

RESEARCH ARTICLE

Immunopreventive Effects against Murine H22 Hepatocellular Carcinoma *in vivo* by a DNA Vaccine Targeting a Gastrin-Releasing Peptide

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Abstract

There is a continuing need for innovative alternative therapies for liver cancer. DNA vaccines for hormone/growth factor immune deprivation represent a feasible and attractive approach for cancer treatment. We reported a preventive effect of a DNA vaccine based on six copies of the B cell epitope GRP18-27 with optimized adjuvants against H22 hepatocarcinoma. Vaccination with pCR3.1-VS-HSP65-TP-GRP6-M2 (vaccine) elicited much higher level of anti-GRP antibodies and proved efficacious in preventing growth of transplanted hepatocarcinoma cells. The tumor size and weight were significantly lower ($p < 0.05$) in the vaccine subgroup than in the control pCR3.1-VS-TP-HSP65-TP-GRP6, pCR3.1-VS-TP-HSP65-TP-M2 or saline subgroups. In addition, significant reduction of tumor-induced angiogenesis associated with intradermal tumors of H22 cells was observed. These potent effects may open ways towards the development of new immunotherapeutic approaches in the treatment of liver cancer.

Keywords: Liver cancer - naked DNA - anti-GRP - anti-angiogenesis - anti-tumor

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Introduction

Hepatocellular carcinoma (HCC) is reported to be the fifth most frequently diagnosed cancer in the world and the second leading cause of cancer related-death worldwide (Jemal et al., 2011). However, notwithstanding great advances, no systemic chemotherapeutic protocol have yet been successful in HCC treatment (Ozturk and Oter, 2011). Potential surgical or local ablative therapies ever applied are having poor prognosis (Ray, 2013). Therefore, there is a pressing need for novel strategies to impact development and recurrence of HCC.

Great efforts are being made towards developing targeted therapies that may contribute in cancer control; in that respect construction of nucleic acid vaccines targeting hormone and/or growth factor have recently emerged as a promising approach (Luigi and Gennaro, 2011; Bele 2012). DNA vaccines have become an attractive approach in generating antigen-specific immunity. The naked plasmid DNA is safe, has low immunogenicity, and can be repeatedly administered. In addition, DNA vaccines can be easily prepared on large scale with high purity and are highly stable relative to proteins and other biological polymers (Donnelly et al., 1997). Extensive studies by many investigators established that angiogenesis has a central role in the invasion, growth and metastasis of

solid tumors. Thus, the inhibition of tumor growth by attacking the tumor vascular supply offers a primary target for anti-angiogenic intervention (Matejuk et al., 2011; Zhu et al., 2013). Rinaldi et al. (2009) revealed a prominent preventive effect of dendritic cell-based tumor vaccines against HCC. Gastrin-releasing peptides (GRP) are neuroendocrine peptides that have been previously implicated as regulators of angiogenesis and therefore can be potential target for anti-angiogenic therapies.

Previous investigations of gene therapy approaches for liver cancer have been targeting endothelial cells (Greten et al., 2006; Butterfield, 2007). Our approach is based on a vaccine taking interest in both endothelial cell and tumor cells. In this work, we reported a preventive strategy based on application of a DNA vaccine targeting gastrin-releasing peptide. The efficacy of the anti-GRP DNA vaccine was tested on the murine H22 hepatocarcinoma model. In addition, the inhibition of tumor-induced angiogenesis induced by active immune responses was evaluated in an intradermal tumor model *in vivo*.

Materials and Methods

DNA vaccine construct and preparation

The vaccine of interest pCR3.1-VS-HSP65-TP-GRP6-M2 (TP-GRP6-M2) uses six tandem repeats

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of the human gastrin-release peptide (GRP) gene, from amino acid 18 to 27 (GRP6) as immunogen. The vaccine was constructed using the eukaryotic expression vector pCR3.1 (Invitrogen Corp., CA) bearing on its backbone, the VEGF183 signal peptide (VS) under the control of CMV promoter and eight 5'-GACGTT-3' CpG motifs. In view of immunogenicity optimization, chemically synthesized DNA fragments encoding for immunoadjuvants including, mycobacterial heat shock protein 65 kDa (HSP65) (Yankai et al., 2006), tetanus toxoid 830-844 (T) and pan HLA-DR-binding epitope (PADRE) (P) respectively were sequentially inserted downstream the cytomegalovirus promoter on the plasmid. GRP6 was then placed downstream PADRE, followed by two repeats of mycobacterial HSP70 fragment 407-426 (M2). Beside, the plasmid pCR3.1-VS-HSP65-TP-M2 (TP-M2), lacking the GRP18-27 gene, and the plasmid pCR3.1-VS-HSP65-TP-GRP6 (TP-GRP6), lacking M units were used as the vaccine control. All constructed DNA vaccine vector were verified by DNA sequencing. Structure of the DNA vaccine (TP-M2) is shown in the Figure 1.

Plasmid DNA used for animal immunization were purified using Qiagen Plasmid Mega Kit (Qiagen) and suspended in sterile saline at a concentration of 0.5 µg/µl.

Mice and hepatocarcinoma tumor cell line

Male BALB/c mice (5 to 6 weeks old) were purchased from the Chinese Academy of medical Sciences (Beijing, China) and housed in pathogen-free conditions. All animal procedures were performed according to approved protocols and in accordance with recommendation for the proper use and care of laboratory animals. H22 cell line was obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Beijing, China). Tumor cells were transferred into the abdomen cavity of mice. The ascites were taken from the mouse in the super clean bench and then diluted with physiological saline to 1×10⁷ ml⁻¹. A total of 2×10⁶ ml⁻¹ H22 cells in 0.1 ml were subcutaneously inoculated at the right armpit of mouse to establish a solid tumor model.

Immunization procedure

Purified plasmids DNA were resuspended in sterile saline at a concentration of 0.5 mg/ml. A total of six BALB/c male syngeneic mice were used for each experimental group (one group for each of the three DNA vaccine and saline control). Mice were first anesthetized via i.m. injections in the anterior thigh with 100 µl of 0.25% bupivacaine hydrochloride solution. 72 hours later, mice received i.m. injections of 100 µl each of the plasmid DNA vaccine or saline solution. Booster administrations were given every other week (week 3 and 5) using the same protocol. Sera were taken at various time points for later analysis of antibodies titers.

ELISA for serum anti-GRP antibodies

The formation of antibodies against GRP in immunized mice was tested using ELISA as described by Yankai et al. (2006). Briefly, 96-well flat-bottomed ELISA plates (Costar, USA) were coated with 100 µl/well of

recombinant hVEGF-GRP18-27 proteins (2 µg/well) in 0.1 mM carbonate-bicarbonate buffer and kept overnight at 4°C. Plates were blocked with PBS containing 5% (w/v) bovine serum albumin (BSA; Sigma, USA) for 1 h and then incubated at 37°C for another hour with 100 µl/well 1:100 dilution of serum collected from immunized animals in PBS containing 2% BSA. Thereafter, wells were washed three times by PBST (PBS containing 0.1% Tween 20), and then incubated 1 h at 37°C with 100 µl/well HRP-conjugated goat anti-mouse IgG (Sigma, USA) diluted 1:20,000 in PBS containing 1% BSA. Wells were washed intensively six times in PBST and followed by 20 min incubation at 37°C with 100 µl/well of the peroxidase substrate 0.01% 3,3',5,5'-tetramethylbenzidine (TMB) and 0.24% (w/v) H₂O₂-urea solubilized in 0.2M Na₂HPO₄ 0.1M citrate buffer (pH 5.5). The reaction was stopped with 50 µl/well of 2M H₂SO₄. The ELISA plate was read with a standard ELISA reader at 450 nm. Each measurement was carried out in duplicate. To determine the antibody titer, sera collected at week 10 with different dilutions were tested using ELISA method. Pre-immune serum was used as negative control. Antibody titers was defined as the maximum serum dilution that manifested an ELISA optical density (OD) reading that was at least 5 standard deviations greater than the mean OD obtained in wells that contained pre-immune serum.

In vivo tumor protection experiments

In order to study the antitumor effect of the anti-GRP, mice (six per group) were immunized four times every 2 weeks followed by subcutaneous injections of 1×10⁶ H22 cells into the left flank. After 14 days, animals were sacrificed, and solid tumors were excised and weighed.

In vivo angiogenesis assay

As shown in Figure 3, a vaccination protocol was designed for each experimental group (four mice per group). To visualize the induction of angiogenesis by a tumor *in vivo*, an intradermal tumor model was used. In this model, neovasculature observed predominantly at the tumor periphery can be quantified by vessel counting (Kreisle and Ershler, 1988). At day 0, mice were anesthetized (inhalant 4% Halothane-vet), and 2.10⁶ H22 viable cells in 100 ml of PBS were implanted intradermally at two sites in the abdominal region. Two additional injections of PBS (50 ml) were performed on each mouse as a control. All implant sites were marked with indelible ink to aid identification at the end of the experiment. When the intradermal tumor grew up to 5 mm in diameter, a section of the abdominal wall skin encompassing all implant sites was removed and spread onto filter paper. Sections were examined by light microscopy (×10 magnification), and the total number of blood vessels (major vessels and branching points) was determined within a 1 cm² area around each implant site. Vessel counts from implants of PBS were consistent irrespective of treatment; the mean value was therefore subtracted from control and vaccines immunized vessel counts to give a more accurate indication of tumor-induced blood vessel formation and to ascertain the effect of treatment.

Statistical analysis

Data were expressed as means (\pm SD) and the differences between groups were analyzed by Student's *t* test. Findings were regarded as significant if *P* values were <0.05 .

Results

Induction of anti-GRP antibodies in immunized mice

An ELISA was performed to detect the GRP-specific immunoglobulin G (IgG) level in sera from immunized mice as described in section 2.4. Samples were collected on week 2, 4, 6, 8 and 10 after the initial immunization. TP-GRP6-M2 and TP-GRP6 DNA vaccines, compared with the control vaccine (TP-M2) or the saline control, greatly increased titers of specific anti-GRP antibodies 4 weeks post-inoculation, and the antibody levels remained high for up to 10 weeks post-inoculation (Figure 2). Among the two GRP-based vaccines, the anti-GRP antibody titers in mice immunized with the plasmid containing two copies of the mycobacterial HSP70407-426 gene (M) were highest at 10 weeks post immunization.

These data clearly demonstrated that two copies of HSP70407-426 gene were highly potent in augmenting humoral response and immunogenicity of the anti-GRP vaccine.

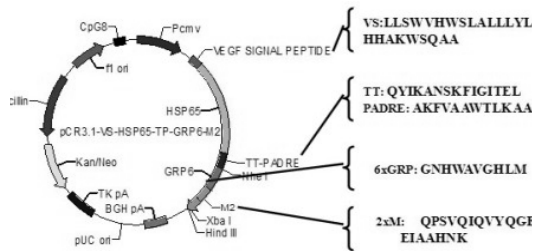


Figure 1. DNA Vaccine Construct. The plasmid pCR3.1 bearing eight CpG motifs and containing the VEGF83 signal peptide (VS, under the control of promoter CMV) was used as backbone. The genes encoding for HSP65 (HSP65), tetanus toxoid830-844 (T), PADRE (P), 6 tandem repeats of hGRP18-27 (GRP6) and 2 copies of mycobacterial HSP70407-426 (M2) were sequentially cloned in the MCS

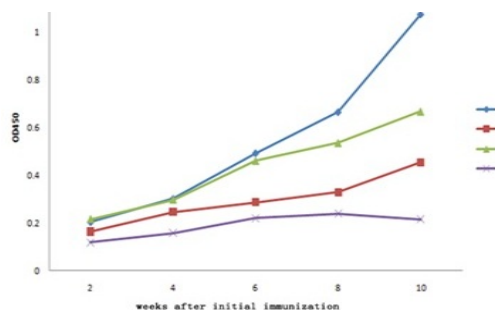


Figure 2. Detection of specific anti-GRP Antibodies. We compared the levels of anti-GRP antibodies in the sera collected from mice 1 to 10 weeks post-initial immunization with pCR3.1-VS-HSP65-TP-GRP6-M2 (TP-GRP6-M2); pCR3.1-VS-HSP65-TP-M2 (TP-M2); pCR3.1-VS-HSP65-TP-GRP6 (TP-GRP6); and saline. TP-GRP6-M2 produced the highest titer of anti-GRP antibodies than any other groups, especially at week 10 after immunization ($p < 0.05$)

The anti-GRP vaccine suppressed the growth of subcutaneous H22 tumors

We determined whether vaccination with TP-GRP6-M2 could protect mice against hepatocarcinoma. H22 cells injected subcutaneously formed large solid tumors in nonimmunized mice (saline) or in mice injected

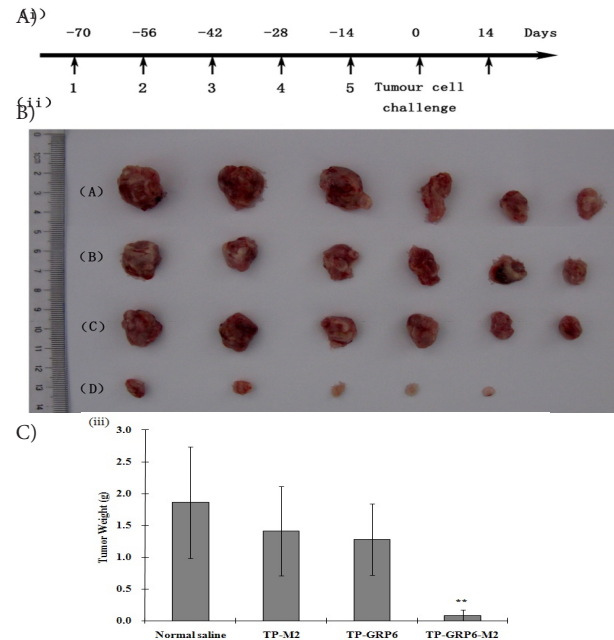


Figure 3. Effects of Anti-GRP DNA Vaccine on H22 Tumor Model. **A)** The immunization scheme indicates the days of vaccination by arrows. Mice were immunized with anti-GRP DNA vaccines day -70, -56, -42, -28, and -14. At day 0, 2.106 H22 hepatocarcinoma cells were injected subcutaneously for tumor cell challenge. Fourteen days later, all mice were killed and tumors were excised and weighted. **B)** The tumors from immunized mice. The picture shows the tumors extracted from mice immunized with saline (A, n=6), pCR3.1-VS-HSP65-TP-M2 (B, n=6), pCR3.1-VS-HSP65-TP-GRP6 (C, n=6), pCR3.1-VS-HSP65-TP-GRP6-M2 (D, n=6). **C)** Comparison of tumor weight; resulted were presented as means \pm SD and significance at $p < 0.05$

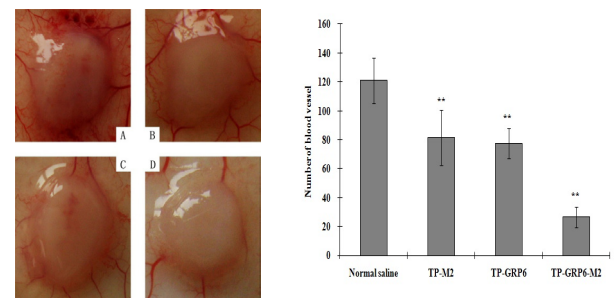


Figure 4. Effects of Anti-GRP Vaccine on the Angiogenesis of Intradermal Tumors. (Left): Light microscopical picture of H22 tumor cells implanted intradermally in the anterior abdominal wall and the development of new blood vessels. Tumor-associated angiogenesis (2/mouse, 4 mice/group) in mice injected with saline **A)** and pCR3.1-VS-HSP65-TP-M2 control vaccine **B)** appeared to be higher than in mice immunized with the anti-GRP vaccine pCR3.1-VS-HSP65-TP-GRP6-M2 **D)**. Representative images were taken at 10x objective. (Right): The total number of blood vessels (major vessels and branching points) was determined within the precise 1 cm² area around each implant site

with a non-GRP-containing plasmid (TP-M2). The size and weights of tumors removed from all immunized mice on day 14 after tumor cell challenge of the three experimental groups were measured. The tumor sizes decreased progressively in mice immunized with the anti-GRP vaccine (TP-GRP6-M2) (Figure 3). The average weight of solid tumors in mice immunized with TP-GRP6-M2 was significantly lower than that for mice in the saline group (0.099 ± 0.085 g vs 1.609 ± 0.99 g, $p=0.03$). However, tumor weight in mice injected with TP-GRP6 ($p=0.466$) or TP-M2 ($p=0.828$) was not significantly lower than that of the saline (Figure 3). A highly inhibition was achieved in TP-GRP6-M2 group. It was concluded that this DNA vaccine had strongly elicited anti-tumor immunity against the growth of H22.

Intradermal tumor angiogenesis

To assess the effect of the immune response on tumor-associated angiogenesis induced by anti-GRP DNA vaccine TP-GRP6-M2, H22 tumor cells were implanted intradermally at two sites in the abdominal region. Four groups of mice were immunized with saline, vaccine control TP-M2, TP-GRP6 or anti-GRP DNA vaccine TP-GRP6-M2 respectively, as described earlier. As shown in Figure 4, tumor cells implanted intradermally were found to induce significant angiogenesis when the intradermal tumor grows up to 5 mm in diameter.

The total number of blood vessels around each implant site from mice immunized with anti-GRP DNA vaccine TP-GRP6-M2 was lower than blood vessels from saline (26.5 ± 7.17 vs 121 ± 15.69) and mice immunized with vaccine control TP-M2 (26.5 ± 7.17 vs 81.6 ± 19.05).

Discussion

Cancer mortality is related to the spread of neoplastic cells to distant loci where the cells, supported by existing blood vessels and angiogenesis, proliferate and give rise to secondary tumors. Tumor angiogenesis is up-regulated by a number of conditions including hypoxia, hypoglycemia, mechanical disruption, and genetic and inflammatory alterations (Rosen, 2009) that lead to activation of growth factors and pro-angiogenic genes (Gordan and Simon, 2007; Gruber and Simon, 2006). Angiogenesis has a critical role in physiologic and disease processes. For the growth of tumors, angiogenesis must occur to carry sufficient nutrients to the tumor. In addition to growth, development of new blood vessels is necessary for invasion and metastases of the tumor. A number of strategies have been developed to inhibit tumor angiogenesis and further understanding of the interplay between tumors and angiogenesis should allow new approaches and advances in angiogenic therapy. One such promising angiogenic approach is to target and inhibit angiogenesis with vaccines (Matejuk et al., 2011). Targeting the tumor vasculature, over targeting tumor cells, with vaccines as well as other immunotherapies may have several potential advantages namely a more accessibility to the immune system; the genetic stability and as a result the reduction of the risk of resistance to developing immunotherapies (Boehm et al., 1997; Huang

et al., 2004) a more potent CD8+-mediated response favored by less frequency of down-regulation of MHC I in tumor endothelial cells; finally, since inhibition of a single endothelial cell can inhibit up to 100 tumor cells (Folkman, 1996; Folkman, 1997), immunotherapies directed toward tumor endothelial cells have the potential of an amplifying inhibitory effect.

Over the past 20 years, abundant evidence has been collected to suggest that GRP and its receptors play an important role in the development of a variety of cancers (Hohla and Schally, 2010). These observations have inspired researchers to find a suitable approach to treat cancers by taking advantage of blocking the GRP to GRPR signal pathway. GRP is a strong mitogen that augments the growth of several types of tumors including breast (Ni et al., 2012), prostate (Beer et al., 2012), lung (Egloff et al., 2012), anal cancer (Martins et al., 2013), and neuroblastomas (Pirtha et al., 2013, Lee et al., 2013). By activating its receptors (GRPR) located on both tumor and endothelial cells, GRP is also a direct angiogenic factor as shown by *in vitro* and *in vivo* angiogenic assays (Martinez et al., 2005). In addition, GRP is able to contribute indirectly to tumor angiogenesis by augmenting expression of pro-angiogenic factors such as IL-8 and VEGF (Kang et al., 2007; Lee et al., 2013). The GRP antagonist, 77427, markedly inhibits endothelial cell cord formation *in vitro* and inhibits angiogenesis *in vivo* (Levine et al., 2003). Several therapeutic strategies targeting GRP/GRPR, such as peptide antagonists and monoclonal antibodies, have demonstrated their antitumor efficacy *in vitro* and *in vivo* (Cornelio et al., 2007). Recently, vaccines targeting GRP displayed an effective anti-tumor response by inducing humoral and cell mediated immune responses. A recombinant chimeric protein vaccine (HG6) containing six tandem repeats of a GRP fragment (18 to 27 amino acids) fused to the 65-kDa heat shock protein (HSP65) was used to treat mice challenged with breast cancer (Wu et al., 2008). Results from this treatment revealed significant prophylactic and therapeutic activities in term of reduction of tumor size, along with a marked anti angiogenic effect in mice administered with the GRP fusion HG6 protein. In an analogous approach, a DNA vaccine encoding the GRP-HSP65 fusion product showed anti-tumor activities in a prophylactic setting for subcutaneous implanted tumors and a pulmonary metastasis model (Fang et al., 2009). Furthermore, proofs have been given that introduction of two T-helper epitope (HSP70406-426) downstream the immunogen in anti-GRP DNA vaccine augment humoral response and immunogenicity of the vaccine against murine prostate carcinoma (Lu et al., 2009). Our results demonstrated efficient protection against growth of H22 liver tumor cells injected subcutaneously along with a reduced neoangiogenesis.

The mechanism underlying the ability of the anti-GRP vaccine to inhibit the proliferation of H22 cells *in vivo* is yet to be elucidated. It is clear that the humoral immune response has been elicited by vaccination of DNA vaccine with a high titer of GRP antibodies detected. This phenomenon suggests that the GRP antibodies neutralize the self-epitope GRP and lower the concentration of GRP

around the tumor, whereas the activation of GRPR may be interrupted and thus blocking the signaling pathways.

On the other hand, Gastrin-releasing peptide is known to stimulate pro-angiogenic gene expression as well as the expression of various angiogenic markers such as vascular endothelial growth factor (Kang et al., 2007). In addition, it has been shown that GRP or GRPR silencing significantly suppress tumor progression and vascularization (Cornelio et al., 2007). HCC is known as a highly angiogenic cancer, with critical implication of VEGF (Zhu et al., 2011). The results from this study clearly demonstrate that vaccination with the DNA vaccine pCR3.1-VS-HSP65-TP-GRP6-M2 leads to significant reduction of the tumor-associated angiogenesis and vascularization of H22 solid tumor. This effect can contribute to prophylactic approach in developing immunotherapy in liver cancer. DNA vaccine targeting GRP have also shown therapeutic effect (Wu et al., 2008); and due to some immune escape events reported, it will be interesting that our vaccine be tested against well formed H22 tumors.

In conclusion, in researching new alternatives in immunotherapy of liver cancer, we relied on a DNA vaccine targeting Gastrin-Releasing peptide (GRP). The vaccine was applied for its immune preventive effects against H22 hepatocarcinoma cells, suggesting that it could inhibit tumor growth through anti-angiogenic effects. The strategy offers a novel approach to be developed in the treatment. However, further research need to be carried out, such as therapeutic activity, to assess the efficiency of the vaccine in the control of liver cancer.

References

- Beer M, Montari M, Gerhard J, et al (2012). Profiling gastrin-releasing peptide receptor in prostate tissues: Clinical implications and molecular correlates. *Prostate*, **72**, 318-22
- Bele T (2012). Treating cancer with vaccine. *Web Med Central Immunopathol*, **3**, 3267.
- Boehm T, Folkman J, Browder T, et al (1997). Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature*, **390**, 404-7.
- Butterfield LH (2007). Recent advances in immunotherapy for hepatocellular cancer. *Swiss Med Wkly*, **137**, 83-90.
- Donnelly JJ, Ulmer JB, Shiver JW, et al (1997). DNA vaccines. *Ann Rev Immunol*, **15**, 617-48.
- Egloff AM, Davis AG, Shuai YL, et al (2012). Gastrin-releasing expression in non-cancerous bronchial epithelia is associated with lung cancer: a case-control study. *Respiratory Research*, **13**, 9.
- Fang F, Lu Y, Ouyang K, et al (2009). Specific antibodies elicited by a novel DNA vaccine targeting gastrin-releasing peptide inhibit murine melanoma growth *in vivo*. *Clin Vaccine Immunol*, **16**, 1033-39.
- Folkman F (1996). Fighting cancer by attacking its blood supply. *Sci Am*, **275**, 150-4.
- Folkman F (1997). Antiangiogenic Therapy. In Principles and Practice of Oncology. 9th Ed DeVita V T, Hellman S, Rosenberg SA, Lippincott Williams & Wilkins (LWW) Philadelphia: Lippincott-Raven pp 3075-85 Number 978-2.
- Gordan JD, Simon MC (2007). Hypoxia-inducible factors: central regulators of the tumor phenotype. *Curr Opin Genet Dev*, **17**, 71-77.
- Greten TF, Papendorf F, Bleck JS, et al (2006). Survival rate in patients with hepatocellular carcinoma: a retrospective analysis of 389 patients. *Br J Cancer*, **92**, 1862-8.
- Gruber M, Simon MC (2006). Hypoxia-inducible factors, hypoxia, and tumor angiogenesis. *Curr Opin Hematol*, **13**, 169-74.
- Hohla F, Schally AV (2010). Targeting gastrin releasing receptors New options for the therapy and diagnosis of cancer. *Cell Cycle*, **9**, 1738-41.
- Huang J, Soffer SZ, Kim ES, et al (2004). Vascular remodeling marks tumors that recur during chronic suppression of angiogenesis. *Mol Cancer Res*, **2**, 36-42.
- Jemal A, Bray F, Center MM, et al (2011). Global cancer statistics. *CA Cancer J Clin*, **61**, 69.
- Kang J, Ishola TA, Baregamian N, et al (2007). Chung, Bombesin induces angiogenesis and neuroblastoma growth. *Cancer Lett*, **253**, 273-81.
- Kreisle RA, Ershler WB (1988). Investigation of tumor angiogenesis in an id mouse model: role of host-tumor interactions. *J Natl Can Inst*, **80**, 849-54.
- Lee S, Qiao JB, Pritha P, et al (2013). Integrin $\beta 1$ critical for gastrin-releasing peptide receptor-mediated neuroblastoma cell migration and invasion. *Surgery*, **154**, 369-75.
- Lee SY, Kang TH, Knoff J, et al (2013). Enhanced anticancer immunotherapy with a DNA vaccine by depleting platelets. *J Immunother Can*, **1**, 222.
- Levine L, Lucci JA, Pazdrak B, et al (2003). Bombesin stimulates nuclear factor kappa B activation and expression of proangiogenic factors in prostate cancer cells. *Cancer Res*, **63**, 3495-502.
- Lu Y, Ouyang K, Fang J, et al (2009). Improved efficacy of DNA vaccination against prostate carcinoma by boosting with recombinant protein vaccine and by introduction of a novel adjuvant epitope. *Vaccine*, **27**, 5418-511.
- Luigi A and Ciliberto G (2011). Emerging cancer vaccines: The promise of genetic vectors. *Cancers*, **3**, 3687- 713.
- Martins FF, Contu PC, Meurer L, et al (2013). Expression of gastrin-releasing peptide receptor in epidermoid carcinoma of anal canal. *Appl Immunohistochem Mol Morphol*.
- Matejuk A, Leng Q, Chou ST, et al (2011). Vaccines targeting the neovasculature of tumors. *Vasc Cell*, **3**, 7.
- Ni CS, Zhao XL, Sun T, et al (2012). Role of gastrin-releasing peptide in breast cancer metastasis. *Human Pathol*, **12**, 2342-47.
- Ozturk M and Oters S (2011). Molecular approach to treatment of hepatocellular carcinoma: New hope for therapeutic targets. *J Exper Integr Med*, **1**, 83-4.
- Piritha P, Qiao JB, Kim KW, et al (2013). Targeting gastrin-releasing peptide suppresses neuroblastoma progression via upregulation of PTEN signaling. *PLoS ONE*, **8**, 72570
- Ray K (2013). The promise of new approaches in the management of hepatocellular carcinoma adding to the toolbox? *Nat Rev Gastroenterol Hepathol*, **10**.
- Rinaldi M, Lurescia S, Fioretti D, et al (2009). Strategies for successful vaccination against hepatocellular carcinoma. *Int J Immunopathol Pharmacol*, **22**, 269-77.
- Rosen LS (2009). Clinical experience with angiogenesis signaling inhibitors: focus on vascular endothelial growth factor (VEGF) blockers. *Cancer Control*, **9**, 36-44.
- Wu G, Guo W, Ouyan K, et al (2008). A novel vaccine targeting gastrin-releasing peptide: efficient inhibition of breast cancer growth *in vivo*. *Endocr Relat Cancer*, **15**, 149-59.
- Yankai Z, Rong Y, Yi L, Wentao L, et al (2006). Ten tandem repeats of beta-hCG 109-118 enhance immunogenicity and anti-tumor effects of betahCG C-terminal peptide carried by mycobacterial heat-shock protein HSP65. *Biochem Biophys Res Commun*, **345**, 1365-71.
- Zhu XA, Duda DG, Sahani DV, et al (2011). HCC and angiogenesis: Possible targets and future direction. *Nat Rev Clin Oncol*, **8**, 292-301.