Deficient mismatch repair and *RAS* mutation in colorectal carcinoma patients: a retrospective study in Eastern China

Xiangyan Zhang [1,2] , Wenwen Ran [1] , Jie Wu [1] , Hong Li [1] , Huamin Liu [3] , Lili Wang [1] , Yujing Xiao [1] , Xiaonan Wang [1] , Yujun Li [1] , Xiaoming Xing [Corresp. 1]

[1] Department of Pathology, Affiliated Hospital of Qingdao University, Qingdao, China
[2] Department of Pathology, Qingdao University Basic Medicine College, Qingdao, China
[3] Department of Oncology, Affiliated Hospital of Qingdao University, Qingdao, China

Corresponding Author: Xiaoming Xing
Email address: edithxing@126.com

Objectives: To investigate the frequency and prognostic role of deficient mismatch repair (dMMR) and RAS mutations in Chinese patients with colorectal carcinoma.

Methods: Clinical and pathological information from 813 patients were reviewed and recorded. Expression of mismatch repair proteins was tested by immunohistochemistry. Mutation analyses for RAS gene were performed by real-time polymerase chain reaction. Correlations of mismatch repair status and RAS mutation status with clinicopathological characteristics and disease survival were determined.

Results: The overall percentage of dMMR was 15.18% (121/797). The proportion of dMMR was higher in patients <50 years old (p < 0.001) and in the right side of the colon (p < 0.001). Deficient mismatch repair was also associated with mucinous production (p < 0.001), poor differentiation (p < 0.001), early tumor stage (p < 0.05) and bowel wall invasion (p < 0.05). The overall RAS mutation rate was 45.88%, including 42.56% (346/813) KRAS mutation and 3.69% (30/813) NRAS mutation (including 3 patients with mutations in both). KRAS mutation was significantly associated with mucinous production (p < 0.05), tumor stage (p < 0.05) and was higher in non-smokers (p < 0.05) and patients with a family history of colorectal carcinoma (p < 0.05). Overall, 44.63% (54/121) dMMR tumors harbored KRAS mutation, however, dMMR tumors were less likely to have NRAS mutation. Moreover, dMMR, KRAS and NRAS mutation were not prognostic factors for stage I~III colorectal carcinoma.

Conclusions: This study confirms that the status of molecular markers involving mismatch repair status and RAS mutation reflects the specific clinicopathological characteristics of colorectal carcinoma.

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4 **Xiangyan Zhang,[1,2*] Wenwen Ran,[1*] Jie Wu,[1] Hong Li,[1] Huamin Liu,[3] Lili Wang,[1]**

5 **Yujing Xiao,[1] Xiaonan Wang,[1] Yujun Li,[1] Xiaoming Xing[1]**

6 1 Department of Pathology, Affiliated Hospital of Qingdao University, 16 Jiangsu Road,

7 Qingdao, 266000, China

8 2 Department of Pathology, Basic Medicine College, Qingdao University,38 Dengzhou Road,

9 Qingdao, 266000, China

10 3 Department of Oncology, Affiliated Hospital of Qingdao University, 16 Jiangsu Road, Qingdao,

11 266000, China

12 * These authors contributed equally to this work.

13 Correspondence to: Xiaoming Xing, M.D. Department of Pathology, Affiliated Hospital of

14 Qingdao University, 16 Jiangsu Road, Qingdao, China, 266000

15 E-mail address:edithxing@126.com

17 **ABSTRACT**

18 Objectives: To investigate the frequency and prognostic role of deficient mismatch repair

19 (dMMR) and *RAS* mutations in Chinese patients with colorectal carcinoma.

20 Methods: Clinical and pathological information from 813 patients were reviewed and recorded.

21 Expression of mismatch repair proteins was tested by immunohistochemistry. Mutation analyses

22 for *RAS* gene were performed by real-time polymerase chain reaction. Correlations of mismatch

23 repair status and *RAS* mutation status with clinicopathological characteristics and disease

24 survival were determined.

25 Results: The overall percentage of dMMR was 15.18% (121/797). The proportion of dMMR was

26 higher in patients <50 years old (p < 0.001) and in the right side of the colon (p < 0.001).

27 Deficient mismatch repair was also associated with mucinous production (p < 0.001), poor

28 differentiation (p < 0.001), early tumor stage (p < 0.05) and bowel wall invasion (p < 0.05). The

29 overall *RAS* mutation rate was 45.88%, including 42.56% (346/813) *KRAS* mutation and 3.69%

30 (30/813) *NRAS* mutation (including 3 patients with mutations in both). *KRAS* mutation was

31 significantly associated with mucinous production (p < 0.05), tumor stage (p < 0.05) and was

32 higher in non-smokers (p < 0.05) and patients with a family history of colorectal carcinoma (p <

33 0.05). Overall, 44.63% (54/121) dMMR tumors harbored *KRAS* mutation, however, dMMR

34 tumors were less likely to have *NRAS* mutation. Moreover, dMMR, *KRAS* and *NRAS* mutation

35 were not prognostic factors for stage I~III colorectal carcinoma.

36 Conclusions: This study confirms that the status of molecular markers involving mismatch repair

37 status and *RAS* mutation reflects the specific clinicopathological characteristics of colorectal

38 carcinoma.

40 **INTRODUCTION**

41 Colorectal cancer (CRC) is the fourth most common cancer in China, with 331,300 new cases

42 and 159,300 disease-related deaths in 2012 (Chen et al. 2016). The morbidity has increased

43 steadily due to the growth of an aging population and the change of lifestyle in recent years,

44 however, the exact mechanism and related predicted biomarkers are largely unknown.

45 During the past decades, microsatellite instability (MSI) and *RAS* mutation have been well

46 studied as two prevalent genetic biomarkers involved in colorectal carcinogenesis. The mismatch

47 repair (MMR) system, which includes the proteins MLH1, MSH2, MSH6 and PMS2, can repair

48 incorrect base-pairing or unmatched DNA loops to maintain genomic stability. MSI is caused by

49 a deficient mismatch repair (dMMR) system, which leads to a high rate of mutations in repeat

50 sequences and accounts for approximately 15% of all CRCs as well as virtually all Lynch

51 syndrome (LS) patients (Geiersbach & Samowitz 2011; Marra & Boland 1995; Zhang et al.

52 2016). Tumors with high level microsatellite instability (MSI-H) caused by germ line mutations

53 or epigenetic silencing of MMR genes have unique clinicopathological characteristics

54 (Cunningham et al. 2010). In early stage CRC, patients with MSI-H demonstrated favorable

55 prognosis compared to those with low level of microsatellite instability (MSI-L) and

56 microsatellite stability (MSS) (Ribic et al. 2003; Sinicrope et al. 2011), however, these patients

57 did not benefit from fluoropyrimidine-based adjuvant chemotherapy (Ribic et al. 2003; Sargent

58 et al. 2010).

59   The *RAS* gene family, the other significant biomarker, includes *KRAS*, *NRAS* and *HRAS*, is

60 located downstream in the epidermal growth factor receptor (EGFR) signal pathway. Mutations

61 in the *RAS* gene, which are thought to occur early in the adenoma-carcinoma continuum, activate

62 the *RAS*/MAPK pathway independently of EGFR activation, leading to poor response to EGFR

63 inhibitors (Amado et al. 2008; Punt et al. 2016). Moreover, National Comprehensive Cancer

64 Network (NCCN) clinical practice guidelines suggested that *KRAS* and *NRAS* gene mutations

65 should be detected for metastatic CRC (mCRC) patients before treatment with Cetuximab and

66 Panitumumab (Engstrom et al. 2009).

67   The status of dMMR and *RAS* mutation has been widely studied in western countries. The

68 frequency of dMMR CRCs ranged from 15-20% (Giraldez et al. 2010; Sinicrope et al. 2011;

69 Sinicrope et al. 2012), *KRAS* mutation ranged from 20-50% (De Roock et al. 2010; Naguib et al.

70 2010; Palomba et al. 2016; Rosty et al. 2013; Sasaki et al. 2016) and *NRAS* mutation was noted

71 in less than 5% (De Roock et al. 2010; Palomba et al. 2016; Peeters et al. 2013; Russo et al.

72 2014). However, studies in China showed a lower frequency of dMMR compared with that in

73 western populations, and the clinicopathological characteristics were also inconsistent (Huang et

74 al. 2010; Jin et al. 2008; Ye et al. 2015). Although several studies reported the frequency of

75 *KRAS* mutation in Chinese CRC patients, the number of samples was limited in most of these

76 studies (Shen et al. 2011; Ye et al. 2015; Yunxia et al. 2010). Moreover, information about *NRAS*

77 mutation in Chinese CRC patients was limited. Little has been studied on the association

78 between status of dMMR and *RAS* mutation. Therefore, in the present study, we analyzed the

79 dMMR and *RAS* mutation status of CRC patients to evaluate possible associations between

80 dMMR, *RAS* mutation and the clinicopathological characteristics in primary colorectal

81 carcinoma and we also attempted to explore the prognostic roles of dMMR and *RAS* mutation.

82

Materials and Methods

83

84 Eight hundred and thirteen formalin-fixed, paraffin-embedded tumor specimens from CRC

85 patients who underwent primary surgical resection from 2013 to 2016 in the Affiliated Hospital

86 of Qingdao University were selected for this study. The patients' selection method is presented

87 in a consort diagram (Figure1). Patients who had undergone preoperative radiotherapy,

88 chemotherapy and/or EGFR-targeted therapy were not included in this study.

89 The clinical and pathologic variables were extracted from medical records and pathological

90 reports, which included age, gender, primary locations of tumor, tumor diameter, histological

91 characteristics, TNM stage, smoking status, drinking status and family medication history. The

92 patients were followed up until October 2017, and the data concerning cancer recurrence and

93 patient survival were collected. Patients diagnosed with stage I - III colorectal carcinoma were

94 used to explore the prognostic role of dMMR and *RAS* mutation with disease-free survival (DFS)

95 and overall survival (OS).

96 Primary locations of tumors were divided into the right side colon (from the cecum through

97 the transverse colon), the left side colon (from the splenic flexure through the rectosigmoid

98 flexure) and the rectum. Tumors were staged according to the criteria of the seventh edition of

99 the American Joint Commission on Cancer (AJCC) TNM staging system. Mucinous

100 adenocarcinoma and signet-ring cell carcinomas were recorded as mucin-producing tumors.

101 The study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao

102 University (No.20130049) and all patients had signed informed consent.

Immunohistochemistry for MMR proteins

103

104 As previously described (Lin et al. 2014b), all specimens were fixed in 10% neutral buffered

105 formalin and embedded in paraffin blocks. 3 μm-thick tissue sections were used for

106 immunohistochemical analysis. Immunohistochemical staining was performed on an Automated

107 Staining System (BenchMark XT, Ventana Medical Systems, Inc. Arizona, USA) according to

108 the manufacturer's instructions. The ready-to-use antibodies were used as follows: MLH1

109 (No.M1, Ventana Medical Systems Inc, Arizona, USA, working solution), PMS2 (No.EPR3947,

110 Ventana Medical Systems Inc, Arizona, USA, working solution), MSH2 (No.G219-1129,

111 Ventana Medical Systems Inc, Arizona, USA, working solution), MSH6 (No.44, Ventana

112 Medical Systems Inc, Arizona, USA, working solution).

113 The results were analyzed by two pathologists. Any tumor cell with nuclear staining was

114 recorded as positive staining. Intact expression for all these proteins was regarded as proficient

115 MMR (pMMR). Protein expression was defined as abnormal when nuclear staining of tumor

116 cells was absent in the presence of positive staining in stromal cells and lymphocytes (Figure2).

117 The standard criteria for diagnosis of dMMR was as follows: dMMR in MLH1: loss of MLH1

118 and PMS2; dMMR in MSH2: loss of MSH2 and MSH6; dMMR in MSH6: loss of MSH6;

119 dMMR in PMS2: loss of PMS2 (Richman et al. 2015).

120 **Analysis of *KRAS* and *NRAS* gene mutations by ARMS-PCR**

121 Formalin-fixed, paraffin-embedded tumor sections were deparaffinized and air dried, and DNA

122 was extracted using the Tiangen Blood and Tissue Kit (TiangenInc, Beijing, China). *KRAS*

123 (codons12 and 13) and *NRAS* (codons12, 13 and 61) mutations were detected by amplification

124 refractory mutation system in multiple quantitative polymerase chain reaction (ARMS-multi-

125 qPCR) analysis with the Human *KRAS* and *NRAS* Mutation Detection kit (YuanQi Bio-

126 Pharmaceutical Co., Ltd. Shanghai, China). The mutation points detected by this kit are listed

127 in supplement 1. Codons of *RAS* were amplified as described previously (Dong et al. 2016).

128 Briefly, 3 μl sample DNA was amplified in a 25 μl reaction containing 9 μl of Mix1 and 13 μl of

129 PCRMix3. Positive and negative controls for each sample were run simultaneously. The program

130 for the PCR amplification flanking *KRAS* mutation site was as follows: 1 cycle at 42 °C for 5

131 min; 1 cycle at 94 °C for 3 min; 40 cycles at (94 °C for 15 sec; 60 °C for 60 sec). Fluorescence

132 signals were collected at 60 °C. The program for the PCR amplification flanking *NRAS* mutation

133 site was as follows: 1 cycle at 42 °C for 5 min; 1 cycle at 94°C for 3 min; 40 cycles at (94 °C for

134 45 sec; 60 °C for 80 sec). Fluorescence signals were collected at 60 °C. The mutations were

135 identified with a specific probe labeled with Hydroxy fluorescein (FAM). Amplicons were

136 detected using ABI7500 Fast Real-Time PCR System (Thermo Fisher Scientific Inc, MA, US).

137 **Statistical analysis**

138 Results were analyzed with SPSS 19.0 (SPSS, Inc, Chicago). For comparison of the frequencies

139 among groups, the Chi-square test and the Fisher exact test were used. Survival curves for DFS

140 and OS were estimated using Kaplan–Meier analysis with the log-rank test. Probability (p) value

141 < 0.05 was considered as statistical significance.

142

143 **RESULTS**

144 **Patient characteristics**

145 The main characteristics of the patients are summarized in the Table 1. There were 506 (62.24%)

146 males and 307 (37.76%) females with a mean age of 64 years. The majority of the patients

147 (87.7%) were older than 50 years. 11.69%, 40.84%, 37.15% and 10.33% of patients presented

148 with stage I, Stage II, stage III and stage IV disease, respectively. The primary location was more

149 common in rectum (54.49%). There were 283 (34.81%) patients with a smoking history and 165

150 (20.3%) patients with an alcohol in-taking history, respectively. There were 133 (16.36%)

151 patients with mucin-productive carcinoma.

152 **MMR status and associations with clinicopathological characteristics**

153 MMR status was successfully evaluated in 797 patients. 121 (15.18%) patients exhibited dMMR.

154 The rates of dMMR deficiency in MLH1, PMS2, MSH2 and MSH6 were 9.78% (78/797), 1.25%

155 (10/797), 3.26% (26/797) and 0.87% (7/797), respectively. The rates of deficiency in

156 MLH1/PMS2 and MSH2/MSH6 were 11.92% (88/797) and 4.14% (33/797), respectively. The

157 association of clinicopathological characteristics with MMR status is presented in Table 2. The

158 proportion of dMMR was higher in patients <50 years old (p < 0.001). A higher rate of dMMR

159 was found in stage II cancers (19.02%, p = 0.019). dMMR status was also associated with

160 mucinous production (p < 0.001), poor differentiation (p < 0.001) and localization of the tumor

161 to the right side of the colon (p < 0.001). dMMR patients had a higher propensity to bowel wall

162 invasion (p = 0.018).

163   Although dMMR tumors were present more often in patients with CRC family history, no

164   significant difference (22.92% vs 13.13%, p>0.05) was found in this study. The loss of

165   MSH2/MSH6 expression was more often observed in patients with CRC family history (12.5%

166   vs 3.58%, p = 0.016). In other respects, the patients with tumors exhibiting dMMR were similar

167   to those exhibiting pMMR.

168   **_RAS_ gene mutation and associations with clinicopathological characteristics**

169   *RAS* status was tested from 813 patients. The mutation rates of *KRAS* and *NRAS* were 42.56%

170   (346/813) and 3.69% (30/813), respectively. There were three patients demonstrating mutation in

171   both *KRAS* and *NRAS*. Patients suffering from tumors with mucinous production had a higher

172   incidence of *KRAS* mutation compared with those having tumors without mucinous production

173   (54.89% vs 40.18%, p = 0.002). A higher rate of *KRAS* mutation was found in stage II (48.49%)

174   compared with that in stage I, stage III and stage IV (36.84%, 40.45%, 34.52%, respectively)

175   cancers (p = 0.023) and in non-smokers compared with smokers (46.6% vs34.98%, p = 0.001).

176   Patients with CRC family history also showed higher rate of *KRAS* mutation (54.17% vs 37.39%,

177   p = 0.013). Tumors with *RAS* mutation showed lower propensity to lymph node metastasis (p =

178   0.006) and distant metastasis (p = 0.048). No significant associations between *KRAS* mutation

179   and other clinicopathological characteristics were found in the present study. Meanwhile, *NRAS*

180   mutation was not significantly associated with any clinicopathological characteristics (Table 3).

181   **Correlations between _RAS_ mutation and MMR statu**s

182   *RAS* mutation rate was slightly higher in pMMR tumors than in dMMR tumors, but failed to

183   reach a significant difference (46.3% vs 44.63%, p > 0.05). There was also no obvious

184   correlation between MMR status and *KRAS* mutation (42.3% vs 44.63%, p > 0.05). No *NRAS*

185   mutation was detected in dMMR tumors. Compared with dMMR tumors, pMMR tumors had a

186   higher propensity to harbor *NRAS* mutation (p = 0.009, Table 4). The distribution of MMR and

187   *KRAS* status is shown in supplement 2. Correlation between *KRAS* gene mutation and

188   clinicopathological characteristics in dMMR tumors is summarized in Table 5. No significant

189   association between *KRAS* mutation and any clinicopathological characteristics were found in

190   dMMR tumors.

191   **Prognostic value of dMMR and *RAS* mutation in stage I - III CRC**

192   Of the 813 followed-up patients, 729 patients were diagnosed with stage I - III CRC, including

193   95 stage I patients, 332 stage II patients and 302 stage III patients. dMMR and *RAS* mutation

194   were not prognostic for DFS and OS in stage I - III CRC (Figure.3). Of the 121 dMMR patients,

195   109 patients were diagnosed with stage I - III CRC and 45.87% (50/109) patients harbored *KRAS*

196   mutation. However, *KRAS* mutation was not prognostic factor for these patients (Figure 4).

197

198   **DISCUSSION**

199   As prognostic and predictive biomarkers, MMR deficiency and *RAS* mutation are important for

200   clinical treatment and prognosis of CRC patients. Compared with pMMR, patients with dMMR

201   CRCs are reported to have unique clinicopathological characteristics such as poor differentiation,

202   early stage, increased tumor-infiltrating lymphocytes and better clinical outcome (Brenner et al.

203   2014; Korphaisarn et al. 2015; Ribic et al. 2003). The *RAS* gene is a predictive biomarker for the

204   resistance to anti-EGFR monoclonal antibody (MoAb) treatment in mCRCs (Amado et al. 2008;

205   Punt et al. 2016). However, geographic and racial differences between Chinese and other

206   countries were reported (Huang et al. 2010; Ismael et al. 2017; Kim et al. 2007; Vasovcak et al.

207   2011; Ye et al. 2015), which need to be validated with large sample amounts. Furthermore, data

208   regarding *RAS* mutation frequency and dMMR CRC is not consistent in China. Thus, we

209   designed this study in the Chinese population aiming to explore the relationship between the *RAS*

210   mutation, MMR status and clinicopathological parameters, also expecting to find some

211   prognostic and predictive biomarkers for CRC.

212   Our results demonstrated an overall MMR deficiency rate of 15.18%, which is within the

213   established range of 15-21% (Giraldez et al. 2010; Sinicrope et al. 2012; Carethers et al.

214   2004;Cushman-Vokoun et al. 2013), but slightly higher than that reported from other Chinese

215   populations (Huang et al. 2010; Jin et al. 2008; Ye et al. 2015). Reports from Korea (Jung et al.

216   2012) and Japan (Kadowaki et al. 2015) which used PCR-based MSI testing also showed that the

217   frequencies of MSI-H CRCs were around 10%. This discrepancy can be explained by the

218   different detective methods to some extent. Compared with PCR-based MSI testing examination,

219   immunohistochemistry is thought to be easily available and time-saving. Furthermore,

220   immunohistochemistry may detect MMR-deficient cases that can be potentially missed by PCR-

221   based MSI testing (Shia 2008).

222   Correlations between dMMR status and clinicopathological characteristics were

223   controversial (Ismael et al. 2017; Jin et al. 2008; Ribic et al. 2003; Sinicrope et al. 2011). Reports

224   from three independent Chinese groups (Huang et al. 2010; Jin et al. 2008; Ye et al. 2015)

225   indicated that dMMR had specific associations such as female gender, right sided colon tumors

226   and mucious tumors. In a study including 1063 CRCs, Lin et al observed that MSI was

227   associated not only with gender, tumor location and mucin production, but also with tumor

228   differentiation and tumor stage (Lin et al. 2014a). In our current study, we found patients

229   younger than 50 tended to be dMMR. These diverse findings may be attributed to different

230   criteria for age division, ethnicities, environmental factors as well as the specificity and

231   sensitivity of the detection methods.

232   In our study, there was a correlation between MSH2/MSH6 deficiency and family history of

233   CRC, but not MLH1/PMS2 deficiency. In addition, according to the Bethesda criteria (Burt et al.

234   2010), 12 CRCs were diagnosed with LS. In MSH2/MSH6 deficient CRCs, 33.3% (6/18) were

235   LS, while in MLH1/PMS2 defective cases, 13.95% (6/43) were LS, suggesting MSH2/MSH6

236   deficient patients had higher opportunity to be diagnosed with LS. Some of the recent studies

237   may help to explain this finding: the majority dMMR CRCs were caused by inactivation of

238   MLH1 and more than 70% MLH1 deficiency was caused by *MLH1* promoter hypermethylation

239   (Hampel et al. 2005), which could distinguish sporadic dMMR CRCs from LS cases, therefore,

240   most MLH1 defective tumors were sporadic CRC. Another interesting phenomenon in our

241   investigation is that we found most patients' family medical history was unclear and they did not

242   know whether other family members had polyps removed, moreover, many cancers might be

243   prevented by early stage colonoscopy, so the family history may be deceptive (Hampel 2014).

244   Therefore, screening strategy based on family history may be improper. All patients with newly

245   diagnosed CRC should be screened for LS (Hampel 2014). Inconsistent with previous studies,

246   which indicated that patients with dMMR tumors had significantly better survival than that of

247   pMMR patients (Des Guetz et al. 2009; Korphaisarn et al. 2015; Lanza et al. 2006), our study

248   showed that dMMR was not a prognostic factor for patients with stage I - III CRC, although the

249   incidence of dMMR in stage III disease was lower, suggesting that dMMR tumors had lower

250   propensity to metastasize.

251   In the present study, the mutation rates of *KRAS* and *NRAS* are 42.56% and 3.69%,

252   respectively. The *KRAS* mutation rate is significantly higher than the value of 20.7% among 314

253   CRC patients from Taiwan, China (Liou et al. 2011), 22% among 202 CRC patients from the

254   England (Naguib et al. 2010), 30.1% among 392 CRC patients from Switzerland (Zlobec et al.

255   2010), but similar to that previously reported in Guangzhou, China (43.9%, 25/57) (Mao et al.

256   2012). Several factors may lead to such differences, such as sample size, dietary and lifestyle

257   factors, as well as racial and/or environmental differences. Furthermore, we detected the coding

258   sequence of codon12 and codon13 in exon 2 of the *KRAS* gene, which may help to explain the

259   higher percentage of *KRAS* mutation than those detected in codon12 only. Except for exon 2,

260   recent studies have shown 5-10% of tumors harbored exon 3 or exon 4 mutation (Janakiraman et

261   al. 2010; Lin et al. 2014a), which would also result in resistance to anti-EGFR inhibitors.

262   Therefore, extending the detection spectrum of *RAS* might help to optimize the selection of the

263   CRC patients to receive anti-EGFR MoAbs.

264   The frequency of *KRAS* mutation has been reported to be associated with age, gender,

265   differentiation and tumor stage (Gao et al. 2012; Li et al. 2011; Ye et al. 2015; Yunxia et al. 2010;

266   Zhu et al. 2012). Inconsistent with these results, our study showed that *KRAS* mutation was

267   associated with mucin production, tumor stage, non-smoking and CRC family history.   *RAS*

268   mutated tumors showed lower propensity to lymph node and distant metastasis. No convincing

269   evidence demonstrates that *KRAS* mutation is an independent prognostic factor for CRC (Jin et al.

270   2008; Palomba et al. 2016; Russo et al. 2014; Yunxia et al. 2010). In the present study, no

271   associations of *KRAS* mutation with DFS and OS were found in patients with stage I - III CRC.

272   Further studies based on longer follow-up time and larger sample size are needed to confirm this

273   conclusion.

274   In our study, the percentage of the four tumor subgroups, including dMMR/*KRAS* mutation,

275   dMMR/*KRAS* wild-type, pMMR/*KRAS* mutation and pMMR/*KRAS* wild-type tumors was 6.78%,

276   8.4%, 35.88%, 48.94%, respectively, which is similar to the data reported by a study from

277   Beijing, China (Ye et al. 2015). According to recent reports (Nash et al. 2009; Roth et al. 2010),

278   patients with a MSS/*KRAS* mutant tumor had the worst survival than the other three groups.

279   Therefore, dMMR and *KRAS* markers may provide a foundation for developing a molecular

280   prognostic scoring system for CRC patients in the future.

281   Previous studies have shown that pMMR patients tended to harbor more *KRAS* mutation

282   than dMMR patients (Naguib et al. 2010; Ye et al. 2015). One hypothesis for this result is that

283   *BRAF* and *KRAS* mutations were almost mutually exclusive in CRC and MSI tumors are more

284   likely to harbor a *BRAF* mutation, so MSS tumors might harbor more *KRAS* mutations (Naguib

285   et al. 2010). However, in the present study, we did not find any differences in *KRAS* mutation

286   between pMMR and dMMR tumors, and further studies based on larger sample size are needed

287   to explore this controversy in Chinese CRCs.

288   Additionally, our study provided an opportunity to investigate the status of *KRAS* mutation

289   in Chinese dMMR patients. *KRAS* mutation presented in 44.63% dMMR patients in our study,

290   similar to previous studies in western countries (Cushman-Vokoun et al. 2013; Oliveira 2004).

291   All of these results indicate that *KRAS* mutation could be quite common in dMMR tumors. There

292   were no associations between *KRAS* mutation and clinicopathologic characteristics in dMMR

293   tumors. A study conducted by Nash et al indicated that *KRAS* status was an independent

294   prognostic factor in early stage MSI CRC patients (Nash et al. 2009). Moreover, MSI patients

295   with wild-type *KRAS* and *BRAF* tumors have more favorable prognosis than patients with

296   mutated *KRAS* or *BRAF* tumors in early stage CRC (de Cuba et al. 2016; Phipps et al. 2015).

297   However, we did not find *KRAS* mutation as a prognostic factor for dMMR patients with stage I -

298 III CRC.

299   *NRAS*, as one of the *RAS* family, showed close relations with *KRAS*. Unlike *KRAS*, *NRAS*

300 mutation was rarely detected in CRC patients. In our study, the mutation rate of *NRAS* was

301 3.69%, similar to previous reports (Chang et al. 2016; Irahara et al. 2010; Palomba et al. 2016;

302 Peeters et al. 2013). Moreover, we observed 25/388 *KRAS* wild-type tumors with *NRAS* mutation,

303 which can partially help to explain the resistance to anti-EGFR MoAb in some *KRAS* wild-type

304 patients. Considering the heavy financial burden in MoAb treatment in CRC patients, *NRAS*

305 mutation should be tested before MoAb treatment in *KRAS* wild-type tumors. Another interesting

306 phenomenon is that no *NRAS* mutation was detected in dMMR patients, which suggested

307 *NRAS* mutation might be mutually exclusive with dMMR. Meanwhile, *NRAS* mutation was not

308 significantly associated with any clinicopathologic characteristics in our study.

309   However, our results should be elucidated with consideration of its limitations: first, the

310 sample size was relatively small, rendering some findings inconclusive; second, we used

311 commercially available kit authenticated by China Food and Drug Administration (CFDA) and

312 the mutation subgroups were uncertain. A study conducted by Lin et al, demonstrated that

313 mutation in *KRAS* codon12 was associated with significantly poorer outcome than mutations

314 elsewhere or wild-type *KRAS* (Lin et al. 2014a). Therefore, the subgroup of mutation codons

315 should be carefully explored in future; third, we did not collect data of clinical management,

316 therefore, the influence of clinical treatment for survival was uncertain.

317

318 Conclusion

319   In conclusion, this was an exploratory analysis of correlations between *RAS* mutation and

320 MMR status with clinicopathological characteristics in Eastern Chinese CRC patients. The status

321 of these molecular markers, involving MLH1/PMS2, MSH2/MSH6, *KRAS* and *NRAS* mutation,

322 reflects the specific clinicopathological characteristics of CRC. More comprehensive molecular

323 classification and survival analysis should be explored in future experiments.

324

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326 None

327

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Clinicopathological information of the studied patients (n=813).

Characteristics	Number	(%)
Gender		
Male	506	62.24
Female	307	37.76
Age		
< 50	100	12.3
≥ 50	713	87.7
Location		
Right side colon	181	22.26
Left side colon	189	23.25
Rectum	443	54.49
Mucin production		
With	133	16.36
Without	680	83.64
Tumor differentiation		
Poor	138	16.97
moderate	599	73.68
Well	33	4.06
Unknown	43	5.29
Tumor stage		
I	95	11.69
II	332	40.84
III	302	37.15
IV	84	10.33
Bowel wall invasion (T)		
T1	21	2.58
T2	104	12.79

T3	336	41.33
T4	352	43.3
Lymph node metastasis (N)		
N0	458	56.33
N1	203	24.97
N2	152	18.7
Distant metastasis (M)		
M0	729	89.67
M1	84	10.33
Lymphovascular invasion		
Yes	339	41.7
No	462	56.83
Unknown	12	1.47
Alcohol intake		
Ever	165	20.3
Never	648	79.7
Smoking		
Ever	283	34.81
Never	530	65.19
Colorectal family history		
Yes	48	5.9
No	337	41.45
Unknown	428	52.65

Correlations between mismatch repair protein deficiency and clinicopathological characteristics (n=797).

Characteristics	Number	dMMR		MLH1/ PMS2		MSH2/MSH6	
		Defective (%)	P value	Defective (%)	P value	Defective (%)	P value
Gender							
Male	495	73 (14.75)	0.662	52(10.51)	0.561	21 (4.24)	0.853
Female	302	48(15.89)		36(11.92)		12 (3.97)	
Age							
< 50	99	29 (29.29)	< 0.001	23(23.23)	< 0.001	6 (6.06)	0.284*
≥ 50	698	92(13.18)		65(9.31)		27 (3.87)	
Location							
Right side colon	173	61(35.26)	< 0.001	43 (24.86)	< 0.001	18 (10.4)	< 0.001
Left side colon	185	25(13.51)		18(9.73)		7 (3.78)	
Rectum	439	35 (7.97)		27(6.15)		8 (1.82)	
Mucin production							
With	131	36 (27.48)	< 0.001	25(19.08)	< 0.001	11 (8.4)	0.007
Without	666	85 (12.76)		63 (9.46)		22 (3.3)	
Tumor differentiation							
Poor	134	36 (26.87)	< 0.001	24(17.91)	< 0.001	12 (8.96)	0.012*
moderate	589	71 (12.05)		51(8.66)		20 (3.39)	
Well	31	4 (12.9)		3(9.68)		1 (3.23)	
Unknown	43						
Tumor stage							
I	94	6 (6.38)	0.019	5 (5.32)	0.110	1 (1.06)	0.288*
II	326	62 (19.02)		45(13.81)		17 (5.21)	
III	301	41 (13.62)		30(9.97)		11 (3.65)	
IV	76	12 (15.79)		8 (10.52)		4 (5.26)	
Bowel wall invasion (T)							
T1	20	3 (15)	0.018	2 (10)	0.139	1 (5)	0.067*

T2	102	5 (4.9)		5 (4.9)		0 (0)	
T3	334	59 (17.66)		44(13.17)		15 (4.49)	
T4	341	54 (15.83)		37 (10.85)		17 (4.98)	
Lymph node metastasis (N)							
N0	445	74 (16.63)	0.192	54 (12.13)	0.354	20 (4.49)	0.583
N1	200	31 (15.5)		22 (11)		9 (4.5)	
N2	152	16 (10.53)		12 (7.89)		4 (2.63)	
Distant metastasis (M)							
M0	721	110(15.26)	0.550	80(12.13)	0.88	30 (4.16)	0.929*
M1	76	11 (14.47)		8 (10.53)		3 (3.95)	
Lymphovascular invasion							
Yes	335	47 (14.03)	0.451	35 (10.45)	0.679	12 (3.58)	0.481
No	457	73 (15.97)		52 (11.38)		21 (4.59)	
Unknown	5						
Alcohol intake							
Ever	162	19 (11.72)	0.170	13(8.02)	0.170	6 (3.7)	0.755
Never	635	102 (16.06)		75 (11.81)		27 (4.25)	
Smoking							
Ever	263	35 (13.31)	0.170	24 (9.13)	0.226	11 (4.18)	0.967
Never	534	86(16.1)		64(11.98)		22 (4.12)	
Colorectal family history							
Yes	48	11 (22.92)	0.071	5(10.42)	0.795	6 (12.5)	0.016*
No	335	44 (13.13)		32 (9.55)		12 (3.58)	
Unknown	414						

1   *Fisher's exact test was used

Correlations between *RAS* gene mutations and clinicopathological characteristics (n=813).

Characteristics	Number	RAS		KRAS		NRAS	
		Mutation (%)	P value	Mutation (%)	P value	Mutation (%)	P value
Gender							
Male	506	221 (43.68)	0.105	204 (40.32)	0.097	19 (3.75)	0.9
Female	307	152 (49.51)		142 (46.25)		11 (3.58)	
Age							
< 50	100	38 (38)	0.091	37 (37)	0.23	1 (1)	0.161*
$\geq$ 50	713	335 (46.98)		309 (43.34)		29 (4.07)	
Location							
Right side colon	181	91 (50.28)	0.178	88 (48.62)	0.097	3 (1.66)	0.164
Left side colon	189	77 (40.74)		71 (37.57)		6 (3.17)	
Rectum	443	205 (46.28)		187 (42.21)		21 (4.74)	
Mucin production							
With	133	74 (55.64)	0.014	73 (54.89)	0.002	1 (0.75)	0.087
Without	680	299 (43.97)		273 (40.18)		29 (4.22)	
Tumor differentiation							
Poor	138	55 (39.86)	0.315	54 (39.13)	0.604	1 (0.72)	0.093
moderate	599	276 (46.08)		251 (41.9)		28 (4.67)	
Well	33	17 (51.52)		16 (48.48)		1 (3.03)	
Unknown	43						
Tumor stage							
I	95	41 (43.16)	0.031	35 (36.84)	0.023	6 (6.32)	0.18*
II	332	170 (51.2)		161 (48.49)		9 (2.71)	
III	302	133 (44.04)		122 (40.4)		14 (4.64)	
IV	84	29 (34.52)		28 (34.52)		1 (1.19)	
Bowel wall invasion (T)							
T1	21	9 (42.86)	0.36	8 (38.1)	0.158	1 (4.76)	0.36*

T2	104	40 (38.46)		34 (32.69)		6 (5.77)	
T3	336	154 (45.83)		146 (43.45)		9 (2.68)	
T4	352	170 (48.3)		158 (44.89)		14 (3.98)	
Lymph node metastasis (N)							
N0	458	224 (48.91)	0.006	209 (45.63)	0.079	15 (3.28)	0.265
N1	203	88 (43.35)		83 (40.89)		6 (2.96)	
N2	152	61 (40.13)		54 (35.53)		9 (5.92)	
Distant metastasis (M)							
M0	729	343 (47.05)	0.048	317 (43.48)	0.116	29 (3.98)	0.353*
M1	84	30 (35.71)		29 (34.52)		1 (1.19)	
Lymphovascular invasion							
Yes	339	157 (46.31)	0.763	145 (42.77)	0.825	14 (4.13)	0.623
No	462	209 (45.24)		194 (41.99)		16 (3.46)	
Unknown	12						
Alcohol intake							
Ever	165	67 (40.61)	0.128	63 (38.18)	0.203	5 (3.03)	0.615
Never	648	306 (47.22)		283 (43.67)		25 (3.86)	
Smoking							
Ever	283	109 (38.52)	0.002	99 (34.98)	0.001	10 (3.53)	0.863
Never	530	264 (49.81)		247 (46.6)		20 (3.77)	
Colorectal family history							
Yes	48	28 (58.33)	0.017	26 (54.17)	0.013	3 (6.25)	0.178*
No	337	135(40.95)		126 (37.39)		9 (2.67)	
Unknown	428						

1   *Fisher's exact test was used

Correlations between DNA mismatch repair protein expression deficiency and *RAS* status (n =797).

MMR status	RAS		KRAS		NRAS	
	Mutant/tested cases (%)	P value	Mutant/tested cases (%)	P value	Mutant/tested cases (%)	P value
dMMR	54/121 (44.63)	0.734	54/121 (44.63)	0.635	0/121 (0)	0.009[*]
MHL1/PMS2 deficiency	39/88 (44.32)	0.725	39/88 (44.32)	0.875	0/88 (0)	0.044[*]
MSH2/MSH6 deficiency	15/33 (45.45)	0.999	15/33 (45.45)	0.72	0/33 (0)	0.391[*]
pMMR	313/676 (46.3)		286/676 (42.3)		30/676(4.43)	

1   [*]Fisher's exact test was used

Correlations between *KRAS* gene mutations and clinicopathological characteristics in dMMR tumors (n=121).

Characteristics	Number	KRAS Mutation (%)	P value
Gender			
Male	73	31 (42.47)	0.555
Female	48	23 (47.91)	
Age			
< 50	29	11 (37.93)	0.405
≥ 50	92	43 (46.74)	
Location			
Right side colon	61	26 (42.62)	0.891
Left side colon	25	12 (48)	
Rectum	35	16 (45.71	
Mucin production			
With	36	20 (55.56)	0.116
Without	85	34 (40)	
Tumor differentiation			
Poor	36	10 (27.78)	0.099*
Moderate	71	35 (49.3)	
Well	4	2 (50)	
Unknown	10		
Tumor stage			
I	6	2 (33.33)	0.277*
II	62	33 (53.2)	
III	41	15 (36.59)	
IV	12	4(33.33)	
Bowel wall invasion (T)			
T1	3	2 (66.67)	0.179*

T2	5	0 (0)	
T3	59	26 (44.07)	
T4	54	26 (48.15)	
Lymph node metastasis (N)			
N0	74	38 (51.35)	0.056
N1	31	13 (41.94)	
N2	16	3 (18.75)	
Distant metastasis (M)			
M0	110	50 (45.45)	0.753*
M1	11	4 (36.36)	
Lymphovascular invasion			
Yes	47	21 (44.68)	0.927
No	73	32 (43.83)	
Unknown	1		
Alcohol intake			
Ever	19	9(47.37)	0.855
Never	102	46(45.1)	
Smoking			
Ever	35	14 (40)	0.514
Never	86	40 (46.51)	
Colorectal family history			
Yes	11	5 (45.45)	0.589
No	44	24 (54.55)	
Unknown	66		

1   *Fisher's exact test was used

Figure 1

Consort diagram in patient selection.
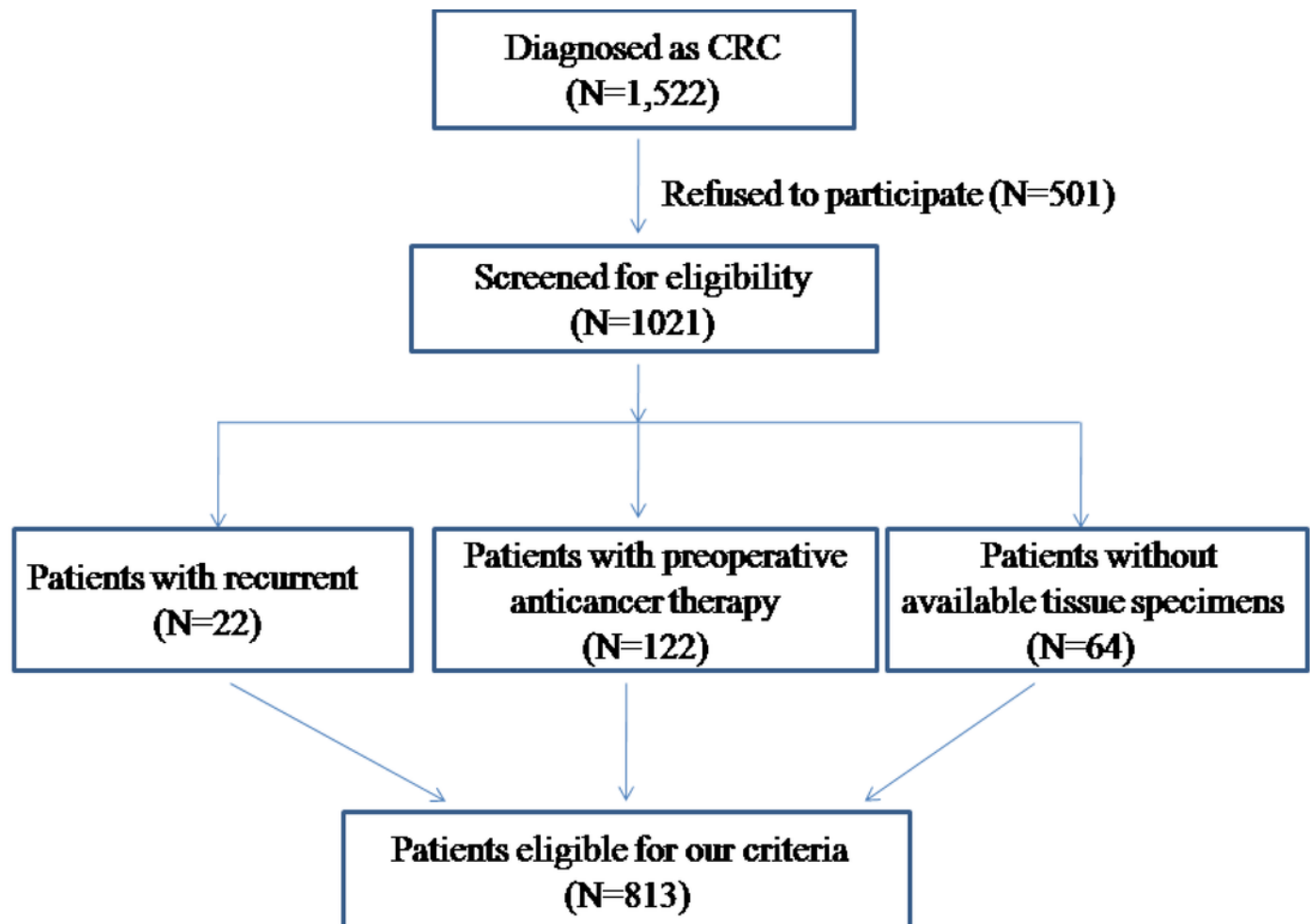
Figure 2

Immunohistochemical staining for mismatch repair proteins in one case of colorectal carcinoma.

Tumor cells with MLH1 (A) and PMS2 (B) expression, and with absent MSH2 (C) and MSH6 (D) expression, which were regarded as deficient MMR. Note the presence of positive staining in stromal cells and lymphocyte serving as internal positive controls.
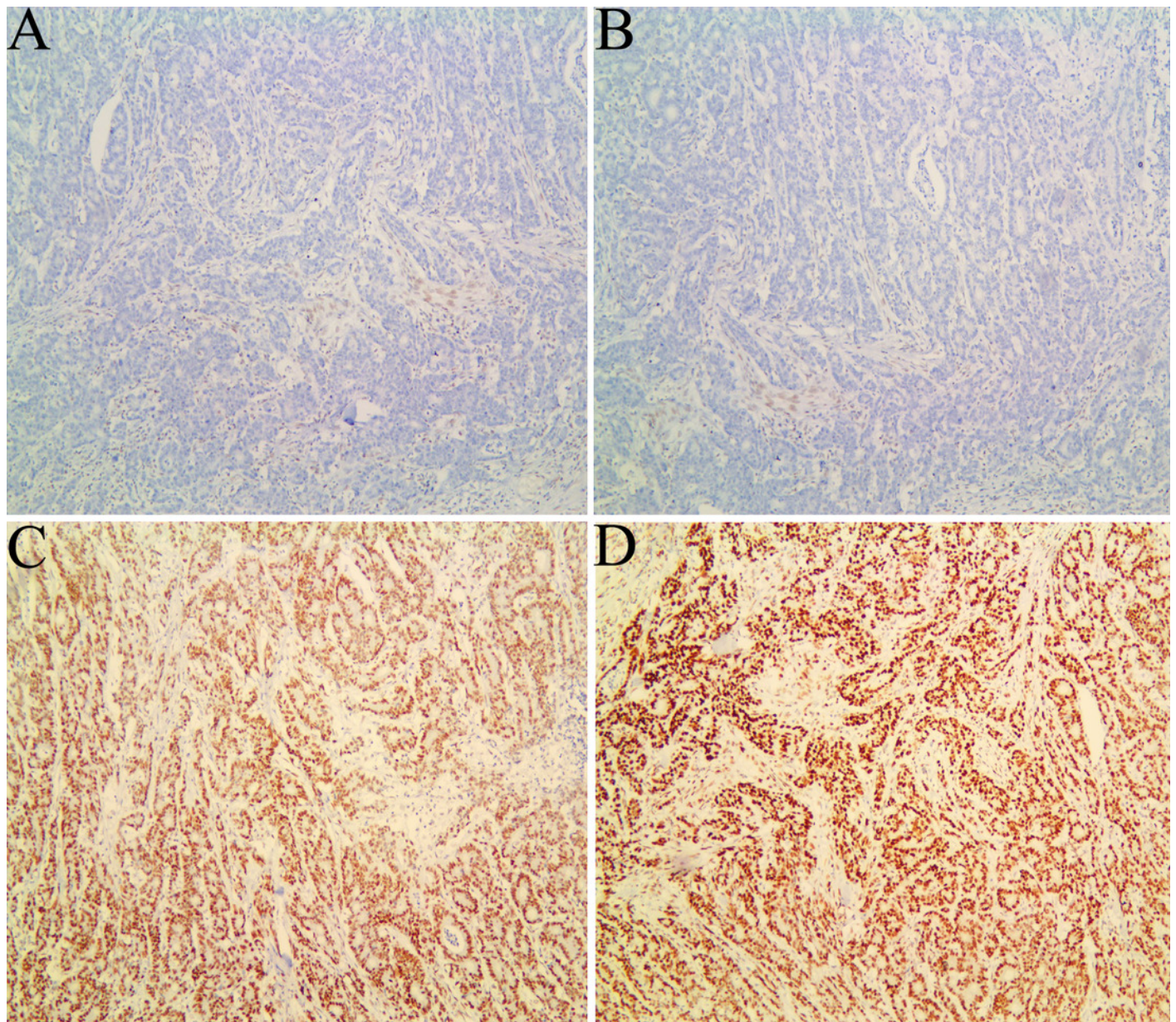
Figure 3

Survival curves for disease free survival (DFS) and overall survival (OS) in stage I - III colorectal carcinoma according to dMMR or RAS status.

A: Disease free survival (DFS) according to dMMR status; B: Overall survival (OS) according to dMMR status; C: DFS according to KRAS status; D: OS according to KRAS status; E DFS according to NRAS status; F: OS according to NRAS status.
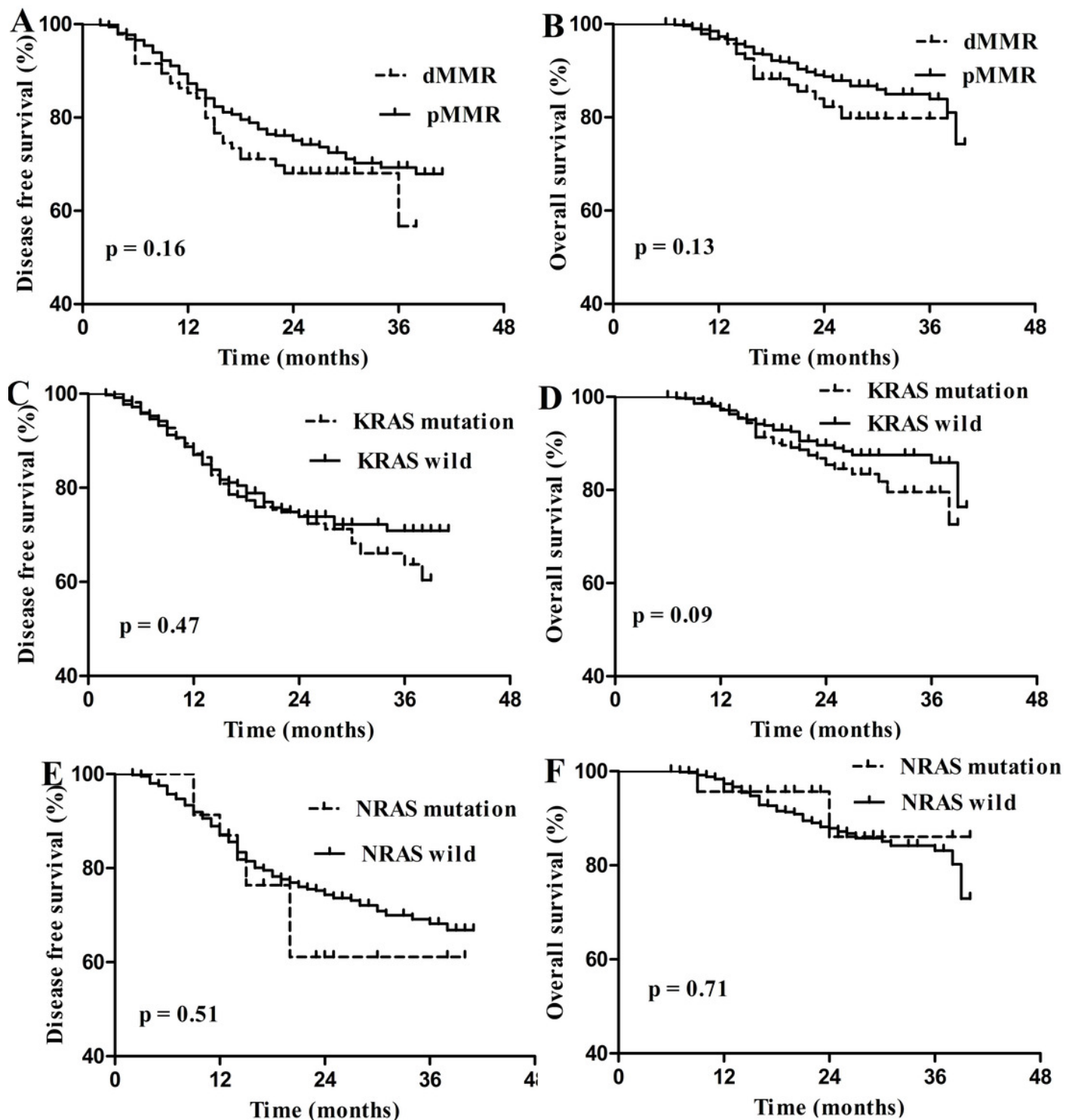
Figure 4

Survival curves for disease free survival (DFS) and overall survival (OS) in stage I - III dMMR colorectal carcinoma according KRAS status.

A: Disease free survival (DFS) according to KRAS status; B: Overall survival (OS) according to KRAS status.