Cytoproliferative activity in colorectal poorly differentiated clusters: Biological significance in tumor setting

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ABSTRACT

Background: Poorly differentiated clusters (PDCs) have gained a significant prognostic role in colorectal carcinomas (CRCs) being associated to high risk of lymph node metastasis, shorter survival time and poor prognosis. The knowledge in PDC biology is not completely clear.
Materials and methods: We assessed Ki-67 LI in 45 CRCs showing ≥10 PDCs. We distinguished PDCs at the periphery of the tumor masses (pPDCs) from those within the tumor masses (cPDCs). We chose 3 cut-offs of Ki-67 labeling index (Ki-67 LI): <10%, 10–50%, and >50% of the labeled cells.
Results: Ki-67 LI in pPDCs was<10% in 37 cases (82%), 10–50% in 6 cases (13%) and >50%in 2 cases (5%); Ki-67 LI in cPDCs was<10% in 4 cases (23.5%), 10–50% in 4 (23.5%) and >50% in 9 (54%). Ki-67 LI in tumor budding foci (TBs) was <10% in 8 cases (32%), 10–50% in 8 (32%) and >50% in 9 (36%). The difference of Ki-67 LI reaches the statistical significance ($p < 0.005$). Ki-67 LI <10% in the pPDCs was associated with nodal metastases (pN+) ($p < 0.0001$), pTNM stage III and IV($p < 0.0001$) and TB ($p < 0.001$). Ki-67 LI > 50% in cPDC was significantly associated withpT3-pT4 and advanced pTNM stages ($p < 0.0001$), N+ ($p = 0.0001$) and LVI ($p < 0.05$).
Conclusion: Different Ki-67 LI detected between cPDCs and pPDCs suggesting a biological difference in PDCs. An actively proliferating central tumor areas can be distinguished from the peripheral portion of the tumors in which the cells interact with the stroma acquiring invasive and metastatic potential.

1. Introduction

Colorectal carcinoma (CRC) is the third leading cause of cancer death in the world with a steadily rising incidence in developing countries [1]. The main unfavorable prognostic factors in CRC patients include advanced pathological TNM stage (pTNM), high histological grade, signet ring cell histology, perineural invasion (PNI), lymph vascular invasion (LVI) and tumor budding (TB) [2,3]. More recently, poorly differentiated clusters (PDCs), *i.e.* aggregates composed of ≥5 cancer cells lacking glandular formation have gained a significant prognostic role in CRC, being associated to high risk of lymph node (N) metastasis, shorter survival time and poor prognosis [4-8]. PDCs are located within the tumor mass (central PDCs, cPDCs) or at its invasive edge (peripheral PDCs, pPDCs) and have been also detected in metastases [9,10]. Because of their morphological similarity to tumor budding foci, which are composed of <5 tumor cells, it was suggested that TBs and PDCs could represent sequential steps in tumor growth [5,11]. They both reflect epithelial-mesenchymal transition [12-14], as highlighted by their reverse pattern of MUC1 expression [15], loss of E-cadherin expression and nuclear internalization of β-catenin [12]. Few data have been reported on the proliferative activity of PDCs; in particular, a recent study explored the proliferation rate in PDCs using Ki-67 labeling index (LI), reporting lower values compared to the tumor mass [11]. To gain knowledge in PDC biology, we assessed Ki-67 LI in a series of CRCs

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enriched in PDCs (>10 clusters) using MIB1 antibody.

2. Materials and methods

2.1. Clinical and pathological features

From the archives of the Institute of Pathology of the University of Modena, Italy, we selected 45 CRCs showing ≥10 PDCs in the microscopic field of a 20× objective lens. PDC counting was performed according to Ueno et al. [13]. PDCs were classified as "central", when found within the tumor mass (cPDCs), and "peripheral" (pPDCs), when found at the invasion front of the tumor (Fig. 1A, B). All cases included in the study were morphologically represented by conventional adenocarcinomas. Data on pTNM stage, location and size of the tumor, World Health Organization(*WHO*)histological grade [2], LVI and TB were available for all case [16].

2.2. Ki-67 labeling index

From a representative paraffin block of each tumor, we cut a 4 μm-thick slide for immunohistochemical analysis against Ki-67 (MIB-1 clone; dilution, 1:100, code M7240; Dako, Glostrup, Denmark) using Bench Mark ULTRA automated stainer (Ventana Medical Systems, Tucson, AZ, USA). Ki-67 LI, representing as the percentage of neoplastic cells positive for Ki-67 over the total number of cells, was separately assessed in pPDCs, cPDCs, TB, and main tumor mass. We chose 3 cut-offs of Ki-67 LI: <10%, 10–50%, and >50%. The evaluation of Ki-67 was done by eyeballing for all PDCs present in the slide and was done at 40×.

2.3. Statistical analyses

The correlation between Ki-67 LI in pPDCs, cPDCs, TBs, and other clinical-pathologic parameters (tumor growth, histological WHO grading, pTNM staging and LVI) was investigated using Fisher test. P-values <0.05 were considered statistically significant. Statistical analyses were performed with Graph Pad PRISMv.6c for Mac (GraphPad Software, San Diego, CA, USA).

2.4. Ethics statement

Histopathological data were anonymized. This study was approved by the "Comitato Etico Area Vasta" [Prat.334/2019/OSS.AOUMO-Prot. AOU0016531/19].

3. Results

The clinicopathological characteristics of the 45 CRCs included in this study are summarized in Table 1. Thirty-onecases were classified as low-grade and 14 as high-grade CRCs, according to the latest WHO classification of tumors of the digestive system [3]; 19 tumors were located in the right colon; and 22 were left-sided and 4 were in sigmoid-rectal junction. Eighteen cases were diagnosed as pTNM Stage II CRCs, 20 as stage III and2 cases as stage IV CRCs. Seventeen cases displayed LVI and 25 showed TB foci. Although pPDCs were present in all tumors (mean pPDC count: 14), cPDCs were found in 17 cases (38%) (mean cPDC count: 4). Tumor cells of the mass were diffusely positive for Ki-67 with a label index always >85%, without significant differences among the 45 tumors examined. Table 2 shows details of Ki-67 LI in PDCs and TBs. Ki-67 LI was significantly lower in pPDCs compared to cPDCs and TBs. Most cPDCs had 50% higher Ki-67, contrary to 5% of pPDCs and 36% of TBs. This explains that the cytoproliferative index of the tumor mass is higher than the peripherical one, demonstrating that at the periphery the cells loose epithelial growth features and gain migratory characteristics.

In detail, pPDCs Ki-67 LI was <10% in 37 cases (82%), 10–50% in 6 cases (13%) and >50%in 2 cases (5%); in cPDCs, Ki-67 LI was <10% in 4 cases (23.5%), 10–50% in 4 (23.5%) and >50% in 9 (54%) respectively. In TB foci, Ki-67 LI was <10% in 8 cases (32%), 10–50% in 8 (32%) and

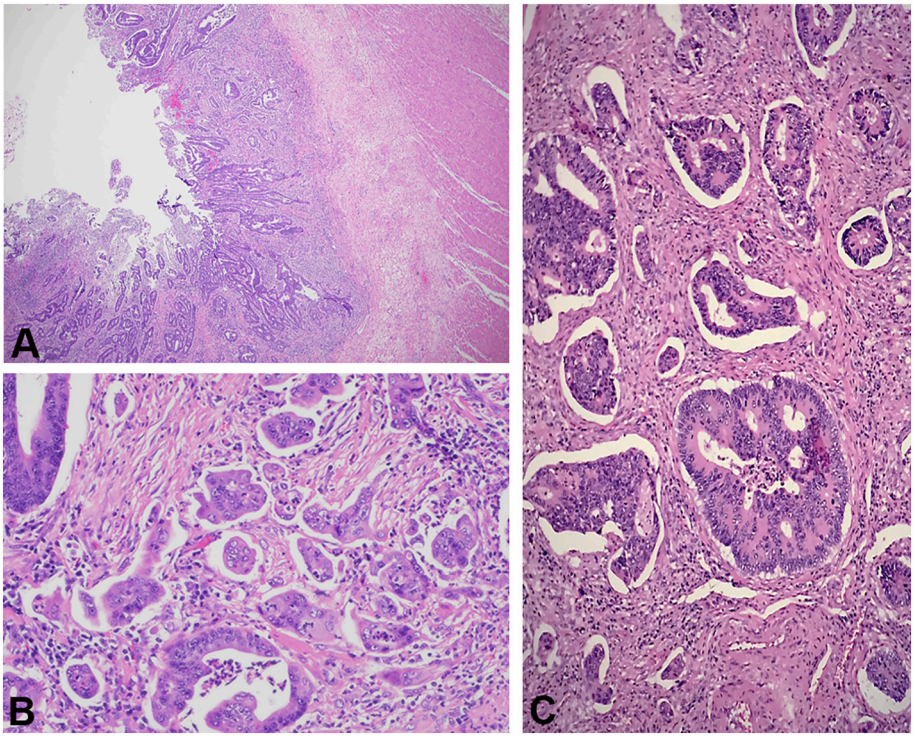

Fig. 1. A. Panoramic images of the tumor masses represented by conventional adenocarcinoma (hematoxylin and eosin stain 2×); B. Higher magnification of A: in all cases in the study cohort, the number of PDCs at the periphery (pPDCs) of the tumor masses was ≥10 at 20× (hematoxylin and eosin stain); C. PDCs within the tumor mass (cPDCs) (hematoxylin and eosin stain 20×).

Table 1
Clinic-pathological features of the 45 studied cases.

Clinic-pathological features		N° of cases
Male/Female		25/20
Age at diagnosis: range (mean)		45–89 (67)
Site	Right colon	19
	Left colon	22
	Sigmoid-rectal junction	4
WHO grade	Low grade	31
	High grade	14
Tumor dimension: range (mean)		18–60 (33)
Gross growth	Vegetant	15
	Ulcerative	30
Histological growth	Infiltrative	45
Lymph-vascular invasion	Present	17
	Absent	28
TB	Present	25
	Absent	20
pPDC[a]	Present	45
	Absent	0
cPDC[b]	Present	17
	Absent	28
pT	1–2	2
	3–4	43
pN	0	21
	1–2	24
pM	0	38
	1	7
Staging	1–2	18
	3–4	27

[a] PDCs at the invasion front of the tumor mass.
[b] PDCs within the tumor mass; TB: tumor budding.

Table 2
Ki-67 labeling index (Ki-67 LI) in PDCs and TB foci of the cohort.

	Ki-67 LI[a]				P-value
	<10%	10–50%	>50%	Total	
pPDCs[b]	37 (82%)	6 (13%)	2 (5%)	45	0.000007
cPDCs[c]	4 (23.5%)	4 (23.5%)	9 (54%)	17	0.00004
TB	8 (32%)	8 (32%)	9 (36%)	25	0.56

[a] n° of stained nuclei in PDC × 100 / total of nuclei in PDC, evaluated with MIB-1 antibody.
[b] PDCs at the invasion front of the tumor mass.
[c] PDCs within the tumor mass; TB: Tumor budding.

>50% in 9 (36%). The difference of Ki-67 LI evaluated in pPDCs, cPDCs, and TBsreach the statistical significance ($p < 0.005$). Immunohistochemistry results for Ki-67 are shown in Fig. 2.

Table 3 shows the correlation between Ki-67 LI in pPDCs, cPDCs, and TB foci and the clinical-pathological features of the study cohort. Ki-67 LI <10% in the pPDCs was significantly associated with pT3–pT4 tumor extension ($p < 0.0001$), presence of nodal metastases (pN+) ($p < 0.0001$), advanced pTNM stage (Stage III and IV) ($p < 0.0001$) and TB ($p < 0.001$). Ki-67 LI >50% in cPDCs was significantly associated with pT3–pT4 tumor extension ($p < 0.0001$). Ki-67 LI >50% in TBs was significantly associated with pT3–pT4 ($p < 0.0001$), pN+ ($p = 0.0001$) and advanced pTNM stage (Stage III-IV) ($p < 0.0001$), LVI ($p < 0.05$).

Table 3
Correlation between Ki-67 labeling index (Ki-67 LI) in pPDCs, cPDCs and TB and the histopathological features.

	Histopathological features			P Value
Ki-67 < 10% in pPDCs	WHO grading	Low-grade	High-grade	n.s.
		28	9	
	ILV	Present	Absent	n.s.
		17	20	
	TB	Present	Absent	<0.0001
		20	17	
	pT	T1-T2	T3-T4	n.s.
		2	35	
	pN	N0	N+	<0.0001
		15	22	
	pM	M0	M+	n.s.
		30	7	
	Stage	I–II	III–IV	<0.0001
		12	25	
Ki-67 > 50% in cPDCs	WHO grading	Low-grade	High-grade	n.s.
		5	4	
	ILV	Present	Absent	n.s.
		5	4	
	TB	Present	Absent	n.s.
		5	4	
	pT	T1-T2	T3-T4	<0.0001
		0	9	
	pN	N0	N+	n.s.
		4	5	
	pM	M0	M+	n.s.
		1	8	
	Stage	I–II	III–IV	n.s
		4	5	
Ki-67 > 50% in TB	WHO grading	Low-grade	High-grade	<0.05
		4	5	
	ILV	Present	Absent	n.s.
		6	3	
	pT	T1-T2	T3-T4	<0.0001
		0	9	
	pN	N0	N+	<0.0001
		0	9	
	pM	M0	M+	n.s.
		1	8	
	Stage	I–II	III–IV	<0.0001
		3	6	

n.s.: not significative; ILV: lymph-vascular invasion TB: Tumor budding.

4. Discussion

PDCs are clusters of neoplastic cells lacking glandular architecture mainly found at the invasive edge of tumor and, less frequently, within the neoplastic mass, recently added to the list of unfavorable prognostic factors in CRC in the WHO Classification of tumors of the digestive system [2,3]. It has been claimed that they may derive from the main tumor mass through the transformation of pre-existing tumor buds.

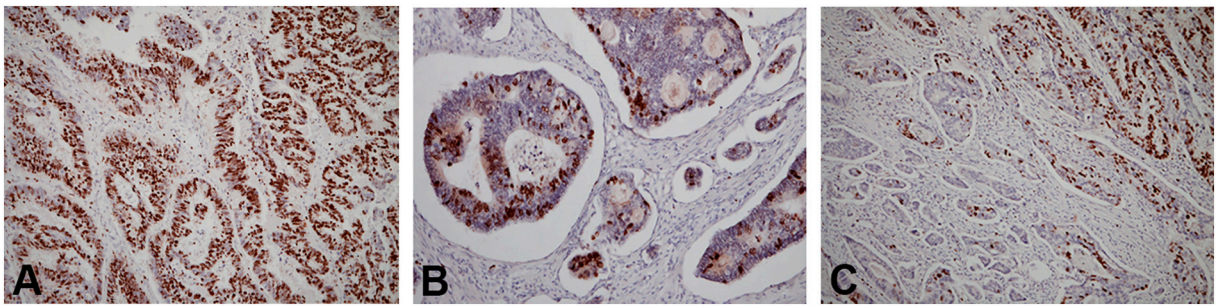

Fig. 2. Ki-67 labeling index in tumor mass (A, 10×), in cPDCs (B, 20×) and in pPDCs (C, 10×) (antibody clone MIB-1 immunohistochemistry stain).

Many studies have reported that PDCs are strongly associated with vascular lymph invasion and lymph node metastases, hence predicting nodal status with higher sensitivity and specificity compared to other traditional, histological prognostic factors [17-19]. This observation has been widely demonstrated for CRCs at all TNM stages, including the early CRC [19,20] encouraging the use of the number of PDCs as a possible tool in the risk assessment of nodal metastases and as a significant predictor of occult micrometastases. Ueno et al. proposed a new grading system for colorectal cancer, based on the number of PDCs counted under a microscopic field of a 20× objective lens, considering tumors with <5 clusters as grade 1, those with 5–9 clusters as grade 2 and those with ≥10 clusters as grade 3 CRCs [21]. However, little is known on their proliferative status as well as their role in tumor growth. Hong et al. observed low proliferative activity in PDCs, describing occasional mitoses in PDC clusters at the periphery of CRC, and a statistically significant lower Ki-67 LI compared to the main tumor mass (71.5% of tumor mass *vs* 31.2% in PDC) [11]. In this study we found that Ki-67 LI in PDCs was lower when compared to that observed in the cells composing the tumor mass and in TB foci. Furthermore, we demonstrated that Ki-67 LI in pPDCs was significantly lower than that observed in cPDCs, suggesting that PDCs have different proliferative status to according to their localization (peripheral *vs* central). The biological difference between pPDC and cPDC has been hypothesized in a previous study in which we found a different expression pattern of β-catenin and E-cadherin in the main tumor mass, cPDC, pPDC, and liver metastases [12]. Probably, pPDCs are mainly involved in tumor invasion, acquire epithelial-mesenchymal transition phenotype and become able to migrate, shifting their biological behavior toward metastatization and vascular invasion. On the other hand, cPDCs are more like the main tumor mass and retain proliferative capability. Several *in vitro* studies demonstrated that tumor cells, singly or in large aggregates, can detach from the main tumor mass and migrate into the desmoplastic extracellular matrix with a mechanism of "cohort-migration" or through a "mesenchymal-amoeboid transformation" [22-28]. Similarly, PDCs lose of pro-adhesion proteins such as cadherin E [29,30] or claudin [31].

Based on our results we suggest segregating PDCs in two distinct categories: a) non-proliferative invasive clusters, localized at the front of the tumor, and b) proliferative clusters, in the center of the tumor mass, with lower invasive potential and probably involved in tumor growth. Low proliferation index has been previously described in tumor budding of CRC [32-35]. Some authors suggested that most of the cells composing the TB at the tumor front probably arrest their cell cycle, blocking proliferation and initiating the stromal invasion and metastatic processes [34,35]. In our cohort, TB cells showed a Ki-67 LI varying from <10% to >50%. However, TB foci were observed in 25 of the cases. In 64% of these, we observed a Ki-67 LI ≤50%, differently from previous reports showing different cut-off of proliferative cells in the buds [32-35].

It was suggested that TBs and PDCs represent different morphological stages of tumor growth and probably derive from the same cellular gems [5]. The significant association with the Ki-67 LI >50% of the cells composing TBs and PDCs (cPDCs and pPDCs) reinforces the concept that buds can grow and progressively transform in PDCs [5].

In our experience, different rates of cytoproliferative activity have been observed in cPDC (within the tumor mass), pPDC and TB (both detected in the peripheral part of the tumor mass). In the tumors we analyzed, pPDCs showed the lowest Ki-67 positivity and represented the category of tumor cells with the lowest cytoproliferative activity. In the pPDC probably occur a later arrest of growth after the acquisition of aggregation capacity, compared to TB. The fact that this occurs in pPDC could be conveyed by the peritumoral microenvironment that favors the epithelial-mesenchymal transition. The coexistence of TB and PDCs in the same tumor mass increases the possibility of tumor cells to spread into the stroma. However, it is also essential to consider another aspect: the cells constituting TB, and even more so those of PDCs, could be in different biological phases: while some are actively growing, others are in arrest growth, supported by the variability of the Ki-67 LI. Therefore, we consider our data preclinical and promising especially as we are increasingly oriented toward the concept of "evolving tumor and spatio-temporal movement".

Although further studies are needed to clear the issue, we believe that the delineated different behaviour of PDCs based on topography (central *vs* peripheric)might deserve proper attention. Although further studies are needed to clear the issue, we suggest that the different PDCs location in tumor masses (central *vs* peripheral) should deserve proper attention in order to clarify their role in the tumor growth and tumor invasiveness.

In conclusion, despite the limited number of cases, the different Ki-67 LI between PDCs and pPDCs confirms the biological difference in PDCs, distinguishing the actively proliferating tumor areas from the peripheral non-/less proliferating portion likely involved in the interaction and destruction of the surrounding stromal matrix and metastatization.

Conflict of interest and source of funding

The Authors have no conflicts of interest directly relevant to this paper. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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