

Review

# Functional Roles of Antigen Receptors Expressed by Cancer Cells and Clinical Applications

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## Abstract

RP215 is a monoclonal antibody generated in mice immunized against OC-3-VGH ovarian cancer extract. The cognate cancer-associated antigen, designated as CA215 was affinity-isolated from cancer cell extract and subject to tryptic peptide analysis by MALDI-TOF MS method. The unique epitope recognized specifically by RP215 was shown to locate mainly on the antigen receptors such as immunoglobulin heavy chains and T cell receptors as well as other glycoproteins expressed by cancer cells, but not those from normal immune cells. The primary carbohydrate structure associated with RP215-specific epitope was elucidated and suggested to be O-glycans (commonly known as Sialyl-T antigen). By extensive IHC studies, RP215 was found to react with most of epithelial cancers, but rarely with normal ones. By using RP215 as the unique probe for cancerous antigen receptors, extensive in vitro studies are performed with culturing cancer cells. Similar to anti-antigen receptors, RP215 was shown to induce apoptosis and complement-dependent cytotoxicity to almost all cancer cells. Gene regulation studies were also performed by using semi-quantitative RT-PCR methods with selected genes involved in the regulations of immunoglobulins, T cell receptors as well as toll-like receptors. RP215 and anti-antigen receptors were shown to affect similarly the gene expression levels of incubated cancer cells with high mutual correlations. Cancerous antigen receptors and RP215 were used separately as the affinity ligands to identify those human serum proteins or fragments interacting with cancer cells. The results indicate that majority of commonly detected human serum proteins ( $\geq 85\%$ ) are those of known pro-cancer or anti-cancer protein components. It was hypothesized from these studies that antigen receptors on cancer cells may serve to interact with those human serum proteins which are essential to growth/proliferation of cancer cells or protection of cancer cells in our normal body environment. By understanding their functional roles, RP215 may serve as a unique tool to target cancer cells in immunodiagnostic applications to monitor serum CA215 levels in cancer patients or in immunotherapeutic applications for cancer therapy or treatments.

Keywords: RP215 monoclonal antibody, CA215, cancerous antigen receptors, CAT-T cell technology, cancer immunotherapy and treatment

## Introduction

### Discovery of antigen receptors in cancer cells

Antigen receptors including immunoglobulins and T cell receptors were known to be expressed by cancer cells for more than two decades through early molecular biological studies [1-3]. More comprehensive biological and immunological studies were reported in early 2000's [3, 4]. It was observed that immunoglobulins can be expressed by

human cancer cells for essential growth/proliferation. Apoptosis can be induced through incubations with anti-immunoglobulins or immunoglobulin-related SiRNA in vitro and in vivo. However, the functional roles of cancerous immunoglobulins are still poorly understood when compared with those of our normal immune system [4].

### RP215 Monoclonal antibody associated with antigen receptors expressed by cancer cells

In 1987, among the monoclonal antibodies generated in mice immunized with OC-3-VGH ovarian cancer cell extract, RP215 was selected and confirmed to react with carbohydrate-associated epitope localized on immunoglobulin heavy chains expressed by cancer cells [5]. Following MALDI-TOF MS analysis, it was documented that the tryptic peptides of the affinity-isolated cancer-associated antigens, CA215 consist mainly of immune-related glycoproteins ( $\geq 85\%$ ). These includes antigen receptors ( $\geq 50\%$ ), antigen presenting molecules, cell adhesion molecules and others related to immunoglobulins superfamily glycoproteins [5, 12].

Judging from this analysis, structure and functional roles of cancerous antigen receptors in cancer cells should be further explored with extensive molecular and immunological studies. RP215 may serve as the preferred immune probe for such investigations and similar results compared with those of anti-antigen receptors [5, 6]. Understanding of their mechanisms of action as well as gene regulations on cancer cells might be essential for future applications in cancer therapy and treatments, when antigen receptors are selected as targets.

### Structural Elucidations of RP215-Specific Carbohydrate-Associated Epitope

RP215 was shown to react mainly with protein bands of 55 KDa, when Western Blot assays were performed either with crude cancer cell extract or with affinity-purified CA215 [5]. Treatments of CA215 or cancer cell extract with NaIO<sub>4</sub> resulted in a loss of RP215-immunoactivity either by Western Blot or other immunoassays, indicating the carbohydrate nature of the RP215-specific epitope. Judging from the analytical results of MALDI-TOF MS method and Western Blot assay [5, 6], it was clearly suggested that RP215 reacts mainly with heavy chains of cancerous immunoglobulins.

The exact epitope locations in heavy chains of immunoglobulins was determined by RP215-based sandwich enzyme immunoassays. By using RP215-coated microwells and purified CA215 as the antigen, RP215-HRP, goat anti-human IgG-Fc, and goat anti-human IgG-Fab were added in dose-dependent CA215 immunoassays. It was clearly demonstrated that goat antihuman IgG-Fc (1 $\mu$ g/ml) has little effect on CA215 signal, but goat antihuman IgG Fab (1 $\mu$ g/ml) resulted in a total inhibition CA215 binding to RP215 in the immunoassays. Therefore, it was judged that RP215-specific epitope is attached to Fab (variable domains) of heavy chains immunoglobulins, but not in the Fc domains. Results of such analysis are presented in Figure 1 A.

The carbohydrate-associated epitope recognized by RP215 can either be N-linked or O-linked glycans. This

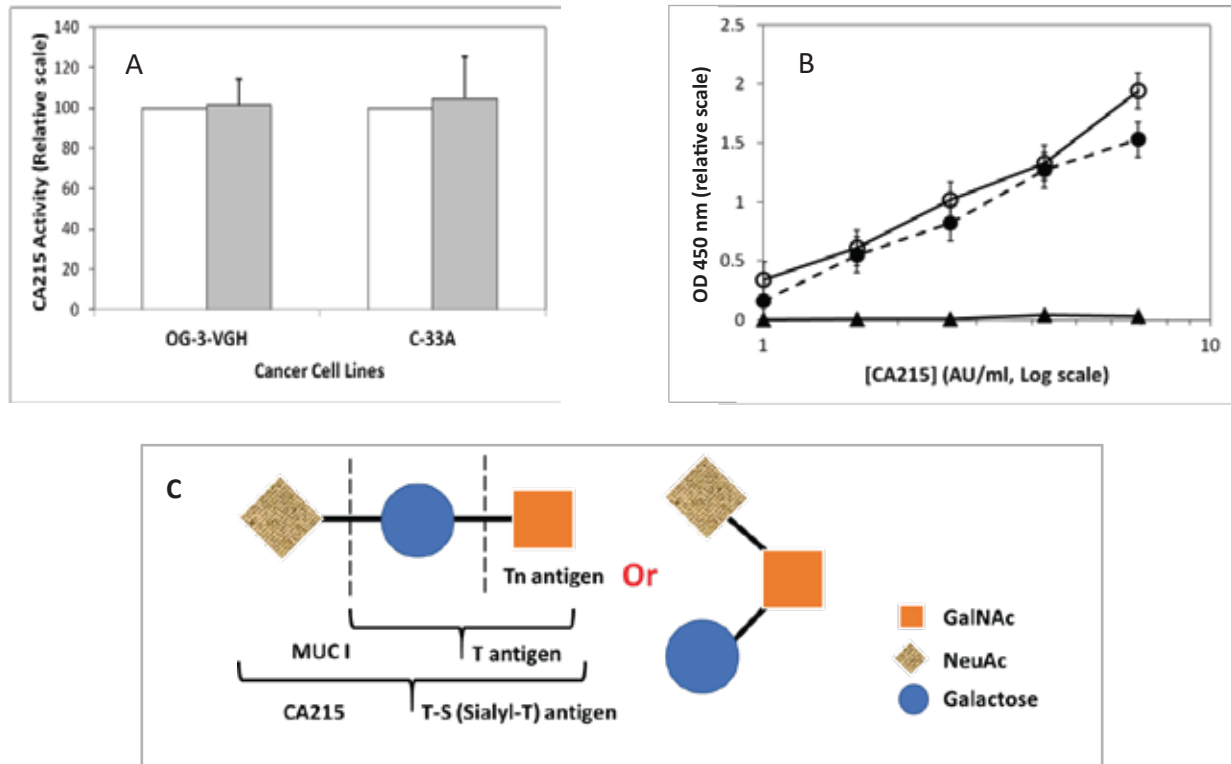
was determined by using co-incubation of culturing cancer cell with tunicamycin which is a known inhibitor of N-glycan [7]. Following 48 hr incubation, with either C33A cervical or OC-3-VGH ovarian cancer cells, it was observed that CA215 immunoactivity was not affected by tunicamycin (Figure 1B). Therefore, we assumed that carbohydrate-associated epitope is O-linked glycan attached on the variable regions of immunoglobulin heavy chains.

Through collaborations with Complex Carbohydrate-Research Centre in Athens, GA, the glycosyl linkage analysis was performed with cancerous immunoglobulins which were sequentially affinity-purified by affinity columns with RP215 or anti-immunoglobulins as the ligands, respectively. The affinity-purified cancerous immunoglobulins were subject to glycosyl-linkage analysis. From the results of such analysis, the only O-linked glycan structures associated with RP215- epitope are Core 1 structure with 3-linked GalNAcitol and/or 3,6-linked GalNAcitol as presented in Figure 1 C. The structure of O-linked glycan appears to be Sialyl T antigen which was known to be associated with many cancer cells due to aberrant termination of truncated O-glycans, attached to proteins in cancer cells. For example, O-glycans of STn, T, Tn in the mucin 1 (MUC 1) glycoproteins have been reported and used as targets for CAR-T cancer therapy [8, 9].

### Tissue Specificity of Cancerous Antigen Receptors

From early studies by using RT-PCR for gene expression of cancer cells, it was revealed that immunoglobulins and T cell receptors can be co-expressed by cancer cells of one single cell origin [1] as well as over 80-90% of established cancer cell lines [10]. Immunohistochemical tissue staining studies with RP215 as the immune probe for cancerous antigen receptors also indicated widespread expressions of RP215-epitope among various cancer tissues [5, 11]. Among these are ovary (n = 87, 64.4%), cervix (n = 51, 84.3%), endometrium (n = 36, 77.8%), stomach (n = 93, 49.5%), colon (n = 87, 43.6%), esophagus (n = 56, 75.7%), lung (n = 58, 31%), and breast (n = 59, 32.2%). In contrast, low percentages of positive tissue staining was observed for the cancer of liver (n = 60, 3.5%) and prostate (n = 22, 10%). In the case of T cell receptors, greater than 80% of the cancer cell lines show positive staining [12]. However, little or no expressions of co-receptors or co-stimulators such as CD3, CD4, and CD8 were detected for almost all cancer cells tested. The results suggest that cancer cells do not have functional T cells commonly observed in normal immune system [12].

### Functional Assessment of Cancerous Antigen Receptors Through Studies of Induced Apoptosis and Gene Regulations



**Figure 1 (A).** Effects of 48 hr tunicamycin treatments (1  $\mu\text{g}/\text{ml}$ ) on CA215 activity in the supernatant of two cultured cancer cells for OC-3-VGH and C-33A. To each cell line the left column is the negative control (assuming 100% activity) without drug-treatment, and the right column is the relative CA215 activity following the drug treatment (expressed in triplicate).

**1 (B).** RP215-based enzyme immunoassays to reveal dose-dependent signal or activity of CA215 (o) expressed in AU/ml) and effects of goat antihuman IgG-Fc (1 $\mu\text{g}/\text{ml}$ ) (●) and goat antihuman IgG-Fab (1 $\mu\text{g}/\text{ml}$ ) (▲) on CA215 signal or activity.

**1 (C).** Elucidated and proposed O-glycan structure associated with RP215-specific epitope as well as that of MUC 1 for comparisons.

### Induced apoptosis and complement-dependent cell lysis by RP215 and anti-antigen receptors

Antigen receptors are known to be expressed on cancer cells and apoptosis can be induced upon 24 and 48 hr incubation of culturing OC3-VGH ovarian cancer cells and many others with RP215 or with anti-antigen receptors such as goat anti-human IgG and anti-T cell receptors [6, 12]. In the case of RP215 at 1  $\mu\text{g}/\text{ml}$  or 10  $\mu\text{g}/\text{ml}$ , as much as 30% to 50% of cancer cells underwent apoptosis following 24 to 48 hr incubation, when TUNEL assays were employed [6]. When goat anti-human IgG (1 and 10  $\mu\text{g}/\text{mL}$ ) was used instead as the ligand, apoptosis of incubated cancer cells was induced to a similar extent as that of RP215. In contrast, the negative control with normal mouse or rabbit IgG of the same concentrations did not result in significant induced apoptosis to cancer cells. Purified anti-T cell receptors  $\beta$  (rabbit IgG fractions) were under the same assay conditions, apoptosis of incubated cancer cells was also induced at concentration of 10  $\mu\text{g}/\text{mL}$ . The apoptosis was similarly induced when several other cancer cell lines, namely, DU145 (prostate), A549 (lung), C33A (cervix) and MDA-MB 435 (breast) were

used instead for studies under similar conditions of TUNEL assays [6, 12]. RP215, antihuman IgG or anti-T cell receptors gave similar results of induced apoptosis, when different cell lines were employed for similar studies. In conclusion, RP215 and the two anti-antigen receptors were confirmed to react with the antigen receptors on cancer cells, and similar degrees of induced apoptosis can be observed to cancer cells. Complement-dependent cytotoxic reactions (CDC) could result in cell lysis in the presence of complement following 2hr co-incubation with all different types of cancer cells [6, 12]. In contrast, normal mouse or rabbit IgG of the same concentrations resulted in little or no complement-dependent cell lysis. Effects of RP215 or anti-antigen receptors on gene expressions of culturing cancer cells

By semi-quantitative RT-PCR, the levels of gene expression change significantly upon the treatments of RP215, or anti-antigen receptors to cancer cells in culture for 24 hours or 48 hours [13]. More than ten different genes were selected for gene regulation analysis. They are generally involved in gene expressions of immunoglobulins and T cell receptors in normal immune systems as

well as toll-like receptors in innate immunity. The selected genes are listed as follows: NF $\kappa$ B-1, IgG, TCR, P21, ribosomal P0, ribosomal P1, cyclin D1, c-fos and EGFR [14, 15] as well as toll-like receptors (TLR-2, -3, -4, -6, -7, and -9) [16,17]. Comparative results of the relative gene regulations changes of cancer cells are given in Table 1. Generally speaking, upon treatments of cancer cells with RP215 or anti-antigen receptors, up-regulations (20 to 25%) of genes expressed by NF $\kappa$ B-1, IgG, TCR, P21, and ribosomal P1 were observed. On the other hand, down regulation of Cyclin D1, c-fos, EGFR, and ribosomal P0 were also consistently observed (10-30%). Gene regulation patterns did not change significantly when C33A cervical cancer cell line was employed instead for similar gene regulation studies [13].

#### Effects on gene regulations of toll-like receptors

#### expressed by cancer cells

RP215 and anti-antigen receptors were also demonstrated to affect the gene regulation patterns of those involved in innate immunity such as toll-like receptors. Gene regulation changes were studied with same cancer cell lines and compared [13]. Similar changes in gene regulation levels were observed for either of these ligands. Generally speaking, upon treatments with RP215 or anti-antigen receptors, significant changes in gene expressions were observed to all of the toll-like receptors (10% to 90%). The up-regulation of TLR-3 gene was found to increase by 226%, 143%, and 219%, respectively for RP215, anti-IgG and T-cell receptors [18]. On the other hand, down-regulations of TLR-4 and TLR-9 genes were consistently observed [19]. Results of such a semi-quantitative RT-PCR analysis of gene regulation changes are semi-qualitatively presented in Table 1 [13].

**Table 1.** Effects of treatments of cultured OC-3-VGH ovarian cells with different antibodies on the expressions of various genes\*.

Gene	G $\alpha$ hIgG	R $\alpha$ TCR $\beta$	mRP215
IgG	0	0	0
TCR	↑	↑↑↑	↑↑↑
NFKB-1	↑↑↑	↑↑↑	↑↑↑
Cyclin D1	↓	↓↓	↓↓↓
P21	↑↑↑	↑↑↑	↑↑↑
c-fos	↓	0	↓
Po	↑↑↑	↓↓↓	0
P1	↑↑	↑	↑
EGFR	↑↑↑	↓↓↓	↓↓↓
TLR-3	↑↑↑	↑↑↑	↑↑↑
TLR-4	↓↓↓	↓↓↓	↓↓↓
TLR-9	↓↓↓	↓↓↓	↓↓↓

\*Effects of treatments of cultured OC-3-VGH ovarian cancer cells with goat anti-human IgG (G $\alpha$ hIgG), rabbit anti-T CR $\beta$  (R $\alpha$ TCR $\beta$ ), and murine RP215 (mRP215), respectively, on the expressions of genes involved in cell proliferation, protein synthesis, cell cycle regulations, and the innate immunity. The expressions of these genes involved were adjusted with that of GAPDH. The negative control with normal mouse IgG was considered 100% in all cases. Antibody concentration of 10  $\mu$ g/ml was used for all parallel studies of comparison. (0) less than 10% gene expression increase or decrease, (↑) 10-20% gene expression increase, (↑↑) 20-30% gene expression increase, (↑↑↑) more than 30% gene expression increase, (↓) 10-20% gene expression decrease, (↓↓) 20-30% gene expression decrease, (↓↓↓) more than 30% gene expression decrease. Obtained and modified from [47] with permission.

### Similar gene regulation profiles of cancer cells by RP215 and cancerous antigen receptors

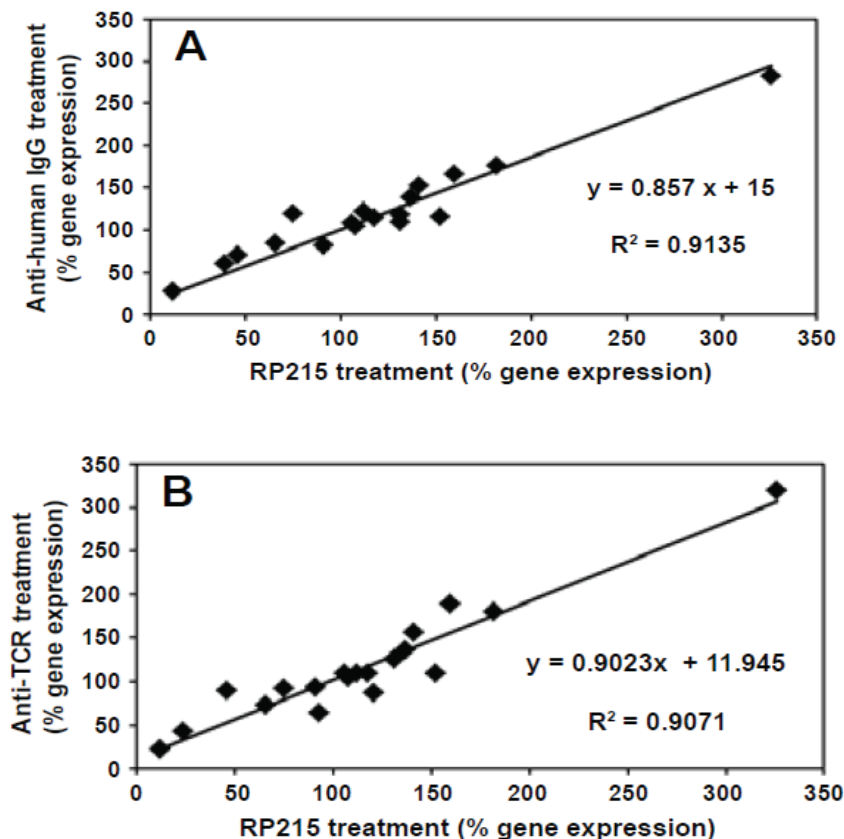
Based on results of semi-quantitative RT-PCR analysis, changes in levels of gene expressions by treated cancer cell lines revealed a high degree of similarity among the three antigen receptor ligands. For example, in response to RP215 treatment of cancer cells, changes in gene expression levels are well correlated with those of anti-human IgG ( $R^2 = 0.9135$ ) or with those of anti-T cell receptors ( $R^2 = 0.9071$ ). They are demonstrated in Figure 2 [13]. These observations strongly support our initial assumptions that RP215 and anti-antigen receptors may affect the treated cancer cells through more or less the same molecular mechanisms of Action [13]. Therefore, cancerous antigen receptors can be targeted by RP215 with similar effectiveness and specificity to cancer cells [13].

Toll-like receptors (TLRs) are known to be a family of receptors in innate immunity involved in pathogen-associated molecular patterns for host-defense against infections [12,16]. They also play important roles in carcinogenesis and proliferation of cancer cells which are consistently regulated by cancerous antigen receptors as demonstrated in this study. Since toll-like receptors are important

components for innate immunity, expressions of cancerous immunoglobulins and/or T cell receptors may in part explain their essential requirements for growth and survival of cancer cells [18, 19]. The involvements of both adaptive and innate immunity in gene regulations of cancer cells are drawing more attentions lately and may have far reaching implications in cancer immunology; not only for basic understanding of cancer, but also for their potential therapeutic applications as well.

### Dual and Distinct functional Roles of Antigen Receptors as Expressed by Cancer Cells Interactions between Cancer Cells and Human Serum Proteins

Under normal human body environments, cancer cells acquire the ability to survive and proliferate and at the same time they are capable of eliminating hostile protein elements in human circulations. Based on this hypothesis, immunology of cancer cells may be different from that of the conventional one [20, 21]. In conventional immune system, the coordinations of separate B cells and T cells are required to generate antibodies and cellular immunity to eliminate or neutralize hostile foreign pathogens in adaptive immunity [20, 21]. However, this may



**Figure 2.** Correlation analysis of changes in gene expression levels with different corresponding pairs of antibody treatments. Genes selected for correlational analysis include IgG, TCRa (TCRa), NFKB-I, cyclin D1, P21, c-fos, Po, P1, P2, EGFR, TLR-2, TLR-3, TLR-4, and TLR-9.

**2-(A):** RP215 and anti-human IgG treatments. The correlation coefficients ( $R^2$ ) is 0.9135

**2-(B):** RP215 and anti-TCR antibody treatments. The correlation coefficients ( $R^2$ ) is 0.9071.

not be the case in cancer cells. Both immunoglobulins and T cell receptors are co-expressed in one single cancer cell to perform somewhat different immunological/biological objectives. We believe that surface bound cancerous antigen receptors are also required to eliminate or neutralize any hostile human serum components or proteins for their survival. At the same time, they are capable of interacting with useful protein components for growth and proliferation of cancer cells. Based on these assumptions, simple experiments were designed to identify those human serum components (anti-cancer or pro-cancer proteins) to meet the expected purposes. Results of such experiments might help us resolve the specific functional roles of these antigen receptors in cancer cells.

CA215 was initially affinity-purified from the shed medium of culturing OC-3-VGH ovarian cancer cells with RP215 as the affinity ligand. Parallely, cancerous immunoglobulins were also affinity-isolated from the shed culture medium with anti-human IgG as the ligand. Following initial purifications, CA215 and cancerous immunoglobulins were used as separate affinity ligands in affinity chromatography. These two affinity columns were then used to capture human serum proteins or components which may interact with or can be adsorbed by either affinity ligands from pooled human serum samples. The affinity-purified human serum proteins from either affinity column were then subject to separate analysis by LC-MS/MS methods following MS analysis of tryptic digests of the affinity-purified human serum proteins or components. Many distinctive serum proteins or their components were unanimously identified by LC-MS/MS methods for further elucidation of mechanisms of action [20, 21].

### Pro-cancer and anti-cancer nature of identified human serum proteins

Out of hundreds of human serum proteins which were isolated and identified by LC-MS/MS, about 85% of these serum proteins were commonly detected from those isolated by either affinity columns [20, 21]. They were selectively listed in Table 2 [20] and classified in general, according to their pro-cancer or anti-cancer nature which has been known through previous studies by other investigators. For example, among the selected human serum proteins of pro-cancer nature are C46-binding protein [26], complement C3 [22], complement factor H [23, 24], serotransferrin [25, 26], and vitronectin [27]. C46-binding can prevent or protect cancer cells from complement attacks [22]. Complement C3 is capable of promoting cancer cell development and progression through various tumorigenic effects [22].

Complement factor H can protect cancer cells from complement-mediated lysis [23, 24]. Serotransferrin has been shown to be a growth factor for cancer cell proliferation, whereas vitronectin was known to be inducer of cancer cell differentiation, spreading, migration, and growth [25-27]. Among the anti-cancer human serum protein (components), anastellin was shown to inhibit tumor growth and metastasis in vivo [28]. Inter- $\alpha$ -trypsin inhibitor heavy chain 4 can lead to down regulation of tumor initiation and progression in multiple solid tumors [29].

Apolipoprotein A was known to suppress tumor growth and metastasis [30, 31]. Fibrinogen  $\beta$  chain has been shown to inhibit tumor vascularization in mouse models [32] whereas keratin type 1 cytoskeleton 9 is known to reduce drug resistance in breast cancer [33]. In summary, from

**Table 2.** Summary of the functional classifications of detected human serum proteins and/or fragments which are recognized by both CA215 and cancerous immunoglobulins according to respective pro-cancer or anti-cancer properties.

Functional activity <sup>a</sup>	Protein	Molecular weight (kDa)	Key notes related to cancer Pro-cancer
<b>Pro-cancer</b>	C4b-binding protein	67	Protects cancer cells from complement activation & attack
	Complement C3	187	Promotes cancer development & progression through various tumorigenic effects
	Complement factor H	139	Protects cancer cells from complement-mediated cytolysis
	Serotransferrin	77	Growth factor for cancer cell proliferation
	Vitronectin	54	Inducer of cancer cell differentiation, spreading, migration, & growth
<b>Anti-cancer</b>	35kDa inter- $\alpha$ -trypsin Inhibitor heavy chain 4	104	Down regulation leads to tumor initiation & progression in multiple solid tumors
	Anastellin	256	Inhibits tumor growth & metastasis in vivo
	Apolipoprotein A-1	31	Suppresses tumor growth & metastasis in animal tumor models
	Fibrinogen $\beta$ chain	56	$\beta$ 43-63 inhibits tumor vascularization in mouse models
	Keratin type I cytoskeletal 9	62	Down regulation is associated with increased drug resistance in breast cancer

a: references see text.

these studies, it can be established that quite a few human serum proteins were commonly detected or identified with known pro-cancer or anti-cancer properties. The interactions between human serum proteins (components) and CA215 or cancerous immunoglobulin ligands could simply be antigen-antibody binding reactions or may be receptor-ligand mediated. The mechanisms of action of these interactions should be individually investigated in the future [20].

Based on the results of these studies, the dual functional roles of cancerous antigen receptors or its associated CA215 can be clearly demonstrated [20, 21]. The hypothesis of dual functional roles of cancer antigen receptors may help to explain why cancer cells under our normal human environment can survive/proliferate and eliminate/neutralize hostile human serum components. Therefore, we believe that there are essential requirements for expressions of cancerous antigen receptors to fulfill specific functional roles for cancer cells.

### Potential Clinical Applications of RP215 Monoclonal Antibody

The universal appearance of antigen receptors on cancer cell surface has been well documented in this review and constituted to be one of important components in cancer cells. The functional roles of antigen receptors have been extensively studied by using RP215 as a unique immune probe for anti-antigen receptors [20, 21]. Therefore, cancerous antigen receptors are unique targets for cancer diagnostic and therapeutic applications, when RP215 was used as the unique probe.

RP215 is specific to carbohydrate-associated epitope of CA215, consisting mainly of cancerous antigen receptors [5]. RP215-based sandwich enzyme immunoassay was established for quantitation CA215 in serum specimens of cancer patients [34]. CA215 antigen standards were prepared from partially purified shed medium of culturing OC-3-VGH ovarian cancer cells. As cancer-associated antigen, CA215 was calibrated and determined in arbitrary units per mL (AU/mL). Typical RP215-based sandwich enzyme immunoassay is a two-hour single step procedure at 37°C with a sensitivity of 0.02 AU/mL [35, 36].

Serum levels of CA215 among patients with ovarian and cervical cancers were evaluated separately by the established enzyme immunoassays [34, 35]. In the case of ovarian carcinoma, positive rates of CA215 were determined to be 58% (n = 24), 86% (n = 7), and 70% (n = 40) for diseases of Stages I, II, and III, respectively [35]. By comparisons, the positive rates of CA215 for those patients with cervical carcinoma were determined to be 66% (n = 35), 94% (n = 18), and 71% (n = 7), for disease of Stage I, II, and III, respectively. In the case of benign tumors, such as endometriosis, and uterine myoma, much lower positive

rates of 10% (n = 10) and 26% (n = 23) were obtained, respectively [35]. Statistical analysis revealed that stage dependence of serum CA215 levels was demonstrated for either cancer, especially at Stage I vs. Stage II or III (P < 0.001). However, serum CA215 levels between Stage II and Stage III are not as clearly defined. Results of these clinical evaluations also revealed that serum CA215 levels remained at relatively high levels during the initial preoperative stages or within one week after surgical operations or after chemo- or radiation therapy [35]. In contrast, serum CA215 levels decreased significantly when determined seven days after surgical operations. The results may indicate that serum CA215 levels reflect the tumor burden of individual cancer patients.

RP215 was shown to react with carbohydrate-associated epitope on cancerous antigen receptors expressed by most of epithelial cancers [5, 6], RP215-based immunoassay should enable us to detect or determine CA215 levels from many cancers of diversified tissue origins [36]. In a large-scale clinical evaluation, serum levels of CA215 were determined from about 500 cancer patients which had been confirmed with either one of twelve cancers [36]. The same clinical specimens were also used to determine marker levels of other known cancer biomarkers including alpha-fetoprotein (AFP), carcino-embryonic antigen (CEA), cancer antigens such as CA125, CA15-3, and CA19-9,  $\beta$ 2-microglobulins and cytoskeleton 19 fragments (cyfra-21) [36]. To given specimens, the positive rates of CA215 were determined and compared with those of other biomarkers for different types of cancer including: lung, colon, liver, ovary, kidney, esophagus, pancreas, breast, and stomach [36]. The results of comparative studies with positive rates of individual biomarkers and those combined with CA215 are summarized and presented in Table 3. Based on such a comparative study, the positive rates of serum CA215 levels are also listed according to different types of cancer: lung (52%), colon (44%), ovary (59%), breast (38%), pancreas (51%) esophagus (61%), stomach (60%), kidney (38%), and lymphoma (83%). Positive rates of serum CA215 levels are also compared with other known cancer biomarkers, each of which is more tissue-specific than CA215 [35, 36]. Among these markers, CA125 is commonly used for detection and monitoring of ovarian cancer [37]. CA15-3 is suitable for cancer of breast [37], whereas CA199 is generally used for monitoring of pancreas, stomach and liver cancer [38]. CEA and  $\beta$  2-microglobulins are cancer biomarkers of broad tissue origins [38]. Cyfra-21-1 is commonly used for monitoring of lung cancer [40].

In Table 3, positive rates of CA215 and other biomarkers, singly and combined are summarized with six types of human cancer (lung, liver, ovary, esophagus, breast, and stomach). It was generally observed that the com-

combined use of CA215 and either one of these cancer biomarkers resulted in higher detection rates to a given cancer [36]. For example, in the case of ovarian cancer, positive detection rates of individual biomarkers are 59% each (n = 68 and 66, respectively). However, the positive rates of two biomarkers combined could increase to 82%. In the case of lung cancer, CA215 and Cyfra 21-1 assays gave positive rates of 52% (n = 112) and 50% (n = 52), respectively. When both biomarkers were combined for determinations, the combined positive rate could reach

77% (Table 3).

The clinical utility of CA215 and other known biomarkers was demonstrated from the immunoassay results of all of these available biomarkers [36]. Combinations of CA215 with other cancer biomarkers are certainly beneficial in increasing positive detection rates during routine monitoring of a given cancer patient. Therefore, potential clinical utility of CA215 as a pan cancer biomarker can be established for routine cancer monitoring [35, 36].

**Table 3.** Comparative positive detection rates of various cancers by CA215-based and other cancer-biomarker-based enzyme immunoassay kits.

CA215 (0.1Au/ml) <sup>d</sup>	Cancer	Lung (n)	Liver (n)	Ovary (n)	Esophagus (n)	Breast (n)	Stomach (n)
CEA (5 ng/ml)	I <sup>a</sup>	52% (112)	74% (58)	-	61% (23)	71% (44)	60% (30)
	II <sup>b</sup>	67% (33)	54% (35)		47% (19)	95% (20)	50% (14)
	III <sup>c</sup>	<b>94%</b>	<b>81%</b>		<b>65%</b>	<b>96%</b>	<b>70%</b>
AFP (20 ng/ml)	I	-	74% (58)	-	-	-	-
	II		50% (40)				
	III		85%				
CA125 (35 Au/ml)	I	52% (112)	74% (58)	59% (68)	61% (23)		
	II	85% (13)	85% (13)	59% (66)	50% (12)		
	III	<b>85%</b>	<b>92%</b>	<b>82%</b>	<b>75%</b>		
CA19-9 (37 Au/ml)	I	-	74% (58)	-	-	-	60% (23)
	II		55% (22)				75% (12)
	III		<b>82%</b>				<b>81%</b>
CA15-3 (30 Au/ml)	I	-	-	-	-	71% (44)	-
	II					83% (6)	
	III					<b>83%</b>	
B microglobulin (2.6 ng/ml)	I	-	74% (44)	59% (68)			
	II		83% (6)	90% (10)			
	III		<b>83%</b>	<b>100%</b>			
Cyfra21-1 (3.3 ng/ml)	I	52% (112)	-	-	-	-	-
	II	50% (52)					
	III	<b>77%</b>					

a: I: percent positive by CA215 only;

b: II: percent positive by other marker only;

c: III: percent positive by combining both CA215 and designated marker.

d: values in parenthesis are cut off value for each marker

### Immunotherapeutic applications of RP215 monoclonal antibody development of RP215-based anti-cancer drugs

Functional roles of cancerous antigen receptors have been described in this review. Anti-cancer properties of RP215 with carbohydrate-associated epitope of O-linked ST glycan has been elucidated through biological studies in vitro and models of nude mouse experiments [5,11]. Similar to anti-antigen receptors, RP215 was shown to induce apoptosis and complement-dependent cytotoxic reactions to culturing cancer cells [13]. Therefore, genes of murine RP215 were humanized to avoid complications

arising from the immunospecificity during human applications [41]. Generally speaking, the genes expressing the Fc domains of murine RP215 (mRP215) are replaced with human IgG, Fc. Modifications of FR domains in the Fab regions of murine RP215 should be made for compatibility of humanized forms, whereas those of CDR domains should be minimized to maintain binding specificity. Genes of humanized RP215 (hRP215) were reconstructed and expressed in CHO cells of high production yield. MRP215 and hRP215 were demonstrated to be bioequivalent or biosimilar in many in vitro biofunctional assays [41]. Basically, hRP215 can be produced in large quantity



for infusion to cancer patients and to achieve anti-cancer efficacy, similar to those available and utilized in many clinical applications.

### RP215 Applications in CAR-T Cell Constructs for Cancer Treatments

The methods described [41] for application of hRP215 as anti-cancer drugs would require infusion of gram or subgram quantities of antibodies as antibody-based anti-cancer drugs. Alternatively, the technology of CAR-T (chimeric antigen receptor – transfected T cells) are also being developed for therapeutic treatments of different human cancer [42, 43]. Generally speaking, genes of hRP215 in the Fab domains are engineered as ScFv and packaged into lentiviral vector for final formulations as chimeric antigen receptor (CAR). Patient's T cells are then isolated and transfected with hRP215-ScFv-linked CAR incorporated in lentiviral vector. Following in vitro expansion of CAR-T cells, the hRP215-CAR-transfected T cells are then infused to the autologous cancer patient for the induction of target-specific cytotoxic killings of cancer cells in vivo and to achieve expected therapeutic objectives [44]. HRP215 (ScFv)-linked CAR-T cells have been successfully constructed. The efficacy of cytotoxic killing of cancer cells was demonstrated through a model study in vitro with C33A cervical cancer cell line.

Typical results of this cytotoxic cell killing assay with concurrent cytokine releases are presented in Figure 3 (A, B, C, and D), respectively. Briefly, C33A cervical cancer cells (Target, T) were cultured for 8 hours with three different concentrations of hRP215-linked CAR-T cells (Effector, E). Under these experimental conditions, dose-dependent lysis of cancer cells was observed as shown in Figure 3A. The validation of hRP215-ScFv-CAR-T cell therapy was further documented by cytokine release assays of IL-2, IL-7, and INF  $\alpha$ , respectively (shown in Figure 3B, C, and D).

By using the same technology, CD19-CAR-T cells have been successfully utilized in the therapeutic treatments of liquid tumor, such as malignancy of blood cells [45]. High degrees of remission rates (50-90%) were observed for different types of lymphomas. However, it remains to be a major challenge during treatments of solid tumors by using this technology. The cancer treatments of solid tumors which consist of the majority of human cancer have only limited success. This is mainly due to the intrinsic problems associated with the immunotherapy of solid tumors [43-46]. With the introduction of hRP215 (ScFv) CAR-T cells, additional progress may be made in the future, when the clinical studies are permitted through FDA's formal IND approval.

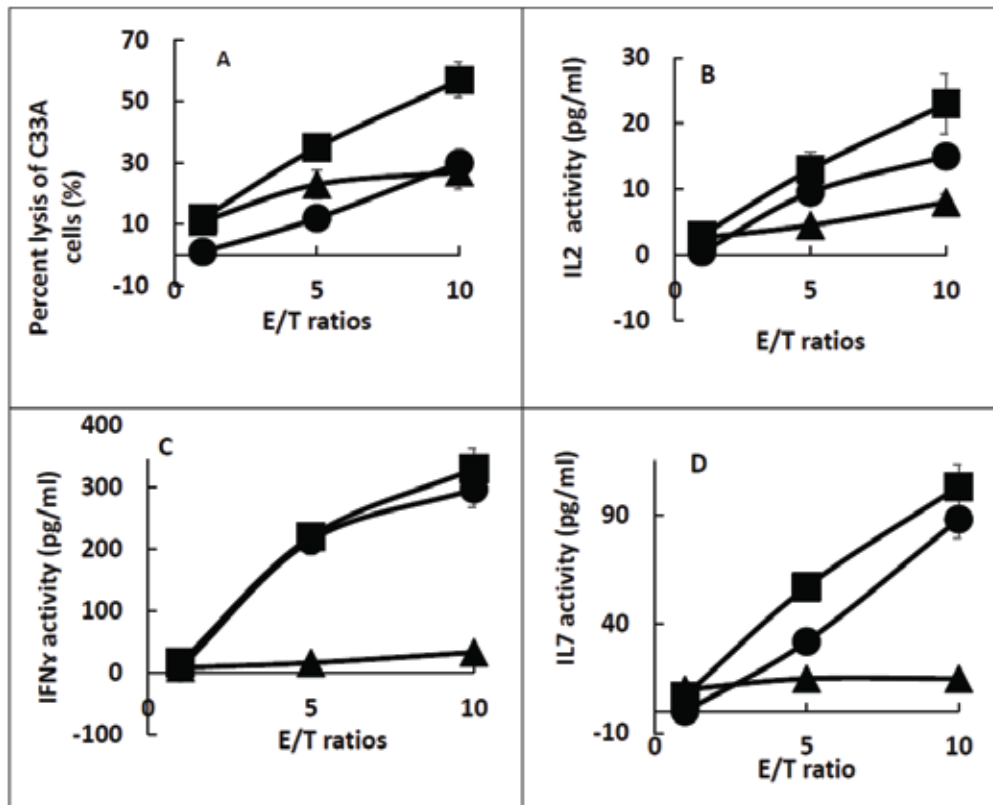
### Conclusions

In this review, we highlight the initial discovery of a

gene expressions of antigen receptors including immunoglobulins and T cell receptors in cancer cells of non-hematological origins [1-4]. In contrast to conventional immunology, both immunoglobulins and T cell receptors are co-expressed on the same cancer cells and do not play the identical functional roles in the cancer immunology as those of the former. These cancerous antigen receptors were demonstrated to be essential for growth/proliferation and survival of cancer cells through interaction studies with human serum proteins [20, 30, 46]. The discovery of RP215 which reacts with carbohydrate-associated epitope (-ST antigen) located in cancerous antigen receptors may represent a major breakthrough for further investigations regarding their respective functional roles. Extensive biological and immunological studies were performed to explore functional roles of cancerous antigen receptors. RP215 has become a unique probe to target cancerous antigen receptors, not only for functional studies, but also for potential immunodiagnostic and immunotherapeutic applications in cancer monitoring and therapy [46]. Therefore, humanized RP215 can be an ideal ligand to suppress vital functions of cancerous antigen receptors, either used as antibody-based anti-cancer drugs or cancer therapy with hRP215 (ScFv)-CAR-T cells constructs in targeting solid tumors in the future [46].

### Abbreviations

A549: lung cancer cell line from ATCC; AFP: alpha fetoprotein; C33A: cervical cancer cell line from; ATCC: CA125: cancer marker 125; CA15-3: cancer marker 15-3; CA19-9: cancer marker 19-9; CA215: tumor-associated antigen 215 recognized by RP215; CAR: chimeric antigen receptor; CAR-T: chimeric antigen receptor transfected T cell; CD3: core receptor of T-Cell; CD4: core receptor of T-Cell; CD8: core receptor of T-Cell; CDC: complement-dependent cytotoxicity reactions; CEA: carcinoembryonic antigen; c-fos: onco gene; CHO: Chines hamster ovary; cIgG: IgG expressed by cancer cells; cyclin D1: A 36-kD protein which regulates cyclin-dependent protein kinase activity; cyfra-21-1:lung cancer marker; Du145: prostate cancer cell line; E/T: effector / target ratio; EGF: epidermal growth factor; EGFR: epidermal growth factor receptor; EIA: enzyme immunoassay; ELISA: enzyme-linked immunosorbent assay; Fab: fragment of antigen-binding domains; Fc: Fc fragment, constant domain of immunoglobulins; GalNAcitol: N-acetyl galactose-linked glycan; hRP215: humanized RP215; IFN $\gamma$  : Interferon  $\gamma$ ; IgSF: immunoglobulin superfamily proteins; IHC: immunohistochemical staining; IL2: Interleukin 2; IL7: Interleukin 7; IND: initial new drug applications; LCMS/MS: liquid chromatography tandem mass spectrometry; LDH: lactate dehydrogenase; MALDI-TOF MS: matrix-assisted laser desorption ionization time-of-flight mass spectrometry;



**Figure 3-A.** Percent cell lysis of target (T) C33A cancer cells upon 8 hours of co-culturing with RP215- linked CAR-T cells (E) at three different E/T (Effect/Target) ratios (1, 5 and 10) expressed in % of tumor cell lysis based on LDH activity; ▲ where is negative control (with virus-only-transduced T cells for co-culturing); ■ is RP215-linked CAR-T cells for co-culturing; and ● is net percent tumor cell lysis after subtraction from the negative control.

**3-B.** Cytokine activity release as determined by EIA following co-culturing of RP215-CAR-T cells and C33A cancer cells for 4-8 hours: Interleukin 2 (IL2) activity (pg/ml) released upon co-culturing of RP215-linked CAR-T cells (E) and target C33A cancer cells (T) at three different E/T ratios of 1, 5 and 10, respectively; where ▲ is negative control (with virus-only-transduced T cells for co-culturing); ■ is RP215-linked CAR-T cells for co-culturing; and ● is net IL2 activity release after background subtraction.

**3-C.** Interferon  $\gamma$  (IFN $\gamma$ ) activity (pg/ml) released upon co-culturing of RP215-linked CAR-T cells (E) with target C33A cancer cells (T) at three different E/T ratios of 1, 5 and 10, respectively; where ▲ is negative control (with virus-only-transduced T cells for co-culturing); ■ is RP215-linked CAR-T cells for co-culturing; and ● is net IFN $\gamma$  activity release after removal of background.

**3-D.** Interleukin (IL7) activity (pg/ml) released upon co-culturing of RP215-linked CAR-T cells (E) and target C33A cancer cells (T) at three different E/T ratios of 1, 5 and 10, respectively; where ▲ is negative control (with virus-only-transduced T cells for co-culturing); ■ is RP215-linked CAR-T cells for co-culturing; and ● is net IL7 activity release after background removal.

MB 231: breast cancer cell line from ATCC; mRP215: murine RP215; MUC 1: mucin 1 proteins; NF $\kappa$ B-1: Nuclear Factor Kappa-B, Subunit 1; OC-3-VGH: ovarian cancer cell line of serous origins from Dept CBS/GYN, VGH, Taipei Taiwan; Po: ribosomal protein; P1: ribosomal protein; P21: cell cycle regulator; PCR: polymerase chain reaction; RT-PCR: reverse transcriptase polymerase chain reactions; RP215: a monoclonal antibody generated against ovarian cancer cell extract; ScFv: Single-chain variable fragment of IgG; SiRNA: small RNA of interference; TCR: T cell receptors; TLR: toll like receptors;

TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling.

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#### Conflict of Interest

The author is co-founder of Vancouver Biotech Ltd. In Vancouver. No conflict of interest regarding the content is involved.

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