Journal of the National Cancer Center 4 (2024) 74–85

Contents lists available at ScienceDirect

Journal of the National Cancer Center

journal homepage: www.elsevier.com/locate/jncc

Full Length Article

The development and implementation of pathological parameters and molecular testing impact prognosis of colorectal adenocarcinoma

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ARTICLE INFO

Keywords:
Colorectal adenocarcinoma
Pathological features
Molecular testing
Trends
Prognosis

ABSTRACT

Objective: This study aims to analyze how changes in pathological diagnosis practice and molecular detection technology have affected clinical outcomes for colorectal cancer (CRC) patients in Fudan University Shanghai Cancer Center (FUSCC).

Methods: This retrospective cohort study analyzed 21,141 pathologically confirmed CRC cases diagnosed at FUSCC from 2008 to 2020. Patients were divided into five groups for different analytical purposes: (1) the before vs. since 2014 groups to analyze the influence of the changes in the classification criteria of pT3 and pT4 staging on the survival of patients; (2) the partial vs. total mesorectal excision (TME) groups to analyze whether evaluation of completeness of the mesorectum have impact on the survival of patients; (3) the tumor deposit (TD)(+)N0 vs. TD(+)N1c groups to analyze the influence of the changes in the pN staging on the survival of patients with positive TD and negative regional lymph node metastasis (LNM); (4) the before vs. since 2013 groups to analyze the influence of the changes in the testing process of deficient mismatch repair on the survival of patients; and (5) the groups with vs. without RAS/BRAF gene mutation testing to analyze the influence of these testing on the survival of patients. Patients' clinicopathological parameters, including age at diagnosis, sex, tumor size, location, differentiation, mucinous subtype, TD, lymphovascular invasion, perineural invasion, tumor depth, LNM and distant metastasis, and tumor-node-metastasis (TNM) stage, were compared between groups. Kaplan-Meier analysis with log rank method was performed for patients' overall survival (OS) and disease-free survival (DFS) analyses.

Results: In pathological reports, there were three parameter changes that impacted patient outcomes. Firstly, changes in the pT staging criteria led to a shift of the ratio of patients with stage pT3 to stage pT4 from 1: 110.9 to 1: 0.26. In comparison to patients admitted before 2014 ($n = 4,754$), a significant difference in prognosis between pT3 and pT4 stages was observed since 2014 ($n = 9,965$). Secondly, we began to evaluate the completeness of the mesorectum since 2016. As a result, 91.0% of patients with low rectal cancer underwent TME ($n = 4,111$) surgery, and patients with TME had significantly better OS compared with partial mesorectal excision (PME, $n = 409$). Thirdly, we began to stage TD (+) LNM (-) as N1c since 2017. The results showed that N1c ($n = 127$) but not N0 ($n = 39$) can improve the prognosis of patients without LNM and distal metastasis. In molecular testing, there have been three and five iterations of updates regarding mismatch repair (MMR)/microsatellite instability (MSI) status and RAS/BRAF gene mutation detection, respectively. The standardization of MMR status testing has sharply decreased the proportion of deficient MMR (dMMR) patients (from 32.5% to 7.4%) since 2013. The

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https://doi.org/10.1016/j.jncc.2024.02.001
Received 1 June 2023; Received in revised form 1 February 2024; Accepted 2 February 2024

prognosis of patients underwent MMR status testing since 2013 (n = 867) were significantly better than patients before 2013 (n = 1,313). In addition, detection of RAS/BRAF gene mutation status (n = 5,041) resulted in better DFS but not OS, for patients with stage I-III disease (n = 16,557).

Conclusion: Over the past few decades, updates in elements in pathological reports, as well as the development of standardized tests for MMR/MSI status and RAS/BRAF gene mutations have significantly improved patient outcomes.

1. Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths worldwide. In China, it has become the second most common cancer,[1] whereas two decades ago it was only the fifth.[2] While systemic and precision therapies bring cancer patient management into multidisciplinary team working, pathologists still have a pivotal role in assessing patients' prognosis and guiding their further treatment. Pathologists not only give a definite diagnosis including all the essential parameters for surgical pathology, but also provide the biomarker test results which are valuable for postoperative treatment and follow-up strategies.

The diagnosis methods and report content for CRC have undergone many changes over time worldwide. For example, the 7th edition of the tumor-node-metastasis (TNM) Classification system by the Union for International Cancer Control (UICC)/American Joint Committee on Cancer (AJCC), which was implemented globally in 2009, introduced "non-anatomical" predictors to determine the prognosis and effectiveness of treatment,[3] such as circumferential resection margin (CRM), tumor regression grade (TRG) score, KRAS gene status, and microsatellite instability (MSI) status. In 2018, The AJCC-8 system improved upon this by including additional factors such as tumor deposit (TD),[4] serum carcinoembryonic antigen (CEA) levels, lymphovascular infiltration, perineural infiltration, MSI and RAS/BRAF gene status as the predictors for both prognosis and therapeutic efficacy. Particularly, the AJCC-7 system suggested that patients with positive TD and negative regional lymph node metastasis (LNM) should be considered as venous invasion with extramural spread (V1/2) and pN0.[3] Subsequently, these patients were considered as pN1c stage in the AJCC-8 staging system.[4] For the detection of RAS/BRAF gene mutation, in 2008, the National Comprehensive Cancer Network (NCCN) Colorectal Cancer Clinical Practice Guidelines recommended testing all metastatic CRC (mCRC) patients for KRAS gene status, and only those with RAS wild-type should be prescribed anti-epidermal growth factor receptor (EGFR) monoclonals.[5] In 2010, the guidelines were revised to include RAS/BRAF mutation analysis for CRC patients,[6] and this test has since become a standard molecular assay in Chinese pathology departments.[7-10] Then, the AJCC-8 suggested that RAS/BRAF mutations not only predict the effectiveness of anti-EGFR-mab, but also serve as a prognostic factor for CRC.[4] Subsequently, four CRC guidelines published by NCCN, American Society of Clinical Oncology (ASCO), European Society for Medical Oncology (ESMO), and Chinese Society of Clinical Oncology (CSCO) all recommended mutation analysis of these genes in surgically resected CRC and mCRC cases, leading to a consensus on this issue. Moreover, patients newly diagnosed with CRC should undergo mismatch repair (MMR) protein expression testing was recommended by CSCO in 2020.[11]

Timely updating the elements of standardized pathology reports, including the biomarker detection, is crucial for patients with CRC to obtain progressive personalized treatment and management. This study aims to analyze how changes in pathological diagnosis practice and molecular detection technology have affected clinical outcomes for CRC patients in Fudan University Shanghai Cancer Center (FUSCC).

2. Materials and methods

2.1. Study registration and participants

The study protocol was approved by the Institute Review Board (IRB) of FUSCC, which is a tertiary teaching hospital, and was performed in accordance with the Declaration of Helsinki. Each participant signed an IRB–approved informed consent. The clinicopathological characteristics and molecular testing results of each patient were retrieved from the hospital information systems. Before analysis, we had reviewed and extracted information including the depth of the primary tumor (pT), the extent of spread to the nearby lymph nodes (pN), and the presence of metastasis (pM) from all pathology reports, and then staged each case according to the 8th AJCC TNM staging system. We selected December 31, 2020 as the administrative end date of the survey that traced back to the launched year of the molecular testing.

The pathology registry includes all patients with CRC who were diagnosed or treated at FUSCC. The following information was collected: time at surgery, sex, personal identification information, residential address, age at diagnosis, and pathological diagnosis. Pathological parameters including tumor location, size, differentiation, TD, lymphovascular invasion, perineural invasion, tumor budding and tumor regression grade (TRG). Right-sided colon cancers were defined as those located in the cecum, ascending colon, hepatic flexure and proximal 2/3 of transverse colon; left-sided colon cancers were defined as those located in the distal 1/3 of transverse colon splenic flexure, descending colon, and sigmoid colon.

2.2. Inclusion and exclusion criteria

The inclusion criteria of the study were as follows: (1) Patients with pathologically confirmed colorectal adenocarcinoma, including hereditary CRC syndromes such as Lynch syndrome, familial adenomatous polyposis, Peutz-Jeghers syndrome, juvenile polyposis syndrome, and serrated polyposis syndrome; (2) Patients with registration data included in FUSCC's pathology registry; (3) Patients diagnosed with CRC in FUSCC between January 1, 2008 and December 31, 2020. Changes in pathological diagnosis practice and molecular detection technology include the classification criteria of pT3/T4 and pN0/N1c staging, evaluation of the completeness of the mesorectum, testing of MMR/MSI status, and RAS/BRAF gene mutation status (Fig. 1A). Therefore, for analysis of the influence of these changes on the survival of patients, patients were divided into five groups. Each group has specific inclusion and exclusion criteria, as detailed in Fig. 1B.

2.3. Follow-up and quality control

Prognosis-related parameters included disease-free survival (DFS), and overall survival (OS). The follow-up information of patients was obtained from the department of Cancer Prevention, Clinical Statistics Center. Quality control, conducted by the Department of Cancer Prevention, involved checking the logic, effectiveness, and completeness of survey data and dataset analysis.

2.4. Study groups

This retrospective cohort study included 21,141 patients with pathologically confirmed CRC from January 2008 to December 2020, using FUSCC's pathology registry. Based on the development and implementation of pathological parameters and molecular testing, we mainly divided patients with CRC into five groups for retrospective cohort studies in this study (Fig. 1B), as follows:

The classification criteria of pT3 and pT4 staging. Patients were divided into the before vs. since 2014 groups to analyze the influence of

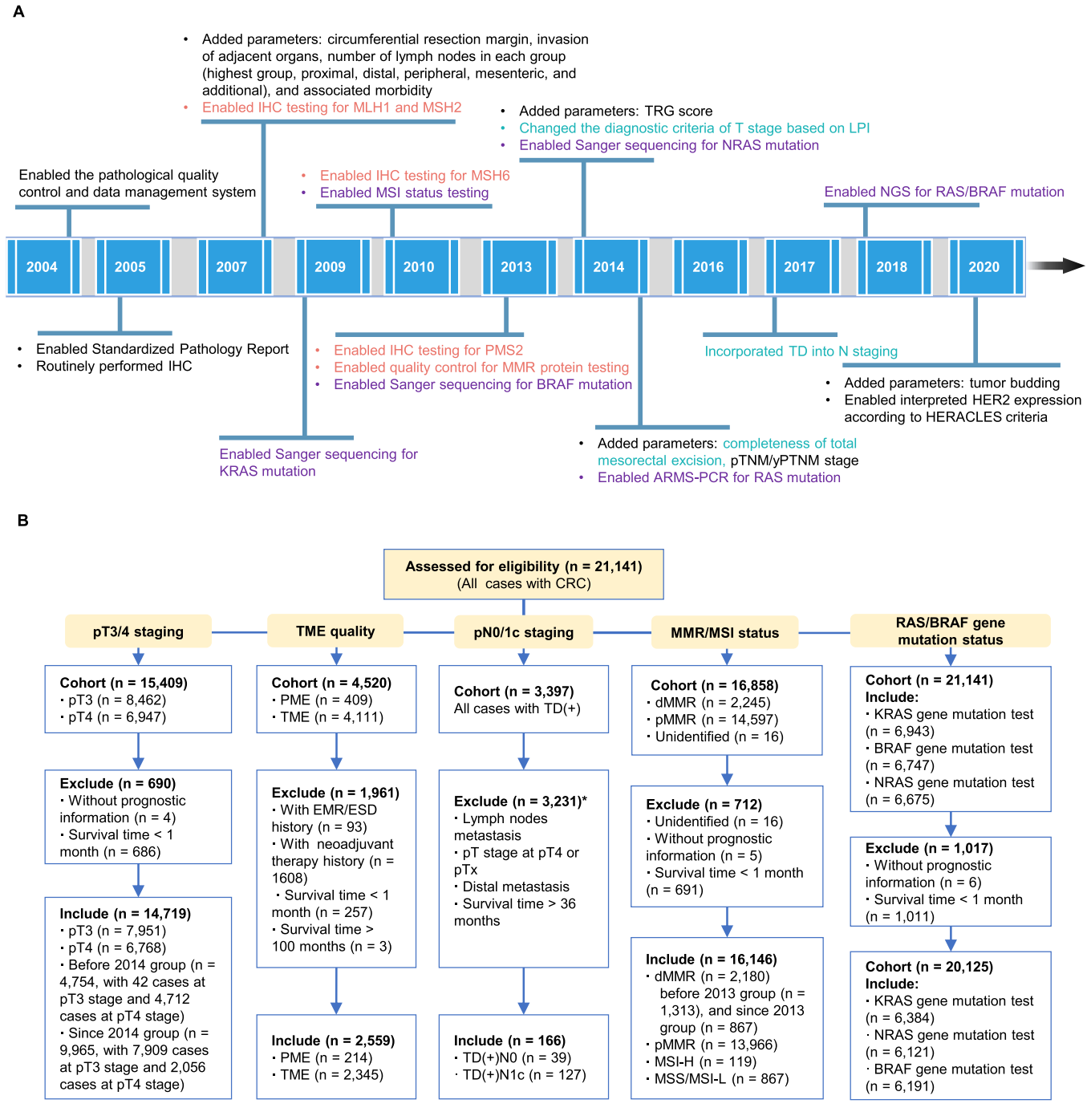

Fig. 1. Flow chart of changes in pathological reports and patient selection process. (A) The progression of pathology reports, IHCs, and molecular tests in FUSCC from 2004 to 2020. Sentences marked with colors are the key changes discussed in the present study. (B) Flow chart of patient selection process. ARMS-PCR, amplification refractory mutation system polymerase chain reaction; CRC, colorectal cancer; dMMR, deficient MMR; EMR/ESD, endoscopic mucosal resection/endoscopic submucosal dissection; IHC, immunochemistry; LPI, local peritoneal involvement; MMR, mismatch repair; MSI, microsatellite instability; MSI-H, MSI-high; MSI-L, MSI-low; MSS,microsatellite stable; NGS, next generation sequencing; PME, partial mesorectal excision; pMMR, proficient MMR; pTNM, TD, tumor deposit; TRG, tumor regression grade, yPTNM, post-neoadjuvant chemotherapy TNM stage .

the changes in the classification criteria of pT3 and pT4 staging on the survival of patients.

The classification criteria of pN0 and PN1c staging. Previously, we diagnosed cases with TD and negative regional LNM as pN0. Starting from January 2017, this type was redefined as pN1c. Therefore, the prognosis of patients with TD (+) pN0 were compared to those with TD (+) pN1c.

Evaluation of the completeness of the mesorectum. Since July 2016, we have been evaluating the completeness of the mesorectum for the lower rectum specimens. Therefore, patients with PME were enrolled for comparison versus patients with TME to analyze whether the evaluation has impact on the survival of patients.

Testing of MMR and MSI status. We divided patients undergoing MMR/MSI status testing into before and since 2013 groups, and

analyzed whether inclusion in the standardized testing process affected the deficiency rate of MMR protein and the prognosis of patients.

Testing of RAS/BRAF status. We compared the prognosis of patients who underwent RAS/BRAF gene mutation testing or not.

2.5. Statistical analysis

All statistical analyses were performed by using SPSS 24.0 (IBM, NY, USA) or R 3.6.0 (https://mirrors.tuna.tsinghua.edu.cn/CRAN/) with default software parameters. Comparisons between groups were determined by paired *t*-test, Mann Whitney test, two-tailed χ^2 test, or one-way ANOVA followed by Tukey's multiple comparison tests, as appropriate. The Kaplan-Meier method was used to compare OS and DFS between the groups. Stratified Cox regression survival analysis was used to explore the risk factors for OS and DFS using the backward stepwise model (likelihood ratio). All *P* values were two-sided, and *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Patient characteristics and survival outcome

We conducted a retrospective analysis of 21,141 patients diagnosed with CRC between 2008 and 2020 (Supplementary Table 1). Of those, 12,593 patients were male (59.57% of the cohort). The mean age at diagnosis was 59.47 years (range: 16–96 years), with a median age of 60 years. Synchronous CRC was observed in 284 patients (1.34%). The most common histopathological type was adenocarcinoma ($n = 12,969$), which accounted for 61.35% of cases, while other types included mucinous adenocarcinoma, signet ring cell carcinoma, and high-grade intraepithelial neoplasia. Approximately 53.70% of patients had rectal cancer (11,352 cases), and LNM was present in 43.2% of patients (9,136 cases). Synchronous distant metastases were observed in 16.39% of patients (3,465 cases), while postoperative recurrence or metastasis occurred in 24.49% of patients (5,179 cases; Supplementary Table 2).

The follow-up period extended until September 30, 2022. We excluded a total of 1,016 patients who were lost to follow-up immediately after their operation or followed up for less than one month from the subsequent survival analysis. The median OS time was 152.033 months ($n = 20,125$). By removing patients at distal metastasis (M1 stage), 13,972 patients were included for DFS analysis. The 1-, 3-, and 5-year OS rates were 94.8%, 82.1%, and 73.4%, respectively, while the corresponding DFS rates were 94.1%, 84.9%, and 80.8%, respectively.

3.2. Changes in pathological diagnosis criteria improved the prognosis of patients

Following the suggestion from CRC Multi-Disciplinary Treatment (MDT) of FUSCC, we revised several pathological parameters in the report content as outlined in Fig. 1A. Among them, we analyzed the influence of changes in three parameters on the prognosis of patients as follows: diagnostic criteria for pT3/pT4 and pN0/N1c staging and assessment of completeness of the mesorectum.

Previously, all tumors that were located close to the peritoneum (<1 mm) with or without a fibrous rim were classified as pT4. From January 2014, we redefined local peritoneal involvement 2 (LPI2) tumors as pT4a (Fig. 2A), while those without the above-mentioned peritoneal involvement signs were defined as pT3. Based on this alternation, we observed a sharp increase in the percentage of patients with pT3 tumors and a rapidly decrease in the percentage of patients with pT4 tumors since 2014 (Fig. 2B). There was a significant difference in the number of patients classified as pT3 and pT4 before and since 2014; the ratio of patients with pT3 and pT4 stage has changed from 1:110.9 to 1:0.26 (Fig. 2C).

To evaluate the significance of changing T staging for guiding clinical practice, we excluded 690 patients without prognostic information or with survival time of less than one month, and divided all 14,719 patients with pT3 ($n = 7,951$) or pT4 ($n = 6,768$) stage into two groups based on surgery time using year 2014 as the time node (Fig. 1B). The group before 2014 consisted of 4,754 patients (with 42 cases at pT3 stage and 4,712 cases at pT4 stage), while the since 2014 group had 9,965 patients (with 7,909 cases at pT3 stage and 2,056 cases at pT4 stage). Of the 14,719 patients with pT3-pT4 stage, although patients with pT3 tumors had better OS and DFS rates compared to those with pT4 tumors in all patients (Supplementary Fig. 1), this difference was only present in the since 2014 group (Fig. 2D-E). We did not observe a statistically significant difference in OS and DFS rates between patients with pT3 and pT4 tumors in the group before 2014 (Fig. 2F-G), indicating that there may be confusion about the classification of pT3 and pT4 cases before 2014, and the invasion depths of many tumors were overestimated. All this suggest that since 2014, the revision of the pT staging criteria for CRC has led to a significant decrease in the number of CRC cases with pT4 stage, and patients at pT3 stage began to show an appropriately different outcome compared to pT4 stage.

Secondly, to evaluate how including TD in N staging affects patient outcomes, we selected 3,397 patients tested TD(+), excluded patients with pT stage at pT4 or pTx, lymph node metastasis or distal metastasis, and survival time less than one month or more than 36 months, resulting in 166 patients whose postoperative clinical management may be influenced by TD (Fig. 1B). Kaplan-Meier analysis revealed that patients in the TD(+)N1c group had significantly better OS ($P < 0.001$) and DFS ($P < 0.001$) than those in the TD(+)N0 group (Fig. 3). These findings suggest that incorporating TD into N staging may improve the prognosis of patients without LNM and distal metastasis, which may be achieved by more proactive postoperative management on patients defined as TD(+)N1c.

Thirdly, we assessed the influence of mesorectum completeness on the prognosis of 4,520 patients with lower rectal cancer. There were 409 cases of PME and 4,111 cases of TME (Fig. 1B, Supplementary Table 2). To eliminate the impact of other factors on the survival, we excluded 93 cases who underwent endoscopic mucosal resection (EMR)/ endoscopic submucosal dissection (ESD) surgery and 1,608 cases who underwent neoadjuvant therapy. For survival analysis, we excluded 257 cases lost to follow-up immediately after their operation or with a survival time less than one month, as well as 3 cases with survival time more than 100 months (Fig. 1B). Out of the remaining 2,559 patients, the Kaplan-Meier analysis results showed that patients with TME had significantly better OS ($P = 0.00024$) than patients with PME (Fig. 4A), whereas there was no significantly difference in DFS between the TME and PME groups (Fig. 4B). The result suggests that TME operation may significantly improve the clinical outcomes of patients with low site rectal cancer.

3.3. Development of testing for MMR and MSI status improved the accuracy of detecting MMR status and the prognosis of patients with CRC

Since 2007, FUSCC has routinely performed immunochemistry (IHC) evaluations of MMR proteins (Fig. 1A). Initially, we only tested for MutL Homolog 1 (MLH1) and MutS Homolog 2 (MSH2) antibodies. Then, we began routinely including MutS Homolog 6 (MSH6) and PMS1 Homolog 2 (PMS2) antibodies in the MMR status detection panel since 2009 and 2012, respectively. Additionally, we introduced routine PMS2 expression detection, and implemented quality control measures and set up positive control for every test in 2013 (Fig. 1A). Therefore, we implemented standardized testing of MMR status in FUSCC in 2013. From 2008 to 2020, a total of 16,858 cases underwent IHC testing for MMR proteins, out of which 2,245 cases were identified as deficient MMR (dMMR, 13.1%), 14,597 cases were identified as proficient MMR (pMMR, 86.6%), and 16 cases were unidentified.

We examined whether the inclusion of standardized testing process affected the deficiency rate of MMR proteins. The deficiency ratios for MLH1 (27.6% vs. 3.2%), MSH2 (14.4% vs. 1.5%) and MSH6 (2.75% vs. 2.55%) proteins were significantly reduced in the since 2013 group

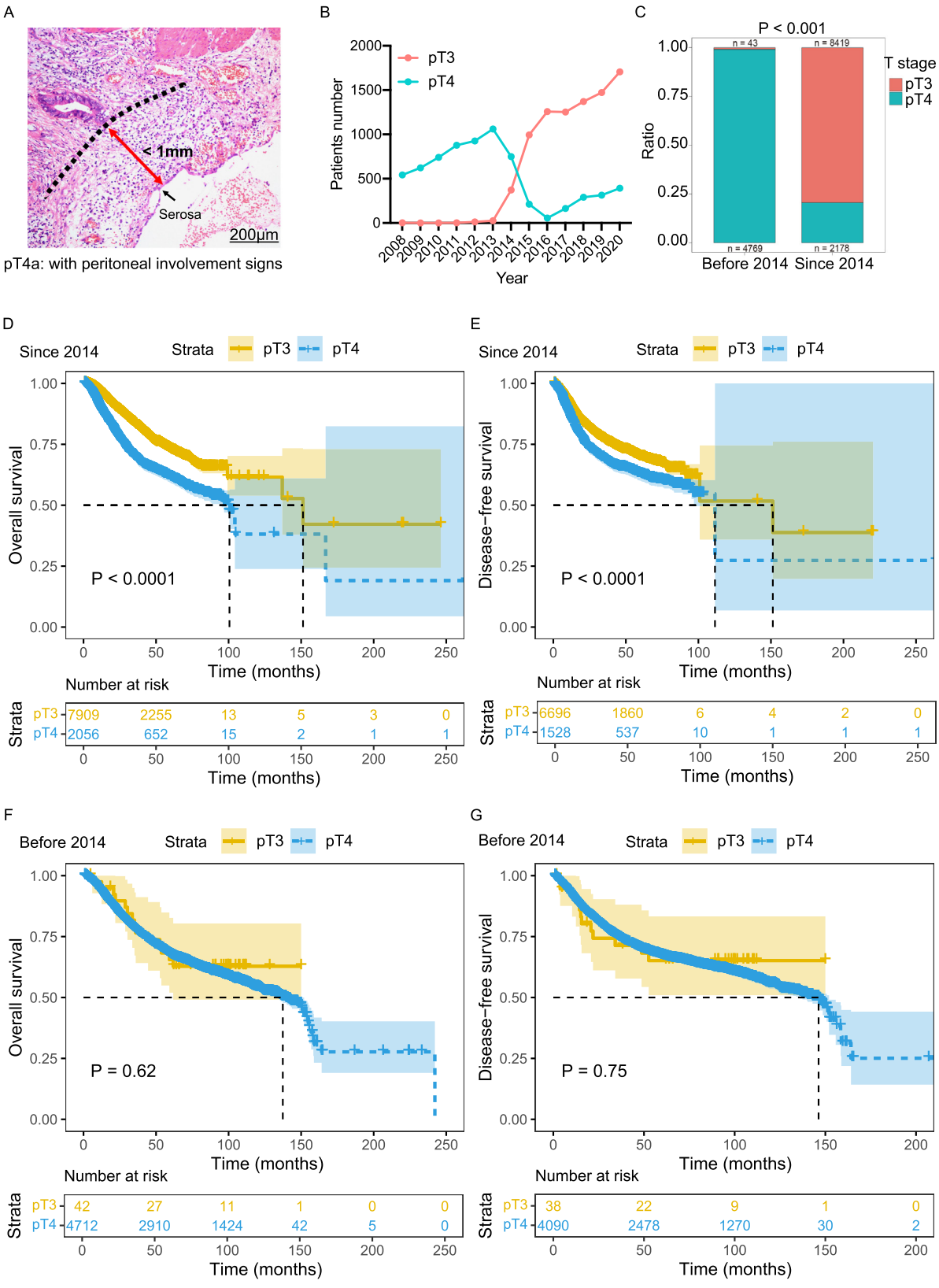

Fig. 2. The impact of changes in diagnostic criteria for pT3/pT4 on the prognosis of colorectal cancer patients. (A) Schematic diagram of pT3 and pT4 staging definitions based on local peritoneal involvement. The dashed line denotes the edge of tumor infiltration. (B) Trends in the number of colorectal cancer patients at pT3/pT4 stage from 2008 to 2020. (C) The difference in the number of patients at pT3/pT4 stage diagnoses before and since 2014. (D-G) Kaplan-Meier curves with log-rank analysis of overall survival (D, F) and disease-free survival (E, G) in patients who underwent surgery after (D-E) and before (F-G) 2014.

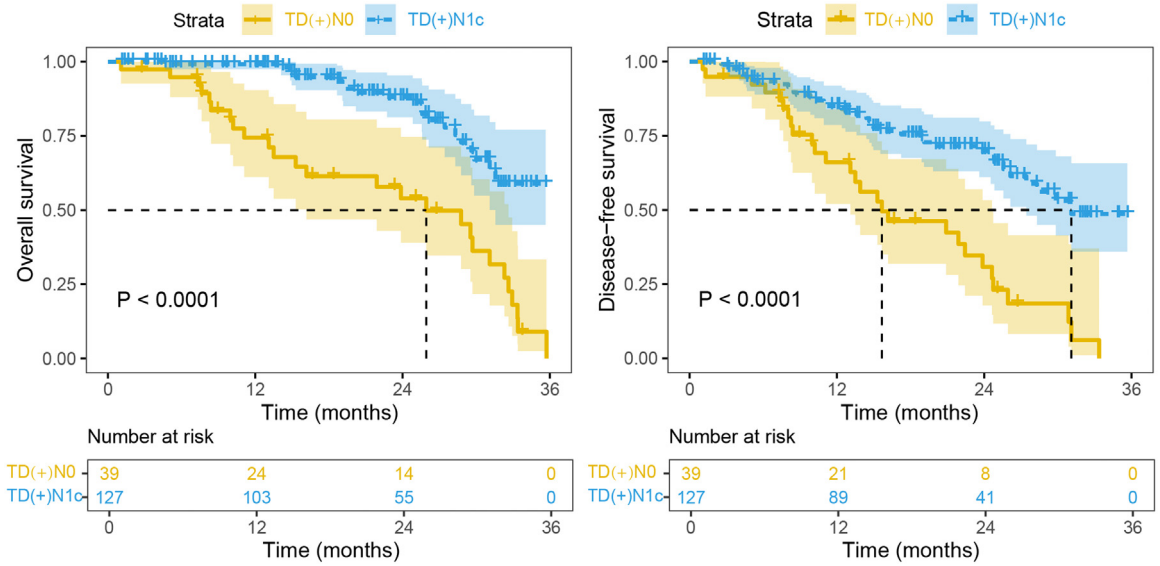

Fig. 3. Definition of pN stage based on TD information affect the prognosis of prognosis of colorectal cancer patients. Kaplan-Meier curves with log-rank analysis of overall survival and disease-free survival in colorectal cancer patients stratified by the TD(+)N0/N1c stage. TD, tumor deposit.

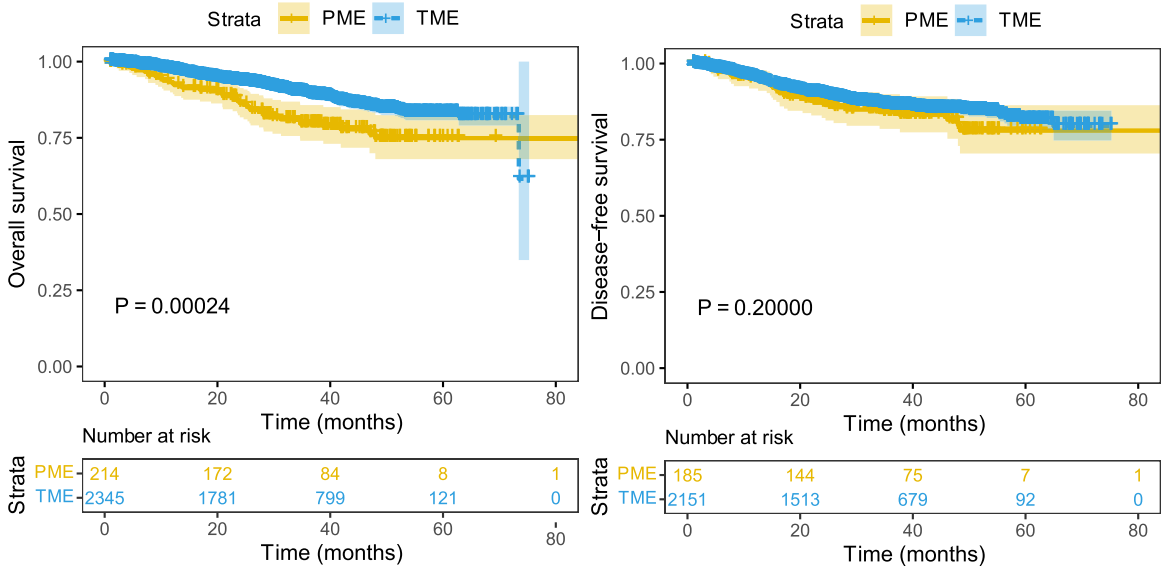

Fig. 4. Evaluation of the completeness of the mesorectum in TME specimen affect the prognosis of prognosis of colorectal cancer patients. Kaplan-Meier curves with log-rank analysis of overall survival and disease-free survival in colorectal cancer patients stratified by the mesorectal excision information. PME, partial mesorectal excision; TME, total mesorectal excision.

compared to the before 2013 group (Fig. 5A). Additionally, the percentage of cases with dMMR also significantly decreased since 2013 (32.5% vs. 7.4%), and the dMMR incidence from 2013 to 2020 ranged from 6.4% to 9.3% (Fig. 5A). These findings suggest that the use of a four-antibody panel and quality control measures for MMR protein IHC testing has significantly improved the accuracy of detecting MMR status in CRC.

Since 2013, FUSCC has been able to use polymerase chain reaction (PCR) or next generation sequencing (NGS) detection to determine the MSI status of patients. Out of 804 cases that underwent MSI testing, 136 (16.9%) were identified as MSI, with 125 (15.5%) classified as MSI-high (MSI-H) and 11 (1.4%) being MSI-low (MSI-L). Although the incidence of dMMR/MSI-H cases varied based on different clinicopathological features, the correlation of MMR/MSI-H status with clinicopathological parameters was not consistent across the four variables of sex, T stage, or NRAS/BRAF gene mutation (Supplementary Table 3). This inconsistency might be due to non-standardized testing procedures for MMR proteins before 2013. Of the 2,245 dMMR cases, MLH1 had the highest proportion of deficiencies, accounting for 67.6% (Fig. 5B). However, in the 703 cases who underwent both MSI and MMR status detection (Fig. 5B-C), there were 117 MSI-H/dMMR cases, among which the proportion of deficiencies of MLH1 was 54.7%, and PMS2 had the highest proportion of deficiencies, accounting for 57.2% (Fig. 5B). Therefore, standardization of the detection process significantly reduced the proportion of deficiencies of MLH1.

There was a discrepancy found between the MMR and MSI status in the 703 patients who underwent both tests (Fig. 5B-C, Supplementary Table 4). Out of the 577 pMMR cases, 6 were identified as MSI-H. Out of the 126 dMMR patients, 111 (88.1%) were identified as MSI-H, 14 cases were identified as microsatellite stable (MSS), and 1 case was identified as MSI-L (Fig. 5B-C). Among these 14 MSS cases (Supplementary Table 4), 2 showed deficiency of both MLH1 and PMS2 proteins, 5 showed

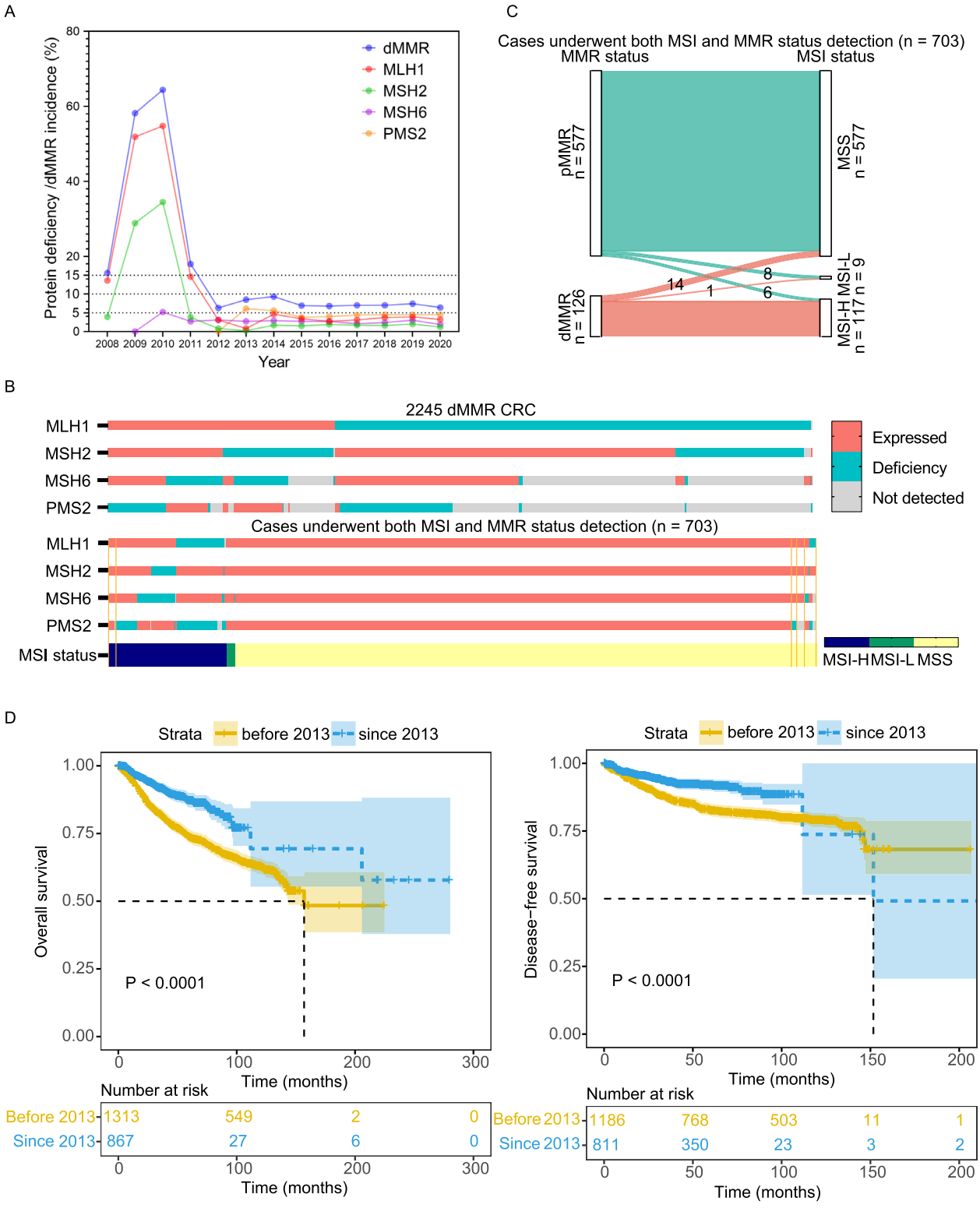

Fig. 5. Trends of MMR/MSI status of CRC patients in FUSCC, 2008–2020. (A) Trends of deficiency ratio of four MMR proteins expression and dMMR incidence of CRC patients in FUSCC, 2008–2020. (B) Sankey of the correlation between MMR and MSI status in 704 CRC patients. (C) Heatmap of four MMR proteins expression in each patient with indicated MMR/MSI status. (D) Kaplan-Meier curves with log-rank analysis of overall survival and disease-free survival in CRC patients with MMR status testing before and after 2013. CRC, colorectal cancer; dMMR, deficient mismatch repair; FUSCC, Fudan University Shanghai Cancer Center; MSS, microsatellite stable; MSI, microsatellite instability; MSI-H, MSI high; MSI-L, MSI-low; pMMR, proficient mismatch repair.

only a deficiency of PMS2 protein, 4 showed only a deficiency of MSH6 protein, and 2 underwent neoadjuvant therapy, which might have affected the expression of MSH6.[12] Additionally, there were three cases that only underwent testing of MLH1 and MSH2 antibodies, and all three showed a deficiency of MLH1 protein (Fig. 5B-C, Supplementary Table 4). Among the 9 MSI-L cases in all 703 cases, only one case with a deficiency of MSH6 protein was identified as dMMR (Fig. 5B-C). Therefore, there were a total of 21 inconsistent cases, and the overall consistency rate between MMR and MSI status was 96.7% (Fig. 5B-C). Moreover, the inconsistency rate was 30.8% (4/13) before 2013, dropping to 2.5% (17/690) after 2013 (Supplementary Table 4). This indicates that standardizing IHC testing methods can enhance the accuracy of MMR status detection.

Regarding the impact of MMR status on patients' prognosis, it was found that the OS and DFS of patients with dMMR were significantly better than those with pMMR ($P < 0.0001$, Supplementary Fig. 2A), which was consistent with previous reports.[13-15] Similarly, the OS and DFS of patients with MSI-H were significantly better than those with MSS/MSI-L ($P < 0.0001$, Supplementary Fig. 2B). This trend was observed even in the since 2013 group ($P = 0.00045$, Supplementary Fig. 3A). However, in the before 2013 group, there was no significant difference in DFS between the dMMR and pMMR groups ($P = 0.084$, Supplementary Fig. 3B). These findings suggest that the prognostic value of MMR status for tumor relapse and metastasis is influenced by the deficiency ratio of MMR proteins, which was significantly decreased since 2013, due to the standardization and quality control of IHC testing process. Therefore, improving the detection process of MMR protein can help to improve the prognosis of patients.

To examine whether the development of standard IHC testing process influenced patients' prognosis, we removed 65 patients who survived less than a month from all 2,245 dMMR patients, and then divided them into the before 2013 ($n = 1,313$) and since 2013 ($n = 867$) groups based on the year of their operation. The Kaplan-Meier analysis results showed that the OS and DFS of dMMR patients admitted before 2013 were significantly worse than those admitted since 2013 (both $P < 0.001$; Fig. 5D), and the stratified Cox regression survival analysis showed a consistent result in most of subgroups (Supplementary Tables 5–6). Although there is no significant difference in prognosis among patients who received neoadjuvant therapy, those with TD(+), and those with high tumor differentiation grade, the sample sizes of these three groups are comparatively small (Supplementary Tables 5–6). These results indicate that standardizing MMR status detection may contribute to improving the prognosis of patients with CRC, considering the significant decrease in dMMR rate since the implementation of the standardized MMR protein detection procedure in 2013.

3.4. Development of testing for RAS/BRAF gene mutations improved the prognosis of patients

In FUSCC, we have the capability to use Sanger sequencing, amplification refractory mutation system PCR (ARMS-PCR) and NGS to detect the RAS/BRAF gene status in CRC patients. Sanger sequencing has been routinely utilized in clinical practice since 2008 for detecting KRAS ($n = 3,553$), BRAF ($n = 3,287$), and NRAS ($n = 3,358$) gene mutations, while ARMS-PCR and NGS were introduced in 2017 and 2018, respectively (Fig. 1 and Supplementary Table 7). The total number of tests and the ratio of CRC patients gradually increased from 2008 to 2013, with two sharp increases in 2014 and 2018 (Fig. 6).

Sanger sequencing was used to test 3,553 specimens, ARMS-PCR for 2,796 specimens, and NGS for 381 specimens (Supplementary Table 7). The proportion of RAS/BRAF mutation tests using Sanger sequencing gradually increased from 2008 to 2018, with a smooth growth tendency before 2013. It then sharply increased in 2014 and 2018, with a growth rate of 265% and 109%, respectively (Fig. 6, Supplementary Table 7). However, the usage of Sanger sequencing gradually decreased from 2018 to 2020 due to the introduction of ARMS-PCR and NGS (Fig. 6, Supplementary Table 7). The proportion of RAS/BRAF mutation testing using ARMS-PCR and NGS sharply increased in 2018 and 2019, with a growth rate of 825% and 706%, respectively (Supplementary Table 7).

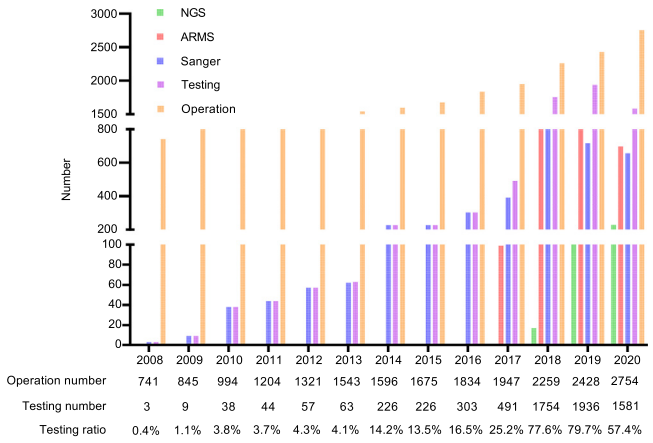

Fig. 6. Trends in RAS/BRAF gene mutations testing of colorectal cancer from 2008 to 2020. ARMS, amplification refractory mutation system; NGS, next generation sequencing.

We conducted a thorough investigation into the rate of KRAS mutations using various methods and found that the average mutation rate was 43.5% from 2014 to 2020, as determined by Sanger sequencing (Supplementary Table 8). In comparison, the positive rates of KRAS mutations were 44.7% in ARMS-PCR results and 41.0% in NGS results from 2018 to 2020 (Supplementary Table 8). During the same time period, the positive rate of NRAS mutations was 3.5%, 3.3%, and 3.5%, and the positive rate of BRAF mutations was 3.9%, 3.8%, and 6.4%, as detected by Sanger sequencing, ARMS-PCR, and NGS methods, respectively (Supplementary Table 8). There was no significant difference in the mutation rate of KRAS and NRAS genes among the three detection methods, while the mutation rate of BRAF was significantly higher in the NGS group compared to the Sanger and ARMS groups (Supplementary Table 8). Notably, among the 24 BRAF-mutant samples identified by NGS, 21 samples had BRAF exon 15 mutations, while the other three had BRAF exon 14, both exon 15 and 17, and exon 18 mutations, respectively. Additionally, the mutation abundance of three among the 12 BRAF exon 15 mutation samples was below 10% (Supplementary Table 9). These results once again confirm the superiority of NGS over the other techniques in terms of sensitivity and specificity.[16]

We finally conducted an analysis to determine the correlation between the detection of RAS/BRAF gene mutation status and patient prognosis. Our data showed that patients who had their RAS/BRAF gene mutation status detected had worse OS and DFS compared to patients who did not undergo detection (all $P < 0.0001$, Fig. 7), and the stratified analysis results showed a consistent result in most of subgroups (Supplementary Tables 10–15). These results indicate that all patients undergoing testing at FUSCC were clinically high-risk individuals. Interestingly, in patients at M1 stage and IV stage, patients who had their RAS/BRAF gene mutation status detected ($n = 1,323$) conferred better OS compared to patients who did not undergo detection ($n = 1,336$, Supplementary Tables 10–12). These results indicate that RAS/BRAF gene mutation detecting clinically improved the postoperative management of these patients at advanced stages, and helped to delay the progression of the disease. However, we found that detecting the RAS/BRAF status ($n = 5,041$) only resulted in better DFS but not OS for patients at stages I-III ($n = 16,557$, Supplementary Tables 10–12). This phenomenon is likely because patients in stages I-III who underwent KRAS/BRAF gene mutation testing after surgery will receive timely guidance for prevention of recurrence and metastasis.

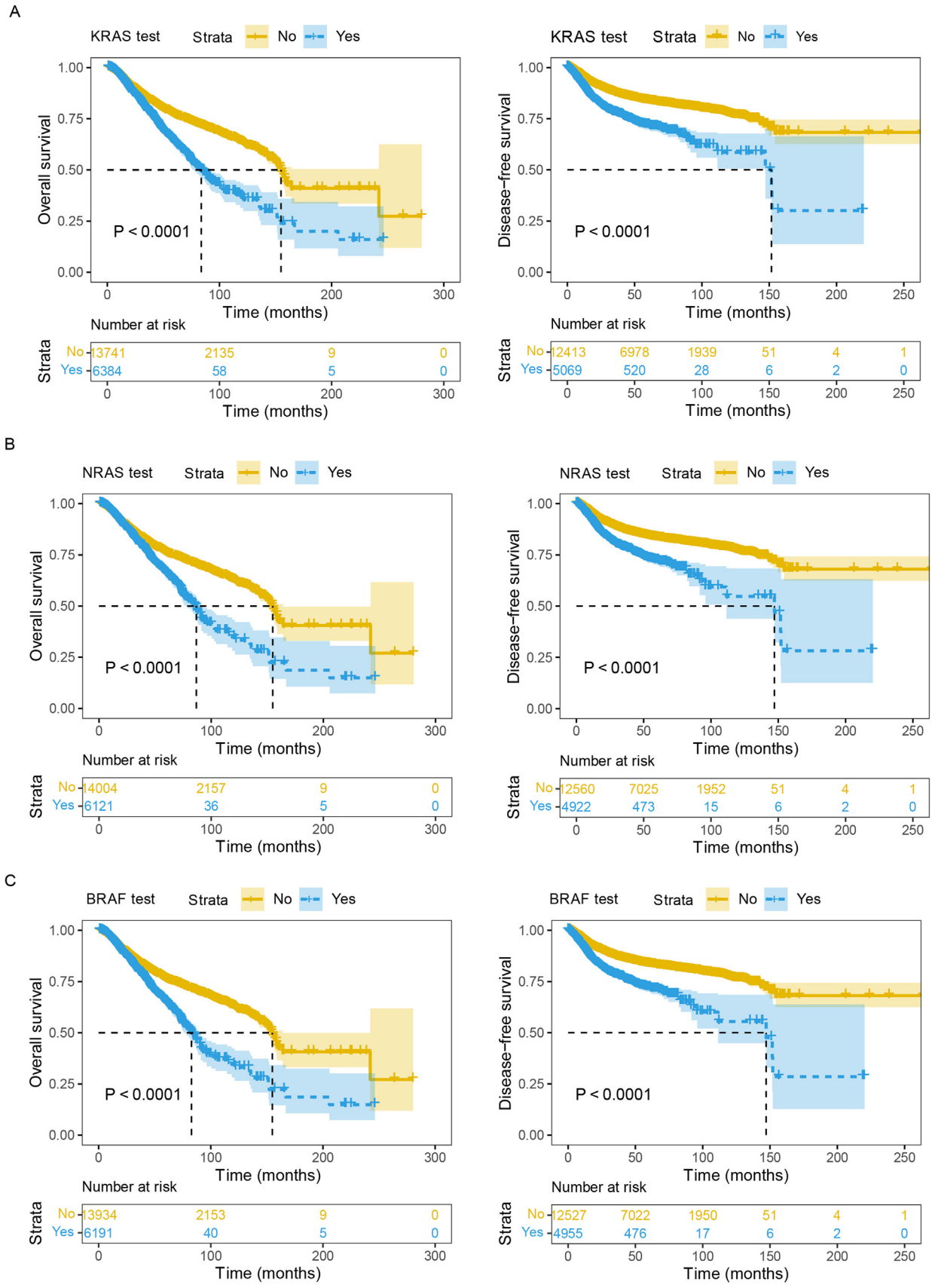

Fig. 7. Overall survival and disease-free survival of colorectal cancer patients with different RAS/BRAF gene mutations testing in Fudan University Shanghai Cancer Center, 2008–2020. (A-C) Kaplan-Meier curves with log-rank analysis of overall survival and disease-free survival in colorectal cancer patients with or without KRAS (A); NRAS (B) and BRAF (C) testing status.

4. Discussion

This retrospective survey, with the longest time span and the largest sample size to date, has showed the substantial development of pathological assessment including biomarker tests of CRC in FUSCC from 2008 to 2020. The gradually updated elements in the formatted pathology report, automated and standardized IHC tests with routine implementation of quality control, and significantly increased breadth and depth of RAS/BRAF gene mutation detection truly reflected the continuous progress with the needs of multidisciplinary and personalized management of CRC patients.[16] Based on a retrospective analysis of pathology reports from the past 12 years, our study reveals that continuous development in pathology report templates, IHC and molecular pathology testing panels, protocols, and technology has resulted in significant improvements in treatment decisions and prognosis for CRC patients.

Since 2013, MDT has been widely developed in FUSCC, and has become the routine diagnosis and treatment guidance for CRC patients. Pathologists began to expand the "key features" in pathology reports according to the needs of clinical practice. In CRC, the depth of tumor invasion beyond the muscularis propria is a crucial prognostic factor. According to AJCC-7, CRCs that invade the pericolic fat tissue are classified as pT3, while those that involve the serosa or adjacent organs or structures are classified as pT4.[3] However, different criteria have caused confusion about the definition of the pT4a category. According to the Shepherd's 1997 LPI classification, mesothelial inflammatory and/or hyperplastic reaction with tumor close to, but not at, the peritoneal surface, was classified as LPI2.[17] AJCC Prognostic Factors Group recommended in 2000 that pT4a should include Shepherd's LPI2,[18] whereas LPI2 was excluded from pT4a in the College of American Pathologists definition since 2008.[19] Before 2014, we diagnosed cases with tumors within 1 mm of the serosal surface as T4, regardless of the presence of inflammatory reaction, mesothelial hyperplasia and/or erosion/ulceration. However, through retrospective observation of clinical cases and literature tracking,[20] we gradually realized that this interpretation method is excessive. Serosal scrape cytology results suggests that only tumors with inflammatory reactions, mesothelial hyperplasia, and/or erosion/ulceration suggest peritoneal involvement.[21] Following continuous discussions with surgeons and radiologists during the MDT, we diagnosed cases without mesothelial inflammation and/or proliferative reactions as T3 since 2014. After conducting our analysis, our data strongly support the value of accurate T staging for guiding clinical treatment and management.

In the AJCC-7 staging system, TD is included in N1c and only considered in the absence of LNM.[3] The AJCC-8 staging system[4] considers TD as prognostic level information and includes it in evidence-based medicine at an evidence level. TD can directly impact the clinical stage and treatment options of patients at T3 stage. If isolated lesions are diagnosed as TD, the tumor stage is considered stage III (T3N1c), and patients must receive postoperative chemotherapy. However, if TD is diagnosed as neurovascular invasion, the tumor stage is considered stage II (T3N0) with high-risk factors, and patients may not require treatment but instead require close surveillance.[22] In clinical practice, we gradually realized that the presence of TD is an important prognostic factor for CRC patients at stage III, and adding TD to N staging may help better determine the duration of adjuvant therapy. After discussing during MDT meetings, we included TD information in the pathology report format since 2017. Furthermore, our data provided supporting evidence for the usefulness of TD as a prognostic indicator for patients with CRC. By incorporating the prognostic adverse factor of TD into the N staging, although it increased the overall TNM staging of the cancer, it also led to more intense postoperative management for these TD(+) patients without LNM and distant metastasis. Therefore, in general, defining these patients as N1c could improve their prognosis.

Additionally, the surgical performance on circumferential radial margin for rectal cancer also showed significant influence on patient prognosis.[23] Pathologists' evaluation on the TME specimen is crucial for assessing the quality of the mesorectal excision and providing feedback to surgeons on their technique. This information is also important in determining patients' risk of local recurrence after surgery. To evaluate the completeness of the mesorectum in TME specimens, we added this content to the standardized pathological report of CRC in 2017.

Using IHC to detect MMR proteins in CRC tissue samples is a straightforward and valuable method for identifying dMMR. Initially, FUSCC only regularly tested for MLH1 and MSH2 proteins, which would overestimate the detection rate of MMR deficiency. However, in the last ten years, we have added MSH6 and PMS2 to the panel. Since several studies have shown the prognostic and predictive value of MMR status in chemotherapy efficacy,[13-15] we have been using IHC to detect all four markers as our routine method for analyzing MMR status since 2013.

Although IHC is a simple and widely used method that can be implemented in almost all pathology departments, it has some limitations regarding subjectivity in clinical practice. These limitations can be caused by the quality of the antibody, detection process (fixation, staining), and other factors, leading to low specificity and repeatability. Moreover, varying degrees of sample quality requirements result in different detection accuracy among centers. To improve this, we implemented routine quality control on the IHC process and set positive control for every test. This significantly decreased the deficiency ratio of MMR proteins and the percentage of dMMR cases in FUSCC from 32.5% to 7.4%. Although the average occurrence of dMMR between 2008 and 2020 was around the same range (12–15%) of MSI-H incidences reported by the Cancer Genome Atlas (TCGA) working group,[24,25] the incidences in FUSCC between 2013 and 2020 were consistently lower (ranging from 6.4% to 9.3%). However, our findings are consistent with a study by Middha et al.,[26] which reported an incidence rate of 8%. In addition, FUSCC observed slightly higher MSI-H incidences in colon adenocarcinoma (20.4%) compared to that reported by Bonneville et al. (19.72%), whereas our data for rectal adenocarcinoma (4.4%) was lower than their report (5.73%).[27] These differences may be due to a larger percentage of advanced stages and rectal cases examined in our study as well as the study conducted by Middha et al.[26] While there was excellent overall consistency between MMR and MSI status, samples tested before 2013 showed lower consistency compared to those tested after 2013.

It is acknowledged that EGFR is a key treatment target for CRC, whereas EGFR blockers are less effective in patients with mutations in the KRAS and NRAS genes or BRAF V600E, as these mutations lead to resistance.[28,29] However, the coverage of RAS/BRAF gene mutation testing in CRC patients varies among hospitals due to differences in detection methods, limited molecular detection platforms, and high costs. The proportion of tests performed also differs between general hospitals and specialized cancer centers or hospitals of different grades.[7-10,30] In response to the CRC MDT's strong demand, our molecular pathology laboratory developed Sanger sequencing procedures for KRAS, BRAF and NRAS gene mutation testing in 2013, and subsequently initiated RAS/BRAF gene mutation testing. As a result, the number of tests conducted in FUSCC increased nearly fourfold in 2014. As RAS/BRAF gene mutation testing became a consensus in clinical practice in 2018,[4] the number and percentage of RAS/BRAF detections at FUSCC increased dramatically in 2018. The detection of RAS/BRAF gene mutations has become critical in guiding precision therapy for CRC, resulting in an increase in the proportion of detection in CRC patients over the years. Patients who undergo testing have greater access to targeted treatment regimens, such as FOLFOX/FOLFIRI ± cetuximab regimen for KRAS wild-type CRC patients and FOLFOX/CapeOx/FOLFIRI ± bevacizumab regimen for RAS-mutated CRC patients.[31] Since KRAS and BRAF mutations are adverse prognostic factors for CRC, detecting these mutations can help screen high-risk populations timely and enable clinicians to carry out early and precise postoperative treatment to prevent recurrence and metastasis. This ultimately improves both the quality of postoperative management and the DFS rate among patients at TNM stages I-III.

However, it should be noted that our study has some limitations. Firstly, despite being ranked as the leading pathology discipline in

the "China Hospital Specialty Reputation Ranking" by the Hospital Management Institute, Fudan University for 10 consecutive years from 2011 to 2020 (https://fdygs.q-health.cn/news222.aspx), we acknowledge that this study only presents data from a single center and cannot provide a comprehensive view of the overall development of CRC pathology discipline in China. Secondly, while our study has a large sample size, we were unable to collect patient therapeutic data for prognostic analysis, which may lead to biased results. Thirdly, this study did not include novel pathological parameters and molecular testing data that are increasingly concerned, such as tumor budding, HER2, and Immunoscore.

With further research and advancements in molecular pathology technology, genetic testing content is increasingly included in NCCN guidelines, and the advent of the immunotherapy era calls for new criteria for clinicopathological reports. Although NCCN Guideline version V1 2021 includes only three multigene tests, namely Oncotype DX, ColoPrint, and ColDx in the chapters on risk of recurrence after CRC surgery and adjuvant chemotherapy, Immunoscore and circulating tumor DNA (ctDNA) analysis are included in version V2.[32] However, considering limited economic and sample resources, pathologists should make informed choices and trade-offs when selecting testing items. They should focus on obtaining the most useful molecular data for patients through a single molecular pathological test.

5. Conclusion

The 12-year period from 2008 to 2020 witnessed substantial advancements in the standardization of reporting and molecular detection of CRC. This progress includes the development of MDT model, pathological diagnostic techniques, and interpretation methods, which have been effective in meeting the increasing medical needs of CRC patients. These advancements also demonstrate the effectiveness of FUSCC's continuous efforts in improving the diagnosis capacity of CRC, and can serve as a useful reference for other centers in China looking to enhance their MDT and pathological diagnosis capabilities. Moreover, given the uneven development of histopathology across hospitals in China and the varying geographic distribution of hospitals with molecular testing capability, our findings could inform future policies and strategies for the pathological department.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics statement and patient consent

This study was approved by the ethics committee of FUSCC (IRB Approval No. 050432-4-2108*), and was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from the patients for publication.

Acknowledgements

We would like to thank the patients and family members who gave their consent on presenting the data in this study. We are greatly thankful to Ke Hu. Qian Gong and Jing Xia (Merck Serono Co., Ltd.) for data cleansing and revision of the manuscript. This work was supported by National Natural Science Foundation of China (grant numbers: 82273370, 82202899, 82172702, 81972249, 81902430, 82002543, 82002946, U1932145), Shanghai Clinical Science and Technology Innovation Project of Municipal Hospital (grant number: SHDC12020102), Natural Science Foundation of Shanghai (grant numbers: 22ZR1413000, 21ZR1414900), Artificial Intelligence Medical Hospital Cooperation Project of Xuhui District (grant number: 2021-017), Shanghai Science and Technology Development Fund (grant number: 19MC1911000), Shanghai Municipal Key Clinical Specialty (grant number: shslczdzk01301), Science and Technology Commission of Shanghai Municipality (grant number: 18401933402), "Chenguang Program" supported by Shanghai Education Development Foundation and Shanghai Municipal Education Commission (grant number: 20CG08).

Author contributions

W.S. acted as the principal investigator, and conducted the trial design and final approval. M.X. and Y.L. accessed and verified the data reported in the manuscript, drafted the manuscript, and performed statistical analyses. D.H. and J.P. gave critical comments and provided methodological support. W.W., C.T. and H.S. performed the data collection. Y.X.L., C.Z. Y.Y. and X.Z. provided suggestions for revisions and reviewed the manuscript. X.W., X.W., M.Z., S.N. and L.W. provided suggestions for revisions and gave administrative Supports. All authors reviewed and revised the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jncc.2024.02.001.

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