Role of Fibrotic Cancer Stroma in Rectal Carcinoma: An Immunomorphological Assessment

Abstract

Background: The aim of the study is to evaluate the role of fibrotic cancer stromal response and tumor budding in ectal adenocarcinoma development and progression. Materials and Methods: Fibrotic cancer stroma was classified into three distinct histological categories, i.e. mature, intermediate, and immature. The number of tumor-budding foci was counted in the low-power field ($\times 10$), and 0–5, 5–9 and ≥ 10 tumor buds were scored as I, II, and III, respectively. All histological and immunohistochemical assessments were made at the invasive front of the tumor. The distribution of T lymphocytes and myofibroblasts was assessed by immunohistochemical reactivity for the cluster of differentiation 3 and anti-smooth muscle antibody actin, respectively. Results: Among 25 cases of rectal carcinoma, 60% (15 cases) of patients had mature fibrotic cancer stroma, whereas 28% (7 cases) of patients had intermediate stroma and 12% (3 cases) of patients had immature stroma. The cancer-specific 5-year survival rate in the groups with mature stroma, intermediate stroma, and immature stroma was 53.34%, 42.8%, and 33.34%, respectively. There was a statistically significant correlation between the category of fibrotic cancer stroma and the tumor budding. Further, on immunohistochemical analysis and counting, the average number of T-cells was 302/400 µm diameter field in the region of mature fibrotic stroma, in comparison with 197/400 μ m and 92/400 μ m in the intermediate and immature fibrotic stroma, respectively (unpaired *t*-test with P < 0.05). Myofibroblasts were observed in 20% of tumors with mature fibrotic stroma compared with 65% in the intermediate fibrotic stroma and 100% of the tumors with immature fibrotic cancer stroma. Conclusions: The histological classification of fibrotic cancer stroma highlights the role of the stromal response with respect to host immune reaction and behavior in rectal adenocarcinoma and acts as a useful tool for predicting patient prognosis and outcome.

Keywords: Colon adenocarcinoma, desmoplasia, myofibroblast

Introduction

Rectal Cancer is common cancer worldwide, but the incidence in India is comparatively low. The geographical variation is significant if we compare the incidence with western countries; however, mortality is higher in the developing country due to inadequate infrastructure and limited resources. According to the Globcan 2020, the prevalence of CRC was 6.3% in males and 3.7% in females in India, and the total number of new cases reported was 65,358 in 2020.^[1]

However, in a country like India, having a population in billions, the absolute number of patients with CRC is large, but the 5-year survival is <40% due to inadequate diagnosis and treatment.

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Over the last two centuries, clinical and pathological observations have established a clear relationship between chronic inflammation, fibrosis, and cancer. Fibrosis can precede or follow cancer development and may participate in multiple stages of tumorigenesis and metastasis. Cancer is characterized by tumor cell invasion which in turn is associated with complex interactions between the neoplastic cells and surrounding host matrix;^[2] previously, it was stated that desmoplasia is a poor prognostic indicator, but recent reports, on the contrary, support the hypothesis that the desmoplastic response limits tumor aggressiveness.^[3] This has not only been studied in colorectal carcinoma but also seen in other tumors such as breast carcinoma, cholangiocarcinoma, pancreatic carcinoma, and pulmonary carcinoma.[3-5] The theory of desmoplastic response having opposing effects on malignant behavior of the tumor suggests that, during the

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process of neoplastic growth, different processes operate independently.^[6,7] The interactions between surrounding host tissue response and neoplastic cells are most frequently assessed quantitatively. However, this complex phenomenon is not assessed by the amount of fibrosis alone but also by its qualitative nature. Further, the role of myofibroblasts in remodeling the tumor microenvironment is increasingly being studied with its upcoming role in the prognostication of colorectal carcinoma. Recently, myofibroblast is also thought to have a role in limiting T-cell locomotive function, apart from its usual contractile function. All these factors have an impact on the prognosis of patients with aggressive malignancies including colorectal carcinomas.^[8-10]

Keeping in view these considerations, we retrospectively analyzed cases of rectal carcinomas with a qualitative assessment of stroma, as well as T-cell distribution and myofibroblast identification in the desmoplastic stroma, and correlated it with usual prognostic indicators and survival.

Materials and Methods

The archives of the department of histopathology were retrospectively reviewed from January 2013 to December 2014. Of the 28,000 pathology case records reviewed, cases of rectal carcinomas who underwent radical surgery were retrieved and included in the study.

Exclusion criteria

1. Patients with synchronous tumors or adenocarcinomas arising in a setting of familial adenomatous polyposis



Figure 1: (a and b) Hematoxylin and eosin-stained sections showing mature fibrotic cancer stroma composed of fine and elongated fibers with fibrocytes; these fibrocytes are stratified into layers. (×40)



Figure 3: (a and b) Hematoxylin and eosin-stained sections showing immature fibrotic cancer stroma showing randomly oriented broad collagen bundles surrounded by myxoid stroma. (x40)

or inflammatory bowel disease were excluded from the study

- 2. Furthermore, patients who had received preoperative chemotherapy or radiation therapy were excluded from the study group
- 3. Patients who died in the postoperative period of 1 month were also excluded.

Following surgical excision, the specimen was fixed in 10% neutral-buffered formalin and sent for histopathological evaluation. The diagnosis was confirmed on paraffin-embedded hematoxylin and eosin-stained (H and E) sections. Follow-up of the patients were done for 5 years or until death whichever was earlier.

Fibrotic cancer stroma was classified into three distinct histological categories, i.e. mature, intermediate, and immature.^[11-13]

- Mature: When composed of fine and elongated fibers with fibrocytes, and the fibrocytes are stratified into layers [Figure 1a and b]
- Intermediate: When broad keloid-like bands of brightly eosinophilic collagen intermingled with mature collagen fibers [Figure 2a and b]
- Immature: Stroma shows broad collagen bundles (keloid-like), randomly oriented surrounded by myxoid stroma [Figure 3a and b].

This histological assessment of stroma was made at the invasive front in the mesorectum or muscularis propria, and the most unfavorable stromal area was considered for categorization.



Figure 2: (a and b) Hematoxylin and eosin-stained sections showing mature fibrotic cancer stroma composed of fine and elongated fibers with fibrocytes; these fibrocytes are stratified into layers. (×40)



Figure 4: (a and b) Tumor budding at the invasive front of the tumor (H and E stain; a, $\times 10$; b, $\times 40$)

How to grade tumor budding?

Furthermore, the tumor budding at the invasive front of the tumor was assessed. Budding focus is defined as an area where there is an isolated single cancer cell or a cluster of tumor cells <5 in number, at the invasive front [Figure 4a and b]. The number of budding foci was counted in one low-power field (×10). Numbers of tumor buds 0–5, 5–9 and \geq 10 were scored as I, II, and III, respectively. Along with the tumor budding, other prognostic features such as tumor type, diameter, and lymph node invasion were also recorded.^[12]

In addition, the distribution of T-lymphocytes and myofibroblasts was assessed by immunohistochemical analysis. A cluster of differentiation 3 (CD 3) immunohistochemical marker was used to assess the density of T-cells at the invasive front of the tumor. The cells were counted on high-power magnification (\times 40). Five areas with the most abundant distribution were selected, and the average number was calculated. Anti-smooth muscle antibody (anti-SMA) actin immunoreactivity was identified the myofibroblasts.^[14,15]

Immunohistochemistry procedure

In brief, sections measuring 3–4-µm thick were cut, deparaffinized with xylene, and brought to water through graded levels of alcohol. Endogenous peroxidase activity was blocked by treating the slides with hydrogen peroxide for 30 min at room temperature. Antigen retrieval was done using the pressure cooker method by immersing the slides in citrate buffer. Then, the slides were incubated overnight with the primary antibody (calretinin, rabbit polyclonal) at 4°C in a humidified chamber. The following day, secondary antibody was added. The sections were then incubated with di-amino-benzidine chromogen for visualization of the peroxidase reaction. After being washed in water, the sections were counterstained with hematoxylin, dehydrated in alcohol, cleared in xylene, and mounted.

CD-3 immunoreactivity for T-cells (membranous) and SMA immunoreactivity for myofibroblasts (cytoplasmic) were then evaluated as mentioned above.

H and E-stained sections and immunohistochemistry slides of all the cases were reviewed by two pathologists independently; then, two histopathologists evaluated the slides together, and occasional discrepancies were resolved



Figure 5: Immunohistochemistry. (a) Smooth muscle actin (SMA) in immature stroma showing diffuse, dense localization of myofibroblasts in the reactive fibrous zone. (×40), (b) Smooth muscle actin in the mature stroma showing very scant smooth muscle actin positive myofibroblasts in the reactive fibrous tissue (×40)

followed by a common consensus score. Finally, the findings were statistically evaluated. Statistical analysis was done using the Kaplan–Meier test, unpaired *t*-test, and Mann–Whitney - test, using IBM SPSS Statistics for Windows, Version 21.0., Armonk, NY, USA: IBM Corp.

Results

Of the 28,000 pathology case records reviewed, there were 45 cases of rectal carcinoma who underwent radical surgery; however, only 25 of these cases of rectal carcinomas were included in the study who satisfied our inclusion and exclusion criteria and in whom follow-up was available. The mean age at presentation was 54.5 years (range 48–65 years). There were 17 male and 8 female patients with a male: female ratio of 2.1:1.

Among the study group, 60% (15 cases) of patients had mature fibrotic cancer stroma whereas 28% (7 cases) had intermediate stroma, and 12% (3 cases) had immature stroma [Table 1]. Cancer-specific 5-year survival rates in the group with mature stroma, intermediate stroma, and immature stroma was 53.34%, 42.8%, and 33.34%, respectively [Table 1].

The number of budding foci of carcinoma cells was maximum with immature stroma and decreased with the maturity of stroma [Table 2]. There was a statistically

Table 1: Survival rates of 25 patients as per the maturation of fibrotic stroma		
Maturation of stroma	Number of cases (%)	5 years survival rate (%)
Mature	15 (60)	53.34
Intermediate	7 (28)	42.8
Immature	3 (12)	33.34
Total	25	

Table 2: Foci of tumor budding in 25 cases of rectal carcinoma Maturation Grade I (n) Grade II (n) Grade III (n) Total of stroma Mature 10 4 1 15 Intermediate 2 2 3 7 Immature 0 0 3 3 7 25 Total 12 6



Figure 6: Immunohistochemistry (a) showing cluster of differentiation three positive lymphocytes, significantly decreased in a case of carcinoma rectum with immature fibrotic cancer stroma at the invasive front. (×40) Immunohistochemistry (b) showing significantly increased cluster of differentiation three positive lymphocytes, in a case of carcinoma rectum with mature fibrotic cancer stroma at the invasive front. (×40)

significant correlation between the category of fibrotic stroma and the tumor budding.

The presence of myofibroblasts based on SMA reactivity was observed in 20% of tumors with mature fibrotic stroma compared with 65% in the intermediate fibrotic stroma and 100% of the tumors with immature fibrotic stroma [Figure 5a and b]. Further, on immunohistochemical analysis and counting, the average number of Tcells was 302/400 μ m diameter field in the region of mature fibrotic stroma, in comparison with 197/400 μ m and 92/400 μ m in the intermediate and immature fibrotic stroma, respectively. Thus, the number of CD 3 lymphocytes were significantly decreased with loss of maturation of the surrounding stroma [Figure 6a and b] (unpaired ttest with *P* < 0.05).

Discussion

Accumulated knowledge, regarding the extracellular matrix over the year, suggests that tumor stroma plays an important role in promoting tumor progression than previously believed. Recent scientific research suggests that there is an important and crucial role of interaction between neoplastic cells and surrounding reactive stromal matrix in the understanding of tumor cell invasion and behavior. It is thought that the process of dissociation of neoplastic cells and dedifferentiation of malignant cells is the initial step in tumor invasion and metastasis by interacting with stroma at the invasive edge.^[11]

Rectal Cancer is one of the most common carcinomas in the developed countries; however, the prevalence in India is less (4.9% of all cancer reported), but 5-year survival is very poor.^[1] Studies have shown that the most common age group affected is the elderly with male preponderance which is in concordant with our present study.^[16-19] In the recent past, tumor-associated stromal microenvironment, which includes proliferating blood vessels, fibroblasts, myofibroblasts, molecules, and other associated extracellular components, which is in the immediate vicinity of the growing malignant tumor cells, are thought to be important regulators of tumor behavior and invasion.^[11,12] In our study, immature stroma was associated with worse prognostic outcomes, while mature stroma was associated with good outcomes. These results suggest that stromal classification can have important prognostic information and more significant than anatomical extent (pTNM) of disease in rectal cancer.

It is important to understand the mechanism of underlying tumor cell invasion, which requires analysis of the complex interactions between the surrounding matrix and the neoplastic cells.^[2,16,17] Furthermore, in-depth analysis of the molecular mechanisms underlying the metastatic process will not only identify individuals at greatest risk of recurrence but also allow the discovery of new tumor targets to prevent disease progression.^[18,19]

The primary step of tumor invasion is the process of dedifferentiation and dissociation of neoplastic cells

at the invasive front of the tumor. While inductive signals from the host microenvironment are thought to be involved in initiating and maintaining this shift by repressing or activating the preformed genetic programme of tumor cells, conversly the collagen-I protein, which is an important microenvironment factor and inhibits the process of dedifferentiation of tumor.^[8,9,11] There is also a significant correlation between the intensity of tumor budding (i.e. dissociation of cancer cells and invasion) and the maturation of fibrous stroma. Similar studies were done by few other authors which are compatible with our present study.^[11,20]

In colorectal carcinoma, many analyses showed a relevant correlation between cancer stroma and patient prognosis. Studies were done by Van Wyk *et al.* and Otranto *et al.* which concluded that mature stroma is the most stable phenotype; the neoplastic as well as invasive activity is very less as compared to the intermediate or immature stroma, whether the tumor is located at the center or in the invasive front.^[20,21] Above studies are concordant with our study of 25 patients in which the mature fibrotic stroma had a better 5-year survival of 53.34% as compared to intermediate and immature stroma where the survival was 42.8% and 33.34%, respectively.

The primary cellular component of the extracellular matrix is the myofibroblast. Myofibroblasts are contractile, secretory cells producing extracellular matrix proteins and cytokines. These cells have now been shown to regulate several tumor-promoting functions including angiogenesis, invasion, and metastasis.^[14,21] Myofibroblast density is usually greatest at the invasive front of the tumor, and several studies have shown that these cells promote tumor invasion by secreting soluble factors.^[10] The latter along with matrix metalloproteinases and their inhibitors alter and remodel the composition of the tumor microenvironment, thereby having a prognostic role in malignancies. These cells are reported to be associated with poor prognosis in several carcinomas, including colorectal carcinoma.^[22-25]

Owing to the contractile properties of myofibroblasts, these are believed to create a physical barrier around the tumor against the immune reaction mounted by the host. This mechanism of myofibroblasts results in the paucity of lymphocytic infiltration and correlates with a poorer prognosis.[26-28] However, this is one of the many proposed hypotheses of tumor-induced inhibition of lymphocyte locomotion. The exact mechanism of the same remains elusive to date. The results of our study, i.e. infiltration of T-lymphocytes, were less in the immature stroma, while myofibroblasts distribution was extensive as compared to mature and intermediate. This was in concordance with the previously explained hypothesis. As mentioned earlier by Ueno et al. in 2004,^[12] we concluded that immature fibrotic stroma inhibits the reach of immune cell to the tumor area, hence facilitates the growth of neoplastic cells. Hence, this

is the most common reason for cancer cocoon by immature stroma, which leads to poor prognosis, which was verified in our cohort of 25 cases. Earlier similar results have been obtained by Ueno *et al.* in a cohort of 862 cases.^[13]

Conclusions

While the prognosis of most malignancies is linked to the features of carcinoma cells, our study focuses on the less studied host stroma. The tumor stroma including fibroblasts and inflammatory cells plays an important role in regulating tumor progression. It is increasingly clear that tumor stroma plays a crucial role in colorectal carcinoma development and progression. Understanding the role of the stromal cells and extracellular matrix will allow us to identify more precise prognostic markers and potentially devise new therapeutic options.

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Conflicts of interest

There are no conflicts of interest.

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