Cytoskeleton Related Protein KIF9A Potential Biomarker of Prognosis, 5-Fluorouracil Chemoresistance and Response to Immunotherapy for Patients with Colorectal Cancer

Zongxian Zhao  
Fuyang People's Hospital

Hongyu Ma  
Bengbu Medical College

Xijie Fan  
Fuyang People's Hospital

Zongju Hu  
Fuyang People's Hospital

Shu Zhu  
Fuyang People's Hospital

Shun Xu  
Fuyang People's Hospital

Qinlingfei Liu  
Fuyang People's Hospital

Yuan Yao  
Fuyang People's Hospital

Fusheng Wang ( wangfs666@163.com )  
Fuyang People's Hospital

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Abstract

One important clinical predicament and treatment challenge of colorectal cancer (CRC) is chemoresistance of 5-Fluorouracil (5-Fu), affecting the prognosis of patients seriously. The resistant of colorectal cancer to 5-Fu based therapy involves multiple intricate molecular mechanisms and unclear pivotal genes. Kinesin family member 9 (KIF9) is one member of KIFs, a kind of cytoskeleton related protein, which has not been studied in colorectal cancer. In this research, we aimed to explore and elucidate the expression level, the clinical characteristics (age, gender, TNM stage, MSI state, BRAF/P53 mutation) and functions (immune infiltration, prognosis) of KIF9 in colorectal cancer. Furthermore, we found that KIF9 high expression was associated with the response to treatment of 5-Fluorouracil and immunotherapy. The gene and protein expression level of KIF9 was detected by using qRT-PCR and IHC for verification. And we evaluated and predicted the biofunction and pathways of KIF9 in CRC by gene set enrichment analysis. Thus, this article provided a comprehensive and systematic understanding of the biofunctions of KIF9 in colorectal cancer, and we elucidated the role of KIF9 as a biomarker for predicting treatment response of 5-Fluorouracil and immunotherapy.

Introduction

Colorectal cancer (CRC), which is characterized by strong heterogeneity and aggressiveness with high mortality, is the most common malignant tumor of the digestive tract\(^1\). In the clinical setting, the American Joint Committee on Cancer (AJCC) classification is a conventional tool used to evaluate the risk and treatment demand of a specific patient based on clinical stage. The stage at diagnosis determines the prognosis of colorectal cancer and the choice of different treatment regimen\(^2\). If diagnosed early stage, endoscopic resection or surgical treatment can achieve the goal of radical tumor treatment and obtain a satisfactory prognosis. On the other hand, comprehensive and systemic treatment (including surgical complete mesocolic excision, chemotherapy, radiotherapy, targeted therapy, immunotherapy et al) play a vital role in the management and treatment of advanced colorectal cancer\(^3,4\). However, although there are multiple options for systemic treatment, a proportion of colorectal cancer patients still experience tumor recurrence, metastasis, and death after comprehensive treatment\(^5,6\). In addition, AJCC classification system has certain limitations due to tumor heterogeneity, unsatisfied sensitivity and specificity. Novel effective and convenient biomarkers, which can provide guidance for the management of CRC, have gradually received attention of clinicians.

As we know, chemotherapy (including adjuvant therapy, neoadjuvant therapy and total neoadjuvant therapy) played an important part in comprehensive management of CRC. In addition, 5-Fluorouracil (5-Fu) based chemotherapy (such as CAPOX, FOLFOX, FOLFIRI et al), which is widely used in the treatment of CRC, can greatly improve the prognosis of CRC patients\(^7\). In mechanism, after 5-Fu is transported into cell, 5-Fu can be converted to several active metabolites and exerts anticancer effects, inhibiting thymidylate synthase and disrupting RNA synthesis\(^8\). Despite the encouraging progress in CRC therapy so far, a current huge challenge is the gradual formation and development of chemoresistance after...
receiving 5-Fu treatment. However, the specific mechanism of 5-Fu chemoresistance, pivotal genes and molecular pathways involved still remained unclear. According to current researches, the resistant of CRC cells to 5-Fu based therapy involves multiple molecular mechanisms. Some influential researches illustrated that drug resistance was driven by genetic mutations, promoted by epigenetic mechanisms and mediated by tumor microenvironment. And tumor heterogeneity belonged to intrinsic factors responsible for chemoresistance, is caused by gene mutation, inheritance, transcriptomic et al. In total, understanding of the mechanism of 5-Fu based chemoresistance still remains limited and need further explored urgently. What’s more, predicting the response of 5-Fu based chemotherapy is essential for guiding treatment of CRC. Thus, the identification, exploration and validation of predictive biomarkers for 5-Fu based chemotherapy can greatly improve the prognosis of CRC patients.

Immune checkpoint therapy has driven a paradigm shift in the treatment of several cancer types and obtain satisfied therapeutic effect. In CRC, immune checkpoint therapy received regulatory approval in 2017 for the treatment of heavily mutated tumors that are mismatch-repair-deficient (dMMR) or have high levels of microsatellite instability (MSI-H) (termed dMMR–MSI-H tumours). However, despite tumor exist heavily mutation, response is highly variable, and a part of patients with hypermutated CRC show no benefit from treatment. And the mechanism of immunotherapy involves multiple immune cells, complex signal pathway and potential key molecule. There is an urgent need for further investigate novel biomarkers to improve immunotherapy response and predict prognosis of CRC patients.

Kinesin superfamily proteins (KIFs) are a class of molecular motors that transport membranous organelles, protein complexes, mRNAs, and take part in mitosis, meiosis and some vital cellular functions. Recently, some powerful and excellent researches have demonstrated that KIFs can effectively affect the development and progress in several cancer types, and have complex molecular mechanism. In hepatocellular carcinoma, KIF4A was overexpressed and associated with tumor cell proliferation, growth and poor prognosis. In breast cancer, KIFs can particular in tumor mitosis. Besides, it was reported that KIF2A, KIF14 and KIF26B was associated with tumor positive lymph nodes in breast cancer patients. Overexpression of KIF1A, KIF3C, KIF5A, KIF12 and KIF14 have been implicated in resistance to docetaxel. Kinesin family member 9 (KIF9) is one member of KIFs, a kind of cytoskeleton related protein, which was first found and reported in 2001. Recently, it was reported that KIF9 might influent the prognosis of some type tumor patients. The mRNA expression level of KIF9 was significantly associated with histologic grade, gene alterations and poor prognosis in glioma and glioblastoma patients. reported that KIF9 could particular in MT1-MMP vesicle transport and regulate cancer cell invasion. Besides, knockdown of KIF9 could increase collagen degradation and invasion. However, it remains unclear that the expression level and related molecular mechanism of KIF9 in colorectal cancer and need further study urgently.

In this study, we comprehensively and systematically analyzed the KIF9 expression level and prognostic value in CRC, along with other clinicopathological features at the first time. Furthermore, KIF9 gene expression level was compared between 5-Fu sensitive group and 5-Fu resistant group. Moreover, gene
set enrichment analysis (GSEA) was performed to evaluate the KIF9-associated biological pathways involved in CRC pathogenesis, providing clues about the function and pathways of KIF9.

**Results**

**KIF9 expression level and associated clinicopathological characteristics**

According to TCGA and GEO, the gene expression levels of KIF9 in colorectal cancer samples were significantly higher than in normal samples \( (P < 0.001, \text{Fig. 1A-C}) \). Meanwhile, the result was further confirmed by comparing by qRT-PCR in 20 paired colorectal tumor tissues and adjacent normal tissues \( (P < 0.05, \text{Fig. 1D}) \). Furthermore, the protein expression levels of KIF9 in CRC tissues and paired normal tissues were obtained and compared by Immunohistochemistry (IHC) (Fig. 1E, F). According to AOD value of IHC image, KIF9 in CRC tissues were significantly higher than in normal tissues \( (P < 0.001, \text{Fig. 1G}) \). In total, the expression of KIF9 in CRC was higher expression than in adjacent normal tissues.

In addition, we divided CRC patients from TCGA into two groups according the median value of KIF9 gene expression. Then, after excluding those patients with missing clinical information, the baseline characteristics of KIF9 high expression group (n = 240) and KIF9 low expression group (n = 238) were shown in Table 1. The results showed that the expression level of KIF9 was not correlated with age, gender, TNM stage et al.
Table 1
Relationship between KIF9 expression level and clinicopathological characteristics in CRC form TCGA

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>KIF9 level</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low (n = 240)</td>
<td>High (n = 238)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>67.57 ± 11.95</td>
<td>65.88 ± 13.07</td>
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<tr>
<td>Gender</td>
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<td>T3 + T4</td>
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<td>184</td>
</tr>
<tr>
<td>N</td>
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<td></td>
</tr>
<tr>
<td>N0</td>
<td>140</td>
<td>147</td>
</tr>
<tr>
<td>N+</td>
<td>100</td>
<td>91</td>
</tr>
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</tr>
<tr>
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<tr>
<td>M1</td>
<td>199</td>
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<tr>
<td>AJCC stage</td>
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<td>135</td>
<td>144</td>
</tr>
<tr>
<td>III-IV</td>
<td>105</td>
<td>94</td>
</tr>
</tbody>
</table>

KIF9 high expression predicting better prognosis in CRC

According to univariate cox regression analysis, age [HR = 1.039, 95% confidence interval (CI): 1.018–1.060, *P* < 0.001], depth of tumor invasion (T) (HR = 2.432, 95%CI: 1.605–3.685, *P* < 0.001), lymph node metastasis (N) (HR = 2.013, 95%CI: 1.567–2.585, *P* < 0.001), distant metastasis (M) (HR = 4.425, 95%CI: 2.843–6.890, *P* < 0.001) and AJCC stage (HR = 2.166, 95%CI: 1.702–2.755, *P* < 0.001) were significantly associated with poorer overall survival (OS) of CRC. Meanwhile, KIF9 high expression (HR = 0.509, 95%CI: 0.329–0.787, *P* = 0.002) was associated with better OS of CRC (Fig. 2A). Furthermore, according to multivariate COX regression analysis, age (HR = 1.042, 95%CI: 1.021–1.036, *P* < 0.001), lymph node metastasis (N) (HR = 1.502, 95%CI: 1.119–2.015, *P* = 0.007) and distant metastasis (M) (HR = 2.781, 95%CI: 1.637–4.723, *P* < 0.001) were associated with poorer OS. Besides, KIF9 high expression (HR =
0.628, 95%CI: 0.403–0.978, \(P = 0.040\) was associated with better OS of CRC (Fig. 2B). The result demonstrated that KIF9 was an independent risk factor for OS of CRC. In addition, survival analysis revealed that KIF9 high expression was associated with better prognosis of CRC, including OS (Fig. 2C/G, \(P < 0.05\)), progression free survival (PFS) (Fig. 3D, \(P < 0.05\)), disease specific survival (DSS) (Fig. 3E, \(P < 0.05\)), disease free survival (DFS) (Fig. 3F, \(P < 0.05\)), and recurrence free survival (RFS) (Fig. 2H, \(P < 0.05\)).

**Correlation between KIF9 expression and tumor subtypes in CRC**

Expression level of KIF9 among different mutation subtypes in CRC were performed to determine whether KIF9 could act as a biomarker to predict the therapeutic effect of targeted therapy and immunotherapy in patients with CRC. According to clinical practice guideline, V-raf murine sarcoma viral oncogene homolog B1 (BRAF), Kirsten rat sarcoma (KRAS), P53 mutation, microsatellite instability (MSI) and vascular endothelial growth factor receptor (VEGFR) were considered into further analysis. KIF9 expression level in BRAF\(^{V600E}\) mutation type was significantly higher than wild type (Fig. 3A, \(P < 0.001\)). Besides, high KIF9 expression was found to correlate with KRAS wild type and high levels of MSI (MSI-H) state of CRC (Fig. 3B, C, \(P < 0.05\)). On the contrary, KIF9 expression was not associated with P53 mutation, VEGFR gene and protein expression (Fig.3D, E and F, \(P > 0.05\)).

**The relationship between KIF9 expression and immune cells infiltration and response to immunotherapy**

Consider the potential biological role of KIF9 in the tumor immune microenvironment, the relationship between KIF9 expression and multiple immune cells infiltration and immune microenvironment was thoroughly studied. The results illustrated that CRC patients who exhibited KIF9 high expression had more T cells CD4 memory activated, T cells follicular helper. On the contrary, KIF9 low expression group had more T cells regulatory (Tregs) and Macrophages M0 (Fig. 4A, \(P < 0.05\)). In immune microenvironment, KIF9 low expression group had higher immune cells scores, stromal scores, ESTIMATE scores and had lower Tumor Purity (Fig. 4B-E, \(P < 0.05\)). In addition, there were some human leukocyte antigen (HLA) genes (HLA-DOA, HLA-DPA1, HLA-DPB1, HAL-DQA1) were upregulated in KIF9 low expression group (Fig. 4F, \(P < 0.05\)). To further analyze the influence of KIF9 expression level on immunotherapy response, the TIDE scores were obtained. Correlation analysis showed that TIDE score was negative correlated with KIF9 gene expression obviously (Fig. 4G, \(P < 0.0001, R=-0.3384\)). Besides, TMB high group had higher KIF9 gene expression than TMB-low group in CRC (Fig. 4H, \(P < 0.05\)). The results implied that KIF9 low expression patients with CRC might have better response to immunotherapy.

**Correlation between KIF9 expression and response to 5-Fu based chemotherapy**
To assess the roles of KIF9 in 5-Fu based chemotherapy of CRC, expression level of KIF9 between 5-Fu response group and non-response group were analyzed. According to GSE87211 dataset, all patients were received 5-Fu based chemoradiotherapy (CRT) before surgery\textsuperscript{21}. After excluding those patients with missing related information, we divided patients into response group and non-response group according to the response to chemotherapy. The response group was identified as descending stage of tumor (both T and N) after CRT. And the rest patients were defined as non-response group. And we found that KIF9 was significantly higher in response group compared to non-response group (Fig. 5A, P < 0.05).

Furthermore, the same result was verified in GSE19860, which all patients received mFOLFOX6 chemotherapy after surgery (Fig. 5B, P < 0.05). Besides, we also found that KIF9 was higher in sensitive group compared to resistant group at the cellular level (Fig. 5C, P < 0.05). Next, we analyzed the correlation between the expression level of KIF9 gene and the response to 5-Fu using the TCGA dataset. The results showed that KIF9 gene expression level was negatively correlated with the sensitivity scores of 5-Fu (Fig. 5D, P < 0.0001), implied that the higher the expression level of KIF9 gene, the more likely it is to response to 5-Fu treatment. Subsequently, we completed external verification with patients from Fuyang City People's Hospital. The response group was identified as occurring descending stage of tumor (both T and N) for those patients who received 5-Fu based neoadjuvant therapy. Besides, as for patients who only received adjuvant therapy after surgery, patients who had not tumor recurrence defined as response group (Table 2). Then, we demonstrated that KIF9 was did higher expression in response group compared to non-response group from Immunohistochemistry (IHC) (Figure. 5E).
Table 2
Clinical characteristics of 50 CRC patients in KIF9-high and -low expression group from Fuyang City People's Hospital.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>KIF9 expression level (IHC)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low (n = 25)</td>
<td>High (n = 25)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>66.00 ± 10.24</td>
<td>65.52 ± 10.31</td>
</tr>
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</tr>
<tr>
<td>Male</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>T</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 + T2</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>T3 + T4</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td>N1-2</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>AJCC stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td>III-IV</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>colon</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>rectal</td>
<td>14</td>
<td>15</td>
</tr>
<tr>
<td>Response to 5-Fu</td>
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<td></td>
</tr>
<tr>
<td>YES</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>NO</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Not receiving</td>
<td>14</td>
<td>16</td>
</tr>
</tbody>
</table>

50 CRC patients were divided into two groups according to the median AOD value of IHC.

GSEA identified functions and signaling pathways

GSEA was performed to identify the biological characteristics shared by tissue samples displaying different levels of KIF9 expression and to identify the functions and pathways in which KIF9 may be involved. Gene Ontology (GO) enrichment analysis indicated that KIF9 high expression was associated with DNA dependent DNA replication, ribonucleoprotein complex biogenesis, ribosome biogenesis,
mitochondrial protein containing complex, structural constituent of ribosome et al (Fig. 6A). In addition, KIF9 low expression was related to phagocytosis recognition, immunoglobulin complex, immunoglobulin complex circulating, antigen binding, immunoglobulin receptor binding et al (Fig. 6B). Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis found that high expression of KIF9 involved in cell cycle, huntingtons disease, parkinsons disease, ribosome, spliceosome et al (Fig. 6C). And low expression of KIF9 was associated with calcium signaling pathway, dilated cardiomyopathy, ECM receptor interaction, focal adhesion, neuroactive ligand receptor interaction et al (Fig. 6D).

Discussion

KIF9, one member of KIF family, is a kind of cytoskeleton related protein. In terms of biological function, KIF9 can provide a kind of pulling force that reels the centrosome up against the nucleus when cells occur mitosis progression22. Besides, Haruhiko Miyata and Masahito Ikawa et al reported that KIF9 is evolutionarily conserved and expressed strongly in mouse testis. And KIF9 is associated with the central pair microtubules and regulates flagellar motility in mice. Lacking KIF protein or KIF9 mutated mice exhibit impaired sperm motility and subfertility23. Recently, it was reported that KIF9 might play a regulatory role in the biological behavior of some tumors. For example, in glioma and glioblastoma, KIF9 gene and protein expression levels were significantly higher than normal control groups. And the mRNA expression levels of KIF9 were increased in the high histologic-grade-group compared to low-histologic-grade. Besides, KIF9 high expression was associated with a poor prognosis in both low-grade glioma and glioblastoma19. However, the roles of KIF9 in colorectal cancer has not been studied.

In this research, we investigated the expression level and potential function of KIF9 in CRC for the first time. We found that gene and protein expression levels of KIF9 were significant higher in CRC compared to normal control tissues. In addition, KIF9 high expression was associated with a better prognosis (OS, DFS, PFS et al) in CRC. Furthermore, univariate/multivariate Cox regression analysis demonstrated that KIF9 was an independent risk prognosis factor in CRC. From the different prognostic effects of KIF9 in CRC and glioma/glioblastoma, KIF9 has different antitumor or tumor-promoting effects in different tumor types. And we further found that KIF9 high expression was associated with sensitive response to 5-Fu based chemotherapy.

5-Fu is a thymidylate synthase inhibitor and the first first-line agents for both single-drug therapy and chemotherapy for CRC. After 5-Fu enters the human body cells, it can convert into 5-fluorouracil deoxynucleotide (5-FdUMP), which can inhibit the activity of thymidylate synthase (TS). In terms of results, 5-Fu inhibits purine synthesis, leading to reduced DNA replication and repair, resulting in inhibition of tumor cell growth24. However, since the congenital or acquired resistance to 5-FU, the advance CRC often occur tumor recurrence and metastasis and lead to worse prognosis and death. Therefore, exploring the related molecular mechanism and identifying molecular biomarker of 5-Fu resistance are important challenges in clinical practice. On the other hand, there were also little articles focusing on the prediction of 5-Fu chemotherapy resistance or sensitivity in CRC basing on gene expression level. Xingxing Huang and Xinbing Sui et al identified that some immune-related genes (HSPA8, RARB, RABEP2, ICAM2, CHGB,
GALP, RBP7 et al) were associated with 5-Fu resistance and poor DFS and OS of CRC patients. And RBP7 may function as a tumor microenvironment regulator to induce 5-Fu resistance, thereby affecting the prognosis of CRC patients\textsuperscript{25}. Besides, Guili Zheng and Qiang Wang et al found and demonstrated that CRC could obtain 5-Fu based chemotherapy by LINC01347/miR-328/LOXL2 axis, and LINC01347 could work as a prognostic biomarker and potential therapeutic target against 5-Fu based chemotherapy resistance of CRC\textsuperscript{26}. In this research, we found that KIF9 was associated with the drug-sensitivity of 5-Fu and had the potential as a molecular marker to predict the response to 5-Fu-based chemotherapy of CRC. From TCGA and GDSC, the sensitivity score to 5-Fu was decreasing with the increase of KIF9 gene expression level, indicating better response to 5-Fu. Besides, this conclusion was confirmed by immunohistochemical analysis of local tissue samples. However, the limitation of this research is that we did not further analyze the predictive efficacy of KIF9 as a predictor of 5-Fu sensitivity and did not compare with other biomarkers.

Furthermore, we analyzed the association between KIF9 expression level and gene mutation state, and tumor location. And we found that KIF9 expression level in BRAF\textsuperscript{V600E} mutation type was significantly higher than wild type, and KIF9 expression level in KRAS wild type was higher than mutation type. Besides, we found that KIF9 gene expression was not correlated with tumor location (left colon, right colon and rectum). According to CRC clinical practice guideline, the BRAF/KRAS mutation state and tumor location can guide the targeted therapy (bevacizumab or cetuximab) of mCRC\textsuperscript{27,28}. In addition, VEGFR2 high gene expression is associated with better prognosis compared with VEGFR2 gene expression levels (median PFS, 13.9 vs. 7.2 months, respectively; \( P = 0.032 \)) among patients with mCRC treated with first line chemotherapy and bevacizumab\textsuperscript{29}. However, the correlation analysis between KIF9 gene expression and VEGFR2 expression was not statistically significant in this research. In general, further deepen study of KIF9 as a biomarker for predicting targeted therapy in colorectal cancer is needed.

In mCRC, immune checkpoint therapy received regulatory approval for treatment heavily mutated tumors that are mismatch-repair-deficient (dMMR) or have MSI-H in 2017\textsuperscript{30}. In this research, we found that KIF9 was high expression in MSI-H CRC compared with MSI-L/MSS CRC and the result implied that KIF9 high expression might associated with the sensitive response to immune checkpoint therapy. For further study, we analyzed the effects of KIF9 on 22 kinds of immune infiltrating cells and immune microenvironment. We found that KIF9 high expression had more T cells CD4 memory activated, T cells follicular helper, and had less T cells regulatory (Tregs). CD4 + T cells can treat tumor through multiple ways, either directly by eliminating tumor cells through cytolytic mechanisms or indirectly by modulating the tumor microenvironment\textsuperscript{31}. T cells follicular helper can provide indispensable help to B cells for potent antibody responses and play an active role in tumor immunotherapy\textsuperscript{32}. In addition, Tregs can suppress aberrant immune response against self-antigens, also suppress anti-tumor immune response. Enriched infiltration Tregs in tumor tissues is often correlated with poor prognosis\textsuperscript{33}. Furthermore, we found that KIF9 low expression was associated with up-regulation of some human leukocyte antigen (HLA) genes (HLA-DOA, HLA-DPA1, HLA-DPB1, HLA-DQA1). Influential studies have shown that the higher the heterozygosity of HLA molecules, the more likely a patient is to benefit from immunotherapy\textsuperscript{34}. By comparing the KIF9 gene
expression level in TMB-high group and TMB-low group, we found that the KIF9 expression level in TMB-high group was significantly increased. And some research discovered that increasing of TMB was associated more neo-antigen and more potential possibility to benefit from immunotherapy. We plotted the correlation analysis drew, and we found that KIF9 expression level was significantly associated with TIDE score, which higher value indicates high potential of tumor immune evasion and less likely to benefits from immune treatment. The above results all showed that KIF9 might be used as a molecular marker for immunotherapy of CRC and KIF9 high expression patients might benefit from immunotherapy. However, the above results are only bioinformatics analysis results, and there is a lack of relevant strong clinical data and related experiment for direct verification, and the role of KIF9 in the immunotherapy of CRC still needs further study.

GSEA showed that KIF9 might participate in calcium signaling pathway, dilated cardiomyopathy, ECM receptor interaction, focal adhesion, neuroactive ligand receptor interaction et al pathways. Hyocheol Bae and Whasun Lim et al demonstrated that polydatin counteracts 5-Fu resistance by enhancing apoptosis via calcium influx in colon cancer. Besides, related research held the views that the tumor microenvironment can protect tumor cells from drug treatment, and extracellular matrix (ECM) can influent chemotherapy resistance by regulating tumor microenvironment. Yoshihiro Nakagawa and Masanori Shinohara et al proved that ECM molecule fibronectin can enhance the 5-Fu resistance by activating integrin-mediated ILK/Akt/NF-κB signaling. However, this research did not further investigate the specific molecular role of KIF9 in 5-FU-based chemotherapy, and we plan to reveal the relevant and concrete roles by in vitro and in vivo experiment in the future.

Methods

Data collection

The gene-expression profiles and associated clinicopathological data of patients with CRC were downloaded from the Cancer Genome Atlas (TCGA) Genomic Data Commons Data Portal (https://portal.gdc.cancer.gov/repository) on Oct 1, 2022. RNA-sequencing gene-expression Counts data for 488 CRC tissue samples and 42 normal adjacent tissue samples were collected for further analysis. The GSE14333, GSE87211, GSE44076, GSE24551, GSE19860, GSE153412, GSE103479 and GSE72970 datasets were obtained from Gene Expression Omnibus microarrays (GEO). Besides, the high quality (level 4) of proteins expression level was obtained by the Cancer Proteome Atlas (TCPA). On the other hand, a total of 50 tumor tissue samples and related clinical characteristics were collected from patients who underwent surgery for CRC at the Fuyang City People's Hospital. All participating patients provided written informed consent, and the study protocol was approved by the ethics review committees of Fuyang City People's Hospital.

Analysis of immune characteristics and response to immunotherapy
To identify immune characteristics between KIF9 high expression group and KIF9 low expression group, TCGA data were analyzed using the “CIBERSORT” packages in R software. The proportion of 22 types of tumor-infiltrating immune cells (TIICs) were obtained. To evaluate the immune microenvironment, immune scores, stroma scores, ESTIMATE scores and tumor purity were obtained by using the “ESTIMATE” packages. Tumor immune dysfunction and exclusion (TIDE) scores were calculated by online tools (http://tide.dfci.harvard.edu/) for predicting immunotherapy response. With the increasing of TIDE scores, immunotherapy tolerance is more likely to occur.

Analysis of drug sensitivity

The sensitivity of drugs was evaluated using “oncoPredict” packages. Accordingly, drug sensitivity scores were estimated and high sensitivity scores mean low sensitivity.

Tumor mutation burden (TMB) data

Tumor mutation burden was calculated by counting the number of synonymous and non-synonymous mutations across a 0.8 to 1.2 megabase region, the somatic alteration information of the TCGA-COAD and TCGA-READ cohort were obtained from TCGA, TMB was calculated by using “maftools” packages in R. TMB is an emerging biomarker for response to immunotherapy. Highly TMB are thought to harbor an increased neoantigen burden, making them immunogenic, and responsive to immunotherapy.

Cox regression analysis and survival analysis

Univariate and multivariate Cox analysis were completed and presented to evaluate the prognostic value of KIF9 expression level and clinical characteristics by using “survival” and “survminer” packages in R software. Furthermore, we divided samples of dataset in to KIF9 high expression group and low expression group according the median value of KIF9 gene expression. Survival analysis was conducted to compare overall survival (OS), disease-free survival (DFS) et al between two groups by using Kaplan-Meier analysis and log-rank test. In addition, KM plotter (http://kmplot.com) was used for further validation directly.

Gene set enrichment analysis

Gene expression data for the TCGA was normalized using “limma” package and GSEA was performed using “clusterProfiler” in R. And the gene datasets (c2.cp.kegg.gmt and c5.go.gmt) were obtained from Molecular Signatures Database (MSigDB). The enrichment terms with \( P < 0.05 \) were considered statistically significant. For better readability, only 10 terms (top 5 high and low normalized enrichment score) were presented by package “enrichplot” in R.

Quantitative real-time polymerase chain reaction (Qrt-pcr)

Total RNA was extracted using TRIzol (absin, abs60154). The concentration and purity of total RNA were determined, 1 µl of total RNA was reverse transcribed into cDNA with 1000 using cDNA Synthesis Mix (absin, abs601510). The samples were subjected to quantitative real-time PCR (qRT-PCR) using 2 × SYBR Green qPCR Master Mix (absin, abs601511) and primers specific for KIF9 (forward, 5’-
GTCGCCCTTTCTCTACC-3’; reverse, 5’-GCCTGAGTGGCTCTTTGA-3’), and GAPDH (forward, 5’-AAGGTGGAGTCAACGGA-3’; reverse, 5’-TTAAAGCAGCCCTGGTGA-3’), with all gene primers obtained from Aoke Dingsheng Biotechnology (Beijing, China). Thermal cycling conditions consisted of an initial denaturation at 95 ºC for 15 s, followed by 40 cycles of denaturation at 95 ºC for 15 s, annealing at 55 ºC for 30 s, and extension at 72 ºC for 30 s. Relative messenger RNA expression levels were calculated using the 2-ΔΔCT method, with the average level of KIF9 expression in the 20 normal colorectal tissues defined as the reference.

**Immunohistochemistry (IHC)**

The fresh tissue samples were collected and fixed with 4% paraformaldehyde (Shanghai Biosharp) immediately. The fixed tissues were trimmed, paraffin-embedded and cut into serial 4 µm section for further immunohistochemical processing. All immunohistochemical procedures were carried out using the Ventana Benchmark Ultra system (Ventana, Tucson, AZ). The antibody of KIF9 protein expression was assessed and detected using a monoclonal rabbit primary antibody (1:100, absin, abs110438). And the secondary antibody (goat anti-rabbit IgG-HRP, absin, abs20040ss) was used. Tissue sections were examined using a light microscope (Olympus CX22; Olympus Life Science). The average optical density (AOD) value was calculated by immunohistochemical morphological analysis software (ImagePro Plus 6.0) for further quantitative analysis.

**Statistical analysis**

The GraphPad Prism 8.0 and R 4.2.1 statistical software were used for statistical analysis and visualization. Parametric continuous variables were compared using Student’s t tests, and non-parametric continuous variables were compared using Mann-Whitney U tests. Spearman correlation coefficients were used to evaluated the correlations between non-parametric variables. Survival curves were plotted using the Kaplan-Meier method and compared using the log-rank test. Cox regression analyses were performed using the R “survival” package. The comparison of proportions between groups were completed by Fisher’s exact test and chi-square test. \( P < 0.05 \) was considered statistically significant.

**CONCLUSION**

The KIF9 gene and protein expression level in CRC tissues were significant higher compared to normal colorectal tissues. In addition, CRC patients with high KIF9 expression had better prognosis (OS, DFS, DSS, PFS) than patients with low KIF9 expression and KIF9 was an independent risk factor for CRC patients. CRC patients with high KIF9 expression were seemly associated with better response to 5-Fu and immune treatment. KIF9 may be a potential prognostic and 5-Fu related CRC-specific molecular marker.

**Abbreviations**

CRC
colorectal cancer
KIF9
Kinesin family member 9
5-Fu
5-Fluorouracil, AJCC: American Joint Committee on Cancer
MIS
microsatellite instability
Kinesin superfamily proteins
KIFs
GSEA
gene set enrichment analysis
GEO
Gene Expression Omnibus
TCGA
the Cancer Genome Atlas
IHC
immunohistochemistry
AOD
average optical density
HR
Hazard Ratio
CI
confidence interval
OS
overall survival
PFS
progression free survival
DSS
disease specific survival
DFS
disease free survival
RFS
recurrence free survival
BRAF
V-raf murine sarcoma viral oncogene homolog B1
KRAS
Kirsten rat sarcoma
VEGFR
vascular endothelial growth factor receptor
HLA
human leukocyte antigen
TMB
tumor mutation burden
CRT
chemoradiotherapy
GO
Gene Ontology
KEGG
Kyoto Encyclopedia of Genes and Genomes
TCPA
the Cancer Proteome Atlas
TIDE
Tumor immune dysfunction and exclusion
MSigDB
Molecular Signatures Database.

Declarations

Data availability

The data generated and/or analyzed during the current study are available from the corresponding author or first author on reasonable request.

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Author information

Authors and Affiliations

Anorectal Surgery Department, Fuyang People's Hospital, No. 501 Sanqing Road, Yingzhou District, Fuyang, Anhui 236000, China.

Zongxian Zhao, Xijie Fan, Zongju Hu, Shu Zhu, Shun Xu, Qinlingfei Liu, Yuan Yao, and Fusheng Wang

Department of Clinical Medicine, Bengbu Medical College, 233000, Bengbu, China.
Contributions

Z.Z. and H.M. participated in all experimental work, analyzed data, and contributed to both artwork design and manuscript writing; X.F., Z.H., Y.Y. conceived the work, analyzed data. S.Z., S.X., Q.L. and F.W. contributed to the revision, polishing and review of this manuscript and figures.

Ethics declarations

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Fuyang People’s Hospital.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Conflicting Interests

The authors declare no conflict of interest.

References


Figures

Figure 1

KIF9 expression levels analysis in CRC tissues and normal tissues. (A) KIF9 gene expression level in 488 CRC tissue samples and in 42 normal tissue samples from TCGA. (B) KIF9 gene expression level in 98 CRC samples, 98 adjacent normal samples and 50 healthy colon tissue samples from GSE44076. (C) KIF9 gene expression in 203 CRC tissue samples and 160 normal colorectal tissue samples from GSE87211. (D) KIF9 gene expression level (by using qRT-PCR) in tumor tissues and adjacent normal tissue samples from 20 CRC patients who underwent tumor resection at Fuyang People's Hospital. The average expression of KIF9 in the 20 normal tissue samples was regarded as a reference. (E) The protein expression of KIF9 in CRC tissues by IHC. (F) The protein expression of KIF9 in adjacent tissues by IHC. (F) The AOD value of KIF9 in 50 CRC tissues and 50 adjacent normal tissues from Fuyang People's Hospital. Asterisks (*) show a significant difference (Mann-Whitney two-sided test, *P<0.05, **P<0.01, ***P<0.001).
Figure 2

Univariate/multivariate Cox regression analysis and survival analysis of KIF9. (A) Univariate Cox regression analysis of CRC prognosis from TCGA. KIF9 expression level was associated with better prognosis (HR=0.509, P<0.01). (B) Multivariate Cox regression analysis from TCGA (KIF9, HR=0.628, P<0.05). (C) KIF9 expression in and overall survival from TCGA (HR=0.53, P=0.01). (D) KIF9 expression in and progression free survival from TCGA (HR=0.68, P<0.05). (E) KIF9 expression in and disease specific survival from TCGA (HR=0.53, P<0.05). (F) KIF9 expression in and disease-free survival from GSE24551 (HR=0.47, P<0.01). (G) KIF9 expression in and overall survival from K-M plotter (HR=0.7, P<0.05). (H) KIF9 expression in and recurrence free survival from K-M plotter (HR=0.2, P<0.01)
Figure 3

Correlation analysis between KIF9 gene expression level and tumor subtypes. (A) KIF9 gene expression level in BRAF^{V600E} mutation type and wild type from GSE103479. (B) KIF9 gene expression level in KRAS mutation type and wild type from GSE103479. (C) KIF9 gene expression level in MSI-H group and MSI-L/MSS group from GSE24551. (D) KIF9 gene expression level in P53 mutation type and wild type from GSE103479. (E) Correlation analysis of KIF9 and VEGFR gene expression level from TCGA. (F) Correlation analysis of KIF9 gene expression and VEGFR protein expression level from TCGA and TCPA. Asterisks (*) show a significant difference (Mann-Whitney two-sided test, *P<0.05, **P<0.01, ***P<0.001).
Figure 4

The relationship between KIF9 expression and immune microenvironment and predicting the response to immunotherapy. (A) 22 types immune cells between KIF9 high expression group and KIF9 low expression group. (B-E) Immune cell scores, stromal scores, ESTIMATE scores and Tumor Purity between different KIF9 expression groups. (F) HLA genes expression level between different KIF9 expression groups. (G) The correlation analysis of KIF9 gene expression level and TIDE score. (H) KIF9 gene expression in TMB-high group and TMB-low group. Asterisks (*) show a significant difference (Mann-Whitney two-sided test, \( *P < 0.05, **P < 0.01, ***P < 0.001 \)).

Figure 5
KIF9 expression level in response group and no-response group after receiving 5-Fu based treatment. (A) KIF9 gene expression level between 5-Fu response group and no-response group from GSE87211. (B) from GSE19860. (C) from GSE153412. (D) The correlation analysis of KIF9 gene expression level and drug sensitivity score of 5-Fu. (E) The AOD value of KIF9 between 5-Fu response group and no-response group by IHC. Asterisks (*) show a significant difference (Mann-Whitney two-sided test, *P<0.05).

Figure 6

Enrichment plots from the gene set enrichment analysis (GSEA). (A) GO enrichment analysis, up-regulated biological function. (B) GO analysis, down-regulated biological function. (C) KEGG enrichment analysis, up-regulated pathways. (D) KEGG analysis, down-regulated pathways.