



DNA ploidy and stroma predicted the risk of recurrence in low-risk stage III colorectal cancer

Yuan Li¹ · Leen Liao¹ · Lingheng Kong¹ · Wu Jiang¹ · Jinghua Tang¹ · Kai Han¹ · Zhenlin Hou¹ · Chenzhi Zhang¹ · Chi Zhou¹ · Linjie Zhang¹ · Qiaqi Sui¹ · Binyi Xiao¹ · Weijian Mei¹ · Yanbo Xu¹ · Jiehai Yu¹ · Zhigang Hong¹ · Zhizhong Pan¹ · Peirong Ding¹

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Abstract

Background For clinically low-risk stage III colorectal cancer, the decision on cycles of adjuvant chemotherapy after surgery is disputed. The present study investigates the use of additional biomarkers of ploidy and stroma-ratio(PS) to stratify patients with low-risk stage III colorectal cancer, providing a basis for individualized treatment in the future.

Methods This study retrospectively enrolled 198 patients with clinical-low-risk stage III colorectal cancer (T1-3N1M0) and analyzed the DNA ploidy and stroma ratio of FFPE tumor tissues. The patients were divided into PS-low-risk group (Diploidy or Low-stroma) and PS-high-risk group (Non-diploid and High-stroma). For survival analyses, Kaplan–Meier and Cox regression models were used.

Results The results showed that the 5-year DFS of the PS-high-risk group was significantly lower than that in the PS-low-risk group (78.6 vs. 91.2%, HR = 2.606 [95% CI: 1.011–6.717], $P = 0.039$). Besides, in the PS-low-risk group, the 5 year OS (98.2 vs. 86.7%, $P = 0.022$; HR = 5.762 [95% CI: 1.281–25.920]) and DFS (95.6, vs 79.9%, $P = 0.019$; HR = 3.7 [95% CI: 1.24–11.04]) of patients received adjuvant chemotherapy for > 3 months were significantly higher than those received adjuvant chemotherapy for < 3 months. We also found that the PS could stratify the prognosis of patients with dMMR tumors. The 5-year OS (96.3 vs 71.4%, $P = 0.037$) and DFS (92.6 vs 57.1%, $P = 0.015$) were higher in the PS-low-risk dMMR patients than those in the PS-high-risk dMMR patients.

Conclusion In this study, we found that PS can predict the prognosis of patients with stage III low-risk CRC. Besides, it may guide the decision on postoperative adjuvant chemotherapy.

Keywords Colorectal cancer · DNA ploidy · Stroma-tumor fraction · Mismatch repair

Yuan Li, Leen Liao and Lingheng Kong contributed equally to this work.

✉ Peirong Ding
dingpr@sysucc.org.cn
Zhizhong Pan
panzhzh@sysucc.org.cn

¹ Department of Colorectal Cancer, State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Sun Yat-Sen University Cancer Center, No. 651, Dongfeng Road East, Guangzhou 510060, Guangdong, People's Republic of China

Background

Colorectal cancer (CRC) is the third most common cancer worldwide [1, 2]. There is no doubt to administer postoperative adjuvant chemotherapy in stage III CRC patients to reduce its recurrence rate, but the cycles of the chemotherapy regime are still disputed [3, 4]. The International Duration Evaluation of Adjuvant Chemotherapy (IDEA) pooled analysis compared 3 to 6 months of adjuvant chemotherapy for stage III colon cancer. Patients were classified into low-risk which referred to T1-3N1 and high-risk referred to T4/N2, suggesting low-risk patients may be offered only 3 months of treatment [5]. However, whether patients with low-risk stage III colon cancer can benefit equally from a reduced adjuvant chemotherapy regime is still controversial [6], suggesting tumor biological heterogeneity in these

patients. The present study investigates the use of additional biomarkers to further stratify patients with low-risk stage III colon cancer, providing a basis for individualized treatment in the future.

Firstly, the DNA Ploidy would reflect the DNA content changes in tumor cells. The abnormal quantity of DNA would be termed as aneuploidy, and aneuploidy in tumors has been implicated as a predictor of a poor prognosis [7]. The aneuploidy may partially cause by chromosome instability (CIN), which is a key process in cancer. CIN would drive the development of tumors and is gaining increasing interest. At present, DNA cytometry is the most widely used approach to detecting DNA ploidy based on nucleus images, and DNA cytometry could be used in FFPE tissues.

The other biomarker is the stroma-tumor fraction, can be assessed from the whole slide images of haematoxylin and eosin (H&E) stained sections. The stroma fraction is described as the ratio of the area occupied by stromal cells to the total occupied by stromal cells and carcinoma cells in the tumor tissues. Patients with higher stroma were observed to have a worse prognosis, and the stroma fraction was observed to be an independent prognostic parameter in CRC and other solid epithelial tumors [8–11]. Usually, the tumors with a stroma fraction higher than 50% were subdivided into the high-stroma groups, and the tumors with a stroma fraction lower than 50% were subdivided into the low-stroma groups.

The combination of ploidy and stroma could predict the prognosis of patients with stage II colorectal cancer, which was reported both in the European and Chinese population [12, 13]. In this study, we would investigate the effect of DNA ploidy and stroma-tumor fraction on the prognosis of low-risk stage III colon cancer.

Method

Patient selection

From 2008 to 2015 at Sun Yat-sen University Cancer Center, patients who underwent radical resection and pathologically confirmed T1-3N1M0 stage III colon cancer were screened continuously. Patients with enough paraffin-embedded pathological tissues for DNA ploidy and tumor stromal ratio detection and no less than 1-year follow-up records were included in this study. The study was approved by the Institutional Research Ethics Committee of Sun Yat-sen University Cancer Center (approval number: B2019-109).

Tumor tissue sample preparation

For DNA ploidy and stroma analyses, the physician selected one representative FFPE tumor block for each patient, and

annotated the whole epithelial tumor region. From the initial tumor block, 5 μ m section was cut and stained with H&E (Harris hematoxylin solution, Vendor: BASO, Cat No.

BA4025; Eosin Y, Vendor: KOHY Path, Ref No. KH-EOYA-05-OT-500) for defining the tumor region, which was annotated the whole epithelial tumor region first. To prepare the nuclear monolayer for DNA ploidy analysis, 50 μ m sections should be contained more than 50% of the representative tumor tissue. The tissue was then sent to MBM Clinical Lab (Ningbo Meishan FTZ) for further processing of DNA ploidy and stroma analyze. The nuclear monolayer protocol was prepared according to the previously described protocol [7]. Briefly, the sections were deparaffinized, washed with Xylene (Vendor: Sinopharm Chemical Reagent Co. Ltd, CAS: 1330-20-7) and Ethanol (Vendor: Sinopharm Chemical Reagent Co. Ltd, CAS: 64-17-5) and digested with protease VIII (Protease from *Bacillus licheniformis*, Vendor: SIGMA, Cat No. P5380-250MG) to disaggregate the cells. The resulting nuclei suspension was then filtered and pelleted, and suspended again in PBS before depositing on a glass slide. The monolayer preparations were air-dried and fixed overnight in 4% formaldehyde before staining using Feulgen's method using Schiff's fuchsin-sulfite reagent (Vendor: SIGMA-ALDRICH, Cat No. S5133-500ML).

Measurement of DNA ploidy

The DNA Ploidy was assessed by the DNA Ploidy Working Station (PWS, Room 4, Kent, UK) based on the Feulgen-stained nuclei images, which was captured by a high-resolution digital scanner (MBM bio-Intelligence 005, China). As previously reported [7], first, the nucleus was automatically grouped into three groups including tumor nuclei, reference nuclei and discarded nuclei by the PWS classifier. Second, the integrated optical density (IOD) of the nucleus was evaluated by the PWS and created the DNA ploidy histograms. The reference nuclei were used as an internal diploid control, and DNA ploidy of tumor cells was reported as diploid, aneuploid, and tetraploid [7]. Aneuploid and tetraploid samples were grouped as nondiploid in this study.

Stroma-tumor fraction

The images of the 5 μ m H&E-stained histological sections were captured by the digital scanner (MBM bio-Intelligence 005, China). An experienced pathologist marked the tumor areas on the scanned images on the interface of the Stroma Analyzer (Room4, Kent, UK), as described by Danielsen et al. [13]. The tumor areas were annotated according to the following criteria: tumor cells are present at all borders; surrounding Stromal tissues are not selected. The stroma fraction in the selected tumor region was automatically calculated by the Stroma software. Tumors with stroma fraction

less than or equal to 0.50 were labelled as low-stroma, while those with stroma fraction greater than 0.50 were labelled as high-stroma.

Statistical analysis

The endpoints of this study were overall survival (OS) and disease-free survival (DFS). OS and DFS were defined as the time from surgery to death from surgery and the time from radical resection to relapse of disease, respectively. The distributions of OS and DFS were estimated using the Kaplan–Meier method, and the significance were tested using the log-rank. All relevant indicators were included in the multivariate model for multivariate analysis. The hazard ratio (HR) of clinical events and corresponding 95% confidence intervals (CIs) were estimated by Cox proportional hazard regression. We set reference categories for each variable accordingly. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics for Macintosh 22.0 software (IBM Corp, Armonk, NY).

Result

Patient demography

256 consecutive T1-3N1M0 stage III colorectal cancer patients were involved at first. The cases who did not have qualified pathological tissue samples or enough follow-up records were excluded. 198 patients with DNA ploidy and stromal ratio test results were included in the analysis. At the end of the follow-up, 161 patients were still alive and 35 patients had a recurrence or metastasis. Median OS and median DFS were 68 months (25–75% quartiles: 55–92 months) and 66 months (25–75% quartiles: 52–91 months), respectively.

There were 106 males and 92 females, and the median age of all patients was 61 years. In addition, 147 patients (74.2%) were diagnosed with stage IIIB (T3N1M0) and 51 (25.8%) patients with stage IIIA (T1-2N1M0) disease. 139 (70.2%) patients were left-side colorectal cancer and 58 (29.3%) patients were right-side. The mismatch repair (MMR) status of 182 (91.9%) patients was evaluated by immunohistochemistry (IHC), among them 34 (18.7%) patients were deficient (d) MMR and 148 (81.3%) patients were proficient (p) MMR. 147 (74.2%) patients were treated with chemotherapy (Capecitabine alone, or Oxaliplatin, leucovorin and 5-FU, or Oxaliplatin and Capecitabine) after surgery, 18 (9.1%) patients did not receive chemotherapy, and 33 (16.7%) patients were unclear. A higher proportion of aneuploidy (74.7 vs 25.3% of diploid) and low-stroma (82.3 vs 17.7% of high stroma) were

observed. Other patients' characteristics and distribution of relevant parameters are listed in (Table 1).

Univariate and multivariate analysis of the prognostic factors

Univariate analysis of DFS and OS in clinical low-risk stage III colorectal cancer is shown in (Table 1). No significant prognostic impact was observed for tumor site, histological grade, number of lymph nodes to be examined, MMR status and utilization of adjuvant chemotherapy in both OS and DFS analysis. In univariate analysis of OS and DFS for DNA ploidy and tumor stromal ratio, P values were also not statistically significant.

Then the DNA ploidy (P) and stroma (S) were combined to classify the patients into two groups: the PS-low-risk group and PS-high-risk group. The patients with diploidy (DD) or low-stroma (LS) tumors were classified as the PS-low-risk group, and patients with aneuploidy (AD) and high-stroma (HS) tumors were classified as the PS-high-risk group.

5-year DFS in the PS-low-risk group ($n = 170$) and PS-high-risk group ($n = 28$) and was 91.2 and 78.6%, respectively. Compared to the PS-low-risk group, the PS-high-risk group had a worse DFS (HR = 2.606 [95% CI: 1.011–6.717], $P = 0.039$) (Fig. 1A). However, as shown in Fig. 1B, the OS did not show significant differences between the PS-low-risk and PS-high-risk groups. The 5-year OS in the PS-low-risk group was 94.1%, compared to 89.3% in the PS-high-risk group (HR = 1.91 [95% CI: 0.53–6.95]; $P = 0.315$).

Multivariate analysis of 5-year DFS showed that only ploidy and stroma ($P = 0.036$) was statistically significant. See Table 2 for details.

The DFS and OS of patients with PS-low-risk tumors were improved after received more than 3 months chemotherapy

As shown in Fig. 2, in the PS-low-risk group, patients who received more than 3 months chemotherapy ($n = 114$) had a better 5-year OS than those ($n = 15$) received less than 3 months chemotherapy (98.2 vs. 86.7%, $P = 0.022$, HR = 5.762 [95% CI: 1.281–25.920]). The DFS showed the similar trend in patients with PS-low-risk tumors, the 5-year DFS of patients who received adjuvant for less than 3 months was significantly lower than those for more than 3 months (95.6 vs 79.9%; HR = 3.7 [95% CI: 1.24–11.04]; $P = 0.019$).

Ploidy and stroma provided prognostic information for patients with microsatellite unstable stage III CRC

There were 34 dMMR patients in our study (Fig. 3), which were divided into PS-low-risk group ($n = 27$) and

Table 1 Univariate analysis of OS and DFS in clinical low-risk stage III patients

| Variables | N (%) | OS | | DFS | |
|----------------------------------|------------|------------------|---------|------------------|---------|
| | | HR (95% CI) | P-value | HR (95% CI) | P-value |
| Age | | | 0.426 | | 0.842 |
| ≤ 61 | 102 (51.5) | – | | – | |
| > 61 | 96 (48.5) | 1.55 (0.52–4.62) | | 1.09 (0.45–2.64) | |
| Gender | | | 0.618 | | 0.585 |
| Male | 106 (53.5) | – | | – | |
| Female | 92 (46.5) | 1.32 (0.44–3.92) | | 1.27 (0.54–2.99) | |
| Tumor site | | | 0.263 | | 0.274 |
| Left | 139 (70.6) | – | | – | |
| Right | 58 (29.4) | 0.43 (0.10–1.96) | | 0.55 (0.19–1.64) | |
| Histological grade | | | 0.215 | | 0.146 |
| Moderately differentiated | 147 (77) | – | | – | |
| Poorly-Moderately differentiated | 44 (23) | 0.30 (0.04–2.30) | | 0.36 (0.08–1.53) | |
| Lymphatic node number | | | 0.788 | | 0.501 |
| ≥ 12 | 144 (72.7) | – | | 1 | |
| < 12 | 54 (27.3) | 1.18 (0.36–3.82) | | 1.36 (0.55–3.38) | |
| MMR status | | | 0.261 | | 0.177 |
| pMMR | 148 (81.3) | – | | – | |
| dMMR | 34 (18.7) | 0.46 (0.12–1.84) | | 0.49 (0.17–1.41) | |
| Stroma | | | 0.575 | | 0.159 |
| Low | 163 (82.3) | – | | – | |
| High | 35 (17.7) | 1.44 (0.40–5.25) | | 1.95 (0.76–5.02) | |
| Ploidy status | | | 0.406 | | 0.497 |
| Diploidy | 50 (25.3) | – | | 1 | |
| Aneuploidy | 148 (74.7) | 1.87 (0.42–8.45) | | 1.46 (0.50–4.32) | |
| Ploidy and stroma | | | 0.315 | | 0.039 |
| Diploidy or low-stroma | 170 (85.9) | – | | – | |
| Aneuploidy and high-stroma | 28 (14.1) | 1.91 (0.53–6.95) | | 2.61 (1.01–6.72) | |
| Adjuvant chemotherapy | | | 0.203 | | 0.056 |
| Yes | 147 (89.1) | – | | – | |
| No | 18 (10.9) | 0.37 (0.07–1.83) | | 0.35 (0.11–1.08) | |
| Adjuvant chemotherapy | | | 0.068 | | 0.088 |
| Less than 3 months | 16 (10.9) | | | | |
| More than 3 months | 131 (89.1) | 4.26 (0.78–23.3) | | 2.96 (0.8–10.9) | |

N patient number, HR hazard ratio, CI confidence interval

PS-high-risk group ($n=7$) by the combinations of DNA ploidy and stroma. It was found that the 5-year OS in PS-low-risk group was 96.3% (1/27), and the 5-year OS in PS-high-risk group was 71.4% (2/7) [HR = 8.448 (95% CI: 0.765–93.277, $P=0.037$)], 5-year DFS in PS-low-risk group and PS-high-risk group was 92.6% (2/27) and 57.1% (3/7), respectively [HR = 6.837 (95% CI: 1.140–41.014, $P=0.015$)]. These results indicated that the combination of ploidy and stroma may be able to assess the recurrence risk of MSI-H patients. However, the sample size in this subgroup was relatively small.

Discussion

Tumor-node-metastasis staging system is currently the best prognostic factor for CRC [3]. Although a growing number of experts recommended a reduced adjuvant chemotherapy regime for clinical low-risk stage III (T1–3N1M0) colorectal cancer patients, it is still in debate [5]. It is necessary to further stratify those patients for personalized treatment in the future, and we sought to identify more accurate prognostic factors to further classify patients with low-risk

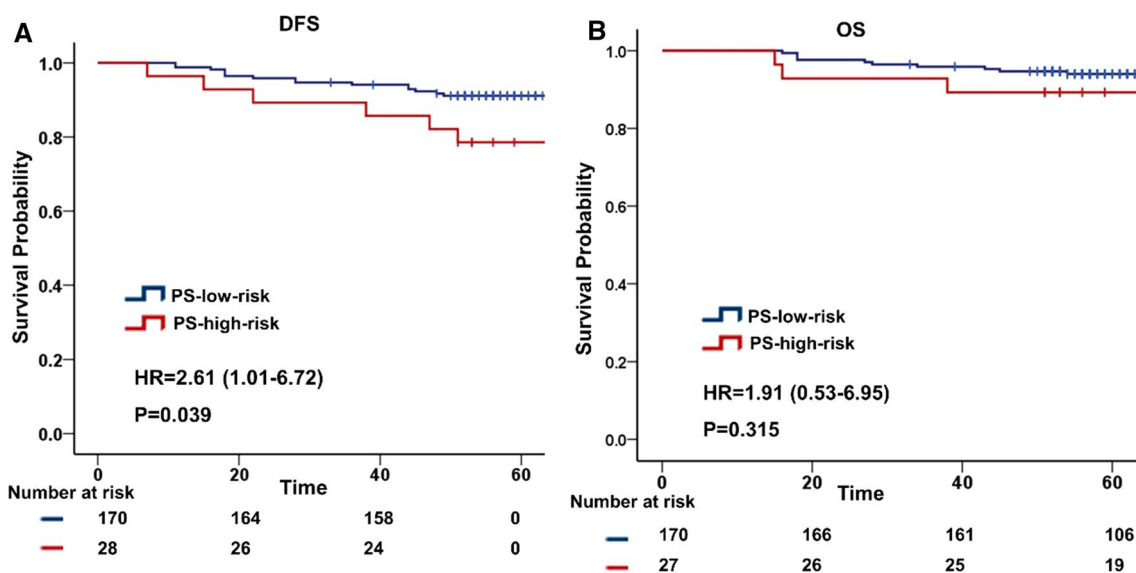


Fig. 1 Kaplan–Meier curves for DFS **A** and OS **B** stage III CRC patients were plotted according to the ploidy and stroma (PS) classifier

Table 2 Multivariate analysis of DFS in low-risk stage III patients

| Variables | 5 year DFS | |
|---|-------------------|---------|
| | HR (95% CI) | P-value |
| Tumor site | 0.14 (0.02–1.12) | 0.063 |
| Right vs left | | |
| Histological grade | 0.79 (0.17–3.74) | 0.769 |
| Poorly-moderately differentiated vs moderately differentiated | | |
| Lymphatic node number | 0.91 (0.25–3.39) | 0.892 |
| < 12 vs \geq 12 | | |
| MMR status | 0.88 (0.23–3.33) | 0.855 |
| dMMR vs pMMR | | |
| Ploidy and stroma | 3.75 (1.09–12.92) | 0.036 |
| Aneuploidy and high-stroma vs diploidy or low-stroma | | |
| Adjuvant chemotherapy (3 months) | 2.19 (0.63–7.60) | 0.215 |
| More than 3 months vs less than 3 months | | |

stage III colorectal cancer. Our study demonstrated that ploidy and stroma was the dominant prognostic factor in low-risk patients with stage III colon cancer. In this study, the ploidy and stroma could classify the stage III low-risk CRC patients into two groups: PS-low-risk and PS-high-risk groups. The DFS of PS-high-risk group (non-diploid and high-stroma) was significantly shorter than that of PS-low-risk (diploid or low-stroma) patients. This was verified both in univariate or multivariable analyses.

Previous studies have demonstrated that ploidy and stroma is an independent prognostic markers in stage II CRC and other solid epithelial tumors [4–9]. The association between ploidy and poor prognosis is well illustrated in a previous study [7]. Aneuploidy could reflect chromosomal instability (CIN) in the nucleus of tumor cells through changes in the DNA content. It has been reported that DNA

ploidy changes were significantly associated with copy number variation [14], this may indicate that DNA ploidy changes were accompanied by genomic instability, which in turn led to tumorigenesis. As previously reported, patients with aneuploidy tumor tissue usually have a poor prognosis. On the other hand, tumor stroma composed of fibroblasts, myofibroblasts, endothelial cells, inflammatory and immune infiltrative cells, is considered to make critical contributions to tumor survival, growth, invasion, and metastatic potential. Studies have reported that the high stroma fraction in solid tumors indicated a worse prognosis [8–11]. In this study, the combination of ploidy and stroma would predict the prognosis of the stage III low-risk CRC patients.

Interestingly, we found that patients in the low-risk group who received more than 3 months chemotherapy had a better 5-year OS than that received less than 3 months

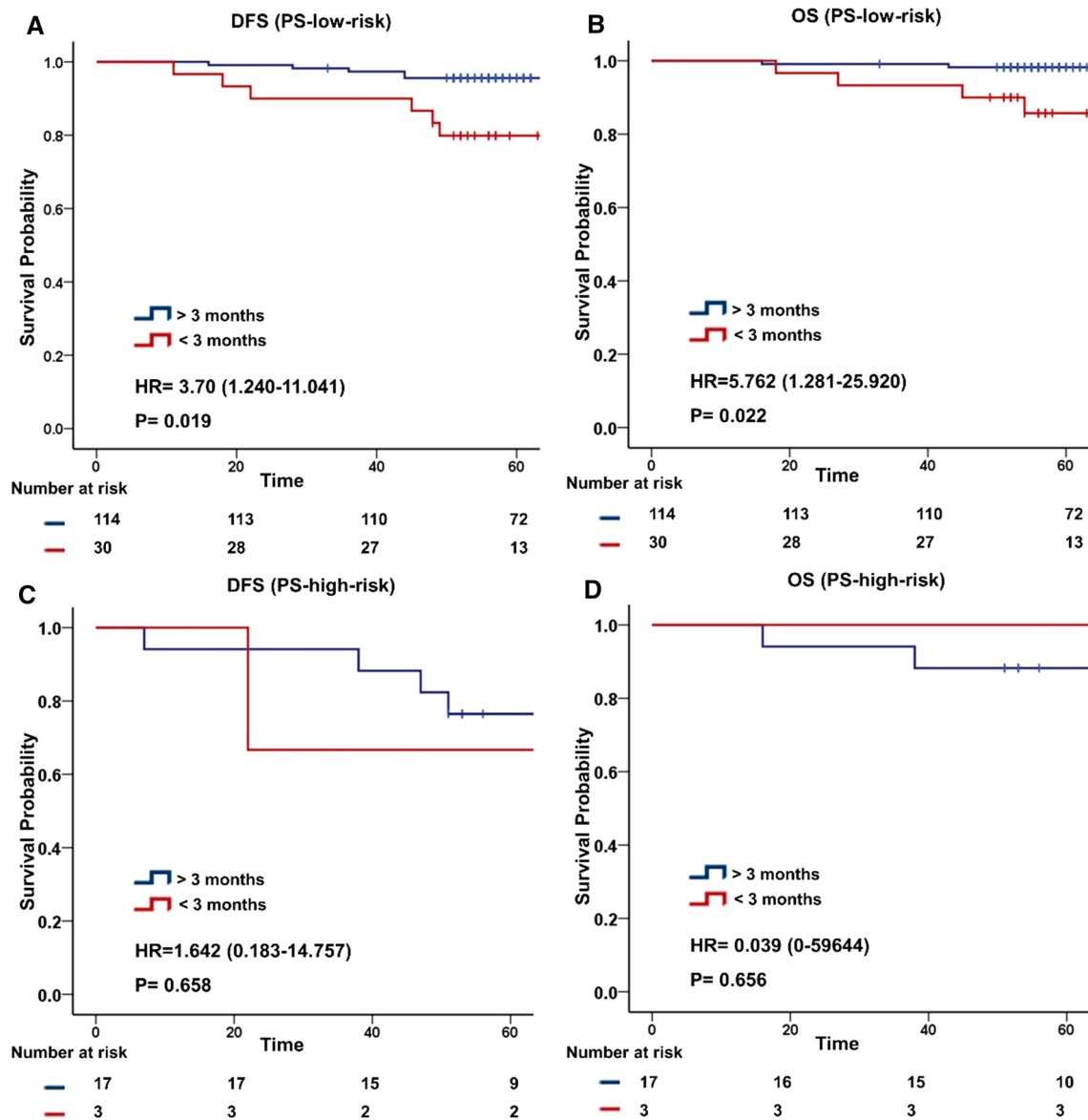


Fig. 2 Kaplan–Meier analyses of disease-free survival and overall survival in patients with stage III CRC after receiving adjuvant therapy for less or more than 3 months. DFS **A** and OS **B** in PS-low-risk patients, DFS **C** and OS **D** in PS-high-risk patients

chemotherapy. This contradicts the traditional concept that low recurrence risk patients could reduce chemotherapy and high recurrence risk patients should receive long chemotherapy. However, from the recommendation of newly established 2020 ESMO clinical practice guidelines for Diagnosis, treatment, and follow-up of locally advanced colorectal cancer, Immunoscore is recommended for patients with low-risk stage III (T1-3N1) colon cancer to determine whether longer chemotherapy is necessary [15, 16]. For patients with low-risk stage III colon cancer, high Immunoscore, predicting low recurrence and benefit from long chemotherapy, 6 months of chemotherapy is recommended for increased survival benefit. Patients with low Immunoscore, predicting

high recurrence may consider receiving 3 months of chemotherapy, which is seen to be equivalent to 6 months of chemotherapy. Interestingly, this recommendation based on recurrence risk stratification is consistent with our present study. Besides, from the result of recent research analyzing chemotherapy response data of tumors in TCGA, it was found that good survival correlated with diploid, which reflects differences in therapeutic response associated with ploidy [17]. The rate of complete or partial response to chemotherapy agents such as Fluorouracil, Oxaliplatin et al. to be considerably higher among MSS compared to MSI tumors, as well as higher among diploid compared to aneuploid tumors [17]. So, the chemotherapeutic drugs may be more effective in

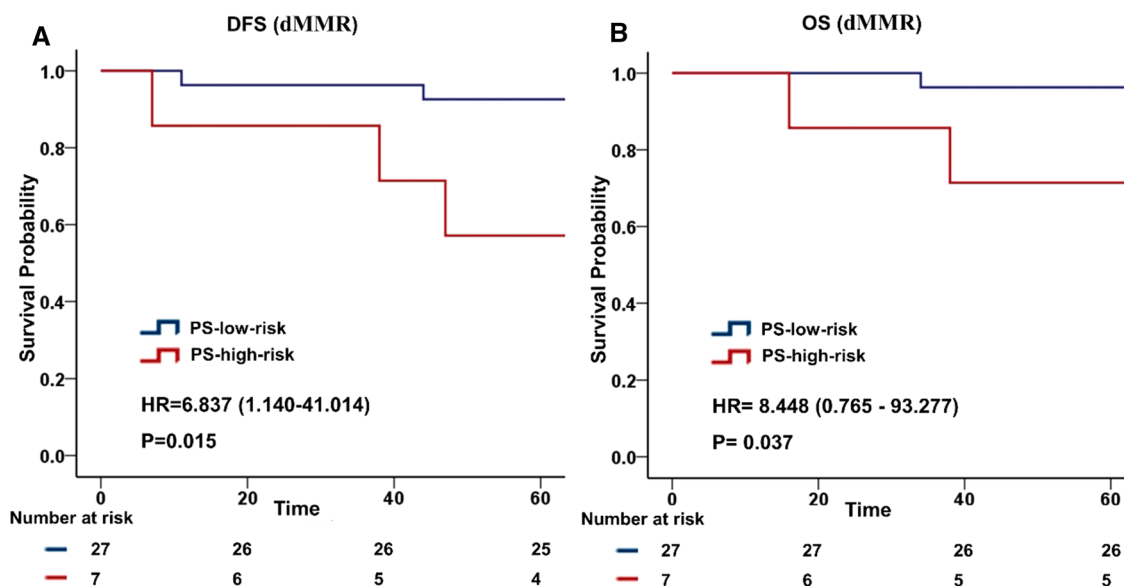


Fig. 3 Kaplan–Meier analyses of disease-free survival and overall survival in patients with microsatellite unstable tumors. DFS **A** and OS **B** in microsatellite unstable tumors stratified by PS-low-risk and PS-high-risk

the low-risk group with diploid tumors. Based on the results of our study and the Immunoscore, it seems more reasonable to give low-risk stage III colorectal patients long-course chemotherapy.

It has been proved that DNA ploidy was negatively correlated with dMMR status [18]. This may be owing to the methylation of many promoters being associated with tumor ploidy and MMR protein [19]. A strong evidence base supports the use of ploidy status for prognostic prediction in patients with stage II colorectal cancer, combined with multivariate analysis, suggesting that tumor diploidy is an even stronger marker of good prognosis than MSI [18, 20, 21]. We also found that DNA ploidy and stroma was stronger marker than MSI in low-risk stage III colorectal cancer from multivariate analyses.

PS-low-risk MSI-H patients had the best OS and DFS in our study. We speculated the chemosensitivity of diploid tumors which mentioned above [17], may help to explain the survival superiority. But as we knew MSI-H was highly predictive of non-response to chemotherapy [22], when the two factors are combined, whether diploid MSI-H patients could benefit from chemotherapy remains to be testified. Whether adjuvant chemotherapy is needed for stage III MSI-H colorectal cancer is still controversial [23, 24]. If our speculation and hypothesis were true, DNA ploidy and stroma may help distinguish a group of MSI-H patients who are suitable for adjuvant chemotherapy.

To our knowledge, this study was the first independently performed prognostic evaluation of ploidy and stroma in clinical low-risk stage III colorectal cancer patients. The

multivariable analysis model displayed with ploidy and stroma as an independent variable which could predict recurrence. Besides, survival analysis showed the benefit of the reduced adjuvant chemotherapy decreased in the PS-low-risk group. Therefore, we believe ploidy and stroma can further stratify existing low-risk stage III colorectal cancer patients, providing a more accurate basis for guiding clinical personalized medicine.

There are certain limitations to our study. First, this study is subject to the limitations and biases inherent in any single institutional retrospective analysis. Another limitation is insufficient information regarding other molecular markers such as KRAS and BRAF V600E mutations, which also play an important role in the prognosis [25]. Information on lymphocytic infiltrates was also not included in the analysis. Details about the duration of chemotherapy are not available in this study. The analysis was primarily based on receipt of any chemotherapy and does not account for early discontinuation of prescribed treatment, which possibly could impact the survival benefit. Better survival and fewer events of recurrence, metastasis and death may reduce the quality of statistical analysis. Future research will be directed in multicenter and expanding following up time to further determine the reliability of the results herein.

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Author contributions YL, LL, ZP and PD: contributed to the conception of the study; BX, QS, YX, WM, JY and ZH: collected data; WJ, LK, JT: and KH: contributed significantly to analysis and manuscript preparation; ZH, CZ, CZ and LZ: designed table and figure; YL and LL: performed the data analyses and wrote the manuscript; ZP and PD: helped perform the analysis with constructive discussions and contributed to critical revision; All authors reviewed the manuscript.

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Declarations

Conflict of interest All authors have no conflicts of interest or financial ties to disclose.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Institutional Research Ethics Committee of Sun Yat-sen University Cancer Center (approval number: B2019-109).

Inform consent For this type of study, formal consent is not required.

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