RESEARCH ARTICLE

Tumour infiltrating lymphocytes in colorectal cancers- correlation with tumour biology and clinical outcome: A cohort study

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Abstract

Objective: To evaluate the pattern of tumour infiltrating lymphocytes in colorectal cancers, and to correlate them with nuclear protein Ki67, vascular endothelial growth factor and clinical outcome.

Method: The retrospective study was conducted at the Nuclear Institute of Medicine and Radiotherapy and the Liaquat University of Medical and Health Sciences, Jamshoro, Pakistan, and comprised data of colorectal cancer patients from January 1, 2008, to December 31, 2018. Whole tumour sections of colorectal cancer were used with haematoxylin and eosin staining, Histological type, grade and infiltrated lymphocytes within the tumour block were assessed. Ki67 and vascular endothelial growth factor were evaluated by immunohistochemistry, while the staining of these biomarkers was assessed by the percentage of cells stained. Data was analysed using SPSS 22.

Results: Of the 201 patients, 110(54.7%) were males and 91(45.3%) were females. Overall median age was 43 years (range 10-85 years). Majority of the tumours 132(65.7%) showed mild to moderate tumour infiltrating lymphocytes, 30(14.9%) had severe tumour infiltrating lymphocytes, while 39(19.4%) did not show any infiltrating lymphocytes. Tumour infiltrating lymphocytes did not show significant association with the histological grade (p>0.05), but high tumour infiltrating lymphocytes were associated with poor survival without being significantly associated with Ki67 pattern and vascular endothelial growth factor (p>0.05).

Conclusion: Majority of colorectal cancer cases showed varying levels of lymphocyte infilteration, and tumour infiltrating lymphocytes were associated with poor survival, without having significant association with Ki67 pattern and vascular endothelial growth factor.

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Introduction

Tumour infiltrating lymphocytes (TIL) are the immune cells which infiltrate tumours in an attempt to kill cancer cells. These cells play a pivotal role in killing abnormal cells, but their activity in cancer has limited understanding. There are multiple types of immune cells with variable functions. Cytotoxic T-cells can potentially play a key role in killing tumour cells directly, and B-cells/plasma cells can also play a role by providing antibodies against tumour antigens.[1] The gastrointestinal tract (GIT) has its own diverse population of lymphocytes, which helps protecting GIT. Main population of the gut lymphocytes are intra-epithelial, which comprises B-lymphocytes and T-lymphocytes.[2] Recent studies have discovered further types of lymphocytes in the gut. Nevertheless, the information of the role of these lymphocytes in cancers is limited. Colorectal cancers (CRCs) are the fourth common cancer and third leading cause of cancer-related mortality around the world[3] The CRC incidence in 2020 in Pakistan was 19.5%, while the global rate was 17.5%. According to Globocon prediction, CRC rate will rise in 2040 by 58% around the world, while in Pakistan it is expected to rise by 81% which means 8688 case in 2040 compared to 4801 in 2020.[3] The survival post-CRC is not promising due to late diagnosis and advanced stage of the disease at the time of diagnosis. Since the CRC rate is expected to rise in the coming years, it is critical to look at potential prognostic and predictive factors.

Available literature suggests that TIL not only showed prognostic significance, but also has links with response to adjuvant radiotherapy and chemotherapy where it appeared to be associated with better survival outcome.[4,5] TIL quantity as well as their function is very important to be studied. A meta-analysis comprising 21,015 patients evaluated TIL presence and showed association with significantly improved survival outcome.[4] The meta-analysis included studies which explored the presence of TIL, different subgroups and location (i.e. centre of the tumour, invasive margins and tumour stroma).[2] Similar results were reported by another systematic review.[6] There were inconsistencies in the scoring method of TIL and their location. Many studies have used only cell lines. There was

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limited literature available to correlate TIL with tumour characteristics and molecular markers.[6]

The current study was planned to evaluate the presence of tumour-infiltrating lymphocytes in whole tumour sections, and to correlate with proliferation marker Ki67 and vasculogenesis marker vascular endothelial growth factor (VEGF) by using immunohistochemistry. Additionally, it was planned to correlate tumour-infiltrating lymphocytes with CRC-specific survival.

Materials and Methods

The retrospective study was conducted at the Nuclear Institute of Medicine and Radiotherapy (NIMRA) and the Liaquat University of Health and Sciences (LUMHS), Jamshoro, Pakistan, and comprised data of CRC patients from January 1, 2008, to December 31, 2018. CRC cases included were those registered at NIMRA and LUMHS whose clinical information, along with good quality tumour sample, was available from diagnosis till death or last follow-up. These patients received primary surgical therapy without any neo-adjuvant systemic or radiotherapy. After their diagnosis, they received treatment as per the institutional policy.

Formalin fixed, paraffin embedded (FFPE) tumour blocks were retrieved and Haematoxylin & Eosin (H&E) staining was done as baseline confirmation of the tumour characteristics. Histological type, grade and infiltrated lymphocytes within the tumour block were assessed. Whole tumour sections were studied.

Guidelines for TIL assessment in solid tumours recommended by the International Immuno-Onoclogy Biomarker Working Group were followed.[7] Tumour centre and periphery were both evaluated (Figure 1).

The invasive margin was defined as a 1mm region cantered on the border separating the malignant cell nests from the host tissue. The central tumour represented the remaining tumour area.

TILs in tumour zones with crush artefacts, necrosis and regressive hyalinization were excluded.[7] The inflammatory cells within tumour were counted. Initially, the areas with high proliferation were identified and cells were counted and average of the five areas was then calculated as the final rate of proliferation. The severity of the inflammatory cells was then compared with the percentage of tumour cells. No proliferation per field (0 in whole slide) was labelled as no proliferation, mild showed <25% of inflammatory cells compared to tumour cells, between 26-50% were moderate, and >50% were severe infiltration. For inter-observer concordance, 25% slides were randomly

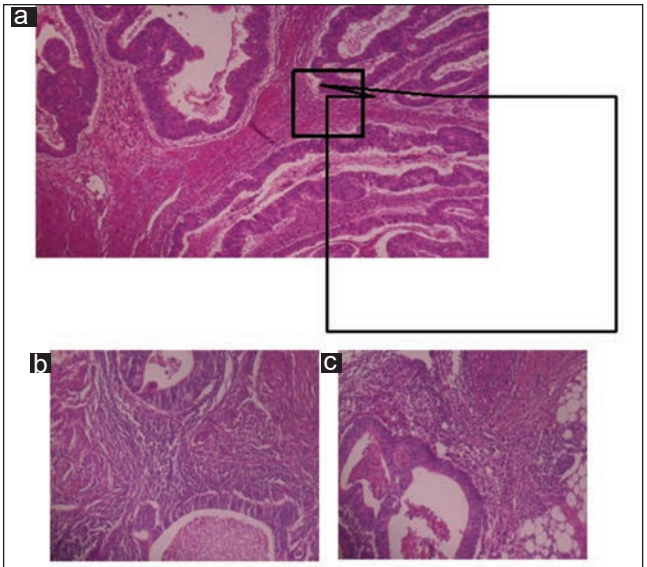

Figure-1: a) Tumour infiltrating lymphocytes pattern (H&E staining), b) Tumour infiltrating lymphocytes pattern (H&E staining), c) Tumour infiltrating lymphocytes pattern (H&E staining).

scored by BMS (Corresponding author). Kappa statistics were performed for all markers for intraobserver and interobserver concordance. For both, the Kappa score was 0.9-1.0 and 0.8-1.0, respectively. Olympus BX40 was used for assessment.[8]

Ki67 and VEGF were evaluated by using indirect immunohistochemistry (IHC) on whole sections. Primary antibodies were Dako ready to use with 30 and 40 minutes of incubation at room temperature, respectively. For secondary antibodies, Abcam horseradish peroxidase (HRP) was used. The method was the same as previously reported.[9]

IHC staining of biomarkers assessed by the percentage of cells stained as well as McCarty's IHC scoring H-score ranged 0-300[10] According to the cut-off percentage of cells used to define positivity/negativity, 10% of cells for Ki67 and 5% for VEGF were considered positive.

Data was analysed using SPSS 22. Mean and median values were used for continuous variables. Chi-square test was used for categorical data. $P<0.05$ was considered significant. Disease-free survival (DFS) and overall survival (OS) was analysed using Kaplan-Meier method. Log-rank and Wilcoxon tests were applied for significant results. For analysis, TIL-positive cases were put together as 1 and no TIL was considering as negative. The correlations were dichotomous as well as according to the categories.

Results

Of the 201 patients, 110(54.7%) were males and 91(45.3%) were females. Overall median age was 43 years (range 10-

Table-1: Pattern of tumour inflammatory lymphocytes (TIL) in colorectal cancer (CRC) cases and correlation with nuclear protein Ki67 and vascular endothelial growth factor (VEGF).

	n (%)			
TIL Pattern				
No infiltration	39(19.4)			
Mild infiltration	78(38.8)			
Moderate infiltration	54(26.9)			
Severe infiltration	30(14.9)			
Grade	**Lymphocytic infiltration**			***p*-value***
	No TIL	**Mild to moderate TIL**	**Severe TIL**	0.179
I (n=59)	9(15.3)	42(71.2)	8(13.6)	
II(n=97)	18(18.6)	68(70.1)	11(11.3)	
III (n=43)	11(25.6)	22(51.2)	10(23.3)	
Status of perforation				
Yes	1(12.5)	6(75.0)	1(12.5)	0.95
No	9(22.0)	27(65.9)	5(12.2)	
Ki67				
Positive	25(20.2)	78(62.9)	21(16.9)	0.50
Negative	14(18.2)	54(70.1)	9(11.7)	
VEGF				
Positive	2(18.2)	9(81.8)	0	0.33
Negative	37(19.5)	123(64.7)	30(15.8)	

*Chi-square test was applied

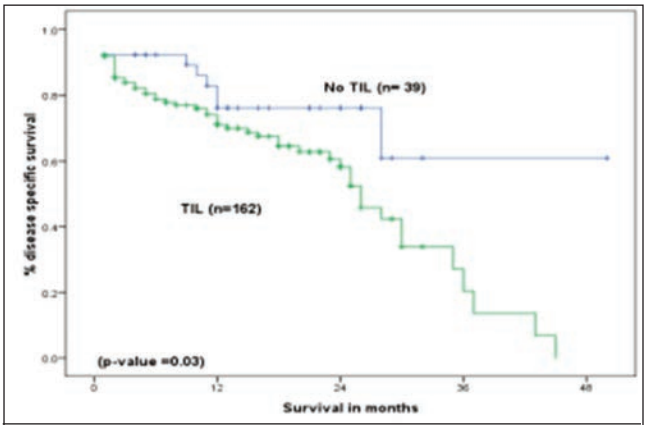

Figure-2: Colorectal cancer (CRC) specific survival in relation to tumour infiltration lymphocytes (TIL) (Kaplan Meier Method was used with application of Log-rank test).

85 years), and mean age was 43.35±17.43 years.

Majority of the tumours 132(65.7%) showed mild to moderate TIL, 30(14.9%) had severe TIL, while 39(19.4%) did not show any infiltrating lymphocytes (Table).

TILs did not show significant association with the histological grade (p>0.05), but high TILs were associated with poor survival without being significantly associated with Ki67 pattern and VEGF (p>0.05).

At the time of analysis, 130(64.7%) subjects were alive, 67(33.3%) died of the disease with metastases, and 4(2%)

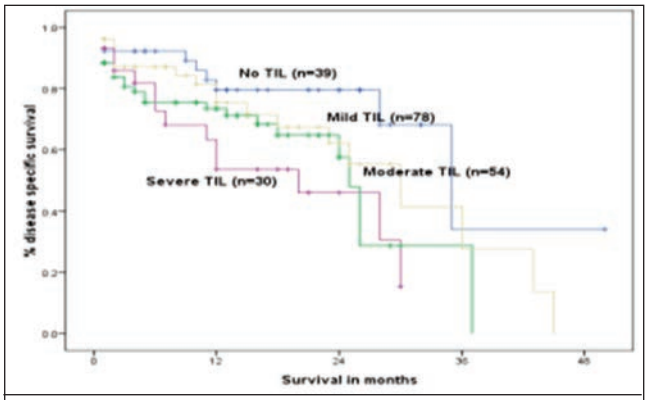

Figure-3: Colorectal cancer (CRC) specific survival in relation to tumour infiltration lymphocytes (TIL) categories (Kaplan Meier Method was used with application of Log-rank test).

patients died of other causes without any sign of metastases at the time of the last follow-up. Median follow-up was 11 months (range: 1-50 months), median overall and DFS survivals were same at 28 months (Range 3- 50 months). Severe lymphocytic infiltration was associated with significantly lower disease-specific survival (p=0.03) (Figures 2-3).

Discussion

The study reported a higher rate of TIL in colorectal treatment-naive tumours, and it was significantly associated with poor survival. The tumours with high TIL were not significantly associated with grade or Ki67 and VEGF.

TILs are the indicator of inflammatory response to cancer which is taken as a foreign antigen. Recent literature has shown growing interest in taking this as a prognostic and predictive marker as high TIL count has been reported to be associated with longer survival.[9] However in the current study, the TIL rate was associated with shorter survival in a population with already poor clinical outcome. This is in contrast with most of the available literature which suggested better survival outcome when there was high TIL count.[11] This raises an important point in immune-therapeutics as high TIL probably suggests aggressive tumour biology or endogenous (intra-tumoral) immunosuppressive mechanisms which suppress TIL function.

A retrospective review of 546 patients showed that overall TIL was not significantly influencing factor, but in N2 stage disease the influence was significant. However it was a subgroup analysis with a small number of patients.[12] These findings are at large consistent with the current findings, and the inconsistencies may be due to the method of defining TIL.

The consistency in the methods of measuring TIL has not been seen. Some studies have reported periphery, others reported centre, and some studies have reported an average of stroma, periphery and centre of the tumour. However, it is very much likely that each location of the TIL accumulation could have different influence on the tumour cells. A study suggested that the peripheral and central invasion in CRC was similar and their presence was associated with lymph node invasion and further progress of the disease.[13] A study comparing the presence of TIL in primary and metastatic cancers showed significant correlation.[14] H. Lee et al. reported in a series on 1532 stage III CRC patients that the TIL density significantly varied according to the size of the tumour, lymph node stage, where larger tumour, higher lymph node stage and poorly differentiated cancers showed high TIL rate.[15] Similarly, correlation of the tumour size was reported in a small series which also reported cytotoxicity of cluster of differentiation (CD)8+ cells in tumour cells.[16]

The concept of the TIL evaluation is not new in cancer biology. Studies have shown that the rate of TIL infiltration was normal, but the function of these cells was lower when natural killer cells were studied.[15,16] However, other studies claimed to have no difference in the TIL and other lymphocytes function.[18] A study presented more insight on the significance of TIL location, where CD3+ cells were strong predictors of survival at peripheral margins.[19] This is an interesting finding that suggests difference in function at different locations of the tumour. Thus, if TILs are characterised according to type and then their functions are defined, this may open up a whole new spectrum of immunotherapeutics in cancer. Another study explored the role of tertiary lymphoid tissue (TLT) with TIL in CRCs and showed coordination between them.[20] Thus, not only TILs but TLTs also play a role in programming of these cells to target tumour cell.

A small-scale but important study identified a number of antigens on cancer cells, and coordinated them on T lymphocytes and highlighted the markers on both sides to evaluate any influence on the outcome[21] The pattern of specific receptors and antigens present on tumour cells as well as on lymphocytes should be studied to identify the cells with the best possible clinical outcome. The role of post-operative TIL or post-systemic therapy TIL has been linked with better treatment outcome.[21-23] Most of the studies reported a significant role of TIL in stage II disease.[23] The current study did not explore specific types of the lymphocytes.

In terms of limitations, the current study did not do IHC determination of TIL, and the subtypes of TIL were not studied. Further studies to evaluate type of cells and their functional status are recommended.

Conclusion

Significant influence of TIL on survival was found. Ki67 and VEGF did not significantly influence the TIL pattern, but tumours with no TIL showed better survival.

Disclaimer: The text is based on an academic thesis.

Conflict of Interest: None.

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