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Original Article

Study of natural IgG antibodies against vascular endothelial growth factor receptor 1 in hepatocellular carcinoma

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Abstract: Natural antibodies have been found to have anti-tumorigenic function. This study was designed to investigate whether natural IgG antibodies against vascular endothelial growth factor receptor 1 (VEGFR1) could suppress the growth of hepatocellular carcinoma (HCC) cells. Three HCC cell lines and A549 lung cancer cells were used for this study. They were grown, respectively, with human plasma positive or negative for anti-VEGFR1 IgG. Cell viability, apoptosis and VEGFR1 gene expression were examined. Three patients with HCC were recruited for a case study. The results showed that plasma anti-VEGFR1 IgG significantly inhibited the proliferation of all three HCC cell lines but not A549 cell line; the proportions of apoptotic cells were significantly higher in HCC cells treated with anti-VEGFR1 IgG positive plasma than those treated with IgG negative plasma. The expression of the VEGFR1 gene was significantly higher in HCC cells than A549 cells. Of three HCC patients who received transfusion of anti-VEGFR1 IgG positive plasma, two cases with stage B showed a good response to the treatment but one with distant metastasis did not. Human plasma IgG against VEGFR1 may be a promising agent for anti-HCC therapy.

Keywords: Hepatocellular carcinoma, VEGFR1, gene expression, natural antibody, tumor immunity, immunotherapy

Introduction

Liver cancer is one of the most commonly diagnosed malignant tumors worldwide, which is the second leading cause of cancer-related deaths in men and the sixth leading cause in women [1] although it has become the third leading cause of female cancer deaths in China [2, 3]. A recent epidemiological study demonstrated that during 2012 there were about 782,500 new cases diagnosed as having liver cancer and 745,500 deaths in the world, with China alone accounting for about 50% of the total number of cases and deaths [1]. Of all cases with liver cancer, more than 90% suffer from hepatocellular carcinoma (HCC) and the number of deaths due to HCC is dramatically increasing each year, with a 5-year survival rate of less than 9% [4, 5]. Patients with late-stage HCC usually have a poor prognosis, and only 30-40% are deemed to be eligible for curative intention with routine treatments, including surgical operation, radiotherapy, chemotherapy and liver transplantation [6, 7]. With advances in surgical techniques and instrumentation as well as the development of molecular target drugs, several potentially curative treatments have become available [8-10], while postoperative therapies for preventing recurrence of HCC remain a key issue to enhance the survival of HCC patients.

Tumor cells have the capability to produce some angiogenic factors such as vascular endothelial growth factors (VEGFs), which can bind to their corresponding receptors on the surfaces of cells, resulting in a variety of biological effects and thereby promoting tumor progression [11]. Accordingly, antiangiogenic therapy has been one of the main anticancer strategies. Bevacizumab (trade name Avastin) is a humanized monoclonal antibody that inhibits the activity of vascular endothelial growth factor A (VEGF-A), and has been clinically used for the treatment of some metastatic cancers [12-14]. Interestingly, bevacizumab has also shown an inhibitory effect on the growth of human HCC both in vitro and in vivo [15], suggesting that HCC cells may express VEGF receptors. Despite its efficacy, systemic anticancer treatments with bevacizumab may have toxic effects on the cardiovascular system, promoting the development of hypertension, cardiac ischemia and congestive heart failure [16]. So there is an urgent need to develop alternative therapies to minimize the cardiovascular toxicity.

Natural antibodies are likely to serve as an important anti-tumorigenic system in the body and their anti-tumor cytotoxicity has been confirmed with in vitro study [17, 18]. It is possible that natural antibody-rich plasma from healthy donors could be used as postoperative therapies to prevent the recurrence of human cancer. In this study, therefore, we detected natural IgG antibodies against VEGF receptor 1 (VEGFR1) in plasma and then analysed the effects of anti-VERGFR1 IgG rich plasma on the proliferation of HCC cell lines. We also recruited three patients with HCC for clinical trial with anti-VERGFR1 IgG rich plasma.

Materials and methods

Detection of anti-VEGFR1 IgG in plasma

Plasma samples were collected from healthy blood donors by the Blood Center of Dongguan, Guangdong Province, China and the Blood Center of Qingdao, Shandong Province, China. Pooled plasma of 20 randomly selected plasma samples was used as a reference sample (RS) for relative quantification of natural anti-VEGFR1 IgG levels in plasma. This work was approved by a local ethics committee based in Qingdao and conformed to the provisions of the Declaration of Helsinki.

An enzyme-linked immune-sorbent assay (ELISA) was used to detect plasma IgG antibody against the extracellular domain of human VEGFR1 protein (NCBI accession NP_002010). The ELISA antibody test kit was supplied by Hailanshen Biotechnology Ltd, Qingdao, China, as described in our previous study [19]. In brief, the antigen-coated plate was washed twice with 200 μl Wash Buffer just before use; 50 μl plasma sample diluted 1:200 in Assay Buffer was then added to each sample well, and 50 μl Assay Buffer was added to each negative control (NC) well. Following incubation at room temperature for 1.5 hours (hrs), the plate was washed three times with 200 μl Wash Buffer and 100 μl peroxidase-conjugated goat anti-human IgG antibody (ab98567, Abcam) diluted 1:30000 in Assay Buffer was added to each well. After incubation at room temperature for 1.5 hrs, color development was initiated by adding 50 μl Stabilized Chromogen (SB02, Life Technologies) and terminated 25 min later by adding 50 μl Stop Solution (SS04, Life Technologies). The measurement of optical density (OD) was completed on a microplate reader within 10 min at 450 nm with a reference wavelength of 620 nm. All the samples were tested in duplicate and the specific binding ratio (SBR) was used to represent plasma anti-VEGFR1 IgG levels. Calculation of SBR is as follows:

$$SBR=(OD_{Sample}-OD_{NC})/(OD_{RS}-OD_{NC}).$$

Cell proliferation assay

Three cell lines derived from human HCC, HepG2, BEL-7402 and BEL-7405 (China Academy of Chinese Medical Sciences, CACMS, China), were used for this study, and A549 cell line (CACMS, China) derived from lung cancer was used as control cells. These cancer cells were seeded in a 96-well plate, 100 μl/well with a density of 2.5×10^4 cells/ml Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 10% fetal calf serum (FCS). After 24-hr incubation in humidified atmosphere with 5% CO_2 at 37°C, the medium was changed and cancer cells were cultured with DMEM containing 15% human plasma either positive or negative for anti-VEGFR1 IgG antibodies for 48 hrs in the same conditions as mentioned above. Cell counting kit-8 (CCK-8, Sigma-Aldrich) was applied to detect cell viability. Briefly, 10 μl CCK-8 solution was added to each well; after incubation at 37°C for 2 hrs, OD of each well was measured on a microplate reader at 450 nm wavelength. The complete medium was used as blank. Cell viability was used to present data and calculated as follows:

Cell viability=$(OD_{positive}-OD_{blank})/(OD_{negative}-OD_{blank})$.

Analysis of apoptosis

HCC cells were seeded in 6-well dishes, 2 ml/well with a density of 2.5×10^5 cells/ml DMEM containing 10% FCS. After 24-hr incubation in humidified atmosphere with 5% CO_2 at 37°C, the medium was changed and HCC cells were cultured with DMEM containing 15% human plasma either positive or negative for anti-VEGFR1 IgG. Cultured cells were then collected at 24 hrs and 48 hrs, respectively, for analysis of apoptosis.

Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, USA) was used to detect apoptosis of HCC cells according to the manufacturer's instruction. Briefly, cultured cells were harvested, washed twice with cold PBS and re-suspended in 400 μl Annexin V-FITC binding buffer. HCC cells were stained with 5 μl of Annexin V-FITC and incubated in the dark at 4°C for 15 min; 10 μl propidium iodide (PI) was added to each well and incubated at 37°C for 5 min. FACSCalibur flow cytometer (Becton-Dickinson, USA) was used to detect apoptosis of cells.

VEGFR1 gene expression assay

Both HCC and A549 cells were seeded in 6-well dishes, 2 ml/well with a density of 2.5×10^5 cells/ml DMEM containing 10% FCS. After 48-hr incubation in humidified atmosphere with 5% CO_2 at 37°C, they were collected for extraction of total RNA using RNAiso reagent (TaKaRa Bio-technology, Dalian, China). Total RNA samples were treated with a DNA-free kit (Fermentas, Hanover, MD, USA) to eliminate DNA contamination, and then reversely transcribed into cDNA using PrimeScript™ RT Master Mix (TaKaRa Bio-technology). A cDNA aliquot equivalent to 40 ng of total RNA was used for quantitative real-time PCR (qRT-PCR) analysis, and SYBR Premix Ex Taq kit (TaKaRa Bio-technology) was used to quantify expression of the VEGFR1 gene on the ABI 7500 real-time PCR system, with a pair of following primers: 5'-TTAGGACCAGGAAGCAGCAC-3' (forward) and 5'-CCGAGGTTCCTTGAACAGTGA-3' (reverse). Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene for normalization, and GAPDH primers used for qRT-PCR amplification were purchased from QIAGEN (Shanghai, China). Relative quantity of gene expression was calculated using the comparative Ct method. Fold change (FC) was used to present data and worked out based on the formula: $FC=2^{-\Delta\Delta Ct}$.

Data analysis

All experimental data were expressed as mean ± standard deviation (SD). Student's *t*-test (two-tailed) was applied to examine the differences in cell viability between cancer cells treated with anti-VEGFR1 IgG positive and negative plasma, in VEGFR1 gene expression between HCC and A549 cells as well as in the percentage of apoptotic cells between HCC cells treated with anti-VEGFR1 IgG positive and negative plasma.

Clinical case study

Three HCC patients with history of hepatitis B and liver cirrhosis were recruited for treatment with anti-VEGFR1 IgG positive plasma; all three patients gave informed written consent to participate this clinical trial. Case one was male and diagnosed as having HCC with multiple nodules at the Second Hospital of Jilin University, Changchun in September 2014, and classified into stage B based on the Barcelona Clinic Liver Cancer (BCLC) staging system [20]. This patient underwent segmentectomy of the liver, followed by chemoembolization treatment three times within a year. Recurrence was identified by intensive CT scan in October 2015, which showed multiple space-occupying lesions in the liver, although there was no sign of regional metastasis. He received the first transfusion of 950 ml anti-VEGFR1 IgG positive plasma at Dalang Hospital based in Dongguan on November 17-19, 2015. Case two was female and diagnosed as having HCC with intrahepatic metastasis at Affiliated Hospital of Qingdao University in June 2015, and classified into stage C based on the BCLC staging system. She received both radiotherapy and chemoembolization before HCC was confirmed to be progressing by PET-CT scan in August 2015; distant metastasis was found in the spleen in December 2016. This patient received the transfusion of 680 ml anti-VEGFR1 IgG positive plasma at the Central Hospital of Qingdao on March 8, 2016. Case three was male and diag-

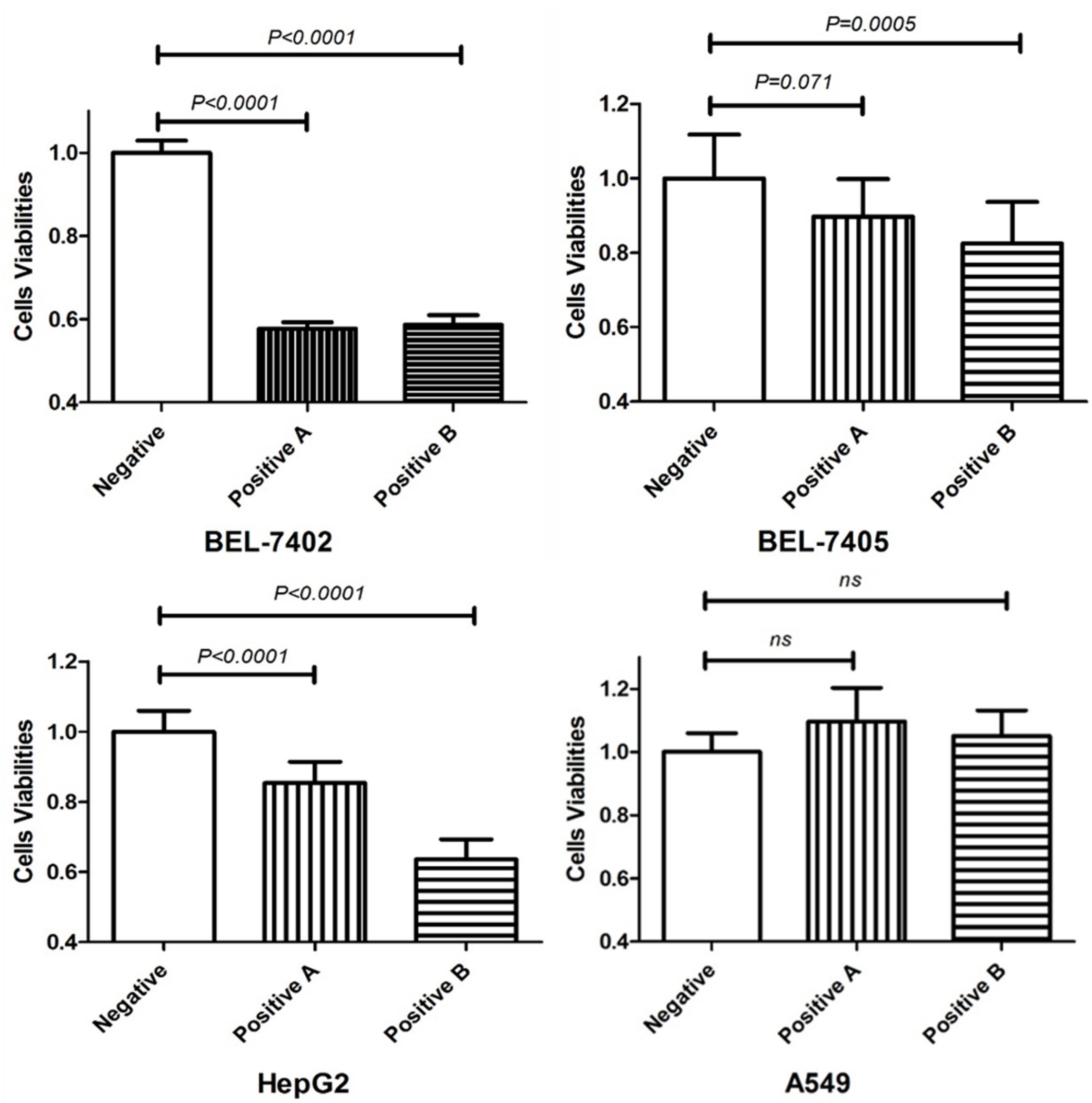

Figure 1. Effects of plasma anti-VEGFR1 IgG on the proliferation of 4 cancer cell lines. Data of cell viability were expressed as mean ± SD; ns: not significant.

nosed as having infiltrative HCC at the Sixth People's Hospital of Qingdao in May 2016; this patient was classified into stage B based on the BCLC staging system and received treatment only with chemoembolization twice since diagnosis was made. He received the first transfusion of 900 ml anti-VEGFR1 IgG positive plasma at the Sixth People's Hospital of Qingdao on September 20-22, 2016.

Results

Plasma with the highest SBR value from two healthy donors was used as anti-VEGFR1 IgG positive plasma (A and B); anti-VEGFR1 IgG negative plasma was taken from three healthy donors with the lowest SBR value and was mixed properly.

Inhibitory effects of anti-VEGFR1 IgG plasma on proliferation of HCC cells

As compared with anti-VEGFR1 IgG negative plasma, anti-VEGFR1 IgG positive plasma had capacity of significantly inhibiting the proliferation of BEL-7402 cells (t=-35.95, df=16, P< 0.0001 for plasma A and t=-35.33, df=16, P< 0.0001 for plasma B), BEL-7405 cells (t=-4.35,

Table 1. Apoptosis induced by plasma anti-VEGFR1 IgG in HCC cells

Time of cell treatment	Percentage of apoptotic cells (%)		T	df	P
	Negative plasma	Positive plasma			
BEL-7402					
24 h	3.192±0.315	5.223±0.343	10.70	10	<0.0001
48 h	8.170±2.392	14.54±3.498	3.68	10	0.0042
HepG2					
24 h	2.275±0.816	4.582±0.679	5.33	10	0.0003
48 h	2.570±0.359	5.153±1.096	5.49	10	0.0003

Data were expressed as mean ± SD.

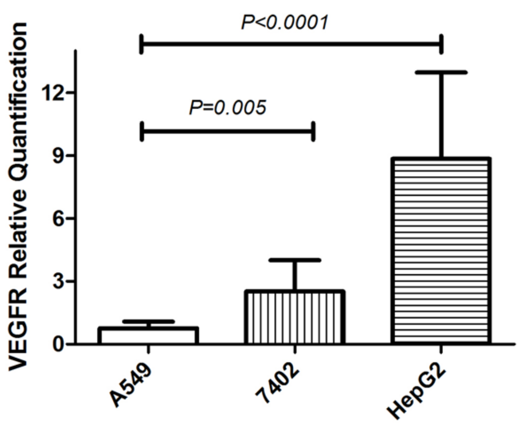

Figure 2. Expression of the VEGFR1 gene in BEL-7402, HepG2 and A549 cells. Data of VEGFR1 gene expression were expressed as mean ± SD.

df=16, *P*=0.0005 for plasma B only) and HepG2 cells (*t*=-6.63, *df*=16, *P*<0.0001 for plasma A and *t*=-15.15, *df*=16, *P*<0.0001 for plasma B). However, anti-VEGFR1 IgG positive plasma did not show any inhibitory effect on the proliferation of A549 cells (**Figure 1**).

Cell apoptosis induced by anti-VEGFR1 IgG plasma

Based on the work on effects of anti-VEGFR1 IgG plasma on the proliferation of HCC cells (**Figure 1**), we used BEF-7402 and HepG2 cell lines to investigate their apoptosis induced by anti-VEGFR1 IgG positive plasma. As shown in **Table 1**, the proportion of apoptotic cells was significantly higher in HCC cells treated with anti-VEGFR1 IgG positive plasma (B) than those treated with anti-VEGFR1 IgG negative plasma (*P*<0.0001 for 24-hr treatment and *P*= 0.0042 for 48-hr treatment in BEL-7402 cells; *P*=0.0003 for both 24-hr and 48-hr treatments in HepG2 cells).

Expression of the VEGFR1 gene in BEL-7402, HepG2 and A549 cells

Student's t-test revealed that the levels of VEGFR1 gene expression were significantly higher in BEL-7402 (*t*=3.33, *df*=14, *P*=0.005) and HepG2 cells (*t*=5.60, *df*=13, *P*<0.0001) than A549 cells, although HepG2 cells had the highest levels of VEGFR1 gene expression in these three cell lines (**Figure 2**).

The outcomes of anti-VEGFR1 IgG positive plasma treatment

The three patients with HCC received treatment with anti-VEGFR1 IgG positive plasma. Case one received plasma transfusion three times between November 2015 and September 2016 (**Table 2**). The condition of this patient had been stable although intensive CT scan showed a nodule of ~10 mm (in diameter) in the segment-8 region, which was 8.7 mm (in diameter) measured in April 2016; this patient therefore received a treatment with radiofrequency ablation (RFA) on August 25, 2016, and also received the third transfusion of 720 ml anti-VEGFR1 IgG positive plasma at the Sixth People's Hospital of Qingdao, China on September 9-12, 2016. However, a magnetic resonance imaging (MRI) scan identified at least 5 new nodules in the liver (segments 2, 3, 4, 6 and 8) on December 2, 2016, as compared to previous follow-up on September 9, 2016. While no further treatment was given, this patient was still alive based on the follow-up on February 15, 2017. Case two had progressed to a late stage of HCC before plasma transfusion was given; she died on April 28, 2016, with a survival of <12 months after first diagnosis was made. Case three had a follow-up examination with intensive CT scan on January 22, 2017 and remarkable improvement was observed by contrasting the CT scan image performed on September 7, 2016.

Discussion

Natural antibodies are defined as the immunoglobulins produced by B lymphocytes in the absence of external antigen stimulation [18]. It has been proposed that the production of natural antibodies appears to be genetically controlled [21]. In function, natural antibodies are

Table 2. Clinical information of three HCC patients who received plasma transfusion

Case	Age (yrs)†	Sex	History of HB	Liver cirrhosis	Distant metastasis	Plasma transfusion‡	Outcome§
One	68	M	Yes	Yes	No	Three times	PFS >12 months
Two	60	F	Yes	Yes	Yes	Once	Died
Three	66	M	Yes	Yes	Yes	Once	PFS >4 months

†Taken at the time of first hospitalization. ‡Case one received the second transfusion of 700 ml anti-VEGFR1 IgG positive plasma at Dalang Hospital based in Dongguan on April 19-21, 2016, and the third transfusion of 630 ml anti-VEGFR1 IgG positive plasma at the Sixth People's Hospital of Qingdao on September 9-12, 2016. §Case one had a progression-free survival (PFS) of >12 months based on an MRI scan that identified at least 5 new nodules in the liver on December 2, 2016. Case two died on April 28, 2016. The follow-up of case three was carried out on January 22, 2017 and remarkable improvement was observed based on intensive CT scan.

physiologically involved in maintaining tissue homeostasis such as elimination of invading pathogenic agents and non-functional proteins, clearance of apoptotic cells as well as destruction of cancer cells [17, 18, 22]. While IgM has been considered as the major isotype of natural antibodies, natural IgG isotype is also abundant in the circulation of most healthy individuals [19, 23].

The present study demonstrated that some healthy individuals had remarkably high levels of natural IgG antibodies against VEGFR1, which could significantly inhibit the proliferation of HCC cells (**Figure 1**). Based on the observations from further study of apoptosis and gene expression in HCC cell lines (**Table 1** and **Figure 2**), anti-VEGFR1 IgG antibodies are likely to target the extracellular domain on the surfaces of HCC cells, inducing apoptosis of these malignant cells. To our knowledge, this is the first report on inhibitory effects of anti-VEGFR1 IgG antibodies on the proliferation of HCC cells in vitro.

In this study, we also carried out clinical trials on three cases with HCC. Case one has been surviving with no apparent progression over a year since recurrence was confirmed in October 2015. However, plasma anti-VEGFR1 IgG failed to show a significant effect on survival of case two as splenic metastasis had been found before this HCC patient received plasma transfusion. Possibly, this therapy is not suitable for the treatment of patients with late stage HCC. The infiltrative type of HCC accounts for 7-20% of all HCC cases worldwide [24] and a much higher proportion in China [25]. Patients with infiltrative type of HCC always have poor prognosis with low survival rates [24, 25]. It is worth noting that plasma anti-VEGFR1 IgG appears to improve infiltrative HCC based on our study. We will continue to follow up cases one and three in order to draw a firm conclusion about the therapeutic effectiveness of anti-VEGFR1 IgG positive plasma on this malignancy. A large-scale clinical trial will be designed based on this initial study.

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Disclosure of conflict of interest

None.

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