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# Functional Roles of Cancerous Immunoglobulins and Potential Applications in Cancer Immunodiagnostics and Immunotherapy

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## ABSTRACT

RP215 is a monoclonal antibody generated against the cell extract of the ovarian cancer cell clone, OC-3-VGH has been shown to react with a carbohydrate-associated epitope located mainly on the heavy chains of immunoglobulins expressed on the surface of most cancer cells. In contrast, these cancerous immunoglobulins, designated in general as CA215, are not found on normal human B cells. Upon isolation from the shed medium of cultured cancer cells, CA215 and cancerous immunoglobulins were found to recognize numerous human serum protein components or fragments, of which both anti- and pro-cancer activities have been identified. These observations strongly support the hypothesis of dual functional roles for immunoglobulins expressed by cancer cells. Through decades of investigations, it was also revealed that apoptosis and complement-dependent cytotoxicity can be induced by RP215 or its humanized forms, as well as by antibodies against antigen receptors, such as those against immunoglobulins or TCRs. For example, RP215 and anti-antigen receptors were found to demonstrate a high degree of correlation in terms of the regulations of many genes involved in the growth and proliferation of cancer cells (e.g. NFκB-1, IgG, P21, cyclin D1, ribosomal P1, and c-fos), as well as for toll-like receptors. Furthermore, significant dose-dependent reductions of implanted tumors were also observed following treatment with RP215 in nude mouse animal models. RP215-based immunodiagnostics were also developed for monitoring of the serum levels of CA215 among cancer patients. Judging from these experimental observations, humanized RP215 may be a suitable candidate for antibody-based anti-cancer drug



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development as it targets cancerous immunoglobulins which are widely expressed on the surface of most cancer cells in humans.

**Content:** 1. Introduction; 2. Biological and immunological studies of cancerous immunoglobulin and RP215; 3. General comparison of normal and cancer immune systems; 4. Functional effect of RP215 on cultured cancer cells; 5. Similar effects of RP215 and cancerous immunoglobulin on the gene regulation of cancer cells; 6. Other studies related to the role of RP215 and cancerous immunoglobulin in cancer immunology; 7. Dual roles of cancerous immunoglobulin in cancer cells; 8. Conclusion.

**Keywords:** RP215; CA215; Cancerous Immunoglobulins; Cancer Immunotherapy.

## ABBREVIATIONS

AFP-Alpha-Fetoprotein; CD44v6-CD44 Variant 6; CDC-Complement-Dependent Cytotoxicity; CDR-Complementarity Determining Region; CEA-Carcinoembryonic Antigen; c-fos-Cellular Proto-Oncogene; Cyclin D1-G1/S Phase Regulator Protein; EGFR-Epidermal Growth Factor Receptor; ELISA-Enzyme-Linked Immunosorbent Assay; hRP215-Humanized Forms of RP215 Monoclonal Antibody; IgA-Immunoglobulin A; IgG-Immunoglobulin G; IgSF-Immunoglobulin Superfamily; IHC-Immunohistochemical; Ki67-Cellular Marker for Proliferation; LC-MS/MS-Liquid Chromatography Tandem Mass Spectrometry; MALDI-TOF MS-Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry; Mab-Monoclonal Antibody; MMP-Matrix Metalloproteinase; mRP215-RP215 Monoclonal Antibody of Murine Origin; MTA1-Metastasis-Associated Gene 1; NFκB-1-Nuclear Factor Kappa-B p105 Subunit 1; P0 and P1-Ribosomal Proteins Protein Synthesis; P21-Cyclin-Dependent Kinase Inhibitor 1; PCNA-Proliferating Cell Nuclear Antigen; PSA-Prostate-Specific Antigen; RT-PCR-Reverse Transcriptase Polymerase Chain Reaction; scFV-RP215-RP215 Single Chain Fragment Variable; sdAb-RP215-RP215 Single Domain Antibody; siRNA-Small Interfering Ribonucleic Acid; SOX2-(Sex Determining Region Y)-box 2; TCRs-T Cell Eeceptors; TLRs-Toll-like Receptors; TUNEL-Terminal Deoxy Nucleotidyl Transferased UTP Nick End Labeling.

## INTRODUCTION

Expressions of immunoglobulins by cancer cells and normal hyperplastic epithelial cells have been observed for more than two decades [1-16]. However, little is known about their mechanisms of action, especially their functional roles during the growth, proliferation, and metastasis of cancer cells in humans. In contrast, the molecular mechanisms of action of B cell expressed immunoglobulins, in response to foreign pathogens, have been well elucidated at the molecular levels, and include notable antigen presentation, maturation, class switching, and somatic hyper mutation [17].

In 1987, a monoclonal antibody, RP215, was generated against the cell extract of the ovarian cancer cell line, OC-3-VGH [18]. RP215 was later shown through MALDI-TOF MS analysis


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to react specifically with a carbohydrate-associated epitope attached to the heavy chains of immunoglobulins expressed by most cancer cells, but is not found on immunoglobulins expressed by B cells [19-22]. Therefore, during the last decade, RP215 was used as a probe to study the mechanisms of action of cancerous immunoglobulins [19-28]. Experimental evidences strongly indicate that cancerous immunoglobulins may serve to capture or absorb certain human serum protein components or fragments, which are required for the growth and proliferation of cancer cells in the human circulation, as well as for neutralizing undesirable human protein components, which may negatively affect the survival of cancer cells in the human body environment [29].

Therefore, in this chapter, we have highlighted the results of our studies, as well as others, which utilize RP215 as a unique probe to demonstrate the functional roles of cancerous immunoglobulins and their implications in cancer immunology. At the same time, we also explore the potential applications of RP215 as an anti-cancer drug to block functional activities of cancerous immunoglobulins, as well as for RP215's applications in cancer immunodiagnostics.

## BIOLOGICAL AND IMMUNOLOGICAL STUDIES OF CANCEROUS IMMUNOGLOBULINS AND RP215

### Structural Aspects for the Expressions of Normal and Cancerous Immunoglobulins

Compared to normal immunoglobulins expressed by B cells, cancerous immunoglobulins were not found to be significantly different in terms of the primary structure, either at the amino acid or mRNA levels, especially in the Fc or constant regions [15,30]. Since the initial discovery of cancerous immunoglobulins, the Fc regions of both normal and cancerous immunoglobulins were compared and determined to be virtually identical at the amino acid level [14]. However, compared to normal immunoglobulins, cancerous immunoglobulins were found to be expressed with limited but distinct somatic hypermutations in the V-(D)-J region, and with no class-switching mechanism [31-34]. Further analysis suggested that seven predominant VHDHJH sets were derived from various cancer types with a different promoter set [14,31]. Therefore, it was hypothesized that immunoglobulin genes expressed by cancer cells may have a distinct repertoire and different functional roles in carcinogenesis [14].

A major breakthrough in research regarding cancerous immunoglobulins came in 2008, when RP215 was initially characterized by its carbohydrate-associated epitope and corresponding glycoprotein, CA215. Initial MALDI-TOF MS analysis of CA215-derived tryptic peptides revealed that RP215 reacts with a carbohydrate-associated epitope located mainly on immunoglobulin heavy chains which are expressed on almost all cancer cells [19-22]. However, RP215 was found to not cross-react with immunoglobulins from B cells, indicating the absence of this RP215-specific epitope in normal immunoglobulins. Therefore, RP215 and its recognized "sugar" epitope mark a clear distinction between cancerous and B cell derived immunoglobulins [30].


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In-depth MALDI-TOF MS analysis revealed that CA215 consists not only of cancerous immunoglobulin heavy chains, but also many other glycoproteins which bear the same RP215-specific epitope. Therefore, the majority of identified CA215 glycoproteins could be grouped into IgSF proteins [19]. These IgSF proteins may include TCRs, antigen presenting molecules, cell adhesion molecules, and several other glycoproteins [19].

## General Comparison of Normal and Cancer Immune Systems

In view of the discovery of immunoglobulins being expressed by almost all hyperplastic epithelial cells or cancer cells of epithelial origins, it has come to attention that there may be two separate and distinct immune systems in our human body environment [1-16]. With RP215 as the unique probe, it is possible to perform further investigations on these two separate systems [19-28]. The comparative results are presented in Table 1.

**Table 1:** Comparison of the normal and cancer immune systems in terms of their structure and functions.

| Structure/functions | Normal immune cells | Cancer cells |
|---|---|---|
| **Expression of antigen receptors** | 1. Immunoglobulins are expressed by B cells<br>2. T cell receptors are expressed by T cells | Both antigen receptors are expressed by cancer cells and are of a single clone [13,14] |
| **Class switching of immunoglobulins** | Yes, in B cells | No [31-34] |
| **Hyper mutation of variable regions of immunoglobulins** | High frequency | Low frequency [31-34] |
| **Glycosylation patterns (unique RP215-specific epitope)** | No O-linked glycans and only one N-linked glycosylation at N297 position of IgG heavy chains; terminal NeuAc only (not recognized by RP215 Mab) | Both O-linked and N-linked glycans are detected in cancerous IgG heavy chains with terminal NeuAc and NeuGc (O-linked glycan recognized by RP215 Mab) [36] |
| **Interactions with TLRs (based on gene expression studies)** | No known interactions with TLRs | Strong interaction with TLRs within cancer cells [46] |
| **Relative immunoactivity** | Normal immunoactivity | Weak immunoactivity (less than 1-5%) due to aberrant glycosylations [37] |
| **Function assays (apoptosis and CDC reactions)** | No known effect | Induced apoptosis and CDC reactions by RP215 and anti-antigen receptors (*in vivo* nude mouse models) [19-28] |

For example, in terms of the expression of antigen receptors, both B cells expressing immunoglobulins and T cells expressing TCRs co-exist separately and exercise separate immune functions [35]. In contrast, both of these antigen receptors can be expressed by cancer cells and originate from a single clone [13,14]. In the case of cancerous immunoglobulins, no class switching and limited hypermutations were observed [31-34]. For both normal and cancerous immunoglobulins, N-glycosylation at the N297 position of the IgG heavy chains was commonly observed, although cancerous immunoglobulins displayed more complicated patterns of N-glycans [36]. In addition, O-linked glycans can only be observed in cancerous immunoglobulins and be recognized by RP215 [36]. Through gene regulation studies, strong interactions with TLRs were observed in cancer cell-expressed immunoglobulins and not in B cell-derived immunoglobulins. Cancerous immunoglobulins were also found to be less immunoreactive to


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anti-human immunoglobulins than normal immunoglobulins due to the presence of aberrant glycosylations in cancerous immunoglobulins [37]. In addition, functionally, apoptosis and CDC reactions are strongly induced upon treatment of cultured cancer cells with RP215, indicating the surface expression of immunoglobulins in cancer cells [38].

## Carbohydrate-Associated Epitope in CA215 and Cancerous Immunoglobulins

Since the RP215-specific epitope was found to be sensitive to treatment by mild periodate solution at neutral pH, it was believed that a carbohydrate moiety may be involved in the epitope recognition of CA215 by RP215 [30]. Upon treatment of cultured cancer cells with tunicamycin, which inhibits the biosynthesis of N-glycans, the activity of the RP215-specific epitope was found to be unaffected. Therefore, it is assumed that the RP215-specific epitope is O-linked and not N-linked [36].

Since the immunoactivity of CA215 with RP215 was strongly inhibited by goat anti-human IgG (Fab), but not by goat-anti-human IgG (Fc), it has been suggested that the O-glycan recognized by RP215 is located on the variable regions (Fab) of the immunoglobulin heavy chains and not on the constant regions (Fc) [22]. The common core 1 O-linked glycan structure of CA215 and cancerous immunoglobulins have been elucidated through comprehensive glycoanalysis and were found to be identical, and a relatively simple trisaccharide (3-linked and 3,6-linked GalNAcitol) was detected [36]. However, it remains to be proved whether additional amino acid residues are involved in the epitope recognition by RP215, which possesses a relatively high binding affinity (Kd ≈ 1nM to CA215) [36].

## Immunohistochemical Studies with RP215 as a Probe

Although immunoglobulins are expressed by almost all cancer cells of epithelial origins in humans, the incidence of its high expression in cancerous tissue sections were investigated by IHC studies [27]. RP215 was employed as a bioequivalent probe for cancerous immunoglobulins in this study. These cancerous tissue sections included those of the ovary (n = 87), cervix (n = 51), endometrium (n = 36), stomach (n = 93), colon (n = 87), esophagus (n = 66), lung (n = 58), breast (n = 59), liver (n = 60), and prostate (n = 22). From the results of these studies, the following types of cancers revealed high percentages of positive staining with the RP215 as a primary antibody probe: esophagus (76%), stomach (50%), colon (44%), ovary (64%), breast (32%), lung (31%), cervix (84%), and endometrium (78%). These results seemed to suggest that RP215 is capable of targeting surface bound immunoglobulins of numerous cancers with diversified tissue origins, especially those of epithelial origins [10].


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## Functional Effects of RP215 on Cultured Cancer Cells

### *In vitro* studies

Since the initial discovery of immunoglobulins expressed by cancer cells, it has been demonstrated that transfection with immunoglobulin-related siRNAs are capable of inhibiting the growth and proliferation of cancer cells *in vitro* and *in vivo* [9,10,39]. It was further shown that treatments of anti-human immunoglobulins could result in apoptosis of cultured cancer cells *in vitro* and reduce the tumor volume of implanted tumors in nude mouse experiments [19-28].

Since RP215 reacts specifically and mainly with the “sugar” epitope of heavy chains of cancerous immunoglobulins, *in vitro* apoptosis and CDC reactions can be induced upon treatments of cultured cancer cells with RP215. A similar result was found when either antibodies against immunoglobulins or T cells receptors were utilized [38]. Results of such studies are presented as Figures 1 and 2, respectively. In Figure 1A, TUNEL assay was used to observe the induced apoptosis of cultured OC-3-VGH ovarian cancer cells after 24 hrs incubation with either 1μg/mL or 10μg/mL RP215 (murine or humanized forms) or anti-antigen receptors [38].

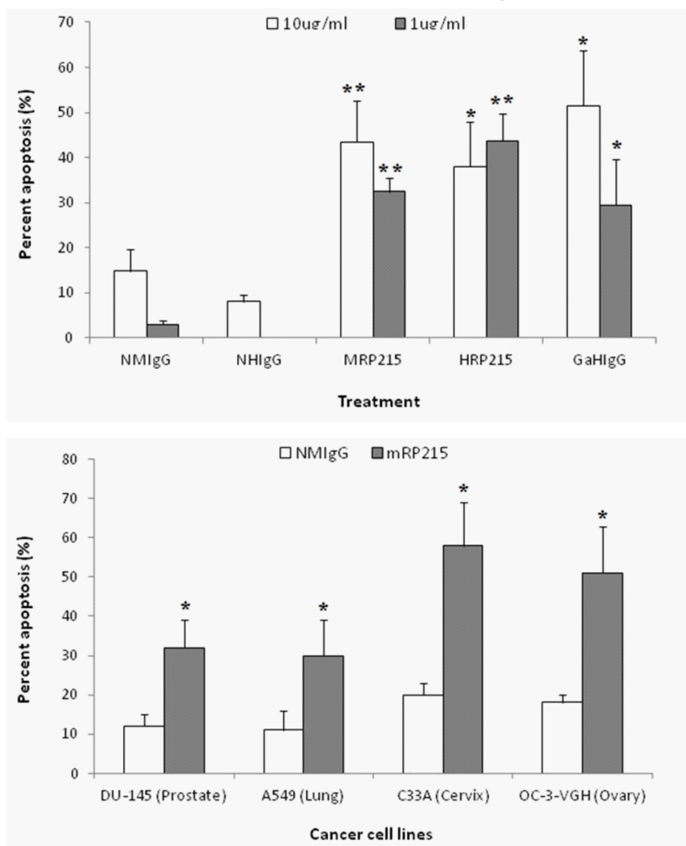


**Figure 1:** Percent induced apoptosis of cultured cancer cells following treatment as assayed by TUNEL assay.



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**(A)** Percent induced apoptosis of OC-3-VGH ovarian cancer cells following 24hrs incubation with various antibodies. Antibodies were added separately for 24hrs incubation and include 10μg/mL and 1μg/mL of normal mouse IgG (NMIgG), normal human IgG (NHIgG), murine RP215 (mRP215), respectively. Cancer cell lines tested include DU-145 (prostate), A549 (lung), C33A (cervix), and OC-3-VGH (ovary). * and ** represent statistical significance of P<0.01 and P<0.001, respectively [38].

**(B)** Percent induced apoptosis following treatment of various cultured cancer cell lines with normal mouse IgG (NMIgG) or murine RP215, respectively. Effects of normal mouse IgG (NMIgG) and murine RP215 on cultured cancer cells of various cancer cell lines, including DU-145 (prostate), A549 (lung), C33A (cervix), and OC-3-VGH (ovary). * and ** represent statistical significance of P<0.05 and P<0.01, respectively [38].

The same observation in Figure 1B was also obtained when other cancer cell lines were employed for similar studies. These cancer cell lines included DU-145 (prostate), A549 (lung), and C33A (cervix). Similarly, CDC reactions were also observed following incubation of cultured OC-3-VGH ovarian cancer cells with RP215 and other anti-antigen receptors in the presence of complement (Figure 2) [38].

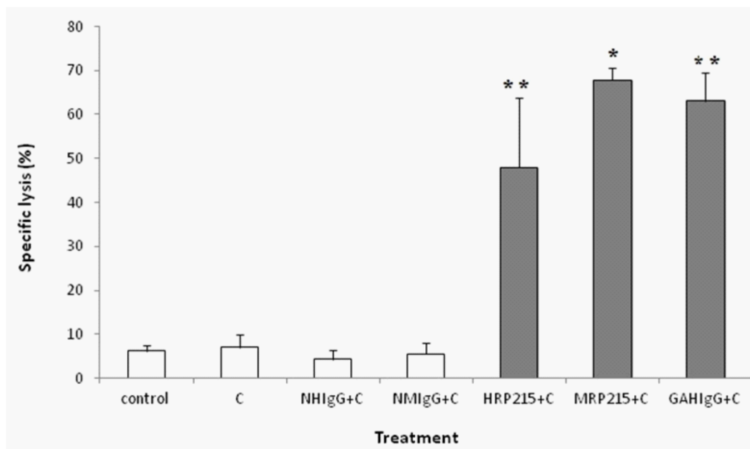


**Figure 2:** Complement-dependent cytotoxicity assay to demonstrate complement-dependent lysis of OC-3-VGH ovarian cancer cells with different treatments in the presence of absence of complement. C represents complement. Negative controls include no treatment (control), complement only (C), normal human IgG plus complement (NHIgG+C), and normal mouse IgG plus complement (NMIgG+C), respectively. Treatments which resulted in a significant percentage of lysis included humanized RP215 plus complement (HRP215+C), murine RP215 plus complement (MRP215+C), and goat anti-human IgG plus complement (GAHIgG+C), respectively.* and ** represent statistical significance of P<0.01 and P<0.001, respectively [38].



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### *In vivo* nude mouse model studies with RP215 treatments

Several nude mouse experiments were performed to reveal the *in vivo* effect of RP215 on implanted tumors following dose-dependent injection of RP215. The results of these studies indicated that RP215 significantly inhibited tumor growth and reduced tumor volume in a dose-dependent manner in nude mouse animal models with their implanted OC-3-VGH (ovarian), SK-MES-1 (lung), or C33A (cervix) cancer cells [25]. Results with implanted OC-3-VGH ovarian or SK-MES-1 lung cancer cells in typical nude mouse models are presented in Figure 3A and 3B, respectively.

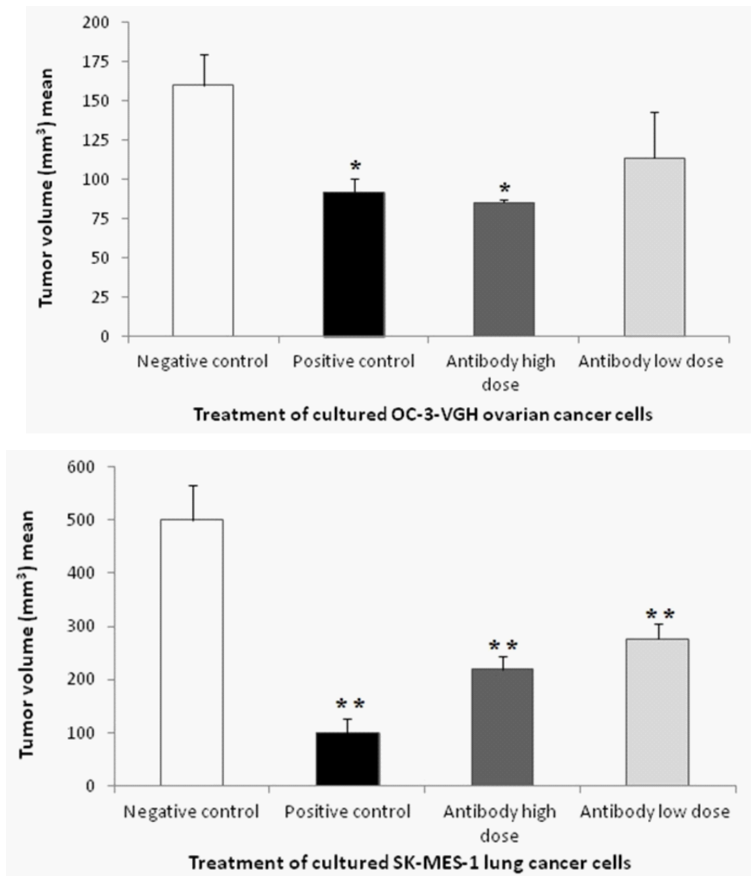


**Figure 3:** Nude mouse experiments to demonstrate the dose-dependent effect of injected RP215 on implanted tumor volumes. **(A)** Effects of injected RP215 in nude mice experiments with the OC-3-VGH ovarian cancer cell line as the model. On day 0, mice were inoculated with OC-3-VGH cancer cells. Mice (n = 4 for each group) were injected with either no antibody (negative control) or drug treatment (positive control, antibody high dose, or antibody low dose). Drug treatment with 60mg/kg cyclophosphamide (positive control) or 10mg/kg RP215 (antibody high dose) or 2mg/kg RP215 (antibody low dose) were given to mice by intraperitoneal injection on the same day. Tumor volumes from mice of various treatments were determined on day 15 [25].


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**(B)** Effects of injected RP215 in nude mice experiments with the SK-MES-1 squamous lung cancer cell as the model. 0.2mL of cultured SK-MES-1 cancer cells were implanted at exponential growth phase into mice underneath the armpit or via the back (SK-MES-1 cell concentration: 1.5-2.5x$10^7$ cells/mL). Three weeks after inoculation, the implanted tumors in each mouse become visible. Mice were then divided randomly into 4 groups (n = 5 for each group) and were injected with phosphate buffered saline (negative control) or drug treatment (positive control, antibody high dose, or antibody low dose). In the positive control group, mice were injected with 1000μg/$m^2$ Genicitabineand cisplatin (80-100mg/$m^2$) in each dose. Both the antibody low dose and antibody high dose had mice injected with either 0.14mg/mouse/dose or 0.75mg/mouse/dose, respectively, of RP215. In all 4 groups, antibody or drug treatments were performed twice in total during the 4$^{th}$ and 5$^{th}$ week following tumor cell implant. At the 6$^{th}$ week mark, the tumors were taken out and their volume measured. Tumor volumes were determined on the 6$^{th}$ week after tumor implant of SK-MES-1 cancer cells and antibody or drug injections with 5 mice for each treatment group. * and ** represent statistical significance of $P < 0.05$ and $P < 0.001$, respectively [25].

Nude mouse experiments with low, medium, and high concentrations of naked and $I^{131}$-labeled RP215 monoclonal antibody were performed on nude mice with implanted OC-3-VGH ovarian cancer cells. Injections of both naked and $I^{131}$-labeled RP215 monoclonal antibody exhibited significant inhibition of tumor growth *in vivo* in a dose-dependent manner, as compared to the negative controls [25]. However, additional anti-tumor cytotoxicity activities were observed upon injection with $I^{131}$-labeled RP215. These results clearly indicate that RP215 could inhibit tumor growth *in vivo* in nude mice, and that RP215 demonstrates potential as an anti-cancer drug for the therapeutic treatments of human cancer.

## Humanization of RP215 for cancer immunotherapy–demonstration of bioequivalence

In view of the fact that RP215 is a suitable substitute or probe which functions like antibodies against cancerous immunoglobulins, RP215 in humanized forms can serve as a suitable candidate for anti-cancer drug development and immunotherapeutic applications[19-28]. To develop RP215-based anti-cancer drugs for human applications, it is essential to modify the original murine RP215 into humanized forms. The humanized versions of RP215 were demonstrated to have affinity and specificity to CA215, comparable or equivalent to the original murine RP215 [40]. The resulting humanized (hRP215) and murine RP215 (mRP215) contain substantially similar or identical CDR regions. CDR domains of the variable regions for either IgG chains were designed and determined by a number of established methods [40].

Substantial equivalence between humanized and murine RP215 can be first demonstrated by sandwich or binding immunoassays with either forms of RP215 as the capturing or detecting antibodies. Affinity-purified CA215 was used for coating on micro wells for comparative binding with hRP215 or


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mRP215. The hRP215 with the highest affinity was shown to have a dissociation constant (Kd) of 4.4nM to CA215, which was comparable to that of the original mRP215 (Kd = 4.2nM) [40]. Both mRP215 and hRP215 were shown to have no cross-reactivity to normal human IgG [40]. Bioequivalence between mRP215 and hRP215 was also established based on other criteria, including SDS-PAGE and Western blot of affinity-purified CA215 by using either mRP215 or hRP215 ligand, as well as by RP215-based sandwich enzyme immunoassays [40]. Bioequivalence between mRP215 and hRP215 was also demonstrated by functional assays of these two monoclonal antibodies on cultured cancer cells. By TUNEL apoptosis assays, both mRP215 and hRP215 were shown to induce significant apoptosis of cultured OC-3-VGH ovarian cancer cells at antibody concentrations as low as 1μg/mL following 24hrs incubation (Figure 1A) [38]. Similarly, CDC reactions could also be induced by incubation of cultured cancer cells with either antibody at 10μg/mL in the presence of complement with comparable effectiveness [38]. Therefore, following the humanization processes, the bioequivalent hRP215 can then be utilized for development of anti-cancer drugs through serial preclinical and clinical studies in humans without the consequence of heterologous immune response in humans.

## Applications of RP215 in cancer immunodiagnostics

In view of the fact that the majority of cancer cell lines and cancerous tissues express the RP215's cognate antigen, CA215, RP215 was utilized to formulate sandwich enzyme immunoassays to determine CA215's potential as a pan cancer marker, and to investigate whether serum levels of CA215 are correlated with stages of tumor progression. These studies indicated that serum CA215 levels did not strongly correlate with stages of tumor progression. However, significantly elevated levels of CA215 could be readily detected among patients with stage I ovarian or cervical cancer (Table 2) [20]. Serum levels of CA215 were also found to correlate with clinical treatments. For example, significantly decreased serum CA215 levels were detected following surgical removal of the tumor or treatment with radio- or chemotherapy [20].

**Table 2:** Serum levels of CA215 (in Au/mL) from normal individuals and from patients with different stages of ovarian or cervical carcinoma.

| | Normal control | Ovarian carcinoma[a] | | | Cervical carcinoma[b] | | |
|---|---|---|---|---|---|---|---|
| | | Stage 1 | Stage 2 | Stage 3 | Stage 1 | Stage 2 | Stage 3 |
| **Number of cases (n)** | 59 | 24 | 7 | 40 | 35 | 18 | 7 |
| **Mean (Au/mL)** | 5.47 | 36.2 | 60.2 | 55.0 | 48.0 | 78.6 | 75.7 |
| **Standard deviation (SD)** | 10.37 | 27.2 | 27.0 | 44.7 | 44.7 | 61.8 | 83.9 |

[a]: for normal control vs. any of the stages 1, 2 or 3 of ovarian carcinoma, P < 0.001. For stage 1 vs. stage 2 and 3 ovarian carcinoma, P < 0.05. For stage 2 vs. stage 3 ovarian carcinoma, P > 0.05; [b]: for normal control vs. any of the stages 1, 2, or 3 of cervical carcinoma, P < 0.001. For stage 1 vs. stage 2 and 3 cervical carcinoma, P < 0.01. For stage 2 vs. stage 3 cervical carcinoma, P > 0.05 [20].


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Positive detection rates of 50% or higher were also found in serum specimens of cancer patients with lymphoma or cancers of the lung, liver, esophagus, stomach, ovary, breast, or cervix [41]. In addition, CA215 and other known cancer biomarkers, including CEA, AFP, CA19-9, CA15-3, CA125, ferritin, β2 microglobulin, Cyfra21-1, and PSA were found to be comparable in detection sensitivity, although the former exhibits little cancer specificity due to its pan-cancer nature [41]. However, simultaneous or combined use of CA215 with many other more tissue-specific cancer biomarkers resulted in higher cancer detection rates, when compared to the use of a single marker alone (Table 3) [41]. Altogether, these results strongly support the use of CA215 as a suitable pan cancer marker for monitoring of early stages of ovarian and cervical cancer, as well as for many other cancers (Figure 4).

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

| CA215 (0.1Au/mL) | | Lung (n) | Liver (n) | Ovary (n) | Esophagus (n) | Breast (n) | Stomach (n) |
|---|---|---|---|---|---|---|---|
| CEA (5 ng/ml) | I[a] | 52% (112) | 74% (58) | - | 61% (23) | 71% (44) | 60% (30) |
| | II[b] | 67% (33) | 54% (35) | | 47% (19) | 95% (20) | 50% (14) |
| | III[c] | **94%** | **81%** | | **65%** | **96%** | **70%** |
| 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 | **85%** | **92%** | **82%** | **75%** | | |
| CA19-9 (37 Au/ml) | I | - | 74% (58) | - | - | - | 60% (30) |
| | II | | 55% (22) | | | | 75% (16) |
| | III | | **82%** | | | | **81%** |
| CA15-3 (30 Au/ml) | I | - | - | - | - | 71% (44) | - |
| | II | | | | | 83% (6) | |
| | III | | | | | **83%** | |
| $B_2$microglobulin (2.6 ng/ml) | I | - | 74% (58) | 59% (68) | | | |
| | II | | 56% (16) | 90% (10) | | | |
| | III | | **81%** | **100%** | | | |
| Cyfra21-1 (3.3 ng/ml) | I | 52% (112) | - | - | - | - | - |
| | II | 50% (52) | | | | | |
| | III | **77%** | | | | | |

[a]: CA215 only; b: other marker only; c: III: combined [41].

## Similar Effects of RP215 and Cancerous Immunoglobulins on the Gene Regulations of Cancer Cells

In order to elucidate the potential mechanisms of action by which cancerous immunoglobulins regulate the growth and proliferation of cancer cells, gene regulation studies were performed


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with cultured OC-3-VGH ovarian and C33A cervical cancer cells. Upon treatments with RP215 or antibodies against antigen receptors, such as anti-human IgG and anti-TCR, to cultured cancer cells, noticeable changes in the gene expressions of several genes involved in cell proliferation, protein synthesis, and cell cycle regulations were observed by using semi-quantitative RT-PCR [38]. For both cancer cell lines, RP215 and antibodies against antigen receptors were found to affect the gene expression levels of similar genes involved in the regulation of cell growth. For example, up regulation of genes including NFκB-1, IgG, TCR, P21, and ribosomal P1, and down regulation of genes including cyclin D1 and c-fos were observed following treatment of cancer cells by either RP215 or anti-antigen receptors. Correlation analysis was also performed between RP215 vs. anti-human IgG or anti-TCR. The results indicated that the changes in the gene expression levels of the selected genes were well correlated [38]. Therefore, these results indicate a common mechanism of action by which RP215 or anti-antigen receptors induce apoptosis or CDC reactions to cancer cells. Treatments of cultured cancer cells with RP215 and anti-antigen receptors may cause downregulation of cyclin D1, c-fos, and EGFR, which, in general, would inhibit cancer cell growth and proliferation [42].

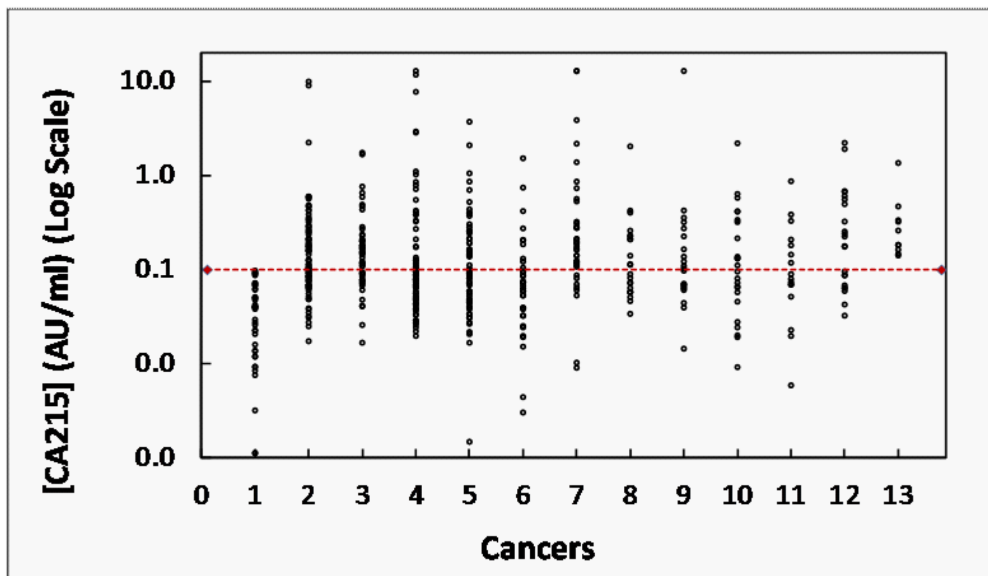


**Figure 4:** Scattergram to reveal the serum CA215 levels and positive rates from normal individuals (n = 52) and cancer patients (total n = 557). 1: normal individuals as the negative control (5%, n = 52); 2: lung cancer (52%, n = 112); 3: liver cancer (74%, n = 58); 4: colon cancer (44%, n = 95); 5: ovarian cancer (59%, n = 68); 6: prostate cancer (40%, n = 40); 7: breast cancer (71%, n = 44); 8: kidney cancer (38%, n = 23); 9: esophageal cancer (61%, n = 23); 10: stomach cancer (60%, n = 30); 11: pancreatic cancer (41%, n = 17); 12: cervical cancer (51%, n = 33); and 13: lymphoma (83%, n = 12). The dash line indicates the cut-off value of 0.1 Au/mL. The positive rates of all different cancers were statistically significant with P <0.05 [41].


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In addition, changes in the expression levels of TLRs following treatment of OC-3-VGH cancer cells with RP215 or antibodies against antigen receptors were also examined. The experimental evidence seems to be consistent with the potential roles of TLRs in carcinogenesis and growth/proliferation of cancer cells [43-45]. Results of semi-quantitative RT-PCR seemed to suggest significant up regulated expressions of the TLR-3 gene and down regulated expressions of the TLR-4 and TLR-9 genes following treatment of cancer cells with RP215 or anti-antigen receptors [46]. In addition, similar results were found when C-33A cervical cancer cells were treated with RP215 or anti-antigen receptors, although with significant quantitative variations [46]. Furthermore, the expression level of the TLR-2 gene was found to be up regulated upon antibody treatments of C-33A cancer cells [46]. It was also found that antibodies against TLR-4 and lipopolysaccharide, exhibited no significant effect on the expression level of cancerous immunoglobulins. This suggests that the effect of cancerous immunoglobulins on the expression level of TLR genes is unidirectional [46]. High correlations of the gene expression changes of TLRs following treatment of cultured cancer cells with RP215 or anti-human IgG were also observed and are demonstrated in Figure 5.

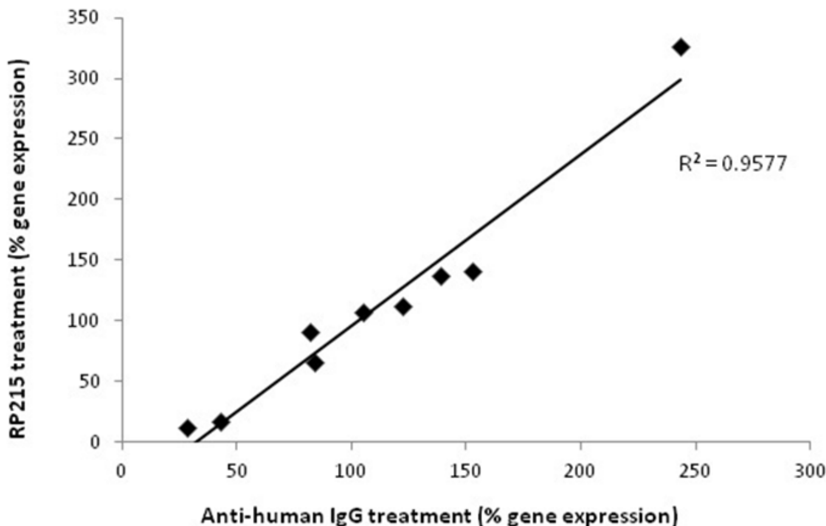


**Figure 5:** Correlation analysis of the changes in gene expression levels of selected genes involved in cellular growth/proliferation and the innate immune response following separate treatments of RP215 and anti-human IgG of the OC-3-VGH ovarian cancer cell line. The data represent the relative gene expression levels of TLR-3 (I), NFκB-1 (II), P21 (III), P1(IV), IgG (V), c-fos (VI), cyclin D1 (VII), TLR-9 (VIII), and TLR-4 (IX)in the OC-3-VGH ovarian cancer cells following separate treatments with RP215 and anti-human IgG and have been plotted for correlation analysis. The correlation coefficient between these two antibody ligands was determined to be $R^2$= 0.9577 [38].


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## Other Studies Related to the Roles of RP215 and Cancerous Immunoglobulins in Cancer Immunology

### RP215 antibodies induce cell cycle arrest at G0/G1 phase in breast cancer and implication in immunotherapy

Potential drawbacks in using monoclonal antibodies for cancer immunotherapy include their large size, low tumor penetration potential, and the possible emergence of human anti-mouse antibodies due to their murine origins [47,48]. Therefore, studies were performed to develop recombinant scFV-RP215 and sdAb-RP215 antibodies of small molecular size in the variable regions of RP215, and their effects on breast and ovarian cancer cells were examined [49].

Both of these recombinant antibodies were found to exhibit similar specific affinity and activity to the RP215-specific epitope. Following treatment of MB231 breast cancer cells with scFV-RP215 or sdAB-RP215, staining with priopidium iodide, and analysis by flow cytometry, a significant increase in the percentage of cells in the G0/G1 phase was observed [49]. Similar results, although to a lesser degree, were also found when cells were treated with RP215 [49]. These results suggested that scFv-RP215 and sdAb-RP215 caused cell cycle arrest of breast cancer cells at the G0/G1 phase [49]. However, sdAb-RP215 was found to only slightly inhibit breast cancer growth and not significantly induce apoptosis of cancer cells [49].

### RP215 as a diagnostic tool to determine metastasis, migration, and prognosis of cancer

Through use of RP215 as a unique probe, it was discovered that cancerous immunoglobulins are strongly expressed in basal cells of ductal carcinoma, basal-like cells near cancer nests of invasive ductal carcinoma, and parenchyma cells within cancer nests [50,51]. The distribution of staining for cancerous immunoglobulins was also found to be in agreement with staining for CD44, a common marker for breast cancer stem cells, and not correlated with cytokeratin five, which is commonly expressed on normal mammary stem cells and myoepithelial cells [50,52,53]. Cancer stem cells exhibit characteristics of both tumor cells and stem cells, and are capable of self-renewal and differentiation [54]. Cancer stem cells also exhibit high migration potential and high levels of resistance to chemotherapeutic drugs [54]. Therefore, these results indicate that cancer stem cells express RP215-specific immunoglobulins, and that RP215 may be used for the identification of cancer stem cells, which play important roles in tumor malignancy [50].

Furthermore, the expression of cancerous immunoglobulins was also found to be correlated with cancer cell proliferation, migration, and drug resistance, all of which are characteristics of adult stem cells [50,51]. For example, expression of cancerous immunoglobulins was found to be significantly higher in migrating cancers than primary tumors, and in ovarian cancer cells with resistance to the anti-cancer drug, taxol [50]. Therefore, RP215 may also be utilized as a diagnostic tool in testing for cell proliferation, cell migration, and resistance to chemotherapeutic drugs [50,51].


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Cancerous **immunoglobulins** as a biomarker for diagnosis, classification, and prediction of prognosis of lung cancer

Previously, it has been found that the expression levels of cancerous immunoglobulins were correlated with tumor grade and proliferative markers, such as PCNA, and ki-67 in cancers of the breast, esophagus, and soft tissues [11,51,55]. Through IHC studies of various histological types of lung cancers from human patients with RP215 as a unique probe, it was found that cancerous immunoglobulins are strongly correlated with tumor differentiation, metastasis, and clinical outcome, including cancer cell migration and invasion [56,57]. Strong positive RP215 staining was found in most patients with squamous cell carcinoma [56]. This suggested that cancerous immunoglobulins may represent a novel marker for squamous cell carcinoma, and may be related to the carcinogenesis of squamous cell carcinoma [56].

RP215 staining was also found to be significantly correlated with poor differentiation and metastasis in patients with adenocarcinoma [57]. For example, RP215 staining was found to be strong in metastatic adenocarcinoma and weak in primary adenocarcinoma [57]. Furthermore, through comparative studies with two other biomarkers associated with metastatic adenocarcinoma, CD44v6 and SOX2, it was observed that the expression of RP215-recognized immunoglobulins correlated with these two biomarkers of metastatic lung cancer, but RP215-recognized immunoglobulins exhibited greater specificity to metastatic adenocarcinoma [57-62]. In contrast to CD44v6 and SOX2, RP215-specific immunoglobulins were not recognized from alveolar epithelial cells neighboring cancerous tissues, and lymphocytes of the lymph nodes [57]. The expression of cancerous immunoglobulins recognized by RP215 was also significantly correlated with poor overall survival of adenocarcinoma patients [57].

In order to elucidate the potential mechanisms of action by which cancerous immunoglobulins promote the invasion and metastasis of lung cancer cells, knockdown experiments of cancerous immunoglobulins were performed. In one study, the gene expression changes of epithelial-mesenchymal transition-related molecules were examined. Epithelial-mesenchymal transition is known to be a crucial event in the tumor metastasis process, and involves several molecules, including E-cadherin, MMP-2, and MMP-9 [63]. Following knockdown of cancerous immunoglobulins through siRNA, decreased migration and invasion capacity of lung cancer cells was observed, and up regulation of E-cadherin expression, as well as down regulation of MMP-9 was found [57]. Therefore, cancerous immunoglobulins may play a role in lung cancer cell invasion and metastasis through altering the expression of E-cadherin and MMP-9 [57].

Similarly, in another study, knockdown of cancerous immunoglobulins was found to decrease cellular proliferation, migration, and attachment of cultured lung cancer cells [56]. However, significant changes in the gene expression levels of E-cadherin and MMP-9 were not found. Instead, it was observed that MTA1 was significantly suppressed following knockdown of cancerous immunoglobulins in A549 and SK-MES-1 lung cancer cell lines [56]. MTA1 was also observed to


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be co-localized with cancerous immunoglobulins in lung cancer cells, and knockdown of MTA1 resulted in decreased migration and attachment of lung cancer cells [56]. These findings suggest that cancerous immunoglobulins may exert its effects on metastasis via the MTA1 signaling pathway in lung cancer cells. Therefore, while both studies indicate the potential of cancerous immunoglobulins as a clinical prognostic indicator of lung cancer, the mechanisms of action by which cancerous immunoglobulins affect lung cancer growth and metastasis remain debatable.

### Normal human epidermis expression of IgG with the RP215 epitope

Recent studies have found that functional IgG and IgA are capable of being produced by normal human epidermal cells and the human keratinocyte HaCaTcell line [64]. Strong positive staining of the epidermis, but not of the dermis, was observed when using RP215 as a probe. These results indicate that epidermis-derived IgG contains the unique RP215 "sugar" epitope, which is not found on normal B-cell derived immunoglobulins [64]. Similarly to cancerous immunoglobulins, the heavy chains of epidermis-derived IgG and IgA displayed limited rearrangement patterns and did not originate from the classical class switching mechanism [64]. These epidermis-derived IgG and IgA also exhibited potential antibody activity by binding to pathogens, such as *Staphylococcus aureus* [64].

Under normal physiological conditions, skin basal cells undergo cell division and differentiation into keratinocytes which migrate through the epidermis, playing an important role in wound healing [65]. Because cancerous immunoglobulins have been implicated in the survival, proliferation, and migration of cancer cells, it was hypothesized that epidermis-derived IgG may be involved in the survival, migration, and differentiation of keratinocytes [64]. Furthermore, IgG deposits have been observed in skin lesions of autoimmune disease, including bullous diseases and lupus erythematosus, and therefore may be involved in the pathogenesis of skin autoimmune diseases [64,66-68].

## Dual Roles of Cancerous Immunoglobulins in Cancer Cells

In the traditional immune system, antigen receptors, such as immunoglobulins and TCRs of immune cells serve crucial roles in the adaptive immune response against foreign pathogens and cancer cells [35]. However, it has been known for years that cancer cells are also capable of protecting themselves immunologically from the normal immune system through an unknown mechanism of action which may not be identical to that of the normal immune system [69]. Therefore, it was hypothesized that cancerous antigen receptors, including cancerous immunoglobulins, may play a role in the immune surveillance and protection of cancer cells against the normal immune environment [29,70-72].

Because B cell derived immunoglobulins and cancerous immunoglobulins differ in their expression patterns and activation, the mechanisms by which they exhibit immune protection may greatly differ. For example, unlike B cell derived immunoglobulins, cancerous immunoglobulins


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exhibit no class switching and limited mutations or diversity in their variable regions [31-34]. Cancerous immunoglobulins also exhibit aberrant glycosylation patterns and immunoglobulins of different classes or subclasses may be expressed simultaneously by cancer cells derived from a single clone [13,14,37,73]. The antigens bound by either of these immunoglobulins may also differ [74].

Through various *in vitro* and *in vivo* studies, it has been shown that cancerous immunoglobulins play crucial roles in the growth and proliferation of cancer cells [19-28]. Results from recent studies also demonstrate that cancerous immunoglobulins are capable of binding both pro-cancer and anti-cancer serum proteins within the human circulation [29]. Therefore, it has been hypothesized that cancerous immunoglobulins serve dual roles in cancer cells by serving to preserve the growth and proliferation of cancer cells, as well as by neutralizing harmful agents to cancer cells within the circulation.

## Human serum proteins recognized by CA215 and cancerous immunoglobulins

Attempts were made to determine the possible serum antigens bound by CA215 and cancerous immunoglobulins in order to elucidate the potential molecular mechanisms of action behind the functional roles of cancerous immunoglobulins. The molecular nature of these antigens may be relevant to the growth and proliferation or immune protection of cancer cells from the natural human environment. CA215 and cancerous immunoglobulins were first affinity-purified from the shed media of cultured OC-3-VGH ovarian cancer cells through use of RP215 and anti-human IgG as separate general ligands, respectively. Potential serum antigens with affinity to CA215 or cancerous immunoglobulins were then affinity isolated from human serum by using purified CA215 or cancerous immunoglobulins as general affinity ligands. Captured serum protein components were then analyzed by LC-MS/MS [29]. Approximately 72% of the fifty protein serum components were found to be commonly recognized by either CA215 or cancerous immunoglobulins [29]. Among the serum protein components and fragments detected byLC-MS/MS, more than half were found to be associated with pro- and anti-cancer activities (Table 4) [29]. Therefore, CA215 and cancerous immunoglobulins may serve to promote the growth and proliferation of cancer cells and neutralize harmful agents within the human circulation by interacting with these human serum proteins [29,75].


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**Table 4:** Summary of the functional classifications of detected human serum proteins and/or fragments which are recognized by both CA215 and cancerous immunoglobulins [29].

| Functional activity | Protein | Molecular weight (kDa) | Key notes related to cancer |
|---|---|---|---|
| Anti-cancer | 35kDa inter-α-trypsin inhibitor heavy chain 4 | 104 | Down regulation leads to tumor initiation & progression in multiple solid tumors [76] |
| | Anastellin | 256 | Inhibits tumor growth & metastasis *in vivo* [78,79] |
| | Apolipoprotein A-1 | 31 | Suppresses tumor growth & metastasis in animal tumor models [81] |
| | Fibrinogen β chain | 56 | β43-63 inhibits tumor vascularization in mouse models [80] |
| | Keratin type I cytoskeletal 9 | 62 | Down regulation is associated with increased drug resistance in breast cancer [77] |
| Pro-cancer | C4b-binding protein | 67 | Protects cancer cells from complement activation & attack [86-89] |
| | Complement C3 | 187 | Promotes cancer development & progression through various tumorigenic effects [92-93] |
| | Complement factor H | 139 | Protects cancer cells from complement-mediated cytolysis [86-91] |
| | Serotransferrin | 77 | Growth factor for cancer cell proliferation [82-85] |
| | Vitronectin | 54 | Inducer of cancer cell differentiation, spreading, migration, & growth [94-97] |

## Human serum proteins demonstrating anti-cancer activities

Notable commonly recognized anti-cancer serum components detected by CA215 or cancerous immunoglobulins include 35kDa inter-α-trypsin inhibition heavy chain 4, anastellin, apolipoprotein A1, fibrinogen β chain, and keratin type I cytoskeletal 9 [29]. Of these detected anti-serum components, many have been shown to be down regulated in cancerous tissue. For example, down regulation of 35kDa inter-α-trypsin inhibition heavy chain 4 is associated with tumor initiation and progression in multiple solid tumors [76]. In addition, down regulation of keratin type I cytoskeletal 9 has been observed in drug resistant human breast cancer tissue [77]. Other detected anti-cancer serum components have been found to exert anti-angiogenic effects in order to prevent the growth and proliferation of cancer cells within the human body. These include anastellin, fibrinogen β chain, and apolipoprotein A1. Anastellin, a fragment of the first type II module of fibronectin, exhibits anti-angiogenic properties which result in inhibition of tumor growth and metastasis *in vivo* [78,79]. For fibrinogen β chain, the first 20 amino acids of the N terminus (β43-63) have been found to significantly inhibit tumor vascularization, thereby increasing tumor necrosis in mouse models [80]. Apolipo protein A1, a major protein component of high density lipoprotein, has been found to not only inhibit tumor angiogenesis but also decrease tumor growth, metastasis, invasion and myeloid-derived suppressor cell recruitment [81]. Furthermore, it has been demonstrated that mice lacking apolipo protein A1 develop tumors quicker than wild-type mice, suggesting that apolipo protein A1 act as a tumor suppressor [81]. By interacting with these anti-cancer serum protein components, CA215 and cancerous immunoglobulins may serve to protect cancer cells from these components within the human circulation.


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### Human serum proteins demonstrating pro-cancer activities

Notable commonly recognized pro-cancer serum components detected by CA215 or cancerous immunoglobulins include serotransferrin, C4b-binding protein, complement factor H, complement C3, and vitronectin [29]. Some of these pro-cancer serum components, such as serotransferrin, have been implicated as important growth factors for cancer cell proliferation [82-85]. Other pro-cancer serum components are important members of the complement system and play crucial roles in regulating complement activation. For example, cancer cells have been observed to interact with C4b-binding protein and complement factor H in order to protect cancer cells from complement activation [86-89]. In particular, many primary tumors and cancer cells lines have been found to express and secrete complement factor H [86,88-91]. Downregulation of complement factor H has also been shown to sensitize cells to complement attack, leading to reduction in tumor growth [87-90]. Complement C3, a central protein in the complement cascade, is associated with a variety of tumorigenic effects, leading to cancer cell development and progression. These tumorigenic effects include the production of vascular endothelial growth factor, reorganization and disintegration of the extracellular matrix reorganization, and promotion of cancer cell migration and invasion [92]. Furthermore, compared to wild-type mice, mice deficient in C3 exhibited significantly decreased tumor proliferation [93]. Vitronectin, a glycoprotein found in the extracellular matrix and in human serum, has been shown to be an inducer of cancer stem cell differentiation in breast and prostatic carcinoma, as well as an inducer of cancer cell spreading, migration, and growth [94-97].

## CONCLUSION

In this chapter, updated research observations have been analyzed in order to investigate the functional significance of cancerous immunoglobulins and potential diagnostic and therapeutic applications of the unique RP215 "sugar" epitope associated with these molecules [19-29,50,51,56,57]. A major advancement in the field of cancer immunology arose from the discovery of RP215 and its carbohydrate-associated epitope located on immunoglobulin heavy chains which can be found on the surface of most cancer cells [19-22]. Functionally, it was clearly demonstrated that RP215 can serve in a bioequivalent role as antibodies against antigen receptors, including IgG and TCRs. All three categories of these antibodies were shown to induce apoptosis and CDC reactions to a variety of human cancer cell lines [38]. In addition, dose-dependent volume reductions of implanted human tumors were also observed in nude mouse animal models [25]. Therefore, humanized forms of RP215 may be a suitable candidate for anti-cancer drug development. This assessment would require in-depth preclinical studies, followed by IND enabling approved by FDA, and a series of clinical trials to assess RP215's anti-cancer efficacy [19,25,27,40,98]. In addition, immunodiagnostic applications of RP215-based EIA can also be pursued in routine monitoring of cancer patients.


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In order to determine the potential roles of CA215 and cancerous immunoglobulins in cancer cells, affinity-purified CA215 or cancerous IgG from the shed medium of cultured cancer cells were used as the ligand to capture any interacting human serum proteins components of fragments, which were then detected by LC/MS-MS analysis. It was observed that as many as 72% of the captured or identified human serum proteins were identical when either of CA215 or cancerous IgG was utilized as the affinity ligand [29]. Further analysis revealed that both anti-cancer and pro-cancer components were detected [29]. This observation is consistent with the belief that cancerous immunoglobulins serve dual functional roles in cancer cells, both in supporting the growth and proliferation of cancer cells and in neutralizing hostile antigens in the human circulation [29,70-72]. Therefore in this chapter, the functional roles of cancerous immunoglobulins were demonstrated and the potential of RP215 for development as anti-cancer drugs or for immunodiagnostic applications was presented.

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