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Original Article

Receptors for advanced glycation end products (RAGE) is associated with microvessel density and is a prognostic biomarker for clear cell renal cell carcinoma

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ABSTRACT

The receptor for advanced glycation end products (RAGE) is involved in a variety of biological processes, including tumorigenisis. Previous studies have demonstrated that RAGE regulates the neo-angiogenesis related downstream molecule – vascular endothelial growth factor receptor 2 (VEGFR-2). Here, we investigated the potential relationship between RAGE, VEGFR-2 and angiogenesis in 80 renal cell carcinoma (RCC) patients. Real-time quantitative PCR and ELISA analysis were used to explore the *RAGE* and *VEGFR-2* gene expression levels and the protein of VEGFR-2 expression. Meanwhile, angiogenesis was detected by the semi-quantification of endothelial cell marker CD34 combined with caldesmon, which was detected by microvessel density (MVD) technique and immunohistochemistry. Tumors were classified as low or high RAGE-expressing using the median as the cut-off. Immunofluorescence staining for RAGE protein was performed as well. Additionally, the median gene expression levels of VEGFR-2 in the tumors were significantly lower expressing low levels of RAGE expression, 0.34 (95% CI, 0.28–0.39) compared to the expressing high levels of RAGE expression, 0.45 (95% CI, 0.29–0.61), ($P = 0.03$). The median MVD was significantly lower in the tumors expressing low levels of RAGE, 6.5 (95% CI, 6.21–7.43), compared to the expressing high levels, 7.9 (95% CI, 6.25–8.93), ($P < 0.01$). Further, a positive association was certified with VEGFR-2 protein levels, $P = 0.07$. Besides, RCC with high levels of RAGE expression are associated with high VEGFR-2 mRNA/protein levels and a higher density of microvessels; conversely, Kaplan–Meier survival analysis suggests that a significant correlation of elevated RAGE expression with decreased overall survival and metastasis-free survival. Our results establish that RAGE was identified as a potential prognostic biomarker for disease prognosis of RCC.

1. Introduction

Renal cell carcinoma (RCC) is a common urologic malignancy that accounts for about 3% of all human cancers. Around 200,000 patients are diagnosed with RCC resulting in approximately 100,000 deaths worldwide each year, and its incidence is increasing steadily in recent years [1,2]. One third of patients are presented with metastases, whereas another third will develop metastases. At present, biomarkers for early detection and follow-up of the disease are not available, accounting for late diagnosis and subsequent poor prognosis. Angiogenesis, the development of capillaries from pre-existing blood vessels, is essential for the growth of malignant tumors [3]. Recent studies show the vascular endothelial growth factor (VEGF) plays an important role in regulating the process of angiogenesis, and the clinical benefit from targeting this pathway in tumor patients with metastatic renal cell carcinoma is well documented [4,5].

RAGE, the receptor for advanced glycation end products, is a 35 kD transmembrane receptor of the immunoglobulin super family. RAGE has been implicated in a variety of human disease processes [6]. In view of its inflammatory function in innate immunity and its ability to detect a class of ligands through a

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common motif, RAGE is often referred to as a pattern recognition receptor. Normally, RAGE is expressed at high levels in the lungs, and at lower levels in a variety of other cell types, including neurons, immune cells, activated endothelial and vascular smooth muscle cells. However, in patho-physiological settings, such as chronic inflammation, diabetes or neurodegenerative disorders, RAGE expression is increased significantly in other tissues, such as vasculature, haematopoietic cells and the central nervous system [7]. Due to an enhanced level of RAGE ligands in diabetes or other chronic diseases, this receptor is hypothesized to have a causative effect in a range of inflammatory diseases, such as diabetic complications, Alzheimer's disease and even some tumors [8]. It is interesting to note that there is very low or no RAGE expression in normal tissues, but enhanced expression in chronic inflammation and cancer. It has also been well documented that RAGE ligands bind to RAGE and activate its downstream signalling mechanisms that fuel chronic inflammatory conditions leading to neoplastic stage [9]. Therefore, these features of RAGE make it an ideal candidate for therapeutic strategies against, such as chronic inflammation and diabetic complications, not much is known about its role in renal cancer.

Recently, RAGE has been found to be responsible for cancer progression in several human cancers, including gastric, colon, prostate, pancreatic, billiary, esophageal, lung, etc. [10–16]. In vitro and clinical studies suggest that RAGE activation is associated with proliferation, migration, and invasion of cancer cells [17]. Although clinical usefulness of RAGE expression for estimating inflammation-related carcinogenesis has been reported, the relationship between RAGE expression and tumor angiogenesis is unknown [18].

Recent studies demonstrated roles of RAGE in the regulation of angiogenesis and the VEGF signal pathway [19]. It is interesting to note that RAGE played an crucial role in tumor angiogenesis by modulating VEGFR-2-related signal transduction via regulation of tumor-associated inflammation and contributed to the development of human glioma [20]. The interaction between RAGE and VEGF triggers the activation of key cell signalling pathways, such as NF-κB, p38 and MAPKs pathways, resulting in the cancer progression and metastasis. To the best of our knowledge, there is no data about the relationship between RAGE and the VEGF system in patients of RCC. In this investigation, we will investigate the relationship between RAGE, VEGFR-2 and neo-angiogenesis in patients with RCC.

2. Materials and methods

2.1. Patients and specimens

Eighty consecutive patients with RCC during January 2005 to July 2006 were included, all undergoing surgical resection for histologically verified clear cell renal cell carcinoma and they received care and regularly follow-up at the Department of Transplantation Centre, The First Affiliated Hospital of Wenzhou Medical University (wenzhou, China) for more than 6 years. Pretreatment examinations included a chest X-ray, urological ultrasound and CT scan of the abdomen. Postoperatively, the tumors were histologically classified and staged according to the 1997-revised TNM system and graded according to Fuhrman's nuclear grading system [21]. Information regarding patient characteristics was based on patient records and registries. The study was approved by the local Institutional Review Board and informed consent was obtained from all patients enrolled in the study.

Immediately after surgery, the removed bowel segment was brought to the Department of Clinical Pathology and tissue from the tumor was sampled by a pathologist. Samples for mRNA analyses were placed in RNA*later*™ (Qiagen, CA, USA) and stored at −20 °C. Samples for quantitative protein analysis were frozen and stored at −80 °C. The samples were frozen within 30 min of surgical removal. Samples intended for later immunohistochemistry (IHC) and immunofluorescence staining (IFS) followed routine fixation and paraffin embedding. Samples for quantitative protein analysis and IHC were sampled in close proximity to each other. Based on a microscopic examination of H&E stained FFPE tumor sections, it was semi-quantitatively estimated that the tissue used for quantitative protein analyses was dominated by carcinoma cells (> 50%).

2.2. VEGFR-2 mRNA and protein analyses

The *VEGFR-2* gene expression and protein analyses have previously been described [22]. Briefly, tissue samples were homogenized and total RNA was isolated according to the RNeasy® Mini Handbook of June 2001 (Qiagen, MD, USA). RNA was quantified using spectrophotometry (Eppendorf, Hamburg, Germany) followed by cDNA synthesis using a M-MLV RT kit (Invitrogen Co., Carlsbad, CA, USA). The geometrical mean of β-2-microglobulin and β-actin was used for normalization. Gene expression analyses were performed using fluorescence-based real-time reverse transcription polymerase chain reaction (RT-PCR). The analyses were performed on the ABI PRISM 7900 HT fast real-time PCR system, TaqMan (Applied Biosystems, Foster City, CA, USA).

After protein extraction, VEGFR-2 was analyzed using Quantikine ELISA kits (DVR200; R&D Systems, Minneapolis, MN, USA). The controls were also purchased from R&D Systems. The assay employs the quantitative sandwich enzyme immunoassay technique. Results are presented in pg/mg of total protein. Tissue samples, standards and controls were assayed in duplicate and the mean was recorded. The total coefficients of variation on three levels (low, medium and high concentrations of VEGFR-2) were 10.8, 9.1 and 7.2%, respectively.

2.3. CD34 and caldesmon immunostaining and MVD counting

The staining and counting procedures have previously been described [23]. Briefly, MVD was measured using tissue sections stained by antibodies against CD34 and caldesmon in order to visualize immature microvessels. A kit from NeoMarkers (NeoMarkers, San Francisco, CA, USA) was used for the detection of primary antibodies. The anti-CD34 antibody was obtained from Novocastra (Bristol, UK; endoglin NCL-CD34, clone QBEND/10) and used at a dilution of 1:350. The anti-caldesmon antibody was obtained from Dako (code M3557, clone h-CD) and used at a dilution of 1:50.

Microvessels were counted at the invasive tumor front by two observers unaware of the clinical parameters. The mean MVD from three hotspots was used for statistical analysis. Any stained endothelial cell or endothelial cell cluster clearly separated from adjacent microvessels by tumor cells and/or stroma elements was considered a single countable microvessel. Vessel lumen was not necessary for a structure to be counted as a microvessel. Consensus counts resolved any discrepancy between the observers. Regarding the CD34 (brown) and caldesmon (red) staining, only microvessels without associated red staining were counted in order to preferentially estimate the MVD of the immature vessels.

2.4. Immunofluorescence staining for RAGE

Tumor samples from seven patients with RCC and peritumoral "normal" tissue from the same patient were collected and 8 μm thick frozen sections were cut using cryostat (Leica Microsystem Inc., Bannockburn, IL), sections were blocked with BSA (0.5% for