Role of CD34, vascular endothelial growth factor, and p53 in neoangiogenesis as correlated with stage of disease in colorectal carcinoma

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ABSTRACT

BACKGROUND

Angiogenesis is critical for the growth of colorectal carcinoma (CRC). Vascular endothelial growth factor is the most important angiogenic growth factor.

METHOD

Fifty cases of CRC operated at INHS Asvini were studied by using immunohistochemical labelling of the tumours by using CD34, p53, and vascular endothelial growth factor (VEGF).

RESULTS

Out of the 50 cases, 31 (62%) were positive for p53; of which 80.7% showed high expression. Significant staining (>20% tumour cells showing positivity) was seen in 33 patients (66%), while 34% were negative. Of the 33 positive cases, 57.58% showed high-grade VEGF positivity.

CONCLUSION

Vascular endothelial growth factor correlated significantly with the stage and grade of disease. Intratumours microvessel density as calculated from CD34 expression increased with the stage and grade of the CRC.

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Key Words: angiogenesis; colorectal carcinoma; immunohistochemistry

INTRODUCTION

Colorectal carcinoma (CRC), one of the most common cancers causing death worldwide,¹ is treated with surgery, and patients with stage III (Dukes' C) and a subset of stage II CRC (Dukes' B2) are treated with adjuvant therapies. Still the locoregional

recurrence after curative resection remains a problem. The main prognostic factors in CRC are lymph node involvement, size of the tumour and stage of disease.² However, these factors do not fully predict individual clinical outcome. Adjuvant treatment is hampered by a lack of reliable prognostic factors in addition to the clinicopathologic staging system.³ Folkman first proposed that tumour angiogenesis could serve as a potential target for anticancer therapy. Normal tissue homeostasis is maintained by balancing cell proliferation with physiologic deletion of senescent cells by apoptosis. Disruption of apoptotic pathway may confer a selective growth advantage, upsetting this homeostatic state.⁴ In about 50% of CRC, however, mutant p53 aids cancer cell survival.

Angiogenesis is studied by calculating the microvessel density (MVD) i.e. number of vessels per unit area or per field. MVD, as a surrogate marker of tumoural angiogenesis, has been proposed to identify patients at high risk of recurrence. It was first developed by Weidner et al in 1991 using immunohistochemical (IHC) staining of microvessels. Some authors used Chalkley count or computerised image analysis systems, both aimed to minimise subjectivity.⁵ Tenderenda et al⁶ carefully counted the microvessels (per 200× field), and graded (1 to 4+), in the most active areas of neovascularisation. They concluded that the number of microvessels per 200× field may be an independent predictor of metastasis. Tomisaki in 1996 studied MVD in CRC and found that microvessel quantification showed a significant correlation with liver metastasis.⁷

Takahashi et al⁸ observed a significant correlation between vascular endothelial growth factor (VEGF) expression and metastatic disease. A separate study evaluated factor VIII, VEGF, basic fibroblast growth factor, proliferating cell nuclear antigen, and the presence of vascular, lymphatic, and perineural invasion in CRC node-negative disease.⁹ Multivariate analysis identified vessel count as a significant correlate with time to recurrence and VEGF expression. VEGF has been reported as an independent prognostic factor by Des et al.¹⁰ Kuniyasu et al¹¹ found VEGF levels significantly higher in hyperplastic adjacent mucosa and suggested that it contributes to neoangiogenesis and progression of tumour.

MATERIALS AND METHOD

Fifty resected cases of CRC were studied by Department of Pathology at a tertiary health care centre between January 2000 and May 2007.

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Figure 1 p53 immunoreactivity by the tumour cell nuclei (400×).

Figure 2 Vascular endothelial growth factor immunopositive high-grade tumour glands of colorectal carcinoma (400×).

Figure 3 High-grade intratumoural microvessel density highlighted by immunohistochemistry for CD34 on tumour sections (400×).

Inclusion Criteria

Adenocarcinoma or its variants with at least a six-month follow-up after completion of chemotherapy were selected. Relevant details like site, size, type of growth, whether ulcerative/proliferative, and enlarged nodes were noted. The blocks with maximum depth of tumour invasion were retrieved for performing IHC.

Figure 4 Correlation between p53, vascular endothelial growth factor, and CD34 (intratumoural microvessel density) expression in colorectal carcinomas. VEGF: vascular endothelial growth factor.

Figure 5 Correlation between CD34 (intratumoural microvessel density), p53, and stage of the colorectal carcinomas.

The tumours were staged according to TNM classification. Relevant data from oncology files regarding follow up was retrieved to note therapy given, relapse and the survival data.

Immunohistochemistry

Immunohistochemistry (IHC) was performed using standard avidin-biotin-complex (ABC) method on poly-L-lysine coated slides. Sections were brought down to water following which endogenous peroxidase blocking was done with 3% hydrogen peroxide solution in methanol for 20 minutes.

Antigen retrieval for p53 (Monoclonal mouse anti-human p53 antibody, DO-7, Dako) was done by microwaving in TRIS-ethylenediamine tetra acetic acid buffer at pH 9.0 for five cycles, each lasting five minutes. The first cycle was at 100% power, while the remaining were at 60% power. Similarly antigen

Table 1 Average intratumoural microvessel density (standard deviation) as seen in various groups.

VEGF		p53		Stage		Grade	
Expression	Mean IMD	Expression	Mean IMD	Group	Mean IMD	Group	Mean IMD
1+ (negative)	65	0	86	I	84	I	111
2+ (low)	129	1+	104	II	153	II	178
3+ (high)	213	2+	126.5	III	206	III	223
–	–	3+	202	IV	234	–	–

IMD: intratumoural microvessel density, VEGF: vascular endothelial growth factor.

Table 2 Expression of p53 and vascular endothelial growth factor.

VEGF/p53	1+ (Negative)	2+ (Low)	3+ (High)	Total
0/1+ (negative)	11 (66.7)	5 (38.5)	3 (15.8)	19
2+ (low)	0 (0)	6 (46.2)	0 (0)	6
3+ (high)	6 (33.3)	3 (15.4)	16 (84.2)	25
Total	17	14	19	50

VEGF: vascular endothelial growth factor.

$P=0.000$.

$\chi^2=33.99$.

retrieval for VEGF (Monoclonal mouse anti-human VEGF, Dako) and CD34 (Monoclonal mouse anti-human CD34, QBE10, Dako) was done. After the antigen retrieval, the slides were allowed to cool to room temperature and were then flooded with TRIS buffer solution pH 7.3 (TBS) for 10 minutes. Endometrium and placenta were used as positive controls for VEGF and CD34, respectively. For negative control, we omitted the primary antibody each time. The IHC was carried out as per techniques described in the literature.¹²

Interpretation of Results

p53 is a nuclear stain and VEGF stains cytoplasm and/or membrane. Tumour “hot spots” which often corresponded to the invasive edge were selected, 500 tumour cells counted, and the number of tumour cells positive for p53 and VEGF were noted individually. They were then graded semi-quantitatively as follows:

zero, no expression; 1+, <20% of tumour cells are positive; 2+, 20–50% positivity; and 3+, >50% are positive. Only tumours that showed >20% staining was taken as positive. The expression below 20% positivity was considered negative. CD34 expression in tumour “hot spots” was identified. Branching structures were counted as a single vessel. A single microvessel was defined as any brown immunostained endothelial cell separated from adjacent microvessels, tumour cells and other connective tissue elements. In this way, the number of vessels was noted in ten continuous fields at 400 $\times$, i.e. an area of 0.152 mm².¹³

Since CD34 expression in the form of intratumoural microvessel density (IMD) is quantitative, and p53, VEGF expressions and grade of the disease are categorical, so to test whether there were significant differences in the IMD value between the different groups of VEGF, p53 and grade of the cancer, ANOVA

(analysis of variance) was carried out after testing for homogeneity of variances. Pair-wise comparisons were carried out using LSD method. To find the correlation between the categorical variables χ^2 -test is carried out using SPSS version 14.0.

RESULTS

In 50 cases of CRC, p53, VEGF, and CD34 immunophenotyping was carried out and their expressions were analysed. Out of the total cases, 34 were males and 16 females with an average age of 58.2 ± 10.0 years. The most common site was rectum (56%). Most patients belonged to stage I, i.e. T1/2 (40%). Sixteen (32%) patients had stage IIA disease, i.e. T3N0. Eleven (22%) patients had locally advanced disease, i.e. T3/4 with lymph node metastasis N1. One patient had stage IIIC disease while two (4%) had distant metastasis.

Twenty-seven (54%) cases showed a moderately differentiated adenocarcinoma. Three cases had a poorly differentiated carcinoma, while 20 cases had a well-differentiated tumour out of which six were mucin secreting. Thirty-seven patients remained free of disease at the endpoint of the study and six patients who died of disease had advanced stage disease, either local or with distant metastasis. Relapses occurred in three patients after 2–5 years of completion of therapy. Out of the three patients that died of unrelated cause, two died of coronary disease while one committed suicide eight months after completion of therapy. One case was lost to follow-up.

Out of the 50 cases, 31 (62%) were positive for p53; of which 19.3% showed low expression while 80.7% showed high expression, i.e. >50% of the cells showed positive immunoreactivity in the tumour cell nuclei (Figure 1). All cases showed staining with VEGF monoclonal antibody. However, significant staining was seen in 33 patients (66%), while 34% were negative (<20% stained tumour cells). Of the positive cases, 42.42% had low VEGF staining while 57.58% showed high-grade staining (Figure 2). The IMD ranged from 65 to a maximum of 213/10 high power fields (hpf) (Figure 3 and Table 1).

Comparing expression of p53 with VEGF, it was found that 66.7% of cases that were p53 negative, were also VEGF negative; while 84.2% of cases with high-grade immunoreactivity, i.e. 3+ VEGF expression also had grade 3 expression of p53. The correlation in the expression of p53 and VEGF was statistically significant ($\chi^2=33.99$, $P=0.001$) Table 2. Association between

p53 and stage using χ^2 -test shows that there is no association between the p53 expression and stage. Whereas association between VEGF expression by the tumour cells and stage shows that there is significant association between VEGF expression and stage of the disease.

Average IMD also increased from 96.8 and 110.2 (107) in p53 negative tumours to 126.2 in grade 2 to 183.3 in grade 3 tumours. ANOVA reveals that there is a significant difference in the mean CD34 value in the three groups of p53 ($P=0.000$). The pair-wise difference in the three groups of p53 was carried out by LSD method. It was found that groups 1 and 2 was not statistically significantly different with respect to CD34 ($P=0.466$), whereas there was significant difference in the mean CD34 value in groups 1 and 3 ($P=0.000$) and groups 2 and 3 ($P=0.031$) (Figure 4). Higher the p53 value, higher was the average IMD value.

The average IMD increased from 65 in VEGF negative tumours to 129 in low-grade and 213 in high-grade expression of VEGF. ANOVA revealed that there is significant difference in the average IMD value in various VEGF grades ($P=0.000$). Again pair-wise comparison using LSD shows that there is significant difference in grades 1 and 2 ($P=0.000$), grades 1 and 3 ($P=0.000$) and also in grades 2 and 3 ($P=0.000$). The value of IMD increases with increase in VEGF grade (Table 1).

Intratumoural microvessel density had differed with the stage of the disease ($P=0.00$) (Figure 5). Mean IMD increased from 84 in stage I tumours to 206 in stage III, and to 234 in stage IV disease. Pair-wise comparisons revealed that there was no significant difference in the IMD value in stages II and III ($P=0.124$) and also in stages III and IV ($P=0.31$). All other pairs showed significant difference. Intratumoural microvessel density also differed significantly with the grade of the tumour (Table 1, Figure 5). ANOVA shows that there is significant difference in the IMD value in various grades of tumours. However pair-wise comparison shows that there is no significant difference in IMD value in grades 2 and 3 ($P=0.066$). Other pairs namely (1 and 2) and (1 and 3) shows significant difference ($P=0.000$). Tumours with high IMD were poorly differentiated and had poor prognosis and vice versa. Another important incidental observation was made with respect to mucinous tumours, all of which were well-differentiated. Most mucinous tumours were negative for both p53 and VEGF expression, or they had lower expression. Only one case had grade 3 positivity for p53. Mean IMD (114.0) correlated with that of well-differentiated tumours (mean, 116.0).

DISCUSSION

Colorectal carcinoma still remains a major cause of morbidity in the form of recurrence and mortality.¹⁻³ In this study, p53, VEGF, and IMD by staining for CD34 are considered and correlated with grade, stage, and survival data. Normally p53 prevents propagation of genetically damaged cells. Vascular endothelial growth factor expression is regulated by local tissue hypoxia, cytokines, oncogenes, tumour suppressor genes, and growth factors.¹⁴ CD34 is constitutively expressed on endothelial cells.¹⁵

p53 was detected in 62% of the cases, of which 19.3% showed low-grade expression while 80.7% had high-grade p53. The incidence of p53 positivity in another study was up to 42%.¹⁶ No significant correlation was found with the stage or grade in our study. This corresponded to the findings of Kressner, who stated that p53 is of limited value in CRCs¹⁷ unlike Goh et al.¹⁸ Similarly Vermuelen et al.³ independently found that p53 and VEGF only when considered together are reliable prognostic indicators.

Vascular endothelial growth factor expression correlated significantly with the stage and grade, i.e. grade 3 VEGF was found in higher stage and higher grade of the tumour. This was similar to other studies.^{9,19} We found an incidence of 66% positivity for VEGF, but it is not an independent prognostic factor in CRCs. Perrone et al also confirmed a strong correlation of expression of p53 with VEGF expression and microvessel density with increasing stage of tumour.²⁰ Galizia et al²¹ found a high four-year recurrence in stage pT1-T3 in cases with high-grade p53 and VEGF expression. In contrast, Takahashi found a direct relation of VEGF with metastasis and poor prognosis.²²

Intratumoural microvessel density correlated significantly with the stage and grade. Kern found the same correlation.²³ Weidner et al⁵ and Vermuelen et al³ independently found that increasing IMD was an independent risk factor for metastasis. In our study, there was a 66.7% correlation between negative p53 and negative VEGF and a statistically significant relation ($P=0.001$) between high-grade expressions of p53 and VEGF (Table 2, Graph 1). Vermuelen³ and Perrone et al²⁰ found a similar direct correlation between p53 and VEGF. Ravi et al²⁴ studied one of the possible mechanisms by which p53 has an effect on VEGF. Pal also found an inverse relation between p53 and VEGF.²⁵ Higher p53 was associated with higher VEGF, which in turn was linked to higher IMD and poor prognosis. Vascular endothelial growth factor and IMD were significantly related ($P<0.00001$) with a correlation coefficient of 0.80. Vermuelen,³ Perrone,²⁰ Galizia et al²¹ have independently found the same.

In our study, p53 and IMD had statistically significant correlation ($P=0.0006$) and a correlation coefficient of 0.31. p53 was not as significantly linked to IMD as VEGF, suggesting the role of other proangiogenic factors. Perrone,²⁰ Galizia,²¹ and Yuan et al²⁶ independently found an association between p53 and IMD. However, Kern²³ did not find a significant association between p53 and IMD. He found that vascularity decreased in higher grade and stage tumours and that it did not have any association with metastatic potential.

Assessing parameters related to tumour angiogenesis has provided valuable prognostic information in different tumour types. Alterations in p53 gene have been found to be responsible for changing the local balance of pro- and antiangiogenic factors in favour of the former. Early-stage colon cancer patients (Dukes A or B; pT1-T3 N0 M0) are excluded from adjuvant chemotherapy following potentially curative surgery because they are expected to have good long-term survival. However, some of these patients ultimately succumb from recurrence indicating that conventional staging may be unable to precisely predict.

To summarise, p53 expression did not have an independent statistically significant correlation with the stage or grade.

Vascular endothelial growth factor expression correlated significantly with the stage and grade of disease but it was not an independent prognostic factor. Intratumoural microvessel density as calculated from CD34 increased with the stage and grade. p53 and VEGF correlated significantly suggesting a stimulating effect of p53 on VEGF. Vascular endothelial growth factor and IMD had very strong correlation. p53 and IMD also correlated significantly. Although definite trends are seen in this study, a larger study of the target group i.e. stages II and III is needed to substantiate our findings.

Our study did not find p53 and VEGF to be independent prognostic factors in CRCs but suggests that, considered together they are reliable predictors of disease and could help in selection of patients at a risk of progression, metastasis or recurrence and selectively subject them to the newer drugs like the antiVEGF drug, bevacizumab, and look for the response in them in the form of longer disease-free and overall survival.

Intellectual Contributions of Authors

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CONFLICTS OF INTEREST

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Book review

Six-legged soldiers: using insects as weapons of warfare. Jeffrey A Lockwood. Publisher: Oxford University Press Inc, New York. Publication: 2009. Pages: 400. Illustrations: 50. Price: \$27.95 (Hardcover), \$17.95 (Paperback). ISBN: 9780195333053.

“This is the tale of one of history’s most potent military alliances: the intelligence of humans and the power of insects.”

The practice of using insects for the purpose of warfare is no new practice and today, we have improvised upon those strategies our cave-dwelling forefathers had conceived. *Six-legged soldiers: using insects as weapons of warfare*, is a fascinating book on this subject and can easily be called a treatise of the evolution of entomological warfare. It has been lucidly written by Jeffery A Lockwood, Professor of Natural Sciences and Humanities at the University of Wyoming, in an inimitable style ensuring that this 400-page book is as absorbing as any top-rated novel.

Reconstruction of history is always an arduous task and Lockwood amalgamates the skill of story-telling with data. Each chapter is tastefully titled, in keeping with the general paradigm of dry humour that underlies the entire book. Although the book is tantalising and mentally stimulating, its USP possibly lies in the fact that it is chillingly real; it is not a fantasy fiction but is what actually happened to someone, somewhere and ... yes, could happen again.

The book paints vivid pictures of entomological warfare down the ages: of Palaeolithic man, who flung nests of bees

into enemy caves to force foes out of hiding; of Egyptians, who deified insects for their extreme courage, selfless sacrifice, and commitment to their kin under the worst circumstances that could push a brawnier opponent into retreat. It then describes the times of the World Wars, of Hitler smuggling Colorado beetles into England farms, the Japanese stocking up *Yersinia pestis* infected fleas to be bombed on the enemy but ending up facing the brunt of their own bugs when security measures broke down, and this is said to have cost Japan more lives than the atomic bombing. The last chapter outlines future trends in entomological warfare: all the sci-fi comes true—fusion of bioengineering and entomology to design biotechnological bugs (appropriately called “robotflies”). An entomologist’s delight, to be sure!

Entomological warfare may be re-defined after reading this and may finally get its due importance. This book’s bottom-line is that one does not need high tech bombs for destruction; some very cleverly manipulated commonplace insects will do the job just as well. It is a wake-up call for all. This reasonably priced book is useful for academicians, public health specialists, entomologists, researchers, and all those engaged in literary pursuits. In fact, a highly recommended page turner for people from all walks of life, particularly the military.

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