Identification of HLA Class I-binding Peptides Derived from Unique Cancer-associated Proteins by Mass Spectrometric Analysis

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Abstract. *Background/Aim: Since antigenic peptides of the cancer-associated antigens presented on human leukocyte antigen (HLA) molecules are recognized by specific cytotoxic T-lymphocytes, they have the potential to becoming effective peptide vaccines for cancer immunotherapy. Materials and Methods: Peptides binding to HLA-A*0201 and HLA-A*2402 obtained from human prostate cancer cells by acid-elution were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and source proteins of the peptides were determined based on the HLA-binding capacity listed on the Syfpeithi. Results: We identified TKLSA possibly derived from absent in melanoma 1-like protein (AIM1L), and RLRYT from trans-membrane protein-191C (TMEM 191C) or c20orf201. Messenger RNAs encoding these proteins were expressed in various cancer cell types but none or very few in non-cancerous tissues except for testis, cerebellum and ovary. Conclusion: HLA class I-binding peptides of unique cancer-associated proteins were identified by MS analysis, and might become a promising tool for the generation of novel cancer vaccines.*

Immune response to cancer-associated antigens expressed in cancer cells, but absent or scarcely expressed in non-cancerous cells, might play an important role for T-cell-mediated cancer cell elimination (1-3). Although immune attack on cancer cells alone is unlikely to suppress tumor progression in advanced stages of cancer, caused by the development of immune-escape by tumors, therapeutics based on the immune response against cancer-associated antigens might have the potential to eliminate tumors, providing that cancer-associated immune suppression can be properly regulated by treatment with bio-modulatory or immune-stimulating agents (4-7).

Cancer-associated antigens which could become suitable targets for T-cell-mediated antitumor immune response and their antigenic peptide, which could become synthetic peptide vaccines against cancer, have been enthusiastically sought (8). Essentially, antigenic peptides naturally present on major histocompatibility complex (MHC) class-I molecules on the surface of cancer cells would be the most relevant targets recognized by specific cytotoxic T-cells. Recently, high-throughput methods for a proteomics-based search for cancer-associated antigens have provided a new trend in the development of cancer immunotherapy (9, 10). Identification of HLA class I-presented cancer-associated antigens using immuno-proteomics has been reported, and antigenicity of the identified peptides, stimulating T-cells was demonstrated (11-13). Besides identification of cancer-associated antigens, proteomic tools are increasingly applied for the discovery of important molecules in cancer-associated immuno-biology or biomarkers for diagnosis and treatment of cancer (14, 15).

Immunoproteomics, using the liquid chromatography-tandem mass spectrometry (LC-MS/MS) platform is useful for the identification of naturally-processed antigenic peptide of cancer-associated antigens presented on MHC molecules (16). We previously reported the LC-MS/MS-based identification of MHC class-II binding antigenic peptide derived from cytochrome *P-450* 2J6 isoform expressed in murine hepatocellular carcinoma (HCC) cells by analyzing MHC class-II binding peptides from dendritic cells loaded with HCC cells (17). Synthetic analogue of the antigenic peptide was shown to be immunogenic and vaccination of mice with the peptide was able to stimulate cluster of differentiation (CD)4^+ T-cells. Morse *et al.* (13) described

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Key Words: HLA class-I, antigenic peptide, cancer associated antigen, prostate cancer, mass spectrometric analysis.

that vaccination of cancer patients with cancer-specific peptides identified by immunoproteomic analysis was successfully-able to augment the immune response towards solid tumors. Proteomics for generation of novel anticancer vaccines might have great value for the development of immune-based therapeutics.

In the late stage of metastatic prostate cancer showing resistance to hormone therapy, treatment modalities are limited and new therapeutic options are required for patients with such advanced disease. Prophylactic immunotherapy to prevent the progression to a hormone-resistant stage might contribute to the improvement of prognosis of prostate cancer (9, 14). In the present study, using immunoproteomics analysis, we identified HLA class I-binding peptides derived from unique cancer-associated proteins expressed in human prostate cancer cells, possibly leading to the generation of novel cancer vaccines for prostate cancer.

Materials and Methods

Cell lines. Human prostate cancer cell lines (LNCaP, PC3 and DU145), bladder cancer cell lines (T24, 5637 and TCCSUP) and bladder inverted papilloma cell line (RT4) were obtained from the American Type Culture Collection (Manassas, VA, USA). LNCaP-expressing HLA-A*24 (LNCaP-A*24) cell line was kind gift from Dr. Tanaka, Department of Urology, Sapporo Medical University, Sapporo, Japan (18). Ovarian cancer cell lines (SKOV3, Hac-2, OV-1063, JHOC-5 and JHOC-9), endometrial cancer cell line (Ishikawa), colon cancer cell lines (LOVO, SW48, LS180,, HCT116, SW480 and DLD-1) and chronic myelogenous leukemia cell lines (K562 and KU812) were kindly provided from Professor Okamoto, Dr. Sasaki (Department of Obstetrics and Gynecology Jikei University, Tokyo, Japan), Dr. Ito (Department of Oncology, Institute of DNA Medicine, Jikei University, Tokyo, Japan) and Dr. Kawano (Department of Molecular Genetics, Institute of DNA Medicine, Jikei University, Tokyo, Japan), respectively.

LNCaP, PC3, DU145, RT4, T24, 5637, TCCSUP, LOVO, SW48, LS180, HCT116, SW480, DLD-1, PANC-1, BxPC-3, SKOV-3, Hac-2, OV-1063, JHOC-5, JHOC-9, BT20, K562 and KU812 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, NICHIREI BIOSCIENCES INC., Tokyo, Japan) and Antibio-Antimyco (Life Technologies, Carlsbad, CA, USA). Ishikawa, AsPC-1, Capan-1, Capan-2, MIA-Paca-2 and MCF7 cells were cultured in DMEM supplemented 10% FBS and Antibio-Antimyco. LNCaP-A24 cell lines were cultured in RPMI 1640 supplemented 10% FBS and Antibio-Antimyco with 500 ng/ml puromycin (Life Technologies).

Acid treatment. LNCaP and LNCaP-A24 cells were grown sub-confluently in four 24-cm square dishes. After washing with Phosphate-buffer saline (PBS), cells were collected with a scraper and centrifuged for 5 min at 190 ×*g*. The cells were further washed with PBS twice, and treated with 10 ml of acid solution (0.13 M citric acid and 0.06 M Na_2HPO_4, pH 3.0) for one minute (19). After centrifugation, the supernatant was collected for MS/MS analysis.

LC-MS/MS analysis. The eluted peptide solution was analyzed *via* electrospray ionization (ESI) liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Q-TRAP triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with TurboionSpray source with information dependent acquisition (IDA). The mass spectrometer interfaced with an Agilent 1100 liquid chromatography and autosampler (Agilent Technologies, Wilmington, DE, USA). The ion source conditions and gas setting were as follows: ion spray voltage=5500 V, ion source heater temperature=500˚C, collision gas setting=4, ion source gas 1 setting=50 and gas 2 setting=50, curtain gas setting=40. de-clustering potential=30 V, collision energy=40 V, collision exit potential=15 V. LNCaP and LNCaP-A24 cell-binding peptides were eluted at a flow rate of 0.2 ml/min from a Synergi MAX-RP 80A column (150×2.0 mm, 4 μm particle size) (Phenomenex, Torrance, CA, USA) using a linear gradient of 1.6% min^{-1} of 5-100% ACN (acetonitorile) containing 1% formic acid (FA). The LC-Q-TRAP mass spectrometer was controlled by the Analyst softoware 1.3.2. (Applied Biosystems/MDS SCIEX, Foster City, CA, USA). All the measured MS/MS data in an IDA file were analyzed by the Analyst software 1.3.2 Mascot script. The script can be used to send data over the Internet to the Matrix Science website. The unknown peptides were identified using Mascot MS/MS ion search engine (Matrix Science, Boston, MA, USA).

Protein identification. Possible source proteins containing the identified peptide sequence were searched using Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, Bethesda MD, USA). Amino acid sequences of these candidate proteins were analyzed by Syfpeithi to determine whether the 9-mer peptide containing the identified peptide shows high binding affinity to HLA-A*2402, HLA-A*01 and HLA-A*0201. Proteins containing the identified peptide sequence with high binding affinity to specific HLA types were defined as candidate proteins.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Cancer cells (2.5×10^6) were seeded in a 10cm Petri dish. The following day, cells were collected and RNA was extracted using PARIS kit (Life Technologies). Normal tissue RNA was purchased from TAKARA BIO INC., Life Technoogies and Agilent (stomach: Human Stomach Total RNA, TAKARA BIO INC., Shiga, Japan; bladder: Life technologies; ovary, breast and pancreas: Agilent; and other tissues: Human Total RNA Master PanelII, TAKARA BIO INC.). One microgram of total RNA was used to synthesize cDNA with PrimeScript® RT reagent Kit with gDNA Eraser (TAKARA BIO INC.). Quantitative-PCR (qPCR) of *18S* ribosomal RNA and *c20orf 201* was performed with Taqman gene expression assay (Life Technologies) and absent in melanoma 1-like protein (AIM1L), trans-membrane protein 191C (TMEM 191C) was performed with Solaris qPCR assay (Thermo Fisher Scientific Inc., Waltham, MA, USA). The expression of mRNA was analyzed by comparative Ct methods using *18S* ribosomal RNA as an internal control.

Results

Among peptides isolated from HLA molecules on the prostate cancer cells, representative 5- or 6-mer peptides were selected and their structural amino acid sequences were determined by LC-MS/MS. The number of candidate proteins and antigenic peptides presented in the context of HLA-A*0201, A*01 and A*2402 are shown in Table I.

Table I. *Identified peptide sequences from LNCaP and LNCaP-A24 cells.*

Peptide	Sequence	Cell line	No. of candidate proteins	No. of Syfpeithi-predicted proteins		
				A*0201	A*01	A*2402
1	TKLSA	LNCaP	29	26	8	-
2	TRAGD	LNCaP	3	2	0	-
3	PARSGA	LNCaP	2	1	0	-
4	RLRYT	LNCaP-A*2402	6	6	3	2
5	SMLAER	LNCaP-A*2402	1	0	1	0

Predictive scores of each peptide for binding affinity to each HLA type are shown in Table II. Possible cancer-associated molecules with high HLA-binding affinity were selected from each candidate. AIM1L was chosen as a possible source protein of TKLSA. TKLSA might be 925-933 amino acid of the AIM1L protein and the antigenic peptide should be PLGTKLSAL. TMEM191C and uncharacterized protein C20orf201 were chosen as possible source proteins of RLRYT. RLRYT might be 325-333 amino acid of the TMEM191C protein and the antigenic peptide should be TLRRLRYTL, or 125-133 amino acid of the C20orf201 protein and the antigenic peptide should be ALRLRYTRM.

AIM1L mRNA expression in several cancer cells lines and non-cancerous tissues are shown in Figure 1. Considerable high *ALM1L* mRNA expression was seen in several cancer cell lines but only placenta and testis exhibited high mRNA expression among noncancerous tissues. *TMEM191C* mRNA was expressed in various kinds of cancer cell, but characteristically high mRNA expression was seen in only testis among non-cancerous tissues (Figure 2). Expression of uncharacterized protein *C20orf201* mRNA was high in several cancer cell lines but only in testis and cerebellum among non-cancerous tissues (Figure 3).

Discussion

Five or six-mer peptides from the human prostate cancer cells obtained by a simple acid elution method were analyzed by LC-MS/MS. Since treatment of the cells with phosphate-citrate buffer at pH 3.3 decomposes the multi-subunit structure of the HLA molecule, antigenic peptides presented on HLA molecules should be released from the HLA (19). Although antigenic peptides recognized by specific Cytotoxic T-Lymphocytes (CTLs) are 8- or 9-mer in general, we did not find any proper candidate peptides composed of 8 or 9 amino acids by LC-MS analysis. Alternatively, 5- or 6-mer peptides were found as a possible decomposed product derived from HLA class I-binding antigenic peptide. These peptides might have been digested and truncated during the dissociation process. In fact, we identified a murine MHC class II-binding antigenic peptide from the peptide composed of four amino acids obtained from dendritic/HCC fusion cells. Based on the specific anchor structure in the context of MHC binding and phenotypic characteristics associated with HCC, we have assumed that this peptide might have been derived from cytochrome *P450* 2J isoform (17). A synthesized 16-mer peptide containing the four amino acid sequence with high affinity to the murine MHC class-II was actually immunogenic and vaccination of mice with this 16-mer antigenic peptide induced interferon-gamma production by stimulated T-cells (17). Accordingly, it seems to be possible to determine the structure of the original antigenic peptide and identify the source protein by analyzing the truncated peptide derived from the source protein.

Two peptides possibly derived from unique proteins, which are scarcely reported in the literature and seemingly a kind of cancer-associated phenotype, were obtained in the present study. AIM1, a novel non-lens member of betagamma-crystallin superfamily, was reported to be associated with chromosome-6 mediated tumor suppression (20, 21). Vainio *et al*. demonstrated that AIM1 was highly expressed in primary prostate cancer and cultured androgen-independent prostate cancer cells and suggested that AIM1 might be a potential drug target for treatment of prostate cancer (22). In the present study, *AIM1L* mRNA was found to be expressed in several cancer cell lines, but only in the placenta and testis among non-cancerous tissues. Although none of the characteristics of *TMEM191C* are known, it was found to be expressed in various cancer cell lines, but only testicular tissue exhibited high expression of *TMEM191C* among normal organs. *C20orf201* was identified by the Mammalian Gene Collection Program (23) without determination of functional property. Although no reports concerning association of *C20orf201* with cancer have been found so far, *C20orf201* was significantly expressed in various cancer cell lines but, here again, only in testis among normal tissues.

According to the expression signature of these three proteins in normal and malignan T-cells, it seems likely that they might have characteristics of a cancer-associated

Table II. *The Candidate proteins and their Syfpeithi scores. Three proteins were analyzed in the following amino acid sequence(†: 966-2920, ‡: 955-2903, §: 1-1669) by Syfpeithi.*

Peptide sequence	Accession No	Protein name	Syfpeithi score		
			A*0201	A*01	A*2402
TKLSA	NP_001034864.2	Absent in melanoma 1-like protein	23	10	
TKLSA	NP_116586.1	Protocadherin-10 isoform 1 precursor	18	14	
TKLSA	NP_065866.1	Protocadherin-10 isoform 2 precursor	18	14	
TKLSA	NP_872579.2	Nucleosome-remodeling factor subunit BPTF isoform 1†	18	11	
TKLSA	NP_004450.3	Nucleosome-remodeling factor subunit BPTF isoform 2‡	18	11	
TKLSA	NP_258260.1	FCH and double SH3 domains protein 1	17	x	
TKLSA	NP_861525.2	Bone morphogenetic protein 8A precursor	14	x	
TKLSA	NP_001711.2	Bone morphogenetic protein 8B precursor	14	x	
TKLSA	NP_938204.2	C-Maf-inducing protein isoform C-Mip	16	22	
TKLSA	NP_085132.1	C-Maf-inducing protein isoform Tc-Mip	16	22	
TKLSA	NP_003491.1	Peroxisomal acyl-coenzyme A oxidase 2	18	15	
TKLSA	NP_997404.1	Reticulon-4 isoform E	18	x	
TKLSA	NP_573400.3	Receptor-type tyrosine-protein phosphatase T isoform 1 precursor	x	x	
TKLSA	NP_008981.4	Receptor-type tyrosine-protein phosphatase T isoform 2 precursor	x	x	
TKLSA	NP_775933.1	RING finger protein 175	17	x	
TKLSA	NP_001697.2	B-cell lymphoma 6 protein isoform 1	11	x	
TKLSA	NP_001128210.1	B-cell lymphoma 6 protein isoform 2	11	x	
TKLSA	NP_004376.2	Versican core protein isoform 1 precursor§	18	x	
TKLSA	NP_001157570.2	Versican core protein isoform 4 precursor	18	x	
TKLSA	NP_005912.1	Mitogen-activated protein kinase kinase kinase 1	18	x	
TKLSA	NP_065393.1	Reticulon-4 isoform A	18	x	
TKLSA	NP_004053.1	Cadherin-16 isoform 1 precursor	25	x	
TKLSA	NP_001191673.1	Cadherin-16 isoform 2 precursor	25	x	
TKLSA	NP_001191674.1	Cadherin-16 isoform 3 precursor	25	x	
TKLSA	NP_001191675.1	Cadherin-16 isoform 4 precursor	25	x	
TKLSA	NP_001035847.1	Serine/threonine-protein phosphatase 4 regulatory subunit 1 isoform a	18	x	
TKLSA	NP_005125.1	Serine/threonine-protein phosphatase 4 regulatory subunit 1 isoform b	18	x	
TKLSA	NP_000316.2	Pituitary homeobox 2 isoform c	22	x	
TKLSA	NP_009058.2	Villin-1	x	x	
TRAGD	NP_001380.2	Down syndrome cell adhesion molecule isoform CHD2-42 precursor	15	x	
TRAGD	NP_005603.3	RE1-silencing transcription factor	14	x	
TRAGD	NP_055400.1	Solute carrier family 40 member 1	x	x	
PARSGA	NP_001073977.1	T-box transcription factor TBX18	15	x	
PARSGA	NP_00101502.1	DNA-3-methyladenine glycosilase isoform b	x	x	
RLRYT	NP_001155250.1	[Pyruvate dehydrogenase (acetyl-transferring)]-phosphatase 1, mitochondrial isoform 1	10	x	x
RLRYT	NP_001155251.1	[Pyruvate dehydrogenase (acetyl-transferring)]-phosphatase 1, mitochondrial isoform 2	14	x	x
RLRYT	NP_060914.2	[Pyruvate dehydrogenase (acetyl-transferring)]-phosphatase 1, mitochondrial isoform 3 precursor	14	x	x
RLRYT	NP_001193981.1	Transmembrane protein 191C	24	x	10
RLRYT	NP_001229242.1	Transmembrane protein 191B	24	x	10
RLRYT	NP_001007126.1	Uncharacterized protein C20orf201	16	x	x
SMLAER	NP_060354.4	PIN2/TERF1-interecting telomerase inhibitor 1	12	x	x

phenotype. Among non-cancerous tissues, only testicular tissue commonly expressed the three identified proteins. Cancer-testis antigen is a well-known cancer-associated molecule of which 8- or 9-mer antigenic peptide presented on HLA molecules is recognized by specific CTLs (24, 25, 26). Various kinds of transiently-expressed genes during spermatogenesis are found in testicular and cancerous tissues (27). Melanoma-associated antigens (28) and cancer/testis antigen-1B (NY-ESO-1) (29) are representative cancer-testis antigens and potential targets of cancer immunotherapy. Thus, it might be conceivable that the three proteins identified in this study might belong to the cancer-testis

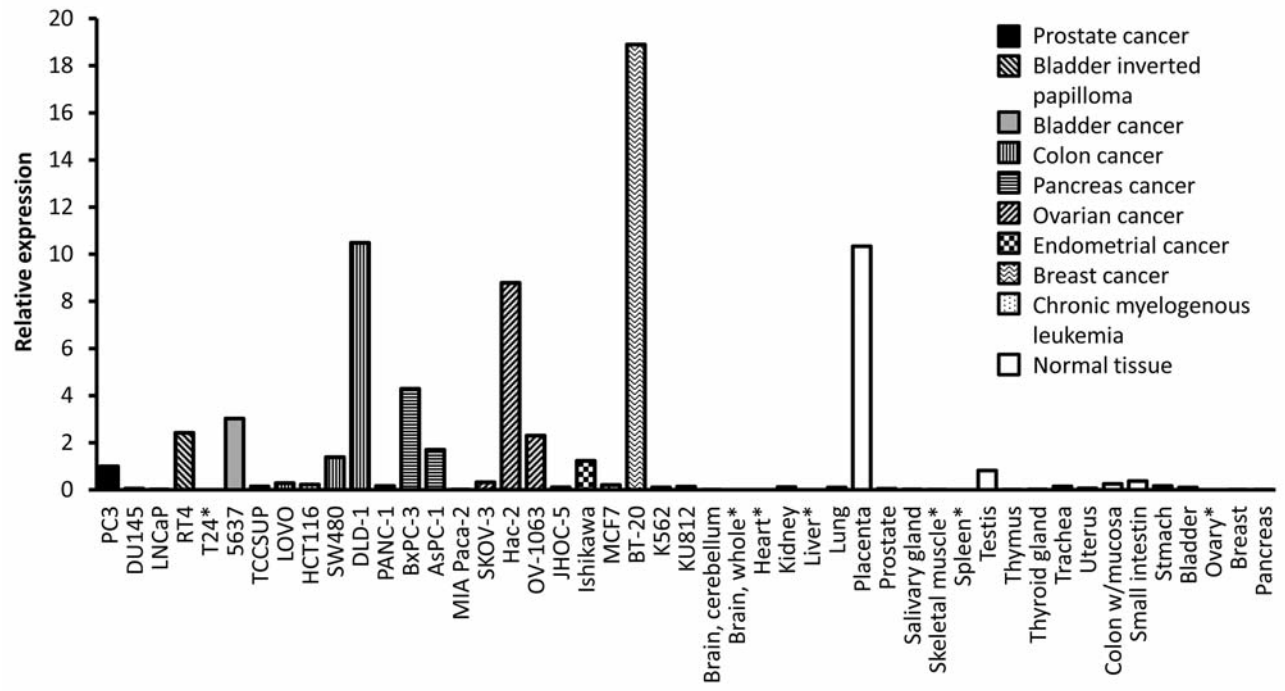

Figure 1. *Expression of absent in melanoma 1-like protein (AIM1L) mRNA in various cancer cell lines and normal tissues. Expression of mRNA encoding AIM1L was analyzed using quantitative reverse transcription-polymerase chain reaction and comparative Ct methods. Each mRNA expression was represented as a value to relative PC3 expression. *Not detected.*

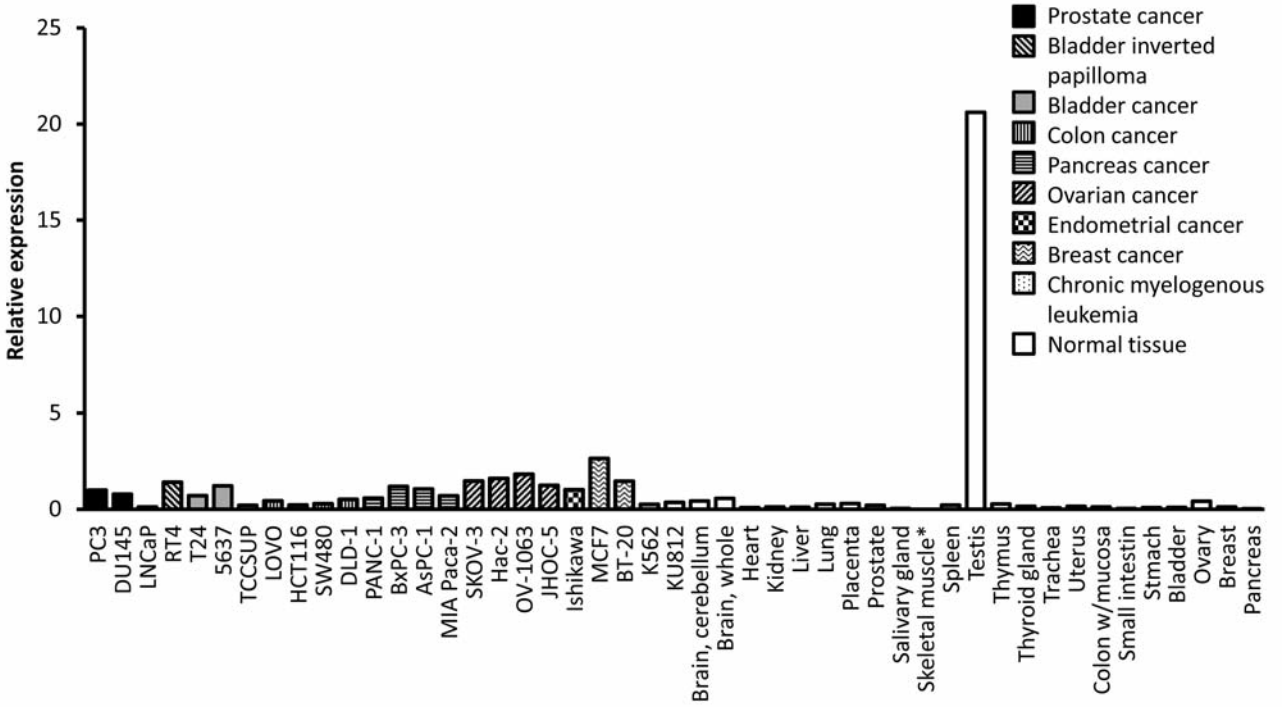

Figure 2. *Expression of trans-membrane protein-191C (TMEM191C) mRNA in various cancer cell lines and normal tissues. Expression of mRNA encoding TMEM191C was analyzed by quantitative reverse transcription-polymerase chain reaction and comparative Ct methods. Each mRNA expression was represented as a value to relative PC3 expression. *Not detected.*

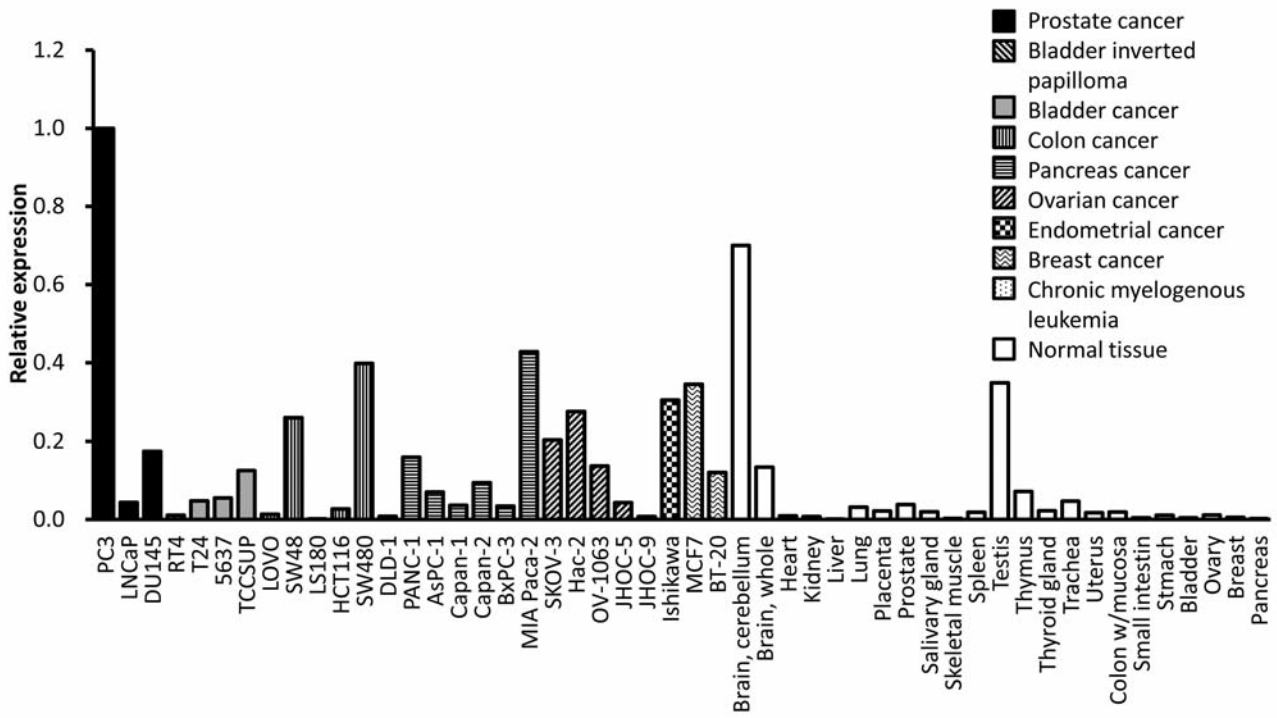

Figure 3. *Expression of C20orf201 mRNA in various cancer cell lines and normal tissues. Expression of mRNA encoding C20orf201 was analyzed by quantitative reverse transcription-polymerase chain reaction and comparative Ct methods. Each mRNA expression was represented as a value to relative PC3 expression. *Not detected.*

antigen family, and, accordingly, peptides obtained by dissociation from HLA molecules might contain a part of antigenic peptide of these cancer-testis antigens. Thus, the structure of 8- or 9-mer peptide containing the identified five or six amino acid sequence might directly indicate the structure for synthetic peptide vaccine which could potentially induce antigen-targeting antitumor immunity. For evaluation of the potential as an immune target recognized by specific CTLs, further studies demonstrating immunogenicity of these peptides in T-cell-mediated antitumor immunity are required.

Identification of novel cancer-associated antigens and determination of the structure of their antigenic peptides have been performed using expression cloning methods or genome-wide exploration with enormous efforts, resulting in generation of many cancer vaccines with low antitumor efficacy. Meanwhile, high-throughput immunoproteomic analysis directly seeking antigenic peptides of cancer associated antigens could provide rapid and efficient generation of effective cancer vaccine because MHC class I peptide actually presented to T-cells are directly collected and analyzed. Immunoproteomic analysis might be also promising in identifying novel biomarkers useful both diagnosis and treatment of cancer.

Acknowledgements

This research was partly supported by The Jikei University Research Fund and KAKENHI (Grant-in-Aid for Young Scientists (B)).

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Received March 14, 2013
Revised April 1, 2013
Accepted April 3, 2013