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The Human VG5Q Gene Transcript is Over Expressed in Colorectal and Bladder Carcinomas

Research Article

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Abbreviations: human umbilical vein endothelial cells, (HUVECS); klippel-trenaunay syndrome;, (KTS); reverse transcriptase polymerase chain reaction, (RT-PCR); TNF-related weak inducer of apoptosis, (TWEAK); tumor necrosis factor (ligand) superfamily, member 12, (TNFSF12); vascular endothelial growth factor, (VGEF); vasculogenesis gene on 5q, (VG5Q)

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Summary

We studied the pattern of the human VG5Q (AGGF1) mRNA expression in both normal and noeplastic colorectal and bladder tissues. VG5Q mRNA was detected by RT-PCR technique. VG5Q is weekly expressed in the majority of normal cases (n=12). Seven of eight colorectal carcinomas (87.5%) overexpressed VG5Q mRNA when compared to their corresponding normal colorectal tissues of the same patient. The level of VG5Q expression in primary tumor is also upregulated in (75%) of the cases when compared to their corresponding liver metastasis. No consistent relationship in the expression level of VG5Q could be deduced when comparing normal colorectal samples to their liver metastasis colorectal tumors. Comparing 4 normal bladder and 16 bladder carcinomas samples reveal that VG5Q expression is also upregulated in bladder carcinomas. The level of VG5Q expression is more frequently upregulated in low grade when compared to high grade bladder carcinomas. These are the first results indicating the association of the newly discovered VG5Q gene transcript with human colorectal and bladder carcinomas. Further studies are needed to evaluate the usage of VG5Q as a complementary histopathologic and a candidate tumor marker among other modalities in both and other types of cancers.

I. Introduction

In the past decade, the field of angiogenesis has greatly widened with the discovery of new factors having either angiogenic or anti-angiogenic activities. Angiogenesis plays a central role in ovulation, implantation of the fertilized ovum, fetal growth and gestation, wound healing and repair following surgery and trauma (Carmeliet, 2005). In many serious disease states, the body loses control over angiogenesis. Excessive angiogenesis occurs in cancer, age-related macular degeneration, rheumatoid arthritis and many other pathological conditions (Carmeliet and Jian, 2000).

VG5Q is a newly discovered angiogenic factor (Tian et al, 2004). Its physiological properties resemble those of the VEGF, but mediate distinct downstream events, probably by interacting with the C-terminal domain of TWEAK (also known as TNFSF12) (Tian et al, 2004). VG5Q colocolizes with TWEAK around the nuclei in HUVECS cultured on plastic dishes. When endothelial tube formation is induced in matrigel, VG5Q and TWEAK moved to the cell surface, and VG5Q detected also outside of cells. Purified wild type VG5Q protein promoted strong angiogenesis in a chick chorioallantoic membrane assay, demonstrating that VG5Q is a potent angiogenic factor. It can bind to endothelial cells and promotes cell proliferation, suggesting that the protein may act in an autocrine fashion. VG5Q shows strong expression in blood vessels and is secreted when vessel formation is initiated. Furthermore, VG5Q was detected in human umbilical vein endothelial cells (HUVECs), human heart fibroblast (HHF) and ovarian cancer cells (OV-3), but low

expression was detected in kidney cancer cells (RP-45), HeLa cells and bladder cancer cells. VG5Q was ubiquitously expressed in human tissues examined, including heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. The VG5Q gene was identified at the 5q13.3 breakpoint of a translocation t(5;11)(q13.3;p15.1) (Tian et al, 2004).

Defects in VG5Q associated with its overexpression, and through mutation render its protein hyperactive are a cause of klippel-trenaunay syndrome (KTS). KTS is a congenital disease characterized by malformations of capillary, venous and lymphatic vessels. Susceptibility to vascular defects typical of KTS is increased either by higher expression of the gene due to chromosomal translocation, or by a mutant protein which is assumed to be hyperactive (Tian et al, 2004).

The association and probably contribution of VG5Q gene product in cancer progression and metastasis is not studied yet, nor do its upstream and its downstream effectors identified. It is the aim of our study to investigate whether VG5Q is differentially expressed in normal and neoplastic states of colorectal and bladder carcinomas. We report here for the first time that the expression level of VG5Q is elevated in primary colorectal carcinomas when either compared to normal tissue or secondary growth tumor that metastasizes to the liver. Moreover, VG5Q is overexpressed in bladder carcinomas when compared to normal bladder tissues. The level of VG5Q overexpression is more frequent in low grade tumor of the bladder when compared to high grade.

Moreover we found that the expression level of VG5Q mRNA is not induced when bladder carcinoma (T24P) and hepatocellular carcinoma (Hep3B) cell lines are exposed to hypoxic stress conditions under different culture confluences.

II. Materials and methods

A. Cell culture

All the human carcinoma cell lines used in this study were obtained from the American type culture collection (Manassas, VA) and were maintained in DMEM-F12 (1:1) medium containing 10% fetal calf serum (inactivated 55 ºC for 30 min), 25 mM HEPES (pH 7.4), penicillin (180 units/ml), streptomycin (100 µg/ml) and amphotericin B (0.2 µg /ml). Approximately $4x10^4$ cells/cm^2 were plated in polystyrene culture dishes (NUNC). Every 4 days, the cells were trypsinized with 0.05% trypsin-EDTA solution (Biet Haemek) for 10 min and re-plated again at the same initial densities.

B. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cultured cell lines, and patient specimens using the TRI REAGENT (Sigma) according to the manufacturer's instructions and treated with DNase I to exclude genomic DNA contamination. The synthesis of cDNA was performed using the p(dT)15 primer (Roche, Germany), to initiate reverse transcription of 2µg total RNA with 400 units of Reverse Transcriptase (Gibco BRL), according to manufacturer's instructions. The PCR reaction was carried out with peQLab Taq-polymerase for 29 cycles (94 °C for 1 min, 52 °C for 45s, and 72 °C for 45s) preceded by 94 °C for 5 min, and a final extension of 5 min at 72°C. The primers used in the PCR reaction were (5'-ACGTACTTGAGCATGGAGATG-3') and (5'-GTCCCCAAGCCTGCATGTGTT-3'), as described by Tian et al. (2004). The PCR products were electrophorized on 2% agarose containing ethedium bromide dye.

C. Hypoxic condition

Hep3B cells (Hepatocellular carcinoma) and T24P cells (Bladder carcinoma) were seeded in 5 ml medium flasks at different conflencies 24 hours pre-treatment. Cells were either placed into Aneoropack rectangular jar (Mitsubishi chemical company Japan) to create a hypoxic conditions within an hour (1% O_2, 20% CO_2), or left into normal oxygen concentration. Incubation lasted for 24 hours before RNA extraction.

D. Specimens

Normal, primary tumor samples from the ceacum and the sigmoid colon and colon, and their corresponding liver metastasis were obtained fresh from surgery from eight patients, and immediately transferred snap frozen in liquid nitrogen, and stored at -80 °C for later RNA extraction. Histological grading was performed on the biopsies by two pathologists who were unaware of our experimental design. Low grade bladder carcinomas used in this study are of grade 1, while those of high grade are of grade 3, according to modern grading classification of bladder cancer (Epstein et al, 1998). All are classified as transitional cell carcinomas of the bladder.

III. Results and discussion

The mechanisms by which the growing tumor tissue recruits new blood vessels has been the subject of intense investigations over the last few years as the acquisition of a functional blood supply seems to be rate-limiting for the ability of a tumor to grow beyond a certain size and to metastasize to other sites. High proliferating tumors frequently outstrip their vascular supply leading to a tumor microenvironment characterized by low oxygen tension, low glucose levels, and an acidic pH (Folkman, 1992; Ellis and Fidler, 1996; Hanahan and Folkman, 1996). Hypoxia is a common feature of solid tumor growth. Reduced pO_2 levels have been found in the majority of human tumors analyzed compared with normal tissue of the corresponding organ (Brown and Giaccia, 1998; Vaupel et al, 1989). A wide range of genes known to be involved in adaptive mechanisms to hypoxia, such as those coding for angiogenic growth factors, enzymes of glucose metabolism, and pH regulation, have classically been associated with tumors. (Semenza, 1998).

Based on this reasoning we studied if VG5Q is a responsive gene to hypoxic stress. Hepatocelluar (Hep3B) and bladder carcinoma (T24P) cell lines were exposed to hypoxic stress under different culture confluences. As shown in (**Figure 1**) hypoxic stress does not affect the expression level of VG5Q mRNA in both cell lines tested even at different confluences. The integrity of the RNA samples was verified by performing a PCR for GADPH housekeeping gene which showed no differences between samples (data not shown). These negative results could indicate that VG5Q promoter does not contain consensus sequence to specific transcription factors involved in hypoxic stress response. However, possibilities of other types of regulation are still possible namely protein stability, activity and secretion.

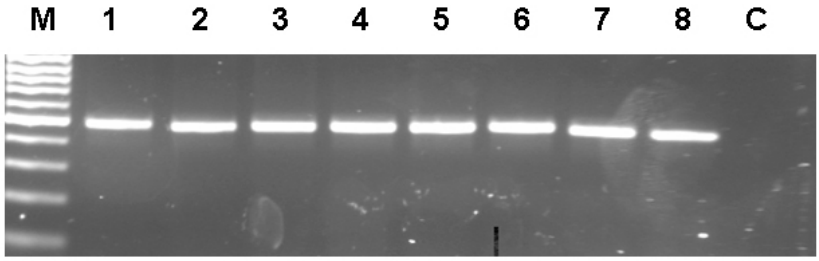

Figure 1. ***The effect of hypoxia on the expression level of VG5Q mRNA in Hep3B and T24P cell lines seeded at different confluences:*** Hep3B and T24P cells were cultured in normal medium conditions for 24 hours at different confluences before hypoxic manipulation. Shown are RT- PCR products for VG5Qin Hep3B cells (1-4), and T24P cells (5-8). C= PCR blank. 1, 2, 5, 6 (Hep3B and T24P cultured at low confluences and grow in normal (1, 6) and hypoxic (2, 7) conditions respectively. 3, 4 7, 8 (Hep3B and T24P cultured at high confluences and grow in normal (4, 7) and hypoxic (5, 8) conditions respectively. Hypoxic manipulation lasted for 24 hours.

Colorectal cancer is one of the most common types of cancer in both men and women. About 6 per cent of the populations in Western countries develop bowel cancer at some time during their lives, making this the second commonest cause of cancer-related death. Approximately 50% of patients diagnosed with colorectal cancer die within 5 years from diagnosis. Prevention and early detection of colorectal cancer will improve the patients' chance of survival dramatically. Altogether, new models based on a deeper molecular understanding of the disease are required to improve screening, diagnosis, treatment, and, ultimately, survival (Bertario et al, 1999).

The clinical value of angiogenesis-related factors as a tumor marker is well established (Sund et al, 2005; Zlobec et al, 2005). In our present study, we explored the status of VG5Q expression in normal versus neoplastic tissues. So we next checked if VG5Q is differentially expressed in normal versus cancer tissues taken from the same patient in colorectal cancer. VG5Q expression levels were assessed by semi-quantitative reverse transcriptase polymerase chain reaction. Samples of colorectal cancers (primary growth) and cancer that metastasize to the liver (secondary growth), and their normal counterpart tissue taken adjacent to cancer primary site from the same patient were analyzed for VG5Q expression. Results show that VG5Q mRNA is upregulated in primary colorectal cancer relative to the normal in seven out of eight samples (87.5%) (**Figure 2a, b**). The status of VG5Q expression in primary tumors does not correlate with its expression in liver metastasic tumors. The level of VG5Q expression in primary tumor is also upregulated in (75%) of the cases when compared to their corresponding liver metastasis. (**Figure 2a, b**).

No consistent relationship in the expression level of VG5Q could be deduced when comparing normal colorectal samples to their liver metastasis colorectal tumors. (**Figure 2a, b**).

A number of disparities between the characteristics of primary tumor tissue and that of metastatic disease have been described suggesting that metastatic tumors are biologically distinct from the primary tumors from which they arose (Agui et al, 2002). Although angiogenesis is needed to sustain growth of primary and metastatic lesions, comparison of microvessel density between primary colorectal cancers and their liver metastases revealed that angiogenesis scores were significantly lower in metastatic lesions compared with their primary tumors (Mooteri et al, 1996). Moreover, the level of VEGF expression may be site specific in patients with metastatic disease, with decreased expression noted in liver metastases relative to primary tumors and abdominal metastases (Berney et al, 1998; Cascinu et al, 2000). Similar results were obtained for VEGFR2, where decreased VEGFR-2 expression was documented in hepatic metastasis compared to primary colon tumors. This could explain why, in our case, the level of VG5Q expression in primary colorectal carcinomas is elevated when compared to their corresponding liver metastases. It was reported that the primary tumor produces a potent antiangiogenic factor, which prevented vascularization and thereby outgrowth of metastasis (O'Reilly et al, 1994; Sckell et al, 1998). The suppression of secondary tumor growth by its primary tumor via inhibition of angiogenesis is a widely accepted phenomenom not only in animal models, but also in human cancer patients (Peeters et al, 2004). Thus in our case we speculate that endogenous inhibitor could be secreted from primary colorectal tumor to suppress the expression of VG5Q angiogenic factor and others in its liver metastatic tumor.

We also checked if VG5Q mRNA expression is elevated in bladder carcinomas and associated with tumor grade. Bladder cancer is the fourth most common malignancy in men, and the eighth most common cause of death from cancer. More than 90% of bladder tumors are urothelial carcinomas. At the time of initial diagnosis, approximately 80% of urothelial carcinomas are confined to the epithelium (pTa, CIS) or lamina propria (pT1), whereas the remaining 20% invade the muscularis propria (pT2, pT3, pT4). Our finding that VG5Q expression is more abundant in low grade bladder carcinoma. pTa tumors are the commonest type of primary bladder tumor. These tumors rarely progress but recur in more than 50% of cases. Because most of these tumors show VG5Q overexpression, the detection of such changes may provide an accurate additional means of follow-up and identification of tumor recurrences. This could be especially useful for low-grade lesions, which are difficult to detect by urine cytology and which harbor VG5Q

overexpression in all of cases tested as shown in (**Figure 3**).

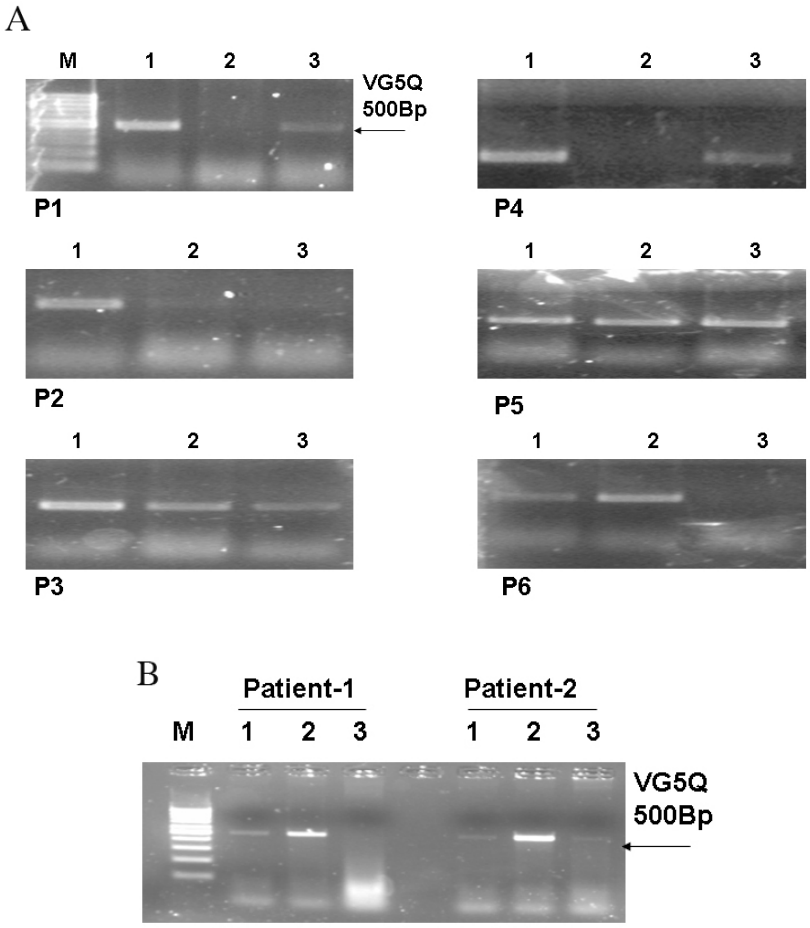

Figure 2. ***VG5Q transcript is differentially expressed in primary colorectal carcinomas when compared to their normal and corresponding liver metastasis.*** Normal, primary tumor and their corresponding liver metastasis biopsies from the ceacum and the sigmoid colon (**A**) and colon (**B**), were obtained fresh from surgery, and immediately transferred snap frozen in liquid nitrogen, and stored at -80 °C for later RNA extraction. RNA extraction and subsequent RT-PCR analysis for VG5Q was performed as described in 'materials and methods'. Shown is the PCR product of VG5Q in 6 patients of sigmoid colon (**A** P1-P4), and caecum (**A** P5-P6). 1-Primary cancer, 2-corresponding liver metastasis, 3-Normal. **(B)-**The expression level of VG5Q in two other patients (Patient 1, 2) of colon carcinomas 1- Normal, 2- Primary cancer, 3- corresponding liver metastasis. M= 100 Bp molecular weight marker. The PCR products were electrophorized on 2% agarose containing ethedium bromide dye.

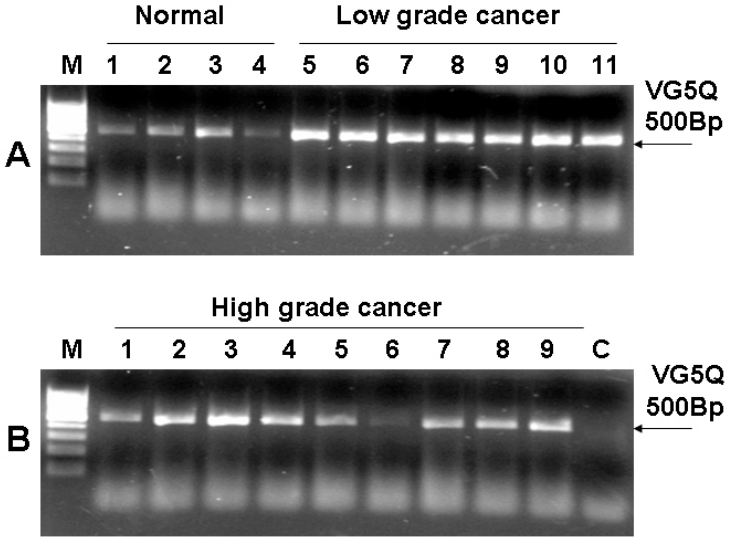

Figure 3. ***VG5Q transcript is elevated in bladder carcinomas when compared to normal bladder with a more pronounced expression in low grade carcinomas.*** Total RNA from normal, low grade bladder carcinomas (grade 1), high grade bladder carcinomas (grade 3) biopsies were obtained and handled as described and subjected to RT-PCR analysis for VG5Q. Shown is the PCR product for VG5Q in 4 normal specimens (**A**, 1-4), 7 low grade carcinomas (A, 5-11), and 9 high grade carcinomas (**B**). C is a PCR blank and M=100Bp molecular weight marker.

To the best of our knowledge, this is the first report that studied pattern of VG5Q expression in normal, primary cancer, and secondary cancer growth, in colorectal cancers, and studied its expression in normal bladder and bladder cancers at different grades. Future studies are required to further elucidate the biological function of VG5Q, especially its role in the tumorigenic process, and to evaluate its diagnostic and prognostic value in larger number of specimens and different tumor types.

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