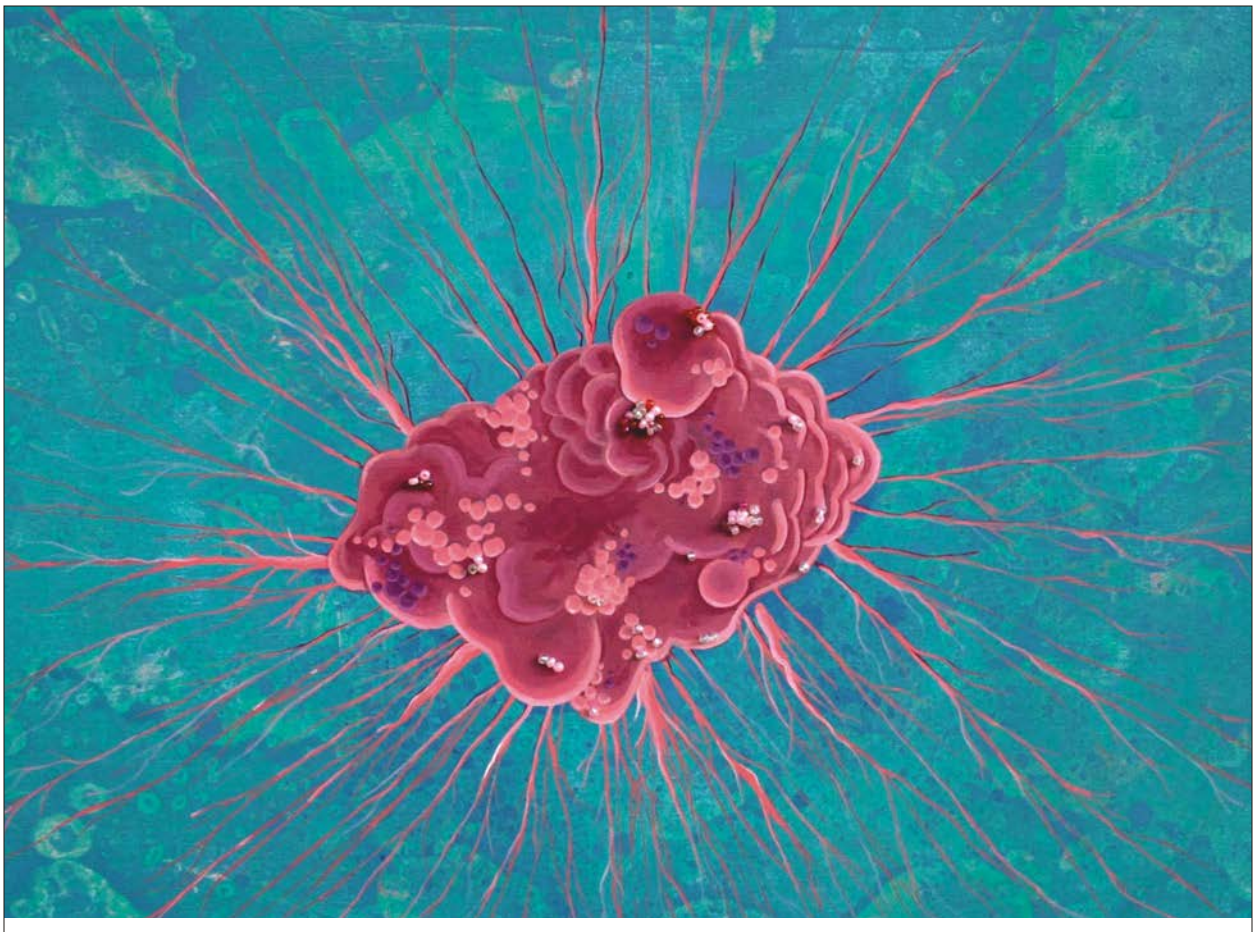

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Level of the Expression of VEGF-A, B, C, D and their Receptors (FLT-1, KDR and FLT-4) and its Correlation with Prognosis in Patients with Colorectal Cancer

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Abstract: This study examined, qualitatively and quantitatively, the expression of VEGFs and their receptors, in a group of patients with colorectal cancer and thier correlation to tumour progression. Human colorectal cancer tissues (n = 48) and normal background tissues (n = 48) were obtained after surgery. RNA was extracted from frozen sections for gene amplification. The expression of VEGF-A, B, C and D and their respective receptors VEGFR-1, VEGFR-2 and VEGFR-3 (FLT-1, KDR and FLT-4) were assessed using RT-PCR and the quantity of their transcripts were determined using real-time-quantitative PCR (Q-RT-PCR). VEGF-B ($p = 0.001$), VEGF-C ($p = 0.02$), VEGFR-1 ($p = 0.019$) and VEGFR-2 ($p = 0.005$) were significantly raised in colon cancer tissues compared with the levels detected in normal background tissues. The expression of VEGF-A, VEGF-D and VEGF-R3 in cancer tissues was not statistically significant ($p>0.05$) from background tissues. The level of the expression of VEGF-C and VEGF-R3 showed no difference in Dukes B and Dukes C. Patients who had cancer penetrating into and through the muscularis propria of the bowel wall and developed nodal involvement (Dukes C), exhibited significantly ($p<0.05$) higher levels of VEGF-B and VEGFR-2 compared with patients who were node negative (Dukes A and B). We conclude that here is aberrant expression of angiogenic factors VEGF-B and VEGF-C, together with their respective receptors VEGF-R1 and VEGF-R2 (FLT-1 and KDR) in colon cancer compared to normal colon tissues. The high level of expression of VEGF-B and VEGF-R2 were correlated to tumour invasion and nodal involvement (Dukes C) and therefore may have prognostic and therapeutic values in colon cancer patients.

Key words: Colon cancer, angiogenesis,VEGFs, Dukes Stage

Introduction

Colorectal carcinoma is one of the world's most common malignancies and the prognosis of patients with colorectal carcinoma is dependent on the presence of lymph node metastasis (Chapuis *et al*., 1985; Dukes and Bussey, 1958; Fielding *et al*., 1986). Due to the metastasis of the primary colon cancer cells to the other organs (mainly liver) through the blood and lymphatic vessels, colorectal cancer is the second -leading cause of cancer-related deaths in Europe and the USA. In case of manifest colorectal cancer, currently the most important factor predicting survival is the regional

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lymph node status at the time of initial surgery. Approximately 90% of patients with Dukes A and B disease (Stage I and Stage II) (without lymph node involvement) will survive for 5 years and nearly 20% of patients with Dukes C disease (with positive regional lymph nodes) will survive for 5 years after curative resection. Patients with Dukes C (Stage III) disease denote lymph node involvement. In these patients, (Dukes C), the number of lymph nodes involved affects prognosis. Patients with 1 to 3 positive nodes have a significantly better prognosis than those with 4 or more nodes involved.

Angiogenesis, defined as the sprouting of new capillaries from pre-existing vessels and is characterized by expansion of the endothelium by proliferation, migration and remodelling and is a key to cancer development and particularly metastasis (Kerbel and Folkman, 2002).

Despite the rapid progression in understanding the biology and the clinical significant of angiogenesis, there is very little information on markers that are specific to tumour endothelium.

The survival of colorectal tumours and thus their metastases are dependent on the balance of endogenous angiogenic and anti-angiogenic factors, such that the outcome favours increased angiogenesis. Angiogenesis has become an attractive target for anticancer drug development, based on its important role in tumour growth, invasion and metastasis. Several growth factors have been identified that regulate angiogenesis in colorectal cancer; the most important of these are Vascular Endothelial Growth Factors (VEGFs) and of the several angiogenic factors, VEGF expression at the deepest invasive site of tumour has been seen as the most statistically significant indicator of prognosis in advanced Colorectal Carcinoma (CRC) (Furudoi *et al*., 2002; Kaio *et al*., 2003; Onogawa *et al*., 2004a).

VEGFs are the most potent angiogenic factors and commonly associated with tumour angiogenesis and lymphogenesis (Jia *et al*., 2004). VEGFs are powerful mitogens and act specifically on endothelial cells, thereby profoundly altering the expression pattern of genes associated with angiogenesis (Crystal, 1999; Dvorak *et al*., 1999; Leung *et al*., 1989; Neufeld *et al*., 1999; Senger *et al*., 1983). VEGFs are induced in tumour cells by factors such as PDGF, bFGF, TNFα, TGFβ, IL-1β and IL-6. Activation of Raf, Ras and Src as well as loss of suppressor genes like p53 correlate with increased VEGFs secretion from tumour cells (Kieser *et al*., 1994). VEGF increases microvascular permeability, leading to protein extravasation, fibrin deposition and formation of a matrix within which tumour cells are likely to sequester (Senger *et al*., 1993). The human VEGF gene is assigned to chromosome 6p12-p21 and is organised into 8 exons separated by 7 introns (Houck *et al*., 1991; Mattei *et al*., 1996; Tischer *et al*., 1991). The coding region spans approximately 14 kb. Alternative splicing from this gene results in production of 5 types of mRNA that encode VEGF variants that differ in their molecular mass and in their biological properties (Neufeld *et al*., 1999). The VEGF family includes VEGF-A (Dvorak *et al*., 1995; Ferrara, 1996) -B (Grimmond *et al*., 1996; Olofsson *et al*., 1996) -C (Joukov *et al*., 1996) and -D (Stacker *et al*., 2001) as well as Placenta Growth Factor (PlGF) (Veilkkola *et al*., 2000). The biological effect of an individual VEGF is mediated through the activation of specific tyrosine kinase receptors expressed mainly on angioblast and endothelial cells (Neufeld *et al*., 1999). VEGF-A and VEGF-B are known ligands for FLT-1/VEGFR-1, VEGF-A and VEGF-C for KDR/VEGFR-2 and VEGF-C and VEGF-D for FLT-4/VEGFR-3 (Hiratsuks *et al*., 1998).

VEGF gene products are markedly increased in certain human tumours, such as lung, thyroid, breast, gastrointestinal tract, kidney, bladder, ovary and uterine cervix. Although, a few studies have been carried out on VEGF and tumorgenesis, there are few articles describing the relationship between all VEGFs (VEGF-B,-C,-D) and their receptors with human colon cancer progression

(Kawakami *et al*., 2003b; Kazama *et al*., 2004; Onogawa *et al*., 2004b). Limited studies have examined the whole family of VEGFs and their respective receptors.

Here we analysed the expression of VEGF-A, -B, -C and -D, their receptors VEGFR-1, R-2 and R-3 in a cohort of patients with colorectal cancer and correlated these molecules with progression of colorectal cancer.

Materials and Methods

Colorectal Tissues (Cancer and Normal) Collection

Colorectal tissues were collected randomly from patients (with the local Research Ethic Committee approval) with colorectal cancer immediately after surgery and stored at -20°C until required (This study was conducted between 2002 and 2004 in UHW Cardiff-UK). The samples consisted of colon tumour tissues (n = 48) and normal background tissues (n = 48) from these patients. Histological information was obtained from the respective histology report. In this study, we used the Dukes staging which was used in the histopatological report in our institution. The Dukes stage matches the UICC TNM staging: Dukes A equals to Stage I T1 N0 M0 or T2 N0 M0, Dukes B to Stage II T3 N0 M0 or T4 N0 M0 and Dukes C to Stage III Any T N1 M0 or Any T N2 M0 (American Joint Committee on Cancer Staging Colon and Rectum, 1988; AJCC Cancer Staging Manual, 1997; International Union Against Cancer Colon and Rectum, 1997; Winawer *et al*., 1997).

RNA Extraction

RNA extraction, reverse transcription Kits and PCR mix were purchased from Abgene (Surrey, UK). Total RNA was isolated using the standard gunanidine isothiocyanate method by following the manufacturer's protocol. The purity and concentration of RNA were determined by spectrophotometer at 260 nm. Reverse transcription was performed and cDNA samples were synthesized in 20 μL reaction volume.

Conventional RT-PCR

Conventional PCR primers were designed using the Beacon Designer software (California, US) and synthesized by Life Technologies (Paisley,UK). The agarose gel extraction kit was purchased from Life Technologies. Primer sequences are given in Table 1. Conventional PCR to amplify the transcripts of VEGF-A, VEGF-B, VEGF-C, VEGF-D and their receptors VEGFR-1, VEGFR-2 and VEGFR-3 was carried out using cDNA from normal colorectal and colorectal cancer tissues. The reaction conditions were: 94°C for 5 min, 36 cycles at 94°C for 40 sec, 54°C for 30 sec, 72°C for 50 sec followed by extension phase of 10 min at 72°C. β-actin was used as an internal housekeeping gene. The PCR products were separated on 2 and 0.8% agarose gel and stained with 10 μL of ethidium bromides prior to examination and photographing under UV light.

Table 1: Primer sequences for conventional PCR

	Sense primer (5' – 3')	Antisenes primer (5'-3')
VEGF-A	attggaggcttgccttgc	gctctatctttctttggtc
VEGF-B	tggtgtcatggatagatgtgtatac	cttggcaacggaggaagc
VEGF-C	ggcttctcctggtgacatctg	ttgcttgggacacattgacattc
VEGF-D	cgatcatctcagtccacattg	cttctggcaggcagcaggtctc
VEGF-R1	gaacgagaaggacggactc	tggtggaactgctgatgg
VEGF-R2	gcctctgtgggtttgcctagtg	ccctctctcctctcccgactttgttg
VEGF-R3	ctgtgcctgcgactgtg	Cagcgtggacaggttgag

Table 2: Primer sequences for quantitative PCR

UniPrimer systems	Sense primer (5'-3')	Z primer (5'-3')
VEGF-B	cacagtcaagggcaccac	actgaacctgaccgtacagatgtgtatactcgagctac
VEGF-C	cctgagtccctggtctct	actgaacctgaccgtacagcttctcctggtgacatc
VEGF-D	gctccagtaatgaacatgg	actgaacctgaccgtacaatctgatgttacgatcgtt
VEGF-R1	ttaaaaggcagcac	actgaacctgaccgtacaatctgctgttcagatcgtt

TaqMan systems	Sense primer (5'-3')	probe	Antisense primer(5'-3')
VEGF-A TAQ	tacctccaccatgccaagtg	Fam- tcccaggctgcacccatggc	atgattctgccctcctccttc
VEGF-R2. TA	Ptgtggctctgcgtggaga	Fam-cgggccgcctctgcgggttt	gggcagatcaagagaaacactagg
VEGF-R3 TAQ	acggcctggtgagtggc	Fam-ccatgacccccccgaccttga	cgtttgactcctccgtgatg

Table 3: Expression of VEGFs and their respective receptors in colon tissues (percentage positive), using conventional PCR

	Normal tissues (n and %)	Tumour tissues (n and %)	p value*
VEGF-A	18 of 48, 38 %	21 of 48, 45 %	0.1
VEGF-B	24 of 48, 49 %	43 of 48, 91%	0.001*
VEGF-C	22 of 48, 46 %	38 of 48, 79 %	0.02*
VEGF-D	22 of 48, 46 %	23 of 48, 48 %	0.1
VEGFR-1(FLT1)	6 of 48, 13 %	19 of 48, 40 %	0.019*
VEGFR-2 (KDR)	19 of 48, 40 %	36 of 48, 76 %	0.005*
VEGFR-3 (FLT4)	16 of 48, 33 %	23 of 48, 47%	0.1*

Chi-square test (χ^2 test)

Real-time Quantitative Polymearase Chain Reaction (QPCR)

We employed the iCycler IQ system (BioRad, Camberley, UK), to quantify the level (shown as copies/µL from internal standard) of the angiogenic factors in the colorectal specimens as we have recently reported (Jiang *et al.,* 2003a; Jiang *et al.,* 2003b). All colorectal cDNA samples were simultaneously examined for each of the VEGFs, VEGF-Receptors along with an appropriate set of plasmid standards and negative controls. Primer sets and probes used in this technique are given in Table 2.

The detection of VEGF-B, VEGF-C, VEGF-D and VEGFR-1 employed a universal probe system (UniPrimer™) (Intergen, Oxford, England). The UniPrimer system use two primers in conjunction with a universal probe (UniPrimer™), which recognised a specific sequence (z sequence), which had been incorporated into the primers (Table 3). VEGF-A, VEGF-R2 and VEGF-R3 detection used the Taqman system, which employs a pair of primers and a FAM-labelled probe that recognises the specific product. A hot-start quantitation master mix (Abgene, Surrey, England) was used for the reactions. PCR conditions for real-time QPCR were as follows: 95°C for 12 min, followed by 50 cycles at 95°C for 15 sec, 55°C for 60 sec and 72°C for 20 sec.

Statistical Analysis

Conventional RT- PCR results were analysed by using Chi-square test (χ^2 test). Quantitative data were analysed using student t-test.

Results

Expression of VEGFs (VEGF-A,-B,-C and –D) and their Receptors (VEGF-R_1, -R_2 and R_3) in Normal and Tumor Colorectal Tissues

VEGF-B was expressed in 91% (43 of 48) colon cancer tissues, compared with in 50% (24 of 48) normal tissues ($p = 0.001$). Similarly, expression of VEGF-C was significantly higher in cancer tissues compared with normal (78.9%, 38 of 48 cases vs 46.4%, 22 of 48 cases, $p = 0.02$). However,

the expression of VEGF-A and VEGF-D was found similar between tumour tissues and normal tissues ($p = 0.15$ and $p = 0.13$, respectively) (Fig. 1 and Table 3).

VEGF-R3 (FLT-4) expression was detected at almost a similar rate in colonic tumours and in normal tissue ($p = 0.14$). In contrast, expression of VEGF-R1 (FLT-1) and VEGF-R2 (KDR) were greater in colonic cancer than normal ($p = 0.01$ and $p = 0.005$, respectively) (Fig. 2).

The level of VEGFs and VEGFRs Transcripts at Different Dukes Stages

We went on to analyse, quantitatively, the levels of transcript in tumour tissues in relation to their Dukes staging. The number of transcripts of VEGF-A and VEGF-D was higher in Dukes A ($n = 16$) compared with Dukes B ($n = 16$) and Dukes C ($n = 16$). The level of VEGF-B and VEGFR-2 was significantly higher in advanced Dukes C tumour compared to Dukes A ($p = 0.02$). The highest level of VEGF-C was found in Dukes B and C ($p = 0.04$) compared to Dukes A tumour (Fig. 3).

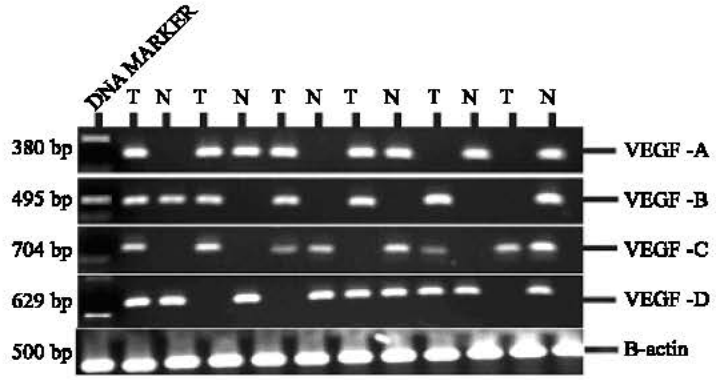

Fig. 1: RT-PCR shows VEGF-A expressed similarly in colon cancer and normal mucosa. VEGF-B expressed highly in colon cancer than normal mucosa. VEGF-C high in colon cancer. No difference in the expression of VEGF-D found between tumours and normal tissues

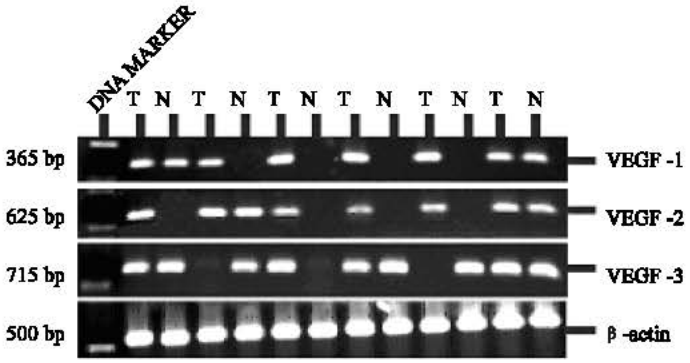

Fig. 2: RT-PCR shows FLT-1(VEGF-R1) expressed greater in colon cancer than in normal mucosa. KDR (VEGF-R2) expressed much higher in colon cancer than in normal. FLT-4 (VEGF-R3) expressed almost equally in colon cancer and in normal mucosa

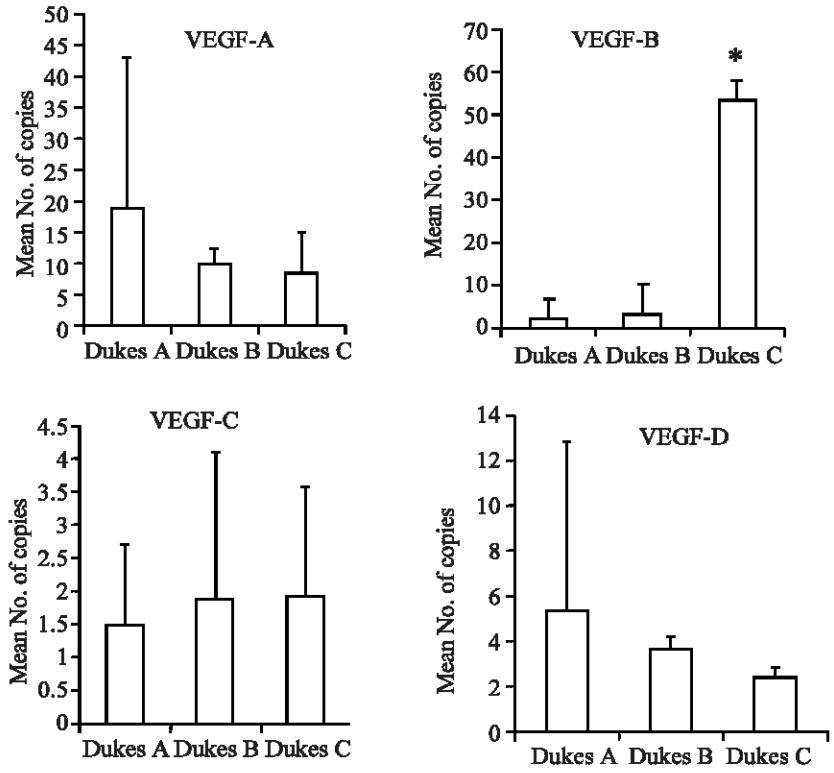

Fig. 3: Levels of expression of VEGFs in colon tissues in tumours with different Dukes stages, using quantitative PCR Q-RT-PCR (Mean copies/µg RNA),VEGF-A E+05 copies,VEGF-B E+09 copies, VEGF-C E+02 copies and VEGF-D E-5 copies. VEGF-B shows highest transcript copies (* p = 0.02) in Dukes C compared to Dukes A tumour

Similar to VEGF-C, the level of VEGFR-1 was found to be similar in Dukes B and C and is higher than that in Dukes A tumours. Dukes C had a higher level of expression of VEGFR-2 than Dukes A and Dukes B. The numbers of copies VEGFR-3 were found to be almost the same in the three Dukes Stages (A, B and C) (Fig. 4).

Discussion

The incidence of cancer metastasis and angiogenesis are closed linked with tumour angiogenesis being associated with poor prognosis (Bricknell and Harris, 1991; Chodac *et al.,* 1980). A vascular network within tumours is generated by budding and sprouting from endothelial cells. It is now known that tumours cells are able to induce angiogenesis and lymphangiogenesis in order to metastasize, partly by producing angiogeneic factors, such as VEGFs. Several growth factors are known to stimulate endothelial cell proliferation and to induce angiogenesis in both normal and tumour cells. Because tumours are dependent on their own blood supply, anti-angiogenic therapy of cancer offers attractive therapeutic approach for targeting tumour cells (Feldman and Libutti, 2000;Papetti and Herman, 2002). There are some limited studies on the role of VEGF expression

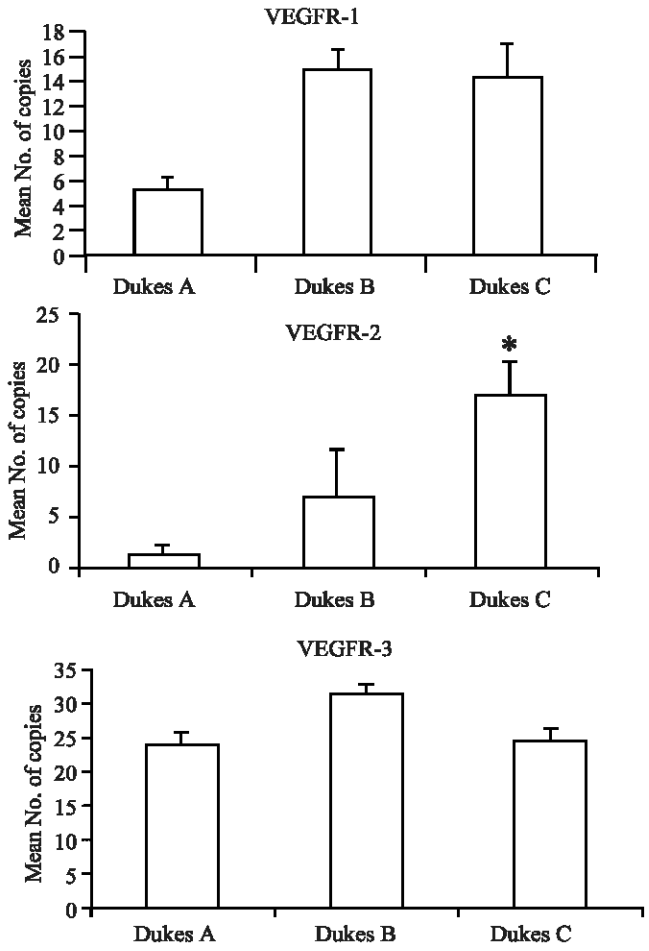

Fig. 4: Levels of expression of VEGF receptors in colon tissues in tumours with different Dukes stages, using quantitative PCR Q-RT-PCR (Mean copies/μg RNA) VEGFR-1 E+04 copies, VEGFR-2 E+05 copies and VEGFR-3 E+02 copies. Dukes C tumour expressed greatest level of VEGFR-2 (* $p = 0.04$) in contrast to early stage Dukes A tumour

in predicting the prognosis of the patients with cancer, especially in colorectal cancer (Kang *et al*., 1997; Kawakami *et al*., 2003a; Kawakami *et al*., 2003b; Werther *et al*., 2000). However, this remains highly controversial (Khorana *et al*., 2003; Lee *et al*., 2000).

In the current study, we investigated the expressions of other VEGFs (VEGF-B, C and VEGF-D) in addition to VEGF-A and their receptors (VEGF-Rs) in colorectal cancer, as well as investigating the transcript expression level of these angiogenic factors in colorectal cancer tissues and correlated that to the tumour progression. The results have shown that VEGF-B, VEGF-C and their receptors FLT-1 and KDR mRNA were expressed at a much higher level in colorectal cancer than in normal mucosa. On the other hand, VEGF-A, VEGF-D and FLT-4 expression were not significantly different between colorectal cancer and normal tissues. These results are in contrast with other studies,

which showed that, the detectable level of VEGF-A189 subtype gene in most of colorectal cancer (Tokunaga *et al*., 1998). However, our results are in agreement with some of the recent reports (Andre *et al*., 2000; Jia *et al*., 2004; Kawakami *et al*., 2003a, 2005) which have reported a higher-level expression of VEGF-C in colorectal cancer and its relationship to lymph node metastasis. Hanrahan *et al*. (2003) has also showed that expression VEGF-A and VEGF-D was high in normal colorectal tissues and suggested an important role of VEGF-C in metastasis spread in advanced colorectal cancer (Hanrahan *et al*., 2003) and these in line with our results.

Present finding is also in agreement with Petri *et al*. (1998) who indicated that VEGF-B and VEGF-C are expressed in most human tumours, such different findings might be due to the different technical approaches used. Such results show that some of these molecules (VEGFs and VEGF-Rs) are higher in normal than cancer tissues and suggest that these molecules could have more important role in physiological angiogenesis rather than tumorigenesis. The other possibility that the lack of sufficient statistical difference with these molecules was the size of samples in the current study. Increased sample number would certainly help to further clarify this issue. Other molecules which have increased expression in cancer might also have a trophic role on tumour cells (Hanrahan *et al*., 2003).

In addition, VEGF-A and VEGF-D levels were high in the early stage of colon cancer ie, no lymph node involvement (Dukes A). VEGF-B and VEGF-R2 levels were found significantly higher in colorectal cancer with regional lymph node involvement (Dukes C). Levels of VEGF-C and VEGF-R1 were similar in Dukes B and Dukes C and showed a possible prognostic value of these molecules in colorectal cancers.

These results suggest that VEGF-A, VEGF-D and their receptors play a role early in tumour development at the stage of adenoma formation and that VEGF-B plays a role in advanced disease when there is more likelihood of metastatic spread. The finding of increased levels of VEGF-A and VEGF-D expression in normal tissues collected from a site distant from the primary tumour indicates changes in the surrounding tumour environment that may enhance the subsequent spread of tumour cells. This is the first study using Q-RT-PCR for measuring the transcript levels of other vascular endothelial growth factors (VEGF-B, C and D) and their receptors in colon cancer and normal background tissues, respectively.

Present data indicates that the persistent expression or elevation of these angiogenic factors and their receptors may be critical for the development of all colorectal cancers. VEGF-B and VEGF-R2 levels were found to be associated with either nodal involvement and/or disease progression and may have a clinical prognostic significance in colorectal cancer development. We conclude that these angiogenic factors, other than VEGF-A (VEGF-B, VEGF-C and VEGF-R2), may have potential prognostic value in colorectal cancer and provide a therapeutic approach. The Avastatin/ Bevacizumab (recombinant humanized monoclonal antibody to VEGF-A) has been approved as front-line therapy for metastatic colorectal cancer; further study on VEGFs and their receptors may provide new targets for anti-angiogenic treatment.

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