Clinicopathological characteristics, molecular alterations and prognosis of mucinous histology in colorectal adenocarcinoma patients

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Research

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Abstract

Background

Mucinous adenocarcinoma (MAC) is conventionally diagnosed by WHO definition when the extracellular mucin is more than 50% of the tumour area. The study is aimed at analyzing the clinicopathological characteristics, mutation spectrum, and prognosis of the colorectal adenocarcinoma (CRC) with any mucinous proportion or feature.

Methods

Clinical and pathological information for 50 patients were reviewed and recorded. Mutation analyses for exon 2–4 of KRAS gene and exon 15 of BRAF gene were performed by Sanger sequencing. Expression of DNA mismatch repairs (MMRs) and P53 proteins was evaluated by immunohistochemistry (IHC). Density of tumour infiltrating lymphocyte (TIL) status was scored. We also evaluated the percentage of glands producing mucin and the morphology of the different tumor cells types in mucin pools. We retrospectively analyzed prognosis of 43 patients with Stage II-III MAC. The primary outcome was progression-free survival (PFS).

Results

The overall frequencies of KRAS and BRAF mutations were 37.9% and 4.4%, respectively. Patients with MAC exhibiting high levels of mucin were related to the increase of tumor diameter ($P = 0.038$), but were not associated with any of the other clinicopathological parameters. The proportion or variable morphology of mucinous component did not stratify PFS in Stage II-III tumours. It is interesting to note that male patients had lower TIL compared with female patients ($P = 0.018$). TIL-low tumors were also correlated with advanced stage ($P = 0.041$). TIL status was a strong independent predictor of PFS in stage II-III mucinous component tumours ($p < 0.001$). All four IV stage patients were also classified into TIL-low case. No parameters were independently associated with outcome after adjusting for tumour stage in multivariate analysis.

Conclusions

TIL status could more accurately determine the biology of the MAC feature for appropriate management, irrespective of quantity or morphology of mucinous component.

Background

Mucinous adenocarcinoma (MAC) is considered a special histologic type of colorectal adenocarcinoma(CRC)with a substantial amount of extracellular mucin. The defining features of this

tumour have been based variously on the presence of ≥ 50% mucinous component of the tumour [1], the mucin pool may contain layers, acini, or cribriform sheets of malignant glands, or scattered individual signet ring cells. This cutoff, while traditional, is largely arbitrary and has not been rigorously evaluated.

MAC is a distinct subtype of CRC. Despite several studies comparing underlying clinicopathological differences between MAC and conventional CRC. Still, controversial results relating prognostic value of mucinous histology have been reported [2–7]. Till present, the prognostic value of MAC remains undetermined when the locations of the tumor, molecular alterations, population characteristics or different treatment plans are taken into account. Moreover, the prognostic significance of ≤ 50% tumour mucinous component remains contentious. Several studies focused on the MAC histopathological features including percentage of mucinous component and signet ring cell component. Williams et al found mucinous tumours and tumours with a mucinous component showed marked similarities for prognostic interactions [8]. Gonzalez et al performed a stage-matched analysis and showed that Stage-matched MAC and adenocarcinoma have similar outcomes, with no prognostic significance to mucinous histological subtype [5]. A study by Inamura et al showed that even when less than 50% component of signet ring cells were present in the tumor, they could still serve as a poor prognostic indicator in CRC, independent of other clinicopathological features [9]. Song et al reported that MAC with more than 50% signet ring cell component and MAC with a greater amount of extracellular mucin were associated with poor progression-free survival (PFS) [10]. Since relatively few studies have analysed the clinicopathologic characteristics of MAC (including histological subtypes and percentage of mucinous component) in relation to patient outcome, we classified the MAC into the following: 1) three categories based on the proportion of extracellular mucin pools. 2) Several histological subtypes based on the mucinous components of different morphology.

In the present study, we explored prognostic interactions between clinico-molecular variables and tumour mucinous component and investigated the prognostic value of TIL status.

Methods

Patients

The medical records of 50 patients who underwent surgical resection at Xuhui District Center Hospital of Shanghai from 2017 to 2020 with a diagnosis of primary MAC. Archived formalin-fixed paraffin-embedded histopathology slides of these patients were retrieved for confirmation of diagnosis and molecular, immunohistochemistry (IHC) and histopathological studies. Patients who received any neoadjuvant chemotherapy or radiotherapy were excluded. Written informed consent was acquired from all the enrolled patients. This study was approved by our institutional review board. Clinicopathological data were collected for analyses of the association with MAC subtypes sex, age, pathological cancer stage, histological type, tumor size, tumor location, lymph nodes involvement and tumor differentiation were included. Two experienced pathologists reviewed all MAC cases using a multi-viewing microscope and were in agreement regarding histological grading and subtyping.

Histopathologic evaluations

Depending on the proportion of extracellular mucin, tumors were separated into three categories: 1) low mucin, extracellular mucin occupying < 50 % of the tumour; 2) moderate mucin, 50-80 % of the tumour; 3) high mucin, ≥ 80 % of the tumour. In terms of the microscopic morphology of tumour cells in mucin pools, three types were defined: 1) strip predominant type, characterised by flattened single-cell layers or strips of tumour cells located along the edge of mucin pools; 2) cluster predominant type, denoted by floating tumour cells forming clusters, acini, or cribriform sheets; 3) predominant signet ring cell type, in which signet ring cells compose ≥ 50 % of tumour cells[8]. The number of poorly differentiated clusters (PDCs) in a single field of highest activity was graded as G1 (< 5 clusters), G2 (5 to 9 clusters), or G3 (≥ 10 clusters) under an objective lens with a magnifification of ×20according to previous article[11].

Mutation analysis

DNA from paraffin-embedded tissue was extracted, and KRAS (exons 2-4), and BRAF (exon15) were amplified by polymerase chain reaction (PCR) using complementary DNA. Amplified products were assessed using the Sanger direct-sequencing method in forward and reverse directions to detect mutations.

Immunohistochemistry staining analysis

Antibody were purchased from Talent Biomedical Technology Co.,Ltd. (Xiamen China). IHC staining for P53 was performed in routinely paraffin embedded specimens. Appropriate positive and negative controls were included in each run of IHC. Microsatellite instability was assessed using IHC staining for mismatch repair (MMR) proteins, including MLH1, PMS2, MSH2 and MSH6. Loss of expression of any of the MMR proteins was considered when there was no corresponding IHC staining in the nuclei in tumor cells, whereas adjacent normal colonic epithelium, lymphocytes, and stromal cells had nuclear staining by contrast.

Evaluation of TIL

TIL were scored blindly and independently by two observers (Xiaoli Jia and Bin Li). Density of TIL for these tumors as scored using H&E stained tissue sections has been reported previously [8,12]. Tumors were classified into TIL-low (< 2 TILs per hpf) and TIL-high (≥ 2 TILs per hpf) cases. For tumors near the cut-point showing a heterogeneous distribution of TILs, scoring was commenced in a hot-spot region identified during low power screening, followed by random fields.

Statistical analyses

We adopted Fisher's exact test to compare the correlations among clinicopathological variables, histological subtypes and gene mutations. PFS was defined as time from surgery to the first confirmed relapse, with censoring done when a patient died or was alive without recurrence at last contact. Survival analysis was performed with Kaplan–Meier method and survival curves were compared using the log

rank test. Cox proportional hazards model was used for univariate and multivariate analysis. Statistical analyses were performed using the SPSS software22.0. P values less than 0.05 were considered statistically significant.

Results

Patient characteristics

We identified 50 patients with MAC that fulfilled the inclusion criteria. There were 19 women and 31 men in this group, ranging in age at diagnosis from 31-90 years of age (median, 67 years). Of the 50 cases, 17 (34 %) involved the right colon, 26 (52 %) the left colon and 7 the rectum (14 %). The average tumor size was 6 cm (range 3-13 cm). The number of patients with pathological stage I, II, III and IV cancer were 3, 20, 23 and 4, respectively (Table 1). 22 % (11 of 50) of cancers were observed with lymph node metastasis and 14 % (7 of 50) exhibited distant metastasis. Lymphatic/vascular invasion and neural invasion were found in 20% (10 of 50) and 50 % (25 of 50) patients, respectively.

Histological subtypes

The clinicopathological characteristics of the mucinous tumours in the series were compared with cases divided according to the volume of the mucinous component. The proportion of extracellular mucin was low in 8 tumours (16 %), moderate in 32 tumours (64 %), and high in 10 tumours (20 %). Patients with MAC exhibiting high levels of mucin were related to the increase of tumor diameter (P=0.038), but were not associated with any of the other clinicopathological parameters. According to the microscopic morphology of tumour cells in the mucin pools, 13 tumours (26 %) were divided into the strip predominant type, 33 tumours (66 %) were the cluster predominant type, and 4 tumours (8 %) were the predominant signet ring cell carcinoma (SRCC) (Table 1). Unfortunately, there were no statistical significance among these groups.

Tumour TIL analysis

In our cohort, TIL status data were available for 50 patients, 28 were classified as TIL-high and 22 as TIL-low respectively. It is interesting to note that men had lower TIL compared with women (P = 0.018). TIL-low tumors were also correlated with advanced MAC stage (P = 0.041) (Fig. 2a). All four IV stage patients were classified into TIL-low cases (Table 2).

Clinico-molecular characteristics

Tumor samples were analyzed for MMR status and somatic mutations in KRAS and BRAF. Five patients (10 %) showed high-frequency MSI (MSI-H) tumors, and 45 patients (90 %) had low frequency MSI (MSI-L) or microsatellite stable (MSS) tumors (2 MSI-L and 43 MSS). There were more early stage tumors (stage I-II) in the MSI-H group (P = 0.048). The remaining clinicopathological factors variables did not significantly differ between the groups. We additionally detected 18 (36 %) KRAS mutations. Among

these, 10 tumors with G12D mutation, 3 with G12V on exon2, 1 with Q61L and 1 with Q61R mutation on exon3, 1 with A146T and 2 with A146V mutation on exon4. BRAF V600E mutation was found in 4 of 50 (8 %). All of these molecular alterations were mutually exclusive. Nonetheless, there was no statistically correlation between KRAS or BRAF mutation and clinicopathological factors.

Comparative analysis of patient outcome

All patients underwent surgical resection.The follow-up period for PFS were available for 50 patients. 14 % (7 of 50) of individuals experienced a disease recurrence. 7 patients developed metastatic disease at more than one site. Liver and lung metastasis were most frequent, uncommon sites of metastatic diseases were bone and pancreas. It is well known that patients with CRC at stage I have a favorable rate of survival, whereas patients with CRC at stage IV show a poor rate of survival. However, it is difficult to predict the survival of patients with stage II or stage III CRC. Therefore, we analyzed the prognostic value of 43 patients with stage II and III MAC. In the univariate analysis, only TIL-low had a negative association with PFS (P = 0.000). In contrast, patient sex, age, tumor size, tumour location, lymph node metasis, gene mutation, MSI result, lymphovascular or perineurial invasion and MAC histological subtypes showed no significant correlation. (Figure 1H). P53 was expressed in 40–80 %–cases and negative in 10–20 %–cases. The prognostic value of P53 negative expression exhibited a tendency toward significance (P = 0.109) (Table 3) (Fig. 2b). Nevertheless, multivariate analysis revealed that none of the parameters showed a statistically significant association with PFS.

Discussion

MAC is relatively infrequent subtype of neoplasm that accounts for 10–20% of all primary CRC [1, 5, 13]. For this reason, this entity is typically underrepresented in CRC research, and a lot of its biology remains poorly understood. Incidence in our department, MAC comprised about 12% (50/400) of surgical resected cases. This study demonstrated that a high frequency of KRAS mutation, but low BRAF and MMR deficiency rates. Among these,the KRAS mutation rate was equal to 38.5%, which was close to most of the previous reports either about Chinese or other ethnicities in CRC patients [2,14.15]. BRAF mutation rates are significantly different in previous studies of Chinese population [16]. Like in other studies, these two gene (KRAS and BRAF) mutations were mutually exclusive [17, 18]. Similar to Xiaodong Li's study [16], we found that both BRAF or KRAS mutations has no correlation with amount of mucinous component in the tumours.

In the present study, the prevalence of MSI was lower than that reported in the literature [19–21]. Our findings, together with Kepil et al reports, suggest that this finding might have arisen from a geographical/ ethnic difference [22]. Besides, our results did not demonstrate a significant relationship between PFS outcome and MMR deficiency. Also, contrary to previous reports [23], we found that P53 negative expression patients were more prone to metastatic relapse, although no significant association was found. This may be partially because the number of MMR deficiency or P53 negative expression case is limited in our study.

The mucinous component of CRC has been drawing attention in recent years. Most currently studies comparing CRC with MAC use this criterion, nevertheless, their impact on patients' prognosis has remained controversial. The present results do not agree with some previous reports [3, 10] but support those of a recent study by Gonzalez et al [5] and Chen et al [2], in which they state that the proportion of mucinous component of MAC has no significant influence on patient prognosis. While mucinous features should still be mentioned, utilizing criteria such as a 50% cut off is not supported by our data. However, we found the bigger the tumor size, the higher the number of mucinous.

In addition to investigating the prognostic significance of the percentage of tumour glands producing mucin, we also evaluated the morphology of the different tumour cells types in mucin pools. MAC tumour cells exhibit highly diverse morphological features, such as layers, acinar or cribriform structures, or individual signet ring cells. SRC is defined by the presence of ≥ 50% of tumour cells with intracytoplasmic mucin. It has been reported that compared with MAC, patients with SRC were more frequently associated with metastatic disease, metastases at multiple sites, and had a poorer survival than MAC patients [24, 25]. Interestingly, during the follow-up period of 32 months, one SRCC patient did not experience any tumor recurrence. The morphology of SRCC case is shown in (Figure. 1c). This patient showed high TIL counts. Our findings suggest that, to the contrary, no correlation was found between the different morphological types and PFS for the SRC or MAC. Additionallly, while one study did find that PDCs grade had the highest accuracy in predicting RFS in CRC patients, no significant results were found for MAC patients. Taken together, we found histologic characteristics of MAC, including percentage of tumor volume comprised of mucus, were not predictive of outcome.

Lymphocytic infiltration is a major immunological defense against tumor cells in solid tumors and is a potential predictor of CRC[26, 27]. Williams et al[8]found TIL status to be a strong independent predictor of PFS in mucinous component tumors, and a superior predictor of prognosis compared with histological grade. The more TIL infiltrates in the tumor microenvironment, the better the prognosis may be. Likewise, we also demonstrated that TIL status was a strong independent predictor of PFS in MAC. Although the histologic subtype of SRCC was once thought to convey additional risk of mortality, our study demonstrates that, SRCC patients with high TIL levels also showed better PFS prognosis. Furthermore, our study found more male patients diagnosed with TIL-low tumors than female patients. The results of our study suggest that using the grade of TIL status instead of WHO grade of the entire tumor can help identify patients with a high risk of recurrence more accurately.

This study still had certain limitations. Firstly, this study was a retrospective analysis, and these patients' long-term survival could not be obtained at present. Secondly, limitations of our study include its small patient sample, as well as the low number of adverse events (recurrences), both factors likely contributed to the lack of statistical significance of TIL status in multivariate analysis. A larger sample size may be needed to adequately detect these issues in the future.

In summary, TIL status showed statistically significant association with PFS. In contrast, other parameters, including KRAS or BRAF mutational status□MMR deficiency□percent of glands producing

mucin and the morphology of the different tumor cell types in mucin pools showed no significant correlation. Consequently, TIL assessment should be considered for introduction into clinical practice and included in the pathology report of MAC. The result also revealed that the current cut off of 50% mucin component to define MAC might be challengeable. To confirm these findings, further studies with larger sample size are needed.

Abbreviations

MAC: Mucinous adenocarcinoma; CRC: Colorectal adenocarcinoma; MMRs: Mismatch repairs; TIL: tumour infiltrating lymphocyte; IHC: Immunohistochemistry;

PFS: Progression-free survival

Declarations

Availability of data and materials

All data generated or analyzed during this study are included in the submission. The raw data are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Institutional and/or national research ethic committee has approved the data collection and management process.

Consent for publication

Not applicable.

Competing interests

The authors state that there are no conflicts of interest to disclose.

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Authors' contributions

Xiaoli Jia and Bin Li wrote the manuscript. Xiaoli Jia designed the study and analyzed all the data. Xiaoli Jia and Bin Li are co-first authors. ZheYan helped with patient follow-up. The authors read and approved the final manuscript.

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Tables

Table 1 Clinico-pathological and molecular data of the patients with MACs according to the proportion of mucinous component

Clinicopathological variables	5 % < MAC < 50 %	50 % ≤ MAC < 80 %	80 % <MAC < 100 %	P value
Age				
< 50	2	4	2	0.625
≥ 50	6	28	8	
Sex				
Male	4	21	5	0.535
Female	4	11	5	
P53				
Positive	7	26	7	0.679
Negative	1	6	3	
Tumour location				
Right colon	3	13	1	0.461
Left colon	4	15	7	
Rectum	1	4	2	
Tumor size				
≤ 4 cm	3	7	0	0.038
> 4 cm	2	22	9	
Lymphovascular invasion				
Present	2	5	4	0.222
Absent	6	27	6	
Perineural invasion				
Present	3	18	4	0.546
Absent	5	14	6	
Primary tumour stage				
I	2	1	0	0.217
II	2	12	6	
III	3	17	3	
IV	1	2	1	

lymph node stage				
0	3	19	5	0.51
1	5	13	5	
Distant metastasis				
Absent	0	4	3	0.188
Present	8	28	7	
MSI result				0.757
MSI-H	0	4	1	
MSI-L	0	1	1	
MSS	8	27	8	
KRAS				
No mutation	5	21	6	1
Mutation	3	11	4	
BRAF				
No mutation	8	29	9	1
Mutation	0	3	1	
Poorly differentiated clusters				
G1	4	12	2	0.178
G2	1	11	1	
G3	3	9	7	
TIL status				
TIL-low	2	14	6	0.318
TIL-high	6	18	4	
Microscopic morphology of tumour cells in mucin pools				
Strip predominant type	1	11	1	0.26
Cluster predominant type	7	21	9	
Signet ring cell carcinoma	0	2	2	0.283
Non-signet ring cell carcinoma	8	30	8	

Table 2 Clinico-pathological and molecular data of the patients with MACs according to TIL status

Clinicopathological variables	TIL-low	TIL-high	P value
Age			0.706
< 50	4	4	
≥ 50	17	25	
Sex			0.018
Male	17	13	
Female	4	16	
P53			0.286
positive	15	25	
negative	6	4	
Tumour location			0.492
Right colon	9	17	
Left colon	3	4	
Rectum	9	8	
Tumor size			0.752
≤ 4 cm	5	9	
> 4 cm	16	20	
Lymphovascular invasion			0.741
Present	4	7	
Absent	17	22	
Perineural invasion			0.567
Present	12	13	
Absent	9	16	
Primary tumour stage			0.041
I	0	3	
II	7	13	
III	10	13	
IV	4	0	
Regional lymph node stage			0.398

0	13	14	
1	8	15	
MSI result			1
MSI-H	2	3	
MSS	19	26	
KRAS			0.551
No mutation	12	20	
Mutation	9	9	
BRAF			
No mutation	20	26	0.63
Mutation	1	3	
Poorly differentiated clusters			
G1	10	8	0.386
G2	4	9	
G3	7	12	
Microscopic morphology of tumour cells in mucin pools			
Strip predominant type	7	11	0.774
Cluster predominant type	14	18	
Signet ring cell carcinoma	1	3	0.63
Non-signet ring cell carcinoma	20	26	

Table 3 Clinico-molecular variables and PFS in patients with stage II/III MACs

Clinicopathological variables	Hazard ratio	95% CI	P value
Age			0.206
< 50	19.52	14.27-27.46	
≥ 50	26.46	20.87-32.3	
Sex			
Male	20.8	14.27-24.77	0.874
Female	23.83	18.79-28.87	
P53			0.109
Positive	25.57	14.14-27.46	
Negative	16	8.75-23.25	
Tumour location			0.693
Right colon	24.47	19.18-29.75	
Left colon	19	15.8-22.2	
Rectum	17.25	7.698-26.8	
Tumor size			0.658
≤ 4 cm	17.25	10.89-23.62	
> 4 cm	22.83	17.89-27.77	
Proportion of mucinous component			0.472
5 % < MAC < 50 %	NA	NA	
50 % ≤ MAC < 80 %	NA	NA	
80 % <MAC < 100 %	NA	NA	
TIL status			0.000
TIL-low	NA	NA	
TIL-high	NA	NA	
Lymphovascular invasion			0.568
Present	20.31	13.55-27.08	
Absent	24.38	19.22-29.54	
Perineural invasion			0.185
Present	15.6	10.82-20.57	

Absent	25.5	21.06-29.94	
Primary tumour stage			0.708
II	22.49	16.09-9.90	
III	22.48	17.42-27.56	
Regional lymph node stage			0.457
0	25.03	19.40-36.66	
1	16.6	12.93-22.28	
MSI result			0.605
MSI-H	27	20.6-33.4	
MSI/MSS	21.08	16.72-25.44	
KRAS			0.822
No mutation	17	13.18-20.82	
Mutation	23.84	18.70-28.98	
BRAF			0.452
No mutation	NA	NA	
Mutation	NA	NA	
Microscopic morphology of tumour cells in mucin pools			
Strip predominant type	22.89	NA	0.775
Cluster predominant type	22.32	NA	
Signet ring cell carcinoma	NA	NA	0.452
Non-signet ring cell carcinoma	NA	NA	
Poorly differentiated clusters			0.811
G1	19.69	12.82-26.56	
G2	18.33	15.67-21.0	
G3	24.3	18.05-30.55	

NA: not available

Figures

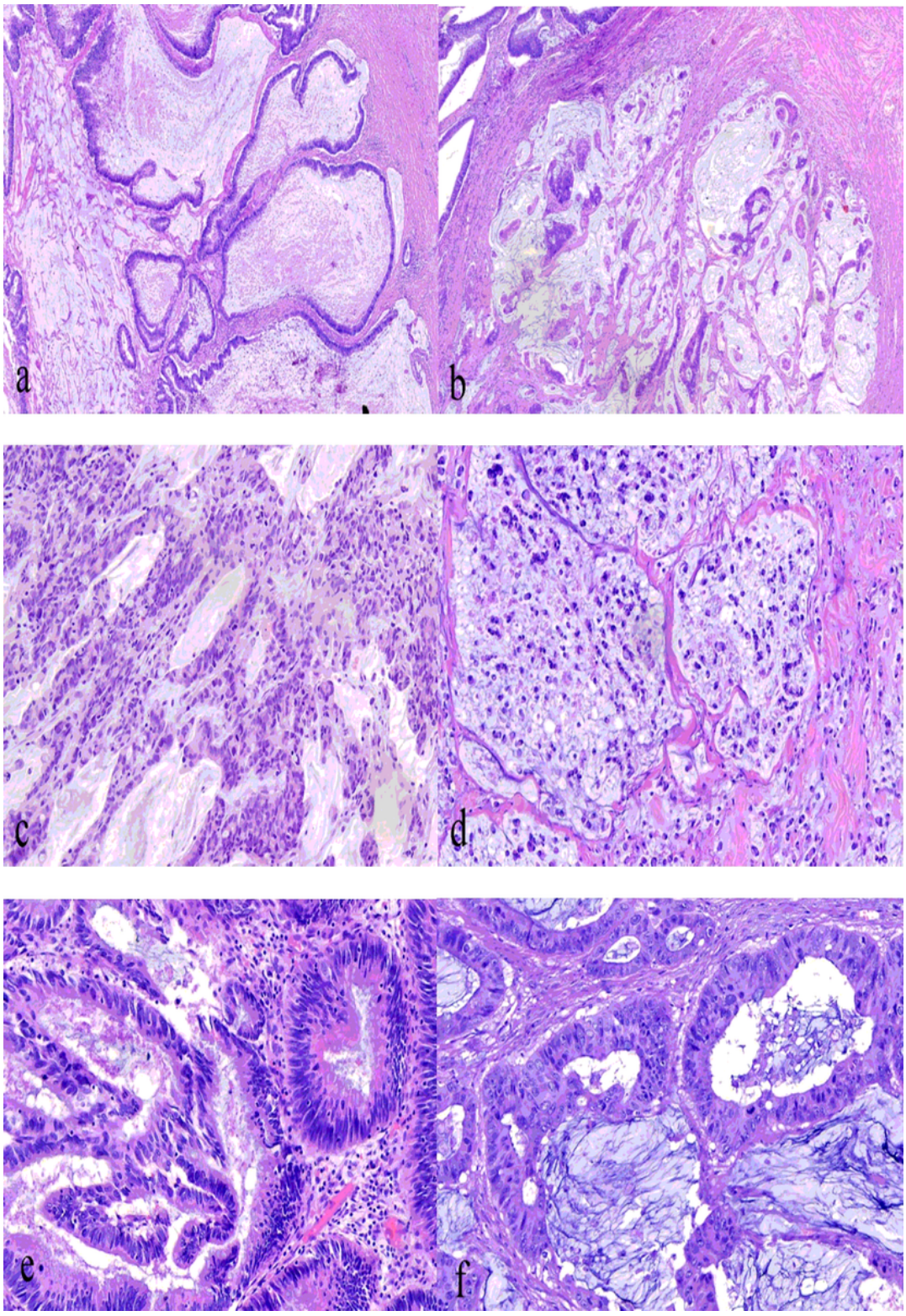

Figure 1

Representative images of MAC . (a-b)Two kinds of histological types of MAC base on the morphology of the floating tumour cells. (HE, original magnification 20×) (a) In the strip predominant type, flattened single cell layers are lining the extracellular mucin pools. (b) In the cluster predominant type, floating tumour cells form clusters sheets. (c-d)Tumor tissue consists of mainly signet ring cells (HE, original

magnification 20×) (e-f)TIL status (HE, original magnification 40×) (e)TIL-high (≥ 2 TILs per hpf) (f)TIL-low (< 2 TILs per hpf)

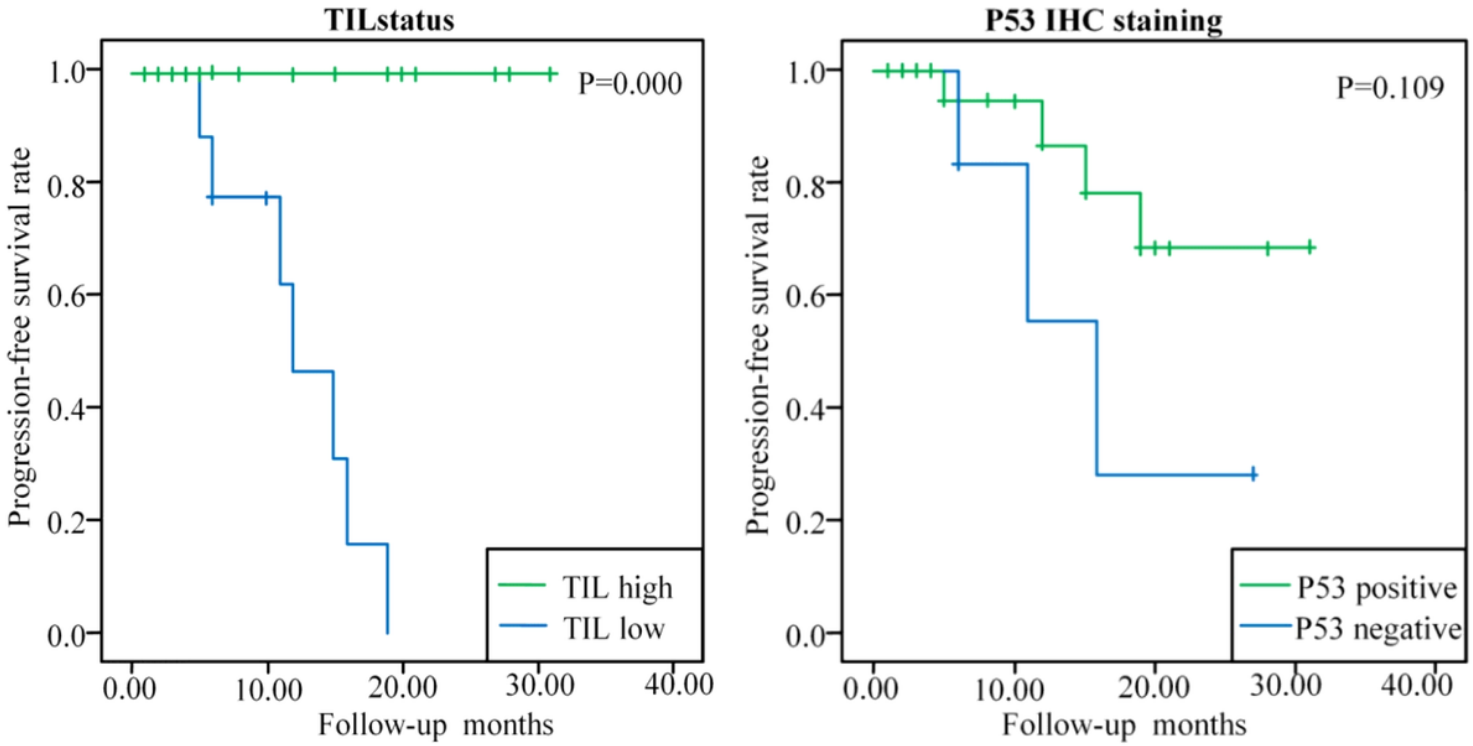

Figure 2

Kaplan-Meier PFS survival curves according to the TIL status and P53 IHC staining in MAC. (a) TIL-low had a negative association with PFS. (b) P53 negative expression exhibited a trend toward association with relapse.