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Original Research Article

Immunohistochemical Detection of Bone Marrow Micrometastasis in Cases of Colorectal Malignancies

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Abstract

Colorectal carcinoma is one of the tumors with high incidence of cancer related death rate mostly due to metastasis. Tumor spread to blood and bone marrow is the most crucial step in systemic tumor spread. Disseminated tumor cells (DTC) / Micrometastasis are defined as occult cancer cell clusters less than 0.2 mm which can be by Immunohistochemistry (IHC) by epithelial specific antigens like cytokeratin. IHC techniques can pick as few as 1 to 2 tumor cells in 1×10^6 hematopoietic cells. Patients with presence of DTC in bone marrow had shown to have a shorter survival time and have shorter distant metastatic disease-free survival time than patients with negative bone marrow status. Many studies have proved the detection of DTC in bone marrow is an independent prognostic marker which can help in planning the appropriate treatment strategy.

Aims and Objectives: To study the incidence of bone marrow micrometastasis and its prognostic significance in colorectal malignancies.

Material and Methods: This is a 3-year study proposed and conducted in the Tirunelveli Medical College Hospital during September 2011 to July 2013. 50 patients with small biopsy proved diagnosis of malignancy in colorectal region were selected. All patients were followed and resected specimens were evaluated. Tumor Sections were studied to note the microscopic type of tumor, grade, depth of invasion and number of nodes positive for metastatic carcinomatous deposits under light microscopy. Patients were followed post operatively and before administration of chemotherapy bone marrow aspirations were taken for all 50 patients. Bone marrow smears were prepared and fixed in methanol, of which 3 smears were stained with Leishman stain and 3 smears were fixed for imunostaining (pancytokeratin).

Observation and Results: Out of 50 cases 11 cases showed presence of disseminated tumor cells (DTC) in bone marrow. 3 cases from T2 stage and 8 cases from T3 stage were positive for DTC. 5 cases of N1 stage and 6 cases of N2 stage were positive for DTC. All 12 cases with well differentiated morphology (12 cases) were negative whereas 4 out of 22 cases with moderately differentiated and 7 out 16 of cases with poorly differentiated morphology were positive for DTC. 11 out of 39 cases at DUKE Stage C were positive but both Stage A (4 cases) and Stage B (7 cases) were negative.

Conclusion: Our study has enumerated the importance of detection of disseminated tumor cells in Bone marrow aspirates of colorectal malignancies using IHC (Cytokeratin). Bone marrow

micrometastasis detection rate increases with increase in grade of tumor, depth of invasion and stage of tumor. Our study also concludes that disseminated tumour cells in Bone Marrow aspirates of colorectal carcinoma could have an independent prognostic value which helps the surgeons and oncologists to plan a appropriate treatment strategy.

Keywords: Colorectal Carcinoma, Bone Marrow Micrometastasis, Disseminated Tumor Cells, IHC (Cytokeratin).

Introduction

Carcinoma of colon and rectum are one among the important causes of death due to cancer and fourth most common cancer in the west [1,2]. Colorectal carcinoma is one of the tumors with high incidence of cancer related death rate and incidence next only to breast cancer among women and comes third after prostatic carcinoma and carcinoma lung in men, accounting for 13.1% and 12.8% of all forms of cancer, respectively [3]. In2006, 412,900 CRC cases were newly diagnosed in Europe, whereas approximately 207,400 deaths from colorectal cancers were certified [3].

Among the treatment modalities of GI cancer, surgery is the most preferable therapeutic option which can be attempted with curative intention in 80% of patients with local or locally advanced cancer. However, a relapse can nevertheless be expected in up to 40% of these patients4. In Dukes' A stage, it can be expected that 10-15% of patients will develop recurrence. In Dukes' B and C stages the probability of recurrent disease development increases to 25% and 65%, respectively [4]. Prognosis depends on pathological staging. This may be based on Dukes classification, Astler – Coller classification. Now universal standardised staging system TNM system is used.

Isolated tumor cells (ITC) /Micrometastasis is defined by sixth edition of TNM classification are cancer cell clusters less than 0.2 mm [5]. They are detected by IHC and molecular methods. Presence of bone marrow isolated tumor cells even in patients with tumor that do not usually cause bone metastasis, indicates that these cells remain in a state of dormancy with a reduced proliferative capacity [6]. Provided an appropriate microenvironment, these dormant cells become dynamic and induces bone metastasis. Presence of disseminated cells in bone marrow is considered a sign of general spread of tumor cells or minimal residual disease rather than indication of early skeletal metastasis [6].

As tumor specific markers to identify the disseminated cells are lacking ,the use of epithelial specific antigens like cytokeratins associated to cytoskeleton , surface adhesion molecules or receptor for growth factor are used for detection of disseminated cancer cells / circulating cancer cells [7,8]. Considering the heterogenicity in antigenic profile of tumor cells, a broad spectrum anti cytokeratin i.e combination of several antibody to various cytokeratin antigens is being used for detection [9]. Immunohistochemical techniques are highly sensitive and can pick as few as one to two tumor cells in 1×10^6 hematopoietic cells [9].

The detection rate of micrometastasis in bone marrow of colorectal carcinoma patient is reported to be 12 percent to 60 percent in contrast to the fact that only 4 percent to 8 percent cases show overt skeletal metastasis [10,11]. The detection rate increased to around 65 percent when sensitive techniques like PCR or methods of enrichments were used [11,12]. With respect to clinical outcome, ITCs detected by immunocytochemical techniques proved to be an independent prognostic marker in multivariate analyses. Numerous studies

have shown that patients with disseminated tumor cells in bone marrow had shorter distant disease free survival time than patients with negative bone marrow status. Patients with presence of DTC in bone marrow had shown to have a shorter survival time [11].

Hence need to detect the occult disseminated cells have gained prognostic significance. We performed study in 50 patients with colorectal malignancies and results are discussed.

Materials & Methods

This is a 3 year study proposed and conducted in the Tirunelveli Medical College Hospital. Patients with small biopsy proved diagnosis of malignancy in colorectal region who came to Surgical and Oncology OP were evaluated. Of these, 50 patients were selected for our study.

All 50 Patients were followed and underwent surgical resection. All 50 Resected specimens were received in Department of Pathology were evaluated and carefully examined grossly for size of tumor, depth of invasion and extensive search for lymph nodes made. Tumor Sections were processed and studied to note the microscopic type of tumor, grade, depth of invasion and number of nodes positive for metastatic carcinomatous deposits under light microscopy.

All 50 Patients were followed post operatively .Before starting the chemotherapy bone marrow aspirations were taken from the all 50 patients after obtaining written consent from the patient or his/her relative. Under local anaesthesia with strict aseptic precautions bone Marrow aspirate was obtained by direct puncture of posterior iliac crest using bone marrow needle. Marrow Smears were prepared and fixed in methanol, of which 3 smears were stained with Leishman stain by conventional standard procedures. Equal number of slides was fixed with for imunostaining (pancytokeratin).

Observation and Results

Of 50 cases studied 11 cases showed presence of disseminated tumor cells (DTC) in bone marrow.

Table 1: Detection rate of disseminated tumor cells in marrow.

Total no of Colorectal carcinoma studied	50
No of DTC (Disseminated Tumor Cells) + cases	11(22%)
No of DTC (Disseminated Tumor Cells) – cases	39 (78%)

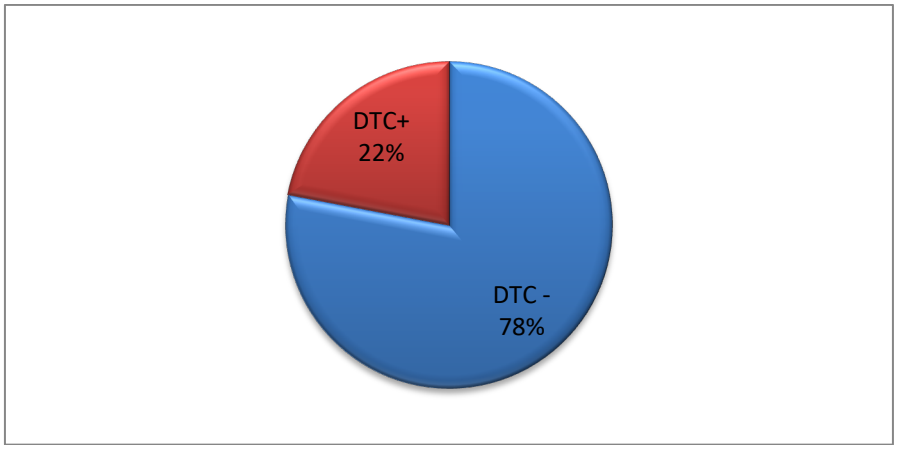

Chart 1: Detection Rate of DTC

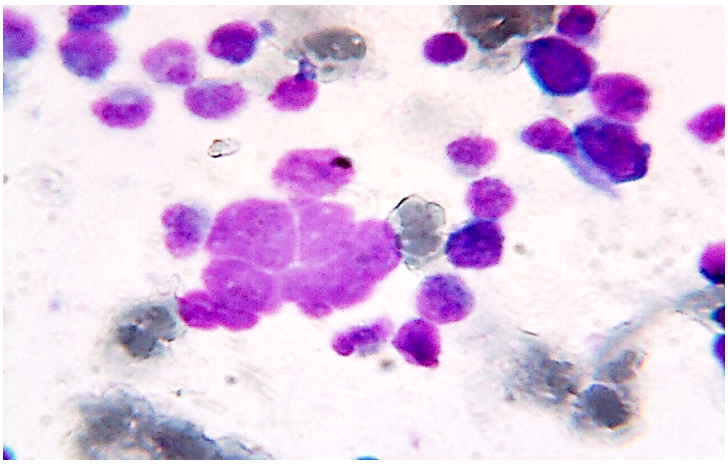

Figure 1: Photomicrograph showing clusters of malignant epithelial cells in background of hematopoietic cells (Leishman stain x100)

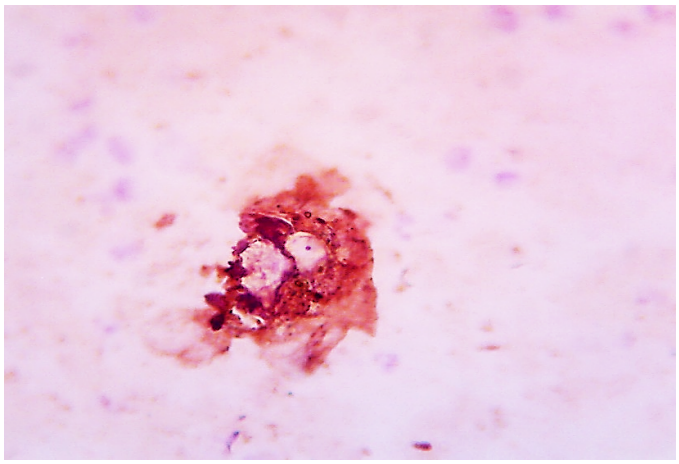

Figure 2: Photomicrograph showing DTC positivity for pancytokeratin x 40

Table 2: Depth (T) of Tumor and DTC:

Tumor (T)	Total No. of cases	No. of DTC + VE cases	No. of DTC - VE cases
T1	4	0	4
T2	24	3(12.5%)	21(87.5%)
T3	22	8(36.36%)	14(63.63%)
T4	-	-	-

Of total 50 cases studied, 4 cases were at T1 stage, 24 tumors were T2 stage and rest 22 were at T3 stage. No cases of T4 were included in study. 3 cases from T2 and 8 cases from T3 stage were positive for DTC.

Table 3: Nodal Status (N) and DTC

Nodal status(N)	Total No. of cases	No. of DTC + VE cases	No. of DTC - VE cases
N0	11	0	11(100%)
N1	21	5(23.80%)	16(76.19%)
N2	18	6(33.33%)	12(66.66%)

11 patients were at N0 stage and 21 were at N1 stage, 18 cases were at N2 stage. No cases of N0 showed positivity for disseminated tumor cells, 5 cases of N1and 6 cases of N2 were positive for disseminated cells.

Table 4: Grade of Tumor and Dtc

GRADE	Total No. of cases	No. of DTC + VE cases	No. of DTC - VE cases
Well Differentiated	12	0	12(100%)
Moderately Differentiated	22	4(18.18%)	18(81.81%)
Poorly Differentiated	16	7(43.75%)	9(56.25%)

Of the 50 cases 12 cases were with well differentiated morphology, 22 cases had moderately differentiated tumor grade and rest 16 had poorly differentiated morphology. No case with well differentiated morphology were positive whereas 4 cases with moderately differentiated and 7 cases with poorly differentiated morphology were positive for DTC.

Table 5: **Stage of Tumor and DTC**

STAGE	Total No. of cases	No. of DTC + VE cases	No. of DTC - VE cases
DUKES A	4	0	4(100%)
DUKES B	7	0	7(100%)
DUKES C	39	11(28.2%)	28(71.8%)

39 cases out of 50 were at DUKES stage C. 11 among them were positive for disseminated cells Whereas stage A and B constituted 4 and 7 cases each with no positivity

Discussion

Solid tumors like colorectal cancers how an extended period of dormancy, attributed to presence of minimal residual disease for many years before overt metastasis may develop. Tumor cells in bone marrow detected by IHC have been correlated with a decrease in disease free interval and prognosis [15]. Detection of these tumor cells can be used for estimating the risk of metastatic relapse, stratifying patients to adjuvant therapy. Surgical resection remains treatment of choice but despite surgery 45% ultimately die of distant metastasis. Detection of disseminated tumor cells may provide a promising approach to improve prognosis and for changing treatment approach in patients [16]. The results of our study were compared with other similar studies. In our study DTC detection rate is 22%. Panabieres *et al* [13] study in the year 2008 enlisted the detection rate of bone marrow disseminated tumor cells in colorectal carcinoma without overt metastasis by immunological methods. In Panabieres *et al* [13] study detection rate is 20-30%. In our study detection rates of disseminated tumor cells in colorectal carcinoma is 16.66%.

Pantel *et al* [8] study in the year 1999 done detailed analysis of bone marrow aspirates from patients with gastrointestinal tract malignancy in various stages and found that colorectal carcinoma patients showed 27.2% positivity in cases without distant metastasis 39% positivity in cases with overt metastasis. This study shows comparatively higher rates of detection from our study which shows 16.66% positivity in colorectal carcinoma patients without metastasis. Our study does not include patients with metastasis.

Sullivan *et al* (1995) [14] done a prospective study in patients who underwent curative surgery for gastrointestinal malignancy, for assessing the prevalence of occult micrometastasis. In Sullivan *et al* (1995) [14] patients with known metastasis

preoperatively were excluded, but patients with metastasis detected during surgery were included in the study. Bone marrow Aspirates were analysed using flow cytometry with antibody to CK-18.

Positive cases of colorectal carcinoma were analysed based on Dukes staging. No patients with Dukes stage A were positive. 24% in Dukes B, 46% in Dukes C, and 25% in Dukes D were positive. In our study no cases from both Dukes A and B were positive, 25% with Dukes C were positive. Patients with metastasis were not included in our study. It appears to have trend towards increased presence of micrometastasis with increasing stage but number of patients included in each stage were not sufficient for statistical comparison.

Shetye *et al* [6] (2004) performed a study including fifty six patients with resected colorectal carcinoma. Thirty four patients with no overt distant metastasis and 22 patients with distant metastasis were included. In Shetye *et al* [6] study 19 out of 56 patients had bone marrow micrometastasis. Among the 19 patients with bone marrow micrometastasis 10 of them were in patients without metastasis i.e 29.4% and 9 were in advanced disease. Of these 19 patients none were in Dukes stage A, 14.28% were from Dukes stage B, 34.6% from Dukes stage C, 40.9 % from Dukes stage D. In our study rate of detection of disseminated cells in colorectal carcinoma patients is 16.66% in contrast to 29.4 % in the Shetye *et al* study. In our study none of patients from Dukes stage A and B were positive, 25% of Dukes stage C were positive. Patients with distant metastasis were not included in our study.

To summarise our study results are correlating with other studies we discussed earlier. In our study percentage of detection rate of bone marrow micrometastasis is low compared with other studies. Increased Detection rate in other studies may be due to difference in techniques used to detect micrometastasis. Lower detection rate in our study may be due to factors lack of enrichment techniques and smaller study population. However our study confirms the significance of detection of Bone Marrow metastasis by IHC and its prognostic value significance of detection of Bone Marrow metastasis by IHC and its prognostic value.

Conclusion

Our study has enumerated the importance of detection of DTC by IHC(Cytokeratin) in Bone marrow aspirates of colorectal malignancies. Bone marrow micrometastasis detection rate increases with increase in depth of invasion and stage of tumor. With increase in grade of tumors i.e poorly differentiated carcinomas have increased incidence of Bone marrow micrometastasis (DTC). Our study concludes that disseminated tumour cells in Bone Marrow aspirates of colorectal carcinoma could have an independent prognostic value which helps the surgeons and oncologists to plan a appropriate treatment strategy.

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