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RP215 and GHR106 Monoclonal Antibodies and Potential Therapeutic Applications

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Abstract

During the last two decades, two distinct monoclonal antibodies, RP215 and GHR106 were generated, respectively and extensively characterized, biologically and immunologically. Both antibodies target separately specific pan cancer markers and are being evaluated preclinically for potential therapeutic applications in cancer immunotherapy and/or fertility regulations. RP215 was shown to react specifically with carbohydrate-associated epitope located in the heavy chain variable regions of cancer cell expressed specific immunoglobulins, designated as CA215 which are distinct from those of normal B cell origins. The cancerous immunoglobulins may function to react with specific human serum proteins to facilitate growth/proliferation as well as protection of cancer cells in circulations. RP215-based enzyme immunoassays were designed to monitor serum CA215 levels among cancer patients. On the other hand, GHR106 was generated against N1-29 oligopeptide located in the extracellular domains of human GnRH receptor found either in the anterior pituitary or in most of the cancer cells. *In vitro* culture of cancer cells revealed that either of these two antibodies can induce apoptosis of cancer cells following 24 - 48 hours incubations. Anti-tumor activities of both antibodies were evaluated by typical nude mouse experiments. Either one was shown to effectively reduce the volumes of implanted tumors, dose-dependently. Humanized forms of either antibody were made available in CAR (chimeric antigen receptor)-T cell constructs. They were shown separately to induce cytotoxic killings of cancer cells *in vitro* by releasing cytokines upon incubations of tumor cells with either of CAR-T cell constructs. In addition, GHR106 also acts as GnRH antagonist by a specific targeting to pituitary GnRH receptor for reversible suppressions of reproductive hormones such as LH, testosterone or estradiol. Based on the above preclinical assessments, it can be generally concluded that both RP215 and GHR106 are restricted in normal tissue expressions and suitable for targeting cancerous immunoglobulins and GnRH receptor, respectively for cancer immunotherapy. Furthermore, specific tar-

geting of pituitary GnRH receptor may suggest that the long acting GHR106 (5 - 21 days half-life) is an adequate GnRH antagonist for numerous gynecological treatments including ovulation inhibition in IVF/ART, endometriosis, premenstrual syndrome, precocious puberty, uterine fibroids and/or polycystic ovarian syndrome.

Keywords

RP215, GHR106, Cancer Immunotherapy Fertility Regulation, CAR-T Cell Constructs, CA215

1. Hybridoma Technology and Origins of RP215 and GHR106 Monoclonal Antibodies

Since the emergence of hybridoma technology in 1975, numerous monoclonal antibodies were generated mainly from mice for numerous basic research and clinical applications [1].

Monoclonal antibodies were produced from hybrid cell lines originally derived from cell fusions between myeloma cells and spleen cells of mice immunized with antigens [2]. Compared to the polyclonal antisera, monoclonal antibodies are epitope-specific with defined binding specificity and can be mass produced from established hybrid cell lines [1] [2]. In view of these unique characteristics, monoclonal antibodies have become the primary choices for studies of antigen-antibody interactions and for relevant immunodiagnostic and immunotherapeutic applications [3].

1.1. RP215 Monoclonal Antibody

Alternatively, monoclonal antibodies can be selectively generated from immunization with mixture of antigens in mice such as the cancer cell extract. The resulting hybridomas can then be screened for their respective ability to secrete specific antibodies against cancer-associated antigens or pan cancer biomarkers [3]. By immunizing mice against cancer cell extract from OC-3-VGH ovarian cancer cell line (from VGH, Taipei, Taiwan region), the antibody-secreting hybridomas were screened/selected by the established immunoassays and immunohistochemical (IHC) methods. Among thousands of monoclonal antibodies (~3000) generated and screened, the one designated as RP215 was identified to fulfil the criteria of pan cancer biomarker [4]. Through numerous biological and immunological studies during the last decade, RP215 was finally established as the monoclonal antibody for immunodiagnostic and immunotherapeutic applications, especially in cancer immunotherapy [5] [6].

1.2. GHR106 Monoclonal Antibody

GHR106 monoclonal antibody was generated from hybridomas through immunizations of mice with N1-29 oligopeptide as the immunogen corresponding to

the main extracellular domain peptide of human GnRH receptor [7] [8]. GHR106 was found to be specific to GnRH receptor located in the anterior pituitary and on the surface of many human cancer cells. GHR106 was extensively evaluated and established as GnRH antagonist for clinical applications in fertility regulations as well as in cancer immunotherapy.

In this review, biological and immunological studies of both RP215 and GHR106 monoclonal antibodies during the last two decades are highlighted to document their respective potential clinical applications either in cancer immunotherapy or in fertility regulations [8] [9].

2. Molecular Identity of Cancer-Associated Antigens (CA215) Recognized by RP215 Monoclonal Antibody

2.1. Cancerous Immunoglobulins Recognized by RP215

Following the generation of RP215 monoclonal antibody, it was initially established that the epitope recognized by RP215 is carbohydrate-associated due to the loss of epitope structure by NaIO4 [4]. Comprehensive epitope analysis was conducted by MALD-TOF MS method [4]. Affinity purification with RP215 as the ligand was performed and RP215-specific cancer-associated antigens (designated as CA215 from OC-3-VGH ovarian cancer cells) were isolated from cancer cell extract [4].

The purified cancer-associated antigen mixture (CA215) was subject to analysis by MALDI-TOF MS procedure [4] [5]. It was clearly revealed that the majority of CA215 were found to have high degrees of homology with those of human immunoglobulin heavy chains [4] [5]. By using Western blot assay, RP215 was shown to react mainly with proteins of 55 ± 5 KDa molecular weights which appear to be identical to that of immunoglobulin heavy chains. Furthermore, through MALDI-TOF MS analysis, the RP215-specific epitope was also found in many other minor proteins of similar molecular size [5]. These include antigen-presenting molecules (MHC-I, MHC-II), cell adhesion molecules, T cell receptors and many other related or unrelated immunoglobulin superfamily proteins, such as mucins [10].

2.2. Widespread Expressions of T Cell Receptors in Cancer Cells and Recognized by RP215

During the analysis of molecular identity of CA215, it was noticed that significant percentage of CA215 antigens are those of T cell receptor [10]. Further studies revealed that when antibodies against T cell receptor β was used as the probe, minor broad protein bands of 40 - 55 KDa were detected in either CA215 or crude cancer cell extracts, similar to those observed with RP215 as the probe [10].

2.3. Glycoanalysis of Carbohydrate-Associated Epitope Recognized by RP215

Since the RP215-specific epitope in cancerous immunoglobulins is carbohy-

drate-associated, glycoanalysis was performed in collaboration with NIH Complex Carbohydrate Research Center (CCRC) [11]. Initial analysis by MALDI-TOF MS revealed that RP215 recognized epitope is located in the immunoglobulin heavy chains. The exact structure and location of RP215 specific epitope either in Fc or Fab regions of heavy chain needs to be further clarified.

Upon treatment of culture cancer cells with tunicamycin to inhibit biosynthesis of N-glycans, the binding activity of RP215-specific epitope was not affected. Therefore, it can be suggested that RP215-specific epitope is associated with O-linked glycan, but unlikely the N-linked ones [11].

By using RP215-coated microwells and purified CA215 as the antigen, sandwich bindings of CA215 with enzyme immunoassays were designed. Three anti-domain-specific detection probes of human IgG including goat anti-human IgG-Fab (alkaline phosphatase labeled), goat anti-human IgG-Fc (alkaline phosphatase-labeled) and RP215—(horseradish peroxidase-labeled) were employed for comparative binding studies [6].

As indicated in **Figure 1(a)**, goat anti-human IgG-Fc probe has little effect on CA215 binding signal. However, with goat anti-human IgG-Fab as the probe, a strong dose dependent signal of CA215 binding can be clearly demonstrated, similar to that of enzyme immunoassay by using RP215 as the probe.

Based on results of these comparative studies, it can be ascertained that RP215-specific epitope is located at the Fab region (or variable domains) of the heavy chain immunoglobulins, but not in the Ig Fc domains. Results of such comparative analysis are presented in **Figure 1(a)**. Based on this comparative study, it can be demonstrated that carbohydrate-associated epitope is likely located in the Fab or variable regions of heavy chain immunoglobulins [11], but not in the Fe domains.

Therefore, in collaboration with NIH, Complex Carbohydrate Research Center (CCRC), the O-linked glycoanalysis of cancerous immunoglobulins was performed [11]. Affinity-purified CA215 was further purified by goat anti-human IgG affinity column [12]. Cancerous immunoglobins with RP215-specific epitope containing O-linked glycans were isolated and subject to glycosyl-linkage analysis. Based on the result of such analysis, the O-linked glycan structures associated with RP215-specific epitope are core 1 structure with 3-linked GalNAcitol and/or 3,6-linked GalNAcitol as presented in **Figure 1(b)**. The structure of the identified 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 which were found to be attached to immunoglobulins of heavy chain in cancer cells. For example, O-glycans of S-Tn, T, Tn in the mucin 1 (MUC1) glycoproteins have been reported and used as targets for CAR-T cancer immunotherapy in a CAR-T cell construct [13] [14].

A comprehensive glycan analysis of RP215-specific carbohydrate-associated epitope in the immunoglobulin heavy chain of cancer cells is diagrammatically presented in **Figures 1(a)-(c)**, respectively.

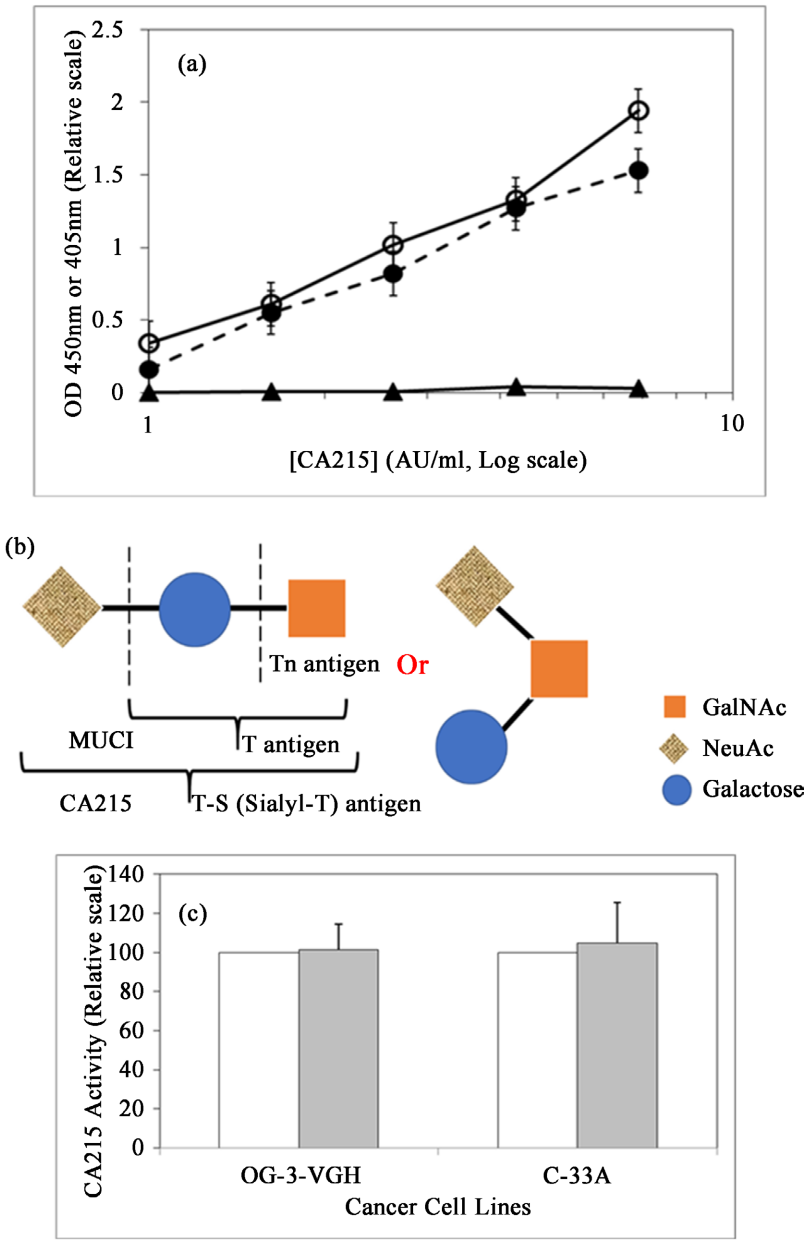

Figure 1. (a) RP215-based enzyme immunoassays to reveal dose-dependent signal or activity of CA215 (o) expressed in AU/ml and effects of goat antihuman IgG-Fc (1 μg/ml) (●) as well as goat antihuman IgG-Fab (1 μg/ml) (▲) on CA215 signal or activity; (b) Elucidated and proposed O-glycan structure associated with RP-215-specific epitope; (c) Effects of 48 hr tunicamycin treatments (1 μg/ml) on CA215 activity in the supernatant of two cultured cancer cells for OC-3-VGH and C-33A.

3. Tissue Specificity and Distribution of CA215 in Cancer Cells

In previous sections, the generations of RP215 monoclonal antibody was disclosed and extensively characterized with respect to the structural identity of the target antigens designated as CA215. As the target antigens, CA215 including cancerous immunoglobulins and several others in cancer cells were identified to

contain carbohydrate-associated epitope recognized specifically by RP215 [5].

To detect the presence of CA215 in human cancerous tissues, extensive immunohistochemical staining (IHC) studies were performed to determine statistically meaningful positive staining rates, when compared with those of negative staining of normal tissues [15]. These cancerous tissue sections with positive staining rates and the case number included those of ovary (64.4%, n = 87), cervix (84.3%, n = 51), endometrium (77.8%, n = 36), stomach (49.5%, n = 93), Colon (43.6%, n = 87), esophagus (75.7%, n = 56), lung (31%, n = 58), breast (32.2%, n = 59), Liver (3.5%, n = 60) and prostate (9.1%, n = 22) [15].

For benign ovarian tumor (n = 31), the positive rate of 6.5% was observed. The positive rates of cancerous tissues ranged from 84.3% for cervical cancer to 31% for lung cancer. In contrast, liver and prostate cancer as well as benign ovarian tumor showed less than 10% of positive staining.

In separate IHC studies, more than 20 different normal human tissue sections were used for IHC staining. Those with high RP215 positive staining rates are normal epithelial cells of hyperplastic nature [4] [15]. The IHC staining rates of these hyperplastic normal tissues included esophagus (83.3%, n = 6), uteri (75%, n = 4), skin (100%, n = 7) and cervix (80%, n = 5) [15]. However, the majority of normal tissue sections revealed negative staining results, when using RP215 as the probe. During the analysis of CA215, molecular identity, significant percentages of CA215 antigens are those of T cell receptor (TCR) origins [10]. By Western blot assays, it was revealed that antibodies specific to TCR also detect protein bands of 40 - 50 KDa molecular weight similar to those using RP215 as the probe [15]. Among different cancer tissue sections including those of endometrium, colon, breast, liver, throat, lung, ovary, kidney and esophagus, the positive staining rates by using RP215 and antiβ TCR as the staining probes ranged from 85% (17/20) and 75% (15/20), respectively. In contrast, the IHC staining of four different normal human tissue sections including kidney, brain, liver, and placenta revealed negative results with either primary antibody probe [15].

Therefore, it becomes apparent that T cell receptors are widespreadly expressed (≥80%) on the surface of cancer cells of many tissue origins, similar to those of cancerous immunoglobulins [10]. These observations may also indicate that cancer cells have evolved to create a separate and unique immune system in which both the immunoglobulins and TCR are co-expressed on the surface of cancer cells except that no expressions of CD-3 and CD-4 co-receptors were detected [10]. This observation may suggest that the immunology of cancer cells is quite different from that in our conventional immune system [10].

4. RP215 as a Probe to Study Functional Roles of Cancerous Antigen Receptors and Biological Implications

4.1. Induced Apoptosis by RP215 to Most Human Cancer Cells

RP215 reacts specifically cancerous immunoglobulins on the surface of almost all cancer cells, especially those of epithelial origins [16]. Apoptosis can be in-

duced specifically upon 24 to 48 hrs incubation with culturing cancer cells. Both RP215 and goat anti-human IgG induce apoptosis of cancer cells to a similar extent at a concentration range of 1 to 10 μg/ml. In contrast, the negative control with normal mouse or rabbit IgG of the same concentrations did not result in significant apoptosis to cancer cells. Since RP215 also recognizes T cell receptors β (rabbit IgG) on the cancer cell surface, apoptosis can also be induced in the presence of anti-T cell receptor β (rabbit IgG fractions) under the same assay conditions as those of RP215 [10]. Several established cancer cell lines including DU145 (prostate), A549 (lung), C33A (cervix) and MDA-MB 435 (breast) were also assessed for induced apoptosis. By using the same TUNEL assays, RP215 and anti-T cell receptor β or goat anti-human IgG was employed as separate probes, similar degrees of induced apoptosis was observed for various cancer cells [17]. Complement-dependent cytotoxic reactions (CDC) were also examined with the culturing cancer cells and either of the antigen receptor probes. In the presence of complement, lysis of cancer cells was observed following 2 hr co-incubation with cancer cells of different cell lines. In contrast, no complement-dependent cell lysis was observed, when normal mouse or rabbit IgG served as the negative control.

4.2. Immunodominant Epitope of RP215 Monoclonal Antibody

RP215 was shown to react with an epitope structurally associated with O-linked glycan located mainly in the variable regions of cancerous immunoglobulins heavy chains. Anti-idiotypic monoclonal antibodies (Aid) were generated against Fab-idiotypic domains of RP215 in rat. These rat monoclonal antibodies were generated and found to bear internal images of RP215-specific epitope [18]. Mice were immunized with Aid to generate Ab3 (anti-aid) [18]. Ab3 obtained from sera of immunized mice and were shown to induce apoptosis of cancer cells, similar to that of RP215. Based on results of these experimental observations, it can be assumed that these rat ant-idiotypic monoclonal antibodies or its Fab fragments can bear internal images of RP215-specific epitope [18].

On the other hand, the affinity-purified CA215 was used as immunogens to generate additional monoclonal antibodies from immunized mice [19]. Unexpectedly, all the newly generated monoclonal antibodies were found to react with the same or similar epitope to that of RP215, indicating that RP215-specific epitope is immunodominant [19]. Among the newly generated monoclonal antibodies RCA10 and RCA100 were found to have the same primary amino acid sequence as that of RP215. The other three (RCA104, RCA110 and RCA111) were found to react with confirmational domains of RP215-specific epitope. These antibodies can be paired mutually in RP215-based immunoassays for quantitative determinations of CA215 [6]. It remains to be explained why the immunodominant epitope exists and was associated with O-glycan (ST antigen) in CA215 or cancerous immunoglobulins, similar to those of MUC1 antigen epitope [19].

4.3. Effects of RP215 on Gene Regulations of Cancer Cells

It was revealed by MALDI-TOF MS analysis that CA215 consists mainly of immunoglobulin super-family proteins, particularly antigen receptors such as immunoglobulins, and T cell receptors, which were commonly recognized by RP215-specific epitope. Both RP215 and anti-antigen receptors were shown to induce apoptosis to culturing cancer cells *in vitro*. Therefore, their respective interactions on gene expressions of cancer cells were investigated [20]. Two cancer cell lines, namely OC-3-VGH (ovary) and C33A (cervix) were used as the model for such gene regulation studies [20]. A dozen of genes involved in the growth and regulations of the immune system were selected for studies and analyzed by semi-quantitative RT-PCR. Among these are NFkB-1, IgG, P21, EGF, Cyclin D1, Ribosomal P, c-fos and toll like receptors (TLR-2, -3, -4, -6, -7 and -9) [20]. RP215 and anti-antigen receptors were found to regulate similarly these gene expression levels upon interactions with cancer cells. High degrees of correlations among the different binding ligands (≥90%) were observed. RP215 and antigen receptors were also demonstrated to affect the gene regulation patterns of those involved in the innate immunity system including toll-like receptors [20]. Changes in gene regulation levels of toll-like receptors upon incubation with RP215 or antigen receptors were commonly observed. Up-regulation of TLR-3 gene was found to increase by 226%, 143% and 219%, respectively upon incubation cancer cells with RP215, anti-IgG and anti-T cell receptor, respectively. However, down regulations of TLR-4 and TLR-9 genes were consistently observed [20].

4.4. Distinct Structure and Expression of Normal vs. Cancerous immunoglobulins

In contrast to immunoglobulins expressed by normal B cells, the molecular mechanisms of actions regarding the cancerous immunoglobulins are not fully understood. In the case of conventional immune system, expressions of antigen receptors (immunoglobulins and T cell receptors) are differentially expressed by B cells and T cells, respectively. However, both cancerous immunoglobulins and T cell receptors are co-expressed by those derived from one single cell clone of cancerous origins. By comparisons, no class switching and few hypermutations were observed during the expressions of immunoglobulins on cancer cell surface. In addition, immu-noglobulins of different classes (IgG, IgA and IgM) and subclasses (IgG1, IgG2) were shown to be co-expressed by a single cancer cell or clone. Both normal and cancerous immunoglobulins were found to have the same location of N-linked glycosylations of immunoglobulin molecules. In contrast, O-glycans are attached and can be detected in the variable domain of heavy chain cancerous immunoglobulins, but O-glycans are rarely found in normal immunoglobulins.

In separate studies, gene expressions for cancerous immunoglobulin were investigated with more than 30 different cancer cell lines of human origin by

semi-quantitative RT-PCR [21]. Results of these studies revealed that high level expressions of immunoglobulin gene were obtained in all of the cancer cell lines tested. Furthermore, we also observed expressions of RAG1/RAG2 and AID genes as well as those of transmembrane domains for anchoring of cancerous immunoglobulins [22]. Therefore, it can be assumed that immunoglobulin genes are universally expressed by all types of cancerous immunoglobulins [21]. Molecular biological studies were performed to study the primary structures of V-(D)-T gene rearrangements of heavy chain immunoglobulin genes [22]. It was generally concluded that cancerous immunoglobulin genes are distinctly expressed as compared to those of normal B cells, especially in V-(D)-J regions of heavy chain domains. Due to limited but distinct somatic hypermutations as well as lack of class switching, only seven predominant VHDHJH set of cancerous immunoglobulins are expressed among cancer of different origins [22]. Parallel comparisons between normal and cancerous immunoglobulins are summarized in Table 1.

5. Dual and Distinct Functional Roles of Cancerous Antigen Receptors

In conventional immune system, antigen receptors such as immunoglobulins and T cell receptors expressed by immune cells were known to play crucial roles in the adaptive immune response against foreign pathogens and/or cancer cells [22]. However, it has also been known that cancer cells are capable of protecting themselves immunologically through unknown evasion mechanisms under our normal body environments. Therefore, we believe that antigen receptors expressed by cancer cells may play an important role in the immune surveillance and/or protections of cancer cells against the normal immune system [22]. B cells derived immunoglobulins and cancerous immunoglobulins were shown to differ in their expression patterns and activation as well as protection mechanisms [23]. Furthermore, cancerous immunoglobulins express aberrant glycosylation patterns in the heavy chain variable regions which are recognized as RP215-specific epitope described in Table 1. Therefore, RP215 was used as a unique probe to isolate cancerous immunoglobulins from the cancer cell extract. The human serum proteins which interact with cancerous immunoglobulins can be isolated, identified and characterized. Interactions between these human serum proteins and cancer cells may serve as the indicators regarding the functional roles of cancerous immunoglobulins expressed by cancer cells [23].

6. Anti-Cancer and Pro-cancer Activities of Human Serum Proteins Interacting with Cancerous Immunoglobulins or CA215

Attempts were made to determine possible serum antigens recognized by cancerous immunoglobulins and to elucidate their potential functional roles in natural human environment [23]. CA215 and/or cancerous immunoglobulins were

initially affinity-purified from the shed media of cultured OC-3VGH ovarian cancer cells by using RP215 and anti-human IgG as separate general ligands. Specific serum antigens with affinity to CA215 or cancerous immunoglobulins were isolated from human serum and subject to analysis by LC-MS/MS method [23].

As many as 72% of the fifty serum protein components were commonly recognized by either CA215 or cancerous immunoglobulins as the affinity ligands. Among these identified proteins, more than half were formed to be associated with pro-cancer or anti-cancer proteins (**Table 1**) [23]. The interactions between CA215 or cancerous immunoglobulins and these serum proteins may be critical to the growth and proliferation of cancer cells and at the same time neutralize harmful proteins or fragments in human circulations. A list of human serum proteins detected by CA215 or anti-human IgG were presented in **Table 2** according to the nature of anti-cancer and pro-cancer properties.

6.1. Human Serum Proteins Demonstrating Anti-Cancer Activities

The detected serum proteins with anti-cancer properties were found to include: Inter-α-trypsin inhibition heavy chain 4, anastellin, apolipoprotein A1, Fibrinogen β chain, and keratin type 1 cytoskeletal protein [23]. Most of these proteins were down regulated in cancerous tissues [24] [25] [26]. Some exert anti-angiogenic effects to inhibit growth and proliferation of cancer cells within human body [23], such as anastellin fibrinogen β chain and apoliprotein A1. For fibrinogen β-chain, the N-terminal fragment was found to inhibit tumor vascularization and increase tumor necrosis [27] [28] [29] in mouse models. Furthermore, it was

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	I. Immunoglobulins are expressed by B cells II. T cell receptors are expressed by T cells	Both antigen receptors are expressed by cancer cells and are of a single clone
Class switching of immunoglobulins	Yes. In B cells	No
Hypermutation of variable regions of immunoglobulins	High frequency	Low frequency
Glycosylation patterns (unique)	No O-linked glycosylation and only one N-linked glycosylation at N297 position of IgG heavy chains; terminal NeuAc only (not	Both O-linked and N-linked glycans are detected in cancerous IgG heavy chains with terminal NewAc and NuGc (O-linked glycan recognized by RP215 Mab
Interactions with TLRs (based on gene expression studies)	No known interactions with TLRs	Strong interaction with TLRs within cancer cells
Relative immunoactivity	Normal immunoactivity	Weak immunoactivity (less than 1% - 5%) due to aberrant glycosylations
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)

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[a]	Protein	Molecular weight (kDa)	Keynotes related to cancer
Pro-cancer	C4b-bindingprotein	67	Protects cancer cells from complement activation & attack
	ComplementC3	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
Anti-cancer	35 kDa inter-α-tryps in Inhibitor heavy chain 4	104	Down regulation leads to tumor initiation &progression in multiple solid tumors
	Anastellin	256	Inhibits tumor growth & metastasis *in-vivo*
	ApolipoproteinA-1	31	Suppresses tumor growth & metastasis in animaltumor models
	Fibrinogen β chain	56	β43-63 inhibits tumor vascularization in mouse models
	Keratintype I cytoskeletal9	62	Down regulation is associated with increased drug resistance in breast cancer

demonstrated that mice lacking apolipoprotein A1 develop tumors faster than the wildtype, suggesting that this serum protein may serve as a tumor suppressor [28] in our normal human environment (Table 2).

6.2. Human Serum Proteins with Pro-Cancer Activities

Serum proteins that showed pro-cancer properties are serotransferrin, C4b-binding protein, complement factor H, complement C3 and vitronectin [27] [28] [29]. Serotransferrin has been implicated as an important growth factor for cancer cell proliferation. C4b-binding protein and complement factor H are important factors to prevent cancer cells from complement activation. Secretion of complement factor H [29] was also observed in many cancer cells. Down regulation of complement factor H has been shown to sensitize cells to complement attack, leading to reduction in tumor growth [25]. Complement 3 is a central protein in the complement cascade, and associated with a variety of tumorigenic effects, leading to cancer cell development and progression. Compared to wild-type mice, C3-deficient mice showed significant decrease in tumor proliferation. Vitronectin is a glycoprotein found in the extra cellular matrix and human serum. It was shown to be an inducer of cancer stem cell differentiation, migration or growth in breast and prostatic carcinoma [23].

6.3. Biological Implications

In summary, many human serum proteins were frequently detected to interact with CA215 on the cancer cell surface. Some were known to exhibit anti-cancer or pro-cancer properties in human circulations [23]. Those with pro-cancer

properties may help to promote growth/proliferation of cancer cells in circulation [23]. On the other hand, serum proteins with anti-cancer in nature can also be neutralized or captured by the surface bound cancerous immunoglobulins or CA215 to inhibit growth of cancer cells. The exact mechanisms of the interactions mediated by cancerous immunoglobulins remain to be investigated in the future. The dual functional roles of cancerous immunoglobulins or CA215 were clearly demonstrated through their mutual interactions with circulating human serum proteins. These experimental observations may help to explain how cancer cells can survive/proliferate or can be destructed under our normal human environments. Therefore, expressions of immunoglobulins on cancer surface cells may be required to perform such dual functional roles.

7. GHR106 Monoclonal Antibody is Long-Acting GnRH Antagonist

As mentioned, GHR106 was generated against N1-29 oligopeptide corresponding to the extracellular domains of human GnRH receptor. During the last decades, this monoclonal antibody has been extensively characterized [7] [8] [9]. Similar to those of RP215 monoclonal antibody, it was finally established that GHR106 is a long-acting GnRH antagonist having a half-life of 5 - 21 days as compared to those available in the market such as Cetrorelix, Elagolix and Relugolix [30] [31] [32].

7.1. Tissue-Specificity of GHR106 and Distribution of GnRH Receptor

GHR106 was initially generated against an oligopeptide corresponding to N1-29 amino acid residues in the extracellular domain of human GnRH receptor located either in the anterior pituitary or in reproduction-related peripheral tissues of gonadal origins such as ovary, testis, uterus and/or placenta [8]. On the other hand, GnRH receptor is almost universally ex pressed on the surface of many cancer cells [7] [8]. Expressions of human GnRH receptor in cancer cell lines were investigated by RT-PCR, indirect immunofluorescence staining and Western blot assays [7]. Out of more than thirty tested human cancer cell lines, only one (Jurket, T cell lymphoma) showed negative staining results based on these criteria [7]. A high incidence of GnRH receptor expression was observed in cancer of many tissue origins. RT-PCR and the binding assays were used to study the expression of GnRH receptor of normal and cancerous tissues in as early as 1989 [33]. The positive detection rates of different cancerous tissue sections varied from 43% to 100% [34]. It was generally observed that the incidence of positive expressions of GnRH receptor among cancer cells has little to do with their respective tissue origins [35]. Among the cancerous tissues studied, the positive staining/expression rates are given as follows: breast (52%), ovary (78% - 80%), endometrium (77% - 100%), prostate (86% - 100%), kidney (80%), brain (43%) and pancreas (57% - 100%) [34] [35].

7.2. Functional Studies of GHR106 Interaction with GnRH Receptor in Cancer Cells and Anterior Pituitary

During the past decade, our studies have been focused on the functional aspects of GHR106 binding to human GnRH receptor *in vitro* and *in vivo* [7] [8]. Through biochemical and immunological studies, the dissociation constant between the antibody and the N1-29 oligopeptide or GnRH receptor in the cancer cell extract was determined to be 3 ± 1 nM [36]. The half-life of GHR106 (hIgG4) in rabbits was determined to be 5 ± 1 days, whereas its half-life (human IgG4) can be as long as 21 days in human circulations. Dual functions of known human GnRH receptor are known to exist, depending on its tissue origins [7] [8], GnRH receptor located in the anterior pituitary is known to regulate or control the release of gonadotropins including LH and FSH upon the pulsetile binding interactions of GnRH released in hypothalamus [8]. GHR106 was shown to compete with native GnRH for the binding of GnRH receptor [8].

"Proof-of-concept" experiments in rabbits were conducted to demonstrate reversible suppressions of serum reproductive hormones upon a single injection of GHR106 (hIgG4) [36]. In the case of the male, a single subcutaneous injection of 1 - 3 mg/kg of GHR106 was shown to reduce parallelly the serum LH and testosterone concentration by 80% - 90% for a period of one to two weeks. The levels of reproductive hormone return to normal levels within one to two weeks post injections. Similarly, the suppressions of LH and estradiol (E2) were observed within two days upon a single injection of GHR106 (hIgG4) in the female rabbits [36]. The E2 levels decrease to less than 30% of the normal level over a period of one week or longer followed by full recovery of all reproduction hormones [36]. Comprehensive "Proof of Concept" rabbit experiment to demonstrate reversible suppressions of reproductive hormones (LH, Testosterone, Estradiol) were presented in **Figure 2(a)** and **Figure 2(b)**, respectively upon single injections of GHR106 in either male or female rabbits. The results of this study revealed that GHR106 (hIgG4) may be used as antibody-based GnRH antagonist with similar biological action to those of decapeptide GnRH antagonist or Elagolix (oral) [31]. Therefore, the relatively longhalf-life of this class of GnRH antagonist may be beneficial to the treatments of certain gynecological and fertility-related diseases which are caused by abnormal modulations of human GnRH receptor [32].

7.2.1. Apoptosis of Cancer Cells Induced by GHR106 as GnRH Antagonist

Immunohistochemical staining studies of GHR106 revealed that greater than 90% of human cancer cell lines express GnRH receptor on the cell surface [7] [8] and were positively stained with this monoclonal antibody [8] [9]. By TUNEL assay, GnRH, Antide (a decapeptide GnRH antagonist) and GHR106 were shown to induce apoptosis of cancer cells to a similar extent. However, GHR106 had a much longer half-life than those of antagonists of small molecules (e. g. Cetrorelix and Elagolix or Relugolix) (days vs. hours). Furthermore, GHR106 was shown to induce complement-dependent cytotoxicity (CDC) reactions to

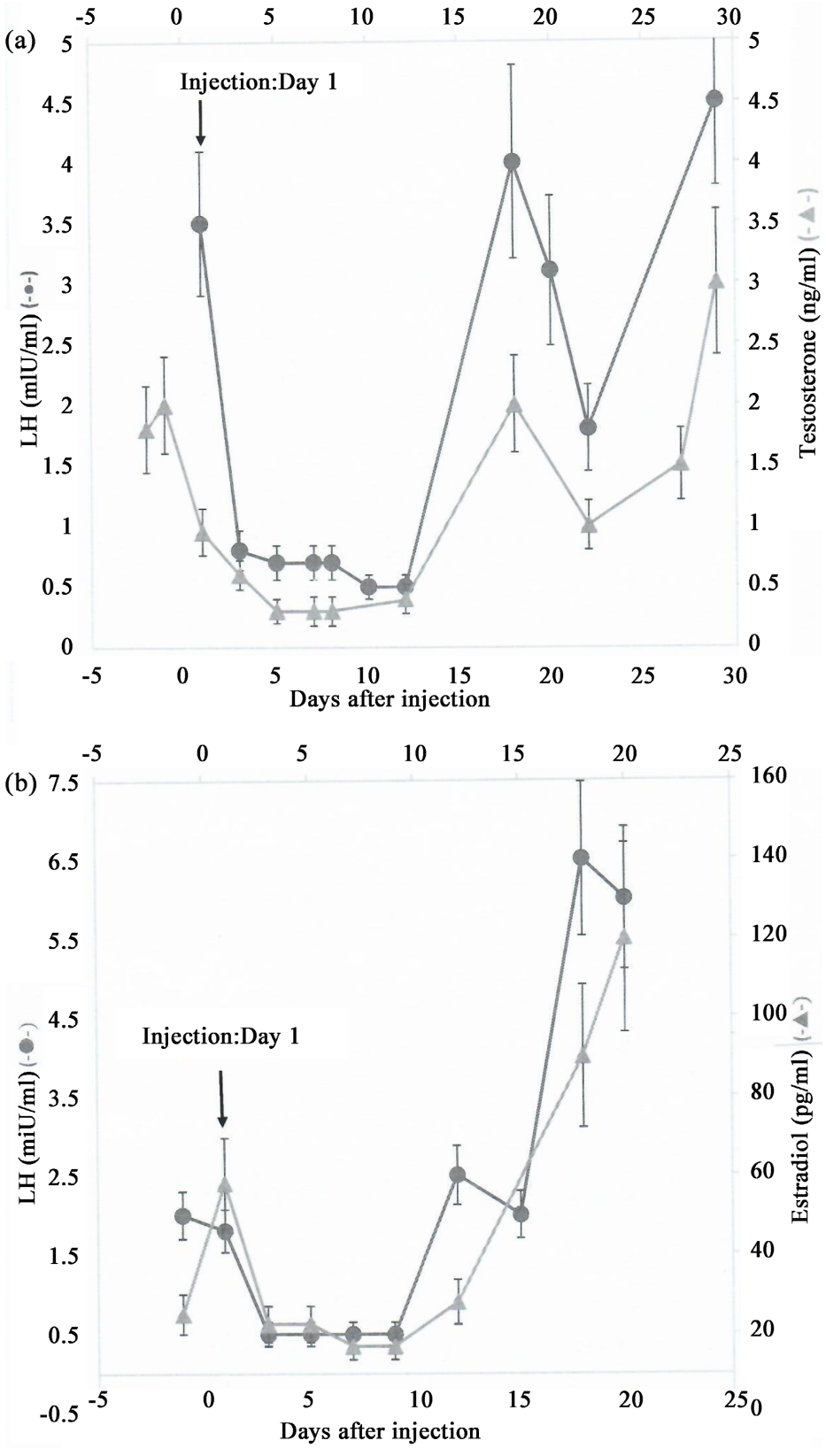

Figure 2. (a) Hormonal profiles (LH and Testosterone) upon a single sc injection of 3 mg/kg of GHR106 (hIgG4); (b) Hormonal profiles (LH and E2) upon a single injection of 3 mg/kg of GHR106 (hIgG4).

cancer cells in culture [8]. Based on this *in vitro* functional studies, we believe that GHR106 can serve as long-acting GnRH antagonist to inhibit the growth of cancer cells *in vivo* for future applications in cancer immunotherapy [9].

7.2.2. Effects of GHR106 and Other GnRH Antagonists on Gene Regulation of Cancer Cells in Culture

Since apoptosis can be induced upon incubation of GHR106 or other GnRH analogs, changes in expressions of several genes involved in cell proliferation or survival of cancer cells were investigated by semi-quantitative RT-PCR method [8]. Interestingly, GnRH peptide analogs, Antide was found to have an identical pattern of gene regulations to those of GHR106 upon treatment of cancer cells *in vitro* [9]. Following 24 to 48 hours of incubations with either GHR106 or Antide, the expression of GnRH receptor gene is unchanged, while the expression of GnRH gene is up regulated by as much as 50%. The EGF (epidermal growth factor) gene, on the other hand, is down-regulated by either ligand. Down-regulation of cell cycle regulator, Cyclin D1 was also found. This observation is consistent with the molecular mechanisms of action by either receptor binding ligand to induce apoptosis of cancer cells *in vitro* [7] [9].

8. Potential Clinical Applications of RP215 and GHR106 Monoclonal Antibodies

Extensive biological and immunological studies have been reviewed in previous sections for these two distinct monoclonal antibodies [4] [5] [6] [7]. Both antibodies showed high binding specificity to cancerous immunoglobulins (CA215) and GnRH receptor, respectively on the surface of most cancer cells in humans, yet rarely on the normal tissues. Therefore, they are considered as pan cancer biomarkers which can be targeted by these two antibodies in cancer immunotherapy for many types of human cancer [7]. In addition, specific binding interactions between GHR106 and GnRH receptor in anterior pituitary can result in reversible suppressions of reproductive hormones [36]. This is similar to other known GnRH antagonists which are being used clinically, except that the former has a longer circulation half-life (5 - 21 days).

8.1. Humanizations of RP215 and GHR106

For clinical applications in cancer immunology and others, humanizations of RP215 and GHR106 are required, since both are of murine origins [37]. Generally speaking, for complete humanizations of antibodies, the constant regions of IgG heavy chains and that of k (kapa)-light chains were replaced. with those of human origins [37]. The resulting humanized forms may contain substantially similar or identical CDR fragments in the Fab variable regions of IgG and were designated as hRP215 and hGHR106, respectively [37]. Binding assays by using CA215-based immunoassays were performed to select the one with the highest binding affinity and similar specificity to that of original mRP215 (Kd~ 4.2 nM) [38]. Similarly, different versions of humanized forms of GHR106 (hGHR106)

were constructed and selected based on the enzyme immune binding assays with cancer cell-coated microwells [38]. Finally, H40 and L42 were selected for the best combinations of heavy/light chains in hGHR106 having the dissociation constant (Kd) comparable to that of murine GHR106 (mGHR106) (~3 ± 1 nM). The finally optimized humanized forms of RP215 and GHR106 (hRP215 and hGHR106) were also constructed and selected. These two humanized monoclonal antibodies have been shown to have the same specificity and similar binding affinity to those of the murine forms and are being utilized for current drug development studies. Both mRP215 and hRP215 were shown to have no cross-reactivity to normal human IgG [38]. Bioequivalence between mRP215 and hRP215 was also demonstrated by functional assays and other established criteria [37]. For example, both mRP215 and hRP215 were shown to induce significant apoptosis and CDC reactions of OC-3-VGH ovarian cancer cells in culture at antibody concentration as low as 1 μg/ml [9].

8.2. Demonstration of Anti-Tumor Effects *in Vivo* by Nude Mouse Experiments

The anti-tumor effects of RP215 and GHR106 on the growth inhibition of implanted tumor were demonstrated *in vivo* by nude mouse experiments [5]. Both goat anti-human IgG (cross-reacting with cancerous IgG) and RP215 were shown to inhibit the growth of implanted tumor in nude mice [5], indicating cancerous immunoglobulins are main target on the cancer cell surface [5].

Three different cancer cell lines including those of ovary (OC-3-VGH), lung (SK-MESH) and cervix (C33A) were used as for generation of implanted tumor in nude mouse experiments. Similar dose-dependent tumor volume reductions were observed with either RP215 or GHR106 injections. The experimental results clearly indicated that RP215 or GHR106 can inhibit tumor growth *in vivo* in nude mice. Therefore, it is reasonable to believe that RP215 and GHR106 can be used as anti-cancer drugs for therapeutic treatments of cancer in humans [6] [7].

8.3. Monitoring of Serum Levels of CA215 among Cancer Patients by RP215-Based Enzyme Immunoassays

CA215 is recognized by RP215-specific epitope and can be detected either in the shed media of cancer cells or from serum specimens of cancer patients [6]. RP215-based enzyme immunoassay was established to determine serum levels of CA215 for monitoring cancer patients [39].

Serum levels of CA215 from patients with ovarian or cervical cancers were determined and evaluated [6] [39]. 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 at stages I, II, and III, respectively [39]. In the case of cervical carcinoma, the positive rates of CA215 for those patients were determined to be 66% (n = 35), 94% (n = 18) and 71% (n = 7) for diseases of stage I, II, and III, respectively [6], in contrast to the benign tumors, such as endometriosis and

uterine myoma, (≤10%). Statistical analysis revealed that stage dependence of serum CA215 levels remained at relatively high levels during the preoperative stages (≤7 days). However, serum CA215 levels decreased significantly when determined 7 days after the surgical operations [6]. These results may indicate that serum CA215 levels reflect the tumor burden of individual patients. Therefore, RP215-based immunoassay should enable us to determine serum CA215 levels from cancer of many tissue origins [39]. In large scale clinical evaluation, serum levels of CA215 were determined from 500 cancer patients which had been confirmed with either one of twelve different types of cancers. The same clinical specimens were also used to determine marker levels of other known cancer biomarkers including alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), cancer antigens such as CA125, CA15-3, and CA19-9, β2-microglobulins and cytoskeleton 19 fragments (Fcyfra-21). 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 [39]. The results of such comparative studies of individual biomarkers and those combined with CA215 are summarized in **Table 3**. Based on these studies, the positive rates of serum CA215 levels are also listed as follows: 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. Among these markers, CA125 is commonly used for detection and monitoring of ovarian cancer. CA15-3 is suitable for cancer of breast, whereas CA199 is generally used for monitoring of pancreas, stomach and liver cancer. CEA and β2-microblubulins are cancer biomarkers of broad tissue origins. Cyfra-21-1 is commonly used for monitoring of lung cancer. Positive rates of CA215 and other biomarkers, singly and combined are summarized in **Table 3**, with six types of human cancer (lung, liver, ovary, esophagus, breast, and stomach). It was generally observed that the combined use of CA215 and either one of these cancer biomarkers resulted in higher detection rates to a given cancer. For example, in the case of ovarian cancer, positive detection rates of individual biomarkers are 59% each (n = 68 and 66, respectively) for CA125 and CA215. 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%. Based on the results of this analysis, the combinations of CA215 and other known cancer bio-markers do improve greatly the positive detection rates for human cancers. They are listed in **Table 3** for comparisons.

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

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

CA215 (0.1 Au/ml)	Cancer	Lung (n)	Liver (n)	Ovary (n)	Esophagus (n)	Breast (n)	Stomach (n)
CEA (5 ng/ml)	Ia	52% (112)	74% (58)	-	61% (23)	71% (44)	60% (30)
	IIb	67% (33)	54% (35)		47% (19)	95% (20)	50% (14)
	IIIc	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 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: percent positive by CA215 only; b: percent positive by other marker only; c:III: percent positive by combining both CA215 and designated marker.

routine cancer monitoring (39) (Table 3).

8.4. Cancer Immunotherapy with Applications of RP215- or GHR106-CAR-T Constructs

CAR-T (chimeric antigen receptor-transfected T cells) technology has been evolved for new applications in cancer immunotherapy [40] [41]. CD19-CAR-T cells have been first utilized in the therapeutic treatments of liquid tumors such as those of blood cell origins [40] [41] [42] [43]. High degrees of remission rates (≥50% - 90%) were observed for different types lymphoma following suitable CD19-CAR-T cell treatments [40]. However, it remains to be a major challenge for treatments of solid tumor by using the same technology [41] [42]. Since the majority of human cancers are solid tumors, the issues of tumor micro-environment and diffusion/penetration

of antibody-CAR-T cell constructs need to be resolved [41] [43]. Therefore, in this study, RP215 and GHR106 were introduced in the format of scFv-CAR-T cell constructs to target pan cancer biomarkers in cancer immunotherapy.

Following humanizations, hRP215 and hGHR106 were produced and scFv (single chain variable fragments) of either antibody was generated. Following insertion into CAR-T cells [40], hRP215 (ScFv)-CAR-T and hGHR106 (scFv)-CAR-T cell constructs were created for *in vivo* expansion. The CAR-transfected T cells can be re-infused back to the same cancer autologus patients to induce cytotoxic killing of cancer cells *in vivo* and achieve therapeutic objectives [41].

For RP215 (scFv)-linked CAR-T cell construct, demonstrations of cytotoxic cell killings of C33A (cervical cancer cells) were presented for IL-2, IL-7 and INF cytokines as shown in **Figure 3**. The cytotoxic cell killings were shown to be dose-dependent depending on the effector (E) to target (T) cells ratios (effector and target cells).

Based on this principle, hRP215(scFv-CAR-T cells and hGHR106(scFv)-CAR-T cells were constructed separately and tested *in vitro* with C33A cervical cancer cells in culture for 8 hours (13,17). Dose-dependent lysis of cancer cells were observed in this model experiments, either with hRP215-CAR-T (**Figure 3**) or hGHR106-CAR-T cells (**Figure 4**). The cell lysis validated by cytokine release assays of IL-2 IL-7 and INF-*γ*, respectively [14]. Similarly, the releases of these three cytokines as a function of (E/T) ratios were demonstrated and presented in **Figure 4** for hGHR106-CAR-T cell construct. The selected cancer targets, CA215 and GnRH receptor are considered as pan cancer biomarker in nature. Therefore, RP215 and GHR106 have unique advantages in clinical applications

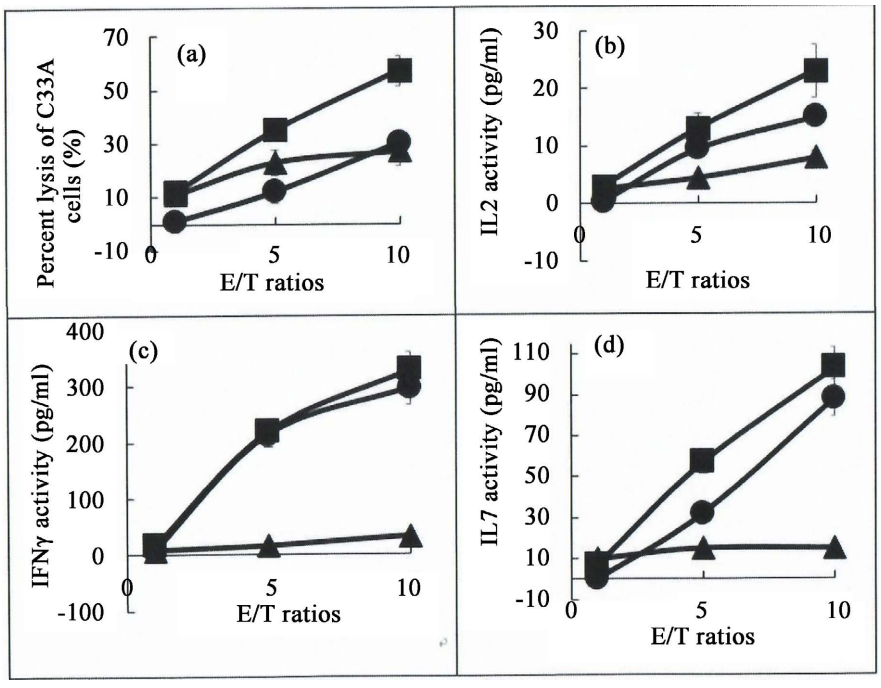

Figure 3. Demonstrations of the lysis of C33A (Cervical) as the target (T) cancer cells upon co-incubation with RP215 (scFv)-linked CAR-T cells (effector cells, E).

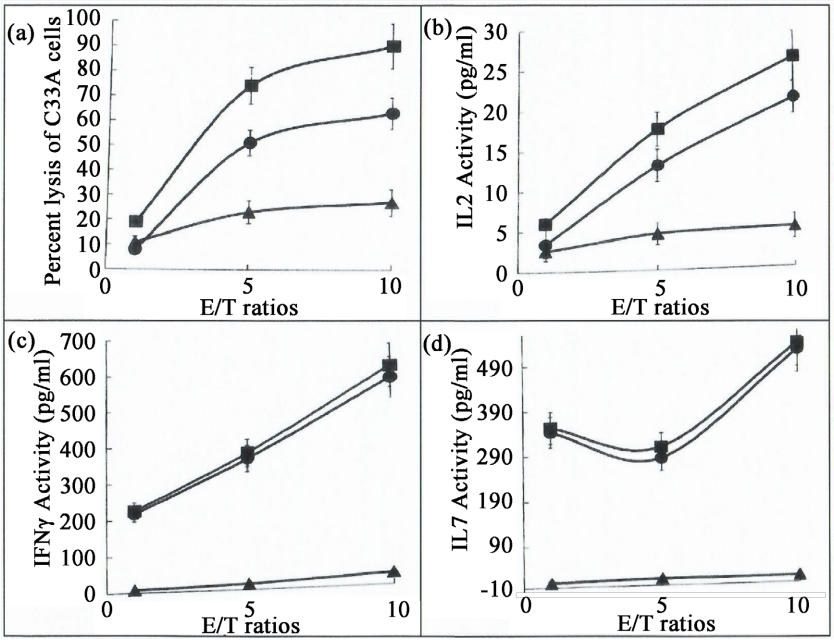

Figure 4. Demonstrations of the lysis of C33A cancer cells (T, target cells) upon co-incubation with hGHR106 (scFv-linked CAR-T cells (E, effector cells) similar to RP215-CAR-T constructs; the cytokine assays were performed with the established ELISA including IL-2 IL-IL7 and IFN-*γ*. E/T ratios varied from 1:1, 5:1 to 10:1 respectively. Cytokine releases were shown to be GHR106-CAR-T cells dependent in all three cytokines, when compared to the negative control with virus-only-transduced T cells for co-culturing.

of cancer immunotherapy for many types of human cancer.

8.5. hGHR106 Is a Long-Acting GnRH Antagonist for Clinical Applications in Fertility Regulations

Since mGHR106 was of murine origin, hGHR106 was created through humanization processes [37]. The resulting hGHR106 retains similar specificity and affinity to mGHR106 in terms of its functions as GnRH antagonist. As described in "proof of concept" rabbits experiments, reversible suppression of reproductive hormones were observed over a period of one to two weeks upon a single injection of hGHR106 (1 - 3 mg/kg) (**Figure 2**) when compared to other known GnRH antagonists such as Cetrorelix and Elagolix,. Besides applications in cancer immunotherapy, hGHR106 can serve as a long-acting GnRH antagonist for therapeutic treatments of many benign gynecological disorders including endometriosis, premenstrual syndrome, precocious puberty uterine fibroids, and polycystic ovarian syndrome. For IVF-ART applications, inhibitions of spontaneous ovulation by a single subcutaneous (sc) injection of hGHR106 may be beneficial to IVF patients.

9. General Conclusion

9.1. RP215 and GHR106 for Cancer Immunotherapy

In this review, two unique monoclonal antibodies, namely RP215 and GHR106

were generated, characterized and recommended for clinical applications in cancer immunotherapy and/or fertility control/regulation. They were shown to target, respectively cancerous immunoglobulins and GnRH receptor which are widespreadly expressed on surface of many cancer cells in humans, but rarely in normal tissues.

Since these two targets are of pan cancer biomarkers in nature, RP215 and GHR106 may be used for clinical therapeutic treatments of many different types of human cancer in various formats (naked, CAR-T or NK-CAR). Based on results of large scale IHC (immunohistochemical) studies, it was established that RP215 and GHR106 positively stain those cancer tissue sections of high cancer incidence in humans [5] [33] [34] [35], including those of cervical endometrial, ovarian, lung, breast, prostate, and stomach. As pan cancer markers, CA215 and GnRH receptor can be targeted specifically by either one of these antibodies, during cancer immunotherapy [40] [41] [42] [43]. Therefore, RP215 and GHR106-based anti-cancer therapy should play important roles in cancer immunotherapy for a broad spectrum of human cancer, especially those of gynecological origins [13] [14].

9.2. GR106 as an GnRH Antagonist for Fertility Regulations

Through "Proof of Concept" rabbit experiments, GHR106 has also been established as long-acting antibody-based GnRH antagonist (5 - 21 days). This is in contrast to the currently available short acting GnRH antagonists (~hours) of small molecular size such as Cetrorelix (decapeptide) and Elagolix (organic chemical) [36].

As a long-acting antibody-based GnRH antagonist, hGHR106 (IgG4) in humanized forms of IgG4 isotype was introduced [36]. Potential clinical market size for GHR106 (IgG4) as GnRH antagonist for treatments of major gynecological diseases can be as much as 35 billion USD per year globally. As long-acting GnRH antagonist, hGHR106 (IgG4) introduced in this review can be used for treatments of any of the following indications in gynecological diseases: 1) Inhibition of spontaneous ovulation in ART, 2) Endometriosis, 3) Uterine fibroids, 4) Precocious puberty, 5) Premenstrual syndrome and/or 6) Polycystic ovarian syndrome.

Therefore, we believe that antibody-based long-acting GnRH antagonist can be a good alternative to currently available ones.

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Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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