

# Implications of apoptosis in cancer immunotherapy\*

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## ABSTRACT

**Inhibitions or blockages of ligand-receptor interactions on cancer cell surfaces by exogenous competitors or antibodies often result in apoptosis or “programmed cell death”. The underlying mechanisms of action for cellular apoptosis depend greatly on the molecular nature of specific ligand-receptor interactions and the signal transduction pathways involved. Two such unrelated systems which are potentially involved in apoptosis of cancer cells are described in this review. They are, respectively, gonadotropin-releasing hormone (GnRH) receptor and cancerous immunoglobulins, or CA215, both of which are widely expressed on the surface of cancer cells from diversified tissue origins. Bindings of GnRH or its decapeptide analogs as ligands to GnRH receptor were known to induce apoptosis of several extrapituitary cell types in gonadal tissues, as well as different cancer cells. Monoclonal antibodies against the GnRH receptor of cancer cells were shown to induce apoptosis, similar to the action of GnRH analogs. In contrast, RP215 monoclonal antibody reacts specifically with the carbohydrate-associated epitope of cancerous immunoglobulins and is known to induce apoptosis of cancer cells *in vitro*. It also causes growth inhibition of tumor cells in nude mouse experimental models. Elucidations of the specific mechanisms of apoptosis in cancer cells of these two molecular interaction systems will not only lead to a better understanding of cancer biology but also benefit patients in cancer monitoring and therapy.**

**Keywords:** Apoptosis; GHR106; RP215; CA215; Pan-Cancer Biomarker; GnRH Receptor

## 1. INTRODUCTION

### 1.1. Gonadotropin-Releasing Hormone versus Gonadotropin-Releasing Hormone Receptor in Cancer Cells

It has been known for decades that gonadotropin-releas-

ing hormone (GnRH), from the hypothalamus, acts as the ligand to the GnRH receptor, located in the anterior pituitary gland, to trigger the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) [1]. Both LH and FSH are essential hormones for sexual maturation and differentiation of gonadal tissues in both sexes, which result in the production and release of either sperm (male) or oocytes (female) for reproduction [1].

It was later observed that GnRH receptor was also found in a number of extrapituitary tissues/organs, especially in reproductive tissues. This extrapituitary GnRH/GnRH receptor system serves completely different physiological functions [1,2]. Basically, GnRH and GnRH receptor in these tissues or cells are expressed in auto-crine/paracrine regulations of cellular growth, apoptosis or differentiation [3]. Under certain normal physiological conditions, apoptosis can be induced through combined expressions of GnRH and GnRH receptor in a given cell type in response to the stimuli of surrounding cellular conditions [3,4]. In the case of cancer cells, GnRH and GnRH receptor are universally expressed and regulated irrespective of their tissue origins [3-5].

Apoptosis can be readily induced with exogenous GnRH or its analogs to cancer cells [1,2]. In view of the fact that the native neuroendocrine hormones, GnRH I and GnRH II, are relatively short in circulation half-life (~minutes), GnRH analogs were synthesized and utilized as a substitute of native GnRH and demonstrated a somewhat longer half-life (~hours) in circulation. These GnRH analogs were further classified as agonists and antagonists, depending on their hormone actions in the pituitary gland [5,6].

Although both the agonists and antagonists have comparable or better affinities to GnRH receptor or longer half-lives than that of GnRH, their respective biological actions are completely opposite to each other. While GnRH agonists show similar functions as those of GnRH, GnRH antagonists can function to inhibit or down-regulate LH and FSH release from the pituitary cells [7]. However, in the case of cancer cells, apoptosis can be induced whether by native GnRH, GnRH agonists, or GnRH antagonists, *in vitro* [8,9].

Recently, monoclonal antibodies (mAbs) against the

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GnRH receptor, especially those specific to the extracellular domain of N1-29 synthetic peptide, have been successfully generated and characterized [10,11]. These mAbs can be considered as long acting and high molecular weight GnRH analogs with an average half-life of 5 - 21 days in circulation.

Through extensive comparative studies with GnRH analogs, it was concluded that anti-GnRH receptor mAb, GHR106, was essentially identical to GnRH or its analogs in terms of their respective biological actions [8,12]. Apoptosis can be induced to cancer cells *in vitro* upon incubation of GHR106 mAb in cultured cancer cells [13]. The apoptotic mechanisms of action of GHR106 mAb were studied through gene expression and regulation studies [12]. The results were then compared with those observed previously for the GnRH analogs. The study results are summarized in this review. Our aim is to evaluate if GHR106 mAb can be a suitable substitute for GnRH agonists and can be used as a long-acting anti-cancer drug, following humanization and clinical studies [13].

### 1.2. Cancerous Immunoglobulins versus RP215 in Cancer Cells

It has been known for two decades [14,15] that immunoglobulins are expressed by cancer cells and certain normal cells other than B cells in humans [16]. It was later demonstrated in several laboratories that cancer cell-expressed immunoglobulins are required for the growth and proliferation of cancer cells *in vitro* and *in vivo* [14,15]. However, the etiology of the expression of cancerous immunoglobulins is still not fully understood. In 2008, a mAb designated as RP215 was shown to react mainly with the carbohydrate-associated epitope of cancer cell-expressed immunoglobulins [17,18]. Through extensive matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and protein BLAST studies, it is now established that RP215 mAb's specific "sugar" epitope can be detected specifically in a number of glycoproteins, generally known as CA215. CA215 represent a group of glycoproteins consisting of immunoglobulins (42%), T-cell receptors (6%) [19], cell adhesion cells (8%), MHC molecules (5%) and several others [12,18]. It was further demonstrated that the RP215-specific epitope is not found in immunoglobulins derived from normal B cells [18,20].

CA215 glycoproteins consist of both membrane-bound and soluble (secreted) forms in cancer cells [18]. The RP215-specific epitope found in CA215 was shown to be universally expressed on almost all cancer cells [20]. Similar to the relationship between GHR106 mAb and GnRH receptor in cancer cells, RP215 mAb was shown to induce apoptosis and complement-dependent cytotoxicity (CDC) reactions to many types of cancer cells [19].

Attempts have been made to elucidate the mechanisms of action regarding the induction of apoptosis through gene regulation and expression studies [12]. These results are briefly summarized in this review.

## 2. APOPTOSIS AND GENE REGULATION OF CANCER CELLS BY GHR106 MONOCLONAL ANTIBODY

### Comparable Biological Activities between GHR106 and Gonadotropin-Releasing Hormone Analogs

As explained in the previous section, GHR106 mAb was generated against the extracellular domain (N1-29 amino acid residue) of the human GnRH receptor [10]. It is in fact a high molecular weight equivalent of GnRH analogs (80 kDa vs 1.2 kDa) in terms of its relative biological actions [11], except with a longer half-life than the latter (5 - 21 days vs hours).

Similar to GnRH or its analogs, apoptosis of cancer cells can be induced upon treatment with GHR106 mAb for 24 to 48 hours. Dose-dependent growth inhibitions of implanted tumors was also demonstrated with GHR106 mAb, as well as GnRH analogs in nude mouse animal models [13].

The mechanisms of actions of GnRH or its analogs (agonists and antagonists) to induce apoptosis of cancer cells have been studied extensively since two decades ago [1-4]. Generally speaking, upon bindings of GHR106 or GnRH analog to GnRH receptor in cancer cells, major changes in the gene expressions of OC-3-VGH ovarian cancer cells were observed [11,21]. The results of such parallel studies with a number of genes involved in the growth regulations of cancer cells are summarized in **Table 1** for comparison.

Based on the results of this study, both GHR106 mAb and GnRH antagonist (Antide, in this case) were found to exhibit identical biological actions on the expressions of a number of genes which are involved in the growth and proliferation of cancer cells [22]. Interestingly, both GHR106 mAb and Antide (GnRH antagonist) cause consistent down-regulation of the epidermal growth factor (EGF) [8], the cell cycle regulator, cyclin D, and some of the ribosomal genes involved in cellular protein synthesis, such as L37 and P0, whereas up-regulation of another cell cycle regulator, P21, was observed.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay [9] has been well established to assess induced apoptosis of cultured cancer cells upon incubation with either GHR106 mAb or GnRH analogs (Antide) [12,13]. A number of cancer cell lines were used for comprehensive analysis, including those of the prostate (PC-3 and DU145), lung (A549), and breast (MDA-

**Table 1.** Effect of treatments of cultured OC-3-VGH ovarian cancer cells with various monoclonal antibodies or GnRH antagonists on gene expressions.

Gene	RP215 (10 µg/mL)	GHR106 (10 µg/mL)		Antide (GnRH Antagonist) (0.1 µg/mL)
		10 µg/mL	20 µg/mL	
GnRH	NT <sup>a</sup>	↑	↑	↑
GnRH receptor	NT	NC	NC	NC
IgG	↑ <sup>a</sup>	NT	NT	NT
NFKB1 <sup>b</sup>	↑	NT	NT	NT
P <sub>0</sub>	↓	↓	↓	↓
P <sub>1</sub>	↓	↑	↑	↑
P <sub>2</sub>	↓	NC	NC	NC
L37	NC <sup>a</sup>	↓	↓	↓
EGF	NC	↓	↓	↓
c-fos	↓	NC	NC	NC
P21	↓	↑	↑	↑
Cyclin D	↓	↓	↓	↓

<sup>a</sup>NT: Not Tested; NC: No Change; ↑ and ↓ indicate significant up and down gene regulation, respectively when compared to that of GAPDH; <sup>b</sup>Abbreviations used: NFKB1—nuclear factor kappa-B p105 subunit 1; P<sub>0</sub>/P<sub>1</sub>/P<sub>2</sub>/L37—ribosomal proteins; EGF—epidermal growth factor; c-fos—cellular proto-oncogene; P21—cyclin-dependent kinase inhibitor 1; cyclin D—G1/S phase regulator protein.

MB-435). The percent of apoptotic cells upon 24 - 48 hour treatments of these ligands were statistically analyzed and presented in **Table 2(a)** for comparisons [12, 13]. It was clearly demonstrated that GHR106 mAb and GnRH antagonist were found to have comparable effects on the induced apoptosis of treated cancer cell lines.

### 3. APOPTOSIS AND GENE REGULATION OF CANCER CELLS BY RP215 MONOCLONAL ANTIBODY

#### 3.1. Induced Apoptosis of Cancer Cells by RP215 Monoclonal Antibody

As mentioned, RP215 mAb was shown to react with the carbohydrate-associated epitope of CA215 found on the surface of different cancer cells, but rarely detected in normal cells [13], except in hyperplastic epithelial cells or cells of the immune privileged sites [16,23,24]. Previous studies by MALDI-TOF MS studies have indicated glycoproteins expressed by cancer cells consist mainly of immunoglobulins, derived from cancer cells [18]. These cancerous immunoglobulins were known to be essential growth factors for the growth and proliferation of different cancer cells [16]. Therefore, it is reasonable to assume that the masking of the surface bound immu-

noglobulins or CA215 can induce apoptosis following RP215 mAb treatments of cancer cells.

Apoptosis of cancer cells can be induced not only by RP215 mAb, but also by goat anti-human immunoglobulin G (IgG) to cultured cancer cells [12,13,16]. Typical studies with different cancer cell lines are summarized in **Table 2(b)** with TUNEL assays. Compared to the negative control, apoptosis induced by RP215 mAb (1 - 10 µg/mL) and goat anti-human IgG to cultured cancer cells were statistically significant [12,13,20]. A list of cancer cell lines were employed in this comparable study including those of the prostate (PC-3 and DU-145), lung (A549), cervix (C33A), breast (MDA-MB-435) and ovary (OC-3-VGH) [13]. Furthermore, apoptosis can be induced at an antibody concentration as low as 1 µg/mL [12,13]. Several RP215-related mAbs, including those of the chimeric form, as well as those derived (RCA104, RCA111) [12], were shown to have similar biological actions in inducing apoptosis of OC-3-VGH cancer cells.

#### 3.2. Effects of RP215 Monoclonal Antibody on Gene Regulations of Cancer Cells

Apoptosis induced by RP215 mAb on cancer cells was evaluated through gene expression studies [12,20,21]. By means of semi-quantitative reverse transcription polymerase chain reaction (RT-PCR), the effects of RP215 mAb was analyzed through the expression of a number of genes involved in cell growth regulations of cancer cells. The results of such studies are summarized in **Table 1**. It was generally observed that treatments of cancer cells with RP215 mAb cause significant down-regulation of several genes responsible for protein synthesis and cell cycle control, whereas expressions of certain genes involved in the expressions of immunoglobulins such as IgG, nuclear factor kappa-B p105 subunit 1 (NFKB-1), and the cellular signal transduction activator, c-fos, are up-regulated [12].

### 4. DISCUSSION

In this mini review, two unrelated ligand/receptor systems were introduced to demonstrate the induced apoptosis of cultured cancer cells and the underlying molecular mechanisms investigated through regulation and expression of a number of genes relevant to cell growth and proliferation [12,13]. In view of the universal expression of the surface bound GnRH receptor and CA215 among almost all cancer cells, GnRH receptor and CA215 can be considered as pan-cancer biomarkers for any potential diagnostic and therapeutic applications in human cancer [12,13].

Both GHR106 and RP215 mAbs fulfill the criteria for the development of anti-cancer drugs. First of all, the antigen distributions are homogeneous and abundant on

**Table 2.** Effect of different antibodies on induced apoptosis of cultured cancer cells. (a) GHR106 monoclonal antibody and GnRH analog (Antide) induces apoptosis of cultured cancer cells; (b) RP215 monoclonal antibody and goat anti-human IgG induces apoptosis of cultured cancer cells.

(a)

Cancer Cell Line (tissue)	Incubation Time (hr)	Antibody	Antibody Concentration ( $\mu\text{g/mL}$ )	% Apoptosis (Negative Control) <sup>a</sup>
PC-3 (prostate)	48	GHR106	10	40 $\pm$ 5 (9 $\pm$ 1)**
		ChGHR106 <sup>b</sup>	10	32 $\pm$ 4 (9 $\pm$ 1)**
		Antide (GnRH antagonist)	0.1	35 $\pm$ 5 (9 $\pm$ 1)**
DU-145 (prostate)	24	GHR106	10	32 $\pm$ 2 (12 $\pm$ 3)*
	48	GHR106	10	46 $\pm$ 9 (12 $\pm$ 3)*
		20		42 $\pm$ 5 (7 $\pm$ 2)*
A549 (lung)	24	GHR106	10	34 $\pm$ 10 (11 $\pm$ 5)*
			20	46 $\pm$ 11 (17 $\pm$ 3)*
		GHR106	10	31 $\pm$ 4 (7 $\pm$ 2)**
MDA-MB-435 (breast)	24	GHR106	20	45 $\pm$ 11 (6 $\pm$ 1)**
	48	GHR106	10	39 $\pm$ 11 (14 $\pm$ 13)*

<sup>a</sup>Statistical significance: \*p < 0.05; \*\*p < 0.01; <sup>b</sup>ChGHR106: chimeric form of GHR106 mAb.

(b)

Cancer Cell Line (tissue)	Incubation Time (hr)	Antibody	Antibody Concentration ( $\mu\text{g/mL}$ )	% Apoptosis (Negative Control) <sup>a</sup>
PC-3 (prostate)	48	RP215	10	31 $\pm$ 7 (9 $\pm$ 1)*
		ChRP215	10	38 $\pm$ 5 (9 $\pm$ 1)**
		Goat anti-human IgG	10	36 $\pm$ 4 (9 $\pm$ 1)*
DU-145 (prostate)	24	RP215	10	32 $\pm$ 7 (12 $\pm$ 3)*
	48	Goat anti-human IgG	10	46 $\pm$ 9 (12 $\pm$ 3)*
		RP215	10	35 $\pm$ 8 (10 $\pm$ 1)*
A549 (lung)	48		20	43 $\pm$ 7 (7 $\pm$ 2)*
		Goat anti-human IgG	10	46 $\pm$ 9 (12 $\pm$ 3)*
		RP215	10	30 $\pm$ 9 (11 $\pm$ 5)*
C33A (cervix)	24		20	36 $\pm$ 12 (17 $\pm$ 3)*
		RP215	10	36 $\pm$ 9 (13 $\pm$ 3)*
			20	38 $\pm$ 10 (12 $\pm$ 2)*
MDA-MB-435 (breast)	24	RP215	10	58 $\pm$ 11 (20 $\pm$ 3)*
	48	RP215	20	37 $\pm$ 8 (6 $\pm$ 1)**
		Goat anti-human IgG	10	44 $\pm$ 4 (7 $\pm$ 2)**
OC-3-VGH (ovary)	48	RP215	20	43 $\pm$ 8 (8 $\pm$ 3)**
		Goat anti-human IgG	20	48 $\pm$ 15 (8 $\pm$ 3)**
		RP215	10	51 $\pm$ 12 (18 $\pm$ 2)
OC-3-VGH (ovary)	48	RCA104 <sup>b</sup>	1	35 $\pm$ 3 (6 $\pm$ 2)*
		RCA111 <sup>b</sup>	1	38 $\pm$ 4 (6 $\pm$ 2)**
		RP215	1	45 $\pm$ 5 (6 $\pm$ 2)**

<sup>a</sup>Statistical significance: \*p < 0.05; \*\*p < 0.01; <sup>b</sup>See reference #12: RCA104, RCA111 mAbs are generated by using CA215 as immunogen.

the cancer cell surface, but not generally found in normal cells or tissues. Furthermore, CDC and antibody-dependent cellular cytotoxicity reaction (ADCC) reactions are effective with these two antibody-based drugs. Finally, cellular apoptosis by either mAb can be induced to almost all cancer cells with known mechanisms of action.

For the initial evaluations of anti-cancer drug candidates, induced apoptosis on cancer cells may serve as a meaningful indicator for future drug development. Nude mouse animal models to demonstrate the growth inhibition of tumor cells upon drug treatment are also required for “proof of concept”. In fact, for both GnRH/GnRH receptor and RP215/CA215 systems, results of TUNEL assay for apoptosis of cancer cells are so far consistent with those of tumor growth inhibition in nude mouse animal models [13].

#### 4.1. GHR106 Monoclonal Antibody and Apoptosis of Cancer Cells

The GnRH and GnRH receptor system, and their roles in induction of apoptosis in cancer cells, have been known for decades [1-4]. This system has been the basis of using GnRH agonists and antagonists for clinical therapy of cancer in the prostate, breast, and ovary [7]. In addition, both GHR106 mAb and the GnRH analog (Antide) were shown to have the same effects on the expression of genes tested so far (see **Table 1**). However, the relatively long half-life of GHR106 mAb (5 - 21 days) might be beneficial clinically for use as anti-cancer drugs, when compared with the GnRH analog, a small decapeptide, which only has a half-life of a few hours. Humanized forms of GHR106 mAb have been successfully generated for preclinical and clinical studies in the therapeutic treatment of human cancer. As a potential antibody-based anti-cancer drug, humanized GHR106 mAb has certain intrinsic benefits over GnRH analogs besides its long half-life. Both CDC and ADCC are effective in using GHR106 mAb as anti-cancer drugs as mentioned previously. By comparison, as a synthetic decapeptide, the GnRH analog has no parallel biological activities [7].

#### 4.2. RP215 Monoclonal Antibody and Apoptosis of Cancer Cells

Cancerous immunoglobulins are universally expressed on the surface of all cancer cells of different tissue origins [14,15,25,26]. RP215 mAb, which reacts specifically with the “sugar” epitope of cancerous immunoglobulins, can be a suitable alternative for goat anti-human immunoglobulins to induce apoptosis, as shown in **Table 2(b)**. Both goat anti-human IgG and RP215 mAb can effectively induce apoptosis of cancer cells [13,16,21]. This observation would imply that the cancer cell surface may

be covered with bound cancerous immunoglobulins for the unproven functions of cell growth, proliferation, and communications [16]. Therefore, these immunoglobulins and other bound CA215 may become unique targets for RP215 mAb when used as an anti-cancer drug.

Bindings or blockages of cancerous immunoglobulins with RP215 mAb to the cancer cell surface seem to affect their critical biological functions for cell growth, resulting in induction of apoptosis of these cancer cells [16]. This phenomenon has become an advantage in terms of the immunotherapy of human cancer with specific mAbs. Dose-dependent inhibitions of tumor growth with RP215 mAb have been demonstrated with several nude mouse animal models with implanted tumor cells from the ovary, cervix or lung, respectively [13,27]. It is expected that humanized forms of RP215 mAb can be a suitable candidate for the development of antibody-based anti-cancer drugs for the immunotherapy of human cancer.

#### 4.3. Effects of GHR106 and RP215 Monoclonal Antibodies on Gene Expression of Cancer Cells

The underlying mechanisms of these two mAbs inducing apoptosis of cancer cells were investigated through gene regulation and expression studies [12,13]. As summarized in **Table 1**, both GHR106 mAb and GnRH antagonist react with the GnRH receptor and cause down-regulation of epidermal growth factor and some ribosomal proteins required for protein synthesis. As clearly indicated in this table, RP215 and GHR106 mAb do not induce apoptosis of cancer cells through the same signal transduction pathways [12].

RP215 mAb, which reacts mainly with cancerous immunoglobulins, was shown to induce apoptosis of cancer cells, similar to anti-human immunoglobulins. This observation would suggest that apoptosis was induced on cancer cells by both antibodies through similar or identical pathways or mechanisms of action [16]. Treatments of this mAb with cultured cancer cells can result in down-regulation of genes responsible for protein synthesis and cell cycle regulations. Previously, it has also been reported that transfection of cancer cells with SiRNA related to IgG can result in apoptosis of cultured cancer cells [25,26]. Furthermore, transfections of stable plasmid constructed with IgG-related small interfering RNA (SiRNA) into cancer cells resulted in significant inhibition of cell growth and proliferation *in vitro* and *in vivo* [25,26]. These experimental observations strongly support that cancerous immunoglobulins are required as growth factors for the growth and proliferation of cancer cells. This phenomenon may also provide important rationale for the induction of apoptosis upon treatments of cancer cells with RP215 mAb. Therefore, it is reasonable

to assume that humanized forms of RP215 mAb may have the potential to be developed as an effective antibody-based anti-cancer drug with multi-indication for different types of cancer in humans.

## 5. CONCLUSION

GHR106 and RP215 mAbs have been evaluated, with respect to their actions, to induce apoptosis on cultured cancer cells, of which the GnRH/GnRH receptor and RP215/CA215 systems are universally and abundantly present. It remains to be shown if the efficacy of these two mAbs in humanized forms can be demonstrated through extensive clinical studies for cancer therapy in humans in the near future.

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