Original Contribution

Poorly differentiated clusters (PDC) in colorectal cancer: Does their localization in tumor matter?

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ABSTRACT

Poorly differentiated clusters (PDC) are aggregates of at least five neoplastic cells lacking evidence of glandular differentiation. By definition, they can be present at the invasive front (peripheral PDC or pPDC) and within the tumor stroma (central PDC or cPDC). In colorectal cancer (CRC), PDC are considered adverse prognosticators and seem to reflect epithelial mesenchymal transition (EMT). In this study, we have investigated the immuno-expression of two EMT-related proteins, E-cadherin and β-catenin, in PDC of primary CRCs and matched liver metastases. pPDC always showed nuclear β-catenin staining and diffusely reduced/absence of E-cadherin expression as opposed cPDC which showed nuclear β-catenin immunoreactivity and E-cadherin expression in about 50% of cases. In addition, the pattern of β-catenin and E-cadherin expression differed between PDC and the main tumor, and between primary CRC and liver metastasis (LM), in a percentage of cases. A discordant pattern of β-catenin and E-cadherin expression between pPDC and cPDC, between main tumor and cPDC, and between primary CRC and LM, confirms that EMT is a dynamic and reversible process in CRC. On the overall, this suggests that pPDC and cPDC are biologically different. We may advocate that PDC develop at the tumor center (cPDC) and then some of them migrate towards the tumor periphery while progressively completing EMT process (pPDC). Based on these results, PDC presence and counting may have different prognostic relevance if the assessment is done at the invasive front of the tumor or in the intratumor stroma.

1. Introduction

Colorectal carcinoma (CRC) is one of the most frequent malignant tumors worldwide [1]. According to the World Health Organization (WHO) Classification, it is graded into low- (LG) and high-grade (HG) based on the percentage of glandular structures composing the tumor, at histological examination (LG: 50% or more; HG: < 50%) [2]. HG is associated with worse outcome [2]. However, the prognostic significance of WHO grading is strongly reduced by fair inter-observer reproducibility [3]. In addition, LG CRCs may also have a sizable minor percent (< 50%) of undifferentiated carcinoma [2], which may reflect

in a higher biological aggressiveness, hence an adverse prognosis.

In 2012, Ueno and co-workers proposed a novel grading system for CRC, based on the counting of clusters of five or more neoplastic cells (despite tumoral budding, where it is defined as a single tumor cell or a cell cluster of fewer than 5 tumor cells) studied predominantly at the invasive front and is referred to as peritumoral tumor budding or within the tumor, which has been referred to as intratumoral tumor budding [4-6] and with no glandular formation which they appellated "poorly differentiated clusters" (PDC) [7]. According to this grading system, CRCs can be subdivided into G1, G2 and G3, when they have a maximum number of < 5, 5–9, ≥10 PDC, respectively, in a x20
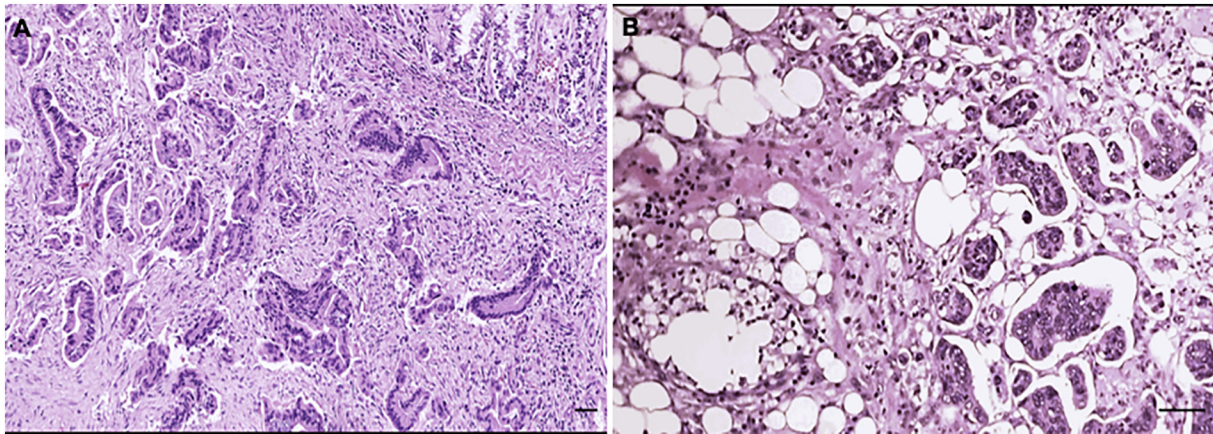
Fig. 1. A. Clusters of poorly differentiated cells growing within the tumor (cPDCs). H&E stain, scale bar – 50 μm, ×10. B. Clusters of poorly differentiated cells at the tumors edge (pPDCs). H&E stain, scale bar – 50 μm, ×20.

microscopic field [7]. PDC grading seems to be more reproducible and prognostically informative than the WHO grading system, and independently from pTNM stage [8-11]. The association between PDC and bad prognosis may be related to the possibility that PDC represent a morphological hallmark of epithelial mesenchymal transition (EMT) [12,13], a process by which neoplastic cells lose their epithelial properties acquire the mesenchymal potential to migrate within extracellular matrix and to metastasize eventually [14]. In support of their relationship with EMT, PDC show inverted MUC1 expression pattern, i.e. MUC1 is expressed along the cell membrane facing the matrix, not the gland lumen [12,13]. The reversed MUC1 pattern may facilitate angioinvasion and metastatization through the interaction of MUC1 with ICAM-1 on the endothelium of vessels [15]. In addition, MUC1 is one of the proteins repressed during EMT [16]. Loss or aberrant (cytoplasmic) E-cadherin expression in PDC further supports the claim that they reflect EMT [12,13]. Indeed, E-cadherin is an adhesion molecule present in the cell membrane of most normal epithelial cells and its loss or aberrant expression seems to be a late event in EMT process [17]. Correct positioning and functioning of membranous E-cadherin seems to depend, among various processes, also on its binding to β-catenin [18]. Thus, when β-catenin is translocated to the nucleus, membranous E-cadherin is lost and cell-to-cell adhesions are disrupted [18].

In a recent study, we have evaluated the presence of PDC in synchronous liver metastases (LMs) from CRC [19]. PDC were localized in the center, at the invasive edge or at both sites of LMs [19]. On the whole, their presence was significantly associated with a worse prognosis, yet we noticed that none of the patients with PDC only at the center of LMs died during the follow-up [19]. Thus, we conjectured that in spite of their morphological overlap, PDC at the center and at the invasive front of LM may have different biological meaning [19].

Along these premises, we have analyzed whether the expression of E-cadherin and β-catenin, which are EMT-related proteins, might be different in PDC located either at the center (cPDC) or at the periphery (pPDC) of primary CRCs and paired LMs.

2. Materials and methods

All procedures were followed in accordance with the Declaration of Helsinki. All cases were preliminarily anonymized; since the study was retrospective and we did not perform any analysis which modified the initial diagnosis, neither patient consent nor formal approval was necessary.

Archival cases of CRCs with surgically resected LM (either synchronous or metachronous) were initially reviewed to identify cases with PDC G3 in the primary tumor (≥10 PDC). Cases treated with preoperative chemotherapy had been excluded due to significant tumor

changes potentially induced by neoadjuvant treatment [20].

Routinely stained H&E sections of the primary tumor were firstly scanned at a low power magnification as to identify the area with the highest number of PDC. Then, PDC were counted using a ×20 objective lens (i.e. a microscopic field with a major axis of 1 mm), with a Zeiss microscope (Carl Zeiss, Oberkochen Germany) [8]. Finally, 20 cases were included in the study. PDC counting was then performed on the corresponding LMs of each case and the greatest PDC count in LM was recorded.

Pertinent information on age/sex, anatomic site (right colon, left colon or rectum), tumor size (cm), lymphovascular invasion, perineural invasion and tumor budding was available in all cases. In addition, for each case the size (cm), the number and site (left or right lobe) of LMs were recorded.

2.1. Immunohistochemistry

Immunohistochemistry (IHC) was applied for E-cadherin and β-catenin on paraffin sections obtained from the complementary H&E section showing the highest number of PDC.

Antibodies and reagents had been obtained from commercial sources and IHC was carried out by using Autostainer BenchMark according to the manufacturer instructions. Specimens of normal colorectal mucosa were used as positive controls.

In each primary CRC and corresponding LMs, E-cadherin and β-catenin immunostains were assessed in the main tumor, in the PDC within the tumor (central PDC or cPDC), and in PDC at their invasive front (peripheral PDC or pPDC).

E-cadherin positivity was recorded as: 1) membranous and complete; 2) membranous and partial; 3) cytoplasmic; 4) absent.

β-Catenin immunostaining was subdivided into 1) membranous and cytoplasmatic; 2) membranous, cytoplasmic and nuclear.

3. Results

In Fig. 1, H&E stain shows the primary CRCs and both cPDC (Fig. 1A) and pPDC (Fig. 1B), as reported in each magnification. Particularly, in 9 primary CRCs both cPDC and pPDC were found, while in 11 only pPDC were observed. None of the primary CRCs had only central PDC (Table 1). The expression of E-Cadherin and β-Catenin in 20 primary CRCs, 20 liver metastasis (LMs), cPDC and pPDC, is detailed in Tables 1 and 2.

3.1. E-cadherin and β-catenin expression in primary CRCs

In 17 primary CRCs, E-cadherin expression was membranous in the

Table 1
Clinical-pathological characteristics and E-cadherin or β-catenin expression in 20 primary CRCs.

Case	Sex	Age	Site primary	Size primary (cms)	WHO grade	LVI	Tumor budding	PNI	E-cadherin stain main tumor	E-cadherin stain peripheral PDC	E-cadherin stain central PDC	β-Catenin stain main tumor	β-Catenin stain peripheral PDC	β-Catenin stain central PDC
1	M	78	Right	3,4	2	Present	Present	Absent	m	Absent	m	m, c, n	m, c, n	m, c, n
2	M	73	Left	2,8	2	Present	Present	Absent	m	Absent	No PDC	m, c	m, c, n	No PDC
3	M	75	Right	3,3	2	Present	Present	Absent	m	c	No PDC	m, c	m, c, n	No PDC
4	M	73	Left	2,7	3	Present	Absent	Absent	Absent	Absent	absent	m, c, n	m, c, n	m, c, n
5	F	68	Right	2,4	1	Present	Absent	Absent	m	c	m	m, c	m, c, n	m, c, n
6	M	71	Left	5	2	Present	Present	Absent	m	Absent	c	m, c	m, c, n	m, c, n
7	F	69	Right	4	3	Present	Present	Absent	Absent	Absent	No PDC	m, c, n	m, c, n	No PDC
8	F	71	Right	3,8	2	Present	Absent	Absent	m	c	No PDC	m, c	m, c, n	No PDC
9	M	70	Rectum	3	2	Present	Present	Absent	m	Absent	No PDC	m, c, n	m, c, n	No PDC
10	M	80	Left	3,5	2	Present	Present	Absent	m	c	m	m, c, n	m, c, n	m, c, n
11	M	71	Right	5	2	Present	Present	Absent	m	Absent	c	m, c	m, c, n	m, c, n
12	F	69	Left	4,4	2	Present	Absent	Present	m	c	m	m, c	m, c, n	m, c, n
13	M	73	Left	3,5	2	Present	Present	Absent	m	Absent	c	m, c	m, c, n	m, c, n
14	F	75	Rectum	5,5	3	Present	Present	Present	Absent	Absent	No PDC	m, c, n	m, c, n	No PDC
15	F	68	Right	2,8	2	Present	Absent	Absent	m	Absent	No PDC	m, c, n	m, c, n	No PDC
16	M	80	Left	3	2	Present	Present	Absent	m	c	No PDC	m, c	m, c, n	No PDC
17	F	72	Left	3	2	Present	Absent	Present	m	c	c	m, c	m, c, n	m, c, n
18	M	76	Left	4,6	2	Present	Present	Absent	m	c	No PDC	m, c, n	m, c, n	No PDC
19	F	67	Left	3,8	2	Present	Present	Present	m	c	No PDC	m, c, n	m, c, n	No PDC
20	F	63	Right	2,5	2	Present	Absent	Absent	m	c	No PDC	m, c, n	m, c, n	No PDC

Abbreviation: LVI, lymphovascular invasion; PNI, perineural invasion; M, male; F, female; m, membranous; c, cytoplasmic; n, nuclear.

main tumor (Fig. 2A). The remaining 3 cases showed no E-cadherin expression in tumor cells. With regard to β-catenin expression, all the evaluated primary CRCs showed a positive cytoplasmic and membranous staining (Fig. 3A). In 10 of these only few cells of the mass displayed also positive nuclear staining (Fig. 3B, Table 1).

3.2. E-cadherin and β-catenin expression in PDC of primary CRCs

As far as E-cadherin expression in pPDC is concerned, the stain was cytoplasmic (10 cases) or absent (10 cases) (Fig. 2A). When evaluating E-cadherin in cPDC, staining was either membranous (4 cases), or cytoplasmic (4 cases) or absent (1 case) (Fig. 2B, Table 1). In 3 primary CRCs, all being identified as HG according to WHO criteria, E-cadherin was negative in the main tumor, pPDC and in cPDC if present (Table 1).

In pPDC and cPDC of primary CRCs, β-catenin expression was in

distributed in cytoplasmic and membranous portions and, in a discrete number of cells, in the nuclei (Fig. 3, Table 1).

3.3. E-cadherin and β-catenin expression in LM and PDC

As reported in Table 2, 16 LMs showed E-cadherin stain in the membrane of the cells of the main tumor, and in the cytoplasm of the remaining 4. In pPDC its expression was cytoplasmic (12 cases) or absent (8 cases), while was membranous (1 case) or cytoplasmic (1 case) in cPDC (Table 2). Regarding β-catenin expression, LMs showed cytoplasmic and membranous stain in the tumor in all cases, with only 10 LMs also exhibiting nuclear labeling in some cells. Diffuse immunoreactivity for β-catenin with cytoplasmic, membranous and nuclear staining was documented in all pPDC (20 cases) and in all cases of cPDC detected (2 cases) (Fig. 4 and Table 2).

Table 2
Clinical-pathological characteristics and E-cadherin or β-catenin expression in matched LM.

Case	n nodules	Site	Size (cms)	PDC (n)	E-cadherin stain main mass	E-cadherin stain peripheral PDC	E-cadherin stain central PDC	β-Catenin stain main mass	β-Catenin stain peripheral PDC	β-Catenin stain central PDC	Status	FU (months)
1	2	Right	2-1	7	m	Absent	No PDC	m, c	m, c, n	No PDC	DOD	8
2	1	Left	1,6	4	m	c	c	m, c, n	m, c, n	m, c, n	DOD	11
3	1	Right	1,5	8	m	c	No PDC	m, c	m, c, n	No PDC	DOD	5
4	2	Both	1–1,5	7	c	Absent	No PDC	m, c, n	m, c, n	No PDC	DOD	7
5	2	Right	2,4–3	2	m	c	No PDC	m, c	m, c, n	No PDC	Alive	103
6	1	Left	1	6	m	Absent	No PDC	m, c, n	m, c, n	No PDC	DOD	12
7	2	Right	1,1–5	6	c	Absent	No PDC	m, c, n	m, c, n	No PDC	DOD	7
8	1	Right	2,3	3	m	c	No PDC	m, c	m, c, n	No PDC	DOD	9
9	1	Left	2,8	8	m	Absent	No PDC	m, c, n	m, c, n	No PDC	DOD	14
10	2	Right	2–2,5	7	m	c	m	m, c	m, c, n	m, c, n	Alive	77
11	2	Right	1,7-2,3	3	c	Absent	No PDC	m, c, n	m, c, n	No PDC	DOD	9
12	2	Both	0,9–3	6	m	c	No PDC	m, c	m, c, n	No PDC	DOD	3
13	1	Left	1,2	2	m	Absent	No PDC	m, c	m, c, n	No PDC	DOD	38
14	1	Left	1,9	7	c	Absent	No PDC	m, c, n	m, c, n	No PDC	DOD	23
15	1	Left	1	8	m	c	No PDC	m, c	m, c, n	No PDC	DOD	27
16	1	Left	1,7-2,3	3	m	c	No PDC	m, c	m, c, n	No PDC	DOD	11
17	1	Left	1–2,9	2	m	c	No PDC	m, c, n	m, c, n	No PDC	DOD	15
18	1	Left	1,5	3	m	c	No PDC	m, c, n	m, c, n	No PDC	DOD	9
19	1	Left	2,2	2	m	c	No PDC	m, c, n	m, c, n	No PDC	DOD	10
20	1	Left	1,6	2	m	c	No PDC	m, c	m, c, n	No PDC	DOD	7

LVI: Abbreviation: m, membranous; c, cytoplasmic; n, nuclear; DOD, died of disease; FU, follow-up.
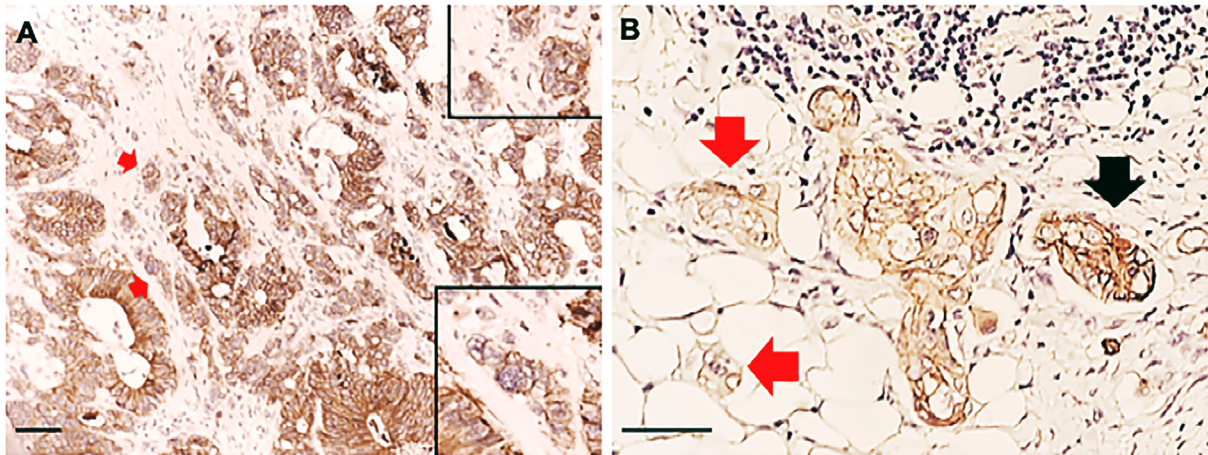
Fig. 2. A. The membranous expression of E-cadherin in PDCs was reduced when compared with tumor (red narrows). IHC stain, scale bar – 50 μm, ×20 (×40 the inserts). B. Some pPDCs showed presence of E-cadherin stain (black narrow), while in other was absent (red narrows). IHC stain, scale bar – 50 μm, ×40. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
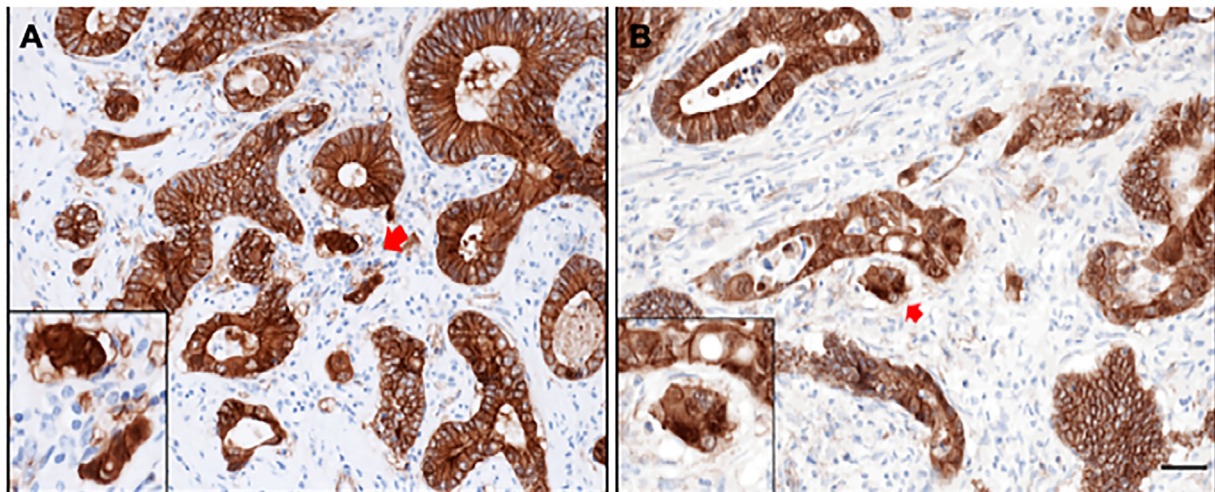

Fig. 3. A. β-Catenin membranous and cytoplasmic expression in tumoral mass PDCs. Nuclear positivity (red narrow) was observed in many cells composing the PDCs, differently from cells composing the main tumor (B). IHC stain, scale bar – 50 μm, ×20 (×40 the inserts).

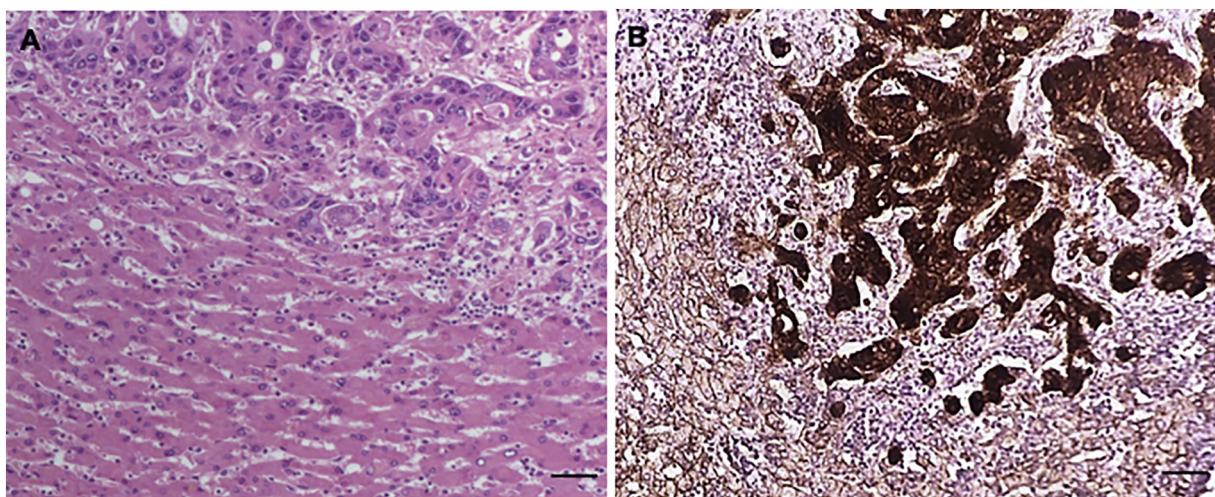**Fig. 4.** Liver metastasis of CRCs. H&E stain, scale bar – 50 μm, ×20 (A). β-Catenin membranous, cytoplasmic and nuclear expression in PDCs in metastasis (B). IHC stains, scale bar – 50 μm, ×20.

3.4. E-cadherin/β-catenin expression pattern combination

The expression pattern of E-cadherin and β-catenin was subsequently analyzed and matched either in primary CRCs or in LMs. As far as this point is concerned, of 17 primary CRCs with membranous E-cadherin in the main tumor, 10 cases showed β-catenin cytoplasmatic and membrane expression whereas 7 cases showed nuclear immunoreactivity as well. In 3 primary CRCs with no E-cadherin stain in the main tumor, β-catenin was detected at nuclear, membranous and cytoplasmic levels.

Regarding LMs, of 16 cases exhibiting membranous E-cadherin immunopositivity in the main tumor, 10 cases had cytoplasmic and membranous β-catenin stain, while 6 cases showed positive labeling also at the nuclear level.

Finally, in all the studied primary CRCs and LMs, pPDC and cPDC showed nuclear, membranous and cytoplasmic β-catenin expression, regardless of E-cadherin expression pattern.

4. Discussion

In this study we have investigated the immuno-expression of E-cadherin and β-catenin in a series of primary CRCs with a high number of PDC and in their paired LMs. Our aim was to establish whether the immuno-expression of those proteins differed between 1) PDC located at the center (cPDC) or at the periphery (pPDC) of primary or metastatic CRC; 2) the main tumor and PDC, in primary CRC or LM; 3) primary CRC and corresponding LM.

The expression of E-cadherin and β-catenin in PDC in comparison to the main tumor had been previously analyzed in primary CRC [12,13], yet we are not aware of similar investigations carried on LMs.

The results of this study can be summarized as follows. Firstly, pPDC had mostly cytoplasmic/absent E-cadherin and nuclear β-catenin pattern. On the other hand, cPDC had mostly nuclear β-catenin, but cytoplasmic/absent E-cadherin in only a few cases. Secondly, in a percentage of primary CRCs and LMs, E-cadherin and β-catenin expression was discordant between the main tumor and PDC. Indeed, compared to the main tumor, pPDC had different E-cadherin expression pattern (i.e. membranous in the main tumor and cytoplasmic/absent in PDC) in 17/20 (85%) primary CRCs and in 16/20 (80%) LMs, and different β-catenin expression pattern (membranous/cytoplasmic in the main tumor and membranous/cytoplasmic/nuclear in PDC) in 9/20 (45%) primary CRCs and in 10/20 (50%) LMs. Similarly, cPDC had different E-cadherin expression pattern in 4/9 (44%) primary CRCs and in 1/2 (50%) LMs, and different β-catenin expression pattern in 6/9 (66%) primary CRCs and in 1 LM. Thirdly, this study showed that E-cadherin and β-catenin expression may differ between primary CRC and its paired LMs. As a matter of fact, the expression of E-cadherin turned from absent or membranous in primary CRC to cytoplasmic in LMs. In addition, primary CRCs with nuclear β-catenin had cytoplasmic/membranous β-catenin in LM, and vice versa.

Interestingly, cytoplasmic/absent E-cadherin was always accompanied by nuclear β-catenin, in the main tumor, pPDC and cPDC. However, membranous E-cadherin was seen in association with either nuclear or only cytoplasmic/membranous β-catenin. This suggests that β-catenin translocation to the nucleus probably precedes E-cadherin disruption, as previously hypothesized [18].

Overall, our findings suggest that β-catenin and E-cadherin expression are dynamic processes which may change during CRC progression and metastatization. This is in keeping with a previous observation that CRCs may have β-catenin over-expression and nuclear accumulation at the invasive front, but not within the tumor center [18,21,22]. Besides, Brabletz et al. demonstrated that primary CRC may have nuclear β-catenin and absent E-cadherin at its invasive front, while its paired LM may lack nuclear β-catenin and show membranous E-cadherin [18]. However, our study shows that pPDC and cPDC may have different EMT phenotype: to the best of our knowledge, this is an unprecedented observation. Indeed, if we consider that absent/cytoplasmic E-cadherin and nuclear β-catenin expression reflect EMT [17,18], we may speculate that pPDC have a tendency to develop a "complete EMT phenotype" (nuclear β-catenin and abnormal E-cadherin), while cPDC develop "complete" (nuclear β-catenin and abnormal E-cadherin), "incomplete" (nuclear β-catenin and membranous E-cadherin) or "absent" (membranous and cytoplasmic β-catenin and membranous E-cadherin) EMT phenotypes. Thus, it is tempting to postulate that PDC firstly originate within the tumor as subclones are mainly involved in the central tumor growth, and then, when they progressively acquire "complete EMT phenotype", they migrate towards the tumor periphery and give rise to invasion and metastatization. Thereafter, in order to proliferate and to produce a metastatic tumor, neoplastic cells need to recover epithelial properties and undergo a reverse process, called EMT [23], which could explain phenotype changing from primary CRC to LM. Finally, even in the metastatic tumor, some cells may undergo EMT and re-acquire invasive and metastatic properties, as reflected by the presence of pPDC with "complete EMT phenotype".

Abnormal E-cadherin expression and nuclear β-catenin accumulation were previously observed also in tumor budding foci of CRC [12,24,25]. This similarity strongly suggests that tumor budding and PDC both represent morphological hallmarks of EMT in CRC, and, according to this development, PDC could represent the evolution of tumor buds or tumor podia, whereas they acquire proliferative and aggressive strength [26]. Besides, their main difference resides in the number of cells they are formed of - at least five in PDC and less than five in tumor budding- and in the fact that, by definition, PDC can be found both in the tumor stroma and at its invasive front, while tumor budding is assessed just at tumor periphery [9].

5. Conclusion

This study suggests that although all PDC are morphologically the same, they can have a different expression of proteins associated with EMT, and, thus they can be at different evolutionary stages towards dedifferentiation. In particular, PDC located at the tumor center seem to be an earlier stage than PDC located at the tumor periphery. On the other hand, it might be argued that the limited number of tested cases represents a major shortcoming. The latter is mostly due to an irreducible bias introduced by the case selection criteria (i.e. PDC G3 stage IV CRCs, not submitted to neo-adjuvant treatment). Future comparable studies are therefore needed in order to validate our assumption whether PDC location (central vs peripheral) and their EMT phenotype (complete or incomplete) is prognostically relevant in patients with CRC.

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