Original Article

Uses of CD31 monoclonal Antibody for the Assessment Of Angiogenesis as a prognostic Factor in Gastric Adenocarcinoma

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Summary:

Background &objectives: *Angiogenesis plays an important role in the growth, progress and the metastasis of solid tumors. This study was done to determine the significance of angiogenesis as another prognostic factor in gastric carcinoma in an attempt to identify the patients at high risk of recurrences after surgical therapy who may benefit from postoperative adjuvant therapy.*

In this study the correlation between the microvessel count(MVC) and the various clinicopathological factors was investigated. A highly significant statistical correlation was found between the mean microvessel count in different histological grades and the stage of gastric adenocarcinoma .

No correlation was found between the MVC and the age and sex of the patients , the size, location and the macroscopical appearances of the tumors

Patients and Methods: *Fifty seven cases of gastric adenocarcinoma were reviewed from private pathology laboratories during the period between 2000-2002 ,including 33 males and 24 females. The age ranged between 20-80 years ,the peak incidence was in the sixth decade . The specimens were formalin- fixed paraffin embedded, and the 5 microns thick sections were stained with Haematoxylin & Eosin stains .Immunohistological staining with CD 31 was performed on all sections. The microvessel count (MCV) was performed on the areas of the highest neovascularization i.e. hot spots. Five hot spots were selected the MVC was done under X200 magnification in light microscope .The relationship between the MVC and the various clinicopathological factors were assessed. (Statistical analysis was done using SPSS system version 10.0 computer software. Student "T" and "F" test were used and the differences were regarded as significant when the P value was less than 0.05.*

Result:: *Using CD 31 an endothelial cells marker monoclonal antibody shows that the MVC was 35 in well differentiated adenocarcinoma and 48.22 in the poorly differentiated tumors. In tumors with no serosal invasion the MVC was 26.5 while in case s with serosal invasion the MVC was 87.7.In cases of negative lymph nodes metastasis the MVC was 27.7 while in cases with positive lymph nodes metastasis the MVC was 43.56.*

Conclusion : *Evaluation of angiogenesis by counting the microvessels using the monoclonal antibody CD 31 as an endothelial cell marker on tissue sections proved to be a helpful and a useful prognostic factor to predict the poor prognosis in gastric cancer patients.*

Key Words*: Gastric cancer ,angiogenesis, microvessel count.CD31.*

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Introduction

Gastric cancer is still the most common fatal malignancy (after lung cancer)in the world for both sexes,(1)The incidence varies geographically being particularly high in countries like Japan ,Chile ,China , Portugal and Russia.While it is four folds or six folds less common in the United States ,United Kingdom ,Canada ,Australia and France, (2) .

In Iraq gastric cancer is one of the commonest ten cancers ,it ranks the seventh among females ,and the eighth among males. (3).

Gastric cancer cells produce angiogenic factors including beta Fibroblastic Growth Factor (b FGF) ,Vascular Endothelial Growth Factor (VEGF) and Interleukin-8 (IL-8)

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Many studies had reported an association between high vascular density i.e. degree of tumor angiogenesis and the presence of tumor metastasis in gastric cancer (4).Maeda et al in 1995 (5) studied tumor angiogenesis as a predictor of recurrence in gastric cancer and they found that there is poor prognosis in patients with hypervascular group than the hypovascular group.(5).

Uses of anti-Human CD31 monoclonal antibody which is called anti-platelets endothelial cell adhesion molecule (PECAM-1),which is abundantly expressed in endothelial cells in normal tissues and in benign and malignant proliferation (6).It is even more sensitive than anti-Human Von-Willbrand Factor (Anti factor 8 RA)/VWF. (7).

In this study Anti-CD 31 was used as an immunohistochemical staining on histological sections to perform the Micro Vessel Count (MCV) of 57 cases of gastric cancer . A statistically significant correlation was found between the (MVC) and the histological grade and stage of gastric cancer.

Materials And Methods

A retrospective study was done including 57 cases of gastric adenocarcinoma were selected from private pathology laboratories in Baghdad for the period between 2000-2002 Including 33 males and 24 females. The age ranged from 20-80 years, the highest incidence was recorded in the sixth decade. The specimens submitted included 43 partial gastrectomies and 14 total gastrectomies. The specimens were formalin-fixed paraffin embedded and 3-5 microns thick sections were cut and stained with Haematoxylin and Eosin stains .The sections were examined carefully to identify the hot spots ,which are defined as those areas with the most dense vessel growth. .Those sections were chosen for immunohistological staining using CD 31 monoclonal mouse anti-human Endothelial cells supplied in liquid form in tissue supernatant isolated from human endothelial vessels of a spleen from patients with Hairy-cell leukemia .The target antigen being the endothelial cells and the platelets glycoprotein The recommended dilution is 28 microliter/ml.(1:20-1/40),the incubation time is one hour and the antigen retrieval method using trypsin(Information taken from DAKO sheets , ,clone CD JC/70 A ,Code No.MO823 Lot.110,Edition 13-11-00 ,DAKO corporation United States of America ,9392 Via Real ,Carpinteria ,CA 93013).

Positive control sections were prepared from a well differentiated adenocarcinoma of human tissue origin and stained by using CD31 antibody.(Figure (1).

Negative control sections were used from similar tissue ,being processed and stained similar to the test samples and the positive control except without adding the antibody.(Figure 2).

The MicroVessel Count (MVC) was then determined by counting any single or clusters of red-stained endothelial cells within the hot spots under light microscope on a X200 magnification (Olympus Microscope).Five hot spots were counted and the mean MVC was calculated (13).

Statistical analysis was done using SPSS (Statistical Package for Social Science) system version 10.0 computer software in association with Excel version 97 .The Student (S) test and (F) test were used to assess the relationship between the MVC and the various clinicopathological factors as the age ,sex ,tumor size , its location ,gross appearance ,the histological type ,the grade ,the depth of invasion ,the stage ,the presence or absence of lymph node and distant metastasis. The differences were considered as significant when the P value was <0.05.

Staging of gastric carcinoma was done by using the TNM staging system (9).

Results:

A total of 57 cases of gastric adenocarcinoma were studied ,including 33 males and 24 females. The age ranged from 20-80 years ,the peak incidence being in the sixth decade. As shown by Table 1 .The tumor was located in the gastric body in 38 cases i.e. (66.7%) of the total ,in 10cases (17.5%) the tumor was located at the pyloric-duodenal junction ,and in 9 cases (15.8&) it was located at the cardio- esophageal junction. In 26 cases ,i .e. 45% of the total ,the tumor was equal or larger than 5cm. in diameter ,and in 31 cases i.e. 54.4% of the total was less than 5cm. in diameter .

Grossly : The tumor was ulcerative type in 42 cases (73.7%) and infiltrative type in 15 cases (26.3%).

Microscopically : In 36 cases (63.2%) the tumor was diffuse type and in 21 cases (36.8%) it was of intestinal type (According to Lauren's classification)(8).

In thirty four cases (59.7%) ,the tumor was poorly differentiated adenocarcinoma ,in 21 cases (36.8%) it was moderately differentiated ,and in two cases only it was well differentiated .

We divided the cases into two groups according to the depth of invasion ,50 cases ,i.e. 87.7% of total were with serosal invasion ,and 7 cases ,i.e. 12.3 % of total were without serosal invasion.

Forty five cases (78.9%) showed lymph node metastasis ,and 12 cases (21.1%) showed no lymph node metastasis. Only one case showed positive distant metastasis i.e. hepatic and peritoneal metastasis.

According to the TNM staging system (9), 5 cases (8.8%) were in stage I,9 cases (15.8%) were in stage II ,and 40 cases (70.2%) were in stage III, only 3 cases (5.2%) were in stage IV.

Microvessel Count (MVC) using CD 31 antibody:

The mean Microvessel count was 40.83 ranging from 12-97.

Figure (1) showed a positive control being stained with CD31 antibody showing positive red-stained endothelial cells with prominent vascularity in a well differentiated gastric adenocarcinoma.

Figure (2) shows a negative control of similar tissue as above without adding the CD31 antibody during staining.

Correlation between the MVCs and the various clinicopathological factors:

1- **Age & Sex:** Table (2) shows that the lowest mean MVC was 32.8 in patients at age group between 20-30 years ,and the highest mean was 49.7 in the age group between 31-40 years ..No statistical significant correlation was seen between MVCs and the age and sex of the studied case. The P value was =0.182 (Using the "F" test).

2- **Tumor Size :** Table (3) Shows that the mean MVCs in tumors less than 5cm. in diameter was 42.6 ,while in tumors equal and larger than 5cm. In diameter was 39.0 . Using the "T" test , there was no statistically significant correlation between the MVCs and the size of the tumors. The P value = 0.565.

3- **Tumor Location :** As shown in Table (4) the mean MVCs was44.6 in tumors located at the cardio-esophageal junction (C) and 39.1 in tumors located at the gastric body (B) and 44.9 in tumors located at the pyloric-duodenal junction (P).There was no statistical significant correlation was found using the "T" test. .The P values were as follow : For C/B = 0.501 ,C/P= 0.97 and B/P = 0.423. respectively.

4- **Tumor Grading :** Table (5) and Figures 3 &4, show that the mean MVC was 35 in well differentiated adenocarcinoma ,29.37 in the moderately differentiated ,and 48.22 in the poorly differentiated tumors . There was a statistically significant correlation between the well differentiated tumors and the moderately differentiated ,the P value was 0.041,and for the well differentiated and the poorly differentiated tumors ,the P value was 0.002 ,and for the moderately and the poorly differentiated tumors ,the P value = 0.001.

5- **Macroscopical Appearances:** Table (6) shows that the mean MVCs was 40.5 in the ulcerative type and 41.8 in the infiltrative type .Using the "T" test there was no significant statistical correlation was found between the MVCs and the macroscopical types. The P value = O.833.

6- **Histological Types of the tumor:** Table (7) and Figures 3&4 showed that the mean MVCs was 29.3 in the intestinal type ,and 47.5 in the diffuse type. There was a highly significant statistical correlation between the mean MVCs in the diffuse and the intestinal types of the tumor. The P value being < 0.001 .

7- **Serosal Invasion:** Table (8) shows that the mean MVCs was 26.5 in cases of negative serosal invasion ,and 42.7 in cases of positive serosal invasion . There is a highly significant correlation between both groups. The P value = 0.007.

8.**Lymph Node Metastasis :** Table (9) shows the mean MVCs was 27.78 in cases of negative lymph node metastasis ,and 43.56 in cases with positive lymph node metastasis. There was a significant statistical correlation between the two groups. The P value = 0.027.

9.**Stage of the tumor:** The mean MVCs was 34 in stages I and II, and was 43.7 in stage III, while in stage IV it was 29 . There was no significant statistical correlation was found between all groups, as show in Table (10).The P values ranged from 0.212 and 1.000.

Discussion :

Over the last decade assessment of angiogenesis has emerged as a potentially useful biological prognostic and predictive factor in human solid tumors. (10).Angiogenesis is needed to sustain growth of both primary and metastatic lesions.(11,12) .Many studies have shown that the greater number of tumor vessels increases the opportunity for tumor cells to enter the circulation ,so the degree of tumor microvessel density correlates the tumor aggressiveness and metastatic potential and it is related to the clinical outcome. (13,14,15) .

Correlation between MVCs and the clinicopathological factors using CD-31 antibody:

Age and sex : In our study the optimum number of the MVCs was 49.7 seen in the age groups between 31-40 years, but no steady increase in the subsequent age groups ,indicating that the correlation is not dependable .(Table 2).

In other studies (Kakeji Y,et al 1999) using CD-31 antibody,(Tomado M.et al 1999) and (Erenoglu C.et al 2000) using anti-vWF antibody ,and (Tanigawa N.et al 1997)using anti-CD 34 antibody they found no correlation between the MVCs and the age and sex of the patients.(16,17,18,19).

Tumor Size: No significant correlation was noticed between the MVCs and the tumor size (Table 3).This is in agreement with other studies as (Kakeji Y.et al Erenoglu C. et al , Tanigawa N.et al ,(16 ,17 ,14).

On the contrary of the study of Xianming C.et al 1998 (20) and Maehara Y.et al 2000 (21) where they found that the tumor size was significantly correlated with the MVcs and that the mean MVCs increases as the tumor size grew larger.

Tumor Location : No significant correlation between the MVCs and the tumor location in the three different sites of the stomach .(Table 4).Our result is similar to the study of Xiangming C.et al

1998 (22).While Erenoglu C.et al 2000 found a significantly higher MVCs in proximally located tumors.(18).

Tumor Grading : A significant statistical correlation was found between the MVCs and the tumor grades suggesting that the higher grade tumors tend to have greater MVCs (Table5)Figures 3 ,4 & 5 .Our results are similar to those of other studies, as Xiangming C.et al 1998 ,Erenoglu C.et al 2000 ,both used the anti-vWF antibody to stain the microvessels and found that the tumor grade is related with the MVCs.(20 ,18). While Tanigawa N. et al 1997 using anti-CD 34 antibody ,Araya M .et al 1997 using anti-vWF antibody ,and Kakeji Y. et al 1999 using anti-CD-31 antibody , found no significant correlation between the grade of the tumor and the MVCs.(19,23 ,16).

Macroscopical Types of tumor : No significant correlation between the macroscopic forms of gastric adenocarcinoma and the MVCs (Table 6).This is similar to the findings of Kakeji Y.et al 1999(16).While Araya M.et al 1997 found that the MVCs was higher in the ulcerative tumors than the other types. (23).

Histological Types of the tumor: The study showed that the MVCs were higher in the diffuse type of gastric adenocarcinoma (47.5) than that in the intestinal type (29.3) .This reflects a highly significant statistical correlation between the two types (Table 7)& Figures 4 &5.. It is in agreement with other studies ;as Erenoglu C.et al 2000 found that the diffuse type tumors have increasing MVCs and also the study of Tanigawa N.et al 1997 (18,19). However, our results were different from those of Maeda K.et al. 1995 and Tomoda M. et al 1999 they found no statistical significant correlation between the MVCs and the histological type of the tumor.(5 ,17).

Serosal Invasion : A significant correlation was found in the MVCs between the group of the negative serosal invasion and the group of the positive serosa invasion (Table 8).This is similar to the study of Xiangming C.et al 1998 and Erenoglu C.et al 2000 ,where they found that the depth on invasion is reflected on the MVCs.(20 ,18).While Maeda K.et al 1995 and Kakeji Y. et al 1999 found no significant correlation between the depth of invasion and the MVCs. (5,16).

Lymph Node Metastasis : As shown in (Table 9)a significant correlation was found between the MVCs in the positive lymph node metastasis group and those with negative lymph node metastasis group. This is similar to the results of other studies done by Maeda K. et al. 1995 ,Araya M. et al 1997 ,Xiangming C.et al 1998 and Erenoglu C. et al 2000 ,(5,23,20,18).

The same results were found in other similar studies done on cancers in other locations as in skin melanoma by Srivastava A. et al.1988 ,Breast cancer by Weidner N. et al 1991 , Lung cancer Macchiarini P. et al.1992 ,prostate cancer by Weidner N. et al 1993.and renal cell carcinoma by Yoshino S. et al 1995.(20,24).

Stage of the tumor: No significant statistical correlation was found between the MVCs and the tumor stage as seen in (Table 10) probably related to the small size sample of cases in stage IV compared to those in stage III. However, this result is in agreement with those of Kakeji Y. et al 1999 (16).On the contrary to the results of Maeda K.et al 1995,and Erenoglu C.et al.2000,who found that the MVCs increase with the stage of the tumor ,suggesting that an enhanced vascular supply reflects an increased malignant potential (5,18).

Distant tumor metastasis : In our study only one case of liver and peritoneal metastasis was recorded and because of the small size of the sample we cannot depend on this statistically.Maeda K. et al 1995 ,Araya M. et al 1997 and Erenoglu C. et al 2000 reported higher MVCs in liver metastasis (23,18).

In conclusion : Counting the microvessel density using anti-CD 31 antibody to demonstrate the microvessels in gastric adenocarcinoma was found to be a useful and a reproducible method that can be applied routinely on tissue sections ,it is important in the assessment of the biological behavior of gastric carcinoma. Higher microvessel counts is correlated to the diffuse type ,higher grade ,serosal invasion and positive lymph node metastasis.No correlation was found between the MVC and the age ,sex of the patients ,the tumor size location ,its macroscopical type and the stage.

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Table (1) The characteristics (clinicopathological factors)of 57 cases of gastric cancer studied

clinicopathological factors	No. of casses
age in years	
20-30	4
31-40	10
41-50	8
51-60	23
>60	12
sex M	33
F	24
Tumor location	
cardio - esophageal junction	9
Body	38
pyloric- duodenal	10
Tumor size	
<5 cm	31
>=5cm	26
Macroscopical type	
Ulcerative	42
Infiltrative	15
Histologic type	
Intestinal type	21
Diffuse type	36
Tumor grading	
Well differentiated	2
Moderately differentiated	21
Poorly differentiated	34
Serosal invasion	
Negative	7
Positive	50
Lymph node metastasis	
Negative	12
Positive	45
Distant metastasis	1
Stage grouping	
Stage I A	3
B	2
Stage II	9
Stage III A	32
B	8
Stage IV	3

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table (6) Correlation between MVCs using CD31 staining antibodyand macroscopical type of gastric cancer

macroscopical type	Patients		MVC using CD31 antibody		
	No.	%	Mean	SD	Range
Ulcerative	42	73.7	40.5	19.96	12-95
Infiltrative	15	26.3	41.8	19.50	20-97
P-value			=0.833(NS)*		
Total	57	100	40.38	19.65	12-97

NS – not significant.

Table (7) Correlation between MVCs using CD31 staining antibodyand histological type of the tumor

Histological type	Patients		MVC using CD31 antibody		
	No.	%	Mean	SD	Range
Intestinal	21	36.8	29.3	8.79	12-46
Diffuse	36	63.2	47.5	20.92	19-97
P-value			<0.001(S)*		
Total	57	100	40.83	19.65	12-97

S – significant.

Table (8) Correlation between MVCs using CD31 staining antibodyand serosal invasion by the tumor

serosal invasion	Patients		MVC using CD31 antibody		
	No.	%	Mean	SD	Range
Negative	7	12.3	26.5	13.52	14-52
Positive	50	87.7	42.7	20.18	12-97
P value			=0.007 (S) *		
Total	57	100	40.5	19.65	12-97

S –significant .

Table (9) Correlation between MVCs using CD31 staining antibodyand the lymph node metastasis

lymph node metastasis	Patients		MVC using CD31 antibody		
	No.	%	Mean	SD	Range
Negative	12	21.1	27.78	7.63	14-52
Positive	45	78.9	43.56	20.34	12-97
P-value			=0.027 (S)*		
Total	57	100	40.38	19.65	12-97

S - significant.

Table (10) Correlation between MVCs using CD31 staining antibodyand the stage of the tumor

stage of	Patients		MVC using CD31 antibody		
	No.	%	Mean	SD	Range
Stage I	5	8.8	34	19.08	14-52
Stage II	9	15.8	34	14.15	19-61
Stage III	40	70.2	43.7	20.64	19-97
Stage IV	3	5.2	29	16.52	12-45
P-value			I-II=1.000 (NS)* I-III=0.435 (NS)* I-IV=0.749 (NS)* II-III=0.212 (NS)* II-IV=0.628 (NS)* III-IV=0.237 (NS)*		
Total	57	100	40.83	19.65	12-97

*NS- not significant.

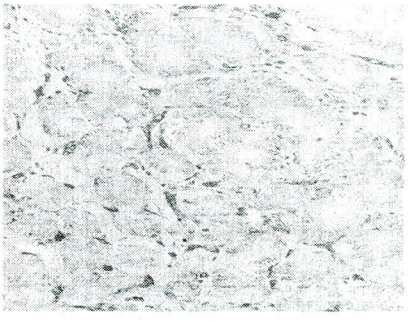

Figure (1) :-Well differentiated gastric adenoCa. As a positive control for CD31 antibody .Micrrovessels (red stained) stand out sharply from other tissue (X200).

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P-value			=0.027 (S)*		
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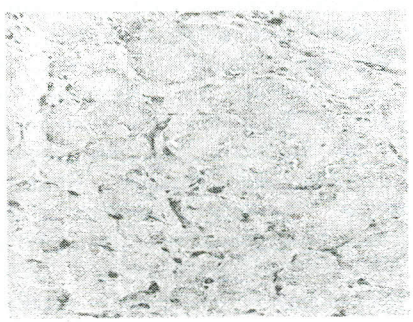

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