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Research article

Discovery, identification and mechanism of chemosensitivity-relate biomarker inter-α-trypsin inhibitor heavy chain 4 in metastatic colorectal cancer

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ABSTRACT

Predictive biomarkers of response to chemotherapy in patients with metastatic colorectal cancer (mCRC) are needed to better characterize tumors and enable more tailored therapies. Here we used serum proteomics to screen for chemotherapy predictive markers. We found that higher baseline serum inter-α-trypsin inhibitor Heavy Chain 4 (ITIH4) expression in newly diagnosed mCRC patients was associated with poorer response to standard first-line chemotherapy. In addition, the higher expression of ITIH4 in CRC tissue also suggested poorer prognosis mCRC patients. Moreover, the overexpression of ITIH4 could promote the proliferation of CRC cells and reduce the sensitivity of CRC cells to 5-fluorouracil (5-FU) by inhibiting apoptosis in vivo and vitro. Through RNA-seq combined with bioinformatics analysis, we speculated that ITIH4 may activate phosphatidyl 3-kinase-protein kinase B (PI3K-AKT) pathway to inhibit apoptosis, thereby reducing the sensitivity of CRC cells to 5-FU. In conclusion, our findings unveil that ITIH4 is associated with CRC resistance to 5-FU, and may serve as a potential predictive biomarker for the sensitivity of advanced CRC patients to standard first-line chemotherapy regimens, and also provide a potential therapeutic target to render 5-FU resistance in CRC patients.

Abbreviations: 5-FU, 5-fluorouracil; ITIH4, inter-α-trypsin inhibitor heavy chain 4; MALDI-TOF-MS, Matrix-assisted laser desorption ionization-time of flight mass spectrometry; mCRC, metastatic colorectal cancer; PI3K-AKT, phosphatidyl 3-kinase-protein kinase B; LMWP, low-molecular-weight proteins; NPSMPs, nanoporous silicon microparticles; PD, progressive disease; PR, partial response; SD, stable disease.

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1. Introduction

Colorectal cancer (CRC) is one of the common digestive tract malignancies. Approximately 25 % of patients with CRC at the initial diagnosis are accompanied with metastasis, while 50 % will develop metastases at advanced stages [1]. Patients with metastatic CRC (mCRC) usually undergo multiple lines of systemic therapy, of which the first-line therapy usually lasts the longest, with objective response rates and disease control rates as high as 38–65 % and 81–90 %, respectively, and progression free survival (PFS) as long as 9–12 months [2]. First-line treatment options include oxaliplatin-based regimens (FOLFOX or CAPOX) or irinotecan-based regimens (FOLFIRI or CAPIRI). These regimens may be administered with or without additional targeted therapy [3]. Even so, 35–62 % of patients with mCRC do not respond well to standard first-line chemotherapy [2]. Therefore, ideal biomarkers are needed to help predict the response and adverse reactions of patients to chemotherapy regimens, so as to accurately identify the beneficiaries of specific treatments and improve treatment efficiency and safety.

For decades, numerous studies have attempted to explore biomarkers that can predict the efficacy of chemotherapy in CRC. These biomarkers mainly involve the following types: 1. key molecules involved in the anti-tumor mechanism of chemotherapeutic agents, for example, altered expression of repair effectors associated with oxaliplatin disruption of DNA replication, which affected the chemotherapy efficacy [4]; 2. enzymes related to the metabolism of chemotherapeutic agents, such as thymidylate synthase (TS) and dihydro pyrimidine dehydrogenase (DPD), which may be potential biomarkers for the efficacy of 5-fluorouracil (5-FU) [5–7]; 3. specific genetic mutations, e.g., patients with Kirsten rats arcomaviral oncogene homolog (KRAS) mutations may respond poorly to standard first-line chemotherapy [8]; 4. microRNAs associated with chemosensitivity [9,10]; 5. various immune cell subpopulations in the tumor microenvironment [11,12]. However, there are currently no established biomarkers that predict treatment response to standard first-line chemotherapy regimens in patients with mCRC.

Serum low-molecular-weight proteins (LMWP) are usually either intact small proteins which have been actively secreted by the cells or small fragments of larger proteins generated by cleavage, degradation, or other cellular processes. LMWP contains rich disease-related information and is a treasure trove of potential biomarkers [13–15]. However, traditional mass spectrometry (MS)-based proteomics analysis is often interfered by a large number of high molecular weight proteins or further degraded during sample collection, resulting in the loss of accurate information about LMWP [16]. The studies found that nanoporous silicon microparticles (NPSMPs) with specific pore sizes were able to capture, enrich, protect and detect LMWP sieved from a high-abundance plasma protein pool [17,18]. In this study, we used NPSMP combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) to screen for potential response-predicting protein markers in the serum of patients with advanced CRC who received standard first-line chemotherapy. The screened target protein was clinically validated, and its potential mechanism of affecting CRC chemotherapy resistance was preliminarily explored.

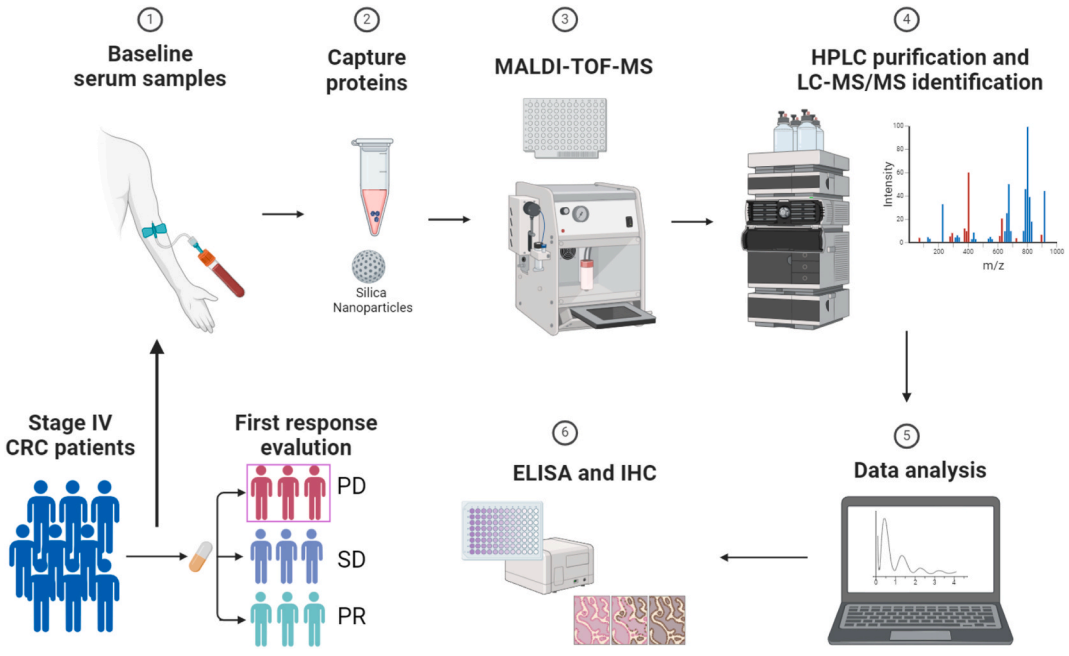

Fig. 1. The study design for screening and identification of efficacy-predictive LMWP biomarkers. Created with BioRender.com LMWP, low-molecular-weight proteins; MALDI-TOF-MS, Matrix-assisted laser desorption ionization-time of flight mass spectrometry; HPLC, high performance liquid chromatography.

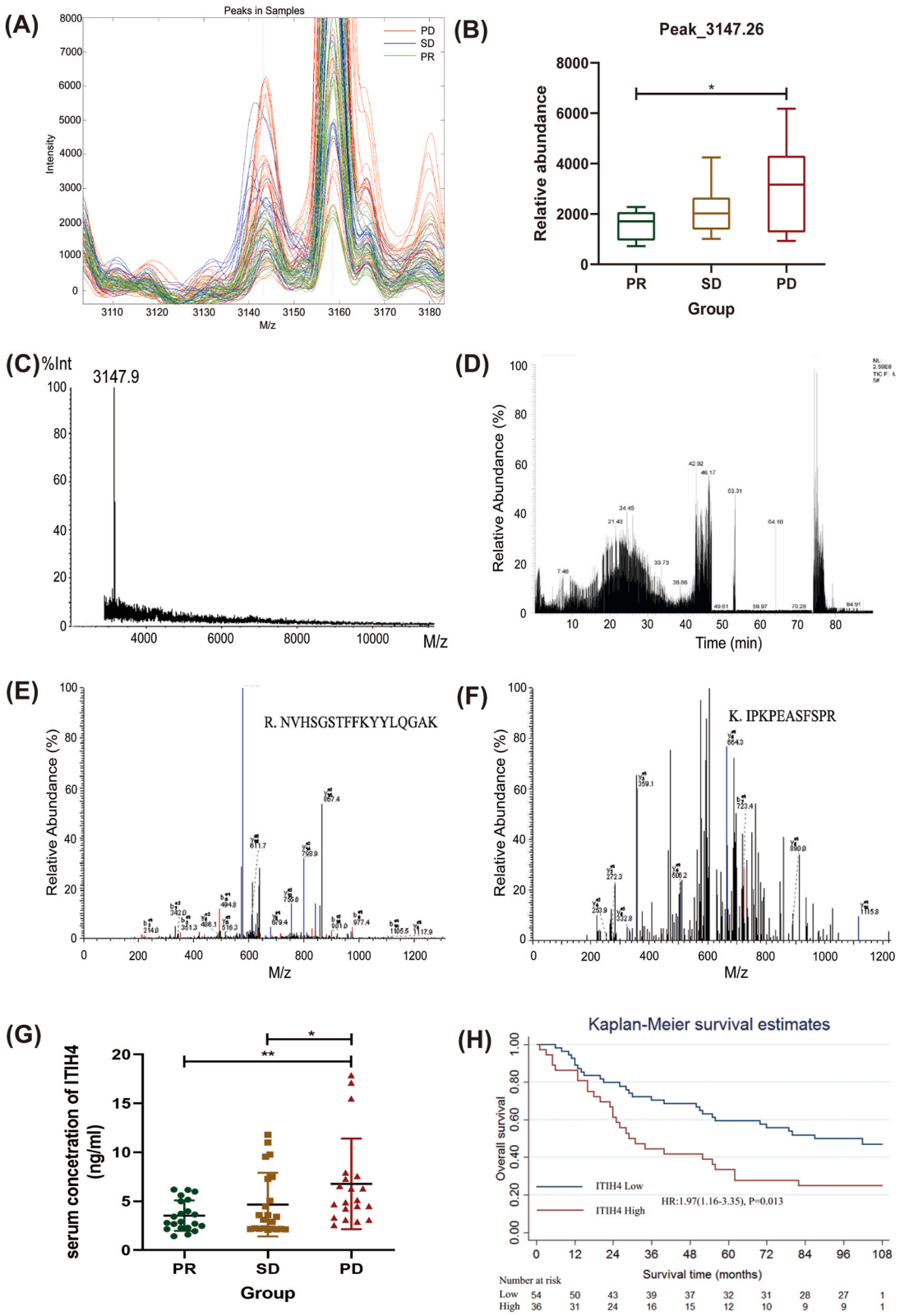

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Fig. 2. Screening and validation of serum chemotherapy efficacy-related markers in newly diagnosed stage IV CRC patients.
A and B. The abundance of peptides characterized by mass spectrometry peak 3147 was significantly increased in PD group; C. The peptides characterized by the mass spectrum peak 3147 were eluted by repeated HPLC separation; D. The peptide sequence was analyzed by LC-MS/MS tandem mass spectrometry; E and F. Two peptides were retrieved: R. NVHSGSTFFKYYLQGAK and K. IPKPEASFSPR. G. The baseline serum ITIH4 level in the PD group (6.77 ng/ml ± 4.52) was significantly higher than that in the PR group (3.53 ng/ml ± 1.52, $P < 0.01$) and SD group (4.35 ng/ml ± 3.18, $P < 0.05$) in stage IV CRC patients; H. Analysis of CRC tissue microarray showed that patients with high expression of ITIH4 in CRC tissue had shorter OS, $P = 0.013$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

2. Results

2.1. MALDI-TOF-MS screening of chemotherapy response related biomarkers for colorectal cancer

To initially screen for potential predictive markers of chemotherapy response, we included 27 patients with mCRC who were treated with standard first-line chemotherapy regimens, then underwent first efficacy evaluation and were classified as partial responses (PR, n = 9), stable disease (SD, n = 9) and progressive disease (PD, n = 9) groups (Table S1). Among them, 40.7 % received FOLFOX, 14.8 % received FOLFIRI, and 44.4 % received CAPOX (Table S2). Baseline blood samples were collected from these patients at the time of initial diagnosis of mCRC.

By analyzing the MALDI-TOF-MS data, a hierarchical clustering algorithm was performed on the high-weight mass spectral peaks. We found that the abundance of mass spectral peak 3147 was significantly increased in PD group compared with PR group and SD group (Fig. 2 A and B). After removing the high-abundance proteins in the serum, the peptides characterized by the mass spectrum peak 3147 were eluted in the high performance liquid chromatography (HPLC) elution time period of 45.10–45.21 min by repeated HPLC separation (Fig. 2 C). The peptide sequence was further analyzed by LC-MS/MS tandem mass spectrometry (Fig. 2 D), combined with the human protein database provided by NCBI, and two peptides were retrieved: R. NVHSGSTFFKYYLQGAK and K. IPKPEASFSPR were all derived from inter-α-trypsin inhibitor H4 (ITIH4) (Fig. 2 E and F). ITIH4 can be cleaved into fragments of different sizes by plasma kallikrein, so we analyzed other fragments of ITIH4 that may exist in the serum of patients by mass spectrometry. Regarding the serum peptide fragment sequences of ITIH4, we referred to data from published articles and identified 19 derived peptides from ITIH4 [19–21]. In addition to 3147, there are 3 peaks (2628, 2726 and 3274) having significantly higher abundance in PD group than that in PR group and SD group, and abundance of peak 3158 in PD group was significantly higher than that in PR group (Fig. S1 A-D), the sequences are shown in Table S3. Results suggested that patients with mCRC with high serum ITIH4 levels at baseline may be less effective after standard first-line chemotherapy.

2.2. High expression of ITIH4 in serum and tissue indicates poor prognosis of CRC patients

To verify whether ITIH4 is associated with chemotherapy efficacy in mCRC patients, we collected baseline serum samples from 63 newly diagnosed mCRC patients. The patient information is shown in Table S4. 42.9 % of patients received FOLFOX regimen, 36.5 % received FOLFIRI regimen, and 20.6 % received CAPOX regimen (Table S5). We detected the serum ITIH4 level of the validation cohort patients by Enzyme-linked immunosorbent assay (ELISA) and the results showed that the baseline serum ITIH4 level in the PD group was significantly higher than that in the PR group and SD group (Fig. 2 G). Therefore, higher baseline serum ITIH4 level is associated

Table 1
COX regression analysis of survival prognosis of 90 CRC patients.

Risk Factors	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P*	HR (95%CI)	*P*
Age				
Younger (≤70)	1			
Older (>70)	1.14(0.67–1.95)	0.619		
Sex				
Male	1			
Female	0.86(0.50–1.47)	0.578		
Grade				
I	1			
II	0.51(0.25–1.04)	0.064		
III	0.77(0.38–1.54)	0.455		
Size				
<5 cm	1			
> = 5 cm	0.87(0.51–1.49)	0.61		
Stage *				
I– II	1		1	
III-IV	2.32(1.35–4.00)	0.002	2.33(1.35–4.01)	0.002
ITH4				
Low	1		1	
High	1.97(1.16–3.35)	0.013	1.88(1.09–3.24)	0.022

with worse response to chemotherapy in stage IV CRC patients.

CRC tissue microarray was used to explore the relationship between tissue ITIH4 expression and CRC prognosis. Firstly, the correlation between tissue ITIH level and clinicopathological information of patients was analyzed, and the results showed that there was no difference between ITIH4 level and age, gender, pathological grade, tumor size, and tumor stage (Table S6). Univariate COX analysis indicated that later tumor stage and higher ITIH4 level had significant adverse effects on overall survival (OS), other factors

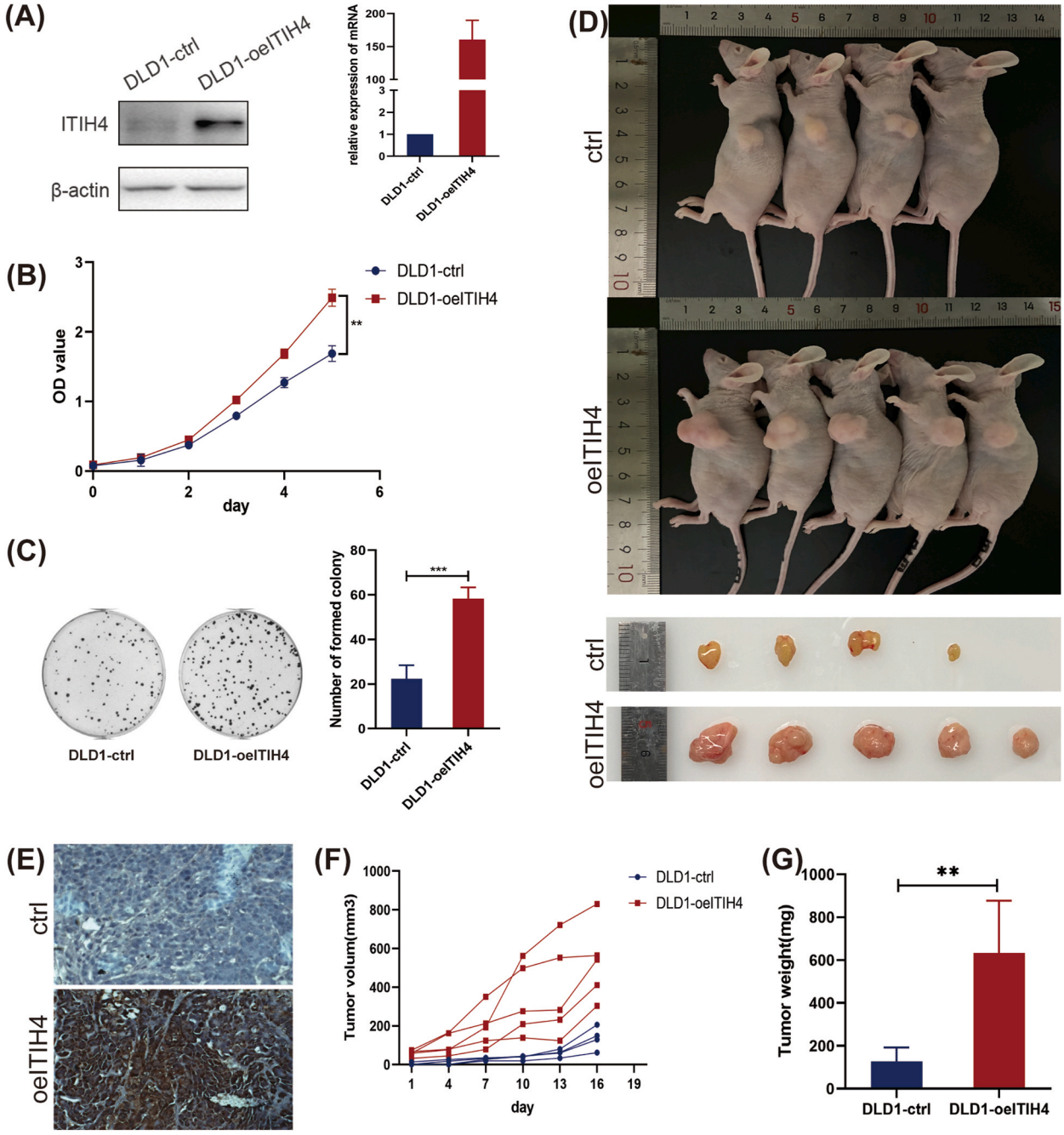

Fig. 3. Overexpression of ITIH4 promoted CRC cell proliferation A. WB and qPCR of ITIH4 in the DLD1 cell line transfected with overexpressing plasmids (Uncropped blots of WB were shown in Fig. S4); B. ITIH4 overexpression promotes the proliferative ability of DLD1 cell lines; C. ITIH4 overexpression promotes the clonogenic ability of DLD1 cell lines; D. Nude mouse subcutaneous tumorigenesis model and corresponding subcutaneous tumor anatomy, the mice were administrated with intraperitoneal injection of PBS; E. The expression levels of ITIH4 in tumors; F and G. ITIH4 overexpression significantly increased the volume (oeITIH4 vs ctrl: 530.6 mm^3 ± 177.0 vs 137.0 ± 51.45 mm^3, $P = 0.007$) and weight (oeITIH4 vs ctrl: 634.5 mg ± 217.8 vs 127.4 mg ± 56.64, $P = 0.005$) of subcutaneous tumors. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

had no significant effect on OS. Multivariate analysis showed that ITIH4 was independently associated with poor prognosis (Table 1). Finally, Kaplan-Meier survival analysis of 90 CRC patients grouped according to tissue ITIH4 expression also showed that high levels of tissue ITIH4 expression were significantly associated with shorter OS, $P = 0.013$ (Fig. 2 H). Analysis of ITIH4 expression levels and patient clinical information in CRC tissues from the TCGA database yielded consistent results (Fig. S1 E and F). In conclusion, high expression of ITIH4 in CRC tissue indicates poor prognosis of patients.

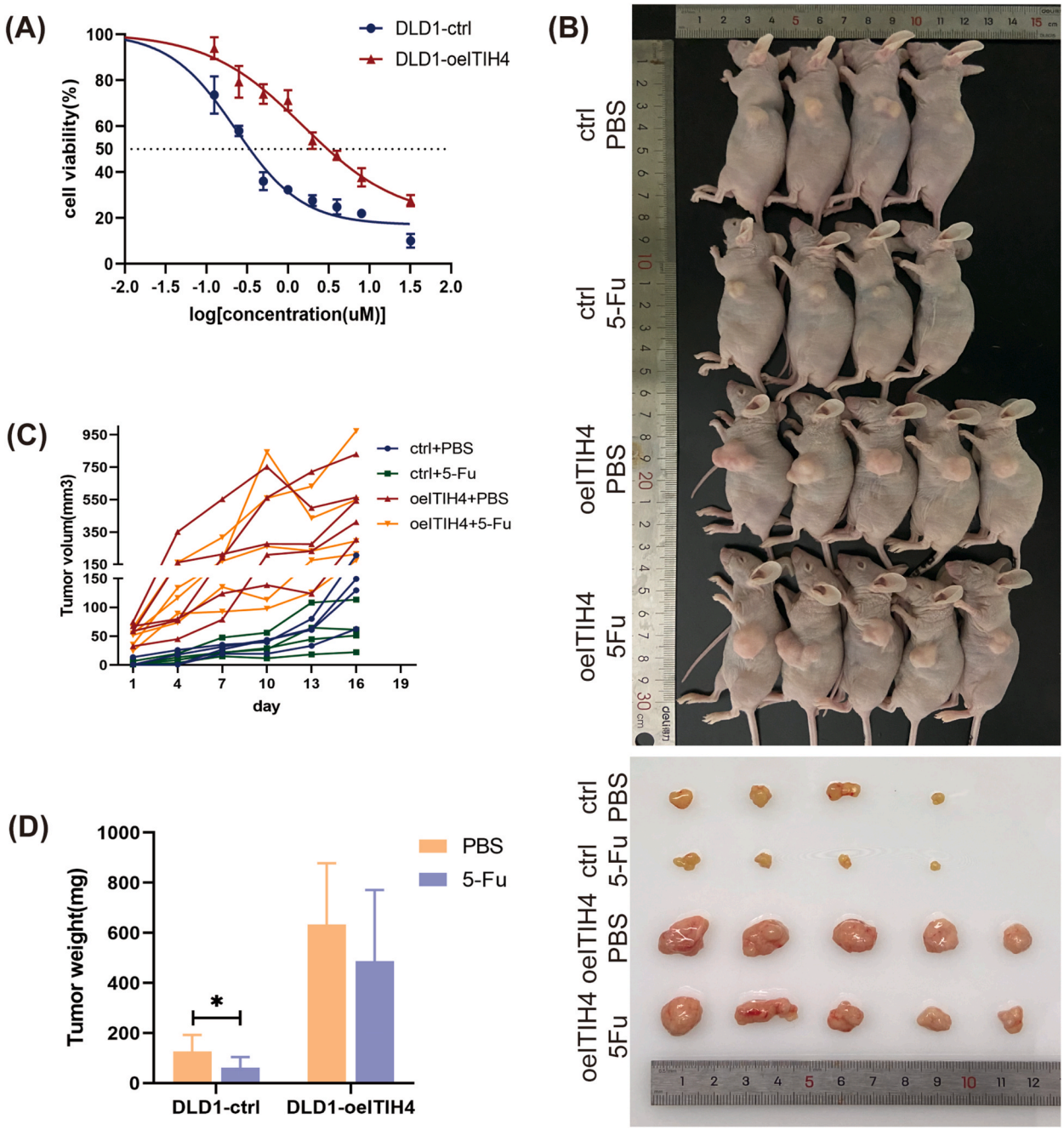

Fig. 4. ITIH4 reduces the sensitivity of CRC cell lines to 5-FU in vivo and in vitro A. ITIH4 increased the 5-FU IC50 of DLD1 cell lines in vitro; B. Nude mouse subcutaneous tumor model and corresponding subcutaneous tumor anatomy; C and D. After 5-FU treatment, the tumor weight and tumor volume in the ctrl group were significantly reduced (volume: 5-Fu vs PBS: 62.02 $mm^3 \pm 32.91$ vs 137.0 $mm^3 \pm 51.45$, $P = 0.008$; weight: 5Fu vs PBS: 62.30 mg ± 36.75 vs 127.4 mg ± 56.64, $P = 0.034$), while there was no significant difference in the oe group (volume: 5-Fu vs PBS, 441.5 $mm^3 \pm 296.2$ vs 530.6 $mm^3 \pm 177.0$, $P = 0.477$; weight: 5-Fu vs PBS, 488.0 mg ± 253.4 vs 634.5 mg ± 217.8, $P = 0.052$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

2.3. Overexpression of ITIH4 promoted CRC cell proliferation

To investigate the little-known function of ITIH4 in the biological behavior of CRC cells, ITIH4 cDNA plasmids packaged in lentiviruses were used to overexpress ITIH4 gene expression in human CRC cell lines DLD1 and RKO. Efficiency was confirmed by qPCR and WB (Fig. 3 A and Fig. S2 A). It showed that overexpression of ITIH4 in DLD1 (Fig. 3 B) and RKO (Fig. S2 B) cells increased cell proliferation. Similar significant difference was found by colony-forming assay as well (Fig. 3 C and Fig. S2 C).

To further confirm our findings, we compared the growth of DLD1-oeITIH4 and DLD1-ctrl cells in a subcutaneously inoculated model. The expression levels of ITIH4 in tumors were detected by immunohistochemistry (Fig. 3 E). Three weeks after injection, subcutaneous tumors formed by DLD1-oeITIH4 were larger and heavier compared with those caused by DLD1-ctrl (Fig. 3 D, F and G). Taken together, these results indicated that ITIH4 could enhance the cell proliferation ability of CRC cells.

2.4. Overexpression of ITIH4 reduces the sensitivity of CRC cells to 5-FU by inhibiting apoptosis in vivo and in vitro

We further analyzed the effects of ITIH4 overexpression on the sensitivity of CRC cells towards 5-FU, a basic chemotherapeutic agent commonly used to treat CRC, respectively. Overexpressing ITIH4 increased the 5-FU half maximal inhibitory concentration (IC50) of DLD1 from 0.2279 μM to 1.530 μM (Fig. 4 A), and 5-FU IC50 of RKO from 1.022 μM to 3.131 μM (Fig. S2 D). Next, nude mice were subcutaneously injected with DLD1-oeITIH4 and DLD1-ctrl cells and received PBS or 5-FU (30 mg/kg every three days) intraperitoneally. 2 weeks later, in DLD1-ctrl tumorigenic nude mice, compared with the PBS group, the tumor volume and tumor weight in 5-FU group was significantly reduced (Fig. 4 B–D), which indicating that DLD1-ctrl-induced tumors remained sensitive to 5-FU. However, In DLD1-oeITIH4 group, neither tumor volume nor tumor weight showed statistical differences between 5-FU and PBS, suggesting that DLD1-oeITIH4-induced tumors are insensitive to 5-FU. Combining the results of in vivo and in vitro, it was shown that ITIH4 overexpression reduced the sensitivity of intestinal cancer cell lines to 5-FU, making them drug-resistant.

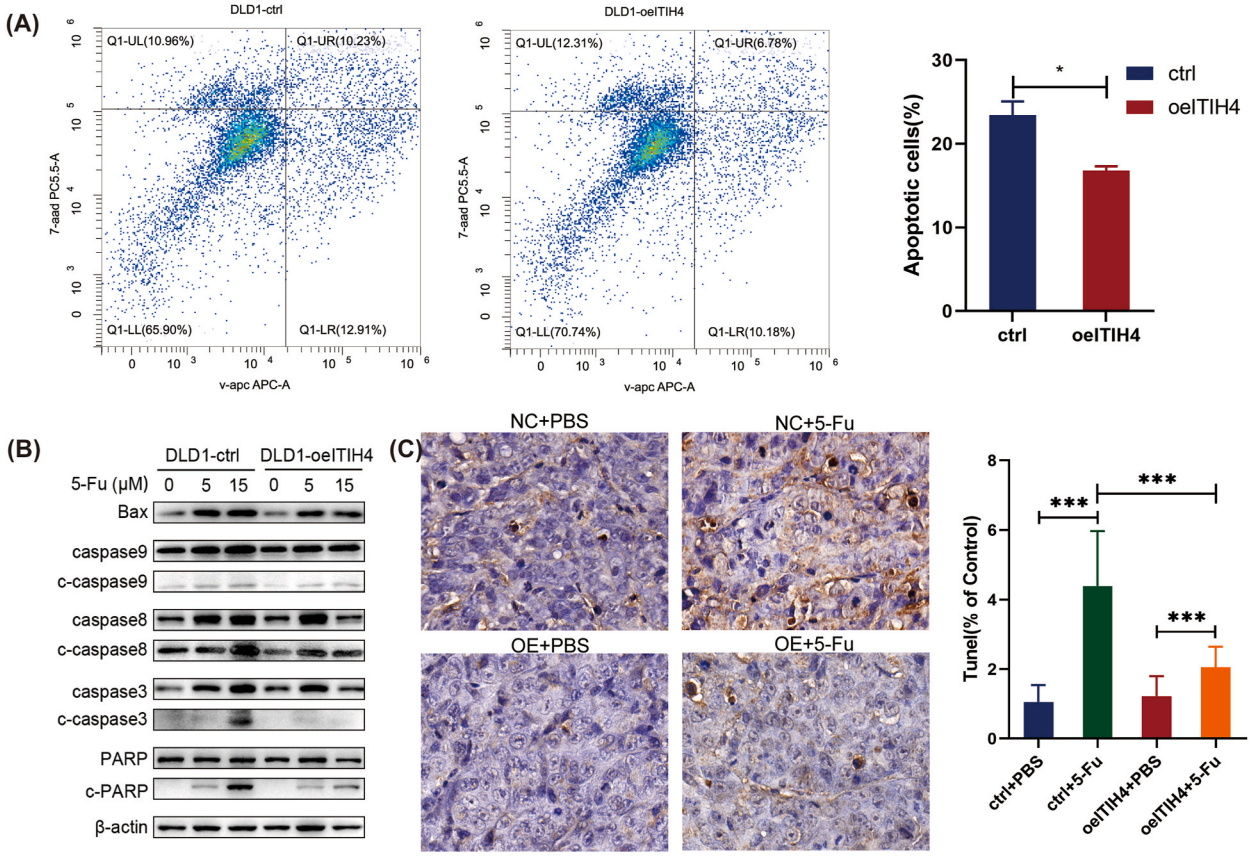

Fig. 5. In vitro and in vivo experiments showed reduced apoptosis in ITIH4-overexpressing CRC cell lines treated with 5-FU A. Flow cytometry showed that the proportion of apoptotic cells in DLD1-oeITIH4 decreased significantly after 48 h of treatment with the same concentration of 5-FU compared with the corresponding control group (DLD1-oeITIH4 vs DLD1-ctrl: 16.27 % vs 22.01 %, $P = 0.011$). B. WB results showed that after 48 h of apoptosis induction by 15 μM 5-FU, compared with the control group, caspase apoptotic signal was attenuated in DLD1-oeITIH4 cells, and the upstream pro-apoptotic protein Bax was inhibited (Uncropped blots of WB were shown in Fig. S5). C. Tunel staining results of subcutaneous tumors. After administration of 5-FU, the positive rate of tunel in DLD1-oeITIH4 tumors was significantly lower than that in DLD1-ctrl group (oe vs ctrl: 2.06 % vs 4.39 %, $P < 0.001$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

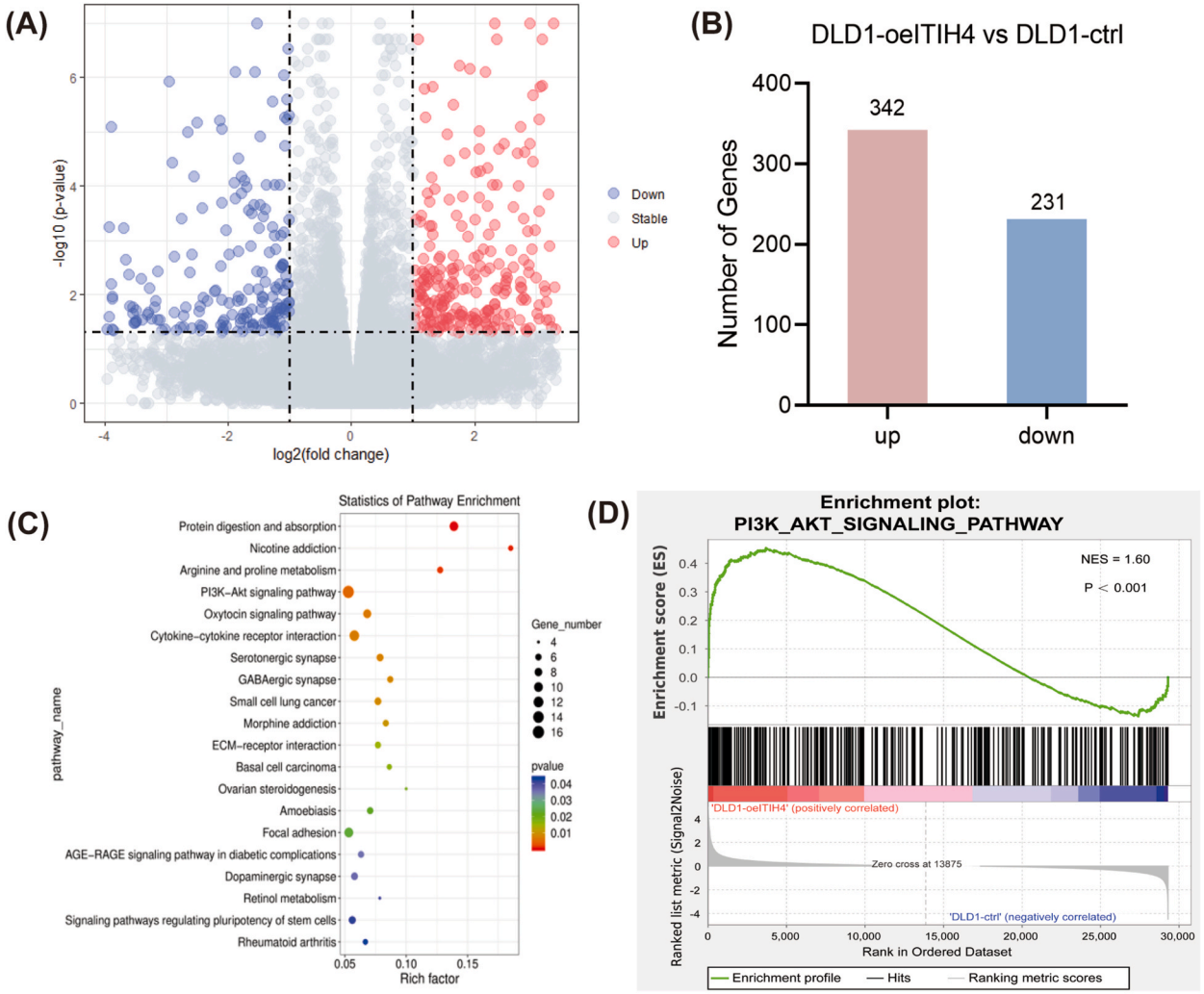

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Fig. 6. Pathway analysis of DLD1-oeITIH4 and DLD1-ctrl cells
A and B. Differentially expressed genes (DGE) including 342 up-regulated and 231 down-regulated genes (FC > 2 or < 0.5, $P < 0.05$); C. Bubble plot of KEGG enrichment analysis of DEGs affected by ITIH4 overexpression; D. GSEA analysis suggesting that the PI3K-AKT pathway is significantly associated with high ITIH4 expression (NES = 1.60, $P < 0.001$); E. PI3K-AKT pathway activity and apoptosis level in DLD1-oeITIH4 and DLD1-ctrl cells treated with 5-FU; F. After adding the PI3K inhibitor LY294002, 5-FU-induced apoptosis of DLD1-oeITIH4 cells increased; G. Co-IP experiments showed that ITIH4 interacts with AKT1 in CRC cells. Uncropped blots of WB were shown in Figs. S6–8.

Moreover, we examined the apoptosis of CRC cells and subcutaneous tumor tissues treated with 5-FU. The apoptosis of CRC cells after 48 h of 5-FU treatment was detected by flow cytometry, and compared with the control group, the proportion of apoptotic cells in DLD1-oeITIH4 was significantly decreased (Fig. 5 A). We also detected the expression of caspase pathway in the cells. Compared with the control, the activation of effector Caspase3 in DLD1-oeITIH4 cells was reduced, and the cleavage of its substrate poly ADP-ribose polymerase (PARP) was correspondingly reduced. In addition, the upstream pro-apoptotic protein Bax and the promoter Caspase8 were inhibited (Fig. 5 B). Then we detected the apoptosis of subcutaneous tumor tissue in nude mice by TUNEL staining. After intraperitoneal administration of 5-FU, the positive rate of TUNEL in DLD1-oeITIH4 tumors was significantly lower than that in DLD1-ctrl group (Fig. 5 C). In conclusion, ITIH4 may reduce the sensitivity of CRC cells to 5-FU by inhibiting apoptosis, thereby resulting in drug resistance.

2.5. PI3K-AKT pathway is positively related to ITIH4

We detected and analyzed the transcriptomes of DLD1-oeITIH4 and DLD1-ctrl cells using RNA-seq. 29,636 genes were quantified, and differentially expressed genes (DEG) included 342 up-regulated and 231 down-regulated genes (Fig. 6 A and B). KEGG enrichment analysis showed that DEG was mainly enriched in protein digestion and absorption, Arginine, and proline metabolism, phosphatidyl 3-kinase - protein kinase B (PI3K-AKT) signaling pathway and other pathways (Fig. 6 C). Subsequent KEGG enrichment analysis of the gene set by GSEA revealed that the PI3K-AKT signaling pathway was significantly associated with ITIH4 overexpression (Fig. 6 D). Next, we verified some genes that were significantly different in the PI3K-AKT pathway, and the results showed that the mRNA levels of AKT3, cyclin-dependent kinase inhibitor 1A (CDKN1A), Collagen Type VI Alpha 1 Chain (COL6A1), and nerve growth factor receptor (NGFR) were significantly up-regulated in DLD1-oeITIH4 cells, while ITGB6 was significantly down-regulated in DLD1-oeITIH4 cells (Fig. S3).

2.6. ITIH4 inhibits 5-FU-induced apoptosis by activating PI3K-AKT pathway

The PI3K-AKT signaling pathway is a classic anti-apoptotic pathway, and several studies have shown that the PI3K-AKT pathway is closely related to the chemoresistance of CRC to 5-FU [22–24]. Combined with the results of RNA-seq, we speculated that the effect of ITIH4 on 5-FU-induced apoptosis may be related to the PI3K-AKT pathway. After 5-FU treatment, the levels of phosphorylated PI3K (p-PI3K) and phosphorylated AKT (*p*-AKT) in DLD1-oeITIH4 cells were significantly up-regulated, indicating that the PI3K-AKT pathway was activated, meanwhile, the apoptosis of DLD1-oeITIH4 cells was inhibited (Fig. 6 E). Subsequently, we used different concentrations of the PI3K inhibitor LY294002 to co-treat DLD1-oeITIH4 cells with 5-FU for 48 h. We observed a gradual increase in cellular apoptosis as the PI3K-AKT pathway was progressively inhibited (Fig. 6 F). In addition, using the Gene MANIA database and the STRING database to analyze the interaction genes of ITIH4, it was found that ITIH4 may interact with AKT1, which was preliminarily verified by Co-Immunoprecipitation (Co-IP) experiments (Fig. 6 G). Taken together, ITIH4 may inhibit 5-FU-induced apoptosis by activating the PI3K-AKT pathway.

3. Discussion

Low-molecular-weight peptides in serum represent a rich source of untapped disease-specific information. Several studies have shown that LMWP profiling by MALDI-TOF-MS combined with bioinformatics can be used to develop diagnostic and therapeutic relevant biomarkers for tumors. Rovithi et al. performed serum peptide analysis using MALDI-TOF-MS on NCSLC patients participating in phase II trials of erlotinib and sorafenib, and created a six-peptide and a combined 13-peptide signature for predicting efficacy and toxicity [25]. Most mass spectrometry studies use various methods to deplete the high abundance protein in order to avoid the LWMP signal being masked by the high abundance protein in the serum [26–28]. The captured LMWP is then eluted from the substrate for mass spectrometric detection. The elution step not only complicates the analytical process, but also reduces the concentration of the analytes [26], especially since some specific peptides may be difficult to elute, resulting in a peptide spectrum that is not fully representative of the true spectrum in serum. Moreover, it has been reported that serum proteins may be degraded very quickly by proteases present in the serum after the blood sample is collected [29]. The NPSMP used in this study control the pore size and surface chemistry of silica particles by electrochemical preparation conditions to specifically fractionate, enrich and protect LMWP sieved from human serum samples and spotted directly onto MALDI plates for detection [17,29]. NPSMP therefore avoids the interference of high protein abundance, elution steps, proteases and other factors, enabling high-fidelity and convenient mass spectral analysis.

Following in vitro and in vivo studies demonstrated that ITIH4 promoted proliferation and 5-FU resistance in CRC cells. Similar to our results, several studies have shown that ITIH4 may have an important role in the development of CRC. Ivancic et al. found that serum ITIH4 levels in $Apc^{Pirc/+}$ rats increased with the number of colonic adenomas [30], and then this team further followed up

patients with colonic adenomas longitudinally and found that compared to healthy controls, serum ITIH4 levels were significantly higher in patients with growing adenomas, which are likely to become high-risk adenomas that then develop into CRC [31]. In addition, Kopylov et al. found that serum ITIH4 was significantly upregulated in patients with early CRC compared to healthy controls [32]. All these results suggest a close association between ITIH4 upregulation and the development of CRC.

This study is the first to investigate the role of ITIH4 in 5-FU sensitivity among mCRC patients. We provided evidence that the process of cancer cell survival under chemotherapeutic treatment is mediated by functional interplay between ITIH4 and PI3K-AKT pathway. We found that the PI3K-AKT pathway was activated in CRC cells overexpressing ITIH4, which consequently made cancer cells become resistant to 5-FU-induced cell death. Phosphorylation of AKT is associated with cell proliferation and anti-apoptotic capacity in CRC. Several studies have found that *p*-AKT inhibits the transcription of several pro-apoptotic genes, such as insulin-like growth factor binding protein 1 (IGFBP-1), Fas ligand (FasL), procaspase-9, and other genes [33,34]. Wang et al. found that CRC patients with PI3K catalytic subunit alpha (PIK3CA) mutations responded poorly to first-line chemotherapy, for which the main mechanism was that *PIK3CA* mutations induced sustained activation of PI3K-AKT signaling, which promoted survival and proliferation of CRC stem cells, thereby inducing chemoresistance [22]. Phosphatase and tensin homolog (PTEN) deletion similarly leads to chemoresistance in CRC cells through activation of the PI3K-AKT pathway [35]. We established a novel link between ITIH4 and PI3K-AKT pathway and ITIH4/PI3K-AKT pathway-induced survival of cancer cells upon 5-FU exposure, which is the first time to be reported.

There remain several limitations of this study. Initially, the serum sample size in our study is very limited and needs further improvement and validation by sample addition. Second, this study lacked treatment targeting ITIH4 and did not explore the possibility of reversing 5-FU resistance by inhibiting ITIH4 expression because the relative low expression of ITIH4 in CRC cell lines. Third, transcriptome analysis did not use cell samples after 5-Fu induction to better characterize the altered transcriptome of CRC cells exposed to 5-Fu toxicity. Finally, the pathway through which ITIH4 activates the PI3K-AKT pathway and thus regulates apoptosis has not been explained.

In conclusion, our data obtained from cancer patients' serum showed that mCRC patients with significantly upregulated ITIH4 levels tend to respond poorly to standard first-line chemotherapy. Furthermore, high ITIH4 in CRC tissue is related to poor overall survival. Overexpression of ITIH4 inhibits apoptosis in CRC cells, thereby enhancing their chemoresistance to 5-Fu. We found that the functional link between ITIH4 and chemoresistance may be related to the PI3K-AKT pathway. Therefore, this study not only substantiates the use of ITIH4 as biomarkers of CRC, but also suggests further exploration of ITIH4 as a target for CRC therapy.

4. Material and methods

4.1. Patients

The filtering route for efficacy-predictive LMWP biomarkers in this study is shown in Fig. 1. This study was approved by the Institutional Review Board of Medical Research of The Second Affiliated Hospital, Zhejiang University School of Medicine, and all experiments were carried out in accordance with the approved guidelines. Baseline serum samples from 90 newly diagnosed stage IV CRC patients admitted to the Second Affiliated Hospital of Zhejiang University School of Medicine were collected, and the baseline period was defined as the period from the initial diagnosis to before any intervention. All cases were diagnosed by pathological confirmation of primary or metastatic lesions, and clinical staging was based on imaging findings including computerized tomography (CT) and magnetic resonance imaging (MRI). All patients subsequently received standard first-line chemotherapy regimens, including: (1) FOLFOX: 5-FU + leucovorin + oxaliplatin; (2) FOLFIRI: 5-FU + leucovorin + irinotecan; (3) CAPOX: capecitabine + oxaliplatin. The first response evaluation was performed by CT or MRI after two cycles of chemotherapy with reference to the standard Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. CRC patients were divided into three groups: (1) partial response (PR); (2) stable disease (SD); and (3) progressive disease (PD). The collection and processing of samples were obtained with the informed consent of the patients and signed written informed consent. The characteristics of patients can be seen in Table S1 and Table S4.

All blood samples were collected in the early morning when the patients were fasting. 5 ml of peripheral blood was collected in a common blood collection tube without anticoagulant at room temperature, incubated for 1 or 2 h, and centrifuged at 2000 rpm for 5–10 min. The supernatant was stored at −80 °C in aliquots of 20–50 μl.

4.2. Detection of peptide profiles by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

Nanoporous silicon microparticles (NPSMPs) (Bio-pSi, Well-healthcare, Hangzhou, China) were used for the Serum peptide and Proteins Captured. Preprocessing steps followed manufacturer's protocol. The pretreated serum samples were detected by MALDI-TOF-MS (ClinMS-Plat I, Well-healthcare, Hangzhou, China), and analyzed by ClinMS Controller (Well-healthcare, Hangzhou, China) and PDAS HJ Cloud (Well-healthcare, Hangzhou, China). Please refer to the Supplementary Methods section for additional details on purification and Identification of candidate protein biomarker.

4.3. Enzyme-linked immunosorbent assay (ELISA)

ELISA tests were used to quantify the protein concentration of ITIH4 in serum samples. Details of the process was shown in the Supplementary Methods section.

4.4. Tissue microarray immunohistochemistry

A tissue microarray (TMA) of 90 primary CRC specimens were purchased from Shanghai Biochip Co. We performed immunohistochemical staining for anti-ITIH4 antibody (1:250 dilution, Abcam 180 139, Cambridge, UK) (Supplementary Methods section). For analysis of ITIH4 transcripts in CRC microarray studies, a Kaplan-Meier curve for overall survival was created using Kaplan-Meier curve with patients grouped according to high and low expression of Trip10. Hazard Ratio (with 95 % confidence interval) and log rank *P* values were calculated and displayed on the graph.

4.5. Cell proliferation, apoptosis and 5-FU sensitivity

Human CRC cell lines overexpressing target protein were constructed by lentiviral vectors. The effect of the target protein on the phenotype and sensitivity to 5-FU of CRC cells in vivo and in vitro was studied through cell function experiments and subcutaneous tumorigenic models in nude mice. Please refer to the Supplementary Methods section for additional details on lentivirus infection and establishment of the overexpression cell lines, quantitative real-time PCR (qPCR), Western blot (WB), cell proliferation assay and 5-FU cytotoxicity, apoptosis, animal and subcutaneously inoculated model, TUNEL assays and Co-Immunoprecipitation (Co-IP).

4.6. RNA sequencing (RNA-seq)

Cells were harvested for total RNA extraction using Trizol reagent (Thermo, 15596018, Massachusetts, USA). Subsequently, mRNA purification and reverse transcription were performed for the construction of cDNA libraries. The sequencing was performed on an Illumina Novaseq™ 6000 (LC-Bio, Hangzhou, China) following the vendor's recommended protocol. The data was analyzed according to the TopHat-HTSeq-DeSeq2 frame. Differential analyses were performed using DESeq2 packages. Differentially expressed genes were then subjected to enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Moreover, gene set enrichment analysis was performed using software Gene Set Enrichment Analysis (GSEA) (v4.1.0).

Statistics

Statistics and graphing of data were performed using GraphPad Prism 9.0 and SPSS 25.0. Cell counting was performed with Image J. Paired samples were compared using a two-tailed paired *t*-test, and unpaired samples were compared using a two-tailed Student's t-test. One-way ANOVA was used to compare variables between multiple groups. Survival curves were drawn by Kaplan-Meier analysis and compared by log-rank test. The evaluation of risk factors was analyzed using univariate and multivariate COX regression models. $P < 0.05$ was considered statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Ethics statement

This human subjects in this study were reviewed and approved by the Institutional Review Board of Medical Research of The Second Affiliated Hospital, Zhejiang University School of Medicine with the approval number: 2019–262, dated 29 July 2019.

All animal experiments in this study were reviewed and approved by the Second Affiliated Hospital of Zhejiang University School of Medicine Animal Care and Use Committee with the approval number: 2024–043, dated 19 March 2024.

Data availability statement

The data associated with this study has not been deposited into a publicly available repository. The data used and/or analyzed during this study are available from the corresponding author on reasonable request.

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Conflict of interest disclosure

The authors have no conflict of interest.

CRediT authorship contribution statement

Yingxin Zhao: Writing – original draft, Visualization, Validation, Methodology, Data curation. **Hong Shen:** Supervision, Project administration, Investigation, Formal analysis. **Jianmin Wu:** Software, Resources, Methodology. **Jiekai Yu:** Writing – review & editing, Supervision, Software, Methodology, Data curation, Conceptualization. **Ying Yuan:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Chenhan Zhong:** Writing – review & editing, Writing – original draft,

Validation, Supervision, Methodology, Funding acquisition, Data curation.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33571.

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