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Cancerous Immunoglobulins and CA215: Implications in Cancer Immunology

Gregory Lee

UBC Center for Reproductive Health, Faculty of Medicine,
The University of British Columbia, Vancouver, BC, V6H 3N1, Canada

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ABSTRACT

Immunoglobulins are typically expressed by B cells in our normal immune system. However, certain normal human tissues, such as hyperplastic epithelial cells, cells of the immunologically privileged sites and the majority of cancer cells, have also been found to be sites of immunoglobulin production. Current research is lacking in regards to the differential immunoglobulin expression, the underling mechanisms of action and the biological implications of these cancerous immunoglobulins in cancer immunology. This article reviews the etiology of atypical immunoglobulin expression in normal non-B cells and cancer cells, with emphasis on the exploration of the possible mechanisms of action and biological function of these atypical immunoglobulins, by means of specific biological probes. In contrast to immunoglobulins of B cell origins, atypical immunoglobulins were found to carry additional post-translational modifications, including a unique carbohydrateassociated epitope recognized by RP215 monoclonal antibody. This unique RP215-specific epitope enables us to differentiate between these two types of immunoglobulins. Atypical immunoglobulins expressed by cancer cells have been a common subject of interest in cancer immunology. Furthermore, the recent accumulation of experimental evidence has indicated that these atypical immunoglobulins are essential for the growth and proliferation of cancer cells under our normal immune environment. RP215 monoclonal antibody also reacts with many other cancer cell-expressed glycoproteins, known as CA215, on the cancer cell surface. Apoptosis of cultured cancer cells can be induced and growth inhibition of implanted tumors can be observed in nude mouse animal models. Therefore, humanized RP215 monoclonal antibody, which reacts mainly with surface bound CA215, may have the potential to be developed as an anti-cancer drug for the treatment of human cancers. A better understanding of cancer cell-expressed immunoglobulins not only improves our knowledge of cancer immunology, but also benefits cancer patients in the field of cancer monitoring and therapeutic treatments.

Keywords: RP215 Monoclonal Antibody, CDC and ADCC Reactions, Apoptosis

1. INTRODUCTION

It has been known for almost two decades that immunoglobulins can be expressed by human cells other than B cells (Cao *et al*., 1991; Chen *et al*., 2009; Hu *et al*., 2008; Kimoto, 1998; Yoshimi *et al*., 2002). Through the early studies of several investigators, it has been well documented that most cancer cells expressed immunoglobulins which were essential for their own growth and proliferation, either *in vitro* or *in vivo* (Li *et al*., 2004; Qiu *et al*., 2003). These observations have brought the attention of many researchers into the molecular mechanisms of action of cancer cell expressed immunoglobulins, as well as the potential benefit in employing this knowledge to create new therapeutic treatments for cancer patients.

In 1987, the monoclonal antibody (mAb), designated as RP215, was identified through immunization of mice with OC-3-VGH ovarian cancer cell extract followed by cell fusions and screening among three thousand hybridomas (Lee *et al*., 1992). RP215 mAb was later shown to react with glycoproteins, designated as CA215, through bindings with a unique RP215-specific carbohydrate-associated epitope (Lee *et al*., 2006). To

search for the molecular identity of CA215, Matrix Adsorption Laser Desorption Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF MS) analysis was performed for the tryptic peptides, which were obtained from affinity-purified CA215 from the shed medium of cultured cancer cells (Lee *et al*., 2008). Subsequently, it was observed that high percentages of the peptides (>40%) were homologous to the heavy chains of immunoglobulins (Lee *et al*., 2008). Through further protein Basic Local Alignment Search Tool (BLAST) analysis, it was concluded that the cancer cell derived CA215 glycoproteins consist mainly of immunoglobulin heavy chains, with a molecular size ranging from 50-75 kDa (Lee *et al*., 2006; 2008). Interestingly, RP215 mAb was shown to have no cross-reactivity with immunoglobulins of B cell origins (Lee *et al*., 2008). This would indicate that the expression of the CA215 epitope is highly linked with that of cancerous immunoglobulins only (Lee *et al*., 2008).

During the last decade, considerable interest has been on the expression of atypical immunoglobulins in the immunologically privileged sites of normal human tissues, including in the cells of the neurons, testes, eyes, placenta and breast. The etiology of immunoglobulins expressed by cancer cells was also actively pursued. In concise reviews in 2008 and 2009, early studies of immunoglobulins expressed in non-lymphoid lineage and neoplastic cells were reported, but with limited information regarding the etiology of cancerous immunoglobulins (Chen *et al*., 2009; Hu *et al*., 2008). During the last several years, a significant progress has been made regarding our current understanding in the expression and biological functions of atypical cancerous immunoglobulins (Chen *et al*., 2009; Hu *et al*., 2008). Therefore, more emphasis of this review will be placed on the implications of recent major developments regarding cancerous immunoglobulins in the field of cancer immunology (Chen *et al*., 2009).

1.1. Expression of Immunoglobulins by Normal and Cancer Cells in Humans

Since over a decade ago, numerous studies have demonstrated that immunoglobulins, or their heavy or light chain subunits, were expressed or detected in either non-lymphoid or cancer cells, in addition to their traditional origins from B cells (Babbage *et al*., 2006; Chen *et al*., 2009; Geng *et al*., 2007; Hu *et al*., 2008; 2003; Kimoto, 1998; Li *et al*., 2001; 2004; 2012; Okabe *et al*., 2001; Qiu *et al*., 2003; Yang *et al*., 2002; Zheng *et al*., 2007b; 2001; Zhu *et al*., 2008). In the case of normal human tissues, the majority of these atypical immunoglobulins were expressed or detected in tissues of the immune-privileged sites, such as the neurons, testes, eyes and placenta (Chen and Gu, 2007; Chen *et al*., 2009; Huang *et al*., 2008; 2009; Niu *et al*., 2011a; 2011b; Zhang *et al*., 2010; Zhao *et al*., 2011). Typically, Immunohistochemical (IHC) assays, Reverse Transcriptasepolymerase Chain Reaction (RT-PCR), in situ hybridization and Western blot assays were employed to detect the presence of immunoglobulins, or their fragments (heavy and/or light chains), in human tissues. In some cases, partial or total DNA or amino acid sequencing was performed to establish the identity of the detected immunoglobulins or their heavy or light chain fragments. In addition, RT-PCR was utilized as a tool to verify the gene expression of the heavy and light chains in these atypical immunoglobulins. Besides demonstrating the gene expression of immunoglobulins, the expression of essential genes necessary for the assembly of immunoglobulin genes, such as Recombinant Activating Gene (RAG1), Recombinant Activating Gene 2 (RAG2) and Activation-Induced cytidine Deaminase (AID) gene, were also documented in each case (Chen and Gu, 2007; Huang *et al*., 2008; 2009; Niu *et al*., 2011a; 2011b; Zhang *et al*., 2010; Zhao *et al*., 2011). However, in general, the mechanisms of action behind the expressions of these atypical immunoglobulins in normal human tissues were discussed with limited experimental evidence (Babbage *et al*., 2006; Chen and Gu, 2007; Geng *et al*., 2007; Hu *et al*., 2003; Huang *et al*., 2008; 2009; Li *et al*., 2001; Liu *et al*., 2009; Niu *et al*., 2011a; 2011b; Okabe *et al*., 2001; Yang *et al*., 2002; Zhang *et al*., 2010; Zhao *et al*., 2011; Zheng *et al*., 2007b; 2001; Zhu *et al*., 2008). In most cases, the expressions of the atypical immunoglobulins in the immunologically privileged sites of normal human tissues were found to only implement the possible functions of immune protection (Chen and Gu, 2007; Huang *et al*., 2008; 2009; Niu *et al*., 2011a; 2011b; Zhang *et al*., 2010; Zhao *et al*., 2011).

The expression of atypical immunoglobulins in various cancer cells or tissues continues to be the subject of major research interest. Besides cancer cells of epithelial cell origins, the majority of soft tissue tumors (Chen *et al*., 2010; Liu *et al*., 2012; Qiu *et al*., 2012b), such as sarcomas, were also found to express immunoglobulins, with the level of these atypical immunoglobulins correlating with markers of proliferation (PCNA, KI67, cyclin D1) and tumor grades (Zhang *et al*., 2012). Based on the results of this study, it was concluded that the expression of atypical immunoglobulins was a common characteristic of all human cancers (Chen *et al*., 2009; Hu *et al*., 2008; Kimoto, 1998; Lee and Ge, 2009). Therefore, these atypical immunoglobulins can be considered as pan cancer biomarkers. However, it remains difficult to

differentiate the source of these atypical cancer immunoglobulins from that of normal lymphoid derived immunoglobulins in human tissues (Chen *et al*., 2009; Hu *et al*., 2008). Lymphocyte markers, such as CD19 and CD20, are required to rule out the cross contamination from lymphocyte infiltration as the source of immunoglobulins in cancer tissues (Chen *et al*., 2009; Hu *et al*., 2008; 2002).

A major breakthrough in research regarding cancer immunoglobulins came in 2008 when RP215 mAb was initially characterized with respect to its carbohydrate-associated epitope and the molecular identity of the corresponding glycoprotein antigens, CA215, was unfolded (Lee *et al*., 2008). Upon the initial MALDI-TOF MS analysis of CA215-derived tryptic peptides, it was generally concluded that RP215 mAb reacts with a carbohydrate-associated epitope of immunoglobulin heavy chains derived from cultured cancer cells. In contrast, RP215 mAb did not cross-react with immunoglobulins produced from normal B cells, indicating the complete absence of this RP215 specific epitope in normal lymphoid derived immunoglobulins (Lee *et al*., 2008). Thus, RP215 mAb and its recognized "sugar" epitope, marked a clear distinction between these two types of immunoglobulin molecules (Zheng *et al*., 2009; 2010).

1.2. Structural Characterization of Cancerous Immunoglobulins and the Etiology of Gene Expression

Since the initial observations that immunoglobulins were also expressed by non-haemotopoietic cells (Cao *et al*., 1991; Chen *et al*., 2009; Hu *et al*., 2008; Kimoto, 1998), efforts have been made to elucidate the primary structures of the V-(D)-J gene rearrangement of expressed immunoglobulin heavy chains from cancer cells (Dudley *et al*., 2005; Early *et al*., 1980; Geng *et al*., 2003; Goebel *et al*., 2001; Jung *et al*., 2006; Zheng *et al*., 2007a). At the same time, the molecular mechanisms of cancerous immunoglobulin expressions were also compared with those from B cells. Generally speaking, expressions of atypical immunoglobulins in normal and cancer cells are quite distinct from those of B cell origins (Zheng *et al*., 2009; Zhu *et al*., 2010). In contrast to normal B cell derived immunoglobulins, cancerous immunoglobulins are expressed with limited but distinct somatic hypermutations in the V-(D)-J region of the heavy chain genes (Honjo *et al*., 2002; Muramatsu *et al*., 2000; Papavasiliou and Schatz, 2002). In addition, in these cancerous immunoglobulins, no class switching mechanism was observed (Zheng *et al*., 2009; Zhu *et al*., 2010). The characteristics of the immunoglobulin heavy chain gene repertoire were investigated by sequencing of the $V_HD_HJ_H$ region of the heavy chain genes. It was generally observed that seven predominant VHDHJH sets were derived from different cancer types. The mechanisms of limited hypermutation in cancer cells were found to be different from antigen selection in normal B cells. The promoter used for the gene expression of cancerous atypical immunoglobulins was also different from the promoter used for the gene expression of immunoglobulins by B cells (Oct. 2 vs. Oct. 1). Therefore, it was concluded that the cancer cell derived immunoglobulin genes may have a distinct repertoire, which may play a role in carcinogenesis (Zhu *et al*., 2010).

So far, experimental evidence seems to indicate that the cancer cell expressed immunoglobulins are essential for the growth and proliferation of cancer cells (Li *et al*., 2004; Qiu *et al*., 2003). Transfection of anti-sense DNA or SiRNA to cancer cells resulted in growth inhibition of cancer cells *in vitro* (Li *et al*., 2012; Qiu *et al*., 2003). In addition, the sense and anti-sense SiRNA were annealed and subcloned into the small hairpin RNA expression vector pSM2 as stable plasmids for transfection into cancer cells. Among the transformed cancer cells, SiRNA mediated knockdown of cancerous immunoglobulins was observed by suppressing tumor growth in an immunedeficient nude mouse model in vivo and inhibiting the growth of cancer cells in vitro. It was hypothesized that cancerous immunoglobulins are capable of reducing Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) against the cancer cells by blocking the Natural Killer (NK) cells' effector function through the Fc receptors of cancerous immunoglobulins (Iannello and Ahmad, 2005; Li *et al*., 2012; Winkel and Anderson, 1991). However, the ADCC-suppression mechanism has not yet been demonstrated to function in vivo since endogenous immunoglobulins derived from B cells might be dominant in the natural immune environment. Despite the ambiguity surrounding the functional mechanism of cancerous immunoglobulins, all the experimental observations have so far been consistent with cancer cell expressed immunoglobulins being a growth requirement and likely being regulated in an autocrine manner (Lee and Ge, 2010).

1.3. CA215 Vs. Cancerous Immunoglobulins

In-depth MALDI-TOF MS analysis was performed with tryptic peptides of affinity-purified CA215 from the shed medium of cultured OC-3-VGH cancer cells (Lee *et al*., 2008). It was further revealed that the cancer cells' CA215 glycoproteins consist not only of immunoglobulin heavy chains, but also a number of other glycoproteins with suitable sites for unique

glycosylations to create a unique epitope that can be recognized by RP215 mAb (Lee *et al*., 2008; 2012e). The details of such analyses are presented in **Table 1**. Besides immunoglobulins, the majority of identified CA215 glycoproteins could be grouped into the Immunoglobulin Superfamily (IgSF) proteins, as well as others. These IgSF proteins include T cell receptors, antigen presenting molecules, cell adhesion molecules and several others under the same category (Kimoto, 1998; Lee *et al*., 2012e).

As expected, it was found that in immunohistochemical staining studies of cancerous tissues, the positive staining patterns between RP215 and anti-immunoglobulins were not always identical (J. Gu, personal communication). However, the results of both immuno-probes demonstrated high correlation, since immunoglobulins are predominant and are common molecules recognized by these two immuno-probes. Both immuno-probes were shown to react with the soluble and membrane bound forms of immunoglobulins expressed by cancer cells (Lee *et al*., 2008). Therefore, RP215 mAb may be an acceptable alternative for study of cancerous immunoglobulins in the field of cancer immunology (Lee *et al*., 2008). Extensive evaluations and development processes on RP215 mAb are necessary in order to determine its biological effects on cancer cells and whether RP215 mAb can be developed as an anti-cancer drug in the future (Lee *et al*., 2009a; 2012a; 2012c).

1.4. Biological and Immunological Studies of RP215 Monoclonal Antibody and CA215

Through years of intensive studies, the molecular nature of CA215 and its mutual relationships with cancerous immunoglobulins have been somewhat established (Lee *et al*., 2008; 2012a; 2012e). This information is briefly summarized in the following sections in order to assess if RP215 mAb is a suitable candidate for development as an antibody-based anti-cancer drug, as well as to evaluate the potential role of CA215 in the immunology of cancer cells (Lee *et al*., 2012e).

1.5. Biochemical and Molecular Characterizations of RP215 mAb and CA215

Through biochemical analysis, it was generally established that RP215 mAb reacts specifically with a unique carbohydrate-associated epitope of cancerous CA215 glycoproteins consisting mainly of immunoglobulin heavy chains (Lee *et al*., 2008; 2012e). This was achieved by extensive MALDI-TOF MS analysis of CA215-derived tryptic peptides followed by the National Centre for Biotechnology Information (NCBI) protein BLAST services (Lee *et al*., 2012e). Through Western blot assay and IHC analysis, it was demonstrated that RP215 mAb reacts with almost all of the thirty established cancer cell lines (**Table 2**) (Lee *et al*., 2012e). This was consistent with RT-PCR and ELISA studies, which revealed the universal expressions of mRNA for the genes of immunoglobulin heavy and light chains, as well as the antigens, CA215 (Lee and Ge, 2009; Lee *et al*., 2012e). The mRNA expression of immunoglobulin-related genes was also demonstrated in selected cancer cell lines through semi-quantitative RT-PCR (Lee and Ge, 2009). In a given cancer cell line, OC-3-VGH (ovarian), these genes included those of the three different immunoglobulin classes (G, A and M) and subclasses (G1, G2 and G3), as well as the membrane domains of surface-bound immunoglobulins such as RAG1, RAG2 and AID. Interestingly, the mRNA expression ratios of different immunoglobulin classes or subclasses were discovered to be similar in the cancer cell lines to those in normal human serum specimens. Following single cell cloning of OC-3-VGH ovarian cancer cells, it was further revealed that IgG, IgM and IgA were co-expressed among cells originally derived from a single clone (Lee and Ge, 2009; Qiu *et al*., 2012a).

Table 1. MALDI-TOF MS analysis of tryptic peptides derived from affinity-purified CA215

Molecule Function/Category	Number of Peptides Matched[a] Total = 124 (%)
I. Antigen receptors	
1. Antibodies and immunoglobulins	52 (42.0%)
2. T cell receptor chains	7 (5.7%)
II. Antigen presenting molecules (MHC I and MHC II)	6 (4.9%)
III. Adhesion molecules	10 (8.1%)
IV. Cytokine and growth factors	8 (6.5%)
V. Receptor tyrosine kinase/phosphatase	7 (5.7%)
VI. Others	
1. IgSF related (e.g., titin)	12 (9.7%) Total with homologyb: 75/124 (60.5%)
2. IgSF unrelated (e.g., mucin)	9 (7.3%)

Obtained from (Lee *et al*., 2012c) with permission; a; Acid-eluded CA215 was used for MALDI-TOF MS analysis with ASCOT Program from http://matrixscience.com. b; Excluding overlapping peptides

1.6. Immunoactivity of Cancerous Immunoglobulins Vs. Normal Immunoglobulins

To elucidate the molecular nature of cancerous immunoglobulins, the cancerous immunoglobulins were affinity-isolated from the shed medium of cultured cancer cells by sequential purifications with RP215-linked and anti-human IgG-linked immunoaffinity chromatography. The resulting purified cancerous Immunoglobulins (IgG) were characterized biochemically and immunologically (Lee *et al*., 2012b). By using typical EIA kit for normal human IgG, it was observed that cancerous IgG exhibited a significantly lower immunoactivity than that of normal IgG (1-5% of the normal activity) (Lee *et al*., 2012b). This phenomenon can only be explained based on the fact that atypical cancerous immunoglobulins are aberrantly glycosylated, resulting in significant alternations in protein structures and immunoactivities of the former. This observation was consistent with an earlier report by other scientists regarding the heterogeneity arising from aberrant immunoglobulin expression in cancer cells (Hu *et al*., 2011).

Table 2. Expressions of human immunoglobulin heavy chain (or CA215) and light chains in different fibroblast[a] and cancer cell lines as detected by Western blot, EIA and RT-PCR

			Methods Used[b] (Relative Signal Strength[c])				
			I			III	
Origins	Designation cell line	ATCC No.	(Hc)	λ/κ	II Hc	Hc	λ/κ
Breast	MCF7[d]	HTB-22	+				
	MDA-MB-231[d]	HTB-26	+	+	+	+	+
	MDA-MB-468	HTB-132	+	-			
	MDA-435	+ ± +					
	T-47D[d]	HTB-133	+	±	+	+	±
Cervical	C-33A	HTB-31	+		+		
	CA33		+			+	
	SiHa	HTB-35	+, -[e]	+		+	+
	ME-180	HTB-33	+		+	+	+
Colorectal	HCT 115		+	±	+	+	±
	HCT 116	CCL-247	+		+	+	
	HT-29[d]	HTB-38	+	±	+		+
	SW-48[d]	CCL-231	+	±	+	+	+
Hepatocellular	Hep3B	HB-8064	+	±	+	+	+
	HepG2	HB-8065	+		+	+	±
	Hep-2		+	±	+		
Kidney	293[d]		+				
Lung	A549[d]	CCL-185	+				
	Calu-6	HTB-56	+	±	+	+	+
	H441	HTB-174	+	±	+	+	+
	H460					+	
	MRC-5[a]	CCL-171	+	±	+	+	±
	WI-38[a]	CCL-75	+	±	+	+	±
Lymphoma	HEL1		±	±		±	±
Melanoma	MMAN		±	±	±	±	±
	MMRU		+	±	+	+	±
	SK-MEL-3	HTB-69	+		+	+	
Neuroblastoma	SH-SY5Y	CRL-2266	+	±	+	+	±
Osteosarcoma	U-2 OS	HTB-96	+				
Ovarian	SK-OV-3	HTB-77	+	±	+	+	±
	OC-3-VGH		+	±	+	+	+
Placenta	JEG-3	HTB-36	+,-[e]	±		+	±
Prostate	DU 145[d]	HTB-81	+	±	+	+	+
	PC-3	CRL-1435	+		+	+	
T-cell leukemia	Jurkat	TIB-152	+,-[e]	±		+	±

Obtained from (Lee and Ge, 2009) with permission. a; Normal hyperplastic and proliferated fibroblast cell lines derived from fetal lung tissue. b; Methods: Method I: Western blot using RP215, goat anti-human IgG, COX-100 (Mouse anti-human IgG heavy chain), or goat anti-human IgG (λ/κ) as a probe. Method II: EIA using coated cells and RP215 or COX-100 as a probe. Method III: RT-PCR using primers for immunoglobulin heavy chain constant region or light chain constant region Igλ and/or Igκ under the same conditions. Detailed conditions are described in the text. c; The strength of the signal in the order of + (strong), ± (weak), - (invisible). d; IgG was observed in these cell lines from the following publications: (Babbage *et al*., 2006; Chen and Gu, 2007; Lee *et al*., 2008; Qiu *et al*., 2003). e; Western blot exhibited a positive band when using anti-human IgG as a probe but showed no band when using RP215 as a probe

Table 3: The TUNEL apoptosis assay to demonstrate the increase in apoptosis of cultured cancer cells from prostate, lung, cervix, breast and ovary, respectively in response to 24-48 hrs of treatment with various antibodies

Cancer cell Line (tissue)	Incubation time (hrs)	Antibody	Antibody Concentration (μg/mL)	% Apoptosis (Negative Control)[a]
PC-3 (prostate)	48	RP215	10	31±7 (9±1)*
		ChRP215	10	38±5 (9±1)**
		Goat anti-human IgG	10	36±4 (9±1)*
DU-145 (prostate)	24	RP215	10	32±7 (12±3)*
		Goat anti-human IgG	10	46±9 (12±3)*
	48	RP215	10	35±8 (10±1)*
			20	43±7 (7±2)*
		Goat anti-human IgG	10	46±9 (12±3)*
A549 (lung)	24	RP215	10	30±9 (11±5)*
			20	36±12 (17±3)*
	48	RP215	10	36±9 (13±3)*
			20	38±10 (12±2)*
C33A (cervix)	24	RP215	10	58±11 (20±3)*
MDA-MB-435 (breast)	24	RP215	20	37±8 (6±1)**
		Goat-anti human IgG	10	44±4 (7±2)**
	48	RP215	20	43±8 (8±3)**
		Goat anti-human IgG	20	48±5 (8±3)**
OC-3-VGH (ovary)	24	RP215	10	51±12 (18±2)
	48	RCA104	1	35±3 (6±2) *
		RCA111	1	38±4 (6±2)**
		RP215	1	45±5 (6±2)**

Modified from (Lee *et al*., 2012a) with permission. a; Single asterisk (*) and double asterisks (**) indicate a statistical significance of $p<0.05$ and $p<0.01$, respectively

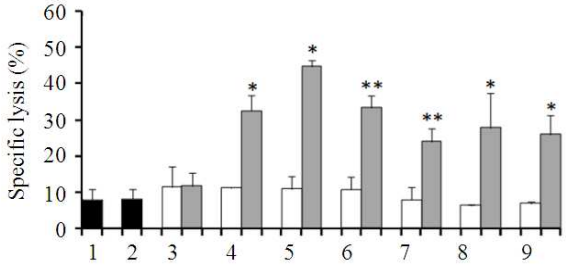


Fig. 1. Complement-dependent cytotoxicity assay to demonstrate the effect of RP215 mAb and its chimeric form, ChRP215, on the complement-dependent cell lysis of OC-3-VGH ovarian cancer cells and PC-3 prostate cancer cells. No treatment (lane 1) or 3 μL freshly prepared rabbit baby complement (lane 2) were used as the negative controls (in black bars). Respective effects of normal mouse IgG (lane 3), RP215 on OC-3-VGH cells (lane 4), chimeric RP215 (ChRP215) ChRP215 on OC-3-VGH (lane 5), RP215 on PC-3 (lane 6) ChRP215 on PC-3 (lane 7), goat anti-human IgG on OC-3-VGH (lane 8) and goat anti-human IgG on PC-3 (lane 9) are shown above. White bars represent the monoclonal antibody (10 μg mL^{-1}) alone, gray bars represent the monoclonal antibody (10 μg/mL) plus complement. Single asterisk (*) and double asterisks (**) indicate statistical significance of $p<0.05$ and $p<0.01$, respectively. Obtained from (Lee *et al*., 2012a) with permission

1.7. Effect of RP215 mAb on the Induction of Apoptosis of Cancer Cells

By using terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) assay, it was clearly demonstrated that RP215 MAb incubated at a concentration of 1-10 μg mL^{-1} for 24-48 h could induce apoptosis of cultured cancer cells, including those from the ovary, cervix, breast, lung and prostate (Lee and Ge, 2010). The chimeric forms of RP215, ChRP215, were found to have the same effects as that of murine RP215. In addition to RP215, apoptosis could also be induced in OC-3-VGH ovarian cancer cells by other RP215-epitope-related monoclonal antibodies (RCA10, RCA100, RCA104, RCA110 and RCA111 to be described later) (Lee *et al*., 2012d). By comparison, goat anti-human IgG also induced apoptosis to a similar extent to that of RP215 mAb, indicating that cancer cell expressed surface bound immunoglobulins are effectively blocked by antibodies against cancerous immunoglobulins (Lee and Ge, 2010; Lee *et al*., 2012d). The results of the apoptosis assay are summarized in **Table 3** for comparison.

1.8. Effect of RP215 mAb on Complement-Dependent Cytotoxicity of Cancer Cells

The Complement-Dependent Cytotoxicity (CDC) assay was employed to study the complement-dependent cell lysis to cultured cancer cells in the presence of complement and

RP215 mAb (10 μg mL^{-1}, 2 h of incubation at 37°C). ChRP215 also demonstrated a similar degree of CDC reaction to cultured cancer cells under the same conditions of incubation (Lee *et al*., 2012a; 2012c; 2012e). Goat anti-human IgG were also found to have similar effects to those of RP215 mAb. This suggested that CDC reactions are induced in the presence of complement to the surface bound immunoglobulins by antibodies, RP215 and goat anti-human IgG. In contrast, complement alone revealed little or no effect on CDC reactions to cultured cancer cells. The effects of specific cell lysis by CDC assays are summarized in **Fig. 1**.

1.9. Effects of RP215 mAb on the Expression of Selected Genes in Cultured Cancer Cells

Through studies of gene regulations by quantitative and semi-quantitative RT-PCR, a number of genes related to the growth of cancer cells were examined for their response to the treatments of cultured cancer cells with RP215 mAb for 48 h. The results of this qualitative analysis with a number of selected genes were summarized for assessment. It was generally concluded that RP215 mAb was found to up-regulate the gene expression of IgG and NFK-B1, but significantly down-regulate genes of the ribosomal proteins, Po, P1 and P2. These genes are essential for protein synthesis in cultured cancer cells (Lee *et al*., 2012c).

RP215 incubation with cultured cancer cells was found to have little effect on Epidermal Growth Factor (EGF), but caused a significant decrease in the gene regulation of c-fos oncogene (Lee *et al*., 2012c). RP215 was also found to down-regulate several genes which are essential for cell cycle regulations, such as p21 and cyclin D.

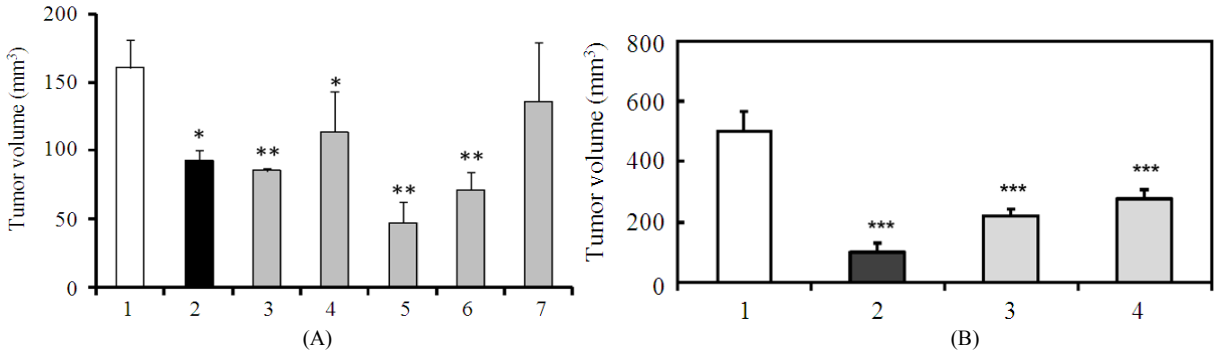


Fig. 2. (A) Nude mouse experiments to demonstrate the dose-dependent effect of injected RP215 mAb on the tumor volumes. Briefly, on day 0, mice were inoculated with OC-3-VGH. Cancer cells were 1 mm^3 in size underneath the dissecting microscope. The mice (n = 4) in the negative control group (Lane 1, NC) were injected with no antibody or drug treatment. RP215 mAb, as well as I^{131}-labeled RP215 mAb was given to mice by intraperituneal injection on the same day. The positive control group (I^{131}-, PC) were injected with 60 mg kg^{-1} of cyclophosphamide. The antibody of the high dose group (lane 3, AH) were injected with 10 mg kg^{-1}) with 10 mg kg^{-1} RP215 mAb, whereas those in the antibody low dose group (lane 4, AM) were injected with 2 mg kg^{-1} of RP215 mAb. In lanes 5, 6 and 7, I^{131}-labeled RP215 mAb (12.5 μCi/mg) was used for injection at a dose of 10 mg mL^{-1} (I-AM), 5 mg mL^{-1} (I-AM), 5 and 2 mg mL^{-1} (I-AL) respectively. The tumor volume and body weight of each mouse were reported every four days. Single asterisk and double asterisks indicate statistical significance of $p<0.05$ and $p<0.01$, respectively. Statistical significance: NC Vs. PC $p<0.05$ (*); NC Vs. AH $p<0.01$ (**); NC Vs. AM $p<0.05$ (*); NC Vs. I-AH $p<0.01$(**); NC Vs. I-AM $p<0.05$ (*) (B) RP215 mAb was also employed for the nude mouse experiment with a human lung squamous cancer cell, SKMES-1 (ATCC: HTB-58) as the model. The cultured cancer cells, in their exponential growth phase, was trypsinized and suspended in PBS and the concentration adjusted to 1.5-2.5×107 cells mL^{-1}. 0.2mL of the tumor cells were implanted under the armpit or via the back. Three weeks after the first inoculation, the implanted tumor in each mouse became visible. The mice were then divided randomly into 4 groups (5 mice/group). The mice were then injected with two different doses of RP215 mAb (high and low doses) in the treatment groups. All the mice in the negative control group (lane 1, NC) were treated with PBS while in the positive control group (lane 2, PC), the mice were injected with Genicitabine (GEM) (1000 μg m^{-2}) and Cisplatin (CDDP) (80-100 mg/m2) in each dose. Both the antibody low dose (lane 3, AL) and antibody high dose (lane 4, AH) had the mice injected with RP215 mAb, by receiving 0.14 mg/mouse/dose and 0.75 mg/mouse/dose, respectively. In all four groups, antibody or drug treatments were performed twice in total during the 4th and 5th weeks following tumor cell implant. At the 6th week mark, the tumors were taken out and their volume measured. Single asterisk and double asterisks indicate statistical significance of $p<0.05$ and $p<0.01$, respectively. Statistical significance: NC Vs. PC $p<0.01$ (***); NC Vs. AH $p<0.01$ (***); NC Vs. AL $p<0.01$ (***) Both A and B were obtained from (Lee *et al*., 2012a) with permission

Based on the results of the gene regulation studies, it can be shown that the molecular mechanism, by which apoptosis of cultured cancer cells is induced, is consistent with the hypothesis that self-expressed cancerous immunoglobulins are essential growth factors for the survival of the cancer cell (Lee *et al*., 2012a; 2012c).

1.10. "Proof of Concept" Nude Mouse Experiments with RP215 mAb

Judging from the effect of RP215 mAb on inducing apoptosis and CDC reactions of cultured cancer cells, it is apparent that CA215 on the surface of cancer cells might be a suitable target for RP215 mAb. "Proof of concept" nude mouse experiments were performed with the implant of tumor cells from three different cancer cell lines, namely OC-3-VGH (ovary), SK-MES-1 (lung) and C33A (cervix) (Lee *et al*., 2009a; 2012a; 2012c). With some differences in experimental design, significant dose-dependent tumor reduction or tumor growth inhibition was observed in each case (Lee *et al*., 2012a). Typical results are summarized in histograms for each cancer line model, including detailed statistical analysis. These are presented in **Fig. 2** for comparisons.

1.11. Anti-Idiotypic Monoclonal Antibodies of RP215 mAb as Anti-Cancer Vaccines and Immunodominant Epitope

RP215 mAb was demonstrated to react with the unique carbohydrate-associated epitope of CA215 glycoproteins identified in many cancer cells (Lee *et al*., 2006; 2008). Anti-idiotypic (aid) monoclonal antibodies can be generated in rats against the Fab-idiotypic domains of RP215. The anti-idiotypic monoclonal antibodies were found to bear the internal images of the RP215-associated epitope (Lee *et al*., 2010; Lee and Ge, 2010). To further characterize the anti-idiotypic monoclonal antibodies, mice were immunized to generate Ab3 (anti-aid). By means of TUNEL assay, Ab3 obtained from mouse immune sera was found to induce apoptosis to cultured cancer cells, similar to that of RP215 mAb. Through Western blot assay, Ab3 antisera and RP215 mAb were found to react with the same protein bands (50-75 kDa) from cancer cell extract, corresponding to those of immunoglobulin heavy chains. By immunohistochemical staining, the majority of cancer cells from different tissue origins were positively stained by both Ab3 antisera and RP215. These experimental observations strongly support the concept that rat anti-idiotypic mAb or its Fab fragments bear the internal images of the RP215-specific epitope (Lee *et al*., 2010). These anti-idiotypic mAbs may be suitable as anti-cancer vaccines which are capable of inducing Ab3 responses in humans to prevent or potentially treat cancer.

During our early studies, additional new monoclonal antibodies were generated against affinity-purified CA215 as immunogens. Unexpectedly, all of the new generation monoclonal antibodies (RCA-related mAbs) recovered were found to react with epitope(s) related to the one recognized by RP215 mAb (Lee *et al*., 2012d). This indicated the existence of an immunodominant carbohydrate-associated epitope recognized originally by RP215 mAb in mice.

Therefore, among the second generation mAbs, two (RCA10 and RCA100) were found to have identical amino acid sequences as those of RP215 and react with the linear structure of the RP215 epitope. The other three (RCA104, RCA110 and RCA111) were considered to react only with conformational domains of the same epitope. RP215 and its RCA related mAbs were shown to react with the same linear or conformational carbohydrate associated epitope(s). This could be demonstrated either by self-pairing or mutual pairing of any of these mAbs in enzyme immunoassays for quantitative determinations for CA215 (Lee *et al*., 2012d). The reason for the existence of immunodominant epitope(s) recognized by RP215 and other CA215-derived mAbs in mice remains to be explained (Lee *et al*., 2012d).

1.12. CA215 and Expression of Immunoglobulin Superfamily Proteins by Cancer Cells

During the initial analysis of tryptic peptides of CA215, it was revealed that over 40% of peptide fragments were homologous to that of immunoglobulin heavy chains (**Table 1**). When other non-immunoglobulin-related peptides were analyzed by NCBI protein BLAST services, as many as 60% of the peptides from CA215 were found to consist mainly of the Immunoglobulin Superfamily (IgSF) proteins, including immunoglobulins (42%), T cell receptors (6%), cell adhesion molecules (8%), mucins (7%), as well as others (Lee *et al*., 2008; 2012e). To investigate if T-cell receptors are widespread among cancer cells, semi-quantitative RT-PCR was performed with the cell extract of over twenty cancer cell lines (Lee *et al*., 2012e). The results showed that as many as 80% of these cancer cells expressed T-cell receptors (α and β subunits), but none were found to express significant levels of co-receptors or co-stimulators, such as CD3, CD4 and CD8. These observations suggested that cancerous T-cell receptors are non-functional, compared to normal T-cells in humans (Lee *et al*., 2012e). In addition, several cell

adhesion molecules with IgSF characters, including CD47, CD54, CD58 and CD147, were found to be highly expressed among all cancer cell lines (Lee *et al*., 2012e). Results of such expression analysis are presented in **Table 4**. Antibodies against cell receptors were found to induce apoptosis and CDC to cultured OC-3-VGH ovarian cancer cells, similar to those of RP215 MAb (**Fig. 3**). Based on the results of this study, it can be concluded that RP215 mAb affects not only the surface bound immunoglobulins of cancer cells, but also the T cell receptor α or β chains and other IgSF proteins as well (Lee *et al*., 2012e). Widespread expressions of nonfunctional T-cell receptors among cancer cells may have implications in cancer immunotherapy via T-cell activation strategy, as well as induced tolerance of T-cells to tumor-associated antigens in humans (Weber, 2007; Witherden *et al*., 2010).

1.13. Glycoanalysis and Glycosylation Site Mapping of RP215-Specific Carbohydrate-Associated Epitope in CA215

Attempts were made to elucidate the possible sugar structure of the carbohydrate-associated epitope(s) ecognized by RP215 detected in CA215 (Lee and Azadi, 2012). The affinity-purified CA215 from both OC-3-VGH ovarian and C33A cervical cancer cell lines was subjected to glycoanalysis. Profiles of N-linked and O-linked glycans were analyzed and compared with those of known glycoproteins. In the case of N-linked glycans, CA215 was found to contain high mannose and complex bisecting structures with terminal N-glycolylneuraminic acid, when compared with those of normal human immunoglobulins. For O-linked glycans, several oligosaccharides were detected in CA215 with structures similar to those of mucins, but also with terminal N-glycolylneuraminic acid.

These results are presented in **Table 5**. To further identify the molecular nature of glycoproteins with linked RP215-specific epitope, glycosylation site mappings of CA215 were performed. From the results of a total ten glycopeptides isolated, two were found to be N-linked and eight O-linked. Protein BLAST search of peptide sequence homology revealed that two N-linked and six O-linked glycopeptides were almost 100% matched to human immunoglobulin heavy chains. The results of one such glycosylation site mapping are summarized in **Table 6**.

Based on this analysis, it can be suggested that immunoglobulin heavy chains could be the main sites for the attachment of the RP215-specific carbohydrate-epitope. N-glycolylneuraminic might not be directly involved in the RP215-epitope recognition. Based on the results of this glycoanalysis, it can be suggested that the RP215-specific epitope is composed of O-linked glycans of simple oligosaccharides, which are located mainly on the variable regions of the cancerous immunoglobulins (CA215D is designated as affinity-purified cancerous IgG in **Table 5**) (Lee and Azadi, 2012). In contrast, O-linked glycans have never been identified in normal human IgG (Arnold *et al*., 2007).

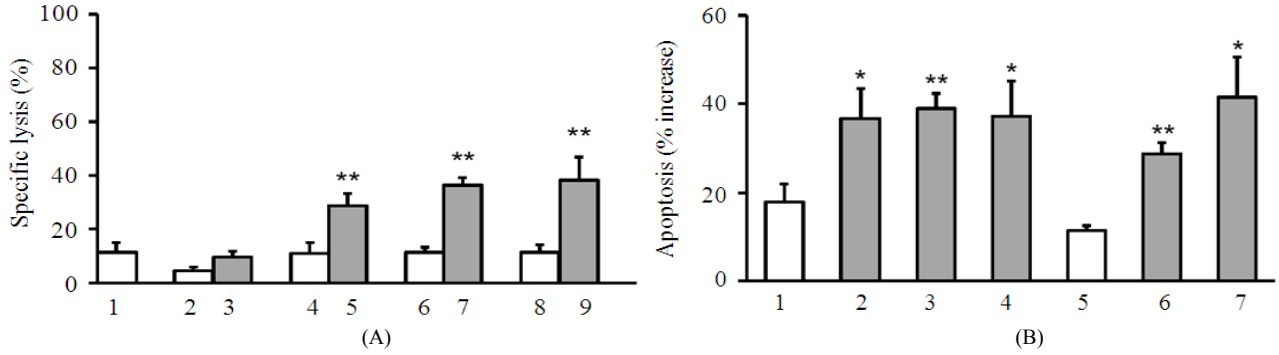


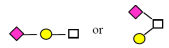
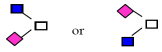
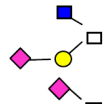
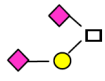
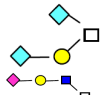
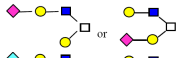
Fig. 3. (A) The Complement Dependent Cytotoxicity (CDC) assay to demonstrate the induction by anti-T cell receptor β subunit (TCRβ) MAb, RP215 and goat anti-human IgG to OC-3-VGH. Lane 1: complement only. Lane 2: normal mouse IgG (NMIgG). Lane 3: NMIgG and complement. Lane 4: anti-TCRβ MAb. Lane 5: anti-TCRβ MAb plus complement. Lane 6: RP215. Lane 7: RP215 plus complement. Lane 7: goat anti-human IgG (GαHIgG). Lane 8: GαHIgG plus complement. Double asterisks indicate statistical significance of $p<0.01$. Both A and B were modified from (Lee *et al*., 2012e) with permission. (B) The TUNEL apoptosis assay to demonstrate the increase in apoptosis of cultured OC-3-VGH cancer cells in response to treatment for 48 antibodies with the following antibodies (10 μg mL^{-1}): lane 1: NMIgG, lane 2: anti-human IgM, lane 3: anti-human IgG, lane 4: RP215, lane 5: normal rabbit IgG, lane 6: rabbit anti-T Cell Receptor β subunit peptide 1 (TCRβ1) and lane 7: rabbit anti-T Cell Receptor β subunit peptide 2 (TCRβ2). Single asterisk and double asterisks indicate statistical significance of $p<0.05$ and $p<0.01$, respectively. Both A and B were modified from (Lee *et al*., 2012e) with permission.

Table 4. Expression of human T Cell Receptor (TCR), IgG and selected CD markers in different cancer cells and two fibroblast cell lines, as detected by RT-PCR[a]

Origin	Designation cell line	ATCC No.	TCR (α)	TCR (β)	IgG (Fc)	CD3[a]	CD4	CD8	Cell Adhesion Molecules (CD47, 54, 58, 147)[b]
Breast	MDA-MB-231	HTB-26	+++[c]	++	+	-	-	-	++
Cervical	C-33A		+	+	+	-	-	-	++
Colorectal	HCT115		++	+	±	-	-	-	++
	SW-48	CCL-231	-	±	±	-	-	-	++
	HT-29	HTB-38	-	±	+	-	-	-	++
Hepatocellular	Hep3B	HB-8064	++	+	+	-	-	-	++
Lung	Calu-6	HTB-56	+++	+	+	-	-	-	++
	H441	HTB-174	++	+	+	-	-	-	++
	WI-38[d]	CCL-75	+++	++	+	-	-	-	++
	A549	CCL-185	+	-	+	-	-	-	++
	MRC-5[d]	CCL-171	-	-	+	-	-	-	++
Lymphoma	Hel		+	±	±	-	-	-	++
MMAN			+	-	+	-	-	-	++
	Raji	CCL-86	+++	+++	++	-	-	-	++
Melanoma	MMRU		++	-	+	-	-	-	++
Ovarian	SKOV-3	HTB-77	+++	+	+	-	-	-	++
	OVCAR-3		+	±	++	-	-	-	++
	OC-3-VGH		++	+	+	-	-	-	++
Prostate	Du 145	HTB-81	+	±	+	-	-	-	++
T-cell leukemia	Jurkat	TIB-152	+++	+++	+	±	-	-	++
Bone marrow	K562	CCL-243	+	±	+	±	-	-	++

Obtained from (Lee *et al*., 2012c) with permission. a; As a positive control, human peripheral blood cells showed expression of CD3, CD4 and CD8. b; All the cell adhesion molecules including CD47, CD54, CD58 and CD147 expressed among all the cancer cell lines listed. c; Relative expression level follows the order of +++ (strong positive), ++, +,±(very weak), -(negative). d; Normal hyperplastic and proliferated fibroblast cell lines derived from fetal lung tissue

Table 5. Comparative profiles of permethylated O-linked glycans of human IgG and five different CA215 samples

Sample ID	Observed Mass m/z [M+Na]+	Proposed structure	Structure
CA215 (lots: A, B and C)[a]	534	GalNAc1Gal1	
CA215 (lots: A and B)	708	GalNAc1Gal1Fuc1	
CA215 (lots: A, B, D, C, E and F)[b]	896	GalNAc1Gal1NeuAc1	
CA215 (lots: C, E and F)	926	GalNAc1Gal1NeuGc1	
CA215C	940c	GalNAc1GlcNAc1NeuAc1	
CA215(lots: A, B and C)	1140	GalNAc1GlcNAc1Gal1NeuAc1	
CA215 (lots: C, D, E and F)	1257	GalNAc1Gal1NeuAc2	
CA215 (lots: C, E and F)	1317	GalNAc1Gal1NeuGc2	
CA215 (lots: A, B, C, E and F)	1345	GalNAc1GlcNAc1Gal2NeuAc1	
CA215 (lots: C, E and F)	1375	GalNAc1GlcNAc1Gal2NeuGc1	

Obtained from (Lee and Azadi, 2012) with permission. a; CA215 lots A, B and C were from OC-3-VGH ovarian cancer cells (CA215-OC-3) lots A and B were obtained through acid elution, whereas lots C, D, E and F were obtained through elution with 3M urea. b; Lot CA215D was obtained by an additional purification of urea-eluted CA215 (S15K-100425) with goat anti-human IgG affinity column followed by the same analysis (CA215D is designated as affinity-purified cancerous IgG). CA215 lots E and F were from C-33A cervical cancer cells (CA215-C33A). c; Detected by MALDI-TOF MS method but not found by NSI-MS method. d; N-acetylgalactosamine (□), N-acetylglucosamine (■), Fucose (▲), Galactose (●), N- acetylneuraminic acid (♦) and N-glycolylneuraminic acid (♦)

A number of experimental observations have been documented to indicate that RP215-specific epitope is carbohydrate-associated in CA215 (Lee and Azadi, 2012). Initially it was observed that RP215 immunoactivity was decreased significantly upon treatments with mild periodate at neutral pH, or high temperatures (100oC, 15 minutes), or at extreme pH (< 2 or < 12.0) (Lee *et al*., 2006; Lee and Ge, 2010). Culture of cancer cells in serum-free medium also resulted in a total loss of RP215 immunoactivity (Lee *et al*., 2010). On the other hand, the deduced amino acid sequences of Fc regions of cancerous immunoglobulins (IgG or IgA) were shown to be almost identical to those of normal human immunoglobulins (Lee and Ge, 2009). This would suggest that RP215-specific epitope is carbohydrated but not peptide structure-associated. Furthermore, RP215-epitope(s) were detected in many other unrelated proteins with no mutual homology in their respective amino acid sequences (Lee *et al*., 2012e). Finally, aberrant glycosylations of cancerous immunoglobulins could result in the creations of RP215-associated "sugar" epitope and low immunoactivity when compared to those of normal human immunoglobulins (Lee *et al*., 2012b).

1.14. RP215-Based Enzyme Immunoassays and Clinical Diagnostic Applications

Judging from the molecular and epitope nature, CA215 should be considered as pan-cancer bio-markers. In view of the existence of multiple RP215-specific sugar epitopes, RP215 alone can be used for sandwich immunoassays for determination of shed or soluble forms of CA215. Therefore, serum levels of CA215 can be determined quantitatively for diagnostics and monitoring of human cancer derived from different tissue origins (Lee, 2009). Patient serum specimens with confirmed cancer diagnosis were subject to analysis by enzyme immunoassays. Normal human serum specimens were used as the negative control for comparative studies. Results of such analysis with more than 500 serum specimens from patients of over ten different cancers are presented as a scattergram in **Fig. 4**. Based on the results of such analysis, the positive rates vary significantly with different types of cancers and also levels among individuals with the same type of cancer. Further in-depth analysis with serum specimens of ovarian and cervical cancers was performed to assess the clinical diagnostic utility of CA215 as pan-cancer bio-markers (Lee, 2009; Lee *et al*., 2009b). It was generally established that serum CA215 levels are well correlated with the cancer staging of patients under consideration (Lee, 2009). Furthermore, the tumor burden of a given cancer patient can also be reflected from the serum CA215 levels, as radiation and surgical operations could significantly reduce the serum level of CA215 in cancer patients after one week (Lee, 2009; Lee *et al*., 2009b).

Table 6. N-linked and O-linked glycosylation site mappings of CA215. Fc refers to the constant region of the immunoglobulins. Fab represents the variable region of the immunoglobulins. Lot CA215C (urea-eluted) was used for this analysis. The source protein BLAST service was: http://blast.ncbi.nlm.nih.gov/Blast.cgi

Accession number	Peptide detected[a]	Peptide sequence homology of proteins (%)
I. CAC12842.1	1. EEQFNSTFR	Immunoglobulin heavy chain (Fc) (100%)
II. CAA04843.1	2. EEQFNSTYR	Immunoglobulin heavy chain (Fc) (100%)
III. AAB60643.2	3. LSVPTSEWQR	Cathepsin S (100%)
IV. AAK68690.1	4. FTCLATNDAGDSSK	Hemicentin (100%) Titin (100%) Palladin isoform 4 (92%) LRN4 (78%) (IgSF proteins)
V. AAD38158.1	5. DTLMISR	Immunoglobulin heavy chain (Fc) (100%)
VI. AAC39746.2	6. GYLPEPVTVTWNSGTLTNGVR	Immunoglobulin heavy chain (Fab) (90%)
VII. AAN76042.1	7. SVSLTCMINGFYPSDISVEWEK	Immunoglobulin heavy chain (Fc) (90%)
VIII. CAJ75462.1	8. QSSGLYSLSSVVSVTSSSQPVTCNV	Immunoglobulin heavy chain (Fab and Fc) (100%)
IX. ABY48864.2	9. VYTMGPPREELSSR	Immunoglobulin heavy chain (Fc) (98%) IgA variable region (89%) IgM (98%)
X. NP_001139647.1	10. TFPSVR	Zinc finger protein 414 isoform I (100%) Forkhead box protein C2 (100%) Immunoglobulin heavy chain variable region (83%)

Obtained from (Lee *et al*., 2012e) with permission. a; Bold letters indicate glycosylation sites

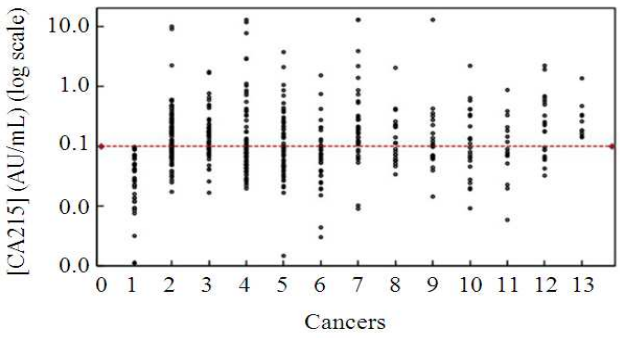


Fig. 4. Scattergram to reveal serum CA215 levels and positive rates from normal individuals (n = 52) and cancer patients (n = 505). Lane 1: normal individuals with no cancer as negative control (5% positive, n = 52). Lane 2: lung cancer (52% positive, n = 58). Lane 3: liver cancer (74% positive, n = 58). Lane 4: colon cancer (44% positive, n = 95). Lane 5: ovarian cancer (59% positive, n = 68). Lane 6: prostate cancer (40% positive, n = 42). Lane 7: breast cancer (71% positive, n = 44). Lane 8: kidney cancer (38% positive, n = 42). Lane 9: esophageal cancer (61% positive, n = 23). Lane 10: stomach cancer (60% positive, n = 30). Lane 11: pancreatic cancer (41% positive, n = 17). Lane 12: cervical cancer (51% positive, n = 33). Lane 13: lymphoma cancer (83% positive, n = 12). The dash line indicates the cut-off value of 0.1 Au/mL. Statistical analysis was completed by the T test for pair-wise comparison. The positive rates of all different cancers were statistically significant at $p<0.05$. Obtained from (Lee *et al*., 2009b) with permission

As a pan-cancer bio-marker, CA215 can be used in combination with more tissue-specific cancer markers for a better clinical diagnosis (Lee *et al*., 2009b). For example, in the case of ovarian cancer, the combined use of CA215 and CA125 could significantly improve the detection diagnostic sensitivity. Similarly, Alpha Fetoprotein (AFP) and CA215 can be combined for a better diagnosis of hepatoma as the detection rate of using both cancer markers is higher than that of a single cancer marker (Lee *et al*., 2009b; Park *et al*., 2011).

To further document that CA215 can be a useful serum pan-cancer bio-marker, Western blot assay was employed to detect cancerous immunoglobulins from cancer patients with high serum levels of CA215. These cancer patient serum specimens included those of the esophagus, stomach, breast, colon and liver. With RP215 as the unique probe, cancerous immunoglobulins (mainly IgG) from these serum specimens can be unambiguously detected by observed proteins bands with a molecular weight of 160 kDa for intact IgG and 50-55 kDa for IgG heavy chain subunits (Lee *et al*., 2009b). The results of this analysis enabled us to make a close link between CA215 and cancerous immunoglobulins (Lee *et al*., 2009b).

1.15. Applications of RP215 mAb for the Clinical Status Assessment of Cancer Cells in Humans

RP215 mAb reacts specifically with the unique "sugar" epitope of cancer cell-expressed CA215, such as a variety of IgSF proteins, including cell adhesion molecules. Bindings of RP215 on these IgSF proteins might affect the proliferation and migration (metastasis) of cancer cells *in vivo* (Barclay, 2003; Lee *et al*., 2012e). Expressions of RP215-specific epitope were found to be well correlated with the status of cancer cells and cancer stem cells. Higher expressions of RP215 epitope seem to take place in the metastatic states of cancer cells (Qiu *et al*., 2012a) than those of cancer in situ. Therefore, it is reasonable to hypothesize that RP215 mAb can be used as a diagnostic tool to study the clinical status of cancer cells in terms of their potential for proliferation, migrations and prognosis *in vivo*.

1.16. Assessment of RP215 Monoclonal Antibody as Anti-Cancer Drugs

Although not completely understood, it is generally accepted based on experimental evidence that cancerous immunoglobulins are essential for the growth and proliferation of cancer cells *in vitro* or in vivo. RP215 mAb recognizes unique "sugar" epitopes of cancer cell-surface expressed CA215 glycoproteins, which consist mainly of cancerous immunoglobulins plus other minor IgSF components. Therefore, it is reasonable to assume that RP215 mAb can be used to target cancer cells by inducing apoptosis, CDC and ADCC reactions in vivo upon antibody binding on the cell surface. Biological and immunological studies summarized in this review may provide meaningful information and "proof of concept" regarding the suitability of humanized RP215 mAb as an anti-cancer drug in humans.

Since most atypical immunoglobulins with RP215-specific epitope were found in immune privileged

sites of normal human tissues, such as the neuron, testes, eyes and placenta (Chen *et al*., 2009; Hu *et al*., 2008). Therefore, infusion of humanized RP215 mAb may not be toxic to the human body. On the contrary, human cancerous tissues may be preferentially targeted due to abundant expressions of RP215-specific epitope on the cancer cell surface.

2. CONCLUSION

2.1. Implications of Cancerous Immunoglobulins and Ca215 To Cancer Immunology

Universal expressions of atypical immunoglobulins by cancer cells of different tissue origins should be generally accepted in cancer research, even though the etiology is unknown (Chen *et al*., 2009; Hu *et al*., 2008). With the identification of RP215-specific carbohydrate-associated epitope, more information about the immunology of cancer cells was unfolded (Lee *et al*., 2008; 2012e). Surface bound CA215 glycoproteins expressed by all cancer cells consist not only of immunoglobulins, but also a number of immunoglobulin Superfamily (IgSF) proteins, including T cell receptors, MHCI and MHCII molecules, cell adhesion molecules, as well as cytokine receptors (Lee *et al*., 2012e). The characteristic secondary domain structures of IgSF proteins enable the additional glycosylations of this specific group of molecules in cancer cells (Barclay, 2003), leading to the additional unique glycosylated epitope recognized by RP215 mAb. Besides the surface bound immunoglobulin molecules, other IgSF proteins can also be targeted by this RP215 mAb to induce apoptosis of cancer cells. Furthermore, CDC and ADCC reactions are also common and effective in the immunotherapy of cancer cells *in vivo*.

During the extensive molecular analysis of cancer cell expression of CA215 with the unique RP215 specific epitope, many glycoproteins involved in the normal immune system were identified and found to be highly expressed among many types of cancer cells, but rarely found in non-immune related cells in humans. Functional significance of these IgSF proteins remains to be explored (Barclay, 2003). It remains to be established if cancerous immunoglobulins play any significant roles in the innate immunity of cancer cells (O'Neill, 2008; So and Ouchi, 2010). The widespread expression of T cell receptors in cancer cells should be addressed in relation to the T cell activation and/or T cell tolerance in cancer immunotherapy (Witherden *et al*., 2010). Finally, the high expression of IgSF-related cell adhesion molecules may be related to the metastasis of cancer cells. Some of these puzzles may have to be resolved for further understanding of cancer immunology, as well as for the effective cancer immunotherapy in humans (Weber, 2007). The preferential attachment of the RP215-specific epitope to cancer cell-expressed CA215 glycoproteins but not the same proteins in the majority of normal cells remains to be investigated (Lee *et al*., 2008; 2012c).

We believe that the unique RP215-specific epitope(s) arises from the aberrant expressions of certain glycosyl transferases among all cancer cells. This question can be answered only when the epitope structures and enzymes involved in the "sugar" attachment (Lee and Azadi, 2012) has been fully elucidated.

Despite a significant advancement regarding our understanding of cancerous atypical immunoglobulins, their general and special roles in the growth and proliferation of cancer cells, as well as their molecular evolutionary origins, remains to be elucidated. Additional experimental and clinical studies would be required to address these questions. Nevertheless, a better understanding of cancerous immunoglobulins and CA215 will not only improve our knowledge in the field of cancer immunology but also benefit cancer patients in the area of cancer monitoring and therapeutic treatments.

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3.1. Conflict of Interest

Gregory Lee is cofounder of Vancouver Biotech Ltd. For the remaining authors, none are declared.

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