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Original Article

Clinical retrospective study on the expression of the PD-L1 molecule in sporadic colorectal cancer and its correlation with K-ras gene mutations in Chinese patients

Jiasheng Xu[1*], Wenpeng Zhao[2*], Kaili Liao[3], Luxia Tu[1], Xiaozhen Jiang[1], Hua Dai[1], Yanqing Yu[1], Qiuying Xiong[1], Zhenfang Xiong[1]

[1]Department of Pathology, The First Affiliated Hospital of Nanchang University, No. 17 Yongwaizheng Street, Nanchang 330006, Jiangxi, China; Departments of [2]Vascular Surgery, [3]Clinical Laboratory, The Second Affiliated Hospital of Nanchang University, No. 1 Minde Road, Nanchang 330006, Jiangxi, China. [*]Co-first authors.

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Abstract: Objective: To detect the expression of PD-L1 and K-ras gene status in colorectal cancer tissues and analyze the relationship between PD-L1 expression and the clinicopathological features and K-ras gene status in colorectal cancer. Methods: Two hundred fifty colorectal cancer tissues were collected from the First Affiliated Hospital of Nanchang University. The normal intestinal mucosal tissues of 20 patients were randomly selected for inclusion in the control group. PD-L1 expression was detected by immunohistochemistry. K-ras gene mutation in colorectal cancer tissues was detected by sequencing. The clinical significance of PD-L1 expression and relationship between PD-L1 expression and K-ras gene mutation were analyzed. Results: The immunohistochemistry assay showed that PD-L1 was highly expressed in colorectal cancer. The positive expression of PD-L1 was increased with lymph node metastasis and high TNM stage. The 5-year survival rate of PD-L1-positive patients was significantly lower than that of PD-L1-negative patients. The K-ras gene mutation rate was 35.6%, and the main mutation site was in codon 12. The positive PD-L1 expression rate in patients with K-ras gene mutations was significantly higher than that in patients with wild-type K-ras gene mutations. Conclusion: PD-L1 is highly expressed in colorectal cancer, and its expression is related to metastasis and tumor stage. PD-L1 expression is closely related to K-ras gene mutation, and the K-ras gene status may affect PD-L1 expression. Trial registration: retrospectively registered.

Keywords: PD-L1, colorectal cancer, prognosis, K-ras gene status

Introduction

Colorectal cancer is one of the most common malignant tumors worldwide, and its incidence is third in both men and women [1-3]. In recent years, its incidence has been increasing among younger age groups, and the 5-year survival rate of patients with advanced colorectal cancer is less than 5% [4, 5]. It is urgent to identify new molecular markers for the diagnosis and prognosis of colorectal cancer, study its molecular mechanism and explore new targets and strategies for colorectal cancer diagnosis and treatment.

PD-L1 is an important negative costimulatory molecule of the b7/cd28 superfamily. Under physiological conditions, PD-L1 is mainly expressed in immune cells. However, high PD-L1 expression can be detected in various human malignant tumors, such as non-small cell lung carcinoma, small cell carcinoma, gastric cancer, ovarian cancer, renal cell carcinoma, glioblastoma, malignant melanoma, bladder cancer [6-12] and breast cancer [12-14]. The PD-L1 expression level is closely related to clinicopathological parameters and prognosis.

Currently, drugs targeting the PD-L1/PD-1 signaling pathway, such as blocking antibodies, are being used in the clinical treatment of lung cancer and advanced malignant melanoma. Clinical trials for treating various malignant tumors, such as liver cancer and kidney cancer, have also shown good safety and efficacy [15-19]. Considering the important role of the

PD-L1 molecule in the tumor immune escape mechanism, its correlation with tumor biological behavior and prognosis is quite different in different types of tumors.

In colorectal cancer, patients with high PD-L1 expression show high tumor differentiation and no lymph node metastasis and have a good prognosis. Some studies have shown that patients with high PD-L1 expression show low tumor differentiation. However, these patients are prone to liver metastasis, a late tumor stage diagnosis, and a poor prognosis, indicating that high-level PD-L1 expression maybe a biomarker for a poor prognosis [20]. Other studies have shown that PD-L1 expression and its effect on prognosis may be related to tumor microenvironmental factors, primary tumor sites in CRC and the dense infiltration of PD-L1^{+} immune cells [21-23]. The biological behavior and prognostic significance of PD-L1 in colorectal cancer remain controversial.

Although the mechanisms of the occurrence and development of colorectal cancer are not fully understood, the important role of mutation in the proto-oncogene K-ras in colorectal cancer was confirmed a long time ago [24]. The K-ras gene has become an important molecular marker for colorectal cancer targeted therapy. Cetuximab combined with chemotherapy has become an important choice for the first- and second-line treatments of wild-type K-ras gene patients [25]. However, in colorectal cancer, a correlation exists between PD-L1 expression and the state of the K-ras gene. Additionally, no relevant study has investigated their relationship to the prognosis of colorectal cancer or their interactions, which are worthy of further investigation.

Thus, this study aimed to analyze the expression characteristics, clinical significance and prognostic value of PD-L1 in colorectal cancer. The correlation and interaction between the PD-L1 molecule and K-ras gene in the development and progression of colorectal cancer were assessed by measuring PD-1 expression in colorectal cancer tissues and detecting K-ras gene mutation.

Materials and methods

Basic patient information

Control group: From the distal intestinal segment removed by surgery in patients with colorectal cancer, the intestinal mucosa beyond 5 cm from the outermost periphery of the tumor tissue was used as the normal intestinal mucosa. Twenty normal intestinal mucosa tissue samples were randomly selected as the control group and included samples from 13 male and 7 female patients, aged 32-72 years, with a median age of 47 years.

All the specimens were fixed in 10% neutral formalin solution and embedded in paraffin. The histological type of all the tumor tissue samples was adenocarcinoma. The 2010 edition of "Who Digestive System Tumor Pathology and Genetics" classification of colorectal tumor by TNM staging was used. All the tissue sections were diagnosed by two highly experienced pathologists, and important parameters, such as histological classification, lymph node metastasis, nerve infiltration, and vascular invasion, were reassessed (**Table 1**).

Chip production [27]

The tissue slides were thoroughly washed and dried and treated with polylysine antibiotics. All the sampled tissues were paraffin-embedded and sectioned, and the sections were subjected to HE staining to evaluate the cancer tissues. The HE slices and corresponding paraffin blocks (donor wax blocks) were marked to make a tissue chip (receptor wax block) of 2.2 cm × 2.5 cm × 2 cm, and a 5 × 9 total 45-point tissue sample was designed on the wax block. To construct the array, 6 pieces of perforated blank wax blocks were made by punching with a tissue microchip, the wax block was softened in a water bath of 39°C~42°C, and the tissue column with a diameter of 2 mm and a height of 4 mm was removed individually using a tissue chip instrument. Each of the six blank wax blocks was pushed into the corresponding holes of the predesigned holes, and the wax blocks were continuously sliced and placed on slides for antistripping treatment.

Immunohistochemical staining

PD-L1 expression in 250 colorectal cancer tissues was detected using the EnVision immunohistochemistry two-step method [28]. Rabbit-derived antibody was purchased from Abcam at a concentration of 0.994-1.009 mg/ml. Additionally, PBS was used instead of the primary antibody as a blank control, and a known positive slice was used as a positive

Table 1. Relationship between PD-L1 protein expression difference and clinicopathological parameters (%)

Clinical pathological parameters	Number of cases	PD-L1 positive	χ^2	*P*
gender				
male	143	60 (41.9)	0.219	0.640
female	107	50 (46.7)		
Age (years old)				
<40	29	10 (34.5)	2.799	0.270
≥40 and ≤60	150	65 (43.3)		
>60	71	35 (49.3)		
Mass type				
Ulcer type	187	79 (42.2)	0.693	0.707
Diffuse infiltration	23	11 (47.8)		
Uplift type	40	20 (50.0)		
Tumor size (cm)				
<5	99	37 (37.4)	1.163	0.559
≥5 and ≤10	113	55 (48.7)		
>10	38	18 (47.4)		
Tumor site				
Right colon	81	35 (43.2)	1.273	0.513
Left colon	79	33 (41.7)		
Transverse colon	10	5 (50.0)		
rectum	80	36 (45.0)		
Differentiation				
Highly differentiated	40	18 (45.0)	1.113	0.573
Medium differentiation	114	55 (48.2)		
Low differentiation	96	37 (38.5)		
Histological type				
Tubular adenocarcinoma	145	60 (41.4)	4.301	0.231
Papillary adenocarcinoma	35	14 (40.0)		
Mucinous adenocarcinoma	40	23 (57.5)		
Signet ring cell carcinoma	30	13 (43.3)		
Lymph node metastasis				
YES	92	58 (52.2)	4.474	0.034
NO	158	62 (39.2)		
Vascular infiltration				
YES	70	30 (42.9)	2.833	0.267
NO	180	80 (44.4)		
Neurological invasion				
YES	63	25 (39.7)	4.756	0.033
NO	187	85 (45.5)		
Liver metastasis				
YES	40	22 (55.0)	5.844	0.015
NO	210	68 (32.4)		
Lung metastasis				
YES	31	13 (41.9)	0.067	0.796
NO	219	97 (44.3)		
TNM staging				
I	20	4 (20.0)	5.61	0.018
II	40	8 (20.0)		
III	80	40 (50.0)		
IV	110	58 (52.7)		

control. The expression of PD-L1 in the tissue microarray was independently evaluated by two highly experienced pathologists, and the results were consistent with the diagnosis results. PD-L1 was positively expressed in the presence of brownish-yellow particles in the cytoplasm and/or cell membrane. The percentage of positive cells in tumor cells was assessed, and 5% positivity was defined as tumor PD-L1 positivity.

Colorectal cancer tissue DNA extraction

The slices were observed, and the appropriate wax block was selected. The block was sliced into 10-15 white tablets, and then, the slices were dyed and the tumor tissue area on the HE slice was marked. The wax was removed, followed by rehydration, manual removal of non-tumor tissue by scraping, collection of the tumor tissue, and extraction of the DNA from the tumor tissue. The extracted DNA was subjected to PCR amplification, and the K-ras gene exon 1 sequencing primer sequence was as follows: K-ras 1F: AGGCCTGCTGAAAATGACTG; K-ras 1R: TCAAAGAATGGTCCTGCACC; the K-ras gene exon 2 sequencing primer sequence was as follows: K-ras 2F: TGTAATAATCCAGACTGTGTTCTCC; K-ras 2R: AGCTTATTATATTCAATTTAAACCCACC.

DNA direct sequencing analysis

The K-ras gene mutation was detected by direct DNA sequencing, and the PCR product was sent to Beijing Liuhe Huada Sequencing Co., Ltd., Shanghai Branch for purification. All the samples were subjected to two-way sequencing using a sequencer system (ABI3730XL) and Big Dye sequencing reagent. The K-ras gene mutation peak map of known colorectal cancer was used as a positive control, and sequence analysis was performed using DNA Star software.

Follow-up

To investigate the postoperative survival of patients with colorectal cancer, we objectively evaluated the clinical treatment effect. To improve the level of scientific research, we followed up with 250 patients with colorectal cancer for which there were complete data.

The follow-up data of the enrolled cases were collected from March 2018 to December 2018 and correlated to patients with colorectal cancer.

Statistical analysis

All the data were analyzed using the SPSS 21.0 software package. The correlation between PD-L1 expression and clinicopathological factors was studied by χ^2 test, Fisher's exact test and Spearman's rank correlation analysis. Survival curve estimates were performed using the Kaplan-Meier method. The count data were analyzed by t-test, and the statistical analysis of the data results was repeated 3 times. The data were expressed as means ± standard error (X ± S). The measurement data were analyzed by χ^2 test, and the difference was considered significant at $P<0.05$.

Results

PD-L1 staining results

Among the 250 colorectal cancer cases, the positive PD-L1 protein expression rate was 44.0% (110/250). PD-L1 protein expression showed positive expression in the membrane or cytoplasm of tumor cells, showing fine or coarse brownish-yellow particles, and the nuclei were not stained; the expression in the tumor tissues was heterogeneous, and the normal mucosa of the cancer was not stained (**Figure 1**). Among the patients, 143 were male and 107 were female, with an age range of 18-88 years and a median age of 52 years. Regarding TNM stage, 20 cases were stage I, 40 cases were stage II, 80 cases were stage III, and 110 cases were stage IV.

Relationship between the PD-L1 expression level and clinicopathological features of colorectal cancer

Among the 250 cases of colorectal cancer, 110 cases were PD-L1-positive, and 140 cases were PD-L1-negative. The PD-L1 expression levels in the colorectal cancer tissues is shown in **Table 1**. Statistical analysis showed that the positive PD-L1 expression rate was significantly higher in the patients with lymph node metastasis than in those without lymph node metastasis (52.2% vs 39.2%), and the difference was significant ($P<0.05$). In patients with liver metastasis, the positive PD-L1 expression rate was significantly higher than that

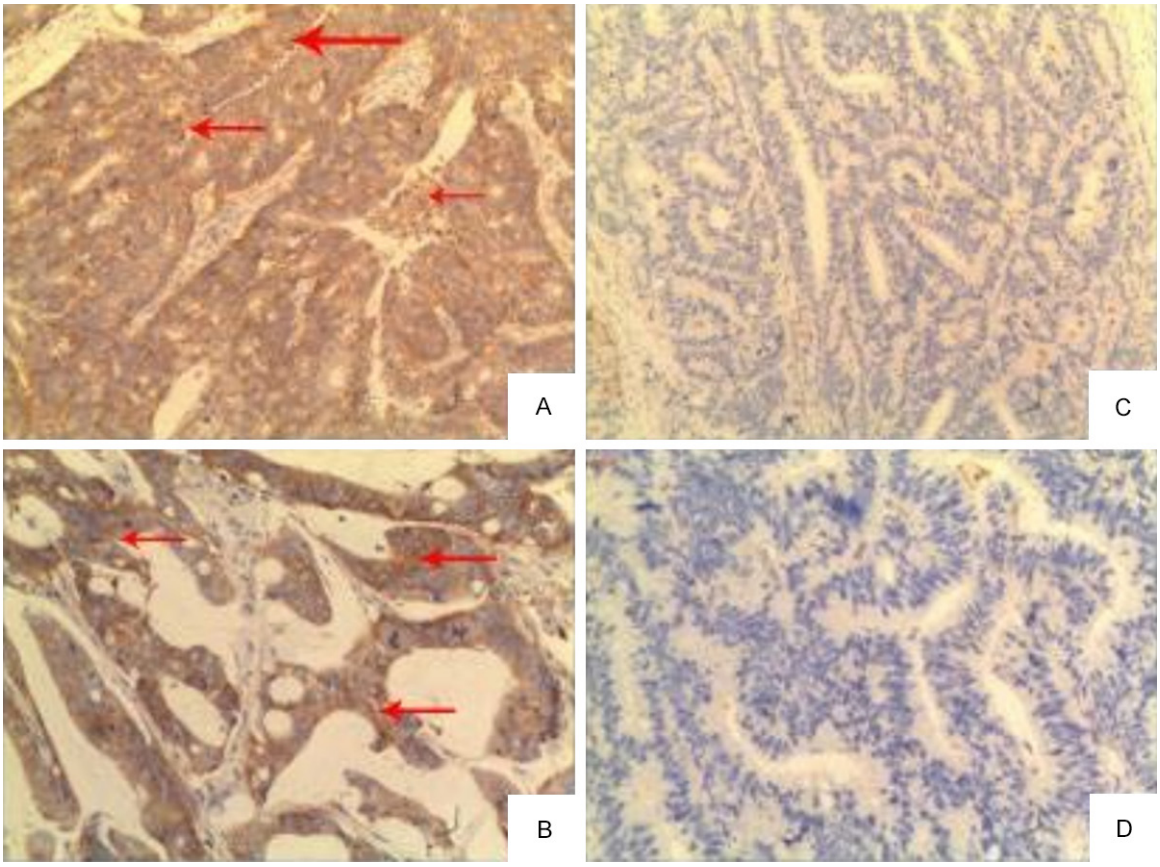


Figure 1. Immunohistochemical staining for detection of PD-L1 expression in colorectal cancer. A: PD-L1 positive expression, × 100; B: PD-L1 positive expression, × 200; C: PD-L1 negative, × 100; D: PD-L1 negative, × 200.

of patients without liver metastasis (55.0% vs 41.9%), and the difference was significant ($P<0.05$). Regarding the TNM stage of tumors from stage I to stage IV, the positive PD-L1 expression rate also increased gradually, and the difference was significant ($P<0.05$). No significant differences were found in the PD-L1 protein expression between patients of different sexes, ages or gross mass sizes, locations, histological types or degrees of differentiation ($P>0.05$) (**Table 1**).

Relationship between the PD-L1 expression level and the prognosis of colorectal cancer patients

We analyzed the relationship between the PD-L1 expression and prognosis of patients with colorectal cancer. All the patients with colorectal cancer had complete follow-up information, and 22 patients were unavailable for follow-up or refused follow-up, accounting for 8.8%. The 5-year survival rate of colorectal cancer patients was 60% (150/250), the overall average survival time was 55.4 months, and the overall median survival time was 48 months (survival range: 1-84 months). The 5-year survival rate of the PD-L1-positive patients was 47.3% (52/110), the average survival time was 45.2 months, and the median survival time was 42 months. The 5-year survival rate of the PD-L1-negative cases was 70.0% (98/140), the average survival time was 57 months, and the median survival time was 55 months. Kaplan-Meier curves and log-rank tests were used to analyze the relationship between PD-L1 expression differences and the overall survival time. The survival time of the PD-L1-positive patients was significantly lower than that of the PD-L1-negative patients, with significant differences observed ($P<0.05$) (**Figure 2**). Using the Cox risk scale model, we analyzed the relationship between PD-L1 expression differences and prognosis in patients with colorectal cancer. PD-L1 was a prognostic factor in the single factor survival analysis.

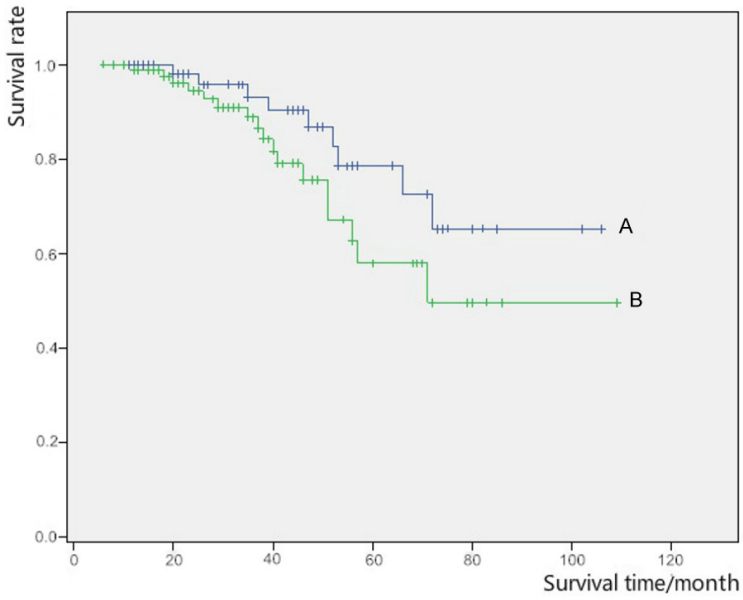


Figure 2. Relationship between PD-L1 expression and survival of colorectal cancer patients. A: PD-L1 negative group; B: PD-L1 positive group.

Table 2. Results of single factor and multivariate Cox regression models

variable	*P value*	95% CI
Univariate analysis		
PD-L1 expression	0.007	(1.009-3.579)
age	0.476	(0.485-1.345)
gender	0.521	(0.853-1.987)
Tumor location	0.765	(0.475-2.875)
Tumor differentiation	0.028	(1.175-2.567)
Infiltration depth	0.14	(1.408-2.989)
Neurological invasion	0.174	(0.896-1.841)
Vascular invasion	0.302	(0.777-1.507)
Lymph node metastasis	0.004	(2.469-4.961)
TNM staging	0.038	(1.284-2.836
multi-factor analysis		
PD-L1 expression	0.008	(1.050-2.988)
Lymph node metastasis	0.006	(2.165-4.519)
TNM staging	0.024	(1.134-3.556)
Tumor differentiation	0.367	(0.811-1.765)

Based on the above results, PD-L1 can be used as an independent indicator to evaluate the prognosis of colorectal cancer. Additionally, tumor differentiation, lymph node metastasis, and TNM staging were associated with survival (**Table 2**). In multivariate survival analysis, PD-L1 was found to be associated with a poor prognosis (**Table 4**). Furthermore, lymph node metastasis and TNM staging were associated with the prognosis of colorectal cancer and were unfavorable factors for prolonged survival (**Table 2**). Based on the above results, PD-L1 can be used as an independent indicator to evaluate the prognosis of colorectal cancer.

Relationship between the K-ras gene status and clinicopathological features of colorectal cancer

Among the 250 cases of colorectal cancer, 89 cases had K-ras gene mutations, and the mutation rate was 35.6%. The specific gene mutation statuses are shown in **Table 3**. No cases of simultaneous mutations were found in codons 12 and 13. At the same time, no mutation was found in codon 61 (**Figure 3** and **Table 3**).

In cases of lymph node metastasis, liver metastasis, and lung metastasis, the K-ras gene mutation rates were 51.1% (47/92), 55.0% (22/40), and 48.3% (15/31), respectively, which were significantly higher than those without lymph nodes. The proportion of metastatic cases was 26.6% (42/158), 31.9% (67/210) without liver metastasis and 29.5% (74/219) without lung metastasis, with a significant difference ($P<0.05$). Regarding TNM staging, the K-ras gene mutation rates in patients with stage I, II, III, and IV disease were 20.0% (4/20), 32.5% (13/40), 32.5% (26/80), and 41.8% (46/110), respectively. Thus, with the increase in TNM staging, the K-ras gene mutation rate was significantly increased. In stage IV patients, the mutation rate of the K-RAS gene was the highest and significantly higher than that of stage I patients ($P<0.05$). No significant differences were found in the K-ras gene mutation rates between different patients based on sex, age, gross tumor morphology, tumor location, tumor differentiation, vascular or nerve infiltration, or different histological types ($P>0.05$) (**Table 4**).

Relationship between PD-L1 expression and K-ras gene status

In 89 cases of K-ras mutant cancer tissues, 52 were positive for PD-L1, and the positive rate was 58.4%. Among 161 k-RAS wild-type cancer tissues, 58 were positive for PD-L1, and the

Table 3. Types and frequencies of 12 and 13 codon mutations in the K-ras gene

Codon	Wild type	Point mutation	Number of cases
12 Codon	GGT (Gly)	GAT (Asp)	20
	GGT (Gly)	GTT (Val)	20
	GGT (Gly)	AGT (Ser)	11
	GGT (Gly)	TGT (Cys)	12
	GGT (Gly)	GCT (Ala)	4
13 Codon	GGC (Gly)	GAC (Asp)	22

positive rate was 36.0%. In the patients with K-ras mutations, the positive PD-L1 expression rate was significantly higher than that of the patients with wild-type K-ras ($P<0.05$). Spearman's rank correlation analysis showed that PD-L1 expression was positively correlated with K-ras gene mutation ($r = 0.73$; $P<0.05$).

Discussion

Some studies have revealed that K-ras gene mutation and status are associated with tumor stage, lymph node metastasis, Dukes' staging and microsatellite instability [29, 30]. Other studies have shown that K-ras mutation is not correlated with age, sex, tumor size, tumor location, TNM staging or other parameters [25]. Notably, the frequency of the K-ras gene mutation in right-side colon cancer was significantly higher than that in left-side colon cancer or rectal cancer, and K-ras mutations were more likely to be detected in men aged 50-60 years and in patients with tumor recurrence. Gao Feng et al. [31] found that K-ras gene mutations were more likely to occur in female patients.

In this study, we found that the PD-L1 expression rate in colorectal cancer was 44%, while PD-L1 expression was not detected in normal colorectal tissues. This finding differs from that reported by scholars studying different populations (34.9%) [32]. The causes may be related to ethnic groups and geographical differences. Additionally, our data showed that positive PD-L1 expression is closely related to the biological behavior of colorectal cancer. Statistical analysis showed that, in patients with lymph node metastasis and liver metastasis, the positive PD-L1 expression rate was significantly higher than that in patients without lymph node metastasis or liver metastasis. With increasing tumor TNM stage, the positive PD-L1 expression rate also increased gradually. No statistically significant differences were found with respect to sex, age, gross mass, size of the mass, location of mass, histological type, degree of tumor differentiation, or expression of the PD-L1 protein. Some studies have shown that positive PD-L1 expression is associated with tumor differentiation, and PD-L1 is highly expressed in poorly differentiated cancer tissues [33]. The cause may be related to an unclear tumor grade, and the subjective factors of different pathologists reporting different degrees of differentiation. Other research data are inconsistent with our results. Some research groups found that a high PD-L1 expression status is negatively correlated with tumor differentiation, lymph node metastasis, distant metastasis and prognosis [34]. Patients with expressed tumors tend to have a high degree of tumor differentiation, no lymph node metastasis and distant metastasis, and a better prognosis. The cause may be that the enrolled cases in this study were rigorously screened, excluding patients who received chemotherapy or were diagnosed with other diseases before surgery. Additionally, the expression of PD-L1 on the surface of tumor cells was affected by the tumor microenvironment. The regulation of various cytokines is related to changes in the microenvironment and the regulatory network of the interaction in different pathological stages [35]. KM survival analysis showed that the risk of tumor progression and tumor-related death in PD-L1-positive patients was significantly higher than that in PD-L1-negative patients. Univariate and multivariate analyses of the Cox risk ratio model showed that the prognosis of PD-L1-positive patients was worse than that of PD-L1-negative patients. PD-L1 can be used as a molecular marker for independently evaluating the prognosis of colorectal cancer patients without knowing factors such as the tumor size, location, degree of differentiation, histological type, or TNM staging.

Therefore, PD-L1 is closely related to tumor invasion, progression, and prognosis. Several clinical trials related to PD-L1 are currently underway. Data from phase I clinical trials of mdx showed that one patient with melanoma and one patient with renal cell carcinoma achieved partial remission, one patient with CRC showed complete remission, and one

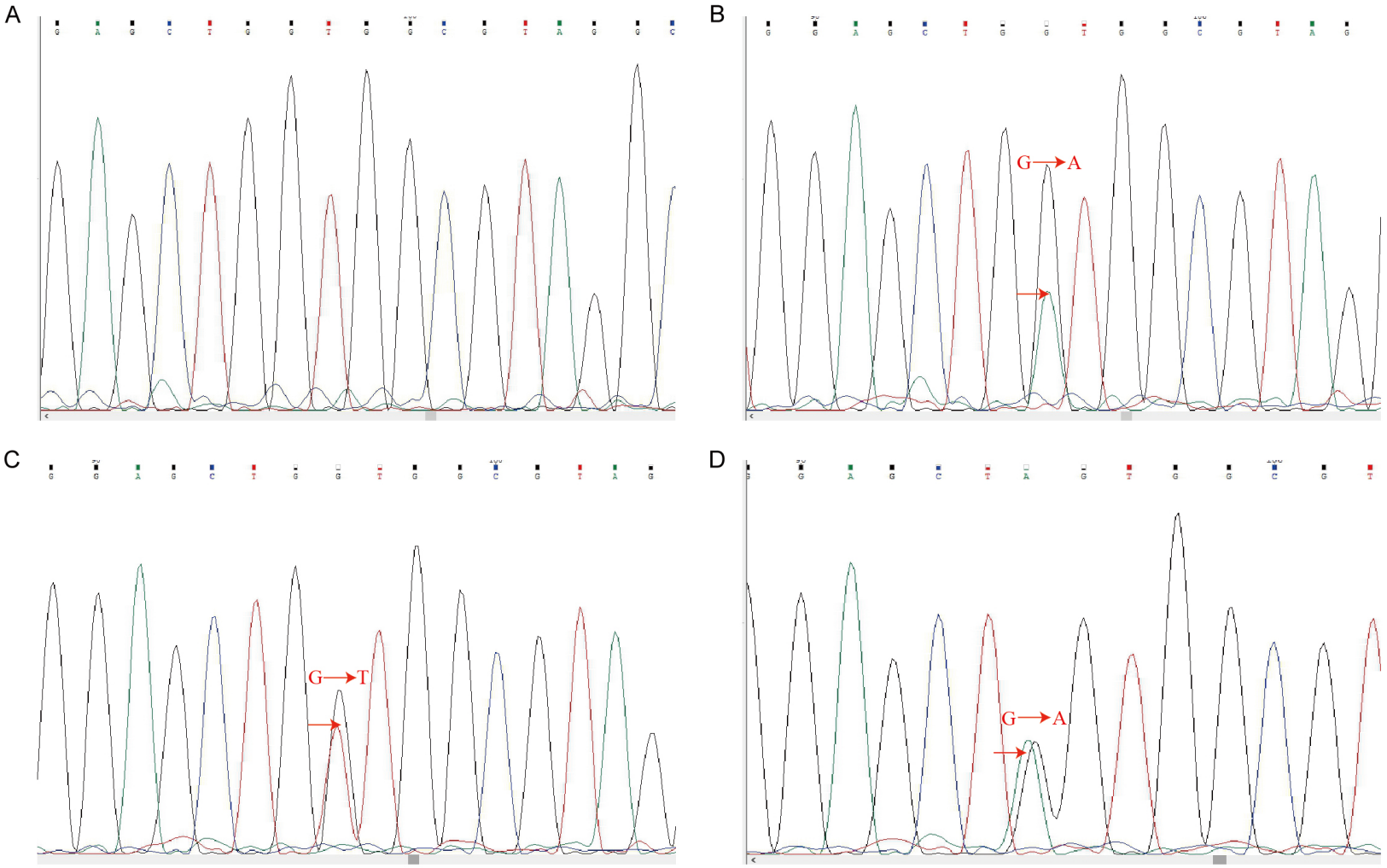
Table 4. Relationship between K-ras gene status and clinical pathological parameters Example (%)

Clinical pathological parameters	Number of cases	K-ras gene mutation	χ^2
gender			0.398
Male	143	46 (32.2)	
Female	107	43 (40.2)	
Age (years old)			2.798
<40	29	10 (34.5)	
≥40 and ≤60	150	50 (33.3)	
>60	71	29 (40.8)	
Mass type			5.907
Ulcer type	187	64 (34.2)	
Diffuse infiltration	23	8 (34.8)	
Uplift type	40	17 (42.5)	
Tumor size (cm)			4.941
<5	99	28 (27.2)	
≥5 and ≤10	113	44 (39.0)	
>10	38	17 (44.7)	
Tumor site			1.277
Right colon	81	27 (33.3)	
Left colon	79	31 (39.2)	
Transverse colon	10	4 (40.0)	
rectum	80	27 (33.8)	
Differentiation			5.473
Highly differentiated	40	16 (40.0)	
Medium differentiation	114	44 (38.6)	
Low differentiation	96	29 (30.2)	
Histological type			4.745
Tubular adenocarcinoma	145	46 (31.7)	
Papillary adenocarcinoma	35	16 (45.7)	
Mucinous adenocarcinoma	40	15 (37.5)	
Signet ring cell carcinoma	30	12 (40.0)	
Lymph node metastasis			14.444
YES	92	47 (51.1)	
NO	158	42 (26.6)	
Vascular infiltration			5.769
YES	70	28 (40.0)	
NO	180	61 (33.9)	
Neurological invasion			0.567
YES	63	26 (41.3)	
NO	187	63 (33.7)	
Liver metastasis			
YES	40	22 (55.0)	7.79
NO	210	67 (31.9)	
Lung metastasis			
YES	31	15 (48.3)	8.478
NO	219	74 (29.5)	
TNM staging			
I	20	4 (20.0)	20.938
II	40	13 (32.5)	
III	80	26 (32.5)	
IV	110	46 (41.8)	

patient with non-small cell lung cancer had significantly reduced tumor size. These data suggest that tumor immunotherapy that blocks the PD-1/PD-L1 pathway may benefit patients and that PD-L1 expression may be used to screen patients who may achieve immunotherapeutic benefit [36]. Recent studies have shown that tumor-associated PD-L1 can also act as a ligand to inhibit the function of T cells in combination with other receptors, such as CD80. Although this view remains controversial, the proposal of this interaction indicates that the function of PD-L1 is complicated and diverse. In tumor-targeted therapy, inhibition of PD-L1 or PD-1 alone may not completely block the PD-1/PD-L1 pathway.

Although the occurrence and development of colorectal cancer is a multistage, multistep process involving multiple genes, the important role of proto-oncogene K-ras mutation in colorectal cancer was confirmed a long time ago [14] and is closely related to the occurrence and development of colorectal cancer.

The K-ras gene is located on human chromosome 12 and has a length of approximately 45 kb. Three common mutation types occur in the gene: point mutation, gene amplification, gene translocation and insertion. The most common mutation type is point mutation, and almost all of the mutation sites are located in codons 12 and 13 of exon 1 [37]. The K-ras gene encodes a 21-kD Ras protein that is an important signaling molecule downstream of the EGFR pathway. When the K-ras gene is mutated, the conformation of the Ras protein changes and remains active independent of the upstream EGFR signal, stimulating unrestricted cell division and proliferation and leading to malignant transformation and distant metastasis [38]. Clinically, the K-ras gene has become an important molecular marker for the targeted therapy of colorectal cancer. Cetuximab combined with chemotherapy has become an important choice for first- and second-line treatments of K-ras gene wild-type patients [39].


A
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G→A
C
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D
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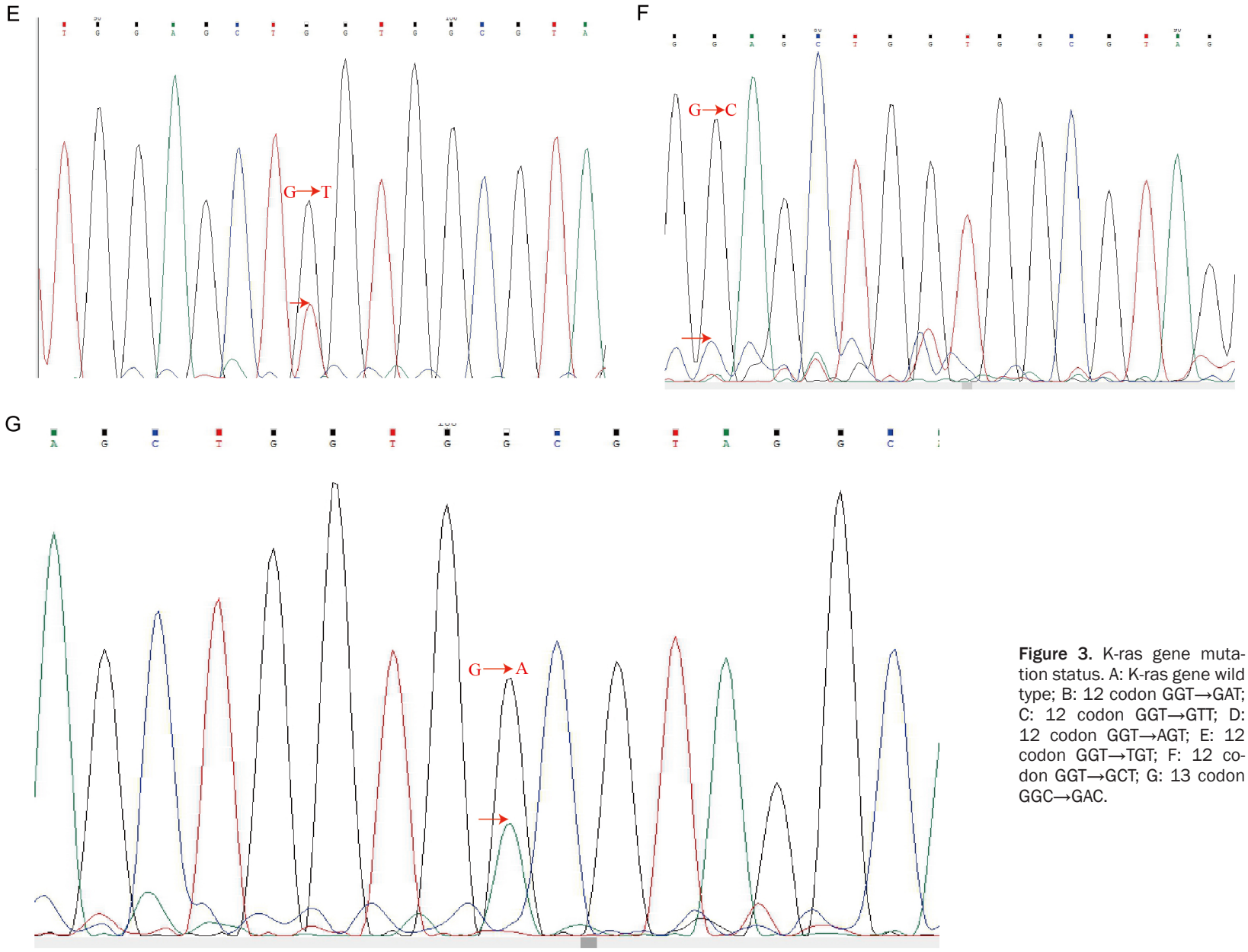

Figure 3. K-ras gene mutation status. A: K-ras gene wild type; B: 12 codon GGT→GAT; C: 12 codon GGT→GTT; D: 12 codon GGT→AGT; E: 12 codon GGT→TGT; F: 12 codon GGT→GCT; G: 13 codon GGC→GAC.

Most reports have shown that the K-ras gene mutation rate in colorectal cancer is 30%-40%, and more than 90% of the mutations are concentrated in codons 12 and 13, a finding that agrees with our results. No consistent conclusions have been drawn about the different reports of K-ras gene mutations and the clinicopathological features of colorectal cancer. Mannan et al. [29] found that K-ras gene mutations were significantly associated with tumor stage and lymph node metastasis. Zlobec et al. [40] showed no correlation between K-ras gene mutation and age, sex, tumor size, tumor location, TNM stage or other parameters. Naguib et al. [27] reported that the K-ras gene status was associated with Dukes' staging and microsatellite instability. Pajkos et al. [28] found that the frequency of K-ras gene mutations in right-side colon cancer was significantly higher than that in left-side colon cancer and rectal cancer; K-ras mutations were more likely to be detected in men aged 50-60 years and in patients with tumor recurrence. Gao Feng et al. [31] found that K-ras gene mutations are more likely to occur in female patients.

Regarding lymph node metastasis, liver metastasis and lung metastasis, we showed that the K-ras gene mutation rate was significantly higher than that without lymph node metastasis, liver metastasis or lung metastasis. As the TNM stage increased, the K-ras gene mutation rate also increased significantly. These conclusions suggest that colorectal cancer with the K-ras mutation is more prone to lymph node metastasis, liver metastasis, and lung metastasis and is associated with a high tumor stage. The detection of the K-ras gene status and analysis of its mutations and types can provide clues for predicting lymph node metastasis, distant metastasis and tumor progression. Thus, mutation of the K-ras gene may endow the tumor with a certain invasion and metastasis potential, but the occurrence and progression of colorectal cancer are processes involving multiple genes, and it is necessary to further clarify the interaction of these genes and expression changes caused by these genes. Gene mutations may lead to the activation of cellular signaling pathways and subsequent lymph node metastasis and liver metastasis. At the same time, attention should be given to follow-up to further evaluate the roles of these markers in predicting metastasis, which is the direction of our future research.

Our previous studies demonstrated that because of the high expression status of PD-L1 in colorectal cancer tissues, the malignant biological behavior of colorectal cancer cells can be effectively inhibited by inducing low PD-L1 expression and by promoting the apoptosis of colorectal cancer cells in vitro to inhibit their invasion and migration. We examined the expression of PD-L1 and the status of the K-ras gene in colorectal cancer cells and showed that PD-L1 expression in K-ras mutant cells was significantly higher than that in K-ras wild-type cells. K-ras mutation detection in colorectal cancer tissues showed that the positive expression rate of PD-L1 in the K-ras gene mutation group was significantly higher than that in the K-ras gene wild-type group, and PD-L1 expression and K-ras gene mutation were positive. The correlation indicates that PD-L1 expression is closely related to K-ras gene mutation in colorectal cancer, and the K-ras gene status may affect PD-L1 expression.

PD-L1 is a negative immunoregulatory molecule, and its regulatory signaling pathways and mechanisms are diverse. The K-ras gene is a hotspot gene in the EGFR signaling pathway, and EGFR mainly transmits signals to cells through the downstream RAS/RAF/MAPK and PI3K/AKT/mTOR cell proliferation signaling pathways, leading to increased nuclear gene transcription and regulation of cell proliferation and differentiation [41]. Following K-ras gene mutation, the PI3K/AKT/mTOR signaling pathway can be directly activated independent of the activation of upstream EGFR [42].

Conclusion

1. The expression of PD-L1 is closely related to the mutation of K-ras gene. The state of K-ras gene may affect the expression of PD-L1.

2. The mutation rate of K-ras gene in colorectal cancer was 35.6%, and the main mutation site was located in 12 codons. Colorectal cancer patients with k-RAS mutations are more likely to develop lymph node metastasis, liver metastasis, lung metastasis and are associated with high tumor stage.

3. PD-L1 is highly expressed in colorectal cancer tissues, and the expression of PD-L1 is associated with clinicopathological features. Positive cases of PD-L1 are more prone to

lymph node metastasis and liver metastasis, which are associated with high tumor stage.

4. The difference in expression of PD-L1 was associated with overall survival. The survival of patients with positive expression of PD-L1 was significantly lower than that of patients with negative PD-L1. PD-L1 could be used as an independent indicator to predict the prognosis of colorectal cancer.

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Disclosure of conflict of interest

None.

Abbreviations

pd-1, programmed death-1; PD-L1, programmed death ligand-1; TNM, Tumor Node metastasis; PCR, Polymerase Chain Reaction; DNA, deoxyribonucleic acid; EGFR, epidermal growth factor receptor; K-Ras, K-rat sarcoma; MAPK, mitogen-activated protein kinase; AKT, protein kinase B; mTOR, mammalian target of rapamycin.

Address correspondence to: Zhenfang Xiong and Qiuying Xiong, Department of Pathology, The First Affiliated Hospital of Nanchang University, No. 17 Yongwaizheng Street, Donghu District, Nanchang 330006, Jiangxi Province, China. E-mail: zhenfangxiongedu@163.com (ZFX); 1224058146@qq.com (QYX)

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