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TECHNICAL REPORT

A mouse model for peritoneal metastases of colorectal origin recapitulates patient heterogeneity

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Abstract

The peritoneum is a common site of dissemination in patients with colorectal cancer. In order to identify high-risk patients and improve therapeutic strategies, a better understanding of the peritoneal dissemination process and the reasons behind the high heterogeneity that is observed between patients is required. We aimed to create a murine model to further elucidate the process of peritoneal dissemination and to provide an experimental platform for further studies. We developed an in vivo model to assess patterns of peritoneal dissemination of 15 colorectal cancer cell lines. Immune deficient mice were intraperitoneally injected with 10,000 human colorectal cancer cells. Ten weeks after injection, or earlier in case of severe discomfort, the mice were sacrificed followed by dissection including assessment of the outgrowth and localization of peritoneal metastases. Furthermore, using a color-based clonal tracing method, the clonal dynamics of peritoneal nodules were observed. The different cell lines showed great variation in the extent of peritoneal outgrowth, ranging from no outgrowth to localized or widespread outgrowth of cells. An association between KRAS pathway activation and the formation of peritoneal metastases was identified. Also, cell line specific tumor location preferences were observed, with similar patterns of outgrowth in anatomically related areas. Furthermore, different patterns regarding clonal dynamics were found, varying from monoclonal or polyclonal outgrowth to extensively dispersed polyclonal lesions. The established murine model recapitulates heterogeneity as observed in human peritoneal metastases, which makes it a suitable platform for future (intervention) studies.

Introduction

Colorectal cancer (CRC) is a highly prevalent disease worldwide, with an estimated incidence of over 1.8 million in 2018 [1]. A common site of dissemination is the peritoneum, being the sole site of recurrence in up to 25% [2]. About 5% of CRC patients present with synchronous peritoneal metastases (PMs) and another 2–19% develop PM along the course of their disease [3–7]. True incidences might even be higher due to the limited accuracy of imaging modalities to detect PM. As a result, PMs are often detected at a late and symptomatic stage, when numerous small peritoneal nodules have developed and treatment options are limited. Recently, much attention is paid to the improvement of diagnostic and treatment strategies for PM. Several clinical trials have been designed in an attempt to prevent PM in high-risk patients, using adjuvant intraperitoneal chemotherapy, or to detect the disease at a subclinical stage in which patients are still amenable for curative intent treatment [8].

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To identify high-risk patients, a thorough understanding of the evolution of PM is required. It is often hypothesized that PMs develop after intraperitoneal exfoliation of tumor cells from a primary tumor with full-thickness invasion of the bowel wall (T4 tumors), or after iatrogenic intraperitoneal tumor spill. The free floating cells then need to preserve their metastatic potential, avoid apoptosis, migrate and adhere to the peritoneal surface, and invade the peritoneum. Finally, tumor cells need to survive and proliferate in the new tumor microenvironment [9]. However, cellular dynamics of the formation of PM of colorectal origin remain unclarified and literature on the topic is limited.

High patient heterogeneity is characteristic for CRC, with differences in driver oncogenes, in chromosomal and microsatellite stability and in dissemination pattern, all potentially impacting prognosis and treatment options [10, 11]. Likewise, a high variety exists in CRC patients regarding PM, including the formation or absence of PM, and the extensiveness and preferential localization in case PM develop. We aimed to create a murine PM model that is able to reflect the heterogeneity found in CRC patients. By quantitatively assessing the in vivo peritoneal outgrowth of extensively characterized human CRC cells, combined with the lentiviral gene ontology (LeGO) labeling method, we attempted to identify CRC subgroups with differential abdominal dissemination ability and preferential localization, as well as to investigate clonal dynamics in PM formation [12, 13]. Hereby, we aim to further elucidate the process of peritoneal dissemination and provide an experimental platform for further studies.

Materials and methods

In vivo model for PMs

To study the ability and pattern of peritoneal dissemination of 15 well-characterized CRC cell lines (for cell line details see Supplemental Table 1), we injected 10,000 human CRC cells in medium containing 50% matrigel (Corning) into the peritoneum of immune deficient mice. Mice were monitored and weighed twice a week. Ten weeks after injection, or earlier in case of severe discomfort (e.g., >15% weight loss), the mice were sacrificed followed by dissection including assessment of PM (Fig. 1a). The extent of PM was assessed with a scoring system equivalent to the peritoneal cancer index (PCI) as used in humans [14]. To this end, the murine peritoneal cavity was divided into seven anatomical regions: right and left subphrenic, (sub)hepatic, subgastric, small bowel/mesentery, pelvic, and back area. Per region, a score of 0–3 was determined based on the amount and size of peritoneal nodules: 0 = no macroscopic tumors, 1 = tumor nodules ≤2 mm, 2 = tumor nodules between 2 and 5 mm or >5 tumor nodules, 3 = tumor nodules ≥5 mm or >10 tumor nodules present. The modified PCI as used in this murine model was calculated by adding the scores of all seven regions with a maximum score of 21 (Fig. 1b, c).

Animals

Female nude (Hsd:Athymic Nude-Foxn1^{nu}) mice (6–12 weeks old) were obtained from Envigo. Animals were randomly assigned to experimental groups, no blinding was performed during these experiments.

Cell culture

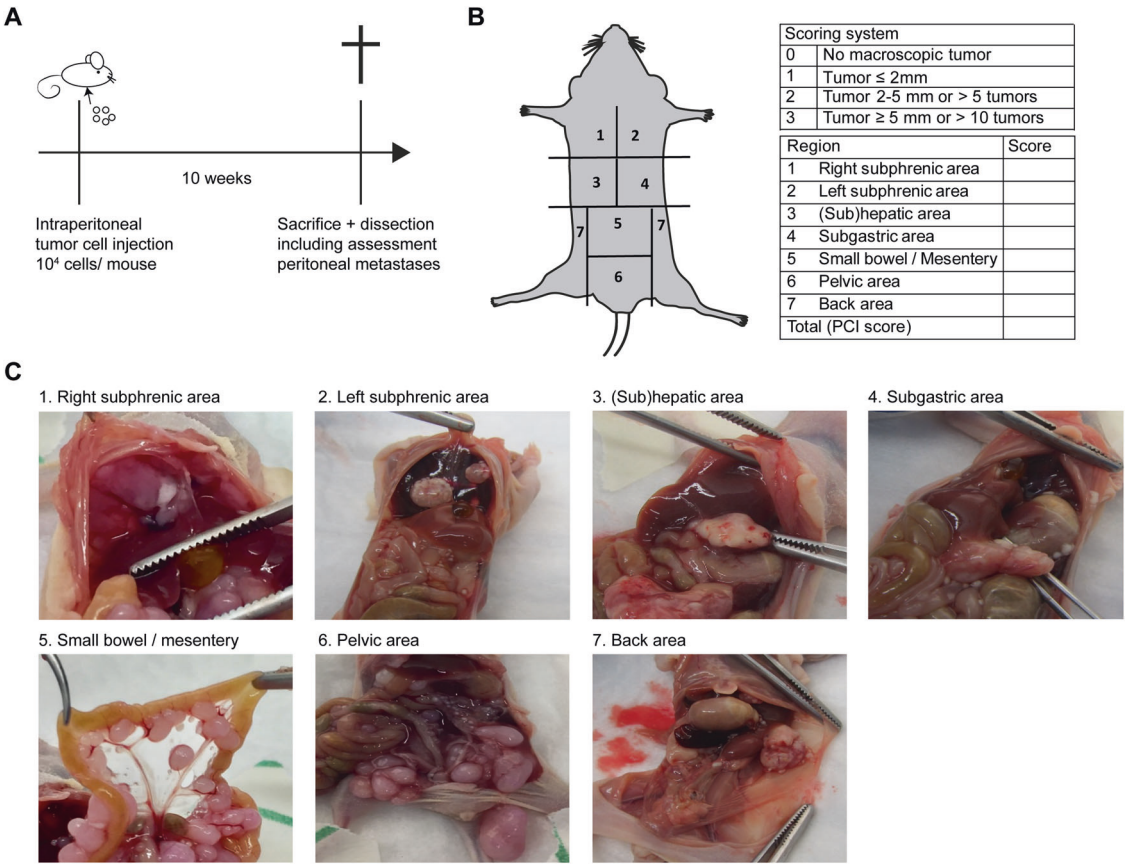
Cell lines T84, SW48, HT55, SW948, LS180, HUTU80, SW620, and OUMS-23 were cultured in Dulbecco's modified Eagle's medium/F-12 medium with L-glutamine, 15 mM HEPES (Thermo-Fisher Scientific, Bleiswijk, The Netherlands) supplemented with 10% v/v fetal bovine serum (Life Technologies), penicillin and streptomycin. Cell lines HCT116, KM12, LS411N, SNU-C1, LS513, MDST8, and NCI-H716 were cultured in RPMI 1640 with L-glutamine, 25 mM HEPES (Thermo-Fisher Scientific, Bleiswijk, The Netherlands) supplemented with 10% v/v fetal bovine serum (Life Technologies), penicillin and streptomycin, 1% D-glucose solution plus (Sigma-Aldrich, Zwijndrecht, The Netherlands) and 100 µM sodium pyruvate (Life Technologies, Bleiswijk, The Netherlands). All cell lines were obtained from the Sanger Institute (Cambridge, UK) and authenticated by STR Genotyping and regularly tested for mycoplasma infection.

Lentiviral LeGO transduction

To study the clonal outgrowth of tumor cells in the peritoneum, we used a series of human serum cultured CRC cell lines (HCT116, KM12, LS411N, SW48, HT55, LS513, SNU-C1, SW620, MDST8, and HUTU80) lentivirally transduced with the LeGO vector set. As described previously, this method combines red, green, and blue (RGB) labeling resulting in cultures in which cells are stably labeled with a wide range of unique colors. This enables tracing of individual cells and their clonal outgrowth by their unique RGB color coding [12, 13]. Of these ten transduced cell lines, five cell lines (HCT116, KM12, SW620, MDST8, and HUTU80) yielded sufficient amounts of peritoneal tumors to perform analysis of clonal dynamics.

Frozen tissue section imaging

Tumors from the different anatomical locations were isolated and immediately fixed using 4%-paraformaldehyde,



Scoring system	
0	No macroscopic tumor
1	Tumor ≤ 2mm
2	Tumor 2-5 mm or > 5 tumors
3	Tumor ≥ 5 mm or > 10 tumors

Region		Score
1	Right subphrenic area	
2	Left subphrenic area	
3	(Sub)hepatic area	
4	Subgastric area	
5	Small bowel / Mesentery	
6	Pelvic area	
7	Back area	
Total (PCI score)		

Fig. 1 In vivo model for peritoneal metastasis and scoring system. **a** 10^4 tumor cells were intraperitoneally injected in immune deficient mice. Ten weeks after injection, the mice are sacrificed followed by dissection including assessment of peritoneal metastases. **b** The extent of peritoneal metastases is assessed with a scoring system equivalent to the PCI as used in humans, by dividing the murine peritoneal cavity into seven regions. Per region, a score of 0–3 is determined based on the amount and size of peritoneal nodules as indicated. The PCI is calculated by adding the scores of all seven regions together with a maximum score of 21. **c** Example images of tumor outgrowth in the different anatomical regions.

followed by 30% sucrose saturation after which tumors were frozen. Prior to imaging, 20-µm-thick sections were mounted with ProLong Gold Antifade Mountant (ThermoFisher Scientific). Tissue sections were imaged using an SP8-X confocal microscope (Leica) and the Leica Application Suite-Advanced Fluorescence software. mCherry, Venus and Cerulean were detected using 587; 510; and 405 nm lasers, respectively. To detect nuclei, sections were counterstained with Hoechst 33342 (Sigma) (405 nm laser), for F-Actin detection ActinGreen-488 (phalloidin) ready probe (ThermoFisher) (488 nm laser) was used. For HE stainings, frozen tumor sections were stained with hematoxylin and eosin.

Image analysis

The number of clones was identified manually by two authors. In case of multiple clones, the same two authors decided whether it concerned a mixed or non-mixed clonal outgrowth pattern.

Geneset analysis R2 platform

The 'R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl)' was used to perform Geneset analysis on datasets GSE36133 and GSE68379 (Sanger and CCLE cell line datasets), using the KRAS.DF.V1_UP signature, (GSE17643) [15].

Statistics

Sample sizes, statistical tests, and definitions of error bars are indicated in the figure legends and calculated using GraphPad Prism 7. All statistical tests were two-sided. *P* values of < 0.05 were considered significant. Clustering analysis was performed using the online ClustVis tool

(https://biit.cs.ut.ee/clustvis/), using Euclidean distance and average linking for both rows and columns [16].

Study approval

All in vivo experiments of this study were approved by the Animal Experimentation Committee at the Amsterdam UMC, location AMC in Amsterdam (LEX227) and performed according to national guidelines.

Results

Outgrowth and localization of PMs

Ten weeks after intraperitoneal injection of 10^4 tumor cells, or earlier in case of severe discomfort, the ability and pattern of peritoneal dissemination of 15 CRC cell lines was systematically analyzed according to the mouse PCI scoring system (Fig. 1a–c). A wide range of peritoneal outgrowth was observed among the different cell lines, varying from no tumors, localized tumor growth or widespread outgrowth of cells (Fig. 2a, b, b example pictures). In contrast, when subcutaneously grafted, these cell lines have been shown previously to be able to successfully form tumors [17]. The growth rate of the subcutaneous xenografts [17] was not correlated with the PCI score, indicating that the ability to form peritoneal lesions is a distinct feature of a cell line, independent of proliferation speed (Supplemental Fig. 1a). Tumor sections demonstrated cell line specific histology and structure (Fig. 2c–d), as has been observed previously in subcutaneous xenograft models of these cell lines [17].

Interestingly, cell line specific preferences for tumor location were observed. Whereas the subgastric area was affected by nearly all tested cell lines, other areas were only populated by specific cell lines (Fig. 3a). For instance, HUTU80 cells seem to have a preference for the subphrenic areas, whereas most other cell lines did not form lesions in this area. Clustering of the peritoneal areas based on the fraction of tumor outgrowth for each cell line revealed very similar patterns in anatomically related areas, such as right and left subphrenic area, or hepatic and gastric regions. Clustering of the cell lines did separate the lines with high and low PCI scores and the cell lines with high PCI scores did tend to grow in most anatomical locations (Fig. 3a).

The development of ascites as a sign of advanced disease is a recognized problem in humans [18, 19]. Likewise, the presence of ascites was associated with PM and higher PCI scores in our model (Fig. 3a and Supplemental Fig. 1b). Also, a high PCI score was usually accompanied by reduced gain or even loss of weight, even when severe ascites was present (Supplemental Fig. 1c). The presence of small (<2 mm) loose tumors throughout the peritoneal cavity (Supplemental Fig. 1d, left panel) was most frequently observed in mice with low PCI scores, especially for cell lines such as LS411N, SW48, LS513, and HT55, although not significantly correlated, since we also observed these loose tumors in mice with high PCI scores, in particular for LS180, SW620, and OUMS-23. In contrast, MDST8 and HCT116, both cell lines resulting in high PCI scores, did almost not form loose tumors at all (Supplemental Fig. 1d, right panel).

No correlation between microsatellite instability or CpG island methylator phenotype and the PCI score was found (Fig. 3a and Supplemental Fig. 1e). Also, there was no difference in PCI score between cell lines that originally have been derived from metastatic lesions or primary tumors (Supplemental Fig. 1e). Notably, SNU-C1, originally derived from a CRC PM did not form any PM, whereas MDST8 and LS180, both originally derived from a primary tumor, did grow out very efficiently when seeded in the peritoneum. Mutations in most common CRC oncogenes did not clearly define the ability of outgrowth in the peritoneal cavity (Fig. 3a and Supplemental Fig. 1f), although cell lines with mutations in the receptor tyrosine kinase (RTK)/mitogen-activated protein kinase cascade (KRAS and BRAF) tended toward higher PCI scores (Fig. 3b). Especially the microsatellite stable cell lines with constitutively active BRAF mutation (OUMS-23 and MDST8) did show a higher ability to form tumors in the peritoneum (Supplemental Fig. 1g), as has been reported in patients before [20–22]. To elaborate on the relation between KRAS/BRAF pathway activation and the establishment of PM, we made use of two publicly available RNA expression datasets (Sanger and CCLE datasets), containing most of the used cell lines (14 and 15 out of 15 lines, respectively). In both datasets, we found a strong correlation of the PCI score with a signature consisting of KRAS upregulated targets (KRAS.DF.V1_UP, GSE17643, Fig. 3c) [15]. Strikingly, the absence of matrigel in the injected cell suspension dramatically reduced the outgrowth of all tested cell lines (Supplemental Fig. 1h), suggesting a requirement for the presence of growth factors and extracellular matrix components, which are abundant in matrigel. It could very well be that tumors with activating KRAS/RTK pathway mutations are less dependent on these external factors for the peritoneal outgrowth.

Clonal dynamics of PMs

Analysis of clonal dynamics by imaging of frozen tissue sections was performed on the five cancer cell lines with the highest PCI scores (HCT116, KM12, SW620, MDST8, and HUTU80). Three distinct patterns of clonal outgrowth were identified. A monoclonal growth pattern, where individual lesions consist of only one color, was characterized by well-

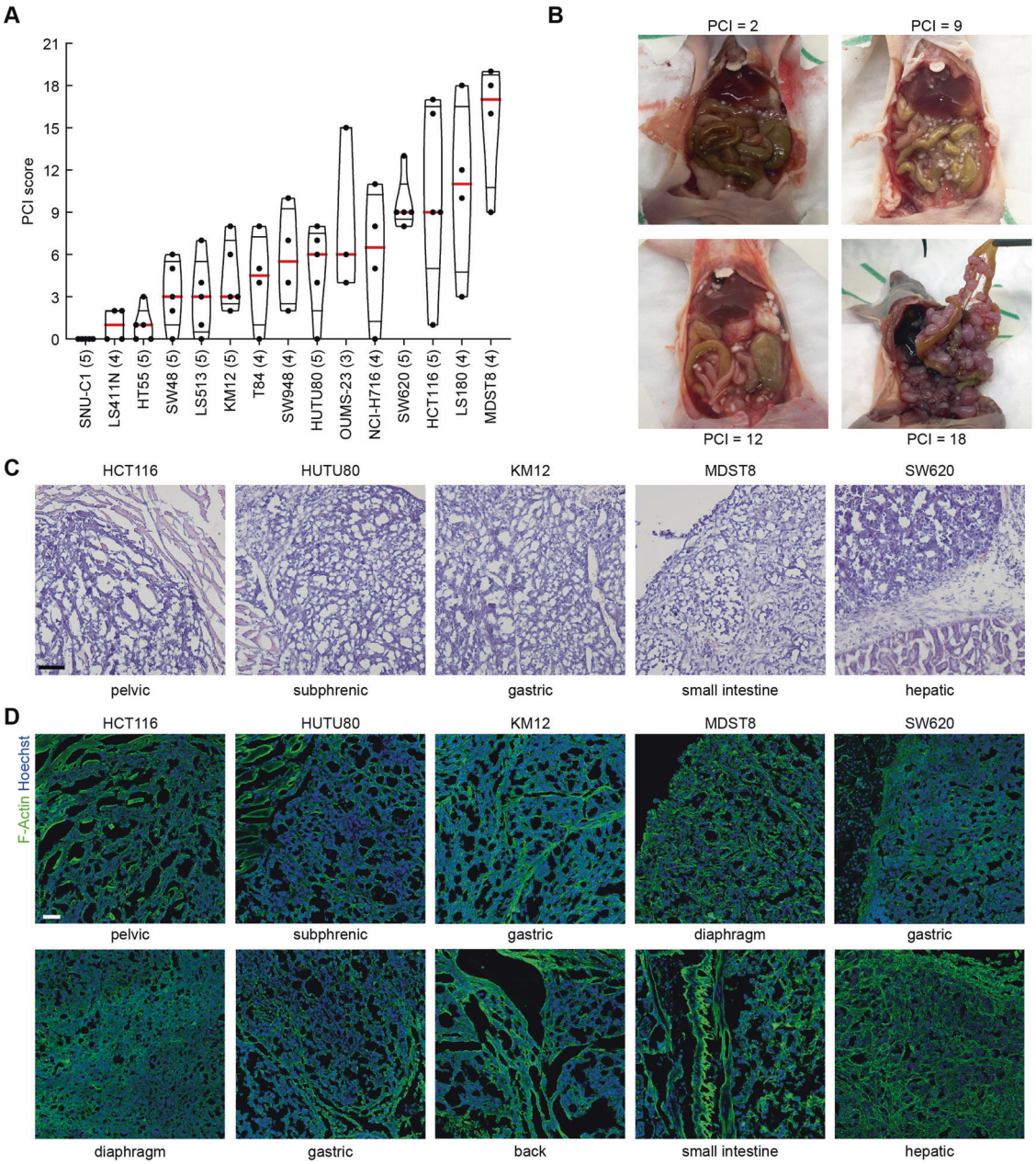


Fig. 2 Peritoneal outgrowth of CRC lines. a PCI score of 15 CRC cell lines, depicted as a violin plot, red lines indicate median PCI scores. Black dots are individual scores per mouse. The number of replicates per cell line is indicated between brackets. **b** Example images of different PCI scores. HE (**c**) and immunofluorescent (**d**) stainings of peritoneal tumor tissue of the indicated cell lines. Scale bars, 100 um. F-Actin (green) and nuclei (blue, Hoechst) are visualized in (**d**).

differentiated morphology with evident glandular structures separated by murine stroma (Fig. 4a, left panel). In contrast, polyclonal tumors contained a mixture of multiple colors. In case clones were still distinguishable, we defined it as a polyclonal non-mixed growth pattern (Fig. 4a, center panels), whereas tumors consisting of regions with a mixture of multiple clones were classified as polyclonal mixed tumors (Fig. 4a, right panel).

We found that peritoneal nodules deriving from the same cell line showed different patterns of clonal outgrowth (Fig. 4b). KM12, SW620, and HUTU80 cancer cell lines showed predominantly monoclonal or non-mixed polyclonal patterns, indicating that single cells are able to initiate tumor nodules, which eventually merge into larger aggregates. On the other hand, MDST8 tumors were all polyclonal, and the majority consisted of regions with homogeneous mixtures of multiple clones. This might indicate polyclonal origins of these nodules, as well as a high tumor cell dispersal. All three patterns were found in the tumor nodules of the HCT116 cell line. Interestingly, MDST8 peritoneal nodules located in

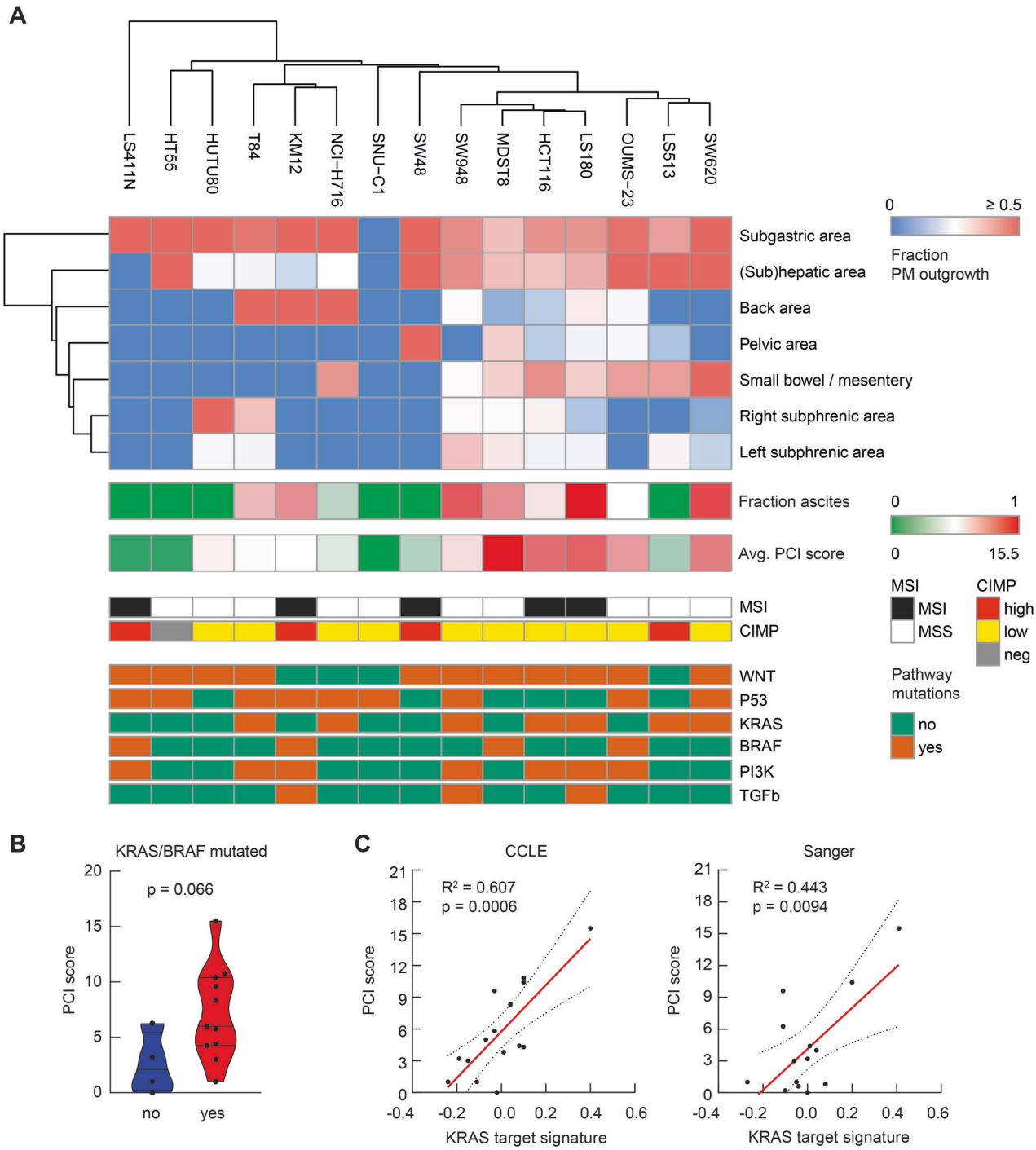


Fig. 3 Clustering analysis of location specific outgrowth. a Fraction of outgrowth of tumors in the seven different regions (rows) for all 15 cell lines (columns), depicted as a heat map. Both rows and columns are clustered using Euclidean distance and average linkage. Below are the corresponding cell line characteristics indicated using color codes. Fraction of ascites: fraction of the mice/cell line that developed ascites after peritoneal tumor cell injection. **b** Violin plots depicting distribution of average PCI score per cell line, specified for the presence of RAS/BRAF mutations. Differences between groups were tested using unpaired, two-tailed *t*-tests. **c** Correlation between PCI score and KRAS target signature expression (*z*-score) (R^2, Pearson correlation), using CCLE (left panel) and Sanger (right panel) cell line gene expression datasets. Red line indicates best fit of linear regression, dotted lines represent 95% confidence intervals.

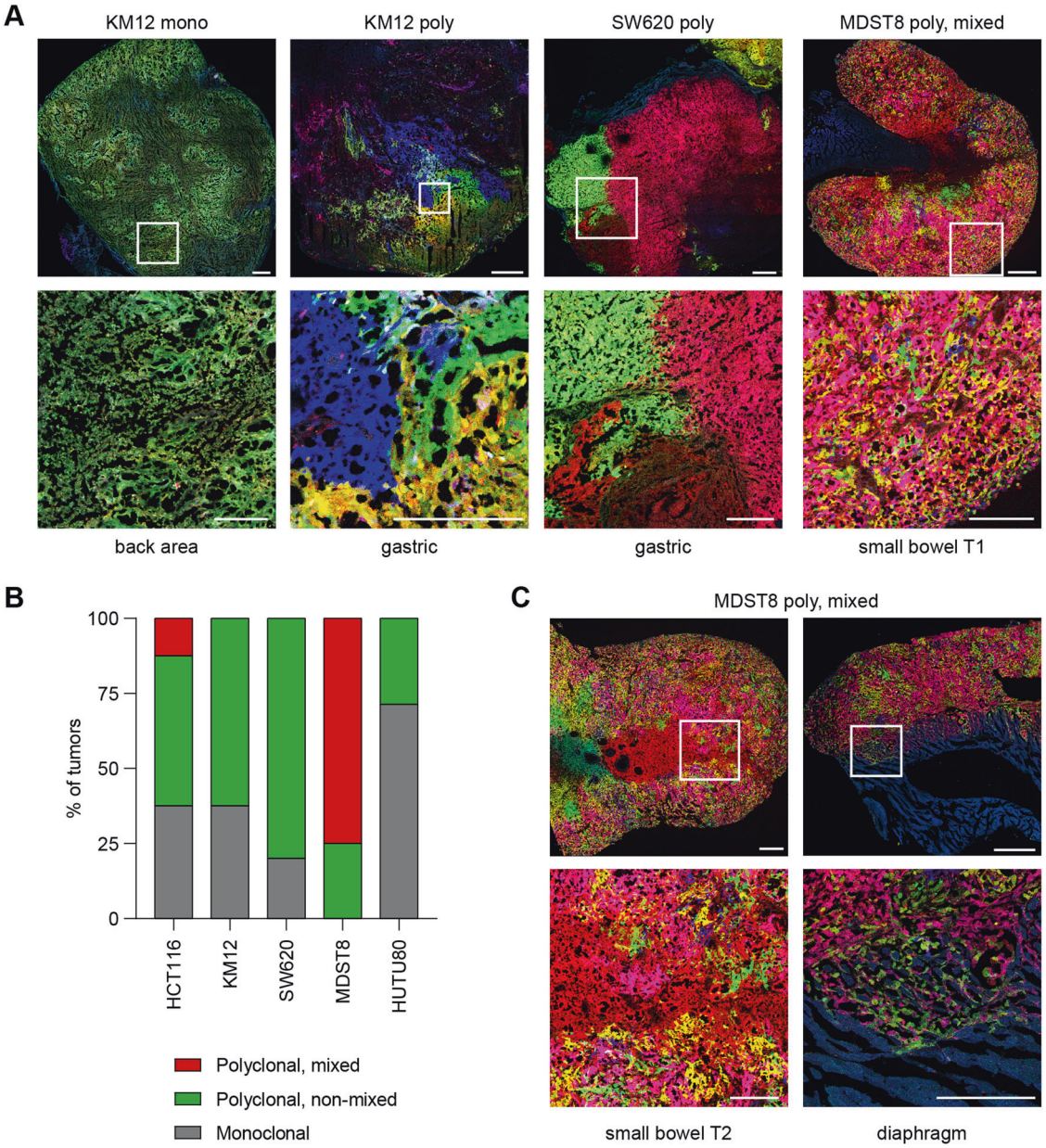


Fig. 4 Clonal dynamics of peritoneal tumor nodules. a Different clonal outgrowth patterns are observed in peritoneal tumor nodules. Cell lines marked with the LeGO tracing system were intraperitoneally injected in immunodeficient mice. Ten weeks after injection, tumor nodules were isolated and processed for confocal imaging. Colors indicate individual clones. **b** Quantification of clonal outgrowth patterns of peritoneal nodules of the indicated cell lines. ($N = 8, 8, 5, 8,$ and 7 tumors for respectively HCT116, KM12, SW620, MDST8, and HUTU80). **c** MDST8 tumor nodules have a highly dispersed polyclonal growth pattern. Different tumors from the same mouse showed similar patterns. **a**, **c** White boxes indicate magnificated area in lower panels. Scale bars, 500 um (upper panels) or 250 um (lower panels).

different areas within one mouse showed highly similar clonal composition patterns (Fig. 4c).

Discussion

Main findings

By quantitatively assessing the in vivo peritoneal outgrowth of intraperitoneally injected human CRC cells, we gained insight in the ability and pattern of peritoneal dissemination of fifteen CRC cell lines. Firstly, the cell lines showed great variation in the extent of peritoneal outgrowth, ranging from no outgrowth to localized or widespread outgrowth of cells. These differences could be correlated to gene expression profiles (KRAS activation), thereby providing potential starting points for targeted therapies. Secondly, cell line specific tumor location preferences were observed, with similar patterns of outgrowth in anatomically related areas.

Thirdly, different patterns regarding clonal dynamics were found, varying from monoclonal or polyclonal outgrowth to extensively dispersed polyclonal lesions, suggesting heterogeneity in the seeding process between cell lines. The observed variation in ability and pattern of peritoneal dissemination demonstrates that the established murine model reflects the heterogeneity as observed in human PM, making it a powerful model system for future (intervention) studies.

Heterogeneity regarding peritoneal dissemination ability

The difference in peritoneal dissemination ability amongst the cell lines in our study, varying from no dissemination to widespread outgrowth, might represent differences in daily clinical practice. It remains unclarified why some patients do and others do not develop PM. Several clinical studies have been conducted in order to identify patients who are at high risk of developing PM. Recognized risk factors include locally advanced tumor stage (T3 and T4), lymph node metastases, right sided tumors, mucinous and signet cell histology, irradical resection (R1 or R2) and tumors with vascular and perineural invasion [5, 7, 23]. Literature is less extensive on the association of genetic profile (i.e., mutations) and the risk of PM. KRAS mutations, present in ~35% of stage IV CRC tumors, has been associated with PM based on small series of patients with already metastasized disease, although the association is not always confirmed [24–26]. BRAF mutated tumors (about 10% of the stage IV CRC tumors) have also been associated with higher rates of PM in stage IV disease (46% vs. 24%, $p = 0.001$) (60% vs. 15%, $p < 0.01$) as compared with BRAF wild-type tumors [20–22]. In line with this (limited) evidence, cell lines with KRAS and BRAF mutations displayed higher PCI scores in our study, although not significant. Nevertheless, transcriptomic analysis of our cell lines did reveal a clear correlation between KRAS upregulated target expression and PCI score. Interestingly, in patients, preferred locations of dissemination (i.e., peritoneum, liver, lung) differ between mutated and wild-type KRAS and BRAF tumors [26]. This might suggest a role of these genes in a location specific mechanistic pathway of dissemination. However, it must be noted that in all studies but one, mutational status was determined in already metastasized CRC, and it is unclear whether the primary tumor already exhibited this genotype before dissemination.

Heterogeneity regarding localization of PM

Heterogeneity was also observed in the pattern of tumor spread in the peritoneum. Some areas were affected by nearly all cell lines (i.e., subgastric and (sub)hepatic), whereas other areas were only populated by specific cell lines (i.e., back and pelvic area) or almost not at all (i.e., small bowel, subphrenic areas). In human, distribution of peritoneal disease is thought to be dependent on gravity, the extent of peritoneal fluid absorption of certain tissues, and peristaltic movement. As a result, peritoneal disease usually concentrates in gravity-dependent areas (e.g., paracolic gutters, pelvis) and on surfaces absorbing peritoneal fluid (e.g., omentum, diaphragmatic peritoneum), while the small intestine is initially spared due to peristaltic movement preventing adhesion of tumor cells [27]. In mice, the omentum is not only a fluid absorbing surface, but due to their horizontal posture also a gravity-dependent area. This might explain the high proportion of PM in the subgastric area representing the murine omentum as observed in this study.

Heterogeneity regarding clonal dynamics

We found heterogeneity in clonal dynamics both between and within cell lines. Roughly three different patterns could be identified: monoclonal, polyclonal non-mixed, and polyclonal mixed. It must be noted that it is possible that the tumors showing a monoclonal growth pattern in 2D sections actually consist of multiple non-mixed clones, which would be visible if all three dimensions were considered. The polyclonal non-mixed tumors seem to be the result of competition of parallel expansion of different clones at distinct rates, or even fusion of multiple individual nodules. Such a pattern was described previously [13]. In contrast, in lesions derived from MDST8, the cell line with the highest PCI score, the mixed phenotype was most prevalent, which might reflect, besides polyclonal metastasis initiation, a high migratory or dissemination ability, properties that are both highly advantageous for peritoneal seeding. These different patterns of clonal dynamics might represent separate theories of the dissemination process. Alternatively to the conventional idea that single cells metastasize after epithelial to mesenchymal transition, it is increasingly assumed that this migration occurs collectively in human carcinomas, leading to polyclonal metastases [28–32]. In line with these findings, a recent study with human ex vivo CRC PM material showed that collective behavior of tumor cells with an epithelial organization predominates in the peritoneal microenvironment, throughout the whole process of peritoneal dissemination [33].

The collective dissemination pattern also suggests, in contrast with the single cell theory, that the microenvironment plays a critical role in the dissemination process. This is depicted in our study by the highly increased peritoneal outgrowth of tumor cells when injecting the cells suspended in matrigel (extracellular matrix of mouse sarcoma cells), as was described previously [34]. Matrigel might enhance peritoneal outgrowth by stimulating tumor cell clustering by

capturing cells in the matrix structure after cell injection. However, also in the cell lines that exhibited a monoclonal growth pattern of peritoneal outgrowth (KM12, SW620, and HUTU80), the presence of matrigel was crucial, implying that matrigel not only promotes peritoneal outgrowth by clustering the cells. Laminin and collagen, the main components of matrigel, have been shown to stimulate motility and adhesion, and also the plethora of growth factors present in the matrigel might benefit peritoneal survival, adhesion and outgrowth of tumor cells [34].

Further implications and limitations

Since the established murine PM model reflects the high heterogeneity as observed in human CRC patients, we believe that this model can be used in future research to study pathophysiological mechanisms of PM. The model allows investigation of the impact of particular mutations on peritoneal dissemination, by ex vivo manipulation (e.g., knockdown/overexpression) of particular genes (e.g., KRAS/BRAF). Furthermore, the model can be used to study different targeted therapy strategies and their effect on the outgrowth of loose tumor cells. Several limitations of the model should be mentioned. The most important drawback of our model is that it does not represent tumor cell dissemination from a primary colorectal tumor. Peritoneal dissemination is a multistep process, which starts with detachment of cancer cells from the primary tumor promoted by several molecules that modulate signaling pathways [35]. By directly injecting cancer cells into the peritoneal cavity, this first step of the metastatic cascade is eliminated. In contrast, tumor cell spill into the peritoneum during primary tumor resection, another way of PM initiation, is captured more representative by our model. In both processes the formation of peritoneal lesions is dependent of the ability of disseminated cells to survive, adhere to the peritoneal lining, and be able to proliferate in this non-native location. Intraperitoneal injection of 10,000 cells might overestimate the number of cells that are released in either process, but this enables the quantitative comparison of peritoneal outgrowth of cell lines, and in the future the exploration of more effective therapies of PMs. In our study, we have used cell lines rather than primary cultures, which has both advantages and disadvantages. In vitro modification of cell lines is more feasible than with primary (polyclonal) tumor material, and ensures proper analysis of the direct effects of these modifications. In addition, it facilitates reproducibility by the use of homogeneous models between laboratories. On the other hand, cell lines might not truly represent the characteristics of the original primary tumor anymore, due to extensive selection by in vitro culturing. Furthermore, due to a lack of an adaptive immune system and a nonspecific residual innate response in the immunocompromised mice, it is impossible to induce an immune response similar as in humans. Next to this, cancer cell-stromal cell interactions might be limited between human cancer cells and murine stroma, while these interactions are believed to play a crucial role in the biology of metastasis. Due to these factors, the underlying biology of the PM in our murine model might be different from spontaneous PM arising from a primary tumor. These limitations should be considered when using the model in future research projects.

Conclusion

We demonstrated that in a murine model for PM of colorectal origin, patient heterogeneity regarding dissemination ability, preferential localization, and clonal dynamics is recapitulated. We found evidence for a role of KRAS pathway activation in the formation of PM. This model can be used in future research to further unravel the pathophysiological mechanisms of PM and to study possible novel drug targets and other therapy strategies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: Globocan estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68:394–424.
2. Jayne DG, Fook S, Loi C, Seow-Choen F. Peritoneal carcinomatosis from colorectal cancer. Br J Surg. 2002;89:1545–50.
3. Koppe MJ, Boerman OC, Oyen WJ, Bleichrodt RP. Peritoneal carcinomatosis of colorectal origin: Incidence and current treatment strategies. Ann Surg. 2006;243:212–22.
4. Lemmens VE, Klaver YL, Verwaal VJ, Rutten HJ, Coebergh JW, de Hingh IH. Predictors and survival of synchronous peritoneal carcinomatosis of colorectal origin: a population-based study. Int J Cancer. 2011;128:2717–25.
5. Segelman J, Granath F, Holm T, Machado M, Mahteme H, Martling A. Incidence, prevalence and risk factors for peritoneal carcinomatosis from colorectal cancer. Br J Surg. 2012;99:699–705.

6. Thomassen I, van Gestel YR, Lemmens VE, de Hingh IH. Incidence, prognosis, and treatment options for patients with synchronous peritoneal carcinomatosis and liver metastases from colorectal origin. Dis Colon Rectum. 2013;56:1373–80.
7. Enblad M, Graf W, Birgisson H. Risk factors for appendiceal and colorectal peritoneal metastases. Eur J Surg Oncol. 2018;44:997–1005.
8. Pinto A, Eveno C, Pocard M. Update on clinical trials in colorectal cancer peritoneal metastasis. Int J Hyperthermia. 2017;33:543–7.
9. de Cuba EM, Kwakman R, van Egmond M, Bosch LJ, Bonjer HJ, Meijer GA, et al. Understanding molecular mechanisms in peritoneal dissemination of colorectal cancer: Future possibilities for personalised treatment by use of biomarkers. Virchows Arch. 2012;461:231–43.
10. Network CGA. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012;487:330–7.
11. Punt CJ, Koopman M, Vermeulen L. From tumour heterogeneity to advances in precision treatment of colorectal cancer. Nat Rev Clin Oncol. 2017;14:235–46.
12. Weber K, Thomaschewski M, Benten D, Fehse B. RGB marking with lentiviral vectors for multicolor clonal cell tracking. Nat Protoc. 2012;7:839–49.
13. van der Heijden M, Miedema DM, Waclaw B, Veenstra VL, Lecca MC, Nijman LE, et al. Spatiotemporal regulation of clonogenicity in colorectal cancer xenografts. Proc Natl Acad Sci USA. 2019;116:6140–5.
14. Jacquet P, Sugarbaker PH. Clinical research methodologies in diagnosis and staging of patients with peritoneal carcinomatosis. Cancer Treat Res. 1996;82:359–74.
15. Barbie DA, Tamayo P, Boehm JS, Kim SY, Moody SE, Dunn IF, et al. Systematic RNA interference reveals that oncogenic KRAS-driven cancers require TBK1. Nature. 2009;462:108–12.
16. Metsalu T, Vilo J. Clustvis: A web tool for visualizing clustering of multivariate data using principal component analysis and heatmap. Nucleic Acids Res. 2015;43:W566–70.
17. Linnekamp JF, Hooff SRV, Prasetyanti PR, Kandimalla R, Buikhuisen JY, Fessler E, et al. Consensus molecular subtypes of colorectal cancer are recapitulated in in vitro and in vivo models. Cell Death Differ. 2018;25:616–33.
18. Sangisetty SL, Miner TJ. Malignant ascites: a review of prognostic factors, pathophysiology and therapeutic measures. World J Gastrointest Surg. 2012;4:87–95.
19. Gasimli K, Braicu EI, Richter R, Chekerov R, Sehouli J. Prognostic and predictive value of the peritoneal cancer index in primary advanced epithelial ovarian cancer patients after complete cytoreductive surgery: study of tumor bank ovarian cancer. Ann Surg Oncol. 2015;22:2729–37.
20. Yokota T, Ura T, Shibata N, Takahari D, Shitara K, Nomura M, et al. Braf mutation is a powerful prognostic factor in advanced and recurrent colorectal cancer. Br J Cancer. 2011;104:856–62.
21. Tran B, Kopetz S, Tie J, Gibbs P, Jiang ZQ, Lieu CH, et al. Impact of BRAF mutation and microsatellite instability on the pattern of metastatic spread and prognosis in metastatic colorectal cancer. Cancer. 2011;117:4623–32.
22. Schirripa M, Bergamo F, Cremolini C, Casagrande M, Lonardi S, Aprile G, et al. BRAF and RAS mutations as prognostic factors in metastatic colorectal cancer patients undergoing liver resection. Br J Cancer. 2015;112:1921–8.
23. Hugen N, van de Velde CJ, de Wilt JH, Nagtegaal ID. Metastatic pattern in colorectal cancer is strongly influenced by histological subtype. Ann Oncol. 2014;25:651–7.
24. Gillern SM, Chua TC, Stojadinovic A, Esquivel J. KRAS status in patients with colorectal cancer peritoneal carcinomatosis and its impact on outcome. Am J Clin Oncol. 2010;33:456–60.
25. Sharma N, Saifo M, Tamaskar IR, Bhuvaneswari R, Mashtare T, Fakih M. KRAS status and clinical outcome in metastatic colorectal cancer patients treated with first-line folfox chemotherapy. J Gastrointest Oncol. 2010;1:90–6.
26. Zihui Yong Z, Ching GTH, Ching MTC. Metastatic profile of colorectal cancer: interplay between primary tumor location and KRAS status. J Surg Res. 2019;4804:30816–3.
27. Nassour I, Polanco PM. Current management of peritoneal carcinomatosis from colorectal cancer: the role of cytoreductive surgery and hyperthermic peritoneal chemoperfusion. Curr Colorectal Cancer Rep. 2017;13:144–53.
28. Aceto N, Bardia A, Miyamoto DT, Donaldson MC, Wittner BS, Spencer JA, et al. Circulating tumor cell clusters are oligoclonal precursors of breast cancer metastasis. Cell. 2014;158:1110–22.
29. Cheung KJ, Ewald AJ. A collective route to metastasis: seeding by tumor cell clusters. Science. 2016;352:167–9.
30. Cheung KJ, Padmanaban V, Silvestri V, Schipper K, Cohen JD, Fairchild AN, et al. Polyclonal breast cancer metastases arise from collective dissemination of Keratin 14-expressing tumor cell clusters. Proc Natl Acad Sci USA. 2016;113:E854–63.
31. Maddipati R, Stanger BZ. Pancreatic cancer metastases harbor evidence of polyclonality. Cancer Discov. 2015;5:1086–97.
32. Fouad YA, Aanei C. Revisiting the hallmarks of cancer. Am J Cancer Res. 2017;7:1016–36.
33. Zajac O, Raingeaud J, Libanje F, Lefebvre C, Sabino D, Martins I, et al. Tumour spheres with inverted polarity drive the formation of peritoneal metastases in patients with hypermethylated colorectal carcinomas. Nat Cell Biol. 2018;20:296–306.
34. Yao Y, Zhou Y, Su X, Dai L, Yu L, Deng H, et al. Establishment and characterization of intraperitoneal xenograft models by co-injection of human tumor cells and extracellular matrix gel. Oncol Lett. 2015;10:3450–6.
35. Hu Q, Ito S, Yanagihara K, Mimori K. Molecular mechanism of peritoneal dissemination in gastric cancer. J Cancer Metastasis Treat. 2018;4:39.