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Original Research Article

Evaluation of Prognostic significance of TF, and β3 integrin by using Immunoflourecent staining method

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Abstract: Angiogenesis, the formation of new blood vessels from pre-existing ones, is enhanced in various pathological conditions including rheumatoid arthritis, diabetic retinopathy, and cancer development. Angiogenic processes are regulated by both growth factors, and adhesion molecules, such as integrin**.** Current study was aimed to investigate predictive role of β3 integrin and TF protein expression in colorectal adenocarcinoma sample from Iraqi patients, through linking its expression with tumor histopathological variables (stage, grade, grade, and lymph node involvement), by using immunoflourecent staining method. Study done on 40 colorectal cancer samples and their respective resection margins. Current study found that the expression rate of integrin β3 and TF score were significantly higher in patients than in control group, (P<0.001; P<0.001)respectively, I Moreover, when CRC samples breakdown according to histopathological variables present study demonstrated that both of integrin β3 and TF count showed significant correlation with tumor stage (P<0.05 and P<0.05), grade (P<0.05, and P<0.05), and L.N involvement (P<0.05, and P<0.05) respectively. From above results one can conclude that high expression of β3 integrin and TF are associated with poor prognosis, and may play a crucial role in invasion and metastasis of colorectal carcinoma. Therefore, they may consider as a prognostic biomarkers and novel molecular therapeutic targets.
Keywords: Angiogenesis, growth factors, β3 integrin, TF protein.

INTRODUCTION:

Pathogenesis of human malignancies is tightly linked with the vascular system and at a number of 'strategically' important levels [1]. In this regard, two processes stand out as particularly ubiquitous and important: formation of new blood vessels (tumour angiogenesis) and activation of the coagulation system (coagulopathy), [2].

Integrin is a transmembrane glycoproteins composed of non-covalently associated α- and β-chains which recognize proteins of the extracellular matrix (ECM).The importance of αvβ3 in angiogenesis has been underscored based on the findings that αvβ3 is prominently expressed on the surfaces of endothelial cells and is highly up regulated on angiogenic blood vessels such as those in solid tumors and in granulation tissue at the base of healing wounds [1]. Recent investigations have shown that high expression of integrin β3 is positively correlated with invasion and metastasis of cancer cells and tumor angiogenesis [2, 3]. Tissue factor (TF) is an important paradigm and one of the central effectors of coagulation system and angiogenesis. TF – also known as coagulation factor III, thromboplastin,– is a 47 kDa transmembrane glycoprotein [4,5]. Constitutive TF expression is restricted to sub endothelial cells that only interact with blood when vascular integrity is compromised [6]. However, it is clear that during tumourigenesis, this strict regulation of TF expression is lost [7, 8]. Different studies showed a correlation between TF and angiogenesis [9, 10]. Zhang and his collegues found that tumor cells transfected to over express TF grew more rapidly, and established larger and more vascularized tumors than control or antisense transfectants in vivo [11]. Moreover, A significant correlation between TF expression and mocrovesseles density (MVD) was also found in prostate carcinomas [12], and hepatocellular carcinoma [13]. Tumors with higher TF staining showed higher MVD [13]. Thus the aim of the present study is to find if there is a prognostic significance for both of αvβ3 integrin and TF through linking its expression level with various tumor pathological variables by using imunoflourecent technique.

MATERIALS AND METHODS

Patients and Sampling:

Fourty patients with colorectal adenocarcinoma, who were confirmed

histopathologicaly, were included in this study. Their age were ranged from 20- 80 years. Paraffin embedded blocks of tumor and resection margins were retrieved along with the histopathological report of each patient from histopathological laboratory. For staging of the cancer, astler-coller staging system was adopted in this study [14]. In addition, resection margins were confirmed again to be free of malignancy. Adequate thin paraffin embedded sections (5µm thick) of tumor and resection margins were prepared on positively charged slides for the immunoflourecent Technique.

Direct Immunoflourecent Detection of TF, and αvβ3 integrin.

Immunofluorescence is an antigen-antibody reaction where the antibodies are tagged (labelled) with a fluorescent dye and the antigen-antibody complex is visualized using ultra-violet (fluorescent) microscope. Fluorochromes are dyes that absorb ultra-violet rays and emit visible light. This process is called fluorescence. the fluorochromes used in this study, is fluorescein isothiocyanate (FITC), which is commonly used flourochrome. When fluorescein (FITC) is excited by a blue (wavelength 488nm) light, it will emit a green (520nm) colour.

The steps involved are: Fixation of smear on the slide, antigen unmasking by heat epitope retreval methods by using water bath and epitope retrival solution with 6pH. Fallowed by treating the samples with the fallowing flourecene labeled monoclona antibody;

1. mouse anti-human TF (Santa cruz, USA),
2. mouse anti-huma αvβ3 integrin (Santa cruz, USA).

After incubation time (1 hour), washing step was done to remove unbound excess labeled antibody and visualization under fluorescent microscope. When viewed under fluorescent microscope, the field is dark and areas with bound antibody fluoresce green. The scoring of posative cells depend on mean expression level per high powe field.

RESULTS

Tumor Sites versus their Resection Margins

According to immunoflourecent staining technique, Table 1 showed the mean score of αvβ3 integrin, mean expression level of TF in colorectal tissues of patients and control group. Mean αvβ3 integrin expression level was significantly higher in patients than in control group 74.31429±2.713466 % versus 22.55556±1.134987 %, (P<0.001), moreover mean TF count was significantly higher in patients than in control group 76.47059± 2.692649 versus 25.33333± 0.987421 (P<0.001) respectively, as shown in table [1].

Table 1.αvβ3 integrin, and TF protein expression in tumor sites and their resection margins, based on t Test

sample	No of samples	Integrin Mean ± SE	TF Mean ± SE
Tumor	**40**	**74.31429±2.713466**	**76.47059± 2.692649**
control	**40**	**22.55556±1.134987**	**25.33333± 0.987421**
P value		**<0.001**	**<0.001**

Correlation among Protein Expression of αvβ3 and VGF with Different Histopathological Variables

αvβ3 and TF protein expression in colorectal adenocarcinoma were also analyzed against the different pathological variables of the tumors based on Spearman's correlation. As shown in Table 2, and 3, current study demonstrated that there were significant correlations between both αvβ3 and TF expression with tumor stage (P< 0.05, P< 0.05); and grade (P<0.05, and P< 0.05) respectively, depending on mean expression level. Moreover, a significant differences were found when we compare the expression level of both markers according to L.N involvement (P<0.05, P<0.05 respectively) table (4).

Table 2: Expression pattern of αvβ3 and TF along with tumor stage of CRC, based on spearman's correlation (rs)

Stage	No.	Integrin Mean ± SE	TF Mean ± SE
A	**7**	**51.33 ± 2.33**	45.00 ± 3.20
B	**11**	**67.60 ± 2.67**	68.60 ± 2.32
C	**14**	**85.45 ± 2.01**	82.36 ± 1.89
D	**8**	**96.50 ± 0.68**	92.39 ± 0.59
* (P<0.05).			* (P<0.05).

Table 3: Expression pattern of αvβ3and TF along with tumor grade of CRC, based on spearman's correlation (rs)

Grade	No.	integrin Mean ± SE	TF Mean ± SE
WD	**10**	**52.80 ± 4.57**	53.50 ± 4.99
MD	**18**	**74.47 ± 3.18**	74.94 ± 3.74
PD	**12**	**92.85 ± 2.84**	85.60 ± 2.57
* (P<0.05).			* (P<0.05).

Table 4: Expression pattern of αvβ3and TF along with L.N involvement of CRC, based on spearman's correlation (rs)

LN	No.	integrin Mean ± SE	TF Mean ± SE
Free	**14**	**64.90 ± 3.85**	59.75 ± 3.46
Involved	**26**	**90.11 ± 1.74**	86.57 ± 1.60
* (P<0.05).			* (P<0.05).

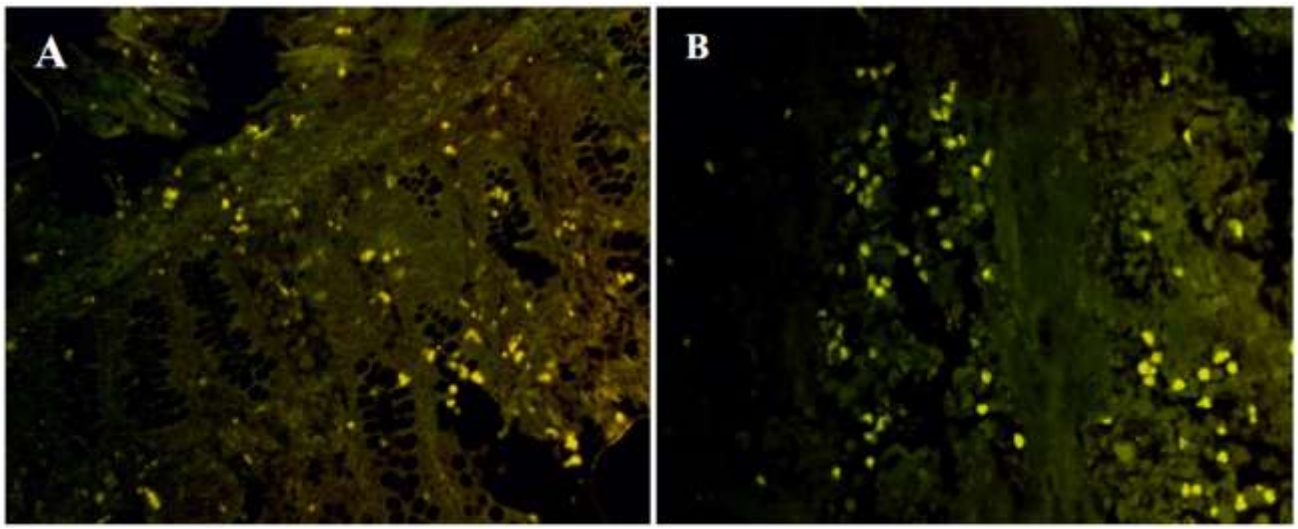

Fig 1: Immunoflourecent staining of TF (A), and β3 integrin (B), in colorectal adenocarcinoma section by FITC flouro chrome (Green color) with dark background. Magnification power (40X).

DISCUSSION

Current study had demonstrated a significant over expression of αvβ3, and TF protein expression in tumor tissue in compares with their resection margins (p<0.05, and p<0.05) respectively, table (1). This observation came in compatible with previously published data which mentioned that αvβ3 is positively correlated with angiogenic activity, invasion, and metastasis, [17]thus, it consider the leading edge of liver metastasis.

Moreover, current study showed that integrin β3 expression rate was significantly associated with tumor stage, poor differentiation, and lymphoid node invasion, (P<0.05, P< 0.05&P<0.05) respectively; table (18-20). Thus, it came with previous studies; they mentioned that an over expression of β3 integrin generally appears to be positively correlated with tumorigenicity. For example, expression of the β3 integrin subunit in melanoma in situ has been found to correlate with tumor thickness, the ability to invade and metastasize, and poor prognosis [21]. Also, increased expression of integrin β3 in gastric cancer influenced the adhesion between tumor cells and ECM. B3 integrin may influence signal transduction, thereby changing the biological behavior of tumor cells, and enhancing the potency of infiltration and migration [22]. Previous studies have shown that integrin αvβ3 is minimally expressed on resting or normal blood vessels, but is significantly up-regulated in vascular cells within human tumors, and has been implicated in tumor-induced angiogenesis [23, 24]. Unligated integrin can act as a negative regulator of cell survival, initiating a process of "integrin mediated death" [25].

The immunoreactivity of TF in colorectal cancer with L.N metastasis (Dukes' stage C) or with liver metastasis (Dukes' D) was significantly higher than in tumors without L.N or liver metastasis (Dukes' A, and B). These data suggest that there is a close relationship between TF and colorectal tumor metastasis. Several reports demonstrated that TF is involved in hematogenous metastasis of cancer cells [26, 27] and indirectly in tumor growth via its effect on

angiogenesis [28]. TF from tumor cells induces fibrin formation where cancer cells are trapped to initiate a new site of tumor growth [29]. TF-mediated thrombin generation activates cell growth by acting on thrombin receptor [30]. Mastuda *et al.;* demonstrated direct receptor function of TF evoked by ligand (FVIIa/VII) binding [31]. Neutralization of TF function in a human melanoma cell by anti-TF antibodies inhibits tumor adherence and local growth in severe combined immuno deficient mice [31]. It is conceivable that the relationship between TF expression and tumor cell progression of CRC depends on increases angiogenesis induced by TF. Collectively, both of β3 integrin and TF expressed by cancer cells appears to act as both a regulatory target and an important mediator of oncogene driven tumor growth and neovascularization. Which might act as a target for immune modulation therapy of cancer patients?

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