiple affinity protein profiling (MAPPing) (Gunawardana & Diamandis 2007; Hardouin et al. 2007). A vast number of potential TAA have been identified, and some of them were characterized in regard to their cellular localization, function, expression profile and reactivity with sera of cancer patients. The main purpose of this study was to characterize 40 antigens that were identified by serological screening of thyroid and colon cancer cDNA libraries by extended allogenic screening with sera of cancer patients and healthy donors.

Methods

Tissue samples

Thyroid and colon primary tumor samples were obtained from the National Cancer Institute (Kyiv, Ukraine) as surgical specimens, frozen in liquid nitrogen and stored at –80°C. The histological classification of tumors and cell differentiation status was confirmed by histopathological examination at the Department of Pathology, National Cancer Institute (Kyiv, Ukraine). Tumor serum samples were obtained at the same Institute from 139 patients (30–65 years old) with different tumor types (benign breast tumor [BBT], 10; thyroid goutre [TG], 11; fibromioma uterus [FU], 11; breast cancer [BC], 42; thyroid cancer [TC], 25; melanoma [M], 10; brain cancer [BrC], 10; colon cancer [CC], 20). Control sera were obtained from 69 individuals who were having annual health examinations and had no evidence of malignancy (27–62 years old). All sera samples were processed in the same way and stored with 50% glycerol at –20°C. Consent forms were obtained from all patients. The study protocol was approved by the Ethics Committee of the Institute of Molecular Biology and Genetics.

Construction and Immunoscreening of cDNA libraries

Total RNA was extracted by the guanidinium thiocyanate method (Chomczynski & Sacchi 1987), and mRNA was purified by the Dynabeads Oligo$(dT)_{25}$ kit (Dynal, Oslo, Norway). Five micrograms of mRNA was used for the construction of oligo(dT)-primed double-stranded cDNA that was ligated into the lambda ZAPII expression vector (Stratagene, La Jolla, CA). The titers of constructed colon and thyroid cDNA libraries were in the range of 0.05–3 × 10^6, and the average inserts size was 1.2–1.5 kb (Table 1). In order to remove the antibodies reactive with bacterial and λ phage proteins before immunoscreening, each sera sample was diluted at 1:10 in tris-buffered saline (TBS) and incubated overnight at 4°C with Sepharose 4B (Pharmacia, Uppsala, Sweden) coupled to protein extracts from XL-1-Blue *Escherichia coli* and λ ZAPII-infected XL-1-Blue *E. coli* cells synthesized as described previously (Garifulin et al. 2003). Final sera dilutions (1:100) were prepared in TBS with 0.2% non-fat milk (Marwell, England) and 0.01% NaN_3 as preservative. Generated cDNA libraries were not amplified before immunoscreening that was performed as described previously (Garifulin et al. 2003).

Allogeneic screening

Pre-absorbed serum samples from 32 patients with benign tumors, 107 patients with different types of cancer and 69 healthy donors were tested in allogeneic screening for the presence of IgG specific to a panel of 40 SEREX-defined antigens. A total of 200 pfu of bacteriophage, encoding each of the individual tumor antigens, were spotted in 0.7 μl volume on the plate's surface with exponentially growing XL-1-Blue *E. coli* cells. In total, 67 clones corresponding to 40 SEREX-defined antigens were spotted in duplicates on 15-cm plate (duplicates were located on different plates). During incubation at 37°C for 7 h, phage plaques became visible and then recombinant proteins were transferred onto nitrocellulose membranes for 12 h. This approach was designed and verified in previous studies and named as plaque-spot serological assay (Scanlan et al. 2002; Garifulin et al. 2003; Kyyamova et al. 2004). Next manipulations were performed by a standard SEREX protocol (Scanlan 1998). Nitrocellulose membranes were blocked by 0.5% non-fat milk, incubated in 10 ml of diluted (1:100) sera at room temperature for 15 h, and then incubated with alkaline phosphatase conjugated Fc-fragment specific goat anti-human IgG (1:3000) (Jackson Immunoresearch laboratories Inc., West Grove, PA). Serum reactivity was detected with the alkaline phosphatase substrate, 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (Sigma, St.Louis, MO).

Table 1. The list of genes identified by SEREX screening of colon and thyroid cancer cDNA libraries.

Gene's acronym	Description of protein product	ID number of clones which were deposited in the Cancer Immunome Database (http://ludwig-sun5.unil.ch/CancerImmunomeDB/)	*N*	Antigens already presented in the Cancer Immunome Database and identified by immunoscreening of cDNA libraries from
Thyroid cancer cDNA library Thy3 (titer 1.05 × 10⁶)				
CTNNBL1	Catenin, β-like 1,	Clone: KY-Thy16	5	
		Clone: KY-Thy18		
		Clone: KY-Thy21		
		Clone: KY-Thy23		
		Clone: KY-Thy30		
PHF20	PHD finger protein 20	Clone: KY-Thy17	3	Renal, colon and ovarian cancers, testis
		Clone: KY-Thy22		
		Clone: KY-Thy29		
CTNNA1	Catenin (cadherin-associated protein) α 1	Clone: KY-Thy19	2	Renal cancer
		Clone: KY-Thy26		
	No significant similarity found	Clone: KY-Thy20	1	
BRAP	BRCA 1 assotiated protein	Clone: KY-Thy24	1	Melanoma, breast, ovarian, colon, head and neck cancers, testis
FXYD3	FXYD domain-containing ion transport regulator 3	Clone: KY-Thy5	1	
GOLGA	Golgi autoantigen GCP16	Clone: KY-Thy27	1	
TOB2	*Homo sapiens* transducer of ERBB2	Clone: KY-Thy28	1	
Thyroid cancer cDNA library Thy4 (titer 1.4 × 10⁶)				
MS12	Musashi gomolog 2 (Drosophila)	Clone: KY-Thy31	1	
		Clone: KY-Thy33	1	
RPS24	Ribosomal protein S24	Clone: KY-Thy32	1	
		Clone: KY-Thy35	1	
SNRPG	Small nuclear ribonucleoprotein polypeptide G	Clone: KY-Thy34	1	
		Clone: KY-Thy39	1	
SQSTM1	Sequestosome1	Clone: KY-Thy36	1	
MLC-B	*H. sapiens* myosin regulatory light chain	Clone: KY-Thy3	1	
		Clone: KY-Thy3	1	
		Clone: KY-Thy42	1	
CTSH	Cathepsin H	Clone: KY-Thy40	1	
PPP1R15A	Protein phosphatase 1, regulatory subunit 15A	Clone: KY-Thy41	1	
COL8A2	Collagen, type V111, α2	Clone: KY-Thy43	1	
GRCh37	*H. sapiens* chromosome 6 genomic contig	Clone: KY-Thy44	1	
		Clone: KY-Thy45	1	
Total number of antigens from two thyroid cancer cDNA libraries = 15		Total number of clones from two thyroid cancer cDNA libraries = 30		
Colon cancer cDNA library 2C (titer 2 × 10⁶)				
RPL18	*H. sapiens* ribosomal protein L18	Clone: KY-CC-1(5′)	1	
CTCL	*H. sapiens* CTCL tumor antigen se2-2	Clone: KY-CC-2	2	
Colon cancer cDNA library 3C (titer 1 × 10⁵)				
COX1	Cytochrome c oxidase subunit I	Clone: KY-CC-3	1	
Colon cancer cDNA library 4C (titer 0.25 × 10⁵)				
TALDO1*	*H. sapiens* transaldolase 1	Clone: KY-CC-4	15	
EEF1A1*	*H. sapiens* eukaryotic translation elongation factor	Clone: KY-CC-5	2	Testis
COL1A1*	*H. sapiens* collagen, type I, α 1	Clone: KY-CC-6	1	Stomach cancer
PDAP1*	*H. sapiens* PDGFA associated protein 1	Clone: KY-CC-7	2	Fibrocarcoma
CG016*	Human BRCA2 region, mRNA sequence	Clone: KY-CC-8	1	
CXCR4*	*H. sapiens* chemokine (C-X-C motif) receptor 4	Clone: KY-CC-9	1	
TRIM2*	*H. sapiens* tripartite motif-containing 2	Clone: KY-CC-10	1	
BTN3A3*	*H. sapiens* butyrophilin, subfamily 3, member A3	Clone: KY-CC-11	1	
FKBP4*	*H. sapiens* FK506 binding protein 4	Clone: KY-CC-12	2	

(Continued)

Table 1. *(Continued)*.

Gene's acronym	Description of protein product	ID number of clones which were deposited in the Cancer Immunome Database (http://ludwig-sun5.unil.ch/CancerImmunomeDB/)	*N*	Antigens already presented in the Cancer Immunome Database and identified by immunoscreening of cDNA libraries from
RPLP0*	Ribosomal protein, large, P0	Clone: KY-CC-13	2	
ACTR1A*	ARP1 actin-related protein 1 homolog A	Clone: KY-CC-14	1	
PLRG1*	*H. sapiens* pleiotropic regulator 1	Clone: KY-CC-15	3	Hepatocellular carcinoma
BRAP*	*H. sapiens* BRCA1 associated protein	Clone: KY-CC-16	2	Melanoma, testis breast, ovarian, thyroid cancers
ACTB*	Actin, β	Clone: KY-CC-17	1	Fibrosarcoma, pancreas adenocarcinoma
GNB2L1*	*H. sapiens* guanine nucleotide binding protein (G protein), β polypeptide 2-like 1	Clone: KY-CC-18(3`),	1	
TSGA2*	*H. sapiens* testes specific A2 homolog (mouse)	Clone: KY-CC-19	1	
IMAGE: 4893383*	The protein product is unknown	Clone: KY-CC-20	1	
Colon cancer cDNA library 1C (titer 2 × 10⁵)				
UACA	Uveal autoantigen with coiled-coil domains and ankyrin repeats	Clone: KY-CC-21	4	
TRIP11	*H. sapiens* thyroid hormone receptor interactor 11	Clone: KY-CC-22(5`)	1	
Colon cancer cDNA library 5C (titer 0.5 × 10⁵)				
HMGN1*	*H. sapiens* high-mobility group nucleosome binding domain 1	Clone: KY-CC-23	1	Breast cancer
HMGN3*	*H. sapiens* high mobility group nucleosomal binding domain 3	Clone: KY-CC-24	2	
XRCC4*	*H. sapiens* X-ray repair complementing defective repair in Chinese hamster cells 4	Clone: KY-CC-25	1	
Total number of antigens from five colon cancer cDNA libraries = 25		Total number of clones from five colon cancer cDNA libraries = 51		
Total number of antigens from thyroid and colon cancers = 40		Total number of colon and thyroid cancers clones = 81		

N = Total number of clones identified for each gene.
*Protein products of these genes were identified and described in this study as potential colon cancer antigens for the first time.

Statistical analysis

Data were statistically analyzed using the χ^2 test.

Results

Previously, we have identified 15 autoantigens from two thyroid cancer cDNA libraries (Thy-3 and Thy-4) (Rodnin et al. 2000; Rodnin et al. 2003) and five autoantigens from three colon cancer cDNA libraries (1C, 2C and 3C) (Garifulin et al. 2003) by SEREX approach. Small-scale allogeneic screening of these TAAs allowed us to identify five antigens (PHF20, FXYD3, TOB2 (ERBB2BP) and CTSH from thyroid cancer, and COX1 antigen from colon cancer) as potential targets for cancer immunotherapy and immunodiagnostics (Garifulin et al. 2003; Kyyamova et al. 2004). Twenty antigens were identified by screening of colon cancer cDNA libraries 4C and 5C and described in this study for the first time (Table 1). Totally, 25 serum positive clones (from 51) corresponding to 25 antigens identified from five colon cancer cases and 30 clones corresponding to 15 antigens from 2 thyroid cancer cases were deposited in the Cancer Immunome database of the Ludwig Institute for Cancer Research under KY-CC1–KY-CC25 (for colon cancer) and KY-Thy16–KY-Thy45 (for thyroid cancer) designations (http://ludwig-sun5.unil.ch/CancerImmunomeDB/) and presented in Table 1.

In this paper, we present the results of expanded allogeneic screening with panel of TAAs and sera of tumor patients using plaque-spot serological assay. In total, 29 clones corresponding to 15 TAAs from 2 thyroid cDNA libraries (Thy-3 and Thy-4) and 44 clones corresponding to 25 TAAs from 5 colon cancer cDNA libraries (1C, 2C, 3C, 4C and 5C) (Table 1) were analyzed with 208 sera including 139 sera from patients with different tumor types and 69 healthy donors' sera. The panel of selected antigens was represented by proteins involved in different cellular processes, including transcription/translation (10 antigens), cytoskeletal rearrangement/cell adhesion (5 antigens), differentiation/development (5 antigens), cell signaling (4 antigens), vesicle transport/targeting (3 antigens), metabolic processes (2 antigens), apoptosis/proteolysis (2 antigens) and 9 antigens with other or unknown

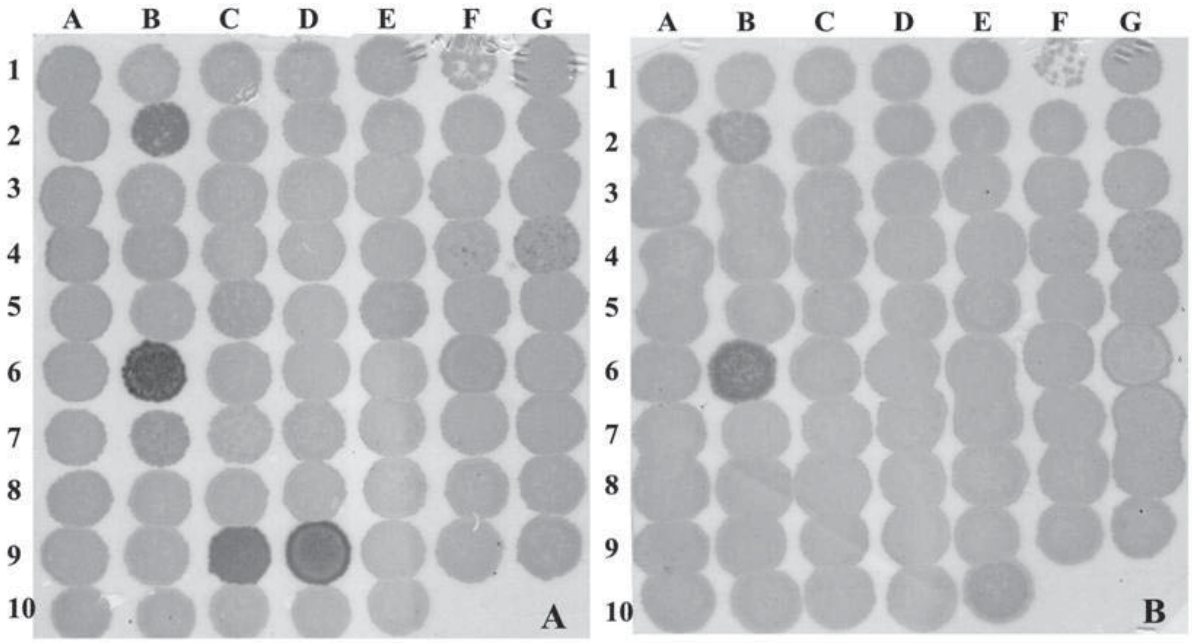

Figure 1. Representative result of a plaque-spot serological assay. (A) 67 clones corresponding to 40 SEREX-defined antigens including positive and negative controls were probed with melanoma patient's sera N4. This analysis revealed: B6 – IgG clone (strong signal, positive control), C6 – non-recombinant clone (negative control), B2 –Thy28 clone (TOB2 antigen) (strong signal), C9 and D9 – clones K9 (KY-CC-7) and K121 (KY-CC-7) (PDAP1 antigen) (strong signals), C5 and E5 – clones K57 (KY-CC-16) and Thy24 (BRAP2 antigen) (weak signals); F6 – RPL18 (weak signal). (B) 67 clones corresponding to 40 SEREX-defined antigens were probed with sera of healthy donor N15. This analysis revealed: B6 – IgG clone (strong signal, positive control), C6 – non-recombinant clone (negative control), B2 – clone Thy28 (TOB2 antigen) (strong signal), E10 – clone 4-2 (KY-CC-25) (XRCC4 antigen) (weak signal).

Table 2. Antigens associated with cancer-related serological response.

		Number of immunoreactive sera/number of sera tested								
		Malignant						Benign		
Name	Clones	N	BrC	BC	M	TC[a]	CC[a]	FU	TG	BBT
CG016	(KY-CC-8)	0/69	2/10 (20%)*							
BTN3A3	(KY-CC-11)	0/69			1/10* (10%)					
FKBP4	(KY-CC-12)	0/69					1/13*(7.7%)			
XRCC4	(KY-CC-25)	0/69				1/25 (4%)	1/13* (7.7%)			
ACTR1A	(KY-CC-14)	0/69		1/42 (2.4%)			1/13* (77%)			1/10* (10%)
TSGA2	(KY-CC-19)	0/69					1/13*(7.7%)			
FXYD3	(KY-Thy25)	0/69				2/25* (8%)				
CTSH	(KY-Thy40)	0/69		1/42 (2.4%)		1/25 (4%)				
Any of eight antigens (%)		0/69 (0%)	2/10* (20%)	2/42 (4.8%)	1/10* (10%)	4/25*(16%)	4/13* (31%)			1/10* (10%)

[a]Not include reactivity with autologous sera used for initial SEREX-analysis.
*Statistically significant difference compared to healthy donors' sera ($p < 0.05$).
BBT, benign breast tumor; BC – breast cancer; BrC, brain cancer; CC, colon cancer; FU, fibromioma uterus; M, melanoma; N, serum of healthy donors; TC, thyroid cancer; TG, thyroid goutre.

functions. Notably, 22 antigens are known to be associated with oncogenic growth (http://harvester.fzk.de, http://www.genecards.org/) and 13 antigens have been previously identified by serological screening of cDNA libraries from ovarian, colon, melanoma, breast, renal, stomach tumors and testis (Table 1). For further evaluation of serological reactivity of SEREX-defined colon and thyroid cancer antigens, qualitative plaque-spot serological assay was used (Figure 1A and 1B).

To monitor the specificity of the plaque-spot serological assay, we used a cDNA clone encoding heavy chain of IgG gene as a positive control, whereas non-recombinant clone was used as negative control (Figure 1A and 1B). During this study, we revealed that among 40 antigens tested, 20 antigens reacted only with autologous serum (data not shown). Six antigens (PPP1R15A, CTCL, COL1A1, RPLPO, GNB2L1 and TRIP11) showed no difference in reactivity with sera from cancer patients and healthy donors sera (data not shown). Remaining 14 antigens revealed preferential serological reactivity with sera from the cancer patients compared with that of sera from the healthy donors and serve as promising antigens for further investigation (Tables 2 and 3). These antigens were grouped according to their reactivity with

Table 3. The list of antigens which showed more frequent reactivity with sera from cancer patients, when compared to sera of healthy donors.

		Reactivity of sera								
		Malignant						Benign		
Name	Clones	N	BrC	BC	M	TC[a]	CC[a]	FU	TG	BBT
TOB2	KY-Thy-28	18/69	3/10	23/42	4 /10	7/25	9/20	5/11	5/11	3/10
		26%	*30%*	*55%**	*40%*	*29%*	*45%*	*45%*	*45%*	*30%*
PHF20	KY-Thy-17	6/69	1/10	3/42		6/25	3/20	2/11	1/11	5/10*
		8.7%	*10%*	*7%*		*24%**	*15%*	*18%*	*9%*	*50%*
	KY-Thy-22	6/69	1/10	3/42		6/25	3/20	2/11	1/11	5/10
		8.7%	10%	7%		24%*	15%	18%	9%	50%*
	KY-Thy-29	1/69	1/10	4/42		5/25	2/20	2/11	1/11	3/10
		1.4%	10%	9.5%*		20%*	10%	18%*	9%	30%*
BRAP	KY-Thy-24	29/69	7/10	18/42	4/10	10/25	8/20	1/11	7/11	1/10
		42%	70%	43%	40%	40%	40%	9%	64%	10%
BRAP	KY-CC-16	20/69	5/10	10/42	4/10	7/25	5/13	1/11	4/11	1/10
		30%	50%	24%	40%	28%	39%	9%	36%	10%
RPL18	KY-CC-1	8/69	1/10	4/42	1/10	7/25	2/13		1/11	1/10
		11%	10%	10%	10%	28%	15%		9%	10%
PDAP1	KY-CC-7	10/69		6/42	3/10	3/25	2/13	4/11	3/11	1/10
		15%		14%	30%	12%	15%	36%	27%	10%
UACA	1C 35**	15/69	3/10	8/42	2/10	7/25	6/13	6/11	6/11	5/10
		22%	30%	19%	20%	28%	46%	54%*	54%*	50%
	1C 5.1**							1/11		1/10
								9		10%
	1C 21.1**						1/13	1/11		2/10
							8%	9%		20%
	KY-CC-21	15/69	2/10	17/42	1/10	10/25	5/13	6/11	7/11	5/10
		22%	20%	40%	10%	42%	39%	54%*	64%*	50%
Any of six antigens		47/69	8/10	33/42	7/10	23/25*	11/13	9/11	10/11	8/10
Percentage		68%	80%	79%	70%	92%	85%	82%	91%	80%

[a]Not include reactivity with autologous sera used for initial SEREX-analysis.
*Statistically significant difference compared to healthy donors' sera ($p < 0.05$).
**Indicates the clones which were not deposited in the Cancer Immunome Database.
BBT, benign breast tumor; BC - breast cancer; BrC, brain cancer; CC, colon cancer; FU, fibromioma uterus; M, melanoma; N, serum of healthy donors; TC, thyroid cancer; TG, thyroid goutre.

sera from healthy donors. The first group comprises of 6 colon cancer-associated antigens (CG016, BTN3A3, FKBP4, XRCC4, TSGA2 and ACTR1A) and 2 thyroid cancer-associated antigens (FXYD3 and CTSH). These antigens revealed exclusively cancer-related profile of reactivity with frequency of antibody response in the sera of cancer patients up to 20% (Table 2). Furthermore, antigens BTN3A3, CG016, FXYD3, TSGA2 and FKBP4 reacted at least with one type of cancer patients' sera, whereas ACTR1A, CTSH and XRCC4 reacted with sera from patients with two different types of cancer (Table 2). The percentage of tumor patients' sera with antibody response to at least one of these 8 antigens ranged from 4.8 to 31% and was statistically significant in brain, melanoma, thyroid, colon cancers and BBT (Table 2). Almost all antigens from the first group did not reveal any reactivity with sera from patients with benign tumors with the exception of ACTR1A antigen (Table 2).

The second group includes six antigens, RPL18, PDAP1, UACA, TOB2, PHF20 and BRAP, which showed more frequent antibody response with sera from cancer patients in comparison with sera of healthy donors (Table 3). The antigens RPL18, PDAP1 and UACA were identified by screening of colon cancers cDNA libraries, TOB2 and PHF20, respectively. Of thyroid cancers, cDNA libraries and BRAP were discovered as antigens by screening of cDNA libraries from both tumors (Tables 1 and 3).

Antibody response towards these antigens in sera of healthy donors varied between 0 (clones 1C5.1 and 1C21.1 of UACA antigen) and 42% (clone KY-Thy-24 of BRAP2 antigen) (Table 3). Each of these six antigens had increased serum reactivity in at least one type of cancer patients' sera compared to sera of healthy donors. Antigens that were found twice as frequent with at least one type of cancer as with sera of healthy donors are: TOB2, PHF20, RPL18 and PDAP1. However, statistically significant frequency of antibody response was shown toward only two antigens – TOB2 and PHF20 – in sera of breast and thyroid cancer patients compared with sera of healthy donors (Table 3, marked by asterisks). It should be noted that all antigens from the second group also reacted more frequently with sera from patients with benign tumors. However, we have only observed

statistically significant antibody reactivity towards UACA and PHF20 antigens in sera from patients with benign tumors when compared with sera from healthy donors (Table 3). The percentage of tumor patients' sera with antibody response to at least one of these six antigens was statistically significant only in thyroid cancer patients' tumor type (92%) (Table 3).

In this screening, we had several clones that represented cDNA fragments of different sizes corresponding to the same genes, such as UACA, PHF20 and BRAP2. Interestingly, they showed a different pattern of recognition with sera from cancer patients and healthy donors (Table 3). For example, two clones of UACA antigen (1C35 and 1C25.1) reacted with sera from healthy donors with a frequency 22%, whereas clones 1C5.1 and 1C21.1 did not exhibit any reactivity with normal sera (Table 3). Furthermore, clone Thy-29 that corresponds to PHF20 antigen showed low serum reactivity (1.4%) in the group of healthy individuals compared with clones Thy-17 and Thy-22 (8.7%). The observed differences could be explained by the different size of cDNA fragments of the same gene and the presence of different autoimmune epitopes in protein products of these cDNA clones (data not shown).

This study revealed 9 from 14 antigens, CG016, BTN3A3, FKBP4, XRCC4, TSGA2, ACTR1A, FXYD3, CTSH and TOB2, which have been identified by SEREX methodology for the first time. Five remaining antigens (RPL18, PDAP1, UACA, PHF20 and BRAP) have been identified by other researches and already deposited in the Cancer Immunome database (http://ludwig-sun5.unil.ch/CancerImmunomeDB/) (Table 1) and some of them (BRAP (Greiner et al. 2003), PHF20 (Behrends et al. 2003; Wang et al. 2002) and UACA (Lee et al. 2003; Devitt et al. 2006) have been described by other authors as SEREX-antigens in research papers. Only antigen PDAP1 from these 14 antigens was identified as potential glioma-associated antigen by another approach – immunoscreening of protein microarray with autologous serum of glioma patient (Ludwig et al. 2009). Notably, no overlap was observed between colon cancer-associated antigens examined in this study (CG016, BTN3A3, FKBP4, XRCC4, ACTR1A, RPL18, PDAP1, UACA and BRAP2) and colon cancer-associated antigens selected during autologous and allogeneic screening by Scanlan et al. (Scanlan et al. 2002).

Discussion

Creating a panel of antigens for serological diagnostics of cancer is a very attractive, but challenging task, because serum antibodies have several advantages compared with other tumor markers. The antibodies to TAAs may be presented at the asymptomatic stage of cancer, be stable over a long period of time, can be amplified by immune system in response to a single molecule of antigen, and might be detected in the sera of patients with simple and accessible techniques (Tan 2009). Several approaches have been used today for the creation of antigenic panels or mini-arrays of multiple TAAs for cancer diagnostics. Some of available panels comprise the same and/or different overlapping sets of antigens for diagnosis of different types of cancer, for example, breast, prostate, lung, liver and ovarian cancers (Zhang et al. 2003; Zhang et al. 2007; Li et al. 2008), whereas those which were created for diagnosis of the specific type of cancer, for example, ovarian cancer, have a different TAAs profiles (Li et al. 2008; Gnjatic et al. 2010). This fact clearly demonstrates that despite the heterogeneous antigenic profile in different types of cancer, there are some antigens that are common for various cancers. On the other hand, some of the antigens may turn out to be specific for a certain type of cancer. Zhang et al. noted that comprehensive analysis and evaluation of various combinations of selected antibody-antigen systems will be useful for the development of autoantibody profiles involving different panels or arrays of TAAs in the future, and the results could be useful for diagnosis of certain types of cancer (Zhang et al. 2007). In this regard, investigation of serological reactivity of TAAs by allogeneic screening is a necessary step for revealing those antigens that may be important for cancer immunodiagnostics. In this paper, we present the results of allogenic screening of 40 TAAs with sera from cancer patients and healthy donors using plaque-spot serological assay.

Extensive allogeneic screening of selected antigens with 208 samples of sera from cancer patients and healthy donors allowed us to identify 14 antigens as potential targets for cancer immunodiagnostics and immunotherapy. In this panel, eight antigens (CG016, BTN3A3, FKBP4, XRCC4, ACTR1A, TSGA2, CTSH and FXYD3) revealed exclusively cancer-related profile. The frequency of humoral immune response toward these antigens in cancer patients varied from 2.4 to 20%. These results correlate well with literature data describing low serological reactivity against cancer-related antigens in sera of cancer patients (Scanlan et al. 2002; Zhang et al. 2003; Zhang et al. 2007; Li et al. 2008; Gnjatic et al. 2010). Bioinformatic analysis of eight antigens with cancer-related serological profile revealed their involvement in tumorogenesis, emphasizing their potential as attractive molecular targets for cancer therapy, diagnostics or prognosis.

For example, FKBP52 mRNA and protein levels were found to be increased in CIS (carcinoma *in situ*) and primary breast cancer compared with healthy breast tissue. Autoantibody assay against a panel of five antigens, including FKBP4, allows for an accurate discrimination between early-stage breast cancer, especially CIS, and healthy individuals (Desmetz et al. 2009a). XRCC4 takes part in the reparation of DNA double-strand breaks and might be considered as potential tumor suppressor gene in several types of carcinomas (Gao et al. 2000). Recently, Bau et al. have reported significant association of SNPs in the XRCC4 gene with colorectal cancer, indicating that the genetic polymorphisms of XRCC4 might be involved

in colorectal carcinogenesis (Bau et al. 2010). The mutations in ACTR1A gene could be causally related to malignant pleural mesothelioma tumors (Sugarbaker et al. 2008). Several studies have found a correlation between elevated cathepsin H levels and increased malignancy in multiple tumor types, including gliomas, colorectal carcinomas, prostate and breast cancers, suggesting that this protease may have important tumor-promoting properties (Gocheva et al. 2010). Some findings suggest a potential role for FXYD3 in the development and progression of certain human malignancies, including breast, prostate and pancreatic cancers. FXYD3 may be a promising novel biomarker for the differential diagnosis of renal urothelial carcinoma (UC) and a promising prognosis marker of UC from bladder (Zhang et al. 2011).

Among TAAs tested, six antigens (RPL18, PDAP1, UACA, TOB2, PHF20 and BRAP) showed reactivity with sera from cancer patients as well as with sera of healthy donors. It should be noted that autoantibodies against potential TAAs in sera of healthy individuals have been described in several studies (Comtesse et al. 2005; Nolen et al. 2009). For example, Comtess et al. described antibody response in sera of healthy donors against an average of 7.8 meningioma antigens per serum; whereas in sera of meningioma patients, antibody response was observed against an average of 14.6 antigens per serum (Comtesse et al. 2005). The authors developed a statistical learning method to differentiate serum response of meningioma patients from that of healthy donors. So, the fact that some antigens during this study revealed reactivity with sera from healthy donors does not reduce their diagnostic value.

Statistically more frequent antibody reactivity was observed in sera from breast and thyroid cancer patients toward only two antigens from the second group – TOB2 and PHF20. TOB2 belongs to the TOB family of anti-proliferative proteins, which are involved in the regulation of cell cycle progression and expected to play an important role in suppressing tumor development (Ikematsu et al. 1999; Matsuda et al. 1996). Gene PHF20 codes a PHD finger protein 20 (TZP, GLEA2, HCA58) that shows transcription factor activities. Recent studies showed that aberrant expression of PHF20 gene is associated with non-small and small cell lung carcinomas (Taniwaki et al. 2006; Bankovic et al. 2010). Increased seroreactivity to glioma-expressed antigen 2 (GLEA2) was found to be associated with prolonged survival for glioma patients (Pallasch et al. 2005) and was described in brain tumor patients under radiation (Heisel et al. 2008).

Statistically significant antibody response towards PHF20 and UACA antigens was observed also in sera from patients with benign tumors compared with sera of healthy donors. The observed difference in frequency of antibody response with respect to UACA antigen was statistically significant between normal and benign thyroid tumor in contrast to normal and thyroid cancer. This phenomenon of declining of serum reactivity with increased malignancy was described in different cancers including meningioma of different grades (Comtesse et al. 2005). The authors described this appearance as a result of possible antigen loss during tumor escape mechanism. UACA was discovered previously as human autoantigen in patients with panuveitis (Vogt-Koyanagi-Harada disease, Behçet's disease, sarcoidosis) (Yamada et al. 2001). It was also demonstrated that the prevalence of anti-UACA IgG autoantibody in patients with panuveitis is significantly higher than in healthy donors (Yamada et al. 2001).

During this study, we observed differential serum reactivity against protein products of cDNA clones representing the same antigens, such as UACA, PHF20 and BRAP. Since these clones have different length of recombinant cDNA inserts, we suggest that they vary also in their epitope profiles that are differentially recognized by sera from tumor patients and healthy donors. One can also speculate that cancer patients and normal individuals could develop antibody response against different epitopes of the same antigen. Further studies should be performed to investigate this phenomenon in more detail.

Totally, 14 antigens were selected during expanded allogenic screening of 40 antigens that were identified by SEREX approach. On the further step of research, we plan to verify results obtained applying large-scale screening using quantitative techniques, in particular ELISA. Taking into account the limited number of sera tested and results of analysis of statistically significant frequency of antibody response in sera of cancer patients compared with that from healthy donors in further investigations, we will focus on thyroid and colon cancer patients with tumors of different grades and stages for validation of data obtained.

Conclusions

Allogenic screening of 40 potential colon and thyroid cancer-associated antigens with sera from patients with different tumor types and healthy donors has uncovered 14 antigens with potential significance for cancer diagnostics, and eight of them (CG016, BTN3A3, FKBP4, XRCC4, ACTR1A, TSGA2, CTSH and FXYD3) had exclusively cancer-related serological profile. These antigens and their cognate autoantibodies could be considered as valuable targets for further analysis as potential cancer biomarkers. To validate these data, screening in large cohorts of thyroid and colon cancer patients using different assay principle such as ELISA has to be performed.

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Declaration of interest

The authors have no financial conflicts of interests. The authors report no conflicts of interest.

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