Format for ANSWERING REVIEWERS

January 14, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name :Hardy manuscript 15758 -review.doc).

Title GRP78 expression beyond cellular stress; a biomarker for tumor manipulation

Author: Hardy Britta, Raiter Annat

Name of Journal: *World Journal of Immunology*

ESPS Manuscript NO: 15758

The manuscript has been revised according to reviewer's comments:

1. Format has been updated.

2. Revision has been made according to reviewer's comments.

The response to reviewers comments point by point:

(1) **Reviewer N: 01560498**

"Please add important articles of cell surface biomarkers, involving GRP78."

We have now added important articles of cell surface biomarkers associated to GRP78 including new references: In the Introduction page 3: "Several possibilities for how GRP78 escapes to the cell surface in tumor cells were suggested [11]. In general, GRP78 trafficking from the ER to the cell surface is not well understood. It was demonstrated that ER stress actively promotes GRP78 localization on the cell surface, however ectopic

expression of GRP78 is also able to cause cell surface relocation in the absence of ER stress [11]. There are also conflicting reports of whether GRP78 is expressed on specific tumor cell lines, such as PC-3 prostate cancer cells [12]. It is reasonable that since ER membrane is a source of plasma membrane, this form of GRP78 could be cycled to the cell surface. Studies also suggested that specific cell types may utilize different proteins for transporting GRP78 to the cell surface. For example, the ER transmembrane protein, MTJ-1 is implicated as the GRP78 carrier protein in macrophages [13]. The tumor suppressor Par-4 is reported to be required for GRP78 cell surface localization in PC-3 cells [14]".

(2) **Reviewer N: 00608272**

Specific comments:

1. **Even though the title wrote: GRP78, a biomarker for tumor: However, there are no any sentences to show author how to think or how to do it, to use GRP78 as a biomarker for diagnose or treatment tools. Please add some sentences for this.**

We have now added information about the use of GRP78 as a biomarker for diagnosis or treatment.

We now described cell surface GRP78 as a target for cancer therapy; **Page 8 last paragraph and page 9**: "Additional applications to cell surface GRP78 induction on tumor cells, as a potential target for cancer therapy were suggested [61]. For example, pro-apoptotic moieties or cytotoxic agents were conjugated onto peptides with a high affinity for GRP78 to successfully target and kill cancer cells [62]. Also, an un-conjugated peptidic GRP78 ligand demonstrated toxicity to prostate cancer cell by an extrinsic apoptotic pathway [63]. A human monoclonal IgM antibody against cell surface GRP78 isolated from a cancer patient was found to be capable of inducing lipid accumulation and apoptosis, probably extrinsic, in cancer cells [64]."

We now also included paragraphs describing cell surface GRP78 as a biomarker that co-localizes with cell surface proteins in different pathologies and that serves as trigger for signal transduction pathways: **In the abstract:** "Although GRP78 is primarily located in the ER, under certain conditions it is transported to the cell surface, where it acts as a receptor inducing pathways of cell signaling such as proliferation or apoptosis". And on

Page 5: "In pathological conditions such as atherosclerotic lesions of the human aorta and in endothelial cells of the tumor vasculature, cell-surface GRP78 co-localizes with T-cadherin in human umbilical vein endothelial cells (HUVECs). Overexpression of T-cadherin in HUVECs mediated cell survival in a GRP78-dependent fashion by increasing phospho-Akt and phospho-GSK3β and decreasing caspase-3 levels [32"]." **And on Page 7 a new section**: "**CELL SURFACE GRP78 ON TUMOR CELLS MADIATES SIGNAL TRANSDUCTION**

How GRP78 escapes to the cell surface in tumor cells is not well understood, but it may also involve

some specific mechanisms adapted by the tumor cells [6]. Cell surface GRP78 was reported as a

receptor to mediate tumor cell signal transduction.

Cell-surface GRP78 was found to be associates with MHC class I, a receptor for the coxsackie A9 and Dengue viruses, and functions as the signaling receptor upon binding to the activated form of the plasma proteinase inhibitor, α2-macroglogulin (α2M*) [54]. Binding of cell-surface GRP78 with α2M* on 1-LN prostate tumor cells induced Akt phosphorylation [54] promoting cell proliferation either by inactivating apoptotic pathways or upregulating activated NFk-B. Up-regulation of NF-B augments inactivation of mitogen-activated protein kinase kinase 7 (MKK7) through its binding, to increase levels of growth arrest and DNA-damage-inducible β (GADD45β), thereby preventing JNK-mediated apoptosis. In addition, inactivation of apoptosis signal-regulating kinase (ASK1) by active Akt attenuates downstream JNK-mediated apoptosis [54].

Another interacting protein with GRP78 receptor is the GPI-anchored oncogene Cripto (Cripto-1, teratocarcinoma-derived growth factor 1). Cripto is expressed at high levels in human tumors and is associated with cell proliferation, migration, invasion and tumor angiogenesis via activation of MAPK/ERK and PI3K/Akt. Binding of GRP78 receptor to Cripto was found to inhibit transforming growth factor-β (TGF-β) signaling and to promote cell proliferation [55].

The Protease-activated receptor 4 also known as coagulation factor II (Par-4) is a tumor suppressor that was also associated with cell-surface GRP78. Binding of Par-4 to GRP78 receptor near its N-terminus elicits the apoptotic pathway by activation the

FADD/caspase-8/caspase-3 pathway [14]. On the other hand, Kringle 5 of human plasminogen binding to the N-terminal domain of GRP78 receptor mediates apoptosis of tumor cells involving activation of caspase-7 [56]."

2. **Page 5, line 2, "Ischemic Vascular Diseases (IVD) there are no necessary use uppercase for first capital letters for first alphabet:**

   We now removed the capital letters.

3. **Sentence "that can might effect the legs" maybe "that can affect the legs"**

   We now corrected the sentence, **Page 5"** Chronic stress conditions due to reduced blood flow in atherosclerosis or diabetes patients [28,29], might induce ischemic vascular diseases (IVD) in mammals that might affect the legs, heart and brain [19,30-31]."

4. **Also in the same page, the 4th paragraph, line 4, the word "reversered" maybe "reversed".**

   We now corrected the word and apologized for the typo mistake.

5. **Page 7, authors may need to consider for adding a figure to summarize some possibilities for how GRP78 escapes to the cell surface in tumor cells that may be very important and interesting.**

   Since how GRP78 escapes to the cell surface in tumor cells is not well understood, we did not add a figure but instead we now added an additional section in the Introduction depicting some of the possibilities that were published for how GRP78 escapes to the cell surface in the tumor cells.

   Page 3: "Several possibilities for how GRP78 escapes to the cell surface in tumor cells were suggested [11]. In general, GRP78 trafficking from the ER to the cell surface is not well understood. It was demonstrated that ER stress actively promotes GRP78 localization on the cell surface, however ectopic expression of GRP78 is also able to cause cell surface relocation in the absence of ER stress [11]. There are also conflicting reports of whether GRP78 is expressed on specific tumor cell lines, such as PC-3 prostate cancer cells [12]. It is reasonable that since ER membrane is a source of plasma membrane, this form of GRP78 could be cycled to the cell surface. Studies also suggested that specific cell types may utilize different proteins for transporting GRP78 to the cell surface. For example, the ER transmembrane protein, MTJ-1 is implicated as the GRP78 carrier protein in macrophages

[13]. The tumor suppressor Par-4 is reported to be required for GRP78 cell surface localization in PC-3 cells [14]."

(3) Reviewer No: 00033009

The authors should include a paragraph in the last section of the manuscript suggesting the role (if any) of GRP78 in the intrinsic or extrinsic cell death pathways.

We now included a new paragraphs describing the role of GRP78 in the intrinsic or extrinsic cell death pathways**.**

Page 8: "The two major apoptotic pathways recognized as the death receptor (extrinsic) and mitochondrial (intrinsic) pathways play crucial roles in tumor progression as well as resistance to therapeutic strategies. Although the mechanisms that cause the biological selection for a specific mode of cell death remain unclear, it seems probable that the results depend on the intensity of the stress [60]. Pharmacological induction of intrinsic apoptosis was achieved by exogenous agents triggering acute ER stress [57,59].

Additional applications to cell surface GRP78 induction on tumor cells, as a potential target for cancer therapy were suggested [61]. For example, pro-apoptotic moieties or cytotoxic agents were conjugated onto peptides with a high affinity for GRP78 to successfully target and kill cancer cells [62]. Also, an un-conjugated peptidic GRP78 ligand demonstrated toxicity to prostate cancer cell by an extrinsic apoptotic pathway [63]. A human monoclonal IgM antibody against cell surface GRP78 isolated from a cancer patient was found to be capable of inducing lipid accumulation and apoptosis, probably extrinsic, in cancer cells [64]."

3. References and typesetting were corrected.

4. English language editing was done by the Felsenstein Medical Research Center Editorial Staff. The manuscript editing was performed according to **BPG's Revision Policies for Minireviews.**

We hope reviewer's comments and editorial requests were properly corrected and answered.

Sincerely yours,

Prof. Britta Hardy, PhD.

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