Original Article

A Study Of Angiogenesis In Human Colorectal Tumors By Using Anti-Cd34 Antibody. (Assessment By Light Microscope And Computer–Aided Image Analysis System)

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Summary:

Background*:*

Angiogenesis plays a crucial role in tumorigensis; several reports have described a significant increase in microvessel density (MVD) in colorectal carcinogenesis There are several methods to measure the angiogenesis in neoplasms, but immunohitochemistry seems to be the mainstay of all. This method enable us to measure the tumor microvessel densities highlighted by using antibodies directed against endothelial cell markers like CD31,CD34 or others; then assessment of MVD by manual count of the number of microvessels in what appears to be the most vascular area of the tumor(called the hot spot) using a protocol described by Weidner et al.Automated cellular imaging system is used to analyze immunohistochemically stained slides .studies have shown that the device offers accurate precision and reproducibility of immunostained slide analysis exceeding that possible with manual evaluation which was the prevalent method .

Aims of the study*:*

To assess the angiogenesis in normal, adenomatous (benign) and malignant colorectal tissues using CD34 and the microvessels will be measured both manually by hot spot method as the MVD and by the use of computerized image analysis system as fraction area ,we correlate between microvessels density and fraction area with various clinicopathological parameters in colorectal cancer (CRC), and to a compare between the results which obtained from both methods.

Methods*:*

Paraffin embedded archival materials from 50 cases including three normal resection (non tumoruos) margins, 12 benign colonic lesions and 35 colonic adenocarcinoma were used . 5mm section were cut and they were stained by anti CD34 antibody Angiogenesis was measured as MVD by two methods : manually by light microscope and by a computer image analysis system (as fraction area). Then the MVD and fraction area were correlated with different clinicopathological parameters.

Results *:*

This study demonstrate that there is a statistical difference in MVD and fraction area in both hot spot method and CIAS respectively between benign and malignant tumors . P value < 0.05 in hot spot method and less than 0.001 in CIAS and there was highly significant correlation between MVD and fraction area with the grade . There was significant increase in MVD and fraction area from well differentiated to moderately differentiated and to poorly differentiated .There was no significant correlation between MVD and lymph node involvement by hot spot method but CIAS proved a significant correlation between fraction area and lymph node involvement.Both methods (hot spot and CIAS) proved no significant correlation with age , sex , size of the tumor , site of the tumor , stage of the tumor and the number of lymph node involverment .

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Conclusions*:*

Assessment of tumor vascularity by counting the microvessels using anti CD34 antibody is useful in quantifying angiogenesis in colorectal adenocarcinoma . Intratumoral microvessels count (by manual and computerized method) is important in the assessment of the biological behavior of CRC. Microvessel count is higher in malignant tumors than benign tumors and it is correlated with the tumor grade and higher MVD is associated with lymph node metastasis . Statistically no significant correlation was found between MVD and age, sex of the patients, tumor size, site, stage, histological type and number of lymph node metastasis . The use of CIAS for assessment of angiogenesis is reliable, reproducible and more precise than the manual method.

Key words*:*

Angiogenesis -colorectal tumors - anti CD34 antibody –CIAS.

Introduction:

Carcinogenesis is a multistep event in which angiogenesis plays an important role in both tumor growth and metastasis .[1] Angiogenesis is defined as the formation of new vessels from the preexisting vessels.[2]

The development of colorectal cancer (CRC) proceeds through a series of genetic alterations involving the activation of oncogen and loss of tumor suppressor genes. Most CRC arise from benign adenomas that gradually increase in size, transform to dysplasia, and change to a villous morphology. The progressive accumulation of genetic alterations (e.g. APC, P53, DCC and K- ras) govern the transition of normal colorectal epithelium to adenoma and the malignant transformation to adenocarcinoma. [3] Angiogenesis plays a crucial role in tumorigensis; several reports have described a significant increase in microvessel density (MVD) in colorectal carcinogenesis [4, 5] and the results of a number of investigations on CRC have shown that there is a strong correlation between high MVD and the presence of distant or lymph node metastasis. [6, 7]

There are several methods to measure the angiogenesis in neoplasm, but immunohitochemistry seems to be the mainstay of all. This method enable us to measure the tumor microvessel densities highlighted by using antibodies directed against endothelial cell markers like CD31,CD34 or others [8]; then assessment of MVD by manual count of the number of microvessels in what appears to be the most vascular area of the tumor(called the hot spot) using a protocol described by Weidner et al.[9]

Nowadays the use of morphometric analysis using fully automatic image analyzers has been presented and such techniques have been upgraded to adapt the personal computers to be used as an image analysis system simply by applying a proper software. Such modification will be with less cost than a full complex morphometric machine and easy to use especially when the software can run under

"Microsoft windows" operating system. [10]

Morphometry offers objectivity, increases precision compared with direct visual appraisal and makes statistical analysis easier. It improves assessment of certain histological changes and their production. Morphometry also allows appreciation of three dimensional parameters such as volume, density or surface area per volume. [11]

Patients, Material and Methods :

Tissue Sample:-

In this retrospective study ,the formalin fixed paraffin embedded tissue blocks were collected from the archived materials from the Department of Pathology in AL-Khadimiya Teaching Hospital Teaching laboratory, Teaching Laboratories of the Medical City Hospital, Gastrointestinal and Liver disease Centre and a private laboratory, in the period between January 2004-October 2005.

The paraffin blocks were prepared from a total of 50 cases of surgical

colorectal specimen (8 colorectal biopsies ,18 hemicolectomy specimen and the other 24 cases were total colectomy specimens). They were from benign and malignant cases. Benign cases includ :2 Juvenile rectal polyps, 2 hamartomatous polyp with adenomatous changes, 1 tubular adenoma, 3 tubulo villous adenoma, 4 villous adenoma ,with a total of 12 , while malignant cases include 35 cases with histologic diagnosis ofcolorectal adenocarcinoma .Three cases taken from the normal (non tumorous) resection margins as a control.

Clinicopathological parameters such as the age and sex of the patients, tumor type, stage, histological grade, tumor location,tumor size and lymph node status were obtained from the available histological reports.

Tumor tissues were pathologically staged according to modified Duke's staging system and histologically graded according to WHO classification [12] as: grade I: well differentiated , grade II, moderately differentiated, grade III: Poorly differentiated and grade IV: undifferentiated.

For each case, 2 sections of 5µm thickness were taken; one representive section was stained with Haematoxylin and eosin (H & E), and the other was stained immunohistochemically for CD34. Haematoxylin and eosin stained sections were examined well for the type of the tumor, histological grading and lymphatic and /or vascular invasion and modified Duke's staging.

Immunohistochemical staining:

It was performed by the streptavidin –biotin method . The antibody used was monoclonal mouse antibody anti-human CD34 (QBEND10, Immunotech) at 1:50 dilution .

Vascular detection and counting :

In order to identify the microvessels in tumor area , a monoclonal antibody directed towards an endothelium-associated antigen was used (anti CD34 antibody). Sections 5 µm were cut from paraffin blocks .The section were deparaffinized in xylol and rehydrated through descending alcohol series .Enogenous peroxidase was inactivated by incubating the section with 3% hydrogen peroxide , nonspecific background stainimg was blocked by incubating the sections in protein block. The sections were incubated with primary antibody over night at 4^0 C.

Sections then were incubated for 30 minutes with biotinylated anti-mouse immunoglobulin (Dako cytomation code K0673).Then strepavidin conjugated to horseradish peroxidase was used, then washing with phosphate buffer solution .Then the section were incubated with diaminobenzidine substrate for 10 minutes . The section were rinsed with distilled water and counter stained with Mayr's haematoxyllin. Negative control slides in the absence of primary antibody were included for each staining.

The evaluation for the microvessels was done by two methods:

1-Light microscope evaluation:-

Each section was evaluated for microvessel count (MVC) which can be defined as the number of microvessels within areas with the most dense vessel growth which is called "hot spot" are encountered predominantly at the peripheral tumor margin. Once the region of interest (the vascular hot spot) is defined a higher magnification is selected in order to be able to count the individual stained microvessels

Tumor sections were scanned at low power x100 (10x10) then count at x200 (20x10) which represent a field size of 0.74 mm^2 provided MVC.Three hot spots were selected for MVC the mean value of all the three fields examined were divided on the high power field area which is 0.74 mm^2 and this represent the MVD.

Any single brown stained endothelial cell or endothelial cells clusters clearly separated from adjacent microvessels, tumor cells and other connective tissue elements were

considered as a single countable microvessel (modified Weidner's method). [9]Large vessels with thick muscular walls were excluded from the count. Branching structures were counted as a single vessel unless there is discontinuity in a structure. Vessel lumens although usually present, were not necessary for a structure to be defined as a microvessel and red cells were not used to define a vessel lumen.

2- Computerized image analysis system (CIAS):

Each picture was analyzed by commercially available software called adobe Photoshop according to the following scheme:-

The software used was Photoshop version 7.0 (Adobe system, mountain view, CA)

The procedure for determination of immunostaining intensity was done by using the magic wand tool in the select menu of Photoshop, the curser was placed on DAB positive cells. The tolerance level of the magic wand tool was adjusted so that the entire positive cells were selected. Using the similar command in the select menu, all immunostained cells were automatically selected. Once the different chromogens are selected, quantification accomplished using the histogram command in the image

menu. This display is rarely if ever used by graphic designer but rather serves as an internal measurement of tonal distribution as the basis for automated image manipulation (map commands), when histogram is selected a display appears on the screen depicting the luminosity (color) of all pixels within the selected area, including median and standard deviation. Further more, this display shows the number of pixels that are covered by the selected area.

Because the number of pixels reflects a surface area on the image, important spatial information can be obtained for the specific chromogen (and have the cells expressing a certain antigen) and can be expressed as percentage of the entire image or in mm^2 which is referred to as the fraction area. The mean value for the fraction area then correlated to the patient age, sex, tumor site, size, histological type, grade, stage and lymph node involvement.

Statistical analysis: Statistcal analysis was performed with SPSS 10.01 statistical package for social science. Data analysis was done by using ANOVA and T test. P value of <0.05 was considered as significant.

Descriptive statistics for the clinical feature of the patients was done using the range , mean and SE.

Results:

We investigated the microvessel count in 50 cases stained by CD34.

In table 1, the detailed data of 50 specimen are shown.

Table 1: Patients character

Clinicopathological parameters		Benign		Malignant	
		Number	Percent	Number	Percent
Age in years	< 35	5	41.6	4	11.4
	35-50	3	25	13	37.1
	> 50	4	33.3	18	51.4
Sex	Male	5	41.66	22	62.9
	Female	7	58.33	13	37.1
Tumor site	Proximal colon	2	16.66	11	31.4
	Distal colon	7	58.3	14	40
	Rectum	3	25	10	28.5
Tumor size in cm	1-4	12	100	0	
	≥ 5	0		17	48.6
Tumor type	Mucinous			7	20
	Non mucinous			28	80
Histological type	TA	1	8.3		
	TVA	3	25		
	VA	4	33.3		
	JRP	2	16.66		
	HP	2	16.66		
	AC			35	100
Tumor grade	WD			8	22.9
	MD			22	62.9
	PD			5	14.3
Tumor stage	Stage B			18	51.4
	Stage C			14	40
	Stage D			3	8.6
Lymph node	Negative			19	54.3
	positive			16	45.7
Positive lymph node	1-3			13	81.3
	≥ 4			3	18.8

The correlation between MVD using anti- CD 34 antibody and various clinicopathological parameters using light microscope:

This study revealed that significant difference in MVD between normal and benign conditions, benign and malignant cases and highly significant difference between normal and malignant conditions , as shown in the table 2. Regarding the tumor grade there was statistically highly significant difference between the three groups (well differentiated ,moderately differentiated and poorly differentiated) where the P value was < 0.001.We did not observe any significant correlation between MVD and age ,sex of the patients , histological type , size, site ,stage of the tumor, lymph node involvement (P value > 0.05)

Table 2:correlation between MVD using anti- CD 34 antibody and various clinicopathological parameters using light microscope:

The variable		MVD range	Mean ± SE	P value
Groups	Normal	5.8-44.6	37.9±3.3	0.04
	Benign	33.8-72.02	55.6±4	
	Malignant	27.2-140.3	66.4±4	
Age	< 35	51.4-108.5	84.3±17	0.23
	35-50	41.9-77.02	59.6±3.3	
	>50	27.02-140.3	69.7±6.7	
Sex	Male	20-75	65.4±5	0.78
	Female	22-118	68.4±7.1	
Tumor site	Proximal colon	47.3-140.3	74.5±9.8	0.16
	Distal colon	33.8-140.3	55.6±4.4	
	Rectum	27.3-77.02	64.7±6.6	
Tumor size in cm	1-4	43.2-112.2	75.1±7.4	0.13
	≥ 5	33.8-140.2	60.3±5.8	
Tumor histology	Mucinous	57.2-93.2	71.5±4.3	0.34
	Non mucinous	33.8-140.3	65.1±4.9	
Tumor stage	Stage B	27.02-112.2	61.3±5.5	0.35
	Stage C	41.9-140.3	69.9±6.6	
	Stage D	58.1- 180.5	80.8±14.7	
Tumor grade	WD	27.02-76.4	50.7±6.2	<0.001
	MD	41.9-108.5	64.1±3.7	
	PD	65.3-140.3	101.5±15.6	
Lymph node	Negative	58.1-108.5	63.8±5.7	0.49
	Positive	41.9-140.3	69.5±5.8	
Positive lymph node	1-3	41.9-140.3	69.3±8.1	0.94
	≥ 4	57.2-93.2	86.5±6.5	

Correlation between fraction area and different clinicopathological parameters:

Measurement of angiogenesis by computerized image analysis system (CIAS) as a fraction area showed a highly significant difference in fraction area between benign and malignant conditions. The fraction area was high in malignant than in benign conditions(P value < 0.001) as shown in table 3.

There was a significant correlation between fraction area and the grade of the tumor .Morever,no correlation was found between fraction area and age and sex of the patients , size , site , histological type of the tumor. P value > 0.05

There was a highly significant correlation between fraction area and lymph node involvement but not with the number of the lymph node involvement as shown in table 3.

Table 3: Correlation between fraction area and different clinicopathological parameters:

The variable		Fraction area range	Mean ± SE	P value
Groups	Normal	0.3-2.8	2.5±0.2	<0.001
	Benign	0.7-4.6	3.3±0.2	
	Malignant	1.3-7.6	3.6±0.3	
Age	< 35	1.3-5	4.03±0.95	0.6
	35-50	1.2-6.1	4.8±0.7	
	>50	1.4-7.6	5.1±0.5	
Sex	Male	1.7-6.6	4.8±0.4	0.82
	Female	1.7-7.6	5.01±0.7	
Tumor site	Proximal colon	1.8-7.7	5.9±1.06	0.06
	Distal colon	1.02-6.1	4.18±0.4	
	Rectum	0.7-6.6	5.5±0.4	
Tumor size in cm	1-4	1.3 -6.5	5.2±0.5	0.98
	≥ 5	1.7-7.7	5.2±0.6	
Tumor histology	Mucinous	1.3-6.5	4.5±0.3	0.28
	Non-mucinous	2.1-7.7	5.1±0.5	
Tumor stage	Stage B	1.3-6.6	4.3±0.5	0.85
	Stage C	1.4-7.6	5.2±0.6	
	Stage D	1.9-6.1	4.8 ±1.3	
Tumor grade	WD	0.3-3.6	3.4±0.2	<0.001
	MD	0.7-5.7	4.6±0.2	
	PD	0.6-7.6	6.7±0.3	
Lymph node	Negative	3.02-4.98	4.03±0.2	<0.001
	Positive	4.95-7.6	6.1±0.4	
Positive lymph node	1-3	1.7-7.7	5.3±0.8	0.76
	≥ 4	0.8-6.2	5.6±0.6	

Discussion:

The development of Colorectal caner (CRC) proceeds through a series of genetic alteration involving the activation of oncogens and loss of tumor suppressor genes.[3] Most CRC arise from benign adenomas that gradually increase in size , transform to dysplasia and change to a villous morphology. The progressive accumulation of genetic alterations (e.g.APC, P53, DCC and K-ras)govern the transition of normal colorectal epithelium to adenoma and malignant transformation to adenocarcinoma

[4].Angiogenesis has been shown to play an important role in transition of normal tissue to pre neoplastic state and eventually to full blown cancer [13]

A tumor blood supply is essential for tumor growth and metastasis since it does not only supply the nutrients and oxygen requirement for the growing tumor but also provides the vascular route for haematogenous spread to distant sites .[14]

Several studies had mentioned a significant correlation between the MVD (as a measure of angiogenesis) with clinicopathological factors and prognosis of patients with various solid tumors. [13, 14] It has been shown that the greater number of tumor vessels , the greater the opportunity for tumor aggressiveness and metastatic potential and is ultimately related to clinical outcome. [15, 16]

The correlations between MVD by using anti CD34 antibody were assessed by two methods light microscope and Computerized Image Analysis System (CIAS) and several clinicopathological factors (age and sex of the patients, tumor size ,site, histological type , grade, stage and lymph nodes metastasis) were investigated. This study has clarified several points regarding the significance of angiogenesis (by measuring MVD) in the assessment of the biological behavior of CRC as appeared from the results.

Correlation between the MVD using anti-CD34 antibody staining and various clinic pathological factors:

Colorectal adenoma and other benign conditions and CRC:

In this study the MVD in the benign colorectal lesions were compared with those in the malignant tumors . MVD was found to be higher in the malignant tumors when compared with those of benign lesions. There was a significant difference in MVD between benign and malignant tumors which reflect more neovascularization in malignant tumors than the benign. These findings suggest that vascular density and remodeling of preexisting vascular network around benign colorectal lesion may serve as a possible tool to assist diagnosis ,to predict the risk of malignant transformation of premalignant lesions and to assign prognosis of early malignant process in colon . This result agrees with the study done by Bossi P. et al [5] using anti CD31 and Kawasaki H . et al [17] using anti CD34 antibody. They evaluated MVD in normal colonic tissue, adenoma and CRC and reported that MVD increased after transition from adenoma to carcinoma and they concluded that angiogenesis play a crucial role in the transition from normal tissue to preneoplastic state and then to cancer.

Similar results also reported in other tumors like breast cancer [1,18], prostatic cancer [19] and endometrial cancer .[20]

All these studies mention that there is significant difference in MVD between benign and malignant tumors and this reflects more neovascularization in malignant tumors than benign tumors.

Similar results obtained by Computerized Image Analysis System (CIAS).

MVD in poorly differentiated adenocarcinoma was significantly higher than those in well differentiated tumors and the MVD were increased from well differentiated to moderately differentiated and poorly differentiated tumors suggesting that a higher grade tumor tend to have greater MVD.

These results are in agreement with other studies of Faviana P. et al . [21] This study used anti-CD34 antibody and proved that tumors of high grade had a higher number of microvessels when compared with cancers of low grade ,but this significant correlation was not found in other studies like Cianchi et al [22], White et al [23] and Tien et al . [6] In which they used anti-CD31 antibody therefore this difference may be due to using of different antibody to stain endothelial cells.

By using CIAS , there was significant difference between fraction area and the grade of the tumor.

In spite of the fact that MVD was higher in patients with advanced disease, a statistically significant correlation was not observed between MVD using anti-CD34 antibody and tumor stage.

This result was in agreement with other studies like Bossi et al [5] ,Cianchi et al [22] ,but not in other studies like .Faviana [21],Tein et al [6], Fenjvesi et al [24] and Yonenaguy et al [25] which found significant correlation between MVD and stage of Colorectal cancer.

This difference between the studies results may be explained by small sample

size, using different antibodies or using different methods in counting.

By using CIAS ,also there was no statistically significant correlation between the fraction area and the stage of the tumor.

Regarding the and the sex of the patients, tumor size , site , tumor histological type there was no significant correlation between MVD and fraction area in both methods (hot spot and computerized image analysis system respectively).

Our study shows no significant correlation between MVD and lymph nodes metastasis ;also there was no significant difference in MVD between the group with less than 4 lymph nodes involvement and those with more than or equal to 4 lymph nodes involvement. These findings are in agreement with Tanigawa et al [4] and Zi-ping Li et al [26] also found that there was no significant correlation between MVD and lymph node metastasis, but not with other studies like Takebayashiy et al [7] ,Tien et al [6]and Faviana et al .[21]

By using CIAS we found a statistically significant correlation between staining area percent (fraction area) and lymph node metastasis, but not with the number of lymph node metastasis.

In our study approximately similar results were obtained from counting microvessels using hot spot method by light microscope and by using CIAS technique except for the correlation with lymph node metastasis which appears to be correlated with fraction area by computerized method but not with MVD by manual method. This may indicate that fraction area by CIAS is more precise and more reproducible than the manual method .

We chose to perform CIAS as a try to decrease the subjectivity and interobserver variability which occur in the manual method of assessing MVD.

Many studies used CIAS for measurement of angiogenesis in different types of tumors and compare the results with the manual method like Fox et al [27] in breast carcinoma. Manual vessel counts were significantly correlated with microvessel area (MVA), microvessel parameters(MVP) and microvessel number assessed by CIAS.

Charpin and his cholleagues 1995 [28] have determined the total CD31 immunostained area or endothelial area (EA) in breast carcinoma.By using automatic screening of the whole tumor section, the result was a significant correlation with tumor size, node status, number of lymph nodes and tumor grade.

Another study by Simpson et al. [29] assessed the total anti-CD34 stained area in breast carcinoma . The number of CD34 positive pixels was recorded in five adjacent fields at X 200 magnification in the manually identified area of greatest vascular density .Image analysis of endothelial area (EA) number of positive pixels was significantly correlated with tumor grade and lymph node positivity.

Our study used a new modified automated scoring system for immunohistochemical staining using commercially available low cost soft ware for image analysis, we can get accurate, precise and reproducible results of immunostained slide analysis exceeding that with manual evaluation which was the prevailing method .

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