Recognition based hormonal 95kDa monoclonal antibody on three human cancer cell lines for developing targeted radio-immuno-imaging and therapy

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Abstract

The present study aimed to explore the possibility of developing a immuno-imaging/therapeutic agent for hCG-expressing tumors by using antibodies raised against them. *Three human cancer cell lines* were selected: lung adenocarcinoma (A549), glioblastoma (U87MG) and breast cancer (MCF7). Anti-β-hCG monoclonal antibody, obtained from ascitic fluid, was purified by affinity chromatography followed by characterization and titration. Ectopic expression of hCG on these cell lines was tested by flow cytometry and in-vitro cytotoxicity with antibodies was tested by MTT assay on the cell line with the highest percentage binding. For positive and negative controls, immortalized trophoblast cells (SW71) and peripheral blood monocytes were used. Antibody was then radiolabeled with lutetium-177 (^{177}Lu) and in vivo biodistribution studies were conducted in murine tumor model. Antibodies could be purified to homogeneity with a concentration 28mg/mL. *Percentage receptor expression* on A549, U87MG and MCF7 cells was 95%, 66% and 55% respectively. About 90% of A549 cells could be killed with antibody at 72h post-treatment. No cytotoxicity was observed on SW71 despite a high binding percentage (96%). Antibodies were radiolabeled with high efficiency (~98%). In-vivo studies using radiolabeled antibodies showed hepato-biliary excretion route and significant uptake in A549 tumor. *In conclusion*, among the 3 cancer cell lines, lung adenocarcinoma significantly expresses β-hCG and shows dose dependent cytotoxicity with anti-β-hCG antibody. Radiolabeling of this antibody can aid in imaging and also has the potential of enhancing its therapeutic potential. This study provides a platform for further studies for targeted radio-immuno imaging and subsequent therapy of hCG-expressing cancers.

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Introduction

The presence of a gonad-stimulating substance in the blood and urine of a pregnant woman was first demonstrated by S. Aschheim and B. Zondek in 1927, the first bioassay for human chorionic gonadotropin (hCG) hormone and it was this named as Ascheim Zondek pregnancy test [1, 2]. Since then this hormone has been studied extensively by various research groups from all over the world resulting in our present day knowledge of hCG. Human chorionic gonadotropin is a non-covalently linked heterodimeric glycoprotein with an alpha (-α) subunit identical to that of luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH), and a beta (-β) subunit unique to hCG conferring biological activity with 2 subunits - α and -β. Its role in implantation of the embryo onto the uterus and in maintenancing the promotion of pregnancy is well established.

However, expression of hCG and its subunits (-α and -β) has, recently, been reported in a variety of cancers such as lung cancer [3], bladder carcinoma [4], colorectal carcinoma [5], pancreatic carcinoma [6], breast cancer [7], cervical carcinoma [8], oral cancers [9], vulva/vaginal cancers [10], prostate cancer [11] and gastric carcinomas [12]. Both germ cell (trophoblastic) and non-germ cell (non-trophoblastic) tumors express hCG. While most placental and germ cell tumors produce holo-hormone hCG, the epithelial tumors mostly express free -β subunit. The free -β subunit (hCGβ) is reported to be the most abundantly expressed form of immunoreactive hCG owing to its unique chain structure unlike hCGα that shares structural similarities with LH and FSH [13-16].

In addition, many authors have commented on the aggressive nature of the hCGβ-positive tumors having the tendency to resist to radiotherapy and to develop metastases [17-18]. The role of hCG on cancer cells is not completely understood and is related to its property of promoting cellular differentiation/proliferation. It is, therefore, postulated that antibodies against hCG have potential of use as new therapeutic options for advanced stage, hCG expressing tumors. Human CG or its subunits secretion is detected in many drug refractory, radio-resistant and hormone refractory cancers. It plays a key role in the

sustenance, and proliferation, migration, and invasiveness of tumor cells.

Moreover, several reports substantiate a strong correlation between hCGβ over-expression and the promotion of angiogenesis and the inhibition of apoptotic signaling pathways such as down-regulation of Fas, Fas ligand, Bax, and p53 in response to hCGβ. Furthermore, hCGβ over-expression has also been reportedly related to the promotion of migration and invasion of carcinogenic cells, essentially, by decreasing E-cadherin expression conferring thus, a more metastatic phenotype of tumor cells [19-21]. E-cadherin, type I transmemberanal protein found in epithelial tissue, is responsible for epithelial cell-cell adhesion and is also an invasion suppressor. Ectopic expression of hCGβ occurs in the 20%-40% of cancers arising from mucosal epithelia, acting more as an autocrine angiogenic and anti-apoptotic growth factor [18] or as a tumor-associated antigen [22].

It is hypothesized that hCGβ acts through antagonizing the TGFβ receptor, [17] since only intact hCG can stimulate the LH/hCG receptor even if the latter is expressed in hCG-positive tumors [18]. The apoptotic effect of TGFβ-1 is known to be counteracted by hCG. High micro-vessel density in hCG expressing cancers is also attributed to hCG and its subunits [23].

More recently hCG has been reported to be involved in activation of intracellular AKT/mTORC1 signalling pathway thus assissting in cell proliferation [24].

Based on these facts, we designed our study aim to evaluate the binding of monoclonal anti-hCGβ antibodies with the surface membrane of three human cancer cell lines and further assess their cytotoxic effect, on them. This antibody has association constant of $3X10^{10}\,M^{-1}$ for hCG and is specific to -β subunit of hCG. It does not recognize LH or FSH and has <5% cross reactivity with LH even though the -α chain sequence is similar for these three hormones. They are postulated to have a potential use as new therapeutic option for advanced stage of hCG-expressing tumors.

For our study we selected terminal cancer cells, which are at present drug and hormone refractory, that is, A549 (human lung adenocarcinoma), U87MG (human glioblastoma) and MCF7 (breast cancer). These are among the most common cancers with a high mortality rate. As positive control immortalized trophoblast cells SW71 were used. Peripheral blood monocytes (PBMC) were used as negative control.

Materials and methods

Monoclonal anti-hCGβ antibodies were kindly gifted by Talwar Research Foundation (New Delhi, India). Protein-A beads were bought from Biodex, BCA kit was bought from Sigma and the radionuclide used lutetium-177 (^{177}Lu) was procured from Bhabha Atomic Research Centre/ Board of Radiation and Isotope Technology (BARC/ BRIT, Mumbai, India). All other chemicals were of analytical grade.

Purification of antibodies

Although *in vitro* techniques are used for mAb production in more than 90% of cases, they are not suitable in all situations, especially if large numbers of antibodies have to be screened for efficacy. Therefore, the mouse ascites method for mAb production was preferred, yielding a higher concentration of antibodies than hybridoma cell culture and thus, meeting the scientific aims of the present study.

The antibodies from mouse ascites were purified by Affinity Chromatography using Protein A column. Briefly, the mouse ascitic fluid was centrifuged at 10,000rpm for 5min. The supernatant was collected and diluted with phosphate buffered saline (PBS, pH 8). The diluted supernatant was incubated overnight at 4°C with protein A beads (Biodex). The beads were then packed in a glass column and washed with PBS. The column was eluted with 10mL of 0.1M glycine (pH 2-3) and neutralized to pH 7. Ten aliquots of 1mL each were collected and characterized.

Characterization

The aliquots collected were pooled together and characterized by 12.5% sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) and by Western Blot technique in order to obtain the protein bands. A medium weight protein marker was used. The concentration of antibody as determined by bicinchoninic acid (BCA kit, Sigma Co.) was 28mg/mL. The protein peaks were analyzed under non-reducing conditions on SDS-PAGE gel and by Western Blot technique at 90-100kV. The aliquots were titrated by BCA assay. The assay was read on ELISA reader (FLUOstar omega BMG labtech) at 540nm with a reference of 690nm.

Immunoreactivity

Four μg of hCGβ was coated in each well of a microtitre ELISA plate by incubating at 37°C for 2h followed by washing with PBS Tween detergent. The wells were then blocked with 1% aqueous solution bovine serum albumin (BSA; 100μL) for 1h at 37°C. Equal amounts of anti-hCGβ monoclonal antibodies were then added to the wells and incubated for 1h at 37°C followed by washing with PBS Tween. Then secondary goat anti-mouse antibodies were added and washed with PBS Tween after 1h incubation at 37°C. The color was developed with orthophenyldiamine (OPD) solution. The reaction was terminated with 5 N sulphuric acid and the plate was read on an ELISA reader (FLUOstar omega BMG labtech) at 490nm.

In-vitro binding of mAb with cell membrane

American Type Culture Collection (ATCC) certified cell lines A549, U87MG and MCF7 were grown in culture in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal calf serum (FCS). The cells were harvested by trypsinization when a single cell suspension was formed.

Flow cytometry is able to assess and quantify hCGβ expression using fluorochromes: In-vitro receptor expression of hCG receptors on these cancer cell lines was tested by Flow Assorted Cell Sorting (FACS) (Becton Dickenson FACS calibur). Briefly, tumor cells ($0.5x10^6$) were incubated for 30min at 4°C with 5μg of anti-hCGβ monoclonal antibody in 100μL of FACS buffer (1% bovine serum albumin, 0.02% sodium azide in PBS). After washing thrice with FACS buffer, the cells were stained by incubating with goat anti-mouse immunoglobulins (IgG) Fc (γ) conjugated to fluorescein isothio-cyanate (FITC) in 1:100 dilution in 100μL FACS buffer for 30min at 4°C. For positive control SW71 cells (immortalized trophoblast cell line) were used while peripheral blood monocytes (PBMC) were used as a negative control and Control cells were similarly processed.

FACS acquisition was carried out on Becton Dickenson FACS Calibur on Cell Quest Pro software and analyzed by FlowJo 6.1.1 software.

In-vitro cytotoxicity

To assess the toxic effect of anti-hCGβ monoclonal antibodies on the A549-cell line, that is with the highest percentage expression binding, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed. Briefly, $3x10^3$cells/well were seeded in a 96-well culture plate. Three wells containing only media were used as control in each plate (representing the blanks for absorbance readings). Once cells adhered to the culture plate (37°C in a humidified atmosphere), antibodies were added in each well starting from 8μg (1:50 dilution) dose and diluted to half subsequently till 1:1600 dilution was achieved. All the doses were added plated in triplicates sets.

The same procedure was followed to test the cytotoxic effect of anti-β-hCG monoclonal antibodies on the non-cancerous SW71 cell line. Processed cells were incubated at 37°C in a humidified atmosphere for 72h, allowing mAb to take effect. The cells were subjected to MTT assay at 72h post-antibody treatment when a visible change was observed under microscope. After removing of 50μL of media from each well and 25μL of MTT reagent solution was added to each well, including controls.

MTT solution was prepared by dissolving MTT salt (Sigma Co.) in MilliQ water at a concentration of 5mg/mL and stored at 4°C in dark. The plate was incubated in dark for 4h at 37°C in a humidified atmosphere, allowing MTT to be metabolized. After 4h, 75μL of dimethyl sulfoxide (DMSO) was added to each well for dissolution of the formazan dye crystals and incubated for 5-10min. The plate was read on an ELISA reader (FLUOstar omega BMG labtech) at 550nm and Optical Density (OD) for each well was recorded, including controls. For accuracy of results, cytotoxic effect was also seen by trypan blue exclusion test. Trypan blue dye exclusion test was performed in order to distinguish between live and dead cells, enhancing assay accuracy.

Radiolabeling and in-vivo imaging

The monoclonal antibody (mAb) was labeled with ^{177}Lu (procured from BRIT /BARC, Mumbai, India) as per the method described by Bander et al [25] and Pan et al [26]. Briefly, the purified antibody was first conjugated with DOTA (1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid), a the macrocyclic chelating agent. Antibody-DOTA conjugate complex was labeled with ^{177}Lu in an ammonium acetate buffer and radiolabeled antibody was sterilized by membrane filtration before administration. The labeling yield was determined by thin-layer chromatography. The labeling efficiency of the ^{177}Lu labeled mAb-DOTA conjugate was found to be ~97.8%; ensuring adequate immunoreactivity.

For the *in-vivo* studies, ethical clearance was obtained from Institutional Animal Ethics Committee. For inoculation of tumor, 4-5x10^6 A549 cells suspended in phosphate buffered saline were injected subcutaneously in the thigh of male wistar albino rats (150-200g) after immune-system suppression by whole body irradiation with high-energy X-rays. Around 7.4MBq of radiolabeled mAb was injected through tail vein. To observe assess the mAb biodistribustion; images were acquired 2h post-injection using a dual head gamma-camera (Symbia by Siemens).

Statistical analysis

Percentage cytotoxicity was expressed by mean ± standard deviation (SD). The difference between the percent binding of cell with antibody and mean percent cytotoxicity was tested by one-way ANOVA test. P value <0.05 was considered significant.

Results

Purification and characterization

The antibodies from the ascitic fluid were purified with protein A column and the aliquots collected were pooled together and characterized by SDS PAGE and Western Blot. Protein bands obtained in SDS PAGE and Western Blot are shown in Figures 1 and 2. The antibodies were purified to a state of homogeneity with distinct light and heavy bands. The H and L band correspond to the heavy and light chains of the IgG antibody. A medium weight protein marker was used. The concentration of antibody as determined by BCA assay was 28mg/mL.

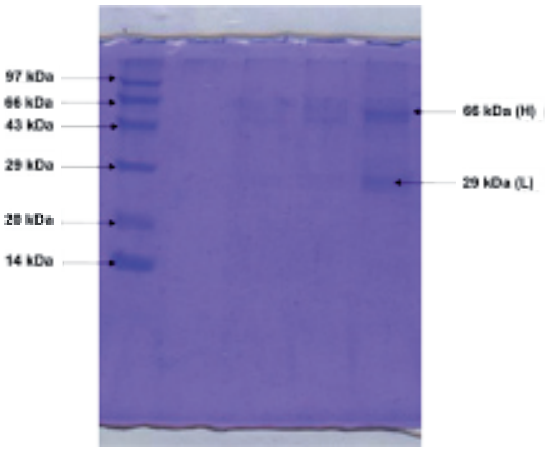

Figure 1. SDS PAGE of anti-hCGβ antibodies (right) and protein ladder (left); H and L correspond to the heavy and light chains of the mAb.

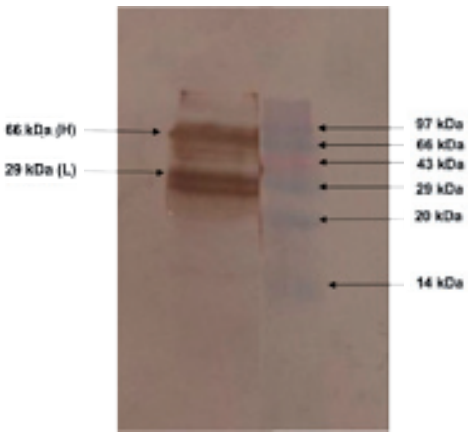

Figure 2. Western Blot of anti-hCGβ antibodies (left) and protein ladder (right).

In-vitro binding of mAb with cell membrane

The overlay graphs of in vitro binding studies done by flow cytometry on A549, U87MG, MCF7, SW71 cells and PBMC are shown in Figures 3, 4 and 5. It was observed that a total of 95.8% of A549 cells showed expression of receptors for anti-β-hCG monoclonal antibody (mAb) (Fig. 3A). SW71 showed 95.3% receptor expression (Fig. 4). However, the negative control cell line PBMC showed very a non-specific

peak pattern of a non-specific binding (~3%-4%) with anti-hCGβ mAb as shown in Figure 5. U87MG cells showed 66% binding with a single peak (Fig. 3B) while MCF7 showed 55% binding (Fig. 3C).

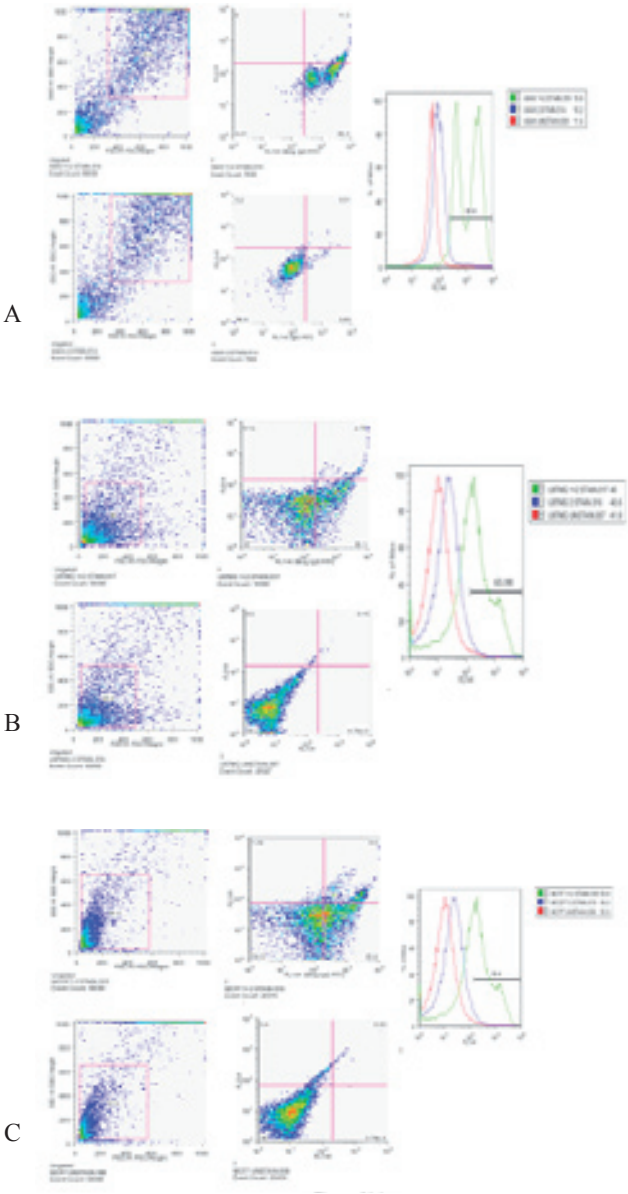

Figure 3. Binding profile of anti-hCGβ antibodies with (**A**) A549 cells (lung adenocarcinoma); (**B**) U87MG (glioblastoma) and (C) MCF7 (breast cancer).

In vitro cytotoxicity

Since A549 had the maximum binding percentage with the antibody, further studies were done with A549 cell line. A dose dependent cytotoxicity was observed with the highest killing of 95.8±4.78% cells was observed at 1:50 dilution (8 μg) (Fig. 6A and B). The micrographs of A549 cells before and after treatment with anti-β-hCG are shown in Figure 7.

On the other hand, compared to A549, the cytotoxic effect of anti-β-hCG monoclonal antibodies on normal trophoblast cells SW71 was very low, that is, 16.94±6.81% (P<0.01) at the same concentration of 1:50 dilution (Fig. 6).

The MTT results were confirmed by trypan blue exclusion test, which yielded similar results.

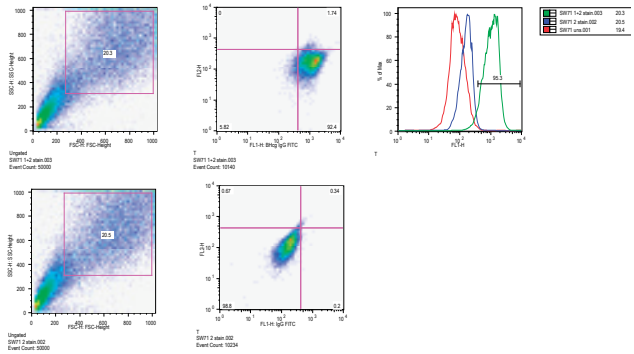

Figure 4. Binding profile of anti-hCGβ antibodies with SW71 cells (trophoblast cells).

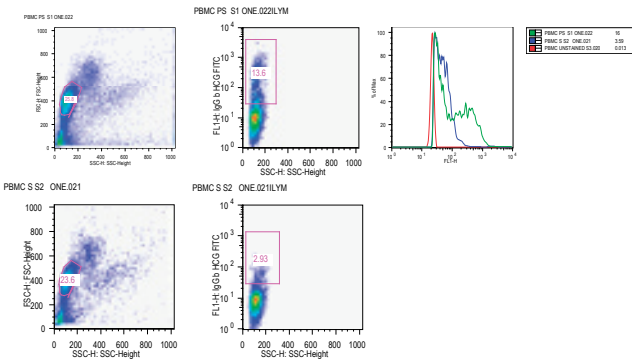

Figure 5. Binding profile of anti-hCGβ antibodies with PBMC cells (peripheral blood monocytes).

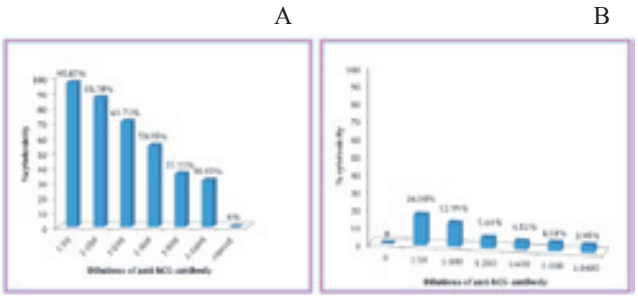

Figure 6. Dose dependent cytotoxicity of anti-hCGβ antibodies on (**A**) lung cancer cells (A549) and (**B**) normal trophoblast cells (SW71).

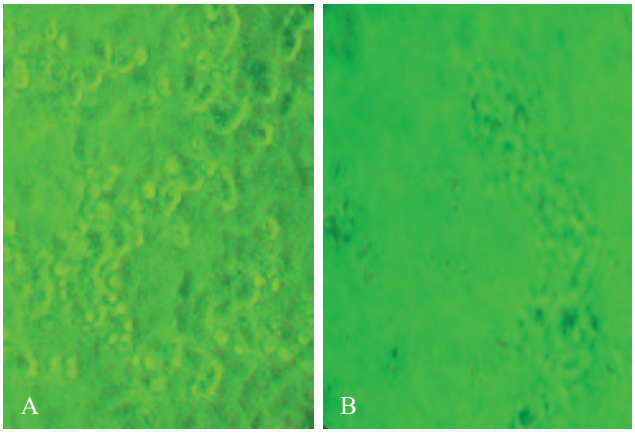

Figure 7. Micrographs of A549 cells before (**A**) and after (**B**) treatment with anti-hCGβ antibodies.

Radiolabelling and imaging

The labeling efficiency of the ^{177}Lu labeled mAb-DOTA conjugate was found to be ~97.8%. The Image acquired showed a significant uptake in gonadal region. The radiolabeled complex had a hepato-biliary route of excretion. The tumor uptake was also significant as can be seen in Figure 8.

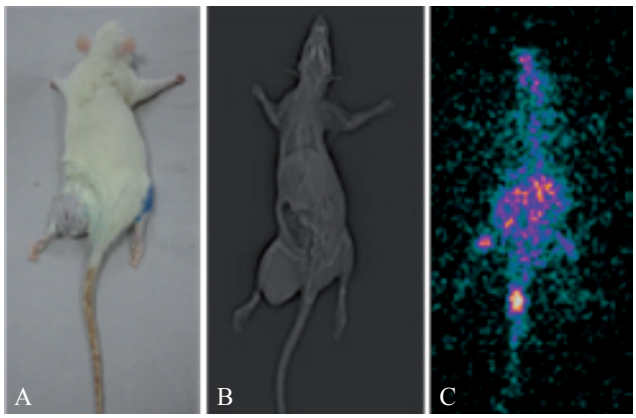

Figure 8. Image showing the tumor growth in the thigh of Wistar rats (**A**), CT image (**B**) and uptake of ^{177}Lu labeled mAb-DOTA conjugate (**C**).

Discussion

The antibodies were purified to a state of homogeneity with distinct light and heavy bands. Our results are comparable to studies done in acute lymphoblastic leukemia and androgen-independent prostate cancer lines [27-29].

Of the three human cancer cell lines, the binding of anti-hCGβ mAb with A549 cell line was the highest and comparable to the positive control cell line SW71. However, as can be seen in Figure 3A two distinct peaks were observed in A549 cells as compared to a single peak in SW71 cells in Figure 4. It can be inferred that the two peaks correspond to two groups of cells, one towards the left (with slightly lower binding) and that the other towards the right of the x-axis. This can be further explained by the inherent heterogeneous nature of cancer cells.

We underline that the difference between the percent binding of A549 cells with anti-hCGβ mAb is significantly higher than that of U87MG ($P<0.01$), MCF7 ($P<0.01$) and PBMC ($P<0.01$). However, the difference between the percent binding of A549 and SW71 was insignificant ($P=1.0$). Furthermore, the difference between the percent binding of U87MG and MCF7 cells with anti-hCGβ mAb was also significant ($P=0.02$). The difference of percent binding of both U87MG and MCF7 from both SW71 and PBMC was also significant with P values <0.01.

In vitro cytotoxicity was tested on A549 because of its highest binding with the mAb. Lung adenocarcinoma is the most common kind of lung cancer, both in smokers and non-smokers. Though many therapeutic approaches are being practised like surgery, chemotherapy, radiotherapy etc, the overall survival rate is less than 10% of people with primary lung cancer five years after diagnosis. While the five-year survival rates can be 35%-40% provided the tumor is localized and is removed at its early stages [30].

The cytotoxic action of antibodies can be exercised by two mechanisms: complement mediated lysis of cells bearing the target antigen on the membrane and antigen dependent cell cytotoxicity. In the present study, since no complement was added to the incubation mixture, so the cytotoxicity action of antibodies observed on A549 cells could be was attributed to antigen-dependent cell mediated cytotoxicity. The lack of 100% cytotoxicity may be due to the inability of all cancer cells to bind with the antibody attributed to their heterogeneous nature. The heterogeneity of expression of hCG on surface of tumor cells is indicated by FACS studies. Some authors have shown killing of leukemia cells by curcumin- anti-hCGβ monoclonal antibody complex [31].

Binding affinity data alone does not determine the overall potency of a drug, thus the in vitro cytotoxicity tests were performed. The cytotoxicity of anti-hCGβ antibody to normal cells expressing holo-hCG was negligible, while the same antibody had a strong (significantly higher $P<0.01$) cytotoxic effect against malignant cells. This might be because of expression of holo-hormone by cells with trophoblastic origin unlike non-trophoblastic cells, which mostly express free β subunit of hCG [12-14]. The mechanism behind this behavior was not studied in the present study. However, this phenomenon is quite favorable advocating the use of anti-hCGβ antibody as a targeted therapeutic agent.

The observed biodistribution of radiolabeled antibodies was in agreement with other studies [32]. Lutetium-177 labeled anti-β-hCG mAb can be used for imaging as well as therapy of lung adenocarcinoma. Furthermore, it can be used for post-therapy response evaluation. Further experimental studies for the 3 specific cancers studied and for other drug-refractory cancers are welcome.

In conclusion, of the selected cancer cell lines, human lung adenocarcinoma had the highest binding affinity for anti-β-hCG mAb. This study indicates that these cells not only express -β subunit of hCG but can also be killed by antibodies raised against it, via antigen dependent cell cytotoxicity mechanism. The antibody could be successfully labeled with ^{177}Lu that could further be used to increase and improve the therapeutic efficacy. The present study establishes in mice the therapeutic role of anti-hCG-antibodies in terminal cancers expressing hCG and provides a platform for the development of an immunotherapeutic or radio-immunotherapeutic drug for human use.

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The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

Bibliography

1. Lunenfeld B. Historical perspectives in gonadotrophin therapy. *Human Reproduction Update* 2004; 10: 453-67.

2. Ascheim S, Zondek B. Hypophysenvorderlappen hormone und ovarialhormone im Harnvon Schwangeren. *Klin Wochenschr* 1927; 6: 13-21.
3. Dirnhofer S, Freund M, Rogatsch H et al. Selective expression of trophoblastic hormones by lung carcinoma: neuroendocrine tumors exclusively produce human chorionic gonadotropin β -subunit (hCG). *Hum Pathol* 2000; 31: 966-72.
4. Dirnhofer S, Koessler P, Ensinger C et al. Production of trophoblastic hormones by transitional cell carcinoma of the bladder: association to tumor stage and grade. *Hum Pathol* 1998; 29: 377-82.
5. Louhimo J, Carpelan-Holmstrom M, Alfthan H et al. Serum hCG β, CA 72-4 and CEA are independent prognostic factors in colorectal cancer. *Int J Cancer* 2002; 101: 545-8.
6. Syrigos KN, Fyssas I, Konstandoulakis MM et al. Beta human chorionic gonadotropin concentrations in serum of patients with pancreatic adenocarcinoma. *Gut* 1998; 42: 88-91.
7. Agnantis NJ, Patra F, Khaldi L, Filis S. Immunohistochemical expression of subunit beta HCG in breast cancer. *Eur J Gynaecol Oncol* 1992; 13: 461-6.
8. Crawford RA, Iles RK, Carter PG et al. The prognostic significance of human chorionic gonadotrophin and its metabolites in women with cervical carcinoma. *J Clin Pathol* 1998; 51: 685-8.
9. Hedstrom J, Grenman R, Ramsey H et al. Concentration of free hCG β subunit in serum as a prognostic marker for squamous-cell carcinoma of the oral cavity and oropharynx. *Int J Cancer* 1999; 84: 525-8.
10. de Bruijn HW, ten Hoor KA, Krans M, van der Zee AG. Rising serum values of β-subunit human chorionic gonadotrophin (β-hCG) in patients with progressive vulvar carcinomas. *Br J Cancer* 1997; 75: 1217-8.
11. Sheaff MT, Martin JE, Badenoch DF, Baithun SI. hCG as a prognostic marker in adenocarcinoma of the prostate. *J Clin Pathol* 1996; 49: 329-32.
12. Zhang W, Yang H, Han S. The effect of ectopic HCG on microvessel density in gastric carcinoma. *Chinese Journal of Oncology* 1998; 20: 351-3.
13. Stenman UH, Alfthan H, Hotakainen K. Human chorionic gonadotropin in cancer. *Clin Biochem* 2004; 37: 549-61.
14. Butler SA, Iles RK. Biological function of the free β -subunit: expression and treatment target in cancer. In: Cole, Larry, (ed.) *Human chorionic gonadotropin (hCG).* Elsevier insights, Elsevier 2010; 153-71.
15. Iles RK. Ectopic hCG β expression by epithelial cancer: Malignant behaviour, metastasis and inhibition of tumor cell apoptosis. *Mol and Cellular Endocrinol* 2007; 260-2: 264-70.
16. Delves PJ, Iles RK, Roitt IM, Lund T. Designing a new generation of anti-hCG vaccines for cancer therapy. *Mol and Cellular Endocrinol* 2007; 260-2: 276-81.
17. Cole LA, Biological functions of hCG and hCG-related molecules. *Repr Biol & Endoc* 2010; 8: 102-16.
18. Iles RK, Delves PJ, Butler SA. Does hCG or hCGβ play a role in cancer cell biology? *Mol & Cell Endocr* 2010; 329: 62-70.
19. Butler SA, Ikram MS, Mathieu S, Iles RK. The increase in bladder carcinoma cell population induced by the free beta subunit of human chorionic gonadotrophin is a result of an anti-apoptosis effect and non cell proliferation. *Br J Cancer* 2000; 82(9): 1553-6.
20. Matsubara H, Ikuta K, Ozaki Y et al. Gonadotropins and Cytokines Affect Luteal Function through Control of Apoptosis in Human Luteinized Granulosa Cells. *J Clin Endo Met* 2000; 85: 1620-6.
21. Wu W, Walker AM. Human chorionic gonadotropin β (HCG- β) down-regulates E-cadherin and promotes human prostate carcinoma cell migration and invasion. *Cancer* 2006; 106: 68-78.
22. He LZ, Ramakrishna V, Connolly JE et al. A Novel Human Cancer Vaccine Elicits Cellular Responses to the Tumor-Associated Antigen, Human Chorionic Gonadotropin β. *Clin Cancer Res* 2004; 10: 1920-7.
23. Jankowska A, Gunderson SI, Andrusiewicz M et al. Reduction of human chorionic gonadotropin beta subunit expression by modified U1 snRNA caused apoptosis in cervical cancer cells. *Mol Cancer* 2008; 7: 26.
24. Palaniappan M, Menon KM. Human Chorionic Gonadotropin stimulates theca-interstitial cell proliferation and cell cycle regulatory proteins by a cAMP-dependent activation of AKT/mTORC1 signaling pathway. *Mol Endocrinol* 2010; 24: 1782-93.
25. Bander NH, Milowsky MI, Nanus DM et al. Phase I Trial of ^{177}Lutetium-Labeled J591, a Monoclonal Antibody to Prostate-Specific Membrane Antigen, in Patients With Androgen-Independent Prostate Cancer. *J of Clin Oncol* 2005; 23: 4591-601.
26. Pan MH, Gao DW, Feng J et al. Biodistributions of ^{177}Lu- and ^{111}In- labeled 7E11 Antibodies to Prostate-Specific Membrane Antigen in Xenograft Model of Prostate Cancer and Potential Use of ^{111}In-7E11 as a Pretherapeutic Agent for ^{177}Lu-7E11 Radioimmunotherapy. *Mol Imaging Biol* 2009; 11: 159-66.
27. Kabeer RS, Pal R, Talwar GP. Human acute lymphoblastic leukemia cells make human pregnancy hormone hCG and expose it on the membrane: A case for using recombinant antibody against hCG for selective delivery of drugs and/or radiations. *Curr Sci* 2005; 89: 1571-6.
28. Talwar GP, Purswani S, Vyas HK. Immunological approaches against human chorionic gonadotropin for control of fertility and advanced stage cancers expressing ectopically hCG. In: *Gonadal and Nongonadal Actions of Gonadotropins*. A Kumar, CV Rao, PK Chaturvedi (Eds). New Delhi, Narosa Publishing House, 2010, pp 183-96.
29. Talwar GP, Gupta R, Gupta SK et al. A monoclonal antibody cytolytic to androgen independent DU145 and PC3 human prostatic carcinoma cells. *Prostate* 2001; 46: 207-13.
30. College of American Pathologists: Lung Cancer, Lung Adenocarcinoma © 2011. http://www.cap.org/apps/docs/reference/myBiopsy/LungAdenocarcinoma.pdf
31. Vyas HK, Pal R, Vishwakarma R et al. Selective killing of leukemia and lymphoma cells ectopically expressing hCG β by a conjugate of curcumin with an antibody against hCG β subunit. *Oncol* 2009; 76: 101-11.
32. Jalilian AR, Khoshdel MR, Garousi J et al. Development of a radiolabeled beta-human chorionic gonadotropin. *Acta Pharm* 2009; 59: 421-9.