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REVIEW

Developments and challenges in neoadjuvant therapy for locally advanced pancreatic cancer

Bo Zhou, Shi-Ran Zhang, Geng Chen, Ping Chen

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) remains a significant public health challenge and is currently the fourth leading cause of cancer-related mortality in developed countries. Despite advances in cancer treatment, the 5-year survival rate for patients with PDAC remains less than 5%. In recent years, neoadjuvant therapy (NAT) has emerged as a promising treatment option for many cancer types, including locally advanced PDAC, with the potential to improve patient outcomes. To analyze the role of NAT in the setting of locally advanced PDAC over the past decade, a systematic literature search was conducted using PubMed and Web of Science. The results suggest that NAT may reduce the local mass size, promote tumor downstaging, and increase the likelihood of resection. These findings are supported by the latest evidence-based medical literature and the clinical experience of our center. Despite the potential benefits of NAT, there are still challenges that need to be addressed. One such challenge is the lack of consensus on the optimal timing and duration of NAT. Improved criteria for patient selection are needed to further identify PDAC patients likely to respond to NAT. In conclusion, NAT has emerged as a promising treatment option for locally advanced PDAC. However, further research is needed to optimize its use and to better understand the role of NAT in the management of this challenging disease. With continued advances in cancer treatment, there is hope of improving the outcomes of patients with PDAC in the future.

Key Words: Neoadjuvant therapy; Pancreatic ductal adenocarcinoma; Locally advanced pancreatic cancer; Chemoradiotherapy; Immunotherapy; Vaccine therapy

Core Tip: In recent years, neoadjuvant therapy (NAT) has emerged as a promising treatment option for many cancer types, including locally advanced pancreatic ductal adenocarcinoma, with the potential to improve patient outcomes. To analyze the role of NAT in the setting of locally advanced pancreatic ductal adenocarcinoma over the past decade, a systematic literature search was conducted using PubMed and Web of Science. Despite the potential benefits of NAT, there are still challenges that need to be addressed. Additionally, there is a need for better patient selection criteria to identify those who are most likely to benefit from this approach.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a malignancy with a poor prognosis, and it is currently the seventh leading cause of cancer-related death worldwide. The number of deaths from PDAC (466000) is almost equal to the number of new cases (496000) each year[1-3]. Experts predict that PDAC will surpass breast cancer as the third leading cause of cancer-related deaths by 2025 in 28 European countries[4]. The 5-year survival rate for patients with PDAC is less than 5%, and locally advanced pancreatic cancer (LAPC) accounts for one-third of all pancreatic cancer cases[5-7]. Unfortunately, approximately 60% of patients with LAPC present with metastatic disease and/or poor performance status, making them ineligible for surgery[8,9]. Despite improvements in diagnosis and treatment, clinical outcomes for these patients remain poor.

In 1992, Evans *et al*[10] first proposed the use of neoadjuvant therapy (NAT) for PDAC and found that patients treated with NAT had superior outcomes compared to those treated with postsurgical adjuvant therapy[11,12]. NAT is administered before surgery to reduce tumor mass, promote tumor downstaging, or eliminate early metastatic cells, thereby improving prognosis. In the last decade, the strategy of NAT followed by conversion surgery has been increasingly employed in the treatment of LAPC[13]. The goal of this review was to summarize and discuss research exploring the use of NAT for LAPC.

DEFINITIONS OF LAPC

Pancreatic cancer is typically classified based on its resectability on preoperative imaging, according to guidelines such as the National Comprehensive Cancer Network 2022 edition, the Chinese guidelines for the Neoadjuvant Therapy of Pancreatic Cancer 2021 edition, and the International Association of Pancreatology[5,14,15]. LAPC is generally defined as local tumor growth with major involvement (> 180° circumferential) or true invasion of the superior mesenteric artery, celiac axis, or hepatic artery and/or involvement of the portal vein/superior mesenteric vein that prevents reconstruction [16]. Table 1 provides a summary of these criteria.

CHEMORADIOTHERAPY FOR LAPC

LAPC, previously thought to be an incurable disease stage and an indication for palliative treatment, is now strongly considered a potentially curable disease. Gemcitabine (GEM)-based chemoradiotherapy is the standard treatment for LAPC, with a reported median survival of 24.2 mo and progression-free survival of 15 mo[17,18]. Polychemotherapy treatment with combined leucovorin calcium (folinic acid), fluorouracil, irinotecan hydrochloride, and oxaliplatin (FOLFIRINOX) and GEM + nab-paclitaxel is recommended[19].

In a database query of patients who received induction FOLFIRINOX for LAPC between 2010 and 2016, nearly 20% responded sufficiently to undergo resection, which improved overall survival compared to that of patients who did not undergo resection[20]. Another study enrolled 485 patients with at least three cycles of first-line chemotherapy with FOLFIRINOX or GEM plus nanoparticle albumin-bound paclitaxel (GA) between 2010 and 2017 and revealed that according to the Response Evaluation Criteria in Solid Tumors a partial response was more common among patients treated with FOLFIRINOX [27 of 140 patients (19%)] than among those treated with GA [8 of 140 patients (6%); $P = 0.001$]. In this cohort of patients, FOLFIRINOX was associated with higher rates of Response Evaluation Criteria in Solid Tumors-defined partial response and subsequent pancreatectomy than GA[21]. The different types of trials evaluating NAT for LAPC in recent years are summarized in Table 2[19,22-25].

Before the administration of chemotherapy, patients diagnosed with LAPC should undergo a thorough preliminary assessment of their performance status based on the Eastern Cooperative Oncology Group (ECOG) score[26]. In addition, it is imperative to evaluate nutritional status, symptom burden, and active comorbidities (with appropriate adjustments made for treatment as warranted) and to assess biliary tract patency while considering the need for diversion or stent

Table 1 Definitions of locally advanced pancreatic cancer by different groups

Group	Definition	
	Arterial	Venous
NCCN [14]	Head/uncinate process: Solid tumor contact > 180° with the SMA or CA Body and tail Solid tumor contact of > 180° with the SMA or CA Solid tumor contact with the CA and aortic involvement	Unreconstructible SMV/PV due to tumor involvement or occlusion (can be due to tumor or bland thrombus)
IAP[15]	Tumor contact/invasion of 180 or more degree CHA Tumor contact/invasion showing tumor contact/invasion of the PHA and/or CA	Bilateral narrowing/occlusion, exceeding the inferior border of the duodenum
CMA[5]	Head Solid tumor contact of > 180° with the SMA or CA Body and tail Solid tumor contact of > 180° with the SMA or CA Solid tumor contact with the CA and aortic involvement	Unreconstructible SMV/PV due to tumor involvement or occlusion (can be due to tumor or bland thrombus). The tumor extensively involves the distal jejunal drainage branch of the superior mesenteric vein

CA: Celiac axis; CHA: Common hepatic artery; CMA: Chinese Medical Association; IAP: International Association of Pancreatology; NCCN: National Comprehensive Cancer Network; PHA: Proper hepatic artery; PV: Portal vein; SMA: Superior mesenteric artery; SMV: Superior mesenteric vein.

Table 2 Summary of trials evaluating neoadjuvant therapy for locally advanced pancreatic cancer

Ref.	Type of study	Years of accrual	No. of patients	Primary endpoint	Arms	Key findings
NCT03652428[19]	Phase 1; Phase 2	2019-2023	24	12 mo after registration or until death	Proton therapy with concurrent GEM + Nab-paclitaxel	Ongoing
NCT02578732[22]	Phase 2	2016-2024	28	Until progression, up to 5 yr	FOLFOX	Ongoing
NCT04247165[23]	Phase 1; Phase 2	2020-2024	20	12 mo	Drug: GEM; Nab-paclitaxel; Nivolumab; Ipilimumab	Ongoing
NCT02873598[24]	Phase 1	2016-2021	15	Up to 5 yr	FOLFIRINOX or GEM/abraxane followed by SBRT	Not yet publicly available
NCT02704143[25]	Phase 2	2016-2020	63	3 yr	Combination of Cyberknife with S-1	Promising efficacy

FOLFOX: Folinic acid, fluorouracil, and oxaliplatin; FOLFIRINOX: Folinic acid, fluorouracil, irinotecan hydrochloride, and oxaliplatin; GEM: Gemcitabine; S-1: Tegafur, gimeracil, and oteracil; SBRT: Stereotactic body radiation therapy.

placement. Geriatric assessment is recommended for patients who are aged 70 years and above[27]. For those patients exhibiting a good performance status (ECOG score between 0-1) along with good nutritional health, first-line chemotherapy is advised, similar to the approach for patients diagnosed with metastatic liver cancer. In contrast, for patients with higher ECOG scores, a GEM-based regimen is preferred due to its lower toxicity profile[28].

The use of stereotactic body radiation therapy (SBRT) with adjuvant chemotherapy in the treatment of LAPC has been a subject of interest among oncologists. While conventional fractionated radiation has been the standard approach, studies have explored the potential of SBRT in downstaging LAPC[29]. One recent study examined the efficacy of sequential SBRT following FOLFIRINOX chemotherapy in patients with stable but unresectable LAPC. The study authors reviewed medical records from 50 patients who were treated with induction FOLFIRINOX for a median of eight cycles, followed by SBRT. The median overall survival and progression-free survival were reported as 26.4 mo (95% confidence

interval: 22.4-30.3) and 16.7 mo (95% confidence interval: 13.0-20.3), respectively[30]. While SBRT appears to have limited utility in the treatment of LAPC compared to conventional fractionated radiation, this study suggested that it may have a role in certain cases. A multidisciplinary approach should be considered when determining optimal treatment strategies for patients with LAPC who are not surgical candidates.

In addition to SBRT, Robert R. Wilson[31] first proposed particle therapy for the treatment of tumors in 1946. After more than 70 years of development, particle therapy has become another well-established tumor treatment method after surgery, chemotherapy, traditional radiotherapy, and immunotherapy[32]. Currently, particle radiotherapy, which includes proton and heavy ion radiotherapy, has been successfully applied to the treatment of cancer. C-ion and proton radiotherapy are the most commonly used types of particle radiotherapy in clinical practice and have higher accuracy and better cell killing effects, especially in high hypoxia areas and radiation-resistant cell cycle phases[33-35]. In addition, particle radiotherapy decreases the viability, proliferation, and migration of cancer cells[36-39]. Therefore, particle therapy is used to treat deeply penetrating and radiation-resistant tumors, especially pancreatic cancers.

IMMUNOTHERAPY FOR LAPC

Currently, the use of immunotherapy for LAPC is supported by limited data. A recent immune checkpoint inhibitor trial investigating anti-PD-L1 therapy in patients with LAPC failed to demonstrate efficacy due to the poor immunogenicity and immunosuppressive tumor microenvironment of pancreatic cancer[40,41]. However, a minority of patients have genetic mutations that may be targeted with specific interventions. Ongoing clinical trials targeting these mutations have led to discoveries[42,43].

Monoclonal antibodies (mAbs) have been an integral tool in cancer treatment for several decades. They possess the ability to directly kill cells through antibody-dependent cytotoxicity and other pathways and to regulate the immune microenvironment by blocking corresponding signaling pathways, reversing immunosuppression, and enhancing the activity of antitumor effector cells. Moreover, mAbs can even be utilized for the delivery of various therapeutic reagents (Table 3)[44-50].

Mesenchymal stem cells (MSCs) are present in some solid tumors, including PDAC, where they represent almost 100% of cells[51-54]. MSCs play a pivotal role in the development of PDAC. By attenuating MSC recruitment into tumors and inhibiting their tumor-supportive activities, therapeutic outcomes for cancer patients can be improved when MSCs are combined with other anticancer drugs, such as immunotherapy[51]. Numerous clinical studies are currently assessing MSC-based cell therapies[55,56].

Mucin 1 (MUC1) is overexpressed in approximately 90% of PDAC cells[57-60]. A study demonstrated that an antibody similar to the anti-MUC1 antibody GP1.4 could inhibit the proliferation and migration of cancer cells[61]. Additionally, Muc1-c, an isoform of MUC1 with the ability to cross membranes and inhibit tumor growth, could be used as a carrier for cytotoxins in the future[62].

The overexpression of vascular endothelial growth factor mRNA is a common feature in most human tumors and is strongly associated with increased invasiveness, vascular density, metastasis, recurrence, and a poor prognosis[63]. The approval of bevacizumab, a mAb that targets vascular endothelial growth factor, has paved the way for the development of other inhibitors targeting this pathway[64,65].

Annexin A6 (AnxA6) is the largest member of the conserved annexin family of proteins and is known for its modular domain organization and interactions with a variety of proteins and lipids[66,67]. Elevated levels of AnxA6 have been documented during the progression of pancreatic cancer[48,68,69]. In a recent study, O'Sullivan *et al*[48] isolated a novel anti-AnxA6 antibody, 9E1, and demonstrated its ability to reduce the invasion capacity of pancreatic cancer cells.

The Notch signaling pathway plays a vital role in the development of embryonic and tissue homeostasis, and it has been implicated in various malignancies. One of the key ligands in mammals is Delta-like ligand 4, which contributes significantly to cancer progression[70]. Demcizumab, a humanized anti-Delta-like ligand 4 antibody, has shown potential for reversing chemotherapy resistance when used in combination with paclitaxel and GEM. However, a recent study showed that while the combination was safe, it did not improve efficacy[71].

Radioimmunotherapy is emerging as a significant treatment option for patients with PDAC[72]. Recent studies have identified CD147 and B7-H3 as potential targets for radioimmunotherapy and have demonstrated the highly promising therapeutic effects of such treatments for PDAC[73,74].

NOVEL THERAPIES FOR LAPC

Vaccine therapy for LAPC

Owing to the intertumoral and intratumoral heterogeneity of pancreatic cancer, immunotherapy, targeted therapy, and other promising treatments have been extensively tested in preclinical studies and clinical trials. However, almost all strategies have shown little significant advantage over conventional chemotherapy against pancreatic cancer, and this issue is often compounded by prevalent therapeutic resistance[75,76]. Cancer vaccines have emerged as a promising therapeutic approach for pancreatic cancer because of their multiple targets, small nonspecific effects, wide therapeutic windows, low toxicity, and induction of lasting immune memory. In particular, mRNA-based vaccines possess numerous advantages over conventional vaccines in terms of factors including efficiency, safety, increased developmental potential, and low production costs. They have facilitated significant technological and conceptual progress in personalized and precise treatment. Hence, they represent a potential choice for novel therapies for pancreatic cancer[77,78].

Table 3 Monoclonal antibody-based therapies targeting non-immune cells for pancreatic ductal adenocarcinoma

Ref.	Type of study	Years of accrual	Target	mAb
NCT01521325[44]	Phase 1	2011-2013	Mesothelin	MORAb-009
Patel *et al*[45]	Preclinical study	2013	MUC-5AC	NPC-1C
NCT03376659[46]	Phase 1/Phase 2	2018-2023	VEGF	Bevacizumab
NCT00614653[47]	Phase 1	2008-2016	VEGF	Bevacizumab
O'Sullivan *et al*[48]	Preclinical study	2017	AnxA6	9E1
Smith *et al*[49]	Phase 1	2008-2011	DLL4	Demcizumab
NCT02722954[50]	Phase 1	2016-2017	DLL4	Demcizumab

AnxA6: Annexin A6; DLL4: Delta-like ligand 4; mAb: Monoclonal antibody; MUC: Mucin; VEGF: Vascular endothelial growth factor.

Cancer gene and signaling pathway therapy for LAPC

Recent evidence has revealed that numerous genes and signaling pathways play critical roles in the pathogenesis and progression of PDAC and thus could be potential valuable therapeutic targets[79,80].

Molecular pathways

Activating mutations of *KRAS* and the phosphoinositide 3 kinase/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling pathway are frequently observed in PDAC and are associated with a poor prognosis[80,81]. In addition, numerous receptor tyrosine kinases have been implicated in the development and progression of PDAC, including tropomyosin receptor kinase, epidermal growth factor receptor, insulin-like growth factor receptor, fibroblast growth factor receptor, vascular epidermal growth factor receptor, platelet-derived growth factor receptor, and others[79,82-84].

Tumor suppressor genes

Tumor suppressor genes play a vital role in regulating cell growth by preventing severe metastasis; in tumors, these genes can be altered *via* mutation or chromosomal rearrangement. In PDAC, several tumor suppressor genes, including cyclin-dependent kinase inhibitor p16, *p53*, and suppressor of mothers against decapentaplegic protein 4, are frequently mutated[80,82].

DNA repair factors

Studies have revealed that PDAC ranks as the third most common cancer associated with mutations in the BReast CAncer gene and mismatch repair genes, following breast and ovarian cancers[85-87]. Novel combination therapies evaluating immune therapies and targeted agents are being tested for patients with PDAC linked to impaired DNA damage repair [88,89].

Epithelial-mesenchymal transition

Epithelial-mesenchymal transition is a critical process in which epithelial cells acquire mesenchymal features[90,91]. In the context of PDAC, epithelial-mesenchymal transition has been associated with tumor initiation, invasion, metastasis, and resistance to therapy[92].

Cancer stem cells

Cancer stem cells (CSCs) play a critical role in tumor initiation, progression, and therapeutic resistance. In PDAC, CSCs express cell surface markers such as CD24, CD44, CD133, epithelial-specific antigen, c-Met, C-X-C motif chemokine receptor 4, and aldehyde dehydrogenase[93]. CSCs have been shown to protect tumor cells from the cytotoxic effects of chemotherapy drugs and are associated with advanced tumor recurrence. However, the mechanisms underlying CSC-mediated drug resistance remain unclear.

EVALUATION OF TREATMENT RESPONSE AFTER NAT

Evaluation of treatment response and prediction of resectability after NAT remains a challenge for patients with LAPC. Pathological assessment of response after surgical resection remains the gold standard, but this approach is limited by its invasiveness[94,95]. Multidetector computed tomography is the most commonly used imaging modality to evaluate the response of LAPC after NAT. Its advantages over other techniques include higher spatial resolution and multiplanar reconstruction capabilities[96]. Other imaging modalities, such as endoscopic ultrasound, diffusion-weighted imaging, positron emission tomography, and perfusion computerized tomography, show significant potential to become powerful tools for assessing tumor resectability and predicting survival after NAT[97]. Tumor markers are also commonly used as evaluation indicators, with carbohydrate antigen 19-9 being the only biomarker currently recommended by the National

Comprehensive Cancer Network guidelines for assessing NAT response. Other promising indicators being studied include circulating tumor cells, circulating tumor DNA, and microRNAs, among others[96].

CONCLUSION

LAPC remains a challenging disease, despite significant progress made in its treatment over the past decade. Notably, immunotherapy has shown remarkable improvements in the management of LAPC. Across all fields of pancreatic cancer research, substantial advancements have been achieved. Basic research has significantly improved the understanding of this disease. Moreover, the advent of advanced DNA and RNA sequencing technologies has enabled The Cancer Genome Atlas consortium to study both the whole genome and transcriptomes of human tumors, thereby facilitating the identification of driver mutations and transcriptional programs implicated in carcinogenesis. These efforts are poised to accelerate the application of precision medicine strategies[98-100]. Administration of NAT for the treatment of LAPC can provide important benefits, although more in-depth studies are needed. Therefore, a multidisciplinary team comprising surgeons, oncologists, radiation oncologists, and radiologists is essential for the optimal treatment of LAPC. The most urgent issues to address include identifying patients who are most suitable for NAT and evaluating treatment effects in a timely and accurate manner to achieve more precise and effective treatments for patients with LAPC.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Regenerating gene 4 promotes chemoresistance of colorectal cancer by affecting lipid droplet synthesis and assembly

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Abstract

BACKGROUND

Regenerating gene 4 *(REG4)* has been proved to be carcinogenic in some cancers, but its manifestation and possible carcinogenic mechanisms in colorectal cancer (CRC) have not yet been elucidated. Our previous study found that the drug resistance of CRC cells may be closely linked to their fat metabolism.

AIM

To explore the role of *REG4* in CRC and its association with lipid droplet formation and chemoresistance.

METHODS

We conducted a meta-analysis and bioinformatics and pathological analyses of *REG4* expression in CRC. The effects of *REG4* on the phenotypes and related protein expression were also investigated in CRC cells. We detected the impacts of *REG4* on the chemoresistance and lipid droplet formation in CRC cells. Finally, we analyzed how *REG4* regulated the transcription and proteasomal degradation of lipogenic enzymes in CRC cells.

RESULTS

Compared to normal mucosa, *REG4* mRNA expression was high in CRC ($P < 0.05$) but protein expression was low. An inverse correlation existed between lymph

node and distant metastases, tumor-node-metastasis staging or short overall survival and *REG4* mRNA overexpression ($P < 0.05$), but vice versa for *REG4* protein expression. *REG4*-related genes included: Chemokine activity; taste receptors; protein-DNA and DNA packing complexes; nucleosomes and chromatin; generation of second messenger molecules; programmed cell death signals; epigenetic regulation and DNA methylation; transcription repression and activation by DNA binding; insulin signaling pathway; sugar metabolism and transfer; and neurotransmitter receptors ($P < 0.05$). *REG4* exposure or overexpression promoted proliferation, antiapoptosis, migration, and invasion of DLD-1 cells in an autocrine or paracrine manner by activating the epidermal growth factor receptor-phosphoinositide 3-kinase-Akt-nuclear factor-κB pathway. *REG4* was involved in chemoresistance not through de novo lipogenesis, but lipid droplet assembly. *REG4* inhibited the transcription of acetyl-CoA carboxylase 1 (ACC1) and ATP-citrate lyase (ACLY) by disassociating the complex formation of anti–acetyl (AC)-acetyl-histone 3-AC-histone 4-inhibitor of growth protein-5-si histone deacetylase;-sterol-regulatory element binding protein 1 in their promoters and induced proteasomal degradation of ACC1 or ACLY.

CONCLUSION

REG4 may be involved in chemoresistance through lipid droplet assembly. *REG4* reduces expression of de novo lipid synthesis key enzymes by inhibiting transcription and promoting ubiquitination-mediated proteasomal degradation.

Key Words: Colorectal cancer; Regenerating gene 4; Aggressive behavior; Prognosis; Chemoresistance; Lipid droplet formation; Epidermal growth factor receptor signal

Core Tip: The roles of regenerating gene 4 (*REG4*) in colorectal cancer (CRC) and the related molecular mechanisms are still unknown. *REG4* may be involved in tumorigenesis and aggressiveness of CRC *via* the epidermal growth factor receptor-phosphoinositide 3-kinase-Akt-nuclear factor-κB pathway, and chemoresistance through lipid droplet assembly. *REG4* might be used as a useful marker for colorectal carcinogenesis and subsequent progression, as well as a potential gene therapy target.

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INTRODUCTION

The regenerating gene 4 (*REG4*) gene family encodes secretory calcium-dependent lectins, which act as acute phase reactants, antiapoptotic factors, and growth agents, and contributes to cellular proliferation and differentiation, inflammation, diabetes, and cancer. To date, human *REG* genes include *REG* I (*Iα* and *Iβ*), *Reg* III (III and *HIP/PAP*), and *REG4*[1-3]. Of these, *REG4* is transcriptionally activated by several transcription factors (*e.g.*, Sp1 transcription factor, GATA binding protein 6 (GATA6) and caudal type homeobox 2), and the *REG4* protein targets, epidermal growth factor receptor (EGF) (EGFR)/Akt/activator protein-1 and Akt/glycogen synthase kinase-3β/β-catenin/T cell factor-4 signaling pathways as a mitogen[4-8]. In pancreatic cancer cells, *REG4* also enhanced invasion by upregulating matrix metalloproteinase expression and macrophage polarization through the EGFR–Akt–c-AMP response element binding protein pathway[9,10]. In gastric cancer cells, the forced expression of *REG4* induced EGFR phosphorylation and inhibited 5-fluorouracil (5-FU)-induced apoptosis[11]. In ovarian cancer cells, *REG4* overexpression as well as recombinant (rh) *REG4* exposure prevented apoptosis, and enhanced proliferation, migration, and invasion with the hyperexpression of Wnt5a, p70s6k, survivin and vascular endothelial growth factor, and reduced expression of Bax[12].

In colorectal tissues, *REG4* mRNA-positive cells are mostly enteroendocrine and goblet cells. Adenomatous and cancer cells positive for *REG4* mRNA exhibited enterocyte-like, mucus-secreting, or undifferentiated features[13]. At the protein level, *REG4* expression was observed in the middle and outer parts of crypts and superficial epithelium, especially goblets[14]. Oue *et al*[15] found that the preoperative serum *REG4* concentration was not elevated in patients with colorectal cancer (CRC) at stages 0–III, but was significantly elevated in those at stage IV. Additionally, *REG4* expression was significantly linked to a worse prognosis in patients with CRC as an independent predictor[15-17]. *REG4* promoted migration and invasion of CRC cells *via* its carbohydrate-recognition domain in both autocrine and paracrine manners, which was significantly decreased by anti-*REG4* antibody[18,19]. Nanakin *et al*[20] found that *REG4* expression was stimulated by tumor necrosis factor (TNF) α, EGF, basic fibroblast growth factor, and hepatocyte growth factor in colon cancer cells, and then promoted cell proliferation and resistance to H_2O_2-induced apoptosis. As for therapy resistance, *REG4* was markedly related to chemoresistance. Violette *et al*[13] discovered that *REG4* protein was strongly expressed in

chemoresistant rectal cancer cells, but expressed weakly in drug-sensitive rectal cancer cells. *REG4* expression was found to correlate with γ-radiation sensitivity in rectal cancer patients receiving radiotherapy[21]. In radiochemotherapy-sensitive CRC cells, *REG4* expression was downregulated, while it was increased in radiochemoresistant cells[22].

Leukocytes have been found to play an important role in shaping the immune microenvironment of CRC[23-25]. Among them, the interleukin (IL)-22-mediated positive effect depended on its ability to induce neutrophil chemokines into the tumor microenvironment[24]. The proinflammatory cytokine group factors (*e.g.* TNF-α, IL-6, IL-12 and IL-23) promoted tumor cell survival, induced angiogenesis, and facilitated cell migration by exerting antiapoptotic activity[25]. Immune checkpoint molecules, including programmed death ligand ½, B7-1/2, B7-acetyl-histone 3 (H3), B7x, V-domain Ig suppressor of T cell activation and galectin-9, were found to be effective regulators of immune activation, which play a key role in immune escape of CRC[26]. Jang[27] found that T regulatory (Treg) cells gradually increased, while $CD8^+$ T cells and $CD8^+$ T cell/Treg cell ratio decreased during progression of CRC. Infiltration of T cells differed between tumor regions, and the proportion in the central region of CRC was the lowest. Unfortunately, there is no report on the relationship between *REG4* and the microenvironment in CRC.

In our previous study, *REG4* expression gradually decreased from gastric intestinal metaplasia, adenoma, and cancer to gastritis at either the mRNA or protein level[28]. Serum *REG4* levels were elevated in patients with gastric cancer, compared to those in healthy individuals. Our group subsequently developed a mouse anti-*REG4* monoclonal antibody for immunohistochemistry, and found that *REG4* was downregulated in CRC[3]. To understand the roles of *REG4* in tumorigenesis and subsequent progression of CRC, we undertook meta-analysis, and bioinformatics, pathological and serological analyses to explore the clinicopathological and prognostic significances of *REG4* in CRC. The effects of *REG4* on the phenotypes of CRC cells were also studied with the detailed mechanisms clarified. We also investigated the impacts of *REG4* on lipid droplet forma-tion and chemoresistance of CRC cells and the regulatory effect of *REG4* on the key lipogenic enzymes in CRC cells.

MATERIALS AND METHODS

Cell culture and transfection

A CRC cell line (DLD-1) was generously donated by Prof. Toshiro Sugiyama, Department of Gastroenterology, University of Toyama, Japan. This line was maintained in RPMI 1640 growth medium supplemented with 11% fetal bovine serum (FBS), 90 U/mL penicillin, and 90 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37 ℃. The cells were transfected at 75% confluence 20-24 h after seeding on culture dishes (Qiagen, Hilden, Germany). The cells were transfected with pcDNA3.1-*REG4* [full-length (FL)-*REG4*], pcDNA3.1-*REG4* [nonsignal peptide (NSP)-*REG4*] or siRNA against histone deacetylase [si histone deacetylase (HDAC); Santa Cruz Biotechnology, Dallas, TX, United States] using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, United States). The cells were treated with high glucose (4.5 mg/L), recombinant human *REG4* protein (rh*REG4*; R and D Systems, Minneapolis, MN, United States) or anti-*REG4* antibody (R and D Systems). The cells were also exposed to 5-FU (thymidylate synthetase inhibitor); cisplatin (DDP), DNA crosslinker; suberoylanilide hydroxamic acid (SAHA); HDAC inhibitor; cycloheximide (CHX), selective inhibitor of protein synthesis to study the degradation and stability of proteins; MG132 (proteasome inhibitor to suppress proteasomal degradation of proteins); acetyl-CoA carboxylase 1 (ACC1) or ATP-citrate lyase (ACLY) inhibitor (Abmole, Chicago, United States). Centrifuged cells were washed with phosphate-buffered saline (PBS), and total protein or RNA was extracted. Some cells were subjected to proteasome extract using a Minute Nuclear or cytosolic proteasome enrichment kit (Invent Biotechnologies, Plymouth, MN, United States). For HDAC silencing, we tested at least two HDAC-specific siRNAs to control for off-target effects.

Assessment of cell proliferation by tetrazolium salt (MTT) assay

As a measure of cell proliferation, MTT was reduced to formazan in a mitochondrial-dependent manner. We seeded cells in 96-well plates. After culture for 24 or 48 h in a 37 ℃ incubator, 50 μL MTT reagent (5 mg/mL) was added to each well. After incubation for 4 h, 150 μL dimethyl sulfoxide was added to each well for coloration. After vigorous shaking of the plates for 30-min to achieve complete solubilization, the optical density was measured on a microplate reader (Model 680; Bio-Rad, Hercules, CA, United States) at a wavelength of 490 nm. We normalized the OD 490 values to the viability of control cells that were not treated with reagents or siRNA. Normalized viability (%) = (OD490 of treated cells/OD490 of untreated control cells) × 100. The normalized viability values were plotted to show the growth inhibition effects of treatments.

Apoptosis assay

We detected phosphatidylserine externalization (on the cell membrane) using fluorescein isothiocyanate (FITC)-labeled Annexin V (Immunotech, Marseille, France) and propidium iodide (PI) to identify early apoptosis. After treatment, any remaining intact cells were collected, washed with cooled PBS at 4 ℃ and centrifuged at 700 × g for 3 min. The cell suspension (490 μL) was gently mixed with FITC-labeled Annexin-V (5 μL) and PI (5 μL). After incubation at 4 ℃ for 10 min in the dark, the cell suspension was detected by flow cytometry.

Wound healing assay

Cells (5.5×10^5 per well) were plated in six-well culture plates. When the cells reached 75% confluence, they were scraped with a tip, washed four times in PBS to reduce the number of broken cells, and cultured in FBS-free RPMI 1640 medium.

We measured the area of the scratch immediately after wounding (0 h) and 24 and 48 h later using Image J. The percentage wound closure was calculated as: (Area of scratch at 0 h-area of scratch at 24 and 48 h)/area of scratch at 0 h) × 100. The values were plotted to show differences in migration between conditions.

Cell migration and invasion assays

Assays for migration were conducted by suspending 1.3 × 10^5 cells per 200 μL in FBS-free RPMI 1640 and seeding the upper chamber of each Transwell (BD Biosciences, Franklin Lakes, NJ, United States). As a chemoattractant, 11% FBS was added to each lower compartment. After incubation for 24 h, we scrubbed the upper surface of the Transwell membrane, washed the Transwell chamber with PBS three times and fixed cells in 100% cold methanol. The membrane was stained with crystal violet for several minutes. For the invasive assay, the same process as above was used except that each Transwell insert was coated with Matrigel (BD Biosciences).

Nile red staining

Cells were cultured on coverslips for 12 h, fixed in 4% paraformaldehyde for 28 min and stained with Nile red (Invitrogen, Carlsbad, CA, United States; 1: 1000) for 13 min. Finally, we stained slides with 4',6-diamidino-2-phenylindole (DAPI) and covered them with SlowFade® Gold anti-fade reagent (Thermo Fisher Scientific). The software programs, Image J and Icy, were used to acquire and analyze images.

Immunofluorescence

Cells were cultured on glass slides, fixed for 10 min with 4% formaldehyde, and permeabilized for 10 min with 0.5% Triton X-100. Cells were rinsed with PBS and treated with anti-goat *REG4* (R and D Systems; 1: 100) antibody for 3 h. The cells were incubated with Alexa Fluor 488 (green) anti-goat immunoglobulin G (IgG) (Invitrogen) for 50 min. Slides were mounted using SlowFade® Gold anti-fade reagent after being stained with DAPI.

Chromatin immunoprecipitation (ChIP)

A Magna ChIP™ G kit (Millipore, Burlington, MA, United States) was used to conduct ChIP. After IP of anti-acetyl (AC) H3, anti-AC-histone 4 (H4), anti-HDAC, anti–inhibitor of growth protein (ING) 5 or anti-sterol-regulatory element binding protein 1 (SREBP1) antibody (Supplementary Table 1), we used ACLY (*5'-AATCGCGGGGCCGTTCTC-3'*, melting temperature (TM): 57.9 ℃; *5'-CGACGAACCCCGCAAAATC-3'*, TM: 55.4 ℃, -43 bp to + 81 bp) primers or ACC1 (*5'-GCCCCGAATGGCAGATCC-3'*, TM: 55.7 ℃; *5'-GCTCAGCGGCAGCCAATG-3'*, TM: 57.4 ℃; -33 bp to + 54 bp) primers for polymerase chain reaction (PCR). IgG was used as a negative control and anti-polymerase II as a positive control. In 20 μL of mixture, DNA was amplified and separated on a 2% agarose gel.

Co-immunoprecipitation

Seven micrograms of primary antibody (Supplementary Table 1) was added to > 1 mg protein and subjected to rotation at 4 ℃ overnight. One hundred microliters of agarose A beads were added, and the mixture was rotated at 4 ℃ overnight. To exclude nonspecific binding proteins, the beads were centrifuged and washed with 1% NP40 Lysis buffer four times. The pellet was mixed using 50 μL 2 × SDS sample buffer, and heated at 100 ℃ for 10 min. The sample supernatant was used for western blotting.

Patients

Colorectal primary cancers (n = 796), adenomas (n = 62), non-neoplastic mucosa [(NNM), n = 667] and metastatic cancers in lymph nodes (n = 179) were sampled at the First Affiliated Hospital of Jinzhou Medical University between 2013 and 2022. One hundred cases of CRC and adjacent NNM were obtained from Liaoning Cancer Hospital and stored at -80 ℃ until RNA and protein extraction. Eighty patients with CRC and 50 healthy volunteers were enrolled to determinate the serum *REG4* level at Liaoning Cancer Hospital and The Affiliated Hospital of Chengde Medical University. Before surgery, none of the patients had undergone radiotherapy or chemotherapy. The ethics committee of our institution authorized the research plan after giving unanimous approval to utilize tumor tissues and patient serum for clinical research.

Pathology and tissue microarray

All tissues were preserved in 10% neutral formalin, embedded in paraffin, and sectioned into 4-mm pieces. Hematoxylin and eosin (HE) staining was used to validate the histological diagnosis and other microscopic features of these sections. In HE-stained sections, representative portions of solid tumors were selected under a microscope. Using a tissue microarray, a 2-mm tissue core from each donor block was punched out and transferred to a recipient block with a maximum of 48 cores (Azumaya kin-1, Nagoya, Japan). From the recipient block, 4-μm–thick sections were sequentially cut and placed on glass slides coated with poly-lysine.

Western blotting

One hundred and seventy microgram samples of denatured protein were separated on 12% SDS-PAGE and then transferred to a Hybond membrane (Amersham, Chicago, IL, United States). The membrane was blocked overnight in 4.5% skimmed milk in Tris-buffered saline with Tween 20 (TBST). The membrane was treated with primary antibody for immunoblotting for 1 h (Supplementary Table 1). Following a TBST rinse, it was incubated for 1 h with anti-goat, anti-mouse or anti-rabbit IgG conjugated to [horseradish peroxidase(HRP); Dako, Glostrup, Denmark]. Bands were observed

using C300 imaging system (AZURE, Peking, China) and ECL-Plus detection reagents (Santa Cruz Biotechnology).

Quantitative reverse transcription polymerase chain reaction (RT-PCR)

CRC cells or tissues were used to extract total RNA with a Qiagen RNase Mini Kit. avian myeloblastosis virus transcriptase and a random primer were used to create cDNA from 2 mg total RNA (Takara, Kusatsu, Japan). The primers for *REG4* were forward: *5′-CCTTTCCACAGTATCCTTCTTCCCT-3′*, TM: 58.4 ℃ and reverse: *5′-TATGGC-CAAAGACCCAGCTGTT-3′*, TM: 58.2 ℃ (104 bp). The primers for ACLY were forward: *5′-AAACTGTGG-GTCCTTTACTCG-3′*, TM: 53.8 ℃ and reverse: *5′-GGATGACGATACAGCCCCTG-3′*, TM: 55.6 ℃ (147 bp). The primers for ACC1 were forward: *5′-GCTGGTCCACATGAACAGG-3′*, TM: 53.8 ℃ and reverse: *5′-GCCTTCTGGATATTCAGGAC-TTT-3′*, TM: 54.5 ℃ (91 bp). The primers for GAPDH were forward: *5′-CAATGACCCCTTCATTGACC-3′*, TM: 52.0 ℃ and reverse: *5′-TGGAAGATGGTGATGGGATT-3′*, TM: 51.7 ℃ (135 bp). RT-PCR amplification was performed using a SYBR Premix Ex Taq™ II kit (Takara).

Immunohistochemistry

After deparaffinization with xylene and dehydration with alcohol, successive slices in a target retrieval solution were microwaved for 17 min (Dako). H_2O_2 at 3% in methanol was used to block endogenous peroxidase activity. To stop nonspecific binding, 4% bovine serum albumin was administered for 5 min. The sections were treated with anti-goat conjugated to horseradish peroxidase (Dako, 1: 100) antibodies for 18 min after being incubated with goat anti-human *REG4* antibody (R and D Systems, 1: 50) for 17 min. To enable previously reported intermittent irradiation, all incubations were carried out in a microwave oven[28]. *REG4* immunostaining was localized in the cytoplasm. For immunohisto-chemistry, according to the degree of color development of cell positive markers, it was divided into: Blue, negative; pale yellow, weakly positive; brown, moderately positive; dark brown, strongly positive. From five typical fields in each region, 100 cells were randomly chosen and counted by two independent observers blinded to the samples (Zhang CY and Zheng HC). Semiquantitative two-tier grading was used to determine the positive proportion of counted cells: Positive, 6%-100% and negative, 0%-5%.

ELISA

We performed an ELISA (Gelatins, Shanghai, China) to determine serum *REG4* concentration. One hundred microliters of standard or serum sample was incubated at 4 ℃ overnight on polystyrene microtiter plates coated with anti-*REG4* antibody. Following the removal of the liquid, we added 100 μL biotin-antibody working solution to each well, which was then incubated at 37 ℃ for 2 h. After aspirating the liquid, 100 μL HRP-avidin working solution was added to each well, followed by washing three times with 350 μL wash buffer, and incubation at 37 ℃ for 1 h. The plates were washed again three times with wash buffer, followed by incubation with 90 μL tetramethylbenzidine substrate at 37 ℃ for 30 min. We dispensed 50 μL stopping solution to each well and measured the absorbance at 405 nm. Recombinant *REG4* (0.312-20 ng/mL) was used as the reference standard.

Meta-analysis

PubMed, Web of Science, BIOSIS, and SciFinder were used to search the literature up to March 14, 2022. The following search phrases were entered: (Colorectal or rectal or colon or rectum) and (*REG4* or REG IV) and (cancer or carcinoma or adenocarcinoma). No limitations on language or publication year were placed on the search. Inclusion criteria for studies were: (1) Studies that used immunohistochemistry to detect changes in *REG4* expression in CRC; and (2) studies that used immunohistochemistry to relate *REG4* expression to pathobiological behavior and CRC prognosis. Exclusion criteria included: (1) Abstracts, comments, reviews and meeting reports; (2) duplication of previous publications; (3) western blotting, RT–PCR, cDNA microarray, or transcriptomic sequencing for *REG4* expression; and (4) lack of sufficient information. Two reviewers (Zhang CY and Zheng HC) independently gathered data from all relevant articles and evaluated the quality of the included studies using the Newcastle-Ottawa Scale (http://www.ohri.ca/programs/clinicalepidemiology/oxford.htm). For survival analysis, we extracted data from Kaplan-Meier curves using an Engauge Digitizer program. We calculated the hazard ratios (HRs) and accompanying 95% confidence intervals (CIs). Twelve papers regarding the correlation between *REG4* expression and cancer risk, and clinicopathological or prognostic factors of CRC were found in PubMed, Web of Science, BIOSIS Citation Index, SciFinder, and CNKI (Supplementary Table 2). Samples of normal colorectal mucosa were only included in four papers[3,29-31]. In 12 investigations, a comparison was made between *REG4* expression and the clinicopathological features of CRC[3,15,17,29-37]. Finally, we covered the importance of *REG4* expression for prognosis in five papers [15,17,30,35,36].

Bioinformatics analysis

We used Oncomine (www.oncomine.org, keywords: *REG4*, Skrzypczak, Sabates-Bellver) to analyze the *REG4* expression level. The Cancer Genome Atlas (TCGA)-assembler in R program retrieved the *REG4* expression and clinicopathological data for a total of 362 patients with CRC from the TCGA database (keywords, *REG4*). Using the Xiantao platform (https://www.xiantaozi.com/, keywords: *REG4*) and The University of Alabama at Birmingham Cancer Data Analysis Portal (UALCAN) database (http://ualcan.path.uab.edu, keywords: *REG4*), we analyzed the expression, methylation, relevant genes, and signaling pathways of the *REG4* gene. A Kaplan-Meier plotter (http://kmplot.com/, keywords: 223447_at) was used to evaluate the prognostic value of *REG4*. Additionally, we discovered the genes that were differentially expressed by Xiantao and subjected these to protein-protein interaction (PPI) network analysis and a search of critical hub genes. Subsequently, gene ontology, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Set Enrichment Analysis (GSEA) analyses were conducted on these genes.

Statistics analysis

Hardy-Weinberg equilibrium was assessed in each study's control group using the χ^2 test. A Z test was used to establish the statistical significance of the pooled odds ratios. A fixed effect model was applied if there was no discernible heterogeneity. Instead, a random effect model was used to analyze prognostic analysis. An I^2 test was used to quantify the heterogeneity impact. To measure the asymmetry of the funnel plot and evaluate publication bias, Begg's and Egger's tests were used to quantify this. Meta-analyses were carried out using Revman software 5.3, and Student *t* tests were used with SPSS 10.0 to handle data from the TCGA database. A log-rank statistic was used to compare survival curves and create Kaplan-Meier survival charts. Cox's hazard proportional analysis was used for multivariate survival analysis. Statistical significance was defined as two-sided $P < 0.05$.

RESULTS

Clinicopathological and prognostic significance of REG4 mRNA expression in CRC

The expression of *REG4* mRNA was higher in colorectal adenoma or CRC than in normal tissues ($P < 0.05$) using quantitative RT-PCR (Supplementary Figure 1A), Xiantao (Supplementary Figure 1B) and Oncomine (Supplementary Figure 1C) datasets. In TCGA data, *REG4* mRNA expression was higher in colonic than rectal cancer ($P < 0.05$) (Supplementary Figure 1D). A positive correlation was noted with microsatellite instability status and *BRAF* mutation, but a negative correlation was found with lymph node metastasis, distant metastasis, and tumor-node-metastasis (TNM) staging in CRC ($P < 0.05$) (Supplementary Figure 1D). Mucinous adenocarcinoma showed higher *REG4* mRNA expression than the other adenocarcinomas ($P < 0.05$) (Supplementary Figure 1E). Kaplan-Meier analysis demonstrated a significantly positive association between *REG4* mRNA expression and the overall survival rate of patients with cancer ($P < 0.05$) (Supplementary Figure 1F), even though Cox's proportional hazards analysis indicated that this relationship was not independent ($P > 0.05$) (643 patients, HR univariate/multivariate analysis 1.436, 95%CI 0.911-2.039). On the basis of a Kaplan-Meier plot, *REG4* mRNA expression had a positive correlation with overall survival of female or white patients, and those with a high mutation burden ($P < 0.05$) (Supplementary Figure 1G). *REG4* methylation [transcription start site (TSS)-289, TSS-46, TSS + 45 and TSS + 2831] and mRNA expression were negatively correlated in CRC ($P < 0.05$) (Supplementary Figure 2A). The *REG4* methylation level was lower in colon cancer than in normal mucosa ($P < 0.05$) (Supplementary Figure 2B), in stage 2 than in stage 4 cancer, in mucinous adenocarcinoma than adenocarcinoma, and in tumor protein p53 nonmutant than mutant cancer ($P < 0.05$) (Supplementary Figure 2C).

REG4-related genes and signaling pathways in CRC

We identified distinct genes in the low and high expression groups of *REG4* mRNA in CRC using a xiantao platform, and constructed a volcanic map (Supplementary Figure 3A). KEGG analysis showed that the top signaling pathway mainly included chemokine activity, taste receptor, protein-DNA and a DNA-packing complex, and nucleosome and chromatin ($P < 0.05$) (Supplementary Figure 3B). GSEA showed that the top signaling pathways were principally composed of a generation of second messenger molecules: programmed cell death protein 1 signaling, HDAC and histone acetyl transferase, and epigenetic regulation and DNA methylation ($P < 0.05$) (Supplementary Figure 3C). The upregulated genes were *CCL19*, *CCL25*, *CIDEA*, *CMA1* and *PLA2G2D*, and downregulated genes were *ANGPTL3*, *CRP*, *H2BC10*, *H4C3* and *H4C6* ($P < 0.05$) (Supplementary Figure 3D). Cytoscape software was utilized to determine the top 10 nodes by degree and STRING software to identify the PPI pairings (Supplementary Figures 4A and B). According to a Xiantao database, *CD3D*, *3D3G*, *CD4*, *HLA-DRA*, *ZAP70*, *ITK*, *CD3E*, *CD247*, *CD28* and *HLA-DRB1* were less expressed in CRC than normal tissues ($P < 0.05$) (Supplementary Figure 4C).

According to the Xiantao database, genes positively correlated with *REG4* in CRC are shown in Supplementary Figure 5A ($P < 0.05$). These genes were mainly involved in transcription repression and activation by DNA binding, and lung epithelial differentiation ($P < 0.05$) (Supplementary Figure 5B). Genes negatively correlated with *REG4* in CRC are shown in Supplementary Figure 5C ($P < 0.05$), and were principally involved in the insulin signaling pathway, sugar metabolism and transfer, and neurotransmitter receptors ($P < 0.05$) (Supplementary Figure 5D). Of the positively correlated genes (Supplementary Figure 5E), *AGR2*, *B3GNT6*, *CREB3L1*, *CTSE*, *FAM177B*, *FCGBP*, *FFAR4*, *MUC2* and *SPDEF* were less expressed in CRC than normal mucosa ($P < 0.05$), while *GRB2* was more expressed in CRC than normal mucosa ($P < 0.05$). Of the negatively correlated genes (Supplementary Figure 5F), *LY6G6E*, *LY6G6D*, *FAM27B*, *PFDN4*, *PPP1R3D*, *LY6G6F*, *RNF43*, *POFUT1* and *DDX27* were more expressed in CRC than normal mucosa ($P < 0.05$), but *MT-RNR1* was less expressed in CRC than normal mucosa ($P < 0.05$).

Relationship between REG4 mRNA expression and immune cell infiltration in CRC

According to xiantao, the infiltration of mast cells, T cells, CD8 T cells, cytotoxic T cells, T helper (Th)1, Th2 and Th17 cells, T follicular helper (TFH) cells, TReg cells, natural killer (NK) $CD56^{bright}$ cells, B cells, interstitial dendritic cells (iDCs), and activated DCs (aDCs) in CRC were positively correlated with *REG4* mRNA expression ($P < 0.05$) (Supplementary Figure 6). Meanwhile, T central memory (Tcm) cell infiltration was negatively correlated with *REG4* mRNA expression ($P < 0.05$) (Supplementary Figure 6).

Clinicopathological and prognostic significance of REG4 protein expression in CRC

Compared to normal mucosa, *REG4* protein expression was low in CRC ($P = 0.03$) (Supplementary Table 3) and positively correlated with lymph node metastasis, TNM staging, and dedifferentiation of CRC ($P < 0.05$) (Supplementary Tables 4-

6). The combined data from five datasets showed a strong correlation between *REG4* expression and overall survival in patients with CRC (Supplementary Table 7) (HR = 0.54, 95%CI: 0.41-0.72, $P < 0.0001$).

According to UALCAN (Supplementary Figure 7A), *REG4* protein expression was higher in mucinous than nonmucinous adenocarcinoma ($P < 0.05$), and negatively correlated with chromatin modifier, p53/Rb, and the HIPPO signaling pathway ($P < 0.05$). Expression of *REG4* protein was higher in CRC than in NNM, according to western blotting ($P < 0.05$) (Supplementary Figure 7B). Serum *REG4* level was higher in patients with CRC than in healthy volunteers after modification by body surface area ($P < 0.05$) (Supplementary Figure 7C). Immunohistochemically, *REG4* expression was higher in colorectal NNM than adenoma and primary cancer, and in primary than metastatic cancer ($P < 0.001$) (Supplementary Figure 7D and E). Mucinous adenocarcinoma showed higher *REG4* expression than well-, moderately and poorly differentiated adenocarcinomas ($P < 0.05$) (Supplementary Table 8).

Effects of REG4 on the phenotype of CRC cells

According to qPCR ($P < 0.05$) (Supplementary Figure 8A), western blotting (Supplementary Figure 8B), immunofluorescence (Supplementary Figure 8C), and ELISA ($P < 0.05$) (Supplementary Figure 8D), DLD-1 cells were successfully transfected with FL-*REG4* and NSP-*REG4* plasmids, as shown by higher *REG4* mRNA and protein expression in transfectants than parental cells. After treatment with rh*REG4*, the viability of DLD-1 cells was increased in a dose-dependent manner ($P < 0.05$) (Figure 1A). Exposure to anti-*REG4* antibody decreased the cell viability of FL-*REG4*-overexpressing DLD-1 cells in a dose-dependent manner ($P < 0.05$) (Figure 1B). Treatment with rh*REG4* or FL-*REG4* overexpression resulted in high viability ($P < 0.05$) (Figure 1C), antiapoptosis ($P < 0.05$) (Figure 1D), migration and invasion ($P < 0.05$) (Figures 1E and F) in comparison with untreated DLD-1 cells ($P < 0.05$). Additionally, FL-*REG4* transfection and rh*REG4* treatment enhanced the expression of *REG4*, EGFR-Tyr992, -Tyr1068, -Tyr1148, -Tyr1173, phosphorylated phosphoinositide 3-kinase (p-PI3K), p-Akt, nuclear factor (NF)-κB, p-NF-κB, Bcl-2, and Bcl-X/L compared with untreated DLD-1 cells by western blotting ($P < 0.05$) (Figure 1G). However, anti-*REG4* antibody blocked the effects of FL-*REG4* overexpression (Figure 1B-G). The effects of NSP-*REG4* on aggressive phenotypes and their related protein expression in DLD-1 cells were not detectable, which differed from the effects of FL-*REG4* expression and were similar to those of *REG4*-nontransfected CRC cells (Figure 1C-F and H).

Effects of REG4 on chemoresistance and droplet formation of CRC cells

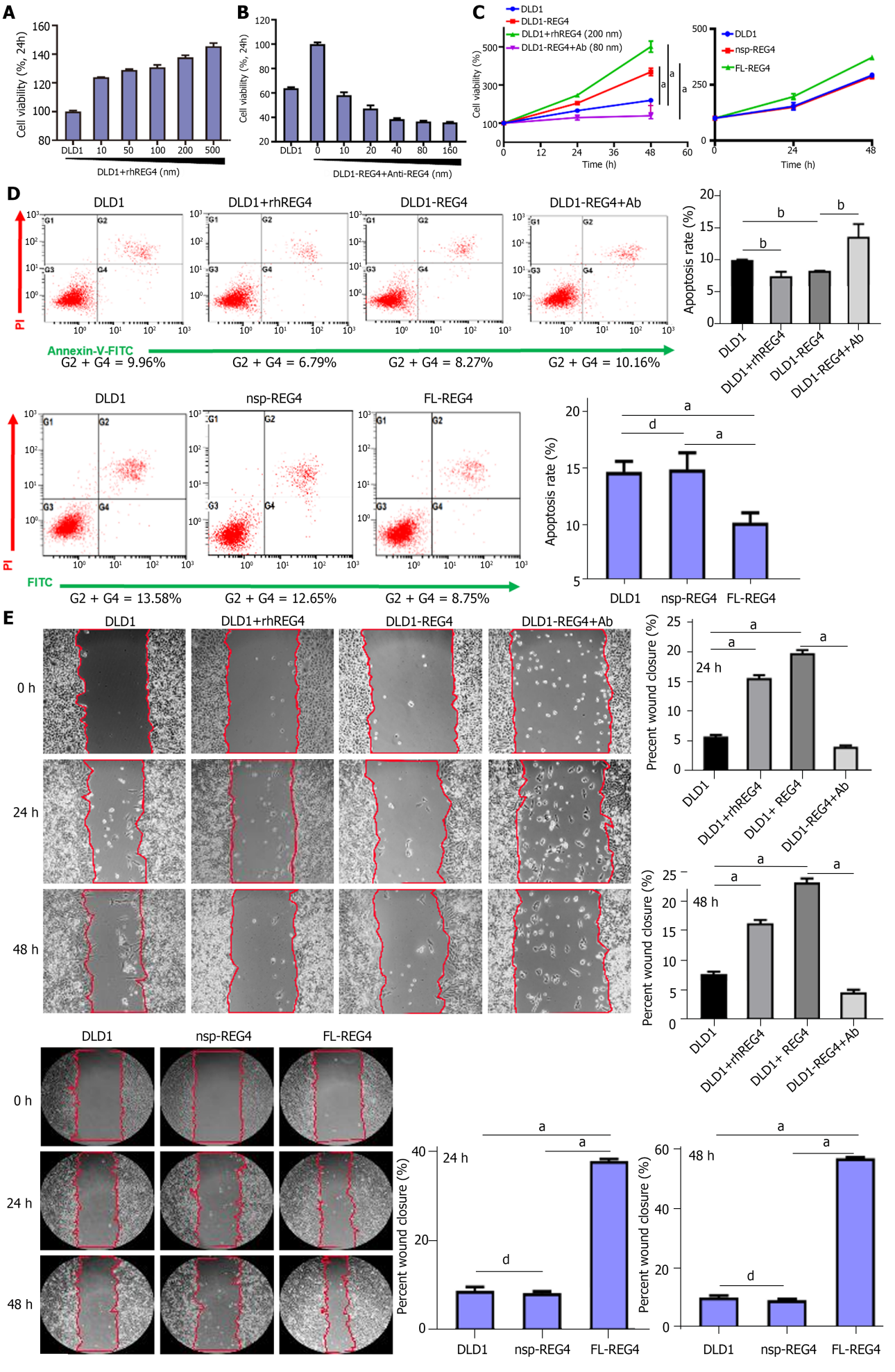
Rh*REG4* treatment and FL-*REG4* overexpression caused DLD-1 cells to become resistant to DDP and 5-FU ($P < 0.05$) (Figure 2A), while anti-*REG4* antibody reversed the chemoresistance of *REG4*-overexpressing DLD-1 cells ($P < 0.05$) (Figure 2A). rh*REG4* treatment and FL-*REG4* increased the formation of intracellular lipid droplets, as shown by Nile red staining ($P < 0.05$) (Figure 2B). *REG4* overexpression was observed in chemoresistant DLD-1 cells. FL-*REG4* overexpression enhanced the expression of acyl coenzyme A-cholesterol acyltransferase (ACAT), perilipin 5, and tail-interacting protein (TIP) 47, but weakened expression of ACLY, ACC1, p-ACC1, HDAC, adipose differentiation-related protein (ADRP), cell-death-inducing DFF45-like effector (CIDE) A, B and C, AC-H3, AC-H4, ING5, and SREBP-1 in DLD-1 cells (Figure 2C). High-glucose treatment significantly increased lipid droplet formation in DLD-1 cells, which was markedly suppressed by ACLY or ACC1 inhibitor ($P < 0.05$) (Figure 2D). DLD-1 cells treated with rh*REG4*, FL-*REG4* transfection, or high glucose had decreased chemosensitivity to 5-FU and DDP, but DLD-1 cells treated with *REG4* antibody, ACC1 inhibitor or ACLY inhibitor had increased chemosensitivity to 5-FU and DDP (Figure 2E). ACC1 or ACLY inhibitor reversed the insensitivity of cells to 5-FU and DDP in the high-glucose treatment group ($P < 0.05$).

REG4 weakened transcription of ACLY and ACC1 via disassociating AC-H3-AC-H4-HDAC-ING5-SREBP1 complex

In DLD-1 cells, FL-*REG4* overexpression weakened the interaction of AC-H3, AC-H4, HDAC, SREBP1 and ING5 proteins with the ACLY or ACC1 promoter, according to ChIP ($P < 0.05$) (Figure 3A). FL-*REG4* overexpression also weakened the interaction of the five proteins ($P < 0.05$) (Figure 3B). Overexpression of FL-*REG4* decreased ACLY and ACC1 mRNA expression ($P < 0.05$) (Figure 3C), and increased lipid droplet formation ($P < 0.05$) (Figure 3D), and increased the half maximal inhibitory concentration (IC_{50}) of cells for 5-FU and DDP ($P < 0.05$) (Figure 3E). After SAHA (siHDAC inhibitor) treatment (2 μM, 24 h) and siHDAC transfection, the interaction between AC-H3, AC-H4, SREBP 1, ING5, HDAC and ACC1, ACLY promoters was weakened compared with the control group ($P < 0.05$) (Figure 3A), and the mRNA level of ACC1 and ACLY decreased ($P < 0.05$) (Figure 3B). After SAHA treatment and siHDAC transfection, the fluorescence intensity of Nile red staining decreased compared with that in the control group ($P < 0.05$) (Figure 3D), and the chemical sensitivity to 5-FU and DDP increased compared with that in the control group ($P < 0.05$) (Figure 3E).

REG4 destabilized ACLY and ACC1 proteins via proteasomal degradation

After treatment with CHX (used to inhibit the synthesis of new proteins), ACC1 and ACLY protein expression was lower in FL-*REG4* transfectants than in DLD-1 cells ($P < 0.05$) (Figure 4A). MG132 (proteasome inhibitor) increased protein expression of ACC1 and ACLY in DLD-1 cells and FL-*REG4* transfectants, which was higher in DLD-1 cells than in FL-*REG4* transfectants ($P < 0.05$) (Figure 4B). Higher levels of ACC1 and ACLY proteins were noted in the nuclear proteasome of FL-*REG4* transfectants than in DLD-1 cells, but this translocation phenomenon was not enhanced by MG132 (Figure 4C). Similar levels of ACC1 and ACLY expression were noted in the cytosolic proteasome of transfectants and parental cells, even after treatment with MG132 (Figure 4C). Regarding ubiquitin ligases, expression of COP1 E3 ubiquitin ligase (COP1), Cbl proto-oncogene (CBL) and NEDD4 like E3 ubiquitin protein ligase (NEDD4L) was higher in FL-*REG4* transfectants than DLD-1 cells, but synoviolin 1 (SYVN1) and NEDD4 were lower in FL-*REG4* transfectants than DLD-1 cells (Figure 4D). Co-immunoprecipitation showed that expression of ubiquitylated ACC1 and ACLY was higher in FL-*REG4* transfectants than in DLD-1 cells, and pretreatment with a small dose of proteasome inhibitor MG132 (5 μM,


A
Cell viability (%, 24h)
DLD1
10
50
100
200
500
DLD1+rhREG4 (nm)
B
Cell viability (%, 24h)
DLD1
0
10
20
40
80
160
DLD1-REG4+Anti-REG4 (nm)
C
DLD1
DLD1-REG4
DLD1+rhREG4 (200 nm)
DLD1-REG4+Ab (80 nm)
Cell viability (%)
Time (h)
DLD1
nsp-REG4
FL-REG4
Time (h)
D
DLD1
DLD1+rhREG4
DLD1-REG4
DLD1-REG4+Ab
PI
Annexin-V-FITC
G2 + G4 = 9.96%
G2 + G4 = 6.79%
G2 + G4 = 8.27%
G2 + G4 = 10.16%
Apoptosis rate (%)
DLD1
DLD1+rhREG4
DLD1-REG4
DLD1-REG4+Ab
DLD1
nsp-REG4
FL-REG4
PI
FITC
G2 + G4 = 13.58%
G2 + G4 = 12.65%
G2 + G4 = 8.75%
Apoptosis rate (%)
DLD1
nsp-REG4
FL-REG4
E
DLD1
DLD1+rhREG4
DLD1-REG4
DLD1-REG4+Ab
0 h
24 h
48 h
Precent wound closure (%)
24 h
DLD1
DLD1+rhREG4
DLD1+ REG4
DLD1-REG4+Ab
Percent wound closure (%)
48 h
DLD1
DLD1+rhREG4
DLD1+ REG4
DLD1-REG4+Ab
DLD1
nsp-REG4
FL-REG4
0 h
24 h
48 h
Percent wound closure (%)
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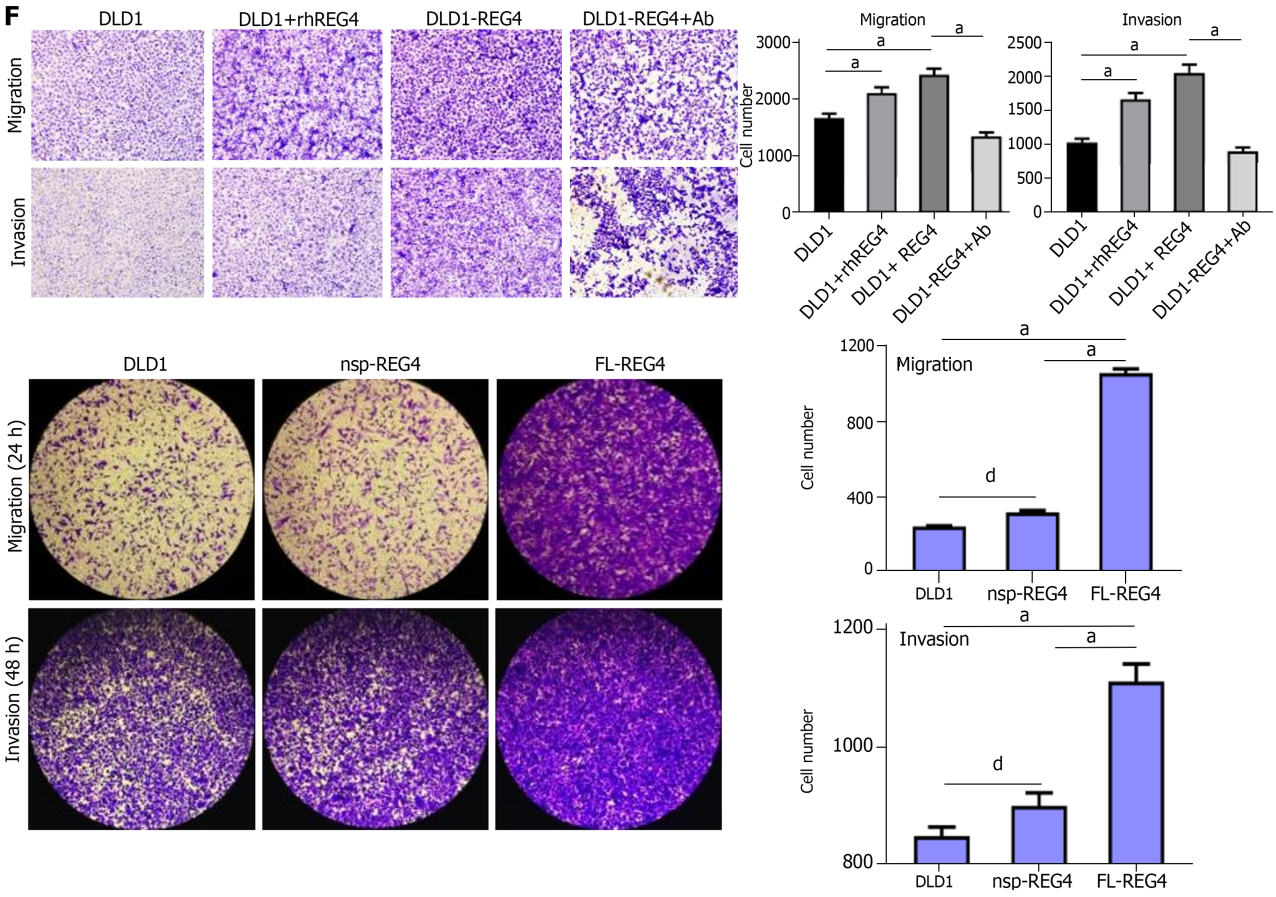
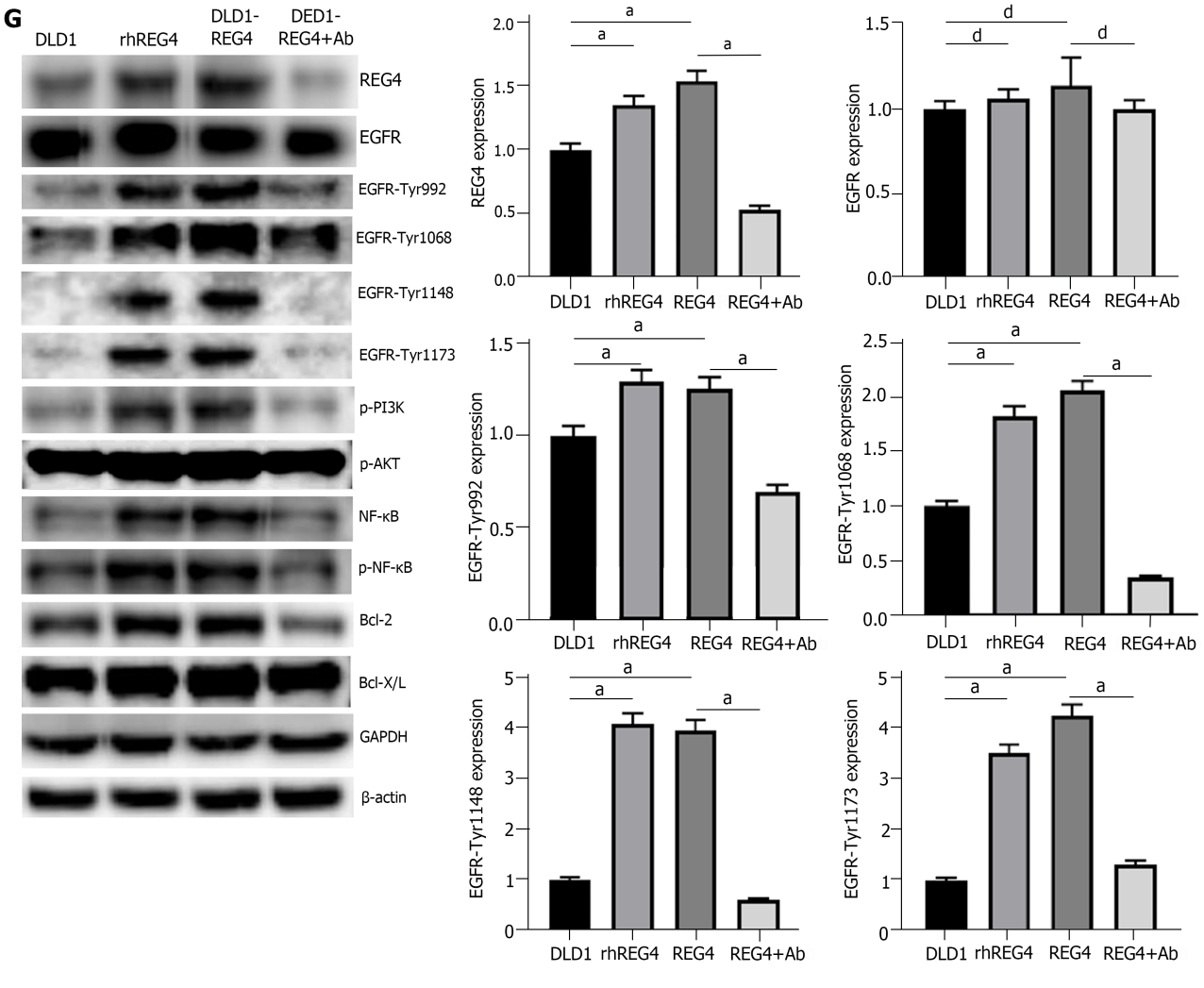
Percent wound closure (%)
48 h
DLD1
nsp-REG4
FL-REG4


F
DLD1
DLD1+rhREG4
DLD1-REG4
DLD1-REG4+Ab
Migration
Invasion
Cell number
DLD1
DLD1+rhREG4
DLD1+ REG4
DLD1-REG4+Ab
nsp-REG4
FL-REG4
Migration (24 h)
Invasion (48 h)


G
DLD1
rhREG4
DLD1-REG4
DED1-REG4+Ab
REG4
EGFR
EGFR-Tyr992
EGFR-Tyr1068
EGFR-Tyr1148
EGFR-Tyr1173
p-PI3K
p-AKT
NF-κB
p-NF-κB
Bcl-2
Bcl-X/L
GAPDH
β-actin
REG4 expression
EGFR expression
EGFR-Tyr992 expression
EGFR-Tyr1068 expression
EGFR-Tyr1148 expression
EGFR-Tyr1173 expression

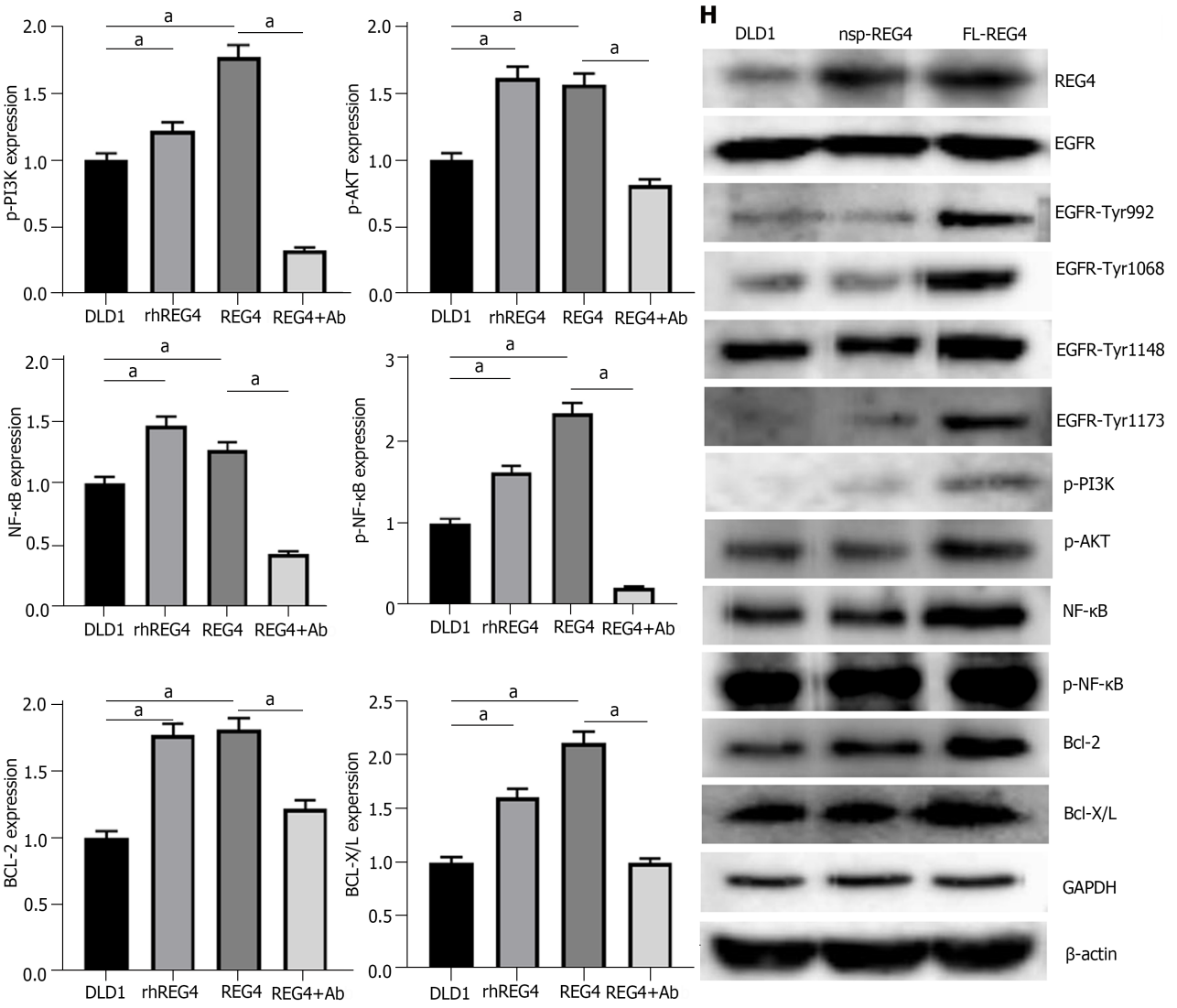


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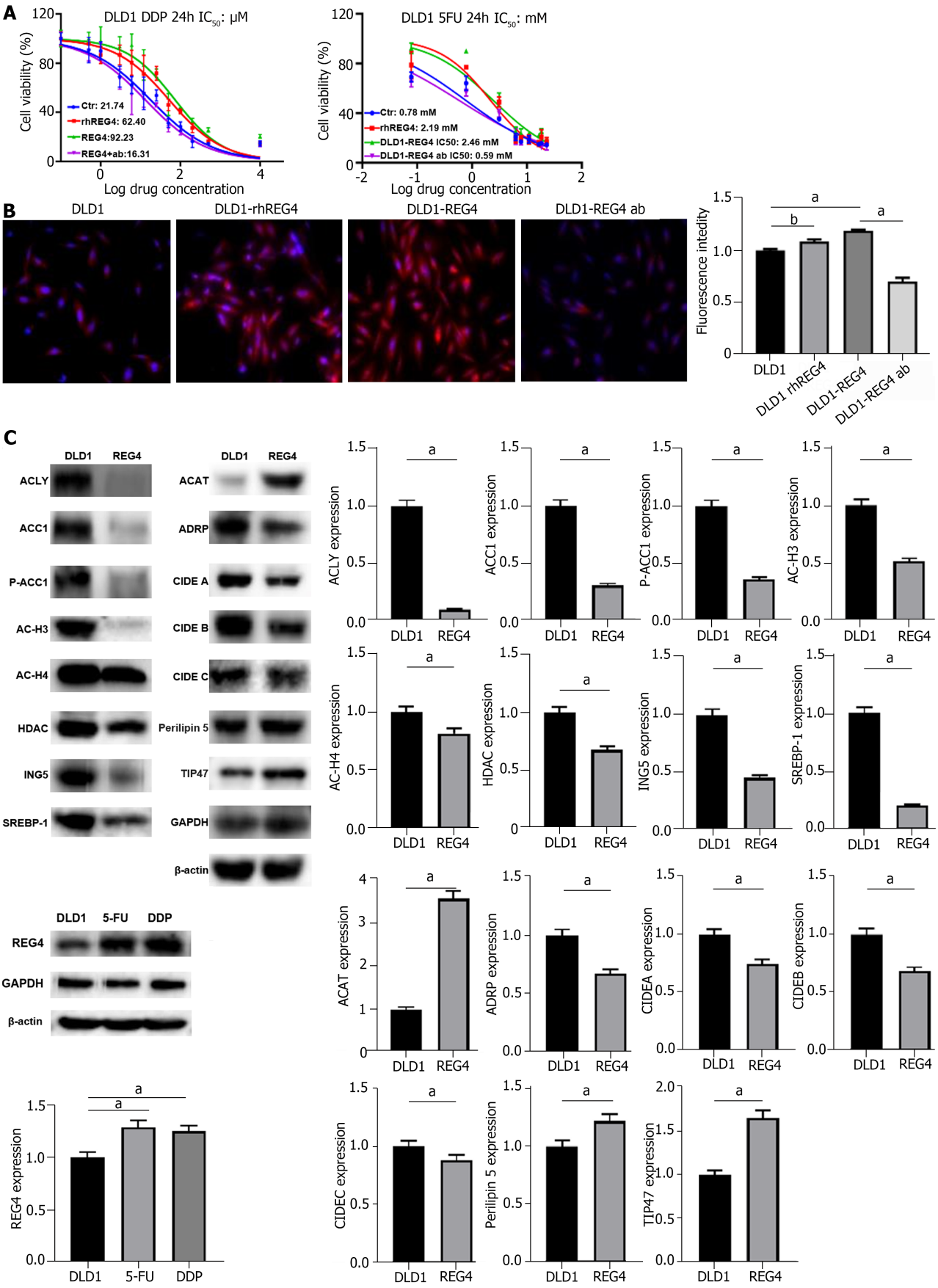
Figure 1 Effects of full-length regenerating gene 4 and nonsignal peptide regenerating gene 4 on phenotypes of DLD-1 cells. A: DLD-1 cells were incubated with increasing doses of recombinant human regenerating gene 4 (*REG4*) (rh*REG4*) (0-500 nmol/L) for 48 h and subjected to cell proliferation assay by tetrazolium salt (MTT) assay; B: Treatment with anti-*REG4* antibody produced a dose-dependent decrease in cell number of full-length (FL)-*REG4*-overexpressing DLD-1 cells; C: MTT assay was used to detect the proliferation of DLD-1 cells transfected with FL-*REG4* or nonsignal peptide (NSP)-*REG4*, and treated with rh*REG4* or *REG4* antibody; D: Apoptosis of DLD-1 cells transfected with FL-*REG4* or NSP-*REG4*, and treated with rh*REG4* or *REG4* antibody detected by flow cytometry; E: Wound healing assay was used to detect migration of DLD-1 cells transfected with FL-*REG4* or NSP-*REG4*, and treated with rh*REG4* or *REG4* antibody; F: Transwell assay was used to detect migration and invasion of DLD-1 cells transfected with FL-*REG4* or NSP-*REG4*, and treated with rh*REG4* or *REG4* antibody; G: Compared with DLD-1 cells, western blotting showed that transfection with FL-*REG4* and treatment with rh*REG4* increased expression of epidermal growth factor receptor (EGFR)-Tyr992, Tyr1068, Tyr1148, Tyr1173, Akt, p-Akt, phosphorylated phosphoinositide 3-kinase (p-PI3K), nuclear factor (NF)-κB, p-NF-KB, Bcl-2 and Bcl-X/L. *REG4* antibody inhibited the effect of FL-*REG4* transfection; H: Western blotting showed that compared with parental cells, DLD-1 cells transfected with NSP-*REG4* had no change in expression of EGFR-Tyr992, Tyr1068, Tyr1148, Tyr1173, AKT, p-AKT, p-PI3K, NF-KB, p-NF-KB, BCL-2, or BCL-XL. $^{a}P < 0.001$; $^{b}P < 0.01$; ^{d}No significance. Ab: Anti-*REG4* antibody; *REG4*: Regenerating gene 4; rh*REG4:* Recombinant human regenerating gene 4; FL: Full-length; NSP: Nonsignal peptide; EGFR: Epidermal growth factor receptor; p-PI3K: Phosphorylated phosphoinositide 3-kinase; NF: Nuclear factor.

9 h) reduced ubiquitination of ACC1 and ACLY (Figure 4E). ACLY bound more to SYVN1, NEDD4 and NEDD4L in FL-*REG4* transfectants than in DLD-1 cells, but bound less to CBL. ACC1 interacted more with COP1 in *REG4* transfectants than in DLD-1 cells, but interacted less with CBL and SYVN1 (Figure 4E). MG132 pretreatment (5 μM, 24 h) aggravated lipid droplet formation ($P < 0.05$) (Figure 4F) and chemoresistance against 5-FU and DDP ($P < 0.05$) (Figure 4G) in DLD-1 cells and FL-*REG4* transfectants.

DISCUSSION

The silencing of *REG4* reduced cellular proliferation, but rh*REG4* had the opposite effect since *REG4* downregulated p21 (Cip1/WAF1) expression and upregulated cyclin D1 expression, which might promote G_1/S transition[38,39]. Anti-*REG4*


A
DLD1 DDP 24h IC50: μM
Cell viability (%)
Ctr: 21.74
rhREG4: 62.40
REG4:92.23
REG4+ab:16.31
Log drug concentration
DLD1 5FU 24h IC50: mM
Ctr: 0.78 mM
rhREG4: 2.19 mM
DLD1-REG4 IC50: 2.46 mM
DLD1-REG4 ab IC50: 0.59 mM
B
DLD1
DLD1-rhREG4
DLD1-REG4
DLD1-REG4 ab
Fluorescence intedity
C
DLD1 REG4
ACLY
ACC1
P-ACC1
AC-H3
AC-H4
HDAC
ING5
SREBP-1
ACAT
ADRP
CIDE A
CIDE B
CIDE C
Perilipin 5
TIP47
GAPDH
β-actin
DLD1 5-FU DDP
REG4
GAPDH
β-actin
REG4 expression
ACLY expression
ACC1 expression
P-ACC1 expression
AC-H3 expression
AC-H4 expression
HDAC expression
ING5 expression
SREBP-1 expression
ACAT expression
ADRP expression
CIDEA expression
CIDEB expression
CIDEC expression
Perilipin 5 expression
TIP47 expression

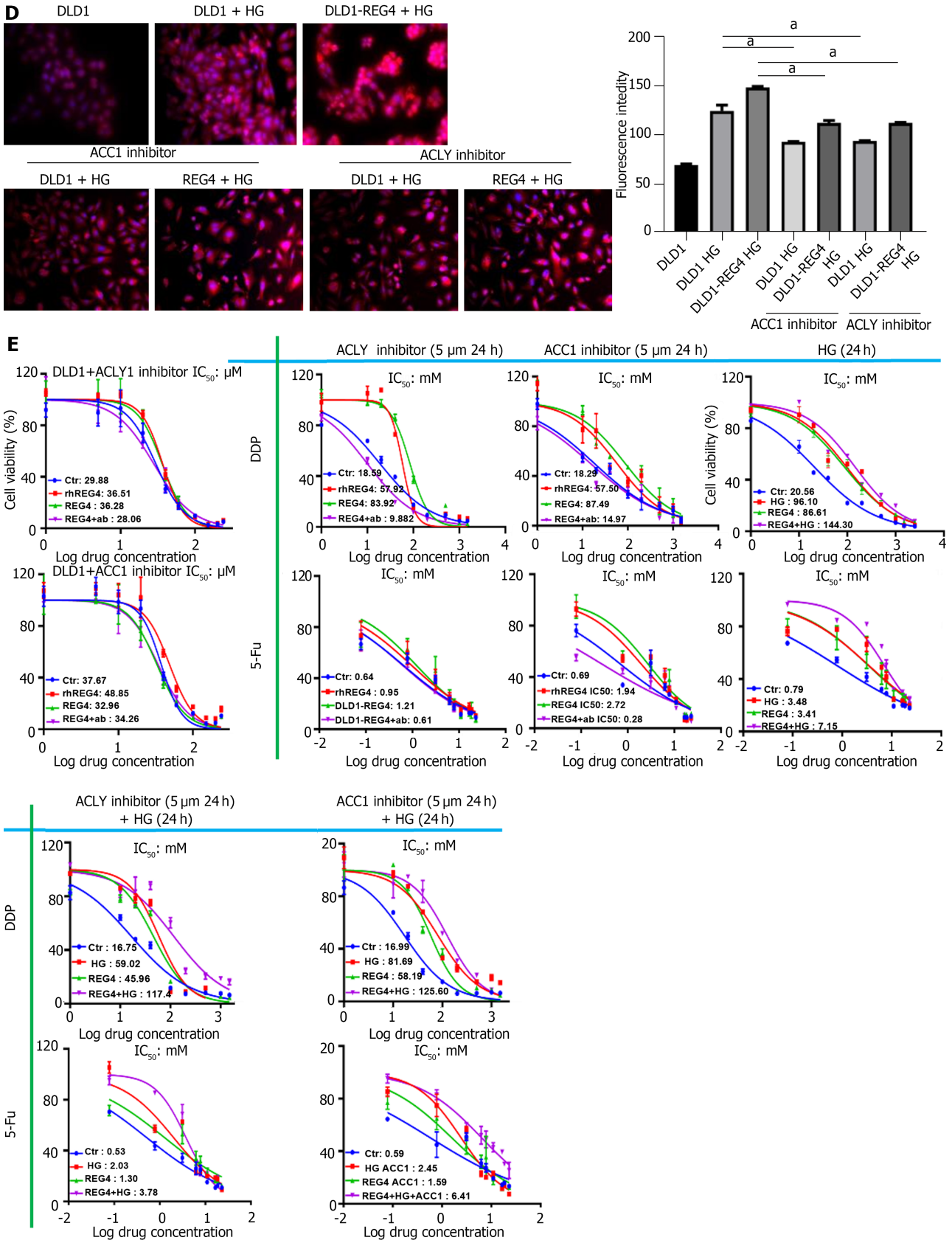


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Figure 2 Effects of regenerating gene 4 on chemoresistance and droplet formation of DLD-1 cells. A: After treatment with cisplatin (DDP) or 5-fluorouracil (5-FU), the viability was measured in DLD-1 cells, DLD-1 cells treated with recombinant human regenerating gene 4 (*REG4*) (rh*REG4*), DLD-1 cells transfected with full-length (FL)-*REG4* plasmid, and DLD-1 cells treated with anti-*REG4* antibody; B: The lipid droplet level was measured in DLD-1 cells, DLD-1 cells treated with rh*REG4*, DLD-1 cells transfected with FL-*REG4* plasmid, and DLD-1 cells treated with anti-*REG4* antibody; C: *REG4* expression was detected in DLD-1, chemoresistant DLD-1 cells to DDP and 5-FU by western blot, proteins related to de novo synthesis or assembly pathway of lipid droplets were also detected in DLD-

1 cells and DLD-1 cells transfected with FL-*REG4*; D: Nile red staining was used to detect the level of lipid droplets in DLD-1 cells and DLD-1 cells transfected with FL-*REG4* after treatment with high glucose (HG), acetyl-CoA carboxylase 1 (ACC1) inhibitor and ATP-citrate lyase (ACLY) inhibitor; E: Tetrazolium salt assay was used to detect half maximal inhibitory concentration of DLD-1 cells for 5-FU and DDP when treated with HG, ACC1 and ACLY inhibitors, alone or in combination with the above conditions. $^{a}P < 0.001$; $^{b}P < 0.01$. Ab: Anti-*REG4* antibody; *REG4*: Regenerating gene 4; rh*REG4:* Recombinant human regenerating gene 4*;* ACC1: Acetyl-CoA carboxylase 1; ACCY: ATP-citrate lyase; AC-H3: Acetyl-acetyl-histone 3; H4: Histone 4; ACC1: Acetyl-CoA carboxylase 1; HDAC: Si histone deacetylase; ING5: Inhibitor of growth protein 5; SREBP1: Sterol-regulatory element binding protein 1; ACAT: A-cholesterol acyltransferase; ADRP: Adipocyte differentiation-related protein; CIDE: Cell-death-inducing DFF45-like effector; TIP: Tail-interacting protein; DDP: Cisplatin; 5-FU: 5-fluorouracil; HG: High glucose.

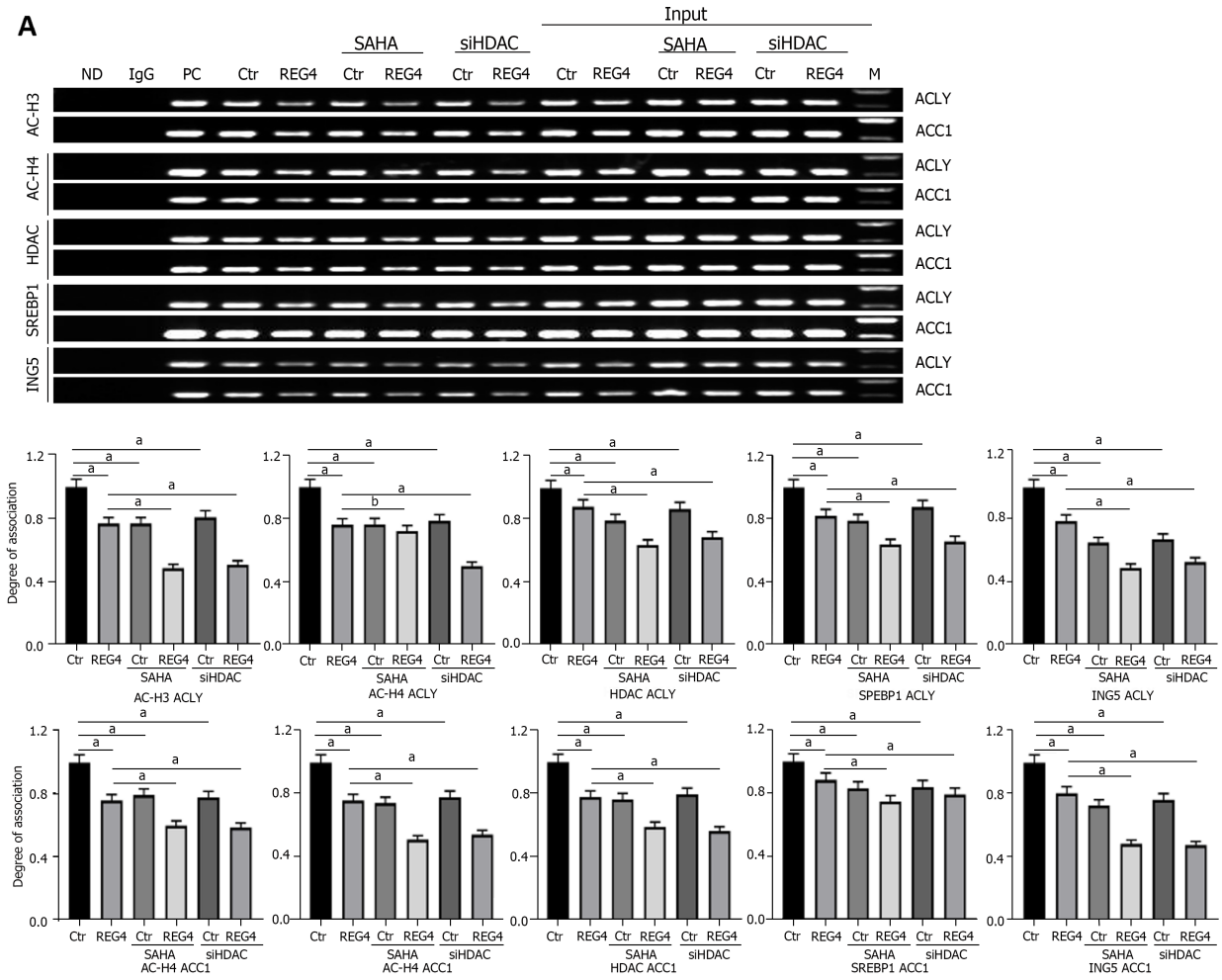
antibody dramatically reduced the autocrine and paracrine effects of secretory *REG4* on the ability of colon cancer cells to invade, migrate and proliferate[19]. *REG4* overexpression may cause resistance to the irradiation-induced apoptosis of colon cancer cells. Animal studies have shown that rh*REG4* increased the expression of the antiapoptotic genes, Bcl-2 and Bcl-xL, and survivin to shield normal intestinal crypt cells against irradiation-induced apoptosis[40].

We conducted bioinformatics analysis, meta-analysis, and pathological and serological studies to evaluate the clinicopathological and prognostic significance of *REG4* expression in CRC. In bioinformatics analysis, we used data of different sizes and from different sources to ensure data heterogeneity, and we used qRT-PCR to verify the reliability of bioinformatics analysis. We discovered that *REG4* mRNA expression was upregulated in CRC, but *REG4* protein expression was downregulated, suggesting that aberrant *REG4* expression could be used as a potential biomarker for colorectal tumorigenesis. In addition, we discovered a negative correlation between *REG4* promoter methylation and mRNA expression in CRC. The correlation of *REG4* methylation with mucinous subtypes or clinicopathological staging was opposite to that of *REG4* mRNA expression. Upregulated *REG4* mRNA expression might be due to promoter hypomethylation in CRC. High *REG4* mRNA expression was discovered in colorectal, pancreatic, hepatic, and prostate malignancies as well as in inflammatory epithelium, dysplasia, and malignant lesions of ulcerative colon tissues[20,41-45], suggesting that upregulated *REG4* mRNA was involved in the early malignant transformation of epithelial cells. Although upregulated *REG4* protein expression occurred in gastric cancer, ovarian cancer, glioma, pancreatic cancer, gallbladder carcinoma, and prostate cancer[12,28,46-49], lower *REG4* expression was found in CRC. In subsequent research, we will use more databases and updated data sets to analyze the expression of *REG4* in CRC to verify the consistency between our studies and strengthen our conclusions.

The tumor microenvironment of CRC is regulated by many factors, such as *CTLA-4*, and the altered expression of these factors can lead to changes in immune responses[50]. So far, there has been no systematic study on the relationship between *REG4* and immune microenvironment in CRC. Here, we studied the correlation between the expression of *REG4* mRNA and the infiltration of immune cells in CRC, and found that high expression of *REG4* was positively correlated with infiltration of mast cells, T cells, CD8 T cells, cytotoxic T cells, Th1, Th2 and Th17 cells, TFH cells, TReg cells, NK CD56^{bright} cells, B cells, iDCs, and aDCs. High expression of *REG4* was negatively correlated with Tcm cell infiltration. In future research, we will focus on the impact of *REG4* expression on the immune environments of primary and metastatic CRC, which is also important for immunotherapy of CRC.

Previously, we discovered that expression of *REG4* was substantially linked with that of mucin-2 and mucin-5AC, and that it was greater in mucinous carcinoma, signet ring cell carcinoma, and intestinal metaplasia that produced mucins [28]. *REG4* was identified as a potential biomarker of mucinous ovarian cancer at both mRNA and protein levels[12,51]. Here, we also found that expression of *REG4* in mucinous adenocarcinoma was greater than that in other histological subtypes at both at the mRNA and protein levels. These findings could account for *REG4* protein overexpression in colorectal mucosa and poorly differentiated, signet ring cell carcinoma and undifferentiated carcinoma. *REG4* expression was positively correlated with lymph node metastasis, TNM staging, poorly differentiated CRC, and had a worse prognosis, in agreement with other studies[12,28,46-49]. The opposite was the case for *REG4* mRNA, suggesting that aberrant *REG4* protein expression might indicate aggressiveness and prognosis of CRC. In CRCs with reduced stroma compared to those with high stroma, *REG4* protein expression was considerably greater[52]. Previously, we performed *REG4* immunostaining on the same tissue microarrays of CRC (Supplementary Table 1), and found similar results. Therefore, the discrepancies about *REG4* mRNA and protein expression might be largely attributable to a complex process from transcription to translation and different methodologies. Serologically, the preoperative *REG4* protein level was higher in CRC than in a healthy population and postoperative patients with cancer, in agreement with the finding that serum *REG4* Levels were greater in pancreatic ductal adenocarcinoma than in chronic pancreatitis[53].

Bishnupuri *et al*[54] found that the transcriptional activator of D-type cyclins, CD44 intracytoplasmic domain, was released after *REG4* connected with transmembrane CD44 and activated γ-secretase to promote proliferation and stemness of colorectal and pancreatic cancer cells. We also found that *REG4* overexpression and rh*REG4* treatment promoted proliferation, antiapoptosis, and migration and invasion of CRC cells. Exposure to anti-*REG4* antibody inhibited the effects of *REG4* overexpression on the phenotypes of CRC cells, which is consistent with our earlier result in ovarian cancer cells[12]. We also found that *REG4* overexpression resulted in resistance of ovarian cancer cells to DDP or taxol by activating the PI3K-Akt-mTOR signaling pathway[55]. As a mutant KRAS-induced factor, *REG4* increased cancer stem cell characteristics *via* Wnt/β-catenin signaling[56]. Jin *et al*[57] showed that *REG4* increased the resistance of gastric cancer cells to 5-FU by stimulating the mitogen-activated protein kinase-ERK-Bim signaling pathway. Here, we found that expression of *REG4*, EGFR-Tyr992, -Tyr1068, -Tyr1148 and -Tyr1173, p-PI3K, p-Akt, NF-κB, p-NF-κB, Bcl-2, and Bcl-x/L was higher in DLD-1 cells treated with rh*REG4* or transfected with *REG4*-overexpressing plasmid than in parental cells. Anti-*REG4* antibody blocked the effects of *REG4* overexpression, indicating that *REG4* promoted the aggressive phenotypes by an EGFR-PI3K-Akt-NF-κB signaling pathway, in line with other studies[7,8,40]. However, the lack of effect of NSP-*REG4* on aggressive phenotypes and related signaling proteins suggested that *REG4* only functioned in


A
Input
SAHA
siHDAC
ND
IgG
PC
Ctr
REG4
M
AC-H3
AC-H4
HDAC
SREBP1
ING5
ACLY
ACC1
Degree of association
AC-H3 ACLY
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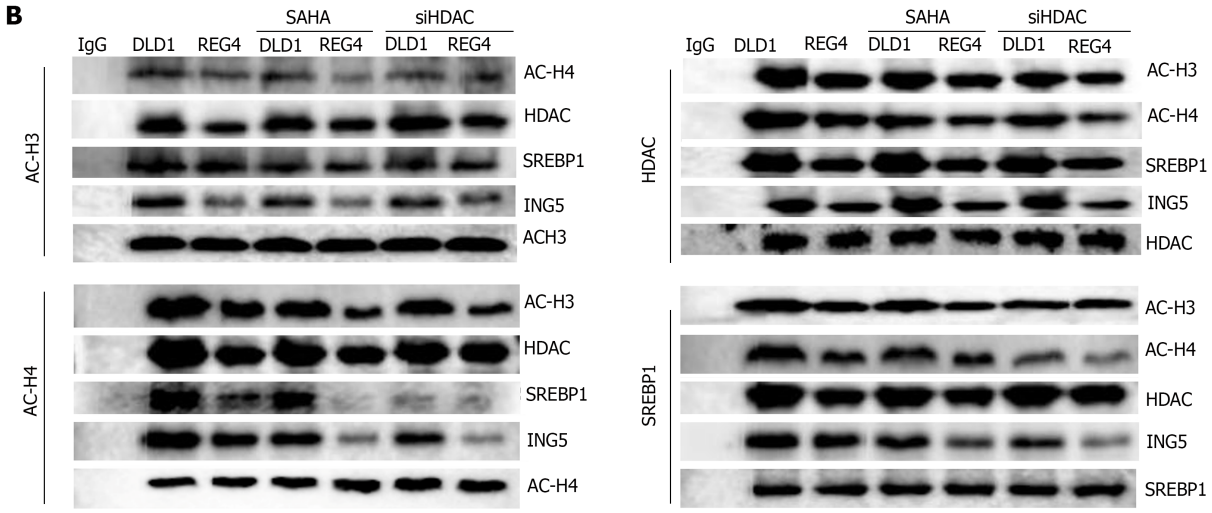
HDAC ACLY
SPEBP1 ACLY
ING5 ACLY
AC-H4 ACC1
HDAC ACC1
SREBP1 ACC1
ING5 ACC1


B
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DLD1
REG4
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AC-H3
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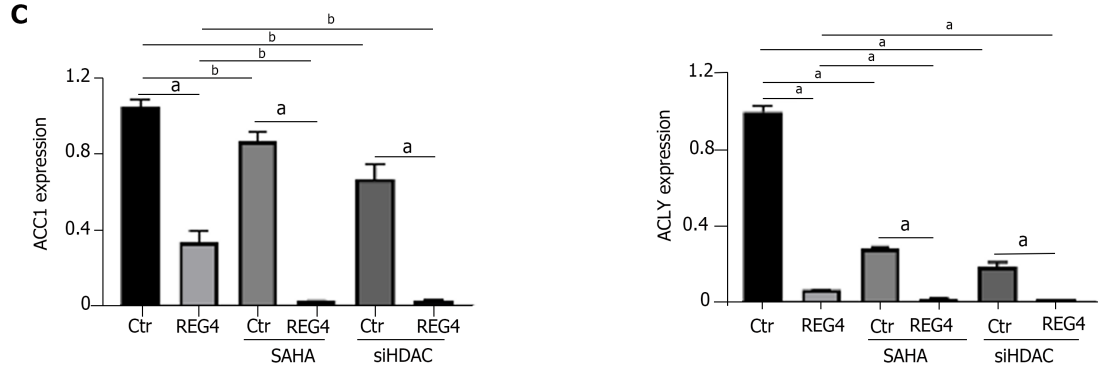
ACH3


C
ACC1 expression
ACLY expression
Ctr
REG4
SAHA
siHDAC

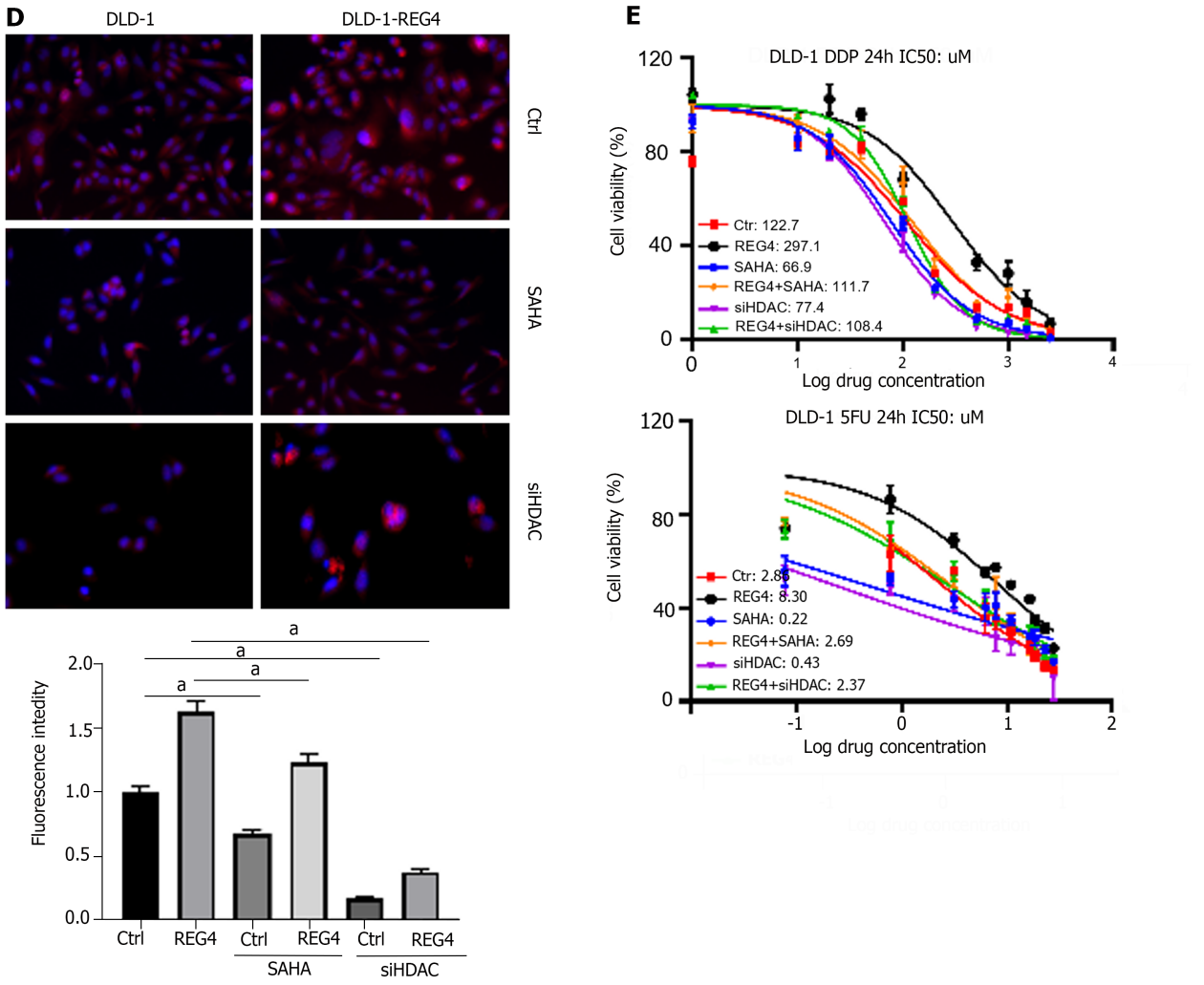


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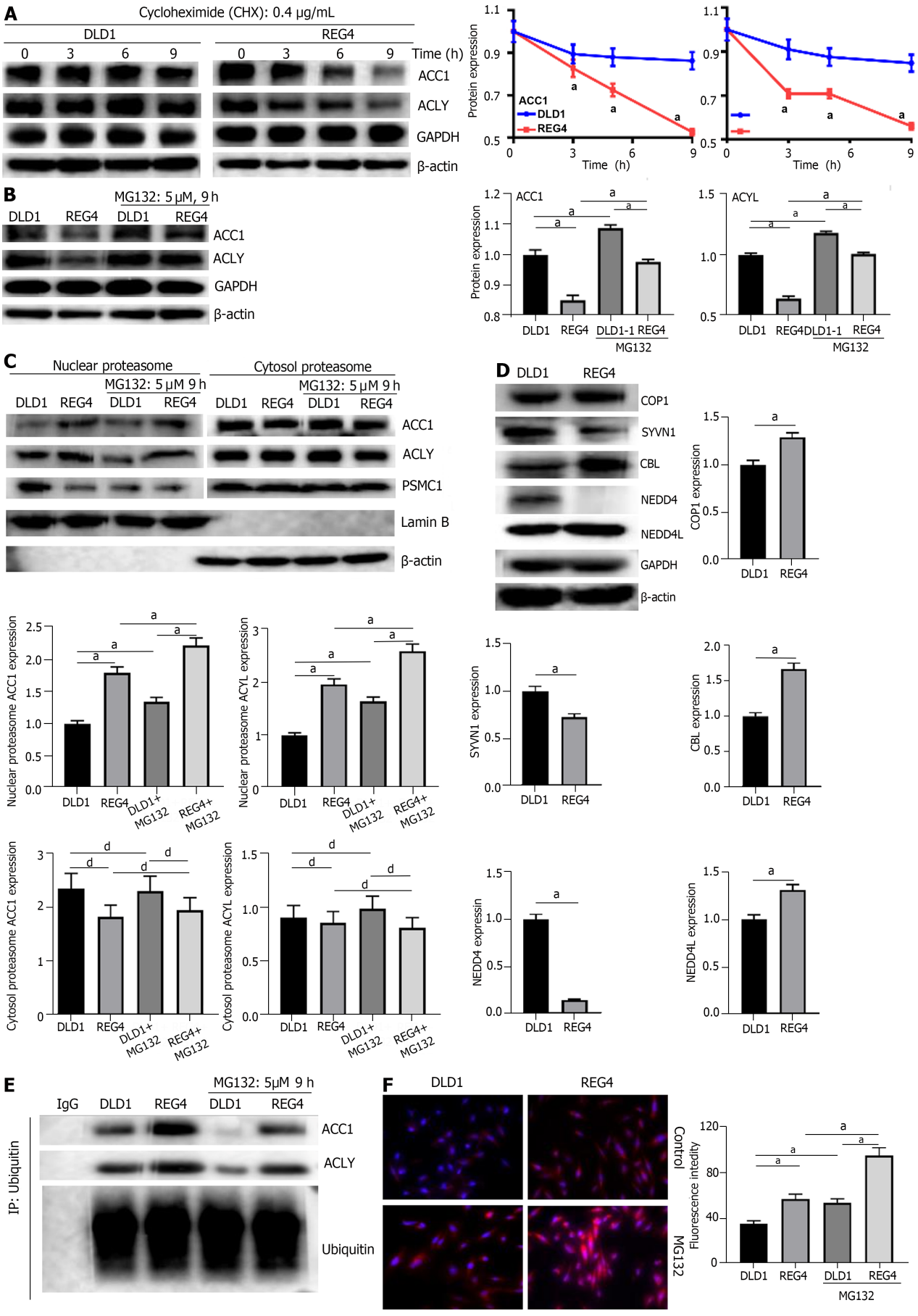
Figure 3 Full-length regenerating gene 4 weakened the transcription of acetyl-CoA carboxylase 1 and ATP-citrate lyase. A: DLD-1 cells and full-length-regenerating gene 4 (FL-*REG4*) transfectants were treated with suberoylanilide hydroxamic acid (SAHA) or small interfering RNA against histone deacetylase (siHDAC), and analyzed by chromatin immunoprecipitation; B: DLD-1 cells and FL-*REG4* transfectants were treated with SAHA, or siHDAC, and analyzed by co-immunoprecipitation assay using anti-anti-acetyl (AC)-acetyl-histone 3-AC-histone 4, anti-HDAC, anti-sterol-regulatory element binding protein 1 or anti-inhibitor of growth protein 5 antibody; C: DLD-1 cells and FL-*REG4* transfectants were treated with SAHA or siHDAC, and analyzed by quantitative reverse transcription polymerase chain reaction; D: DLD-1 cells and FL-*REG4* transfectants were treated with SAHA or siHDAC, and analyzed by Nile red staining; E: DLD-1 cells and FL-*REG4* transfectants were treated with SAHA or siHDAC, and analyzed by half maximal inhibitory concentration assay of 5-fluorouracil and cisplatin. $^{a}P < 0.001$; $^{b}P < 0.01$. *REG4*: Regenerating gene 4; SAHA: Suberoylanilide hydroxamic acid; siHDAC: Small interfering RNA against histone deacetylase; IgG: Immunoglobulin G; ND: No DNA; ACC1: Acetyl-CoA carboxylase 1; ACCY: ATP-citrate lyase; AC-H3: Acetyl-acetyl-histone 3; H4: Histone 4; SREBP1: Sterol-regulatory element binding protein 1; ING5: Inhibitor of growth protein 5; Ctr: Control DLD-1 cells; PC: Positive control.

CRC cells in an autocrine or paracrine manner. These findings demonstrated that *REG4* might be a potential molecular target for gene therapy of CRC.

DLD-1 cells developed resistance to 5-FU and DDP as a result of *REG4* overexpression. 5-FU- or DDP-resistant DLD-1 cells showed *REG4* overexpression, suggesting a role for *REG4* in chemoresistance, in line with previous studies[22,55]. The chemoresistance of CRC cells was produced by lysophosphatidylcholine acyltransferase 2-mediated lipid droplet formation[58], which was also aided by prothymosin α[59], and metastasis-associated in colon cancer[60] through SREBP-1- and fatty acid synthase-mediated and lipogenesis, respectively. Crucial enzymes for de novo fatty acid synthesis are ACC1 and ACLY, which are closely linked to chemoresistance[61]. In the liver and peritoneal tissues, lipid droplet assembly is mediated byadipocyte differentiation-related protein, CIDE, ACAT1, perilipin 5 and TIP47[62-66]. *REG4*-mediated lipid droplet formation might be closely linked to the upregulated expression of ACAT1, perilipin 5 and TIP47 in *REG4* transfectants, but not with de novo lipogenesis, as demonstrated by the downregulated expression of ACC1 and ACLY. *REG4*-mediated lipid droplet formation might account for *REG4*-induced resistance to 5-FU and DDP, which can be reversed by ACC1 or ACLY inhibitor, but deteriorated by high glucose exposure. In combination with these discoveries, we hypothesized that *REG4* may play an important role in chemoresistance, not through de novo lipogenesis, but by lipid droplet assembly, and might be used as a potential target for reversal of chemoresistance in CRC. According to the increased IC_{50} of DLD-1 cells after high glucose treatment, we speculate that high glucose treatment provides more glucose and increases the de novo synthesis of lipid droplets, thereby mediating the drug resistance of CRC cells.


A
Cycloheximide (CHX): 0.4 μg/mL
DLD1
REG4
0 3 6 9
Time (h)
ACC1
ACLY
GAPDH
β-actin
Protein expression
Time (h)
B
MG132: 5 μM, 9 h
DLD1 REG4 DLD1 REG4
C
Nuclear proteasome
Cytosol proteasome
PSMC1
Lamin B
Nuclear proteasome ACC1 expression
Nuclear proteasome ACYL expression
Cytosol proteasome ACC1 expression
Cytosol proteasome ACYL expression
DLD1+ MG132
REG4+ MG132
D
COP1
SYVN1
CBL
NEDD4
NEDD4L
COP1 expression
SYVN1 expression
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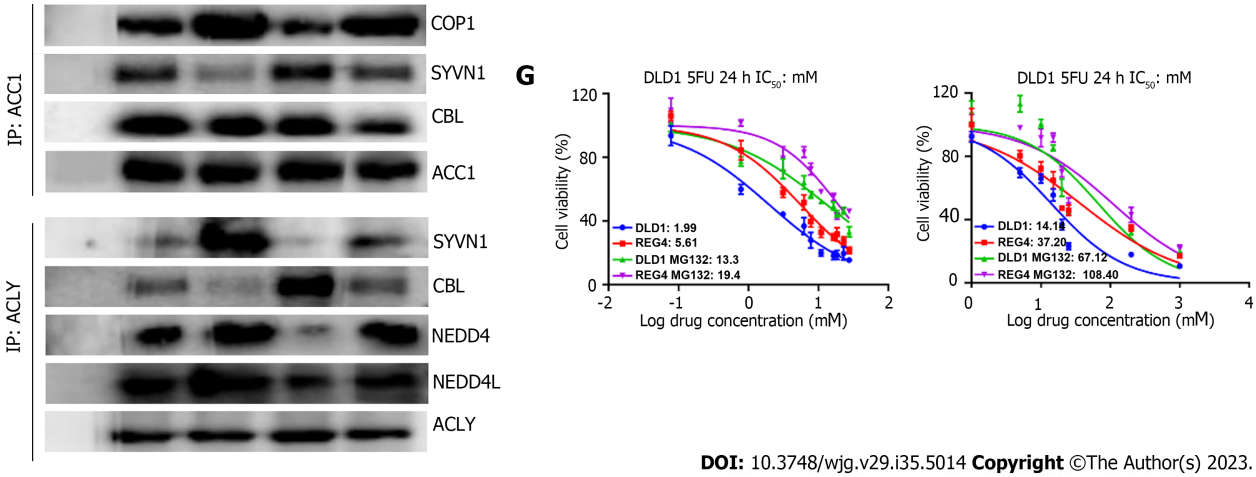

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Figure 4 Full-length regenerating gene 4 destablized acetyl-CoA carboxylase 1 and ATP-citrate lyase proteins *via* proteasomal degradation. A: DLD-1 cells and full-length-regenerating gene 4 (FL-*REG4*) transfectants were treated with cycloheximide (0.4 μg/mL), followed by western blotting to detect acetyl-CoA carboxylase 1 (ACC1) and ATP-citrate lyase (ACLY) expression; B: DLD-1 cells and FL-*REG4* transfectants were treated with MG132 (5 μM, 9 h), followed by western blotting to detect ACC1 and ACLY expression; C: DLD-1 cells, FL-*REG4* transfectants, and MG132-treated DLD-1 cells and FL-*REG4* transfectants were subjected to proteasomal extract and western blotting to detect ACC1 and ACLY expression; D: Ubiquitin transferases (COP1 E3 ubiquitin ligase, synoviolin 1, Cbl proto-oncogene, NEDD4 like E3 ubiquitin protein ligase were detected by western blotting in DLD-1 cells and FL-*REG4* transfectants; E: After co-immunoprecipitation, western blotting was performed in DLD-1 cells and FL-*REG4* transfectants, with or without MG132 treatment; F: Nile red staining of DLD-1 cells, FL-*REG4* transfectants, and MG132-treated DLD-1 cells and FL-*REG4* transfectants; G: Tetrazolium salt assay was performed on DLD-1 cells, FL-*REG4* transfectants and MG132- treated DLD-1 cells and FL-*REG4* transfectants to detect half maximal inhibitory concentration of 5-fluorouracil and cisplatin. [a]*P* < 0.001; [d]No significance. *REG4*: Regenerating gene 4; ACC1: Acetyl-CoA carboxylase 1; ACCY: ATP-citrate lyase; COP1: COP1 E3 ubiquitin ligase; SYVN1: Synoviolin 1; CBL: Cbl proto-oncogene; NEDD4L: NEDD4 like E3 ubiquitin protein ligase; IgG: Immunoglobulin G.

We found that *REG4*-related signaling pathways included chemokine activity, taste receptors, protein-DNA and DNA packing complexes for transcription repression and activation, nucleosomes and chromatin, generation of second messenger molecules, HDAC and histone acetyltransferase for epigenetic regulation, and sugar metabolism. Therefore, we investigated the regulatory effects of *REG4* on the transcription of ACC1 and ACLY. *REG4* decreased expression of AC-H3, AC-H4, ING5, HDAC and SREBP1. *REG4* also weakened the interaction of the five proteins with the promoters of ACC1 or ACLY, or complex formation of the five proteins, and mRNA expression of ACC1 or ACLY. The inhibitory effect of FL-*REG4* transcription can be enhanced by low concentration SAHA (HDAC inhibitor) treatment or transfection with siHDAC. We speculated that HDAC hypoexpression or inactivation might increase the chemosensitivity of CRC cells by inhibiting the de novo lipogenesis, which suggests a potential clinical application for the antitumor drug SAHA. The decrease in lipid droplet formation and increase in chemotherapy sensitivity of DLD-1 cells after SAHA or siHDAC treatment indirectly confirm this view. We hypothesized that *REG4* inhibited the transcription of ACC1 and ACLY by disassociating the complex formation of AC-H3-AC-H4-ING5-HDAC-SREBP1 in their promoters and releasing the combination of ACC1 and ACLY promoters and complex *via* HDAC-mediated deacetylation.

To date, there has been no research about the effects of *REG4* on metabolic reprogramming, histone modification, and microenvironmental stress, and the interaction between *REG4* and metabolic or epigenetic pathways in CRC. With regard to drug resistance, Ying *et al*[67] found that upregulation of *REG4* mRNA was closely linked to the intrinsic drug resistance of gastric cancer cells to 5-FU. All 14 *REG4*-positive patients with gastric cancer showed no change or disease progression when treated with a combination of low-dose 5-FU and DDP[1]. In gastric cancer cells, *REG4* enhanced the resistance to 5-FU through the MAPK–ERK–Bim pathway[1]. Anti-*REG4* antibody significantly inhibited proliferation and chemosensitivity of gastric cancer cells to 5-FU, and *REG4* silencing caused the loss of stemness properties[68,69]. In ovarian cancer cells, *REG4* overexpression or rh*REG4* treatment promoted proliferation, G_2/S progression, antiapoptosis, migration, invasion, and DDP and paclitaxel[12,57]. In future research, we will also explore the potential interaction between *REG4* and metabolic or epigenetic pathways in CRC, which provides a new method for the early diagnosis and targeted therapy of CRC.

Finally, we also analyzed the modulatory effects of *REG4* on the protein stability of ACC1 and ACLY. Firstly, we treated DLD-1 cells and *REG4* transfectants with CHX, and found that *REG4* destabilized ACC1 and ACLY. *REG4* promoted the recruitment of ACC1 and ACLY to the nuclear proteasome for ubiquitylation-mediated degradation, during which ACLY bound to NEDD4, SYVN1 and NEDD4L, and ACC1 bound to COP1. Overexpression of *REG4* mediated lipid droplet formation and chemoresistance in DLD-1 cells. These results suggested that *REG4* facilitated proteasomal degradation of ACC1 and ACLY to suppress de novo lipogenesis. We also speculate that pretreatment of proteasome inhibitor MG132 in DLD-1 cells can increase expression of ACC1 and ACLY by reducing ubiquitination of ACC1 and ACLY, thus increasing formation of lipid droplets and leading to chemoresistance. In the future, we will explore the potential role of *REG4* from metabolic reprogramming and epigenetic regulation.

CONCLUSION

REG4 protein expression was decreased in CRC and positively linked with the degree of invasion, TNM stage, and dedifferentiation, but the converse was the case for *REG4* mRNA expression. *REG4* aggravated aggressive phenotypes in an autocrine or paracrine manner *via* the EGFR-PI3K-Akt-NF-κB signaling pathway. *REG4* may be involved in chemoresistance, not through de novo lipogenesis, but by lipid droplet assembly. *REG4* inhibited the transcription of ACC1 or ACLY by disassociating the complex formation of AC-H3-AC-H4-ING5-histone deacetylase-SREBP1 in their promoters and induced the proteasomal degradation of ACC1 and ACLY proteins. Pretreatment with high glucose might induce chemoresistance of CRC cells, which should be emphasized in clinical practice. SAHA can reverse the chemoresistance of CRC and provide a potential direction for research of DDP and 5-FU resistance of CRC. Finally, *REG4* may be used as a reliable diagnostic marker for the prognosis, aggressiveness and carcinogenesis of CRC and is a potential molecular target.

ARTICLE HIGHLIGHTS

Research background

Regulating gene 4 (*REG4*) has been proved to be carcinogenic in some cancers, but its manifestation and possible carcinogenic mechanism in colorectal cancer (CRC) have not yet been elucidated. Our previous study found that the drug resistance characteristics of CRC cells may be related to their fat metabolism.

Research motivation

With the aging of the world population, the incidence of CRC is increasing. For the treatment of CRC, chemoresistance has always been an urgent problem to be solved.

Research objectives

This study aimed to explore the role of *REG4* in CRC and its association with lipid droplet formation, and the molecular mechanisms involved.

Research methods

We conducted a meta-analysis and bioinformatics and pathological analysis of *REG4* expression in CRC. The effects of *REG4* on the phenotypes and related proteins were also investigated in CRC cells.

Research results

Compared to normal mucosa, *REG4* mRNA expression was high in CRC, but protein expression was opposite. *REG4*-related genes included epigenetic regulation, transcription repression, sugar metabolism and transfer. *REG4* exposure or overexpression promoted proliferation, antiapoptosis, migration and invasion of DLD-1 cells in an autocrine or paracrine manner by activating the epidermal growth factor receptor-phosphoinositide 3-kinase-Akt-nuclear factor-κB pathway. *REG4* was involved in chemoresistance not through de novo lipogenesis, but lipid droplet assembly, which was strengthened by high glucose treatment. *REG4* inhibited the transcription of acetyl-coA carboxylase 1 (ACC1) and ATP-citrate lyase (ACLY) by disassociating the complex formation of anti–acetyl (AC)-acetyl-histone 3-AC-histone 4-inhibitor of growth protein-5-si histone deacetylase-sterol-regulatory element binding protein 1 in their promoters and induced proteasomal degradation of ACC1 or ACLY.

Research conclusions

REG4 may be an indicator of drug resistance and metabolism of tumor cells. *REG4* might be a useful marker for colorectal carcinogenesis, as well as a potential gene therapy target.

Research perspectives

This study provides new insights into a better understanding of the pathogenesis of CRC. *REG4* may be used as a novel therapeutic target. However, the regulatory mechanism needs to be further explored.

FOOTNOTES

Author contributions: Zhang CY and Zheng HC designed the study; Zhang CY participated in data collection; Zheng HC wrote the paper; Zhang R, Zhang L, Wang ZM, Sun HZ and Cui ZG were responsible for revising the manuscript; All the listed authors have contributed and approved the final manuscript.

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Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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Country/Territory of origin: China

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ORIGINAL ARTICLE

Retrospective Cohort Study

Clinical characteristics and outcome of autoimmune pancreatitis based on serum immunoglobulin G4 level: A single-center, retrospective cohort study

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Abstract

BACKGROUND

Autoimmune pancreatitis (AIP) has been linked with elevated immunoglobulin (Ig) G4 levels. The characteristics and outcomes of AIP based on serum markers have not been fully evaluated.

AIM

To compare clinical features, treatment efficacy, and outcome of AIP based on serum IgG4 levels and analyze predictors of relapse.

METHODS

A total of 213 patients with AIP were consecutively reviewed in our hospital from 2006 to 2021. According to the serum IgG4 level, all patients were divided into two groups, the abnormal group (n = 148) with a high level of IgG4 [> 2 × upper limit of normal (ULN)] and the normal group (n = 65). The t-test or Mann-Whitney U test was used to compare continuous variables. Categorical parameters were compared by the χ^2 test or Fisher's exact test. Kaplan-Meier curves

and log-rank tests were established to assess the cumulative relapse rates. Univariate and multivariate analyses were used to investigate potential risk factors of AIP relapse.

RESULTS

Compared with the normal group, the abnormal group had a higher average male age (60.3 ± 10.4 *vs* 56.5 ± 12.9 years, $P = 0.047$); higher level of serum total protein (72.5 ± 7.9 g/L *vs* 67.2 ± 7.5 g/L, $P < 0.001$), IgG4 (1420.5 ± 1110.9 mg/dL *vs* 252.7 ± 106.6 mg/dL, $P < 0.001$), and IgE (635.6 ± 958.1 IU/mL *vs* 231.7 ± 352.5 IU/mL, $P = 0.002$); and a lower level of serum complement C3 (100.6 ± 36.2 mg/dL *vs* 119.0 ± 45.7 mg/dL, $P = 0.050$). In addition, a lower number of cases with abnormal pancreatic duct and pancreatic atrophy (23.6% *vs* 37.9%, $P = 0.045$; 1.6% *vs* 8.6%, $P = 0.020$, respectively) and a higher rate of relapse (17.6% *vs* 6.2%, $P = 0.030$) were seen in the abnormal group. Multivariate analyses revealed that serum IgG4 [(> 2 × ULN), hazard ratio (HR): 3.583; 95% confidence interval (CI): 1.218–10.545; $P = 0.020$] and IgA (> 1 × ULN; HR: 5.908; 95%CI: 1.199–29.120; $P = 0.029$) and age > 55 years (HR: 2.383; 95%CI: 1.056–5.378; $P = 0.036$) were independent risk factors of relapse.

CONCLUSION

AIP patients with high IgG4 levels have clinical features including a more active immune system and higher relapse rate. Several factors, such as IgG4 and IgA, are associated with relapse.

Key Words: Autoimmune pancreatitis; Immunoglobulin G4; Clinical characteristics; Outcome; Relapse; Cohort study

Core tip: Our findings suggested that patients with a high immunoglobulin (Ig) G4 level had different clinical features including a more active immune system and higher relapse rate. Patients with normal IgG4 level still require attention because they have a high incidence of jaundice and pancreatic atrophy. Some factors were identified as risk factors for relapse, such as age > 55 years [hazard ratio (HR) 2.383; 95% confidence interval (CI) 1.056-5.378; $P = 0.036$], high IgG4 level [> 2 × upper limit of normal (ULN) (HR 3.583; 95%CI 1.218-10.545; $P = 0.020$) and high IgA level (> 1 × ULN) (HR 5.908; 95%CI 1.199-29.120; $P = 0.029$).

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INTRODUCTION

Autoimmune pancreatitis (AIP) has recently become a cause of global health concern[1,2]. It is characterized by elevated serum immunoglobulin (Ig) G4 levels and an enlarged pancreas based on diagnostic imaging. AIP is a form of chronic pancreatitis that was initially introduced in 1995 by Yoshida *et al*[3]. However, understanding of the pathology was limited since then until Hamano *et al*[4] introduced the role of IgG4 in AIP.

IgG4 has unique properties, unlike other immunoglobulin subtypes. Among the four IgG subclasses, IgG4 levels are the lowest, accounting for about 5% of total IgG in a healthy adult. IgG4 inhibits the formation of immune complexes through fab-arm exchange and does not affect the classical complement pathway[5]. A correlation between the IgG4 concentration and chronic inflammatory processes and disease severity has been established; the role of IgG4 and disease pathogenicity has also been proposed in previous studies[6,7]. Elevated IgG4 levels have also been observed in autoimmune diseases, such as rheumatoid arthritis, and patients with high IgG4 levels have shown a unique clinical profile[8]. Although the etiology is not fully elucidated, the role of IgG4 in AIP pathogenesis was highlighted as a potential diagnostic marker and predictor of relapse[9-13].

A favorable response despite frequent relapse in the long term is generally seen in patients with AIP receiving steroid therapy. To date, the risk factors of relapse remain controversial. According to an epidemiological survey in Japan, > 20% of patients diagnosed with AIP experienced relapse at least once. It was less likely that the initial steroid doses and serum IgG4 level affected the appearance of relapse[1], while another study reported a higher relapse rate in patients with AIP having high IgG4 levels[14]. In addition, a cohort study highlighted IgG4-related sclerosing cholangitis as a predictor of relapse[15]. In the present retrospective cohort study, we compared the clinical characteristics of patients with AIP based on the serum IgG4 level and analyzed the potential risk factors of relapse.

MATERIALS AND METHODS

Ethics

This was a single-center retrospective study performed at the First Medical Center of Chinese PLA General Hospital. The research protocol was approved by the Ethical Committee of Chinese PLA General Hospital (S2022-330-01).

Study design and population

A total of 308 patients from 2006 to 2021 were reviewed consecutively (Figure 1). Ninety-five patients were excluded due to other chief diagnoses (n = 20), insufficient data (n = 31), and non-fulfillment of the International Consensus Diagnostic Criteria (ICDC; n = 44). Eventually, 213 patients diagnosed with AIP according to ICDC were enrolled. All enrolled patients were divided into two groups based on serum IgG4 levels: normal group [IgG4 ≤ 402 mg/dL, 2 × upper limit of normal (ULN)] and abnormal group (IgG4 > 402 mg/dL)[16].

Demographic characteristics of age, sex, and duration of hospitalization were evaluated. Clinical manifestations included predispositions, symptoms, such as abdominal pain, and involvement of other organs. Weight loss was defined as > 5 kg in the past 3 mo. Extrapancreatic lesions were diagnosed by the IgG4-RD criteria, 2021[17]. Results of blood routine examination, biochemistry, coagulation, and immunological tests were collected. Radiological findings were analyzed from computed tomography (CT), magnetic resonance imaging, and endoscopic retrograde cholangiopancreatography/magnetic resonance cholangiopancreatography. The maximum standard uptake value (SUV-max) of positron emission tomography/CT (PET/CT) was also compared. The effectiveness of treatment was based on patient symptoms and serological and radiological results. Relapse was defined as the reoccurrence of symptoms, abnormal serological results, or the presence of imaging lesions.

Statistical analysis

The t-test or Mann-Whitney U test was used to compare continuous variables, which were presented as mean ± SD or median interquartile range. Categorical parameters were compared using the χ^2 test or Fisher's exact test. Kaplan-Meier curves and log-rank tests were used to assess cumulative relapse rates and univariate and multivariate Cox regression models were performed, respectively. Baseline variables that were considered clinically relevant and showed a univariate relationship with outcome were entered into the multivariate Cox regression model. Variables for inclusion were carefully chosen, considering the number of available events, to ensure the parsimony of the final model. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism 9.0 and SPSS 26.0. The statistical review of this study was performed by a biomedical statistician.

RESULTS

Demographic characteristics

Baseline data are summarized in Table 1 and Supplemental Figures 1-3. There were 65 and 148 patients in the normal and abnormal groups, respectively. There were 189 patients with type 1 AIP and 24 with type 2 AIP. The ratio of men to women was 3.06 and 3.63 in the two groups (P = 0.722), respectively. There was no significant difference in the mean age between the two groups (59.8 ± 11.0 years *vs* 57.4 ± 12.6 years, P = 0.163). However, the average age of men in the abnormal group was higher than that in the normal group (60.3 ± 10.4 years *vs* 56.5 ± 12.9 years, P = 0.047). The duration from onset to diagnosis (78.4 ± 71.7 d *vs* 93.1 ± 124.8 d, P = 0.686) and the days of hospitalization (16.5 ± 8.7 d *vs* 15.6 ± 9.0 d, P = 0.351) were similar between the normal and abnormal groups.

Clinical manifestations

Most patients in the normal and abnormal groups had no obvious causes before their onset (86.2% *vs* 84.5%, P = 0.750; Table 2). The frequency of patients' chief complaints including abdominal pain, jaundice, abdominal distension, and pruritus of patients was similar in the two groups (36.9% *vs* 42.6%, P = 0.440; 30.8% *vs* 28.4%, P = 0.724; 16.9% *vs* 20.9%, P = 0.497; 9.2% *vs* 14.9%, P = 0.263, respectively). It is noteworthy that seven patients in the abnormal group visited a doctor because of diarrhea, whereas no patient in the normal group went to the hospital for diarrhea (4.7% *vs* 0%, P = 0.104). The incidence of weight loss was 52.3% and 50.7% in the normal and abnormal groups, respectively (P = 0.826). With regard to extrapancreatic lesions, sclerosing cholangitis was the most common disease with a similar prevalence rate between the normal and abnormal groups (30.8% *vs* 37.2%, P = 0.368).

Serology results

Laboratory findings are shown in Table 3. The proportion of white blood cells was different between the two groups. Patients in the abnormal group had a higher percentage of eosinophils (6.2 ± 5.4% *vs* 3.9 ± 3.4%, P = 0.001) and a lower percentage of neutrophils (56.1 ± 10.3% *vs* 61.8 ± 11.1%, P = 0.001). The total protein level was higher in the abnormal group (72.5 ± 7.9 g/L *vs* 67.2 ± 7.5 g/L, P < 0.001) and serum albumin level was lower (36.7 ± 4.6 g/L *vs* 39.4 ± 5.8 g/L, P = 0.001). The thrombin time was also longer in the abnormal group (17.4 ± 1.6 s *vs* 16.9 ± 1.2 s, P = 0.012). With regard to the immunological tests, patients in the abnormal group had higher levels of IgE (635.6 ± 958.1 IU/mL *vs* 231.7 ± 352.5 IU/mL, P = 0.002), four IgG subtypes (IgG1, 935.8 ± 319.9 mg/dL *vs* 872.6 ± 371.8 mg/dL, P = 0.045; IgG2, 638.6 ± 241.6 mg/dL *vs* 450.4 ± 174.0 mg/dL, P < 0.001; IgG3, 53.6 ± 58.4 mg/dL *vs* 42.70 ± 43.37 mg/dL, P = 0.034; IgG4, 1420.5 ± 1110.9 mg/dL *vs* 252.7 ± 106.6 mg/dL, P < 0.001), and two subtypes of free light chain [(FLC)-κ, 569.6 ± 252.5 mg/dL *vs* 306.1 ±

Table 1 Demographic characteristics of patients in the two groups

	Normal group (*n* = 65)	Abnormal group (*n* = 148)	*P* value
Male (*n*, %)	49 (75.4)	116 (78.4)	0.722
Ratio (men to women)	3.06	3.63	0.722
Age (yr)	57.4 ± 12.6	59.8 ± 11.0	0.163
Age of men	56.5 ± 12.9	60.3 ± 10.4	0.047[a]
Age of women	60.1 ± 11.8	57.94 ± 12.9	0.572
Duration before diagnosis (d)	78.4 ± 71.7	93.1 ± 124.8	0.686
Lengths of stay (d)	16.5 ± 8.7	15.6 ± 9.0	0.351

[a]$P < 0.05$ *vs* normal group.

Table 2 Clinical symptoms of patients in the two groups (*n*, %)

	Normal group (*n* = 65)	Abnormal group (*n* = 148)	*P* value
Inducements			
No obvious cause	56 (86.2)	125 (84.5)	0.750
Greasy food intake	7 (10.8)	16 (10.8)	0.993
Alcohol intake	2 (3.1)	5 (3.4)	0.910
Initial symptoms			
Abdominal pain	24 (36.9)	63 (42.6)	0.440
Jaundice	20 (30.8)	42 (28.4)	0.724
Abdominal distension	11 (16.9)	31 (20.9)	0.497
Pruritus	6 (9.2)	22 (14.9)	0.263
Diarrhea	0 (0.0)	7 (4.7)	0.104
Accompanied symptoms			
Jaundice	46 (70.8)	86 (58.1)	0.080
Abdominal pain	28 (43.1)	70 (47.3)	0.569
Pruritus	15 (23.1)	37 (25.0)	0.764
Abdominal distension	11 (16.9)	34 (23.0)	0.319
Loss of weight	34 (52.3)	75 (50.7)	0.826
Extrapancreatic lesions			
Sclerosing Cholangitis	20 (30.8)	55 (37.2)	0.368
Salivary and lacrimal gland	7 (10.8)	26 (17.6)	0.207
Retroperitoneal fibrosis	3 (4.6)	6 (4.1)	0.851
Kidney	4 (6.2)	10 (6.8)	0.870
Lung	1 (1.5)	2 (1.4)	0.915

99.7 mg/dL, $P < 0.001$; FLC-λ, 282.5 ± 124.5 mg/dL *vs* 176.4 ± 63.4 mg/dL, $P < 0.001$)]. A lower complement C3 level (100.6 ± 36.2 mg/dL *vs* 119.0 ± 45.7 mg/dL, $P = 0.050$) and a faster erythrocyte sedimentation rate (21.1 ± 20.1 mm/h *vs* 43.4 ± 28.6 mm/h, $P = 0.002$) were noted in the abnormal group. Positive rate of serum antinuclear antibody was similar in the normal and abnormal groups (12.5% *vs* 20.0%, $P = 0.298$).

Radiology examinations

AIP can be categorized as diffuse or focal type based on the extent of pancreatic enlargement in the radiological examinations (Table 4). In the normal group, 33 of 58 (56.9%) cases were diffuse type and 16 (27.6%) were focal type. In the abnormal group, 86 (67.7%) of 127 cases were diffuse type and 24 (18.9%) were focal type. The capsule-like rim was

Table 3 Laboratory results of patients in the two groups (*n*, %)

	Normal group (*n* = 65)	Abnormal group (*n* = 148)	*P* value
Hb (g/L)	126.3 ± 16.8	126.8 ± 14.6	0.828
WBC (× 10^9/L)	6.5 ± 2.2	5.8 ± 1.8	0.026[a]
NEUT	61.8 ± 11.1	56.1 ± 10.3	0.001[a]
LY	26.8 ± 9.8	29.2 ± 8.2	0.071
EOS	3.9 ± 3.4	6.2 ± 5.4	0.001[a]
BASO	0.6 ± 0.4	0.8 ± 0.5	0.003[a]
PLT (× 10^9/L)	212.9 ± 83.3	218.6 ± 75.0	0.621
TP (g/L)	67.2 ± 7.5	72.5 ± 7.9	< 0.001[a]
ALB (g/L)	39.4 ± 5.8	36.7 ± 4.6	0.001[a]
TT (s)	16.9 ± 1.2	17.4 ± 1.6	0.012[a]
IgG1 (mg/dL)	872.6 ± 371.8	935.8 ± 319.9	0.045[a]
IgG2 (mg/dL)	450.4 ± 174.0	638.6 ± 241.6	< 0.001[a]
IgG3 (mg/dL)	42.70 ± 43.37	53.6 ± 58.4	0.034[a]
IgG4 (mg/dL)	252.7 ± 106.6	1420.5 ± 1110.9	< 0.001[a]
IgE (IU/mL)	231.7 ± 352.5	635.6 ± 958.1	0.002[a]
IgG (mg/dL)	1323.6 ± 503.9	2062.1 ± 918.9	< 0.001[a]
FLC-κ (mg/dL)	306.1 ± 99.7	569.6 ± 252.5	< 0.001[a]
FLC-λ (mg/dL)	176.4 ± 63.4	282.5 ± 124.5	< 0.001[a]
C3 (mg/dL)	119.0 ± 45.7	100.6 ± 36.2	0.050
ESR (mm/h)	21.1 ± 20.1	43.4 ± 28.6	0.002[a]
ANA (positive)	5 (12.5)	19 (20.0)	0.298
CA19-9 (abnormal)	33 (53.0)	73 (52.0)	0.887

[a]*P* < 0.05 *vs* normal group. Hb: Hemoglobin; WBC: White blood cell; NEUT: Neutrophil; LY: Lymphocyte; EOS: Eosinophil; PLT: Hemoglobin; BASO: Basophil; TP: Total protein; ALB: Albumin; TT: Thrombin time; FLC: Free light chain; ANA: Antinuclear antibody; CA19-9: Carbohydrate associated antigen 19-9; Ig: Immunoglobulin; C3: Complement C3; ESR: Erythrocyte sedimentation rate.

observed in 10 (17.2%) and 30 (23.6%) cases in the normal and abnormal groups, respectively (*P* = 0.328). Pancreatic duct images showed that the prevalence of abnormalities (stricture or dilatation) was significantly higher in the normal group (37.9% *vs* 23.6%; *P* = 0.045). Hypodense kidney lesions were seen in 11 (8.7%) cases in the abnormal group and four (6.9%) cases in the normal group (*P* = 0.683). The incidence of pancreatic atrophy was significantly higher in the normal group (8.6% *vs* 1.6%, *P* = 0.020). The two groups had similar SUV-max according to PET/CT [normal group: 4.55 (3.725–6.25) *vs* abnormal group: 5.35 (4.025–6.475), *P* = 0.260].

Remission and relapse

Different therapies for AIP were given to patients in the two groups (Table 5, *P* = 0.029). In the normal group, 26 (40.0%) patients received steroid monotherapy, 14 (21.5%) received steroids plus immunomodulators, 12 (18.5%) underwent endoscopy or surgery, and 13 (20.0%) received other therapies such as hepatic protectors. The median duration and dose of prednisolone therapy were 8 (6–9) wk and 40 (30–50) mg/d. In the abnormal group, 81 (54.7%) patients received steroid monotherapy, 30 (20.3%) received steroids plus immunomodulators, nine (6.1%) underwent endoscopy or surgery, and 28 (18.9%) received other therapies. The median duration and dose of prednisolone therapy were 8 (6–9) wk (*P* = 0.200) and 42.5 (40–100) mg/d (*P* = 0.750). Only three patients in the abnormal group did not achieve remission; all of whom received steroid monotherapy: one patient had persistent symptoms and two had prolonged abnormal transaminase levels.

During the median follow-up period of 53 mo, 30 patients experienced at least one episode of relapse in the normal and abnormal groups [4 (6.9%) *vs* 26 (19.1%), *P* = 0.031]. Of the 26 patients in the abnormal group, five (19.2%) had a relapse in extrapancreatic organs (*P* = 0.337). The cumulative relapse rates at 1 and 3 years were 3.8% and 11.0%, respectively, for the abnormal group and 2.0% and 6.9%, respectively, for the normal group (log-rank test, *P* = 0.023, Figure 2). Among the 30 patients with relapse, one (25.0%) of four in the normal group and 15 (57.7%) of 26 in the abnormal group received steroid monotherapy as their initial treatment (*P* = 0.129). Four (15.4%) of 26 relapse cases in the abnormal group

Table 4 Radiological results of patients in the two groups (*n*, %)

	Normal group (*n* = 65)	Abnormal group (*n* = 127)	*P* value
Parenchymal imaging			
Diffuse type	33 (56.9)	86 (67.7)	0.154
Focal type	16 (27.6)	24 (18.9)	0.227
Normal	9 (15.5)	17 (13.4)	0.699
Capsule-like rim	10 (17.2)	30 (23.6)	0.328
Others			
Bile duct enhancement	15 (25.9)	44 (34.7)	0.234
Hypo-dense lesion in kidney	4 (6.9)	11 (8.7)	0.683
Pancreatic atrophy	5 (8.6)	2 (1.6)	0.020[a]
Retroperitoneal fibrosis	3 (5.2)	1 (0.8)	0.057
Pancreatic pseudocyst	1 (1.7)	3 (2.4)	0.782
Pancreatic ductal imaging			
Stricture or dilatation	22 (37.9)	30 (23.6)	0.045[a]
Normal	36 (62.1)	97 (76.4)	
SUV-max	4.55 (3.725-6.25)	5.35 (4.025-6.475)	0.260

[a]$P < 0.05$ *vs* normal group. SUV-max: Maximum standard uptake value.

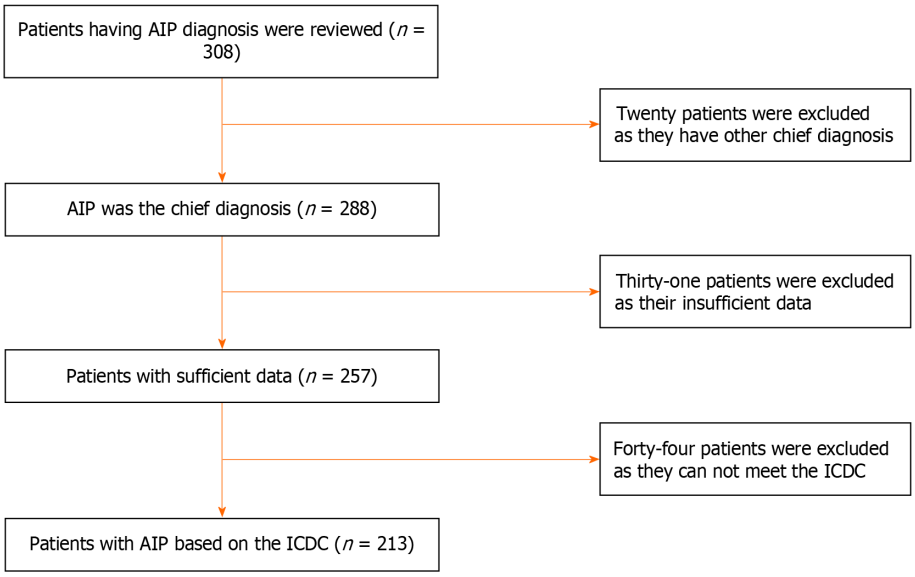

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Figure 1 Flow diagram of the study design. A total of 308 patients were reviewed. Ninety-five patients were excluded due to other chief diagnoses (*n* = 20), insufficient data (*n* = 31), and non-fulfillment of the international consensus diagnostic criteria (*n* = 44). AIP: Autoimmune pancreatitis; ICDC: International consensus diagnostic criteria.

experienced more than one relapse ($P = 0.399$). The median relapse duration in the normal and abnormal groups was 25 (13.5-52.3) and 43 (16.3-72) mo, respectively ($P = 0.442$). Three (75.0%) of four cases in the normal group and 20 (76.9%) of 26 cases in the abnormal group received corticosteroids for relapse ($P = 0.410$). All patients responded well to treatment.

Univariate and multivariate analyses

Univariate and multivariate Cox regression models were performed to predict the risk factors for AIP relapse (Figure 3). Univariate analyses revealed an association between relapse and age > 55 years [hazard ratio (HR): 2.254; 95% confidence interval (CI): 1.074-4.731; $P = 0.032$)]. Similarly, serum IgG4 levels (> 402 mg/dL, 2 × ULN) and IgA levels (> 400 mg/dL,

Table 5 Remission and relapse of patients in the two groups (*n*, %)

	Normal group (*n* = 65)	Abnormal group (*n* = 148)	*P* value
Managements			
Methods			
Steroid monotherapy	26 (40.0)	81 (54.7)	0.029[a]
Steroid and immunomodulator	14 (21.5)	30 (20.3)	
Endoscope or surgery	12 (18.5)	9 (6.1)	
Others[1]	13 (20.0)	28 (18.9)	
Duration of steroid (wk)	8 (6-9)	8 (6-9)	0.750
Dose of steroid (prednisolone, mg/d)	42.5 (40-100)	40 (30-50)	0.200
Remission	65 (100.0)	145 (98.0)	0.555
	Normal group (*n* = 4)	**Abnormal group (*n* = 26)**	***P* value**
Relapse			
Relapse rate	4/58 (6.2)	26/135 (17.6)	0.030[a]
Relapse duration (mo)	25 (13.5-52.3)	43 (16.3-72)	0.442
Lesions of relapse			
Pancreas	4 (100.0)	21 (80.8)	0.337
Extrapancreatic organs	0 (0.0)	5 (19.2)	
Times of relapse			
Once	4 (100.0)	22 (84.6)	0.399
More than one time	0 (0.0)	4 (15.4)	
Initial therapies			
Steroid monotherapy	1 (25.0)	15 (57.7)	0.129
Steroid and immunomodulator	2 (50.0)	2 (7.70)	
Endoscope or surgery	0 (0.00)	2 (7.70)	
Others[1]	1 (25.0)	7 (26.9)	
Therapies for relapse			
Steroid monotherapy	2 (50.0)	18 (69.2)	0.410
Steroid and immunomodulator	1 (25.0)	2 (7.70)	
Endoscope or surgery	1 (25.0)	2 (7.70)	
Others[1]	0 (0.00)	4 (15.4)	

[a]$P < 0.05$ *vs* normal group.
[1]Others: Hepatic protectors, antibiotics and proton pump inhibitors.

1 × ULN) were significant contributors to relapse (IgG4, HR: 3.381, 95%CI: 1.176–9.726, $P = 0.024$; IgA, HR: 6.271, 95%CI: 1.294–30.389, $P = 0.023$). Patients with a thickened bile duct seen on imaging scans also showed a higher risk of relapse (HR: 2.619; 95%CI: 1.096–6.258, $P = 0.030$). The presence of some clinical symptoms such as abdominal pain, type of parenchymal imaging, type of managements, initial dose of steroid and absence of maintenance therapy, were not significantly different between the relapse and non-relapse groups. Using multivariate analyses, we identified three significant independent predictors of relapse, IgG4 levels (> 402 mg/dL; HR: 3.583, 95%CI: 1.218-10.545; $P = 0.020$), IgA levels (> 400 mg/dL; HR: 5.908; 95%CI: 1.199–29.120; $P = 0.029$), and age > 55 years (HR: 2.383, 95%CI: 1.056–5.378; $P = 0.036$).

DISCUSSION

AIP is characterized by elevated IgG4 levels in the blood and abundant infiltration of IgG4-positive plasma cells and

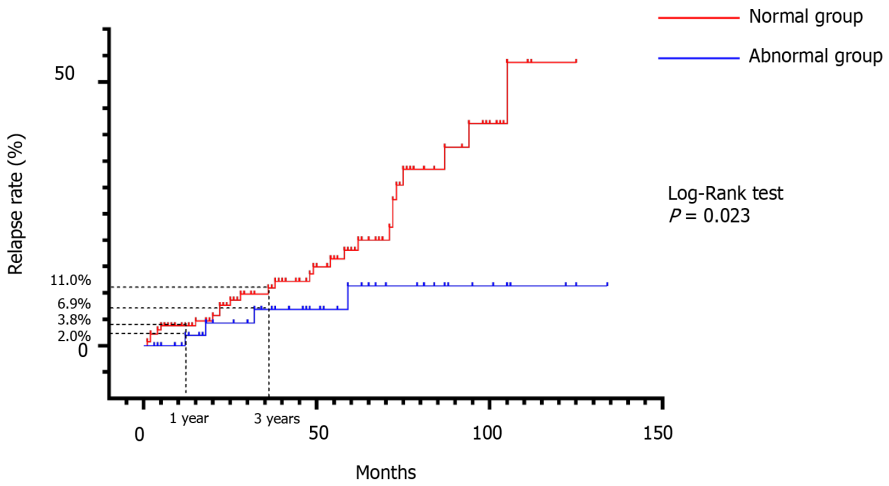

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Figure 2 Cumulative relapse rates in the normal and abnormal groups. The cumulative relapse rates at 1 and 3 years were 3.8% and 11.0%, respectively, for the abnormal group and 2.0% and 6.9%, respectively, for the normal group (Log-Rank test, $P = 0.023$).

lymphocytes in tissues. Serum IgG4 has been recognized as an established diagnostic biomarker for AIP[1,2,18]. In 2011, the ICDC formally defined a cut-off with 2 × ULN of IgG4 as a diagnostic criterion for AIP. A sensitivity of 53%-81% and specificity of 90%-99% were reported in studies adopting this cutoff criterion[16,19-21]. Considering the unique features of IgG4, patients with high IgG4 levels may have a unique clinical profile and prognosis. Previous studies have shown that AIP had a favorable but short-term prognosis following steroid therapy, with a relapse rate of 24%-63%[22-25]. Patients at a higher risk of relapse had higher serum IgG4 and alkaline phosphatase concentrations. The involvement of other organs, such as the bile duct and salivary glands, are also recognized as risk factors[12,23,26].

Epidemiological studies revealed that AIP mainly affected elderly adults, especially those > 60 years, with a male predominance, which was also seen in our study[1,27]. In the abnormal group, the average age of male patients was higher compared with the normal group. No significant differences were noted among women with AIP. Similar findings were reported in previous studies[28,29]. Despite the unclear mechanism, a positive correlation was noted between age and serum IgG4 level with gender preference, which may partly explain the higher incidence rate of AIP in elderly men.

Similar to acute pancreatitis, the enlargement of the pancreas stimulates nerve endings on its capsule, leading the most common symptom of abdominal pain in patients. In addition, jaundice was obvious in patients due to the involvement of other organs, such as the bile duct, or the compression by swollen pancreatic parenchyma. Patients in the normal group had a higher incidence of jaundice with no significant differences. Although little is known about its actual mechanism, it reminds us that patients with obstructive jaundice and normal IgG4 levels are likely to be diagnosed with AIP rather than pancreatic carcinoma. Seven patients in the abnormal group had a rare initial symptom of diarrhea, which partly attributes to the abnormal digestive function of the pancreas.

Analysis of the serological results revealed that patients in the abnormal group had a significantly higher total protein level and lower albumin level, indicating a high globulin concentration, along with higher IgG and IgE levels. IgG is a major component of globulin and can be divided into four subclasses based on its structure. It activates the complement system and regulates phagocytosis[30]. In the abnormal group, except for IgG4, the levels of three subclasses of IgG were significantly higher. Some studies have demonstrated an association between IgG1 and the immunopathogenesis of AIP [31,32]. Further studies about the specific mechanism and the relation between each subclass and AIP are required. A strong relationship between allergic diseases and AIP has been identified[33]. Considering the role of IgE in allergic disorders and high levels noted in AIP, especially in the abnormal group, IgE may play a role in the pathogenesis of AIP [13,30,34]. Patients in the abnormal group also had a higher level of two FLC subtypes (FLC-κ and FLC-λ). FLCs are produced during immunoglobulin synthesis and can be detected under normal physiological conditions as they are not bound to the heavy chains. It is speculated that the accumulation of FLC is attributed to the polyclonal activation of B cells. Previous studies have also proposed their role in other autoimmune disorders; thus, FLCs may be a biomarker for AIP[35]. Additionally, those patients with high serum IgG4 levels had a faster erythrocyte sedimentation rate and a lower complement level. Overall, these immunological findings suggest enhanced antibody biosynthesis and a more active immune system in the patient with high IgG4 levels.

Pancreatic findings such as diffuse enlargement or focal low-density mass in radiology are often the typical features indicative of AIP. Although unusual, a low-attenuating rim-like capsule is also regarded as a specific characteristic of AIP. The proportion of different radiological types was similar between the two groups, which implies that there is no relationship between serum IgG4 levels and radiological types, as reported in a previous study[36]. Imaging scans of the pancreatic duct showed that AIP may have diffuse narrowing of the duct with long or multifocal strictures[12,16]. Unexpectedly, the proportion of patients with an abnormality in the pancreatic duct was higher in the normal group, probably due to the limited sample size. A higher rate of atrophy was seen in the normal group, which requires careful attention to avoid the replacement of parenchyma cells by fibrous tissue as the disease progresses and leads to pancreatic dysfunction.

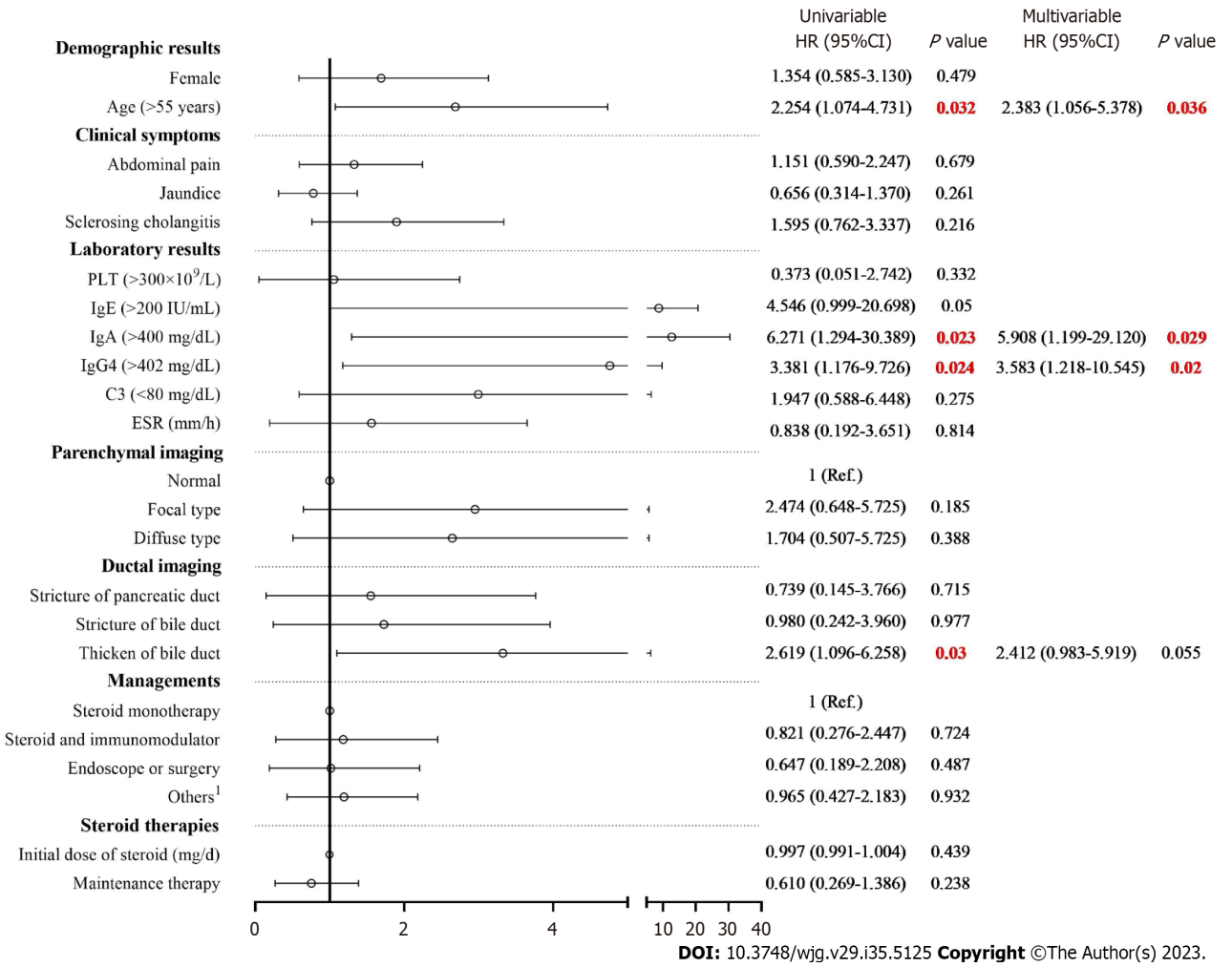

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Figure 3 Univariate and multivariate Cox regression analysis for relapse of autoimmune pancreatitis. Univariate Cox regression analysis indicated that age (> 55 years), immunoglobulin (Ig)A (> 400 mg/dL), IgG4 (> 402 mg/dL), presence of thickened bile duct were risk factors of relapse of autoimmune pancreatitis. Multivariate Cox regression analysis identified three risk factors of relapse, including age (> 55 years), IgA (> 400 mg/dL) and IgG4 (> 402 mg/dL). [1]Others: Hepatic protectors, antibiotics and proton pump inhibitors. PLT: Hemoglobin; Ig: Immunoglobulin; ESR: Erythrocyte sedimentation rate; C3: Complement C3.

Patients in the two groups received varied treatments. In the abnormal group, a higher number of patients received steroid monotherapy, whereas endoscopy or surgery was more common in the normal group. It is possible that the physician's choice of endoscopy or surgery is for biopsy when patients have normal IgG4 levels to exclude cancer diagnosis. Thus, we need simpler and more accurate methods for diagnosis of AIP and to better distinguish it from cancer. To date, the relationship between serum IgG4 level and relapse is controversial. Some studies have demonstrated that an elevated serum IgG4 level is a predictor for relapse[37-39], whereas other studies have reported contrasting findings[23,40]. In the present study, patients in the abnormal group were significantly more likely to experience relapse. Additionally, approximately 20% of patients in the abnormal group had one relapse in the extrapancreatic organs and > 15% of patients had at least two relapses, higher than the normal group. This necessitates further attention to investigate the relationship between high IgG4 levels and AIP.

Previous studies have revealed some controversial predictors of relapse, such as pancreatic enlargement, initial prednisolone dose, and involvement of extrapancreatic organs[1,15,23,41,42]. According to a UK-based study, the relapse rate of patients with elevated serum levels of IgG4 (> 2 × ULN) was twice than that of another group (IgG4 ≤ 2 × ULN) [33]. Similar results were also reported in subsequent studies[42-44]. In the present study, serum IgG4 levels seemed to be an independent risk factor for relapse. Although the specific mechanism remains unclear, the focus on IgG4 should increase owing to its potential effect on the pathogenesis, diagnosis, and prognosis. The concentration of IgA was recognized as an independent risk factor for relapse, which emphasized the association between an active immune system and relapse. IgA is the predominant antibody present at mucosal surfaces, supporting its prominent role in host defense against pathogens. Augmented presence of IgA immune complexes can result in excessive neutrophil activation, potentially leading to severe tissue damage in autoimmune diseases like IgA nephropathy and inflammatory bowel disease[45-47]. A higher IgA level was also reported in IgG4-related disease or calcific pancreatitis[48-50]. However, the elevated serum IgA levels were associated with a lower incidence of relapse of IgG4-related disease in a Japanese study. Multicenter and large sample studies are required to clarify the association between these potential risk factors and AIP. AIP is known to occur more commonly among elderly people. In our analyses, patients over the age of 55 years presented a higher risk of relapse. Maintenance therapy was recommended in various studies to prevent relapse[1,51], while in our

study, the absence of maintenance therapy did not seem to be a predictor of relapse.

There were several limitations to our study. Firstly, it was a single-center retrospective study. Secondly, we only analyzed clinical factors to predict relapse. The possible genetic and environmental factors that may contribute to relapse were not considered. Additionally, the long duration of follow-up increased the risk of recall bias.

CONCLUSION

Patients with high IgG4 levels have a unique clinical profile and are at a higher risk of relapse of AIP. Normal IgG4 levels rendered diagnosis challenging, thereby reinforcing the need for a thorough understanding of the disease. Relapse of AIP is related to age and serum IgG4 and IgA levels. An active immune system is strongly related to high IgG4 levels and the risk of relapse. Long-term and multicenter studies are needed to confirm the risk factors for relapse.

ARTICLE HIGHLIGHTS

Research background

The role of immunoglobulin (Ig) G4 in the pathogenesis, diagnosis and prognosis of autoimmune pancreatitis (AIP) has received attention and patients with high levels of IgG4 have unique properties. There is still controversy about the predictors of AIP relapse, such as high IgG4 level, radiological type and steroid use.

Research motivation

To predict the relapse of AIP and identify the properties of patients with different IgG4 level, especially those with normal IgG4 level.

Research objectives

We determined some predictors of relapse and identified unique characteristics of patients with different IgG4 levels. Further studies about improving the diagnosis algorithm of AIP and predicting the relapse are needed.

Research methods

We conducted a retrospective cohort study, including patients with normal IgG4 level (n = 65) and high IgG4 level (n = 148), and compared their clinical characteristics. We also performed univariate and multivariate Cox regression models to investigate the risk factors of relapse.

Research results

Patients with high IgG4 levels had a higher average male age; a higher level of serum total protein, IgG4, and IgE; and a lower level of serum complement C3. In addition, a lower number of cases with abnormal pancreatic duct and pancreatic atrophy and a higher rate of relapse were noted in the abnormal group. Multivariate analyses revealed that serum IgG4 (> 2 × upper limit of normal, ULN) and IgA (> 1 × ULN) and age > 55 years were independent risk factors of relapse.

Research conclusions

Patients with high IgG4 levels had different clinical features including a more active immune system and a higher relapse rate. Some factors were identified as risk factors of relapse, such as age > 55 years, high IgG4 (> 2 × ULN) and IgA level (> 1 × ULN).

Research perspectives

Further research with large samples should be conducted to verify the predictors of relapse.

FOOTNOTES

Author contributions: Pan F contributed to study conceptualization and reviewed the manuscript; Zhou GZ and Zeng JQ drafted the initial manuscript and reviewed the manuscript; Wang L and Liu M contributed to methodology and formal analysis; Zhou GZ, Yan B and Meng K contributed to data collection; Wang ZK, Zhang XL and Peng LH reviewed the manuscript; All authors approved the final manuscript as submitted, all authors had full access to the data in the present study and accept responsibility to submit for publication.

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ORIGINAL ARTICLE

Retrospective Study

Machine learning-based decision tool for selecting patients with idiopathic acute pancreatitis for endosonography to exclude a biliary aetiology

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Abstract

BACKGROUND

Biliary microlithiasis/sludge is detected in approximately 30% of patients with idiopathic acute pancreatitis (IAP). As recurrent biliary pancreatitis can be prevented, the underlying aetiology of IAP should be established.

AIM

To develop a machine learning (ML) based decision tool for the use of endosonography (EUS) in pancreatitis patients to detect sludge and microlithiasis.

METHODS

We retrospectively used routinely recorded clinical and laboratory parameters of 218 consecutive patients with confirmed AP admitted to our tertiary care hospital between 2015 and 2020. Patients who did not receive EUS as part of the diagnostic work-up and whose pancreatitis episode could be adequately explained by other causes than biliary sludge and microlithiasis were excluded. We trained supervised ML classifiers using H_2O.ai automatically selecting the best suitable

predictor model to predict microlithiasis/sludge. The predictor model was further validated in two independent retrospective cohorts from two tertiary care centers (117 patients).

RESULTS

Twenty-eight categorized patients' variables recorded at admission were identified to compute the predictor model with an accuracy of 0.84 [95% confidence interval (CI): 0.791-0.9185], positive predictive value of 0.84, and negative predictive value of 0.80 in the identification cohort (218 patients). In the validation cohort, the robustness of the prediction model was confirmed with an accuracy of 0.76 (95%CI: 0.673-0.8347), positive predictive value of 0.76, and negative predictive value of 0.78 (117 patients).

CONCLUSION

We present a robust and validated ML-based predictor model consisting of routinely recorded parameters at admission that can predict biliary sludge and microlithiasis as the cause of AP.

Key Words: Acute pancreatitis; Idiopathic acute pancreatitis; Biliary pancreatitis; Microlithiasis; Sludge; Endosonography

Core Tip: Occult biliary lithiasis represents the largest monocausally treatable aetiology group within idiopathic acute pancreatitis cases. The identification of this subgroup protects patients from pancreatitis recurrences and over- or underdiagnosis. Based on 28 easy-to-collect and widely available patient variables, a machine learning-based prediction score can be used to predict the presence or absence of biliary sludge or microlithiasis in the context of pancreatitis hospitalisation. We provide a web-based prediction tool to select patients for endosonography to investigate microlithiasis or sludge as the cause of pancreatitis and treat them accordingly.

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INTRODUCTION

Pancreatitis is a high incidence disease and the underlying cause for the highest number of patients admitted to hospital admission of all benign gastrointestinal-disorders[1]. In approximately 25% of patients with acute pancreatitis (AP), aetiology cannot be established during the first episode of pancreatitis[2,3]. If the aetiology of AP cannot be identified by history, laboratory chemistry, and imaging, it is classified as "idiopathic" [idiopathic AP (IAP)]. Unclassified or idiopathic pancreatitis represents the third largest group of pancreatitis and is therefore of great importance from both a medical and a socioeconomic point of view requiring thorough workup[3,4]. All efforts should be made to elucidate a treatable aetiology to prevent further episodes of AP. A recent meta-analysis has shown that biliary aetiology is the most common cause of idiopathic pancreatitis with a prevalence of 30%[5]. Specifically, in light of morbidity and mortality of AP, it is crucial to differentiate the potentially treatable aetiology of AP triggered by biliary sludge and microlithiasis from idiopathic or other causes of AP. Unfortunately, due to a lack of unifying definition of biliary sludge and microlithiasis, it is currently impossible to assess the risk of sludge and/or microlithiasis as the cause of AP. In the absence of clear evidence, guidelines suggest to treat those patients by cholecystectomy and maybe biliary sphincterotomy. In line, the diagnostic IAP workup requires excluding biliary microconcrements as it is believed that detection and concrement removal and/or cholecystectomy can prevent further episodes of pancreatitis in over 85% of cases[6,7]. To facilitate decision-making on whether the patient should be referred to endosonography (EUS) followed by endoscopic retrograde cholangiopancreatography (ERCP) or cholecystectomy, we developed a predictive tool using a machine learning (ML)-based approach to estimate the probability of the presence of biliary sludge and/or microlithiasis at the time of presentation to the emergency department. The ML tool, which is based on routine laboratory values, will help clinicians to enrich the likelihood to detect microlithiasis or sludge at admission on EUS and hereby reduce the number of EUS exams in presumed acute idiopathic pancreatitis.

MATERIALS AND METHODS

Study design

We retrospectively studied 1340 confirmed and hospitalized patient cases of AP treated at LMU University Hospital

Munich (tertiary care hospital) between January 1, 2015 and October 1, 2020 (ICD-10 codes used: K85.00-K85.91). Patient cohorts with identical inclusion criteria from the University Hospital of the Technical University Munich and the University Medical Center Goettingen served as the validation cohort. The study was conducted in accordance with the updated STARD guideline of 2015[8].

Participants

Only patients meeting the diagnostic criteria for AP as set in the APA/IAP guidelines and adapted in the German S3-Guideline were enrolled in the analysis[9,10]. The first classifier used was whether patients received an EUS during their initial hospital stay, reducing the number of patients for further analysis to 360. The endosonographies were each performed by an experienced endoscopist. In the majority (79%) of pancreatitis stays, EUS was performed on days 1-3. Of the 360 patients with EUS, a total of 142 cases were excluded from further analysis due to incomplete records or missing coding. Two hundred and eighteen patient cases with AP and EUS were then further stratified into a cohort (47 patients) with no other cause of pancreatitis than endosonographically detected biliary microconcrements (biliary sludge/microlithiasis; detection of concrements in the common bile duct or gallbladder and common bile duct) and 171 patients with other causes of AP (Figure 1). In the two study groups [AP + EUS: 47 × microlithiasis *vs* 171 × non-microlithiasis (other cause); Supplementary Table 1], history, alcohol consumption, sonography, ERCP, or EUS findings, start or change of existing medication, known hereditary pancreatitis (available genetic testing of most prevalent susceptibility genes), and laboratory findings [lipase levels, immunoglobulin G subclasses, liver enzymes, triglycerides, and calcium level (corrected for blood serum albumin level)] were retrospectively evaluated. In the context of the laboratory value analyses, the values from the first blood analysis after admission of the respective patient stay was used in each case. The aim was to select patients in which microlithiasis/sludge was likely to subject them to EUS to reduce the number of EUS as an invasive, expensive procedure burdened with complications. To independently validate our machine-based algorithm, we obtained identical clinical data and inclusion criteria from two high volume German pancreas centers (University Hospital of the Technical University Munich: 22 × microlithiasis-AP, 51 × other-AP; University Medical Center Goettingen: 14 × microlithiasis-AP, 30 × other-AP; Supplementary Table 1). The definitions of the entities "biliary sludge" and "biliary microlithiasis" were taken from the endoscopic reports during the retrospective data evaluation and were not re-evaluated due to the current lack of an accepted unifying definition. Due to the differences between the participating centers in the use and partial equation of the two terms biliary sludge and microlithiasis, sludge-triggered pancreatitis was subsumed as biliary AP caused by microlithiasis.

Test methods

All aspects of data reporting, predictive modeling, and validation reporting were performed in accordance with the TRIPOD guidelines[11]. A diagnostic reference standard for laboratory or imaging-based prediction of biliary sludge or microlithiasis in the context of AP has not yet been published. To derive the ML-based predictor model (index test), the following steps were performed (Figure 2): (1) Baseline variables (n = 192) were filtered leaving out variables with zero and near zero variance; (2) All numeric variables were classified into within limit, above upper limit, and below lower limit, based on clinical reference limits. All categorised variables were retained; (3) The training cohort was divided into a training (80%) and a test set (20%). Endpoint balancing was achieved by stratifying the classes by inducing the sampling rate of patients with microlithiasis and reducing the sampling rate of patients with other-AP. ML was performed based on all filtered baseline variables and data from the training set, resulting in a predictor based on all variables (base predictor model); and (4) To improve robustness and interpretability, low-impact variables were iteratively removed. An iterative predictive model with a reduced number of variables (n = 26) was obtained based on the performance in the test set.

All predictor models were constructed using the H_2O.ai platform (https://www.h2o.ai) selecting (with h2o.automl) the best suitable ML method in the training set. The parameters of each method were optimized by employing an internal ten-fold cross-validation on the training set. The optimal method was then applied to the test set to assess the final performance. In each loop, the best performing predictor model was identified from all predicted outcomes obtained using the performance measure logloss. Variables with a higher proportion of missing data (> 25% missing data) were also not excluded *per se* in order to base the final model on the broadest possible number of routinely available variables in the early phase of AP. The iterative predictive model obtained was externally validated in an independent retrospective dataset.

Statistical analysis

All data processing, modeling, and assessment of performances were done using R [version 4.0.4 (2021-02-15, "Lost Library Book")] and visualized in R-studio (version 1.3.9.59). No unique algorithm was developed for this study. All data R scripts or functions are available online at the following link: https://github.com/mayerlelab/microlithiasisPredict. P values of < 0.05 were considered statistically significant if appropriate for the tests used.

RESULTS

Microlithiasis predictive score - results of the identification cohort

Between January 1, 2015 and October 1, 2020, 218 patients with AP received an EUS during their initial admission with AP at LMU University Hospital meeting the study inclusion criteria (Figure 1). In 47 of 218 pancreatitis patients, no causal

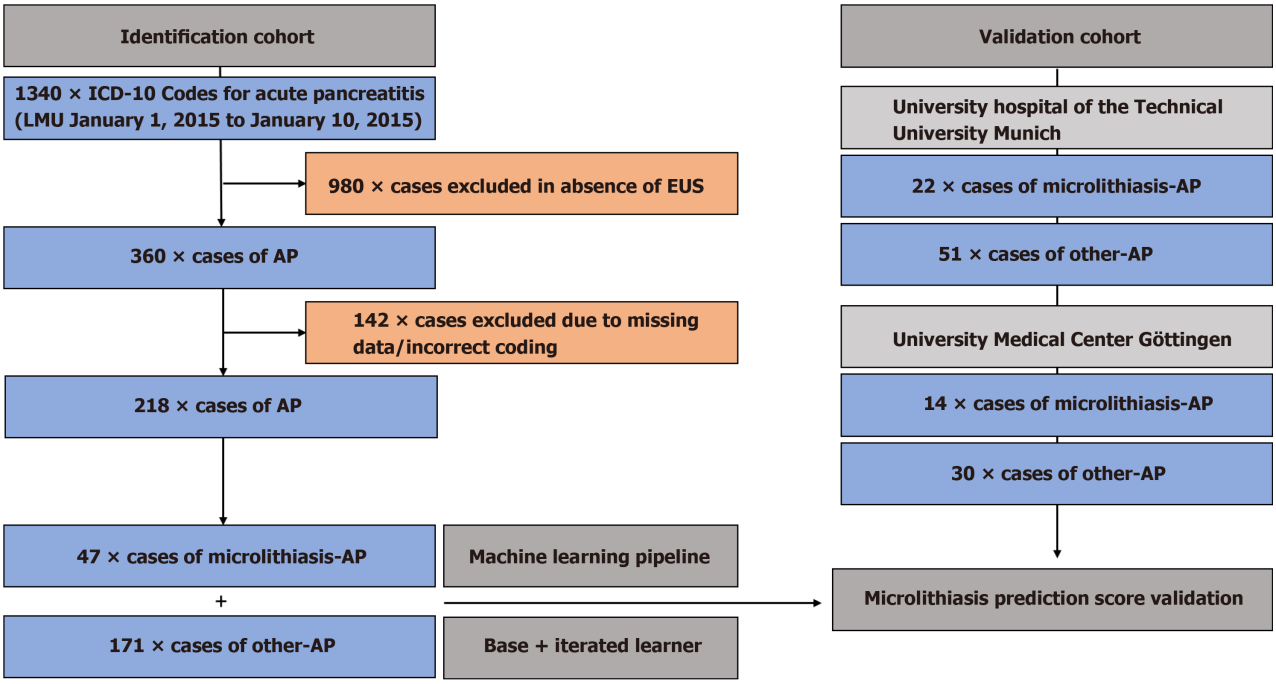

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Figure 1 Flow chart for development and external independent validation of microlithiasis prediction score. In the Ludwig-Maximilians-Universität in Munich identification cohort, 218 acute pancreatitis patients treated as inpatients between 2015-2020 were included in the final machine learning-based score survey. The validation cohort, consisting of 117 pancreatitis cases, was composed of patient data from the University Hospital of Göttingen and Technical University Munich. The microlithiasis predictive model was trained using data from both biliary sludge and biliary microlithiasis patients to cover the entirety of biliary microconcrements and to reflect the current lack of uniform definitions of biliary sludge and biliary microlithiasis in clinical practice. EUS: Endosonography; AP: Acute pancreatitis.

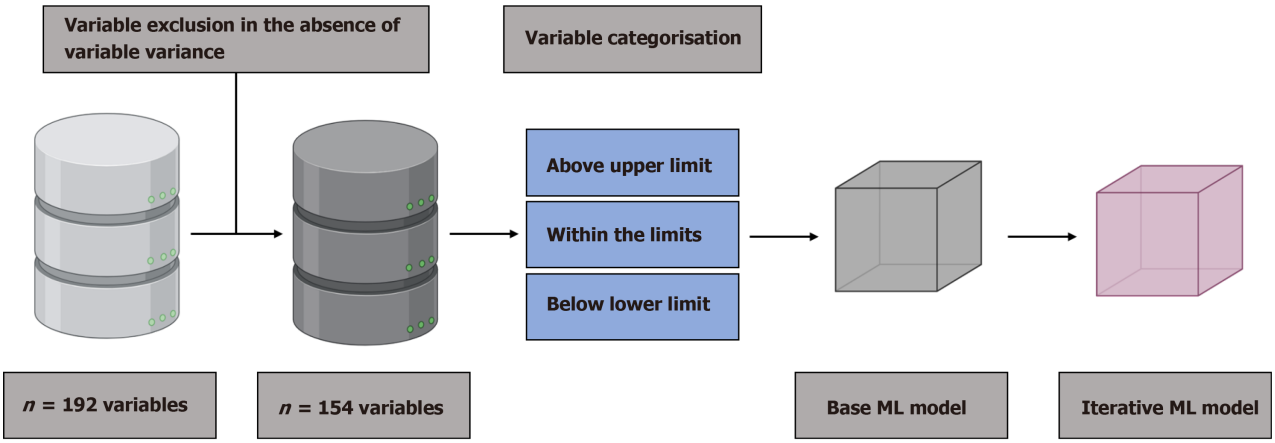

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Figure 2 Machine-learning based model for the prediction of biliary sludge and microlithiasis in the context of acute (presumed) idiopathic acute pancreatitis. Of the initial 192 variables analysed, 154 were included in the categorisation step after excluding those variables without evidence of variable variance. Using an auto-machine learning approach, the final (iterative) predictive model was developed *via* the base model step. ML: Machine learning.

pancreatitis aetiology other than endosonographically detected biliary microconcrements/sludge was found during the respective inpatient stay. Among 171 out of 218 pancreatitis patients with EUS, 52.6% (90/171) were classified as 'idiopathic', 21.6% (37/171) as acute on chronic, and 15.2% (27/171) with macrolithiasis as of biliary aetiology (Supplementary Table 1). Mean age in the microlithiasis/sludge cohort was 59.1 (SD 18.8) years in comparison to that of patients with AP of other aetiologies [54.6 (SD 17.1) years; $P = 0.122$]. Gender distribution was not statistically different in the two cohorts, with a male predominance in both cohorts [31/47 (66%) of microlithiasis patients and 103/171 (60.2%); $P = 0.475$] (Table 1). 76.6% of microlithiasis-AP patients were assessed as mild pancreatitis cases according to the revised Atlanta classification [36/47; 19.1% moderate (9/47) and 4.3% severe (2/47)]. In the other-AP cohort, 71.9% of patients were assessed as mild pancreatitis cases according to the revised Atlanta classification [123/171; 25.7% moderate (44/171) and 2.3% severe (4/171)]. A total of 29 variables from serum samples and 5 from urine were used to develop the ML-based

Table 1 Variables distribution at the Ludwig-Maximilians-Universität University Hospital Munich (identification cohort)

Variable	Microlithiasis (*n* = 47)	Other (*n* = 171)	Total (*n* = 218)	*P* value
Age (yr)				0.122
mean ± SD	59.1 ± 18.8	54.6 ± 17.1	55.6 ± 17.6	
Range	30-92	24-90	24-92	
Sex				0.474
Female	16/47 (34%)	68/171 (39.8%)	84/218 (38.5%)	
Male	31/47 (66%)	103/171 (60.2%)	134/218 (61.5%)	
Albumin				0.706
N-Miss	32/47 (68%)	90/171 (52.6%)	122/218 (55.9%)	
LLN	2/47 (4.2%)	14/171 (8.1%)	16/218 (7.3%)	
WL	13/47 (27.6%)	67/171 (39.1%)	80/218 (36.6%)	
Alkaline phosphatase				0.667
N-Miss	1/47 (2.1%)	5/171 (2.9%)	6/218 (2.7%)	
LLN	0/47 (0%)	1/171 (0.5%)	1/218 (0.4%)	
ULN	22/47 (46.8%)	69/171 (40.3%)	91/218 (41.7%)	
WL	24/47 (51.0%)	96/171 (56.1%)	120/218 (55.0%)	
Total bilirubin				0.110
N-Miss	0/47 (0%)	2/171 (1.1%)	2/218 (0.9%)	
ULN	23/47 (48.9%)	61/171 (35.6%)	84/218 (38.5%)	
WL	24/47 (51.0%)	108/171 (63.1%)	132/218 (60.5%)	
Calcium				0.033
N-Miss	20/47 (42.5%)	60/171 (35.0%)	80/218 (36.6%)	
LLN	6/47 (12.7%)	7/171 (4.0%)	13/218 (5.9%)	
ULN	0/47 (0%)	2/171 (1.1.%)	2/218 (0.9%)	
WL	21/47 (44.6%)	102/171 (59.6%)	123/218 (56.4%)	
Creatine kinase				0.073
N-Miss	29/47 (61.7%)	107/171 (62.5%)	136/218 (62.3%)	
ULN	0/47 (0%)	10/171 (5.8%)	10/218 (4.5%)	
WL	18/47 (38.2%)	54/171 (31.5%)	72/218 (33%)	
CRP				0.391
ULN	37/47 (78.7%)	124/171 (72.5%)	161/218 (73.9%)	
WL	10/47 (21.3%)	47/171 (27.5%)	57/218 (26.1%)	
Total protein				0.743
N-Miss	30/47 (63.8%)	104/171 (60.8%)	134/218 (61.4%)	
LLN	0/47 (0%)	1/171 (0.5%)	1/218 (0.4%)	
ULN	3/47 (6.3%)	16/171 (9.3%)	19/218 (8.7%)	
WL	14/47 (29.7%)	50/171 (29.2%)	64/218 (29.3%)	
Erythrocytes				0.880
N-Miss	1/47 (2.1%)	0/171 (0%)	1/218 (0.4%)	
LLN	14/47 (29.7%)	53/171 (30.9%)	67/218 (30.7%)	
ULN	3/47 (6.3%)	8/171 (4.6%)	11/218 (5.0%)	
WL	29/47 (61.7%)	110/171 (64.3%)	139/218 (63.7%)	

Gamma-GT				0.108
N-Miss	0/47 (0%)	1/171 (0.5%)	1/218 (0.4%)	
ULN	37/47 (78.7%)	113/171 (66%)	150/218 (68.8%)	
WL	10/47 (21.3%)	57/171 (33.3%)	67/218 (30.7%)	
AST/GOT				0.444
N-Miss	17/47 (36.1%)	51/171 (29.8%)	68/218 (31.1%)	
ULN	21/47 (44.6%)	75/171 (43.8%)	96/218 (44.0%)	
WL	9/47 (19.1%)	45/171 (26.3%)	54/218 (24.7%)	
ALT/GPT				0.016
ULN	34/47 (72.3%)	90/171 (52.6%)	124/218 (56.9%)	
WL	13/47 (27.7%)	81/171 (47.4%)	94/218 (43.1%)	
Urea				0.429
N-Miss	31/47 (65.9%)	80/171 (46.7%)	111/218 (50.9%)	
LLN	0/47 (0%)	7/171 (4%)	7/218 (3.2%)	
ULN	3/47 (6.3%)	11/171 (6.4%)	14/218 (6.4%)	
WL	13/47 (27.6%)	73/171 (42.6%)	86/218 (39.4%)	
Hematocrit				0.304
N-Miss	1/47 (2.1%)	0/171 (0%)	1/218 (0.4%)	
LLN	0/47 (0%)	2/171 (1.1%)	2/218 (0.9%)	
ULN	41/47 (87.2%)	160/171 (93.5%)	201/218 (92.2%)	
WL	5/47 (10.6%)	9/171 (5.2%)	14/218 (6.4%)	
Haemoglobin				0.574
N-Miss	1/47 (2.1%)	0/171 (0%)	1/218 (0.4%)	
LLN	12/47 (25.5%)	45/171 (26.3%)	57/218 (26.1%)	
ULN	0/47 (0%)	4/171 (2.3%)	4/218 (1.8%)	
WL	34/47 (72.3%)	122/171 (71.3%)	156/218 (71.5%)	
INR				0.443
N-Miss	3/47 (6.3%)	9/171 (1.1%)	12/218 (5.5%)	
ULN	8/47 (17.0%)	22/171 (12.8%)	30/218 (13.7%)	
WL	36/47 (76.5%)	140/171 (81.8%)	176/218 (80.7%)	
Potassium				0.270
N-Miss	9/47 (19.1%)	2/171 (1.1%)	11/218 (5%)	
LLN	1/47 (2.1%)	7/171 (4%)	8/218 (3.6%)	
ULN	0/47 (0%)	10/171 (5.8%)	10/218 (4.5%)	
WL	37/47 (78.7%)	152/171 (88.8%)	189/218 (86.6%)	
Serum creatinine				0.738
N-Miss	6/47 (12.7%)	0/171 (0%)	6/218 (2.7%)	
LLN	1/47 (2.1%)	5/171 (2.9%)	6/218 (2.7%)	
ULN	5/47 (10.6%)	29/171 (16.9%)	34/218 (15.5%)	
WL	35/47 (74.4%)	137/171 (80.1%)	172/218 (78.8%)	
LDH				0.020
N-Miss	7/47 (14.8%)	19/171 (11.1%)	26/218 (11.9%)	
ULN	30/47 (63.8%)	83/171 (48.5%)	112/218 (51.8%)	

WL	10/47 (21.2%)	69/171 (40.3%)	79/218 (36.2%)	
Leukocytes				0.347
N-Miss	1/47 (2.1%)	0/171 (0%)	1/218 (0.4%)	
LLN	1/47 (2.1%)	3/171 (1.7%)	4/218 (1.8%)	
ULN	16/47 (34%)	80/171 (46.7%)	96/218 (44%)	
WL	29/47 (61.7%)	88/171 (51.5%)	117/218 (53.6%)	
Lipase				0.653
N-Miss	0/47 (0%)	1/171 (0.5%)	1/218 (0.4%)	
LLN	0/47 (0%)	3/171 (1.7%)	3/218 (1.3%)	
ULN	44/47 (93.6%)	157/171 (91.8%)	201/218 (92.2%)	
WL	3/47 (6.4%)	10/171 (5.8%)	13/218 (5.9%)	
MCH				0.498
N-Miss	1/47 (2.1%)	0/171 (0%)	1/218 (0.4%)	
LLN	3/47 (6.3%)	20/171 (11.6%)	23/218 (10.5%)	
ULN	4/47 (8.5%)	10/171 (5.8%)	14/218 (6.4%)	
WL	39/47 (82.9%)	141/171 (82.4%)	180/218 (82.5%)	
MCHC				0.108
N-Miss	1/47 (2.1%)	0/171 (0%)	1/218 (0.4%)	
LLN	0/47 (0%)	13/171 (7.6%)	13/218 (5.9%)	
ULN	1/47 (2.1%)	8/171 (4.6%)	9/218 (4.1%)	
WL	45/47 (95.7%)	150/171 (87.7%)	195/218 (89.4%)	
Triglycerides				0.004
N-Miss	27/47 (57.4%)	110/171 (64.3%)	137/218 (62.8%)	
ULN	1/47 (2.1%)	24/171 (14%)	25/218 (11.4%)	
WL	19/47 (40.4%)	37/171 (21.6%)	56/218 (25.6%)	
RDW				0.329
N-Miss	5/47 (10.6%)	36/171 (21%)	41/218 (18.8%)	
LLN	1/47 (2.1%)	11/171 (6.3%)	12/218 (5.5%)	
ULN	5/47 (10.6%)	21/171 (12.2%)	26/218 (11.9%)	
WL	36/47 (76.5%)	103/171 (60.2%)	139/218 (63.7%)	
MCV				0.893
N-Miss	1/47 (2.1%)	0/171 (0%)	1/218 (0.4%)	
LLN	4/47 (8.5%)	13/171 (7.6%)	17/218 (7.7%)	
ULN	4/47 (8.5%)	12/171 (7.0%)	16/218 (7.3%)	
WL	38/47 (80.8%)	146/171 (85.3%)	184/218 (84.4%)	
Sodium				0.020
N-Miss	8/47 (17.0%)	1/171 (0.5%)	9/218 (4.1%)	
LLN	1/47 (2.1%)	29/171 (16.9%)	30/218 (13.7%)	
WL	38/47 (80.8%)	141/171 (82.4%)	179/218 (82.1%)	
Quick's value				0.479
N-Miss	3/47 (6.3%)	7/171 (4%)	10/218 (4.5%)	
LLN	8/47 (17%)	23/171 (13.4%)	31/218 (14.2%)	
ULN	20/47 (42.5%)	65/171 (38%)	85/218 (38.9%)	

WL	16/47 (34%)	76/171 (44.4%)	92/218 (42.2%)	
Thrombocytes				0.434
N-Miss	1/47 (2.1%)	0/171 (0%)	1/218 (0.4%)	
LLN	8/47 (17%)	22/171 (12.8%)	30/218 (13.7%)	
ULN	4/47 (8.5%)	26/171 (15.2%)	30/218 (13.7%)	
WL	34/47 (72.3%)	123/171 (71.9%)	157/218 (72%)	
TSH				0.567
N-Miss	27/47 (57.4%)	118/171 (69%)	145/218 (66.5%)	
LLN	2/47 (4.2%)	4/171 (2.3%)	6/218 (2.7%)	
ULN	4/47 (8.5%)	6/171 (3.5%)	10/218 (4.5%)	
WL	14/47 (29.7%)	43/171 (25.1%)	57/218 (26.1%)	
Bilirubin-urine				0.027
N-Miss	26/47 (55.3%)	102/171 (59.6%)	128/218 (58.7%)	
Normal	8/47 (17%)	45/171 (26.3%)	53/218 (24.3%)	
Abnormal	13/47 (27.6%)	24/171 (14.0%)	37/218 (16.9%)	
Total protein-urine				0.231
N-Miss	26/47 (55.3%)	102/171 (59.6%)	128/218 (58.7%)	
Normal	10/47 (21.3%)	43/171 (25.1%)	53/218 (24.3%)	
Abnormal	11/47 (23.4%)	26/171 (15.2%)	37/218 (16.9%)	
Ketones-urine				0.020
N-Miss	26/47 (55.3%)	106/171 (61.9%)	132/218 (60.5%)	
Normal	21/47 (44.6%)	51/171 (29.8%)	72/218 (33%)	
Abnormal	0/47 (0%)	14/171 (8.1%)	14/218 (6.4%)	
Leukocytes-urine				0.162
N-Miss	26/47 (55.3%)	102/171 (59.6%)	128/218 (58.7%)	
Normal	7/47 (14.8%)	35/171 (20.4%)	42/218 (19.2%)	
Abnormal	14/47 (29.7%)	34/171 (19.8%)	48/218 (22%)	
Specific gravity-urine				0.918
N-Miss	29/47 (61.7%)	113/171 (66%)	142/218 (65.1%)	
mean ± SD	1018.33 ± 5.941	1018.103 ± 8.777	1018.158 ± 8.159	
Range	1005.000-1025.000	1005.000-1030.000	1005.000-1030.000	

All variables that were used for the final predictive model are listed. For laboratory or urine values, the first available value during the inpatient stay was used. For the investigated groups of microlithiasis-induced acute pancreatitis (n = 47) *vs* the group of pancreatitis induced by other aetiologies (n = 171), the variables were categorised as whether collected or not (N-Miss), in the case of laboratory values whether below the lower limit value, within the limit values, or above the upper limit value. For urine values, in addition to the rate of missing variables (N-Miss), it was categorised whether normal or abnormal. For the P value calculation using χ^2 test, variables with missing data shares of > 25% were not excluded. LLN: Lower limit value; WL: Within the limit value; ULN: Upper limit value; CRP: C-reactive protein; Gamma-GT: Gamma-glutamyl transpeptidase; AST: Aspartate aminotransferase; GOT: Glutamic oxalacetic transaminases; ALT: Alanine transaminase; GPT: Glutamic pyruvic transaminase; INR: International normalized ratio; LDH: Lactate dehydrogenase; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDW: Red blood cell distribution width; MCV: Mean corpuscular volume; TSH: Thyrotropin.

microlithiasis prediction algorithm. All variables listed corresponded to the values measured at admission for each individual pancreatitis inpatient (see Table 1 for the list of variables used). To move from the base ML to the iterated ML model, weighting was done, taking scale variance into account. For the LMU identification cohort, age, triglycerides, sodium, glutamic pyruvic transaminase, erythrocytes, potassium, thyrotropin, protein (total), and leukocytes in descending order were of greatest importance in predicting microlithiasis/sludge. Using the iterated learner-based model, an accuracy of 0.8361 [95% confidence interval (CI): 0.791-0.9185; odds ratio = 20.88 (95%CI: 2.08-209.27)] with a sensitivity of 97.92% and positive predictive value (PPV) of 83.93% could be achieved for the prediction of microlithiasis as the trigger of pancreatitis [negative predictive value (NPV) = 0.80; specificity: 0.31; Table 2].

Table 2 Performance matrix (identification cohort *vs* validation cohort)

Accuracy	Sensitivity	Specificity	PPV	NPV
ID: 0.8361; 95%CI: 0.7191-0.9185	ID: 0.9792	ID: 0.3077	ID: 0.8393	ID: 0.800
VD: 0.7607; 95%CI: 0.673-0.8347	VD: 0.9630	VD: 0.3056	VD: 0.7573	VD: 0.7857

The performance values for the auto-machine-learning-based predictive model are listed. The identification cohort of the Ludwig-Maximilians-Universität in Munich University Hospital (ID) *vs* the data of the predictive models from the validation cohort with patient data from the University Hospital Göttingen and the Klinikum Rechts der Isar (VD). CI: Confidence interval; PPV: Positive predictive value; NPV: Negative predictive value.

Microlithiasis predictive score - results of the validation cohort

Data from two large-volume university pancreas centers were used for score validation. In total, a validation cohort of 36 patients with microlithiasis and 81 non-microlithiasis AP patients were retrieved from the clinical database at the University Hospital of the Technical University Munich (22 × microlithiasis-AP, 51 × other-AP) as well as the University Hospital Göttingen (14 × microlithiasis-AP, 30 × other-AP; Figure 1 and Table 3). In the Technical University Munich cohort, the group of other-AP patients was mainly alcohol-related [31/51 (60.8%)], while in the Göttingen cohort biliary macrolithiasis was held responsible for the majority of AP patients [16/33 (53.3%)]. Idiopathic aetiology was named as the second most frequent aetiology group in both external cohorts with about 30% each [Technical University Munich 17/51 (33.3%), Göttingen 10/33 (33.3%)] (Supplementary Table 1). Microlithiasis patients in the validation cohort were on average 60.1 (SD 18.4) years old, while patients from the other-AP cohort were 55.3 (SD 16.8) years old. In both groups (microlithiasis + other-AP), the majority of patients were male [24/36 (66.7%) and 46/81 (56.8%), respectively], resembling the identification cohort. 63.9% of microlithiasis-AP patients were assessed as mild pancreatitis cases according to the revised Atlanta classification [23/36; 27.7% moderate (10/36) and 8.3% severe (3/36)]. In the other-AP cohort, 59.2% of patients were assessed as mild pancreatitis cases according to the revised Atlanta classification [48/81; 27.2% moderate (22/81) and 12.5% severe (11/81)]. Using automated ML, the best-fitting model for iterative reduction of variables was used to achieve external validation of the microlithiasis predictive score using the optimized iterative ML model. For the validation cohort, based on the variables ordered by scaled importance in Figure 3, an accuracy of 0.7607 (95%CI: 0.673-0.8347), PPV of 0.7573, and NPV of 0.7857 were achieved (sensitivity: 0.96, specificity: 0.31; Table 2). The robustness of the model is shown in the alluvial plot in Figure 3, with only 3 out of 81 patients being misclassified as having microlithiasis and not as having other-AP, corresponding to the discretely higher NPV (compared to the PPV) in the validation cohort (Table 2).

DISCUSSION

Previous and more recent studies on idiopathic pancreatitis still report a proportion of idiopathic pancreatitis stably at 20%-30%[12,13]. However, it has been suspected for decades and is increasingly supported by evidence that a large proportion of pancreatitis patients classified primarily as idiopathic actually suffer from a biliary aetiology and that detecting these patients during the first episode of pancreatitis is restricted due to the lack of availability of timely and high quality EUS exams[14]. Furthermore, there is a lack of reliable data on when, during an inpatient stay of an IAP-labeled patient, an EUS could detect biliary microconcrements as the trigger for pancreatitis without causing an unnecessary burden for the patient through overdiagnosis. This is an important question as we know from Oría *et al*[15] that common bile duct stones usually pass within 48 h, suggesting that microconcrements might even pass more rapidly and might not be detected on EUS. Prospective study data showed a corresponding variance of EUS-based biliary concrement detection rate of 19% in the low risk group, but 58% in the moderate risk group and 50% in the high risk group (grouping according to ASGE recommendation[16]). Risk stratification in terms of pre-test probability for EUS use < 48 h after hospital admission to rule in or out the presence of biliary concrements is warranted before intervention to overcome the lack of availability and reduce costs and side effects[12]. Diagnostic evaluation is complicated by the fact that biliary microconcrements could be a coincidental finding in the context of pancreatitis-induced gallbladder hypomotility, and therefore must always be understood in the individual patient's setting, taking into account a PPV of a biliary pancreatitis origin greater than 85% with elevation of the alanine transaminase (ALT) above three times the upper limit of normal [17]. However, no causally effective drug for pancreatitis therapy is available in 2023 and the detection of causally remediable pancreatitis causes such as biliary microlithiasis or sludge will continue to play a decisive role in the prevention of further pancreatitis attacks. The efficacy of cholecystectomy in the cohort of IAP patients was shown in a meta-analysis with a recurrence rate of 11% compared to 38.9% in conservatively treated patients (risk ratio = 0.41; 95%CI: 0.16-1.07) [18]. Our ML-based approach of predicting biliary microlithiasis and sludge should therefore be understood as an approach to make up for the lack of evidence from prospective studies on the optimal timing of EUS in IAP patients as this score is based on widely available laboratory values and can be used to determine the probability of the presence of biliary microconcrements at admission. Our score helps to select patients for EUS with a high sensitivity and very high NPV and thus will reduce costs and complications of unnecessary EUS exams as well as allow to subject patients to further treatment to prevent recurrence of biliary pancreatitis at the time of presentation in the emergency department. Preliminary work on ML-based algorithms and prediction models in the context of AP has focused on severity assessment and prediction of complications[15]. A multicenter retrospective study used an auto-ML-based approach to

Table 3 Variable distribution in the validation cohort (UMG + Technical University Munich)

Variable	Microlithiasis (*n* = 36)	Other (*n* = 81)	Total (*n* = 117)	*P* value
Age (yr)				0.162
mean ± SD	60.1 ± 18.4	55.3 ± 16.8	56.8 ± 17.4	
Range	23-93	21-87	21-93	
Sex				0.315
Female	12/36 (33.3%)	35/81 (43.2%)	47/117 (40.2%)	
Male	24/36 (66.7%)	46/81 (56.8%)	70/117 (59.8%)	
Alkaline phosphatase				0.032
N-Miss	1/36 (2.7%)	12/81 (14.8%)	13/117 (11.1%)	
ULN	23/36 (63.8%)	30/81 (37%)	53/117 (45.2%)	
WL	12/36 (33.2%)	39/81 (48.1%)	51/117 (43.5%)	
Total bilirubin				0.003
ULN	21/36 (58.3%)	24/81 (29.6%)	45/117 (38.5%)	
WL	15/36 (41.7%)	57/81 (70.4%)	72/117 (61.5%)	
Creatine kinase				0.498
N-Miss	8/36 (22.2%)	32/81 (39.5%)	40/117 (34.1%)	
ULN	5/36 (13.8%)	6/81 (7.4%)	11/117 (9.4%)	
WL	23/36 (63.8%)	43/81 (53%)	66/117 (56.4%)	
CRP				0.199
ULN	32/36 (88.9%)	64/81 (79%)	96/117 (88.8%)	
WL	4/36 (11.1%)	17/81 (21%)	21/117 (17.9%)	
Total protein				0.405
N-Miss	31/36 (86.1%)	73/81 (90.1%)	104/117 (88.8%)	
WL	5/36 (13.8%)	8/81 (9.8%)	13/117 (11.1%)	
Erythrocytes				0.650
N-Miss	0/36 (0%)	1/81 (1.2%)	1/117 (0.8%)	
LLN	10/36 (27.7%)	25/81 (30.8%)	35/117 (29.9%)	
ULN	4/36 (11.1%)	5/81 (6.1%)	9/117 (7.6%)	
WL	22/36 (61.1%)	50/81 (61.7%)	72/117 (61.5%)	
Gamma-GT				0.082
N-Miss	0/36 (0%)	2/81 (2.4%)	2/117 (1.7%)	
ULN	32/36 (88.8%)	59/81 (72.8%)	91/117 (77.7%)	
WL	4/36 (11.1%)	20/81 (24.6%)	24/117 (20.5%)	
AST/GOT				0.079
N-Miss	0/36 (0%)	4/81 (4.9%)	4/117 (3.4%)	
ULN	28/36 (77.8%)	47/81 (58%)	75/117 (64.1%)	
WL	8/36 (22.2%)	30/81 (37%)	38/117 (32.4%)	
ALT/GPT				0.052
N-Miss	0/36 (0%)	2/81 (2.4%)	2/117 (1.7%)	
ULN	28/36 (77.8%)	43/81 (53%)	71/117 (60.6%)	
WL	8/36 (22.2%)	30/81 (37%)	38/117 (32.4%)	
Urea				

N-Miss	7/36 (19.4%)	20/81 (24.6%)	27/117 (23%)	
LLN	13/36 (36.1%)	41/81 (50.6%)	54/117 (46.1%)	
ULN	0/36 (0%)	1/81 (1.2%)	1/117 (0.8%)	
WL	16/36 (44.4%)	19/81 (23.4%)	35/117 (29.9%)	
Hematocrit				< 0.001
N-Miss	0/36 (0%)	1/81 (1.2%)	1/117 (0.8%)	
ULN	36/36 (100%)	80/81 (98.7%)	116/117 (99.1%)	
Haemoglobin				0.725
N-Miss	0/36 (0%)	1/81 (1.2%)	1/117 (0.8%)	
LLN	9/36 (25%)	18/81 (22.2%)	27/117 (23%)	
ULN	3/36 (8.3%)	4/81 (4.9%)	7/117 (5.9%)	
WL	32/36 (88.9%)	64/81 (79.0%)	96/117 (82%)	
INR				0.440
ULN	2/36 (5.5%)	8/81 (9.8%)	10/117 (8.5%)	
WL	34/36 (94.4%)	73/81 (90.1%)	107/117 (91.4%)	
Potassium				0.985
LLN	2/36 (5.5%)	4/81 (4.9%)	6/117 (5.1%)	
ULN	1/36 (2.7%)	2/81 (2.4%)	3/117 (2.5%)	
WL	33/36 (91.6%)	75/81 (92.5%)	108/117 (92.3%)	
Serum creatinine				0.909
LLN	2/36 (5.5%)	6/81 (7.4%)	8/117 (6.8%)	
ULN	7/36 (19.4%)	14/81 (17.2%)	21/117 (17.9%)	
WL	27/36 (75%)	61/81 (75.3%)	88/117 (75.2%)	
LDH				0.018
N-Miss	6/36 (16.6%)	28/81 (34.5%)	34/117 (29%)	
ULN	26/36 (72.2%)	33/81 (40.7%)	59/117 (50.4%)	
WL	4/36 (11.1%)	20/81 (24.6%)	24/117 (20.5%)	
Leukocytes				0.143
N-Miss	0/36 (0%)	1/81 (1.2%)	1/117 (0.8%)	
LLN	1/36 (2.7%)	0/81 (0%)	1/117 (0.8%)	
ULN	16/36 (44.4%)	47/81 (58%)	63/117 (53.8%)	
WL	19/36 (52.7%)	33/81 (40.7%)	52/117 (44.4%)	
Lipase				0.237
N-Miss	0/36 (0%)	2/81 (2.4%)	2/117 (1.7%)	
ULN	32/36 (88.9%)	75/81 (92.5%)	107/117 (91.4%)	
WL	4/36 (11.1%)	4/81 (4.9%)	8/117 (6.8%)	
MCV				0.315
N-Miss	0/36 (0%)	1/81 (1.2%)	1/117 (0.8%)	
LLN	3/36 (8.3%)	7/81 (8.6%)	10/117 (8.5%)	
ULN	1/36 (2.7%)	9/81 (11.1%)	10/117 (8.5%)	
WL	32/36 (88.9%)	64/81 (79%)	96/117 (82%)	
Triglycerides				0.582
N-Miss	26/36 (72.2%)	43/81 (53%)	69/117 (58.9%)	

ULN	3/36 (8.3%)	15/81 (18.5%)	18/117 (15.3%)	
WL	7/36 (19.4%)	23/81 (28.3%)	30/117 (25.6%)	
RDW				
N-Miss	36/36 (100%)	81/81 (100%)	117/117 (100%)	
False	0/36 (0%)	0/81 (0%)	0/117 (0%)	
True	0/36 (0%)	0/81 (0%)	0/117 (0%)	
Sodium				0.154
LLN	2/36 (5.5%)	12/81 (14.8%)	14/117 (11.9%)	
WL	34/36 (94.4%)	69/81 (85.1%)	103/117 (88%)	
Quick's value				0.130
LLN	2/36 (5.5%)	6/81 (7.4%)	8/117 (6.8%)	
ULN	13/36 (36.1%)	44/81 (54.3%)	57/117 (48.7%)	
WL	21/36 (58.3%)	31/81 (38.2%)	52/117 (44.4%)	
Thrombocytes				0.627
N-Miss	22/36 (61.1%)	51/81 (62.9%)	73/117 (62.3%)	
LLN	2/36 (5.5%)	3/81 (3.7%)	5/117 (4.2%)	
ULN	2/36 (5.5%)	2/81 (2.4%)	4/117 (3.4%)	
WL	10/36 (27.7%)	25/81 (30.8%)	35/117 (29.9%)	
TSH				0.773
N-Miss	6/36 (16.6%)	13/81 (16%)	19/117 (16.2%)	
LLN	0/36 (0%)	1/81 (1.2%)	1/117 (0.8%)	
ULN	1/36 (2.7%)	3/81 (3.7%)	4/117 (3.4%)	
WL	29/36 (80.5%)	64/81 (79%)	93/117 (79.4%)	

All variables that were used for the final predictive model are listed. For laboratory or urine values, the first available value during the inpatient stay was used. For the investigated groups of microlithiasis-induced acute pancreatitis (n = 36) *vs* the group of pancreatitis induced by other aetiologies (n = 81), the variables were categorised as whether collected or not (N-Miss), in the case of laboratory values whether below the lower limit value, within the limit values, or above the upper limit value. For the P value calculation using χ^2 test, variables with missing data shares of > 25% were not excluded. LLN: Lower limit value; WL: Within the limit value; ULN: Upper limit value; CRP: C-reactive protein; Gamma-GT: Gamma-glutamyl transpeptidase; AST: Aspartate aminotransferase; GOT: Glutamic oxalacetic transaminases; ALT: Alanine transaminase; GPT: Glutamic pyruvic transaminase; INR: International normalized ratio; LDH: Lactate dehydrogenase; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; RDW: Red blood cell distribution width; MCV: Mean corpuscular volume; TSH: Thyrotropin.

predict pancreatitis severity, comparable to our ML approach, and achieved an area under the curve (AUC) of > 0.90 in the GBM model with a specificity and accuracy of both > 0.95 in the early detection of patients with a subsequently severe course of pancreatitis[19], outperforming clinically established non-ML-based scoring systems such as BISAP or Ranson underlying the relevance of ML approach over an educated guess[20,21]. ML-based prediction scores with regard to biliary microconcrements have not yet been published. Non-ML-based multivariate logistic regression models using widely available laboratory values have previously shown that an ALT level more than three times above the norm at patients' admission [specificity of 82%, sensitivity of 60%, receiver operating characteristic (ROC)-AUC 0.733; P < 0.001] and age > 69.5 years (specificity 92%, sensitivity 57%, ROC-AUC 0.759; P < 0.001) act as the best predictors of biliary aetiology[17,22]. Here, our ML-based prediction score achieves a higher sensitivity (96.30%), whereby ALT and, above all, age also rank 4th and 1st in the weighting of our score, thus confirming the existing evidence in the area of non-ML laboratory value-based prediction of biliary aetiology of pancreatitis (Figure 3). Contrary to previously published studies on laboratory-based prediction of biliary pancreatitis aetiology, our prediction tool is based specifically on microlithiasis and sludge and not primarily on gallstones and occult microlithiasis/sludge subsumed in this cohort.

Our study has several limitations. First, the retrospective study approach did not allow us to generate a uniform definition of the two entities microlithiasis and sludge. Even after extensive literature research, we were unable to delineate a uniform but distinct definition of biliary microlithiasis and sludge. We thus decided to use the terms as synonyms between the endoscopy centers of the three participating university hospitals. This might impose a significant bias. The macrolithiasis, which was again clearly listed in the endoscopy findings across the universities, ensured quality of EUS. Likewise, the patient cohort declared as other-AP in terms of aetiology varied greatly between the participating centers (Supplementary Table 1). Ultimately, this probably reflects the individual diagnostic scope and the question of

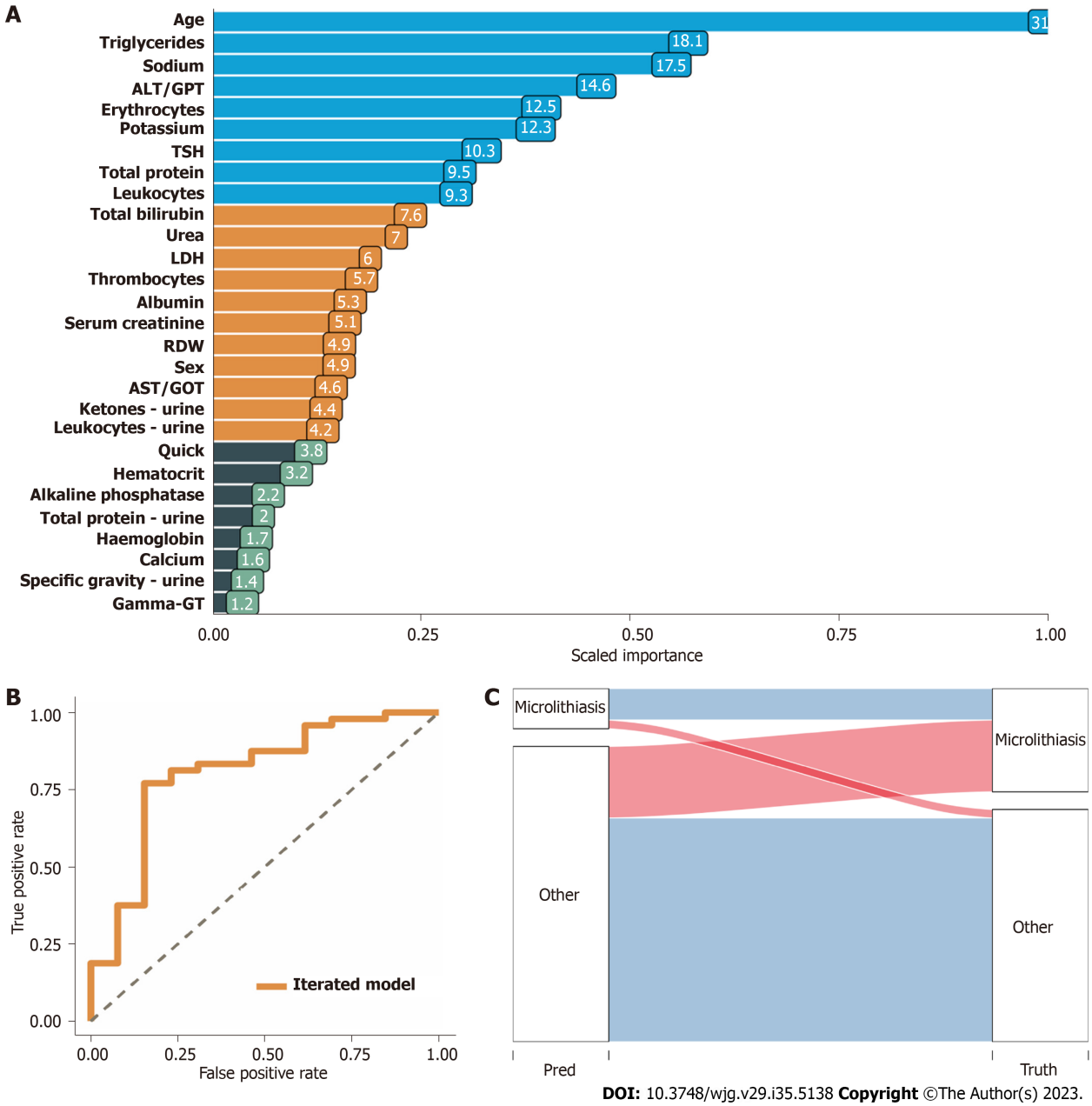

Figure 3 Graphical representation of the prediction model variables according to importance of scale. A: Variables of the final (iterated) auto-machine learning prediction model are ordered by scale of importance; B and C: Precoat diagram showing robust positive and negative prediction (3/81 patient cases were misclassified as microlithiasis and not other-acute pancreatitis). Gamma-GT: Gamma-glutamyl transpeptidase; AST: Aspartate aminotransferase; GOT: Glutamic oxalacetic transaminases; ALT: Alanine transaminase; GPT: Glutamic pyruvic transaminase; LDH: Lactate dehydrogenase; RDW: Red blood cell distribution width.

whether EUS can generate added value in the context of the individual patient. Also, due to the retrospective study design, no attempt could be made to increase the degree of purity of biliary (microlithiasis and sludge) triggered pancreatitis by uniformly fulfilling laboratory chemistry tests prior to EUS. This resulted in a proportion of patients of 36.6% with, for example, missing calcium values in the laboratory chemistry pancreatitis workup.

Our study is convincing in presenting for the first time a robust ML-based and externally validated prediction model for pancreatitis patients declared idiopathic early in the diagnostic workup and may be helpful as a noninvasive decision tool by combining simple and widely used laboratory values to decide for or against EUS. In order to make the microlithiasis predictive score and the relatively high number of underlying variables usable, a user-friendly interface is available online at the following link for use in the research context: https://github.com/mayerlelab/microlithiasisPredict. To illustrate the performance of the microlithiasis predictive score, we designed a graphical user interface for a quick entry of the values of the necessary patient variables, followed by the prediction of the need for EUS. The user-friendly interface (Video core tip, currently not deployed on Web) provides the user with the model-based estimated probability of the patient stratification to microlithiasis/sludge and other-pancreatitis. Moreover, it provides several graphical presentations to illustrate the impact of the specific variables on the decision. A multicenter prospective score validation with harmonised predefinition of biliary sludge and microlithiasis is currently being planned.

CONCLUSION

We present for the first time an ML-based tool, externally validated in two sets of data from tertiary pancreatic referral centers, to predict the presence of biliary sludge and microlithiasis in patients with an initial label of idiopathic pancreatitis with an accuracy of 0.7607 (95%CI: 0.673-0.8347), PPV of 0.7573, and NPV of 0.7857. Upon prospective validation, the prediction score will aid in decision-making on which patient to subject to EUS for diagnostic workup at a first episode of pancreatitis.

ARTICLE HIGHLIGHTS

Research background

About 30% of acute pancreatitis (AP) cases classified as idiopathic actually have a biliary and thus monocausally treatable origin.

Research motivation

To date, there is no predictive score to differentiate between idiopathic and sludge- and microlithiasis-triggered acute biliary pancreatitis. Undiagnosed biliary pancreatitis aetiology poses the risk of overdiagnosis and additional patient burden. AP triggered by small biliary concrements (microlithiasis and sludge) is a particularly challenging diagnosis.

Research objectives

The aim of this study was to develop a machine-learning based prediction score for the presence of microlithiasis and sludge in AP patients. External score validation was performed at two university pancreas centres.

Research methods

The clinical and laboratory parameters of 218 AP patients were used to calculate a machine-learning based prediction model for the presence of sludge and microlithiasis. Forty-seven patients with endosonographic evidence of sludge and microlithiasis (and no other possible underlying pancreatitis aetiology) were used in the identification cohort and compared with 171 AP patients without endosonographic evidence of sludge and microlithiasis. We trained supervised machine learning classifiers using H_2O.ai automatically selecting the best suitable predictor model to predict microlithiasis/sludge. An external pancreatitis cohort from two university pancreas centres with 117 patients was used for validation.

Research results

The score, constructed from a total of 28 simple variables to be collected in the early phase of pancreatitis-associated hospitalisation and validated externally at two university pancreas centres, can predict the presence of biliary sludge and microlithiasis with an accuracy of 0.7607 (95% confidence interval: 0.673-0.8347), positive predictive value of 0.7573, and negative predictive value of 0.7857.

Research conclusions

For the first time, we present a machine-learning based prediction score to differentiate between sludge- and microlithiasis-triggered AP and idiopathic pancreatitis. By using it in the early phase of pancreatitis-related hospitalisation, patient selection for or against the use of endosonography can support clinical decision-making.

Research perspectives

Upon prospective validation, the prediction score will aid in decision-making on which patient to subject to endosonography for diagnostic workup at a first episode of pancreatitis specifically to differentiate between sludge/microlithiasis-triggered and idiopathic AP.

FOOTNOTES

Author contributions: Sirtl S, Żorniak M, Beyer G, Schulz C, Schirra J, Mayerle J, and Mahajan UM designed this study; Sirtl S, Żorniak M, Hohmann E, Dibos M, Wandel A, Phillip V, Ammer-Herrmenau C, Neesse A, Mayerle J, and Mahajan UM contributed to the data acquisition; Sirtl S, Żorniak M, Mayerle J, and Mahajan UM were involved in the data analysis, and manuscript and figure preparation; Mahajan UM participated in the algorithmic programming and statistical analysis; Beyer G, Schulz C, and Schirra J contributed to the technical advice; and all authors approved the final version of the manuscript.

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Institutional review board statement: The study was approved by the Ethics Committee at LMU Munich (Project no.21 - 0126) and was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. The Ethics Committees of the Technical University of Munich and the University Hospital of Göttingen gave their approval for the study to be conducted under the reference

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Conflict-of-interest statement: All the authors report no relevant conflicts of interest for this article.

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ORIGINAL ARTICLE

Clinical Trials Study

Heparanase inhibition leads to improvement in patients with acute gastrointestinal injuries induced by sepsis

Ting-Ting Chen, Jia-Jun Lv, Ling Chen, Min Li, Li-Ping Liu

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Abstract

BACKGROUND

Patients with sepsis are at high risk for acute gastrointestinal injury (AGI), but the diagnosis and treatment of AGI due to sepsis are unsatisfactory. Heparanase (HPA) plays an important role in septic AGI (S-AGI), but its specific mechanism is not completely understood, and few clinical reports are available.

AIM

To explore the effect and mechanism of HPA inhibition in S-AGI patients.

METHODS

In our prospective clinical trial, 48 patients with S-AGI were randomly assigned to a control group to receive conventional treatment, whereas 47 patients were randomly assigned to an intervention group to receive conventional treatment combined with low molecular weight heparin. AGI grade, sequential organ failure assessment score, acute physiology and chronic health evaluation II score, D-dimer, activated partial thromboplastin time (APTT), anti-Xa factor, interleukin-6, tumour necrosis factor-α, HPA, syndecan-1 (SDC-1), LC3B (autophagy marker), intestinal fatty acid binding protein, D-lactate, motilin, gastrin, CD4/CD8, length of intensive care unit (ICU) stay, length of hospital stay and 28-d survival on the 1st, 3rd and 7th d after treatment were compared. Correlations between HPA and AGI grading as well as LC3B were compared. Receiver operator characteristic (ROC) curves were generated to evaluate the diagnostic value of HPA, intestinal fatty acid binding protein and D-lactate in S-AGI.

RESULTS

Serum HPA and SCD-1 levels were significantly reduced in the intervention group compared with the control group ($P < 0.05$). In addition, intestinal fatty

acid-binding protein, D-lactate, AGI grade, motilin, and gastrin levels and sequential organ failure assessment score were significantly decreased ($P < 0.05$) in the intervention group. However, LC3B, APTT, anti-Xa factor, and CD4/CD8 were significantly increased ($P < 0.05$) in the intervention group. No significant differences in interleukin-6, tumour necrosis factor-α, d-dimer, acute physiology and chronic health evaluation II score, length of ICU stay, length of hospital stay, or 28-d survival were noted between the two groups ($P > 0.05$). Correlation analysis revealed a significant negative correlation between HPA and LC3B and a significant positive correlation between HPA and AGI grade. ROC curve analysis showed that HPA had higher specificity and sensitivity in diagnosis of S-AGI.

CONCLUSION

HPA has great potential as a diagnostic marker for S-AGI. Inhibition of HPA activity reduces SDC-1 shedding and alleviates S-AGI symptoms. The inhibitory effect of HPA in gastrointestinal protection may be achieved by enhanced autophagy.

Key Words: Sepsis; Acute gastrointestinal injury; Heparanase; Autophagy

Core Tip: Heparanase (HPA) plays an important role in the occurrence and development of septic acute gastrointestinal injury (S-AGI). Our experimental results show that HPA has great potential as a diagnostic marker for S-AGI. Inhibition of HPA activity reduces syndecan-1 shedding, reduces inflammatory response, improves coagulation and immune function, and alleviates S-AGI symptoms. The inhibitory effect of HPA on gastrointestinal protection may be achieved by increasing the level of autophagy.

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INTRODUCTION

Sepsis, a life-threatening condition caused by the host's dysfunctional response to infection, is a common condition in the intensive care unit (ICU) and is associated with acute organ dysfunction and a high risk of death[1]. Sepsis has become an important public health problem worldwide due to its extremely high prevalence and mortality[2-4]. The intestine is one of the organs most vulnerable to dysfunction caused by sepsis[5]. It has been reported that sepsis causes acute gastrointestinal injury (AGI) in more than 90% of patients[6] and that gastrointestinal function is an important determinant of outcome in ICU patients[7]. Thus, AGI is the central link of sepsis. During sepsis, increased cytokine levels lead to increased intestinal mucosal permeability, in which activated myosin light streptokinase increases paracellular permeability and leads to contraction or opening of tight junctions in the apical region. Increased intestinal permeability subsequently leads to increased systemic inflammation through a positive feedback loop, forming a vicious cycle[8,9]. Treatment of septic AGI (S-AGI) currently consists mainly of prevention and correction of intestinal flora disorders, administration of intestinal mototropic agents, and early restoration of intestinal nutrition. However, these treatments do not necessarily have satisfactory therapeutic results[10]. Therefore, it is of great significance to explore treatment for S-AGI.

Heparanase (HPA) is the only enzyme in the body that can degrade heparin/heparin sulfate. HPA exists in lysosomes in the form of protonase and is widely activated in the context of tumours, inflammation, injury, hypertrophic lesions and immune reactions[11,12]. HPA degrades the heparin sulfate side chain of heparan sulfate proteoglycan (HSPG) and destroys the extracellular matrix and basement membrane, thereby damaging the structural integrity of cells[13]. In addition, HPA exhibits nonenzymatic functions, including cell signaling, adhesion, and differentiation[14]. HPA plays an important role in sepsis. A recent study demonstrated that HPA expression increases during sepsis and is associated with mortality[15]. In our previous review, we reasonably hypothesized that HPA is involved in the occurrence and development of S-AGI[16]. However, the mechanism is unclear, especially in clinical practice, and needs further investigation.

Low molecular weight heparin (LMWH) derived from common heparin is widely used due to its excellent efficacy, good predictability, low risk of bleeding, and reduced number of side effects[17]. With deepening of research, LMWH has been used in other applications in addition to anticoagulation as an anti-inflammatory, anti-fibrosis, antitumour, or antiviral agent[18-20]. These actions are all achieved by inhibiting HPA. As an inhibitor of HPA, LMWH is widely used in sepsis and inflammatory bowel disease[21,22]. Therefore, LMWH was selected as the intervention drug for the intervention group. In this study, we aimed to explore whether the gastrointestinal symptoms of S-AGI patients improve after HPA suppression and whether indicators of inflammation, coagulation, immunity, and survival status improve. The

possible mechanism was also explored.

MATERIALS AND METHODS

Patients

This study was a prospective double-blind randomized controlled trial approved by the Ethics Committee of the First Hospital of Lanzhou University. The ethics number is LDYYLL2022-270. S-AGI patients in the ICU of the First Hospital of Lanzhou University were selected from March 2022 to February 2023. The flow chart is presented in Figure 1, and 95 patients were finally included in the study.

Inclusion criteria: (1) Age ≥ 18 years old, sex unrestricted; (2) Patient meets the diagnostic criteria for sepsis 3.0 [positive or suspected infection with Sequential Organ Failure Assessment (SOFA) ≥ 2 points][1]; (3) Patient meets the AGI diagnostic criteria [(ESICM) 2012 recommendation AGI severity rating][6]; and (4) Informed consent signed by the patient or his or her family.

Exclusion criteria: (1) Combined with underlying gastrointestinal diseases (tumour, tuberculosis, inflammatory diseases, *etc.*); (2) Gastrointestinal surgery; (3) Patients with terminal disease expected to die within 24 h; (4) Patients with neurogenic shock, cerebrovascular accident, or craniocerebral trauma; and (5) Patients with definite haemorrhagic disease.

Groups and treatment

Patients who met the inclusion criteria were randomly assigned to the control group or the intervention group by hierarchical randomization generated by SAS statistical software. A letter for each random number was prepared in duplicate in a blind manner and sealed. At the time of statistical analysis, the blinding was exposed twice, the first blinding involved dividing the patients into groups, and the specific drugs in each group were determined at the second blinding. However, if the patient's condition recurred or haemodynamic instability affected the patient's prognosis during the study, it was terminated, and the blinding was urgently removed.

The control group included 48 patients who received conventional treatment; 47 patients in the intervention group were treated with LMWH in addition to conventional treatment. The control group received special intensive care as needed, including oxygen or mechanical ventilation, antimicrobial therapy, vasopressor administration, fluid resuscitation, blood glucose control, nutritional support, analgesia, sedation, or renal replacement therapy. The control group did not receive heparin as the standard of care for S-AGI patients. In the intervention group, patients were administered LMWH sodium (4000 U qd, subcutaneous injection) for 7 consecutive days in addition to receiving standard treatment as described above. The control group was given the same dose of saline (subcutaneous injection) for 7 consecutive days.

Research indicators and outcome measurement

Baseline data, such as age, sex, body mass index, source of infection, indicators of infection, AGI grade, SOFA score, and Acute Physiology And Chronic Health Evaluation II (APACHE II) score, of all patients were collected at admission. Gastrointestinal functional status was observed at 1, 3 and 7 d after treatment. Specifically, AGI grading assessment, SOFA score, APACHE II score, D-dimer, activated partial thromboplastin time (APTT), and anti-Xa factor coagulation index data were collected. Serum interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), HPA, syndecan-1 (SDC-1), LC3B, intestinal fatty acid binding protein (IFABP), D-lactate, motilin and gastrin levels were measured by enzyme-linked immunosorbent assay (ELISA). CD4 and CD8 T cells were detected by flow cytometry. The length of ICU stay and length of hospital stay were assessed, as was survival status at 28 d of all patients.

ELISA

Serum samples were diluted at an appropriate ratio, and the standard working solution was configured according to the kit instructions (Elabscience, Shanghai, China). Standard, blank and sample wells were established. Then, 100 μL of standard, standard and sample diluent and serum samples to be tested were added and incubated at 37 °C for 90 min. The biotinylated antibody working solution, enzyme binding working solution, substrate solution and termination solution were added successively. After the reaction was terminated, the optical density (OD value) of each well was immediately measured based on an enzyme label at 450 nm.

Flow cytometry

FITC-labelled (the reagents were purchased from Boster, Wuhan, China) mouse anti-human CD3 antibody (2 μL), APC-labelled mouse anti-human CD4 antibody (1 μL), and PerCP/Cy5.5 mouse anti-CD8B monoclonal antibody (1 μL) were placed into flow cytometry test tubes. One hundred microlitres of whole peripheral blood was obtained and incubated at room temperature for 15 min after shaking and mixing. Then, 500 μL of haemolysin, 200 μL of phosphate buffered saline and 100 μL of fully mixed microspheres were added, and the specimens were assessed by flow cytometry. Cells were analysed by Kaluza Analysis software to obtain CD4 and CD8 T-cell counts.

Statistical analysis

Normally distributed data are expressed as the mean ± standard deviation (SD) and were compared with a t test. Nonnormally distributed data are expressed as the median (interquartile range) and were compared using the Mann-

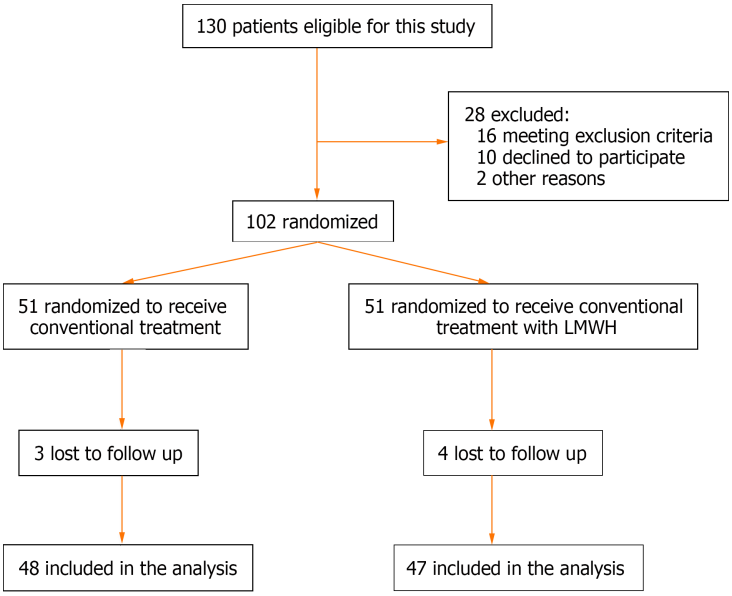

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Figure 1 Flow diagram of the participant selection. LMWH: Low molecular weight heparin.

Whitney *U* test. Counting data were tested using χ^2 tests. The Kolmogorov-Smirnov test was used to test the normal distribution of data. To take into account the repeated nature of the variables, analysis of variance for repeated measurements of the general linear model was implemented. Correlations were analysed using the Pearson method. The Kaplan-Meier method was used to generate a survival curve within 28 d after inclusion. The diagnostic value of HPA was evaluated by receiver operator characteristic (ROC) curve analysis. Graphs were generated using GraphPad Prism 8.0.2 software (SYSTAT, United States), and $P < 0.05$ was considered statistically significant.

RESULTS

A total of 130 patients were screened during the trial (Figure 1). Regarding loss to follow-up, 7 patients were transferred to hospitals for treatment or contact was lost after discharge and could not be followed up. In total, 95 patients with S-AGI were finally included. Of these patients, 48 were randomly assigned to the control group and 47 to the intervention group. The baseline data and clinical parameters of the patients at admission are presented in Table 1. The mean age of the control group was 59.90 ± 18.81 years old, and 68.75% were male. The mean age of patients in the intervention group was 60.98 ± 14.10 years old, and 70.21% were male. In the control group, 9 patients (18.75%) were classified as having AGI grade I, 13 patients (27.08%) as having AGI grade II, 20 patients (41.67%) as having AGI grade III, and 6 patients (12.50%) as having AGI grade IV. In the intervention group, 8 cases (17.02%), 10 cases (21.28%), 22 cases (46.81%) and 7 cases (14.89%) were classified as AGI grades I, II, III and IV, respectively. No significant differences in serum white blood cell counts or procalcitonin, HPA and SDC-1 levels were noted between the two groups ($P > 0.05$). Overall, the two groups were well balanced in terms of baseline characteristics.

LMWH effectively inhibits serum HPA and SDC-1 in S-AGI patients

The serum HPA concentration in the control group was significantly higher than that in the intervention group on the 3rd and 7th d of treatment (Figure 2A) ($P < 0.05$). Serum SDC-1 also showed a difference between the two groups on the 7th d of treatment (Figure 2B) ($P < 0.05$). The above data indicate that serum HPA and SDC-1 levels were effectively inhibited in S-AGI patients in the intervention group.

HPA inhibition improves gastrointestinal function in S-AGI patients

AGI ratings were assessed on the 1st, 3rd and 7th d after treatment (Figure 3A). The AGI grades of both groups decreased and were significantly lower in the intervention group than in the control group on the 7th d ($P < 0.05$). As shown in Table 2, the number of AGI II, III and IV patients in the intervention group was significantly lower after 7 d of treatment than after 1 and 3 d of treatment. In addition, the number of AGI II, III and IV patients were significantly lower in the intervention group than in the control group. IFABP and D-lactate are intestinal barrier biomarkers. Figures 3B and C shows that serum IFABP and D-lactate concentrations on the 7th d were significantly lower than those on the 1st d, with the concentrations in the intervention group being significantly lower than those in the control group ($P < 0.05$). Motilin and gastrin are indicators of gastrointestinal motility. As shown in Figures 3D and E, motilin and gastrin levels increased significantly in the intervention group after 7 d of treatment ($P < 0.05$). All the above data indicate that inhibition of HPA significantly improved gastrointestinal function, the intestinal barrier and gastrointestinal dynamics in S-AGI patients.

Table 1 Changes in the acute gastrointestinal injury grades of the patients in the two groups

Variable	Control group (*n* = 48)	Intervention group (*n* = 47)	*P* value
Age, mean (SD), yr	59.90 (18.81)	60.98 (14.10)	0.752
Sex, male, *n* (%)	33 (68.75)	33 (70.21)	0.877
BMI, mean (SD), kg/m^2	22.62 (4.08)	23.89 (5.10)	0.788
MODS, *n* (%)	33 (68.75)	34 (72.34)	0.701
Septic shock, *n* (%)	32 (66.67)	31 (65.96)	0.942
APACHE II score, median (IQR)	22 (19, 29)	23 (19, 35)	0.966
SOFA score, median (IQR)	9 (7,10.75)	9 (7, 13)	0.871
Infection score, *n* (%)			
Lung	10 (20.83)	16 (34.04)	0.149
Urinary tract	2 (4.17)	1 (2.13)	0.57
Intra-abdominal	14 (29.17)	16 (34.04)	0.609
Central nervous system	13 (27.08)	7 (14.89)	0.145
Blood/vascular access	3 (6.25)	4 (8.51)	0.673
Other	5 (10.42)	2 (4.26)	0.25
Confirmed unknown	1 (2.08)	1 (2.13)	0.988
Initial AGI grade, *n* (%)			
I	9 (18.75)	8 (17.02)	0.826
II	13 (27.08)	10 (21.28)	0.509
III	20 (41.67)	22 (46.81)	0.614
IV	6 (12.50)	7 (14.89)	0.734
WBC, mean (SD), (10^9/L)	19.20 (9.91)	15.92 (9.65)	0.424
PCT, mean (SD), (ng/mL)	10.77 (21.64)	11.19 (17.58)	0.919
HPA, mean (SD), (ng/mL)	10.10 (0.91)	9.81 (0.72)	0.095
Syndecan-1, mean (SD), (ng/mL)	31.77 (7.49)	31.45 (8.29)	0.845

BMI: Body mass index; MODS: Multiple organ dysfunction syndrome; APACHE II: Acute physiology and chronic health evaluation II; SOFA: Sequential organ failure assessment; AGI: Acute gastrointestinal injury; HPA: Heparanase; WBC: Blood cell count; PCT: Procalcitonin; IQR: Interquartile range.

Table 2 Changes in the acute gastrointestinal injury grades of the patients in the two groups

	AGI I, n (%)		AGI II, n (%)		AGI III, n (%)		AGI IV, n (%)	
	Control group	Intervention group	Control group	Intervention group	Control group	Intervention group	Control group	Intervention group
Day 1	0 (0)	0 (0)	6 (12.50)	6 (12.77)	29 (60.42)	29 (61.70)	13 (27.08)	12 (25.53)
Day 3	0 (0)	0 (0)	14 (29.17)	20 (42.55)	30 (62.50)	26 (55.32)	4 (8.33)	1 (2.13)
Day 7	7 (14.58)	17 (36.17)	31 (64.58)	28 (59.57)	10 (20.83)	2 (4.26)	0 (0)	0 (0)

AGI: Acute gastrointestinal injury.

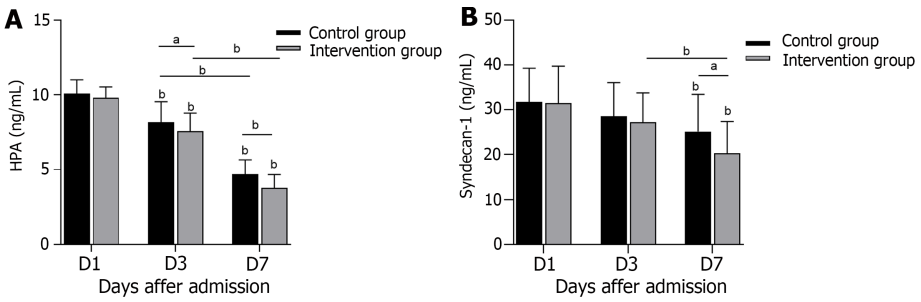

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Figure 2 Comparisons of heparanase and syndecan-1 levels between the two groups. A: Heparanase; B: Syndecan-1. $^{a}P < 0.05$, $^{b}P < 0.001$. HPA: Heparanase.

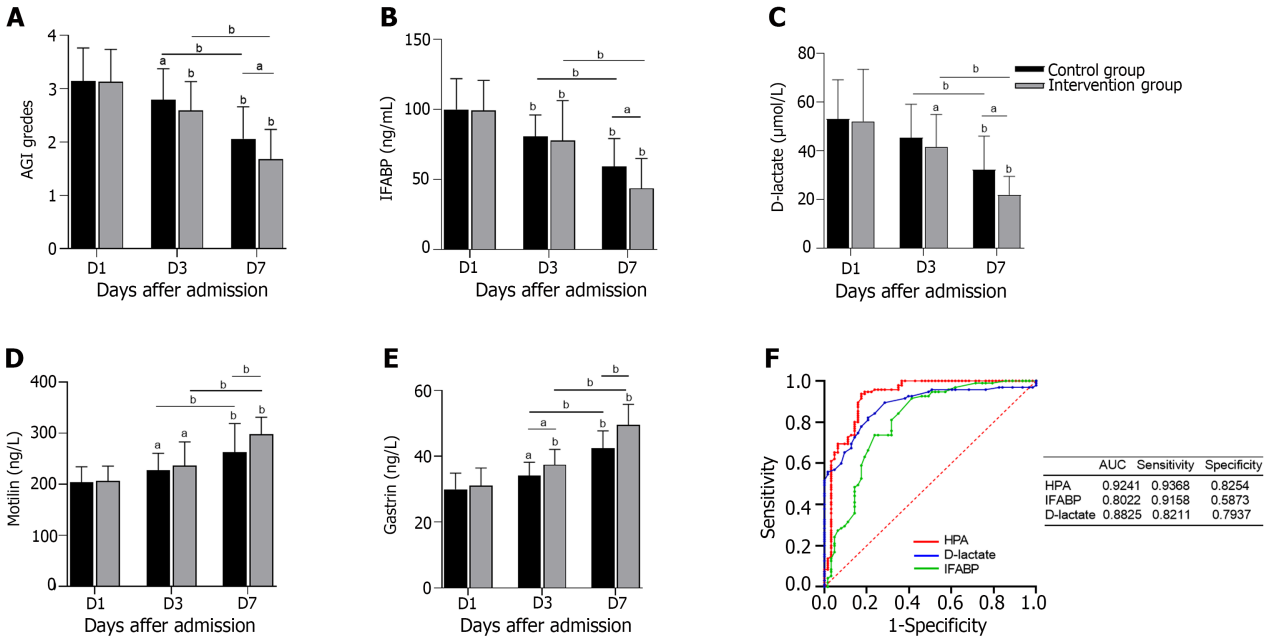

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Figure 3 Comparisons of acute gastrointestinal injury grades, intestinal fatty acid binding protein, D-lactate, motilin, and gastrin levels between the two groups. Receiver operating characteristic curves of heparanase, D-lactate and intestinal fatty acid binding protein. A: Acute gastrointestinal injury grades; B: Intestinal fatty acid binding protein; C: D-lactate; D: Motilin; E: Gastrin; F: Receiver operating characteristic curves of heparanase, D-lactate and intestinal fatty acid binding protein. $^{a}P < 0.05$, $^{b}P < 0.001$. AGI: acute gastrointestinal injury; HPA: Heparanase; IFABP: Intestinal fatty acid binding protein; AUC: Area under the curve.

As shown in Figure 3F, we plotted ROC curves for HPA, IFABP and D-lactate and calculated their AUC values. IFABP and D-lactate are biomarkers of septic AGI, but the AUC for HPA of 0.9241 (95% confidence interval: 0.8690-0.9707) was the largest of the three. The sensitivity and specificity of HPA were 93.68% and 82.54%, respectively, and compared with the sensitivity of D-lactate (82.11% and 79.37%) and the sensitivity of IFABP (91.58% and 58.73%), HPA was still highest. These results indicate that HPA has better diagnostic efficacy in S-AGI. Overall, HPA exhibits great potential as a biomarker for S-AGI.

HPA inhibition induces anticoagulant effects and enhances immune function

Figure 4 shows the inflammation, coagulation and immune indices of the two groups after treatment. As illustrated in Figures 4A and B, IL-6 and TNF-α serum levels decreased significantly on the 7th d of treatment compared with on the 1st d ($P < 0.05$). Despite the lack of a significant difference between the two groups, levels of inflammatory cytokines in the intervention group were reduced. After 7 d of treatment, APTT and anti-Xa factor levels in the two groups increased significantly compared with those on the 1st d of treatment ($P < 0.05$), whereas D-dimer levels decreased significantly ($P < 0.05$). APTT and anti-Xa factor levels increased significantly in the intervention group compared with the control group ($P < 0.05$) (Figures 4C-E). The anticoagulation effect in the intervention group was better than that in the control group. As shown in Figure 4F, the intervention group exhibited significantly more CD4/CD8 cells than the control group ($P < 0.05$). In conclusion, compared with the control group, the intervention group exhibited better anticoagulant effects and immune enhancement effects.

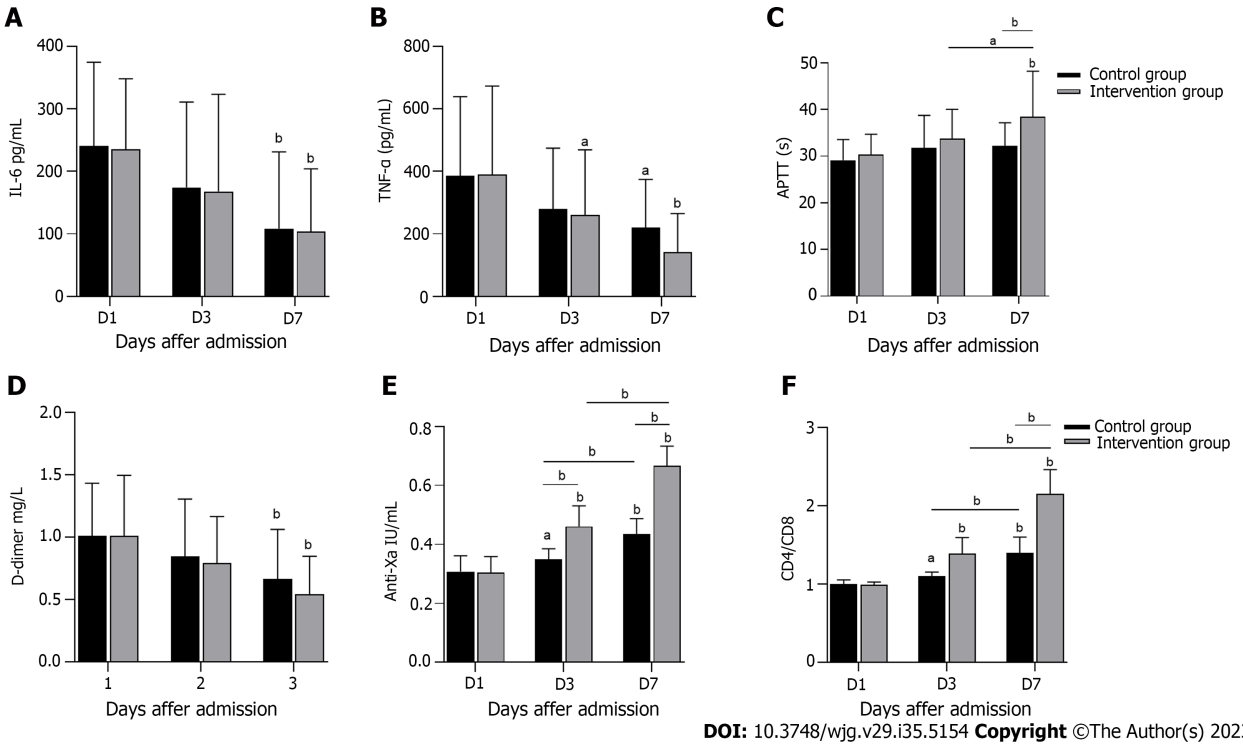

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Figure 4 Comparisons of interleukin-6, tumor necrosis factor-α, activated partial thromboplastin time, D-dimer, anti-Xa, and CD4/CD8 levels between the two groups. A: Interleukin-6; B: Tumor necrosis factor-α; C: Activated partial thromboplastin time; D: D-dimer; E: Anti-Xa; F: CD4/CD8. $^{a}P < 0.05$, $^{b}P < 0.001$. IL-6: Interleukin-6; TNF-α: Tumor necrosis factor-α; APTT: Activated partial thromboplastin time.

HPA inhibition improves gastrointestinal function in S-AGI patients through modulation of autophagy

To explore the possible mechanism by which HPA inhibition improves gastrointestinal symptoms in S-AGI patients, autophagy was assessed (Figure 5). The LC3B level of the intervention group was significantly higher than that of the control group ($P < 0.05$). As shown in Table 3, a significant negative correlation was noted between HPA and LC3B and a significant positive correlation between HPA and AGI grade. Thus, the decrease in serum HPA and SDC-1 is critical for S-AGI patients, and HPA correlates significantly with autophagy and gastrointestinal functional status.

HPA inhibition partially improves the severity score of S-AGI patients but does not shorten the length of hospital stay or improve the survival status

Within 7 d of ICU treatment, the APACHE II score and SOFA score of the two groups had significantly decreased compared to those before ICU treatment ($P < 0.05$), and the SOFA score of the intervention group was significantly lower than that of the control group on the 7th d ($P < 0.05$). However, APACHE II scores did not significantly differ between the two groups (Figures 6A and B). Figures 6C and D shows the length of ICU stay and the length of hospital stay. Although no significant difference was noted between the control group and the intervention group, both stays were shorter in the intervention group. The 28-d survival curve presented in Figure 6E demonstrates no significant difference between the two groups ($P > 0.05$). These results indicated that HPA inhibition improves the clinical severity score of patients but does not significantly improve the length of hospital stay or survival rate.

DISCUSSION

S-AGI is easily missed clinically. Complex assessment of AGI grading is not based on specific symptoms but rather includes subjective assessment of the overall development of the patient's disease. The ideal approach is to replace this grading system with one or two biomarkers[23]. Therefore, it is important to explore potential biomarkers and effective therapeutic agents for S-AGI. In this study, we selected LMWH as an intervention drug to reduce HPA levels (Figure 2). Our results indicate that HPA inhibition significantly improved the gastrointestinal functional status of S-AGI patients, reduced the AGI score, improved the intestinal mucosal barrier and gastrointestinal dynamics of patients (Figure 3 and Table 2), and contributed to their early recovery. Regarding the specific mechanism of LMWH in treatment of S-AGI, we hypothesized that LMWH inhibits HPA, protects the glycocalyx, and alleviates damage to the intestinal barrier, thus improving symptoms. This activity is not related to the direct anticoagulant properties of LMWH. Similarly, Tang *et al*[24] reported that heparin prevents caspase-11-dependent coagulation activation and reduces mortality in sepsis, regardless of its direct anticoagulant properties.

Table 3 Correlation between heparanase and LC3B and acute gastrointestinal injury grade in the two groups

		Control group				Intervention group			
		LC3B (μg/mL)		AGI grade		LC3B (μg/mL)		AGI grade	
HPA (ng/mL)	Day 1	r = -0.8394	P < 0.001	r = 0.8441	P < 0.001	r = -0.8456	P < 0.001	r = 0.7106	P < 0.001
	Day 3	r = -0.9545	P < 0.001	r = 0.7670	P < 0.001	r = -0.8882	P < 0.001	r = 0.8135	P < 0.001
	Day 7	r = -0.8258	P < 0.001	r =0.7657	P < 0.001	r = -0.8724	P < 0.001	r = 0.7839	P < 0.001

AGI: Acute gastrointestinal injury; HPA: Heparanase.

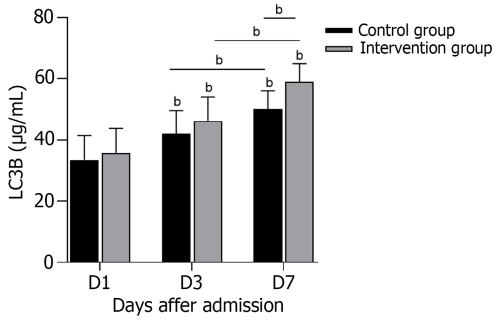

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Figure 5 Comparisons of LC3B levels between the two groups. ^{b}P < 0.001.

The glycocalyx is a complex, negatively charged gel layer on one side of the lumen of endothelial cells. During sepsis, the glycocalyx becomes degraded through activation of various enzymes and/or release of reactive oxygen species[25,26]. A degraded glycocalyx induces white blood cell binding and extravasation as well as platelet recruitment, resulting in increased inflammation and increased risk of thrombosis. In addition, loss of calyx can lead to capillary leakage, which leads to oedema and reduced blood volume throughout the body. Together with thrombosis, these effects lead to tissue hydroperitoneum and organ failure[27,28]. Thus, protection of glycocalyx integrity and the intestinal barrier is essential for treatment of S-AGI. SDC-1 is a biomarker for the glycocalyx and is a transmembrane HSPG that is expressed primarily by intestinal epithelial cells; this protein is strongly associated with inflammatory processes and the integrity of the intestinal mucosa[18]. A recent meta-analysis showed that SDC-1 levels may be a useful predictor of sepsis-related complications and mortality[29]. Therefore, SDC-1 plays a crucial role in S-AGI. HPA is closely related to SDC-1, which degrades the heparin sulfate side chain of HSPG[13], accelerates shedding of SDC-1 from endothelial cells, and increases serum SDC-1 concentrations. LMWH inhibits HPA activity and prevents endothelial cell injury[28]. Therefore, our intervention results also revealed high HPA and SDC-1 levels in the context of decreased S-AGI after treatment. As HPA was significantly inhibited after conventional treatment combined with LMWH treatment, the concentrations of HPA and SDC-1 decreased more significantly (Figures 2A and B). This finding is consistent with previously reported conclusions [15,30].

Our correlation analysis revealed a significant positive correlation between HPA and AGI levels, with AGI levels decreasing significantly after LMWH inhibited HPA (Tables 2 and 3). Additionally, ROC curve analysis suggested that HPA may serve as a biomarker for S-AGI given that HPA is more specific and sensitive than IFABP and D-lactate (Figure 3F). In conclusion, our results indicate that the gastrointestinal symptoms of S-AGI patients are improved and AGI scores are reduced after HPA inhibition. HPA is expected to serve as a diagnostic biomarker for S-AGI.

In sepsis, extensive cross-talk occurs between inflammatory and clotting pathways, accompanied by overactivity and immunosuppression of the inflammatory and clotting responses, which interferes with microcirculation perfusion and leads to organ failure[31,32]. Patients with S-AGI also exhibit excessive inflammation, hypercoagulability, and immunosuppression, and these conditions improve after treatment, as shown in Figure 4. Unfortunately, there was no significant difference in inflammation between the two groups. HPA activates macrophages, leading to secretion of monocyte chemoattractant protein-1, TNF-α, and IL-1β, independent of heparin sulfate degradation activity[33], and these cytokines appear to be elevated in coronavirus disease 2019 patients[34]. It is worth mentioning that LMWH targets factor Xa to play an anticoagulant role and exhibits high anti-Xa activity[35]; hence, the anticoagulant effect in the intervention group was significantly better than that in the control group. In addition, according to the LMWH dose in our treatment plan, no associated bleeding risk was noted during patient treatment, indicating that LMWH is safe and effective. In this study, we found that CD4/CD8 levels in the intervention group were significantly increased. Therefore, HPA inhibition inhibits hypercoagulability and improves immune function in S-AGI patients.

To further investigate the possible mechanism by which HPA is reduced to improve S-AGI, we measured changes in serum LC3B levels in patients during treatment. The intervention results showed that the LC3B level was increased in the intervention group after treatment, with a significant negative correlation noted between HPA and LC3B (Figure 5,

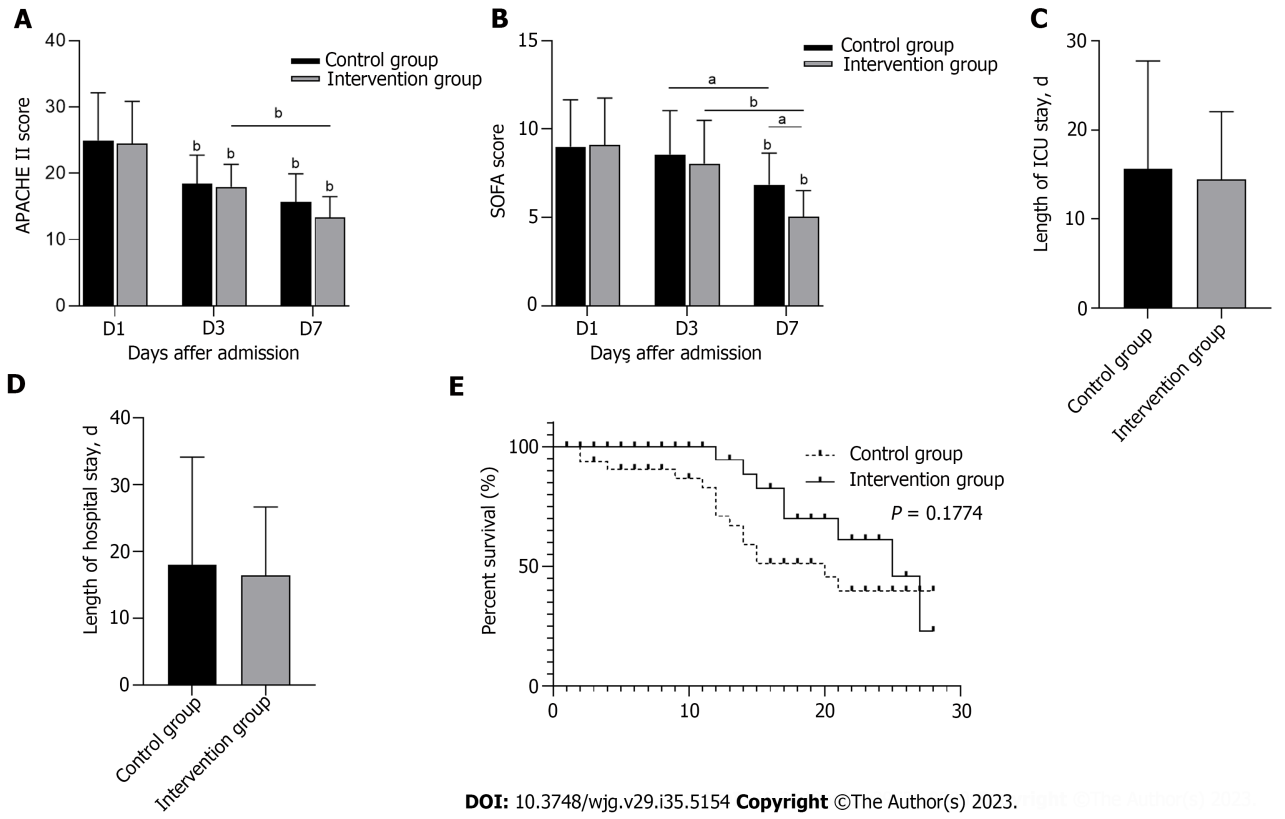

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Figure 6 Comparisons of Acute Physiology and Chronic Health Evaluation score, Sequential Organ Failure Assessment score, length of intensive care unit stay, length of hospital stay, and survival probability within 28 d between the two groups. A: Acute Physiology and Chronic Health Evaluation score; B: Sequential Organ Failure Assessment score; C: Length of intensive care unit stay; D: Length of hospital stay; E: Survival probability within 28 d. $^{a}P < 0.05$, $^{b}P < 0.001$. APACHE II: Acute Physiology and Chronic Health Evaluation II; SOFA: Sequential Organ Failure Assessment; ICU: Intensive care unit.

Table 3). LC3B is a marker of autophagy. Autophagy is the process by which bacteria and viruses that have escaped from phagosomes or damaged mitochondria are enclosed in vesicles, which fuse with lysosomes to form autophagosomes, followed by degradation of the contents[36]. In the early stage of sepsis, autophagy occurs in the heart, brain, lung, liver, kidney and other important organs and plays a protective role in the body. With the progression of sepsis, the body enters a period of continuous immunosuppression, and autophagy activity decreases[37]. This finding is consistent with our results. However, the results for LC3B are only indirect evidence and cannot directly show that HPA correlates completely with autophagy. Therefore, we hypothesize that HPA might aggravate S-AGI by inhibiting autophagy, and we are performing further basic experiments to test this hypothesis. LMWH inhibits HPA, thus enhancing the level of autophagy and playing a protective role in the gastrointestinal tract.

Although HPA inhibition offers many advantages, it did not significantly reduce the length of hospital stay or increase the 28-d survival rate of S-AGI patients (Figure 6). We hypothesize that the reason may be the complex aetiology of ICU patients, critical conditions, mixed interference factors during treatment, and/or the small study sample. Thus, the intervention group did not achieve our expected effect.

Finally, our experiment has some limitations: (1) Given our single-centre design and small sample size, the results may not be generalizable, and the conclusion needs to be confirmed by large-scale clinical prospective trials; (2) LMWH is not a specific HPA inhibitor, but a safe and effective specific HPA inhibitor is currently not available in clinical practice. Therefore, further development of new drugs is needed; and (3) Inhibition of HPA may enhance the level of autophagy and thus protect the gastrointestinal tract in sepsis, and this mechanism needs to be verified by basic experiments.

CONCLUSION

Our intervention results showed that LMWH inhibits HPA activity in S-AGI, reduces SDC-1 shedding, prevents endothelial cell damage, maintains intestinal epithelial cell integrity and barrier function, actively exerts anticoagulant effects, improves patients' immune function and gastrointestinal symptoms, and reduces SOFA scores. Mechanistically, HPA inhibition may play a protective role in the gastrointestinal tract by enhancing the level of autophagy. HPA represents a potential biomarker of S-AGI, and HPA inhibitors may also serve as drugs for treatment of S-AGI.

ARTICLE HIGHLIGHTS

Research background

Patients with sepsis are at high risk for acute gastrointestinal injury (AGI), heparanase (HPA) plays an important role in septic AGI (S-AGI), but its specific mechanism is not completely understood, and few clinical reports are available.

Research motivation

This study is to explore the effect and mechanism of HPA inhibition in S-AGI patients.

Research objectives

To prove the role of HPA in S-AGI and search for effective biomarkers and therapeutic targets for the diagnosis of S-AGI.

Research methods

The therapeutic effect of S-AGI patients in control group and low molecular weight heparin group was compared by a prospective double-blind randomized controlled trial. To evaluate the feasibility of HPA as a diagnostic biomarker for S-AGI.

Research results

HPA inhibitors can significantly improve AGI score, gastrointestinal function, coagulation function and immune function in S-AGI patients. The inhibitory effect of HPA in gastrointestinal protection may be achieved by enhanced autophagy.

Research conclusions

HPA has great potential as a diagnostic marker for S-AGI. Inhibition of HPA activity reduces syndecan-1 shedding and alleviates S-AGI symptoms. The inhibitory effect of HPA in gastrointestinal protection may be achieved by enhanced autophagy.

Research perspectives

HPA has great potential as a diagnostic biomarker for S-AGI, and its inhibitor is a good therapeutic drug choice in clinical practice.

FOOTNOTES

Author contributions: Chen TT collected the clinical data for data analysis and mapping and drafted the manuscript; Lv JJ collected the blood samples from the patients and performed the flow cytometry; Chen L collected blood from the patients and completed the enzyme-linked immunosorbent assays; Li M screened the research subjects and carried out clinical interventions; Liu LP participated in the experimental design, supervised the experimental process and reviewed the experimental results; and all the authors have read and approved the final manuscript.

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ORIGINAL ARTICLE

Observational Study

Lowering the threshold of alanine aminotransferase for enhanced identification of significant hepatic injury in chronic hepatitis B patients

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Abstract

BACKGROUND

The clinical and histological features of chronic hepatitis B (CHB) patients who fall into the "grey zone (GZ)" and do not fit into conventional natural phases are unclear.

AIM

To explore the impact of varying the threshold of alanine aminotransferase (ALT) levels in identifying significant liver injury among GZ patients.

METHODS

This retrospective analysis involved a cohort of 1617 adult patients diagnosed with CHB who underwent liver biopsy. The clinical phases of CHB patients were determined based on the European Association for the Study of the Liver 2017 Clinical Practice Guidelines. GZ CHB patients were classified into four groups: GZ-A (HBeAg positive, normal ALT levels, and HBV DNA ≤ 10^7 IU/mL), GZ-B (HBeAg positive, elevated ALT levels, and HBV DNA < 10^4 or > 10^7 IU/mL), GZ-C (HBeAg negative, normal ALT levels, and HBV DNA ≥ 2000 IU/mL), and GZ-D (HBeAg negative, elevated ALT levels, and HBV DNA ≤ 2000 IU/mL). Significant hepatic injury (SHI) was defined as the presence of notable liver inflammation (≥

G2) and/or significant fibrosis (≥ S2).

RESULTS

The results showed that 50.22% of patients were classified as GZ, and 63.7% of GZ patients developed SHI. The study also found that lowering the ALT treatment thresholds to the American Association for the Study of Liver Diseases 2018 treatment criteria (35 U/L for men and 25 U/L for women) can more accurately identify patients with significant liver damage in the GZ phases. In total, the proportion of patients with ALT ≤ 40 U/L who required antiviral therapy was 64.86% [(221 + 294)/794]. When we lowered the ALT treatment threshold to the new criteria (30 U/L for men and 19 U/L for women), the same outcome was revealed, and the proportion of patients with ALT ≤ 40 U/L who required antiviral therapy was 75.44% [(401 + 198)/794]. Additionally, the proportion of SHI was 49.1% in patients under 30 years old and increased to 55.3% in patients over 30 years old (P = 0.136).

CONCLUSION

These findings suggest the importance of redefining the natural phases of CHB and using new ALT treatment thresholds for better diagnosis and management of CHB patients in the GZ phases.

Key Words: Chronic hepatitis B; Grey zone; Indeterminate phase; Alanine aminotransferase; Antiviral therapy

Core Tip: In clinical practice, 27.8%-55% of chronic hepatitis B patients fall into the "grey zone" or "indeterminate phase" that does not meet the diagnostic criteria of the traditional stages. Additionally, there is still debate regarding how best to treat these grey zone (GZ) patients and the advantages of antiviral therapy. Hence, we evaluated the clinical and histological characteristics, and additionally explored the impact of adjusting the threshold of alanine aminotransferase (ALT) in identifying significant liver injury among GZ patients. Based on these data, lowering ALT thresholds can more accurately identify patients with significant hepatic injury at an earlier stage and reduce the need for unnecessary liver biopsies.

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INTRODUCTION

Hepatitis B poses a significant global public health challenge, as evidenced by the estimated 316 million individuals worldwide who were afflicted with the hepatitis B virus (HBV) in 2019. The impact of HBV-related diseases is substantial, as they result in approximately 555000 fatalities globally, which constitutes 48.8% of all hepatitis-related deaths. Notably, hepatitis B stands as the primary cause of mortality in cases of liver cancer and ranks as the third leading cause of death in cirrhosis cases[1]. The progression of chronic hepatitis B (CHB) is a multifaceted interplay involving viral, host, and environmental factors. HBV interacts with the immune system of the host, and the infection status undergoes continuous changes as the disease progresses[2,3]. According to the European Association for the Study of the Liver (EASL) 2017 Clinical Practice Guidelines, CHB can be categorized into five phases: HBeAg-positive chronic HBV infection, HBeAg-positive CHB, HBeAg-negative chronic HBV infection, HBeAg-negative CHB and HBsAg-negative phase[4]. Antiviral treatment is recommended for patients with HBeAg-positive and HBeAg-negative CHB, while regular monitoring is suggested in HBeAg-positive and HBeAg-negative chronic HBV infection phases[4-6].

Nevertheless, in clinical practice, a considerable proportion of CHB patients (27.8%-55%) fall into the "grey zone (GZ)" or "indeterminate phase" that does not meet the diagnostic criteria for the five stages previously indicated[7-9]. Additionally, there is still debate regarding how best to treat these GZ patients and the advantages of antiviral therapy. A study from the United States showed the incidence of hepatocellular carcinoma (HCC) is higher in GZ patients than in HBeAg-negative chronic HBV infection patients (2.67% *vs* 0.61%)[7]. Furthermore, Huang *et al*[10] demonstrated a significant decrease in the risk of HCC among GZ patients who underwent antiviral therapy[10]. In contrast, another study found that none of the patients progressed to advanced fibrosis or cirrhosis, and only a small proportion (6.3%) of GZ patients transitioned to HBeAg-negative CHB, necessitating the use of antiviral therapy[11].

There is a crucial indication for receiving antiviral therapy when patients with CHB have significant liver fibrosis and/or inflammation, which are risk factors for HCC and liver-related mortality[4-6]. However, few studies have explored liver histological injury in the GZ phase[12]. Therefore, assessing the clinical and histological features may provide useful recommendations for managing the GZ phase. Moreover, an issue that complicates the management of CHB is the disagreement regarding the appropriate treatment threshold for alanine aminotransferase (ALT) levels. The American Association for the Study of Liver Diseases (AASLD) guidelines suggest that the upper normal limit (ULN) for ALT

should be 35 U/L for men and 25 U/L for women, while the EASL guidelines consider 40 U/L as the ULN[4,5]. In China, a recent publication titled "Expert opinion on expanding anti-HBV treatment for chronic hepatitis B" proposed a lower ALT threshold (30 U/L for males and 19 U/L for females) as initiating antiviral therapy in CHB patients[13]. However, the efficacy of reducing ALT thresholds in accurately identifying patients with significant liver damage at an earlier stage and mitigating the necessity for superfluous liver biopsy remains uncertain.

Therefore, using a retrospective cohort of treatment-naive CHB patients who underwent liver biopsy, we evaluated the clinical and histological characteristics, and additionally explored the impact of adjusting the threshold of ALT in identifying significant liver injury among GZ patients.

MATERIALS AND METHODS

Methods

Patient selection: Between January 2008 and December 2020, a total of 1617 consecutive adult patients (age, ≥ 18 years) diagnosed with CHB (hepatitis B surface antigen positive > 6 mo) who had undergone liver biopsy at the Third Affiliated Hospital of Sun Yat-Sen University were included in this retrospective analysis. The exclusion criteria were as follows: (1) Viral coinfection (hepatitis C virus, hepatitis D virus, or HIV); (2) Alcohol abuse (≥ 30 g of alcohol per day for men, ≥ 20 g of alcohol per day for women), nonalcoholic fatty liver disease (diagnosed by liver biopsy) and autoimmune liver disease; (3) Decompensated cirrhosis, HCC, and nonliver cancer; (4) Liver transplantation; and (5) Prior or current antiviral treatment (Supplementary Figure 1). The study was approved by the Ethics Committee of The Third Affiliated Hospital of Sun Yat-sen University. Written informed consent was obtained from each participant prior to the liver biopsy.

Definitions: The clinical phases of patients with CHB were determined in accordance with the EASL 2017 clinical practice guidelines, taking into consideration the highest HBV-DNA levels and ALT levels observed in at least two determinations within the 12 mo preceding enrolment (Supplementary Table 1)[4]. Patients who did not meet the criteria for any of the five phases were classified as GZ, with subcategories including GZ-A (HBeAg positive, normal ALT levels, and HBV DNA ≤ 10^7 IU/mL), GZ-B (HBeAg positive, elevated ALT levels, and HBV DNA < 10^4 or > 10^7 IU/mL), GZ-C (HBeAg negative, normal ALT levels, and HBV DNA ≥ 2000 IU/mL), and GZ-D (HBeAg negative, elevated ALT levels, and HBV DNA ≤ 2000 IU/mL)[9,12]. We used ALT and gamma-glutamyl transpeptidase (GGT) levels of 40 U/L and 60 U/L as the ULN, respectively[4]. The calculations were formulated as follows: Aspartate aminotransferase (AST)-to-platelet ratio index (APRI) = [AST (U/L)/ULN]/[PLT (10^9/L)] × 100; fibrosis score based on four factors (FIB-4) = [age (years) × AST (U/L)]/[PLT (10^9/L) × √ALT (U/L)]; GGT-to-platelet ratio (GPR) = [GGT (U/L)/ULN]/[PLT (10^9/L)] × 100.

Histological assessment: Ultrasonography-guided percutaneous liver biopsy was conducted using a 16-gauge disposable needle. The minimum sample length required was 15 mm, with a minimum inclusion of 6 portal tracts. Inflammation grade (G0-G4) and fibrosis stage (S0-S4) were estimated according to Scheuer's classification[14]. In accordance with the pathological staging system, significant liver inflammation and significant fibrosis were defined as ≥ G2 and ≥ S2, respectively. Significant hepatic injury (SHI) was defined as ≥ G2 and/or ≥ S2[12,15]. The biopsy samples were subjected to blind and independent observation and interpretation by two proficient pathologists. In cases where discordance arose between the two pathologists, a third pathologist, Jianning Chen, was consulted for additional evaluation, leading to a consensus being achieved through subsequent discussion.

Statistical analysis

SPSS version 25.0 software (SPSS Inc., Chicago, IL, United States) was used for statistical analyses. Continuous variables are expressed as the median and interquartile range, and categorical data are expressed as counts and percentages. Kruskal-Wallis tests and Pearson's chi-squared tests were applied to compare variables that were significantly different between groups. The independent predictors of SHI were determined by univariate and multiple logistic regression analyses. Areas under the receiver operating characteristic curve (AUROC) were calculated to investigate the diagnostic performance of the noninvasive scores and were compared by the Delong test. All P values were two-sided, and $P < 0.05$ was deemed statistically significant.

RESULTS

Distribution, clinical characteristics, and liver histological features of patients in different immune states

The baseline characteristics of the 1617 treatment-naive patients are presented in Table 1. Based on the defined criteria, 161 (9.96%) patients were classified as having HBeAg-positive chronic HBV infection, 203 (12.55%) patients were categorized as HBeAg-positive CHB, 171 (10.58%) patients were identified as having HBeAg-negative chronic HBV infection, and 270 (16.70%) patients were classified as HBeAg-negative CHB. Interestingly, 812 (50.22%) patients did not meet the criteria for any of the aforementioned phases and were therefore designated as GZ. Notably, there were significant variations in clinical characteristics across the different phases. The average age of patients with GZ was 36.0 years, with 74.1% being male. These patients had a mean HBV DNA level of 5.24 Log_{10} IU/mL and an intermediate ALT level of 37.0 U/L.

Table 1 Baseline characteristics of chronic hepatitis B patients among different immune phases

Clinical characteristics	HBeAg-positive chronic infection (*n* = 161)	HBeAg-positive chronic hepatitis (*n* = 203)	HBeAg-negative chronic infection (*n* = 171)	HBeAg-negative chronic hepatitis (*n* = 270)	Grey zone (*n* = 812)	*P* value
Age (year)	31.0 (26.0-36.0)	33.0 (27.0-39.0)	37.0 (32.0-42.0)	39.0 (33.0-45.0)	36.0 (30.0-42.0)	< 0.001
Male (%)	97 (60.2)	168 (82.8)	133 (77.8)	221 (81.9)	602 (74.1)	< 0.001
BMI (kg/m^2)	21.3 (19.1-23.0)	22.5 (20.3-24.9)	22.6 (20.3-24.5)	22.8 (20.4-25.1)	22.0 (20.0-24.4)	< 0.001
Diabetes (%)	0 (0)	3 (1.5)	4 (2.4)	10 (3.7)	14 (1.7)	0.071
HBV DNA (log_{10} IU/mL)	8.12 (7.60-8.23)	6.17 (5.38-6.61)	2.52 (2.00-2.89)	5.47 (4.50-6.31)	5.24 (3.92-7.50)	< 0.001
PLT (10^9/L)	211.0 (179.5-238.0)	185.0 (144.0-217.0)	191.0 (156.0-230.0)	185.0 (154.8-219.3)	199.0 (160.0-234.0)	< 0.001
ALT (U/L)	27.0 (21.0-33.0)	69.0 (51.0-111.0)	25.0 (19.0-31.0)	61.5 (50.0-100.3)	37.0 (27.0-59.0)	< 0.001
AST (U/L)	24.0 (21.0-31.0)	48.0 (36.0-73.0)	24.0 (20.0-29.0)	44.0 (33.0-66.0)	31.0 (24.0-45.0)	< 0.001
GGT (U/L)	18.0 (14.0-30.0)	51.0 (30.0-93.0)	25.0 (17.0-36.0)	40.0 (27.8-80.5)	29.0 (20.0-50.0)	< 0.001
Tbil (μmol/L)	12.7 (9.3-17.6)	14.1 (11.0-19.5)	12.6 (9.9-16.5)	14.4 (10.3-21.6)	13.0 (9.6-18.0)	< 0.001
ALB (g/L)	45.2 (43.3-47.6)	44.1 (41.0-46.2)	45.8 (43.7-48.0)	44.5 (41.2-46.9)	44.9 (42.3-47.0)	< 0.001
AFP (ng/mL)	2.4 (1.7-3.5)	5.1 (3.1-14.6)	2.3 (1.6-3.8)	3.8 (2.5-8.0)	3.1 (2.1-5.3)	< 0.001
PT (s)	13.4 (13.0-13.8)	13.4 (12.8-14.1)	13.4 (12.9-14.0)	13.5 (12.9-14.1)	13.4 (12.9-14.0)	0.073
APRI	0.29 (0.24-0.39)	0.69 (0.47-1.12)	0.32 (0.24-0.43)	0.63 (0.42-0.99)	0.40 (0.28-1.27)	< 0.001
FIB-4	0.72- (0.55-1.08)	1.05 (0.71-1.55)	0.92 (0.71-1.33)	1.18 (0.77-1.81)	0.93 (0.67-1.40)	< 0.001
GPR	0.23 (0.16-0.30)	0.71 (0.39-1.44)	0.32 (0.22-0.52)	0.56 (0.34-1.23)	0.37 (0.23-0.68)	< 0.001
Inflammation						< 0.001
G0-1	103 (64.0%)	35 (17.2%)	94 (55.0%)	76 (28.1%)	351 (43.2%)	
≥ G2	58 (36.0%)	168 (82.8%)	77 (45.0%)	194 (71.9%)	461 (56.8%)	
Fibrosis						< 0.001
S0-1	119 (73.9%)	64 (31.5%)	95 (55.6%)	98 (36.3%)	378 (46.6%)	
≥ S2	42 (26.1%)	139 (68.5%)	76 (44.4%)	172 (63.7%)	434 (53.4%)	
SHI						< 0.001
No	95 (59.0%)	32 (15.8%)	80 (46.8%)	63 (23.3%)	295 (36.3%)	
Yes	66 (41.0%)	171 (84.2%)	91 (53.2%)	207 (76.7%)	517 (63.7%)	

PLT: Platelet; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; GGT: Gamma-glutamyl transpeptidase; Tbil: Total bilirubin; ALB: Albumin; AFP: α-fetoprotein; PT: Prothrombin time; APRI: Aspartate aminotransferase-to-platelet ratio index; FIB-4: Fibrosis score based on four factors; GPR: Gamma-glutamyl transpeptidase-to-platelet ratio; SHI: Significant hepatic injury.

The clinical and liver histological characteristics are presented in Table 1 and Figure 1. In the GZ group, the proportion of significant liver inflammation (≥ G2) was 56.8%, and the proportion of significant fibrosis (≥ S2) was 53.4%. Among GZ patients, 63.7% had SHI. Higher proportions of SHI were observed in HBeAg-positive (84.2%) and HBeAg-negative (76.7%) CHB patients. However, HBeAg-positive (41.0%) and HBeAg-negative chronic HBV infection (53.2%) had lower but still relatively high proportions of SHI.

Distribution, clinical characteristics, and liver histological characteristics of patients in different GZs

The clinical characteristics of 812 GZ patients are shown in Supplementary Table 2. Among these patients, the proportion of GZ-C was the highest (41.1%), followed by GZ-B (34.6%), GZ-A (15.8%), and GZ-D (8.5%). Notably, patients in the GZ-A (35.0 years) and GZ-B (31.0 years) subgroups were younger than those in the GZ-C (40.0 years) and GZ-D (36.0 years) subgroups. Furthermore, higher HBV DNA levels were observed in GZ-B (7.86 Log10 IU/mL), followed by GZ-A (5.39 Log10 IU/mL), GZ-C (4.40 Log10 IU/mL), and GZ-D (2.40 Log10 IU/mL).

As shown in Supplementary Figure 2, HBeAg-positive GZ patients exhibited a significantly higher rate of significant liver inflammation (≥ G2) (66.1% *vs* 47.4%, $P < 0.001$) and SHI (70.4% *vs* 56.8%, $P < 0.001$) than HBeAg-negative GZ patients. However, there was no statistically significant difference in fibrosis stages between HBeAg-positive and HBeAg-

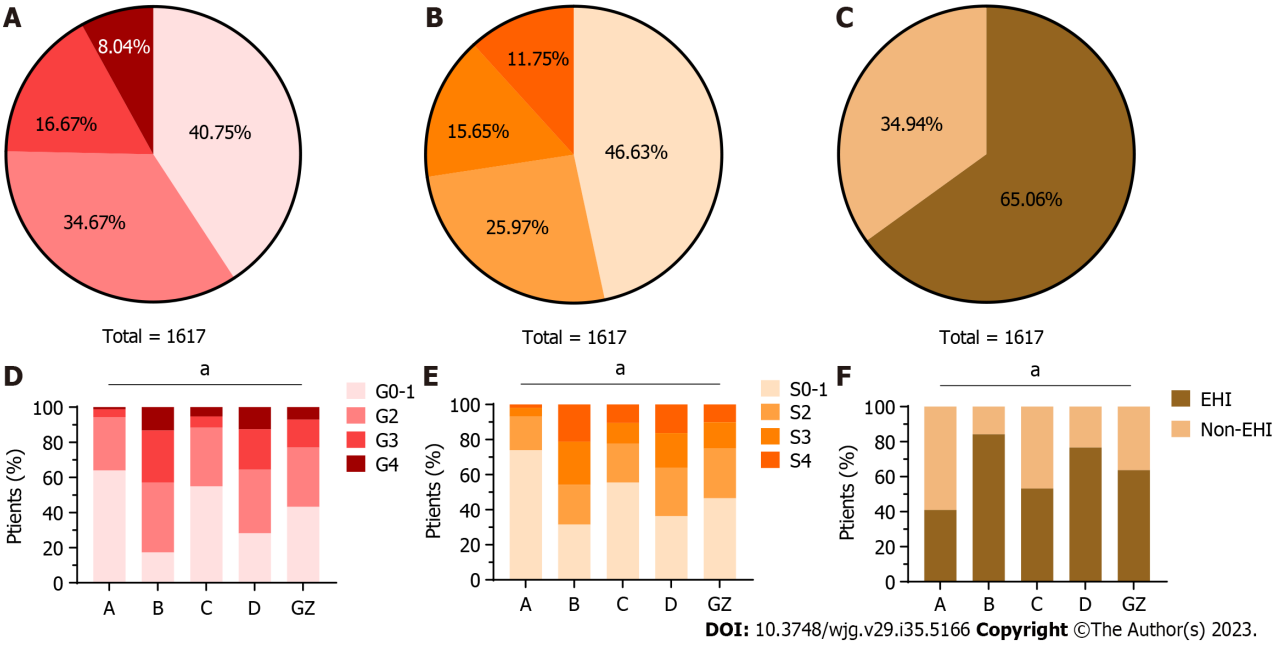

Figure 1 Distribution and liver histological features of chronic hepatitis B patients in different immune states. A and D: Proportions of liver inflammation grades in different immune status groups; B and E: Proportions of fibrosis stages in different immune status groups; C and F: Proportions of significant hepatic injury in different immune status groups. A: HBeAg-positive chronic hepatitis B virus (HBV) infection; B: HBeAg-positive chronic hepatitis B; C: HBeAg-negative chronic HBV infection; D: HBeAg-negative chronic hepatitis B; GZ: Grey zone; EHI: Evidenced hepatic injury. $^{a}P < 0.001$.

negative GZ patients (55.8% *vs* 51.2%, $P = 0.186$). The distributions of liver inflammation grades, fibrosis stages, and SHI are shown in Supplementary Table 2 and Supplementary Figure 2. The highest prevalence of significant liver inflammation (≥ G2) was observed in GZ-B (66.5%), while GZ-D (62.3%) had the highest proportion of significant fibrosis (≥ S2). In terms of SHI, the highest proportion was found in patients from GZ-B (70.5%), followed by GZ-A (70.3%), GA-D (69.6%), and GZ-C (54.2%).

Diagnostic performance of APRI, FIB-4, and GPR to detect SHI in patients with GZ

SHI was observed in 517 (63.7%) GZ patients. Of those, 288 were HBeAg-positive GZ patients, and 229 were HBeAg-negative GZ patients. In the HBeAg-positive cohort, univariate analysis indicated that PLT, ALT, AST, GGT, total bilirubin (Tbil), albumin (ALB), and prothrombin time(PT) were associated with SHI. However, multiple logistic regression analysis indicated that only PLT, AST, GGT, and ALB remained significantly associated with SHI. For the HBeAg-negative cohort, female sex, HBV-DNA, ALT, AST, GGT, Tbil, and PT were associated with higher SHI, whereas PLT and ALB were negatively associated with this event by univariate analysis. By multiple logistic regression analysis, female sex, HBV-DNA, GGT, and PLT were associated with SHI (Supplementary Table 3 and Figure 2).

Further investigation was conducted to assess the diagnostic efficacy of established scoring systems such as APRI, FIB-4, and GPR in predicting SHI (Figure 2). The AUROCs for these three tests showed no significant difference in the HBeAg-positive GZ ($P > 0.05$). However, in HBeAg-negative GZ, APRI demonstrated superior performance compared to FIB-4 ($P < 0.001$) and was comparable to GPR ($P = 0.30$).

Effect of lowering the treatment threshold of ALT on identifying SHI

To examine the significance of lowering the treatment threshold for ALT, a total of 794 patients with normal ALT levels (ULN ≤ 40 U/L) were chosen for further investigation. The distribution of their immune states was as follows: 161 (20.28%) patients had HBeAg-positive chronic HBV infection, 128 (16.12%) patients fell within GZ-A, 171 (21.54%) patients had HBeAg-negative chronic HBV infection, and 334 (42.07%) patients were categorized under GZ-C.

Based on the AASLD 2018 criteria of the ALT antiviral treatment threshold (35 U/L for males and 25 U/L for females), we subsequently evaluated the ratio of SHI in different groups. Among the 794 chronic HBV infections, more than one-quarter of them (221/794, 27.8%) were above the AASLD criteria (Figure 3A). Of these 221 individuals, 29.2% (47/161) with HBeAg-positive chronic HBV infection, 36.7% (47/128) with GZ-A, 21.1% (36/171) with HBeAg-negative chronic HBV infection, and 27.2% (91/334) with GZ-C were above this ALT threshold (Figure 3B). It is worth noting that 54.8% (121/221) of patients had significant liver inflammation (≥ G2), which was significantly higher than that of patients below the ALT threshold (43.1%, 247/573) ($P = 0.003$) (Figure 3C). In addition, the proportion of SHI in the high ALT group was significantly higher than that in the low ALT group (60.6% *vs* 51.3%, $P = 0.018$). In total, the proportion of patients with ALT ≤ 40 U/L who required antiviral therapy was 64.86% [(221 + 294)/794] according to the AASLD 2018 Clinical Practice Guidelines.

Obviously, the SHI value in patients with HBeAg-positive chronic HBV infection below the new ALT threshold was substantially lower than that of GZ-A patients above the ALT threshold (36.8% *vs* 57.4%, $P = 0.016$). The former was in the

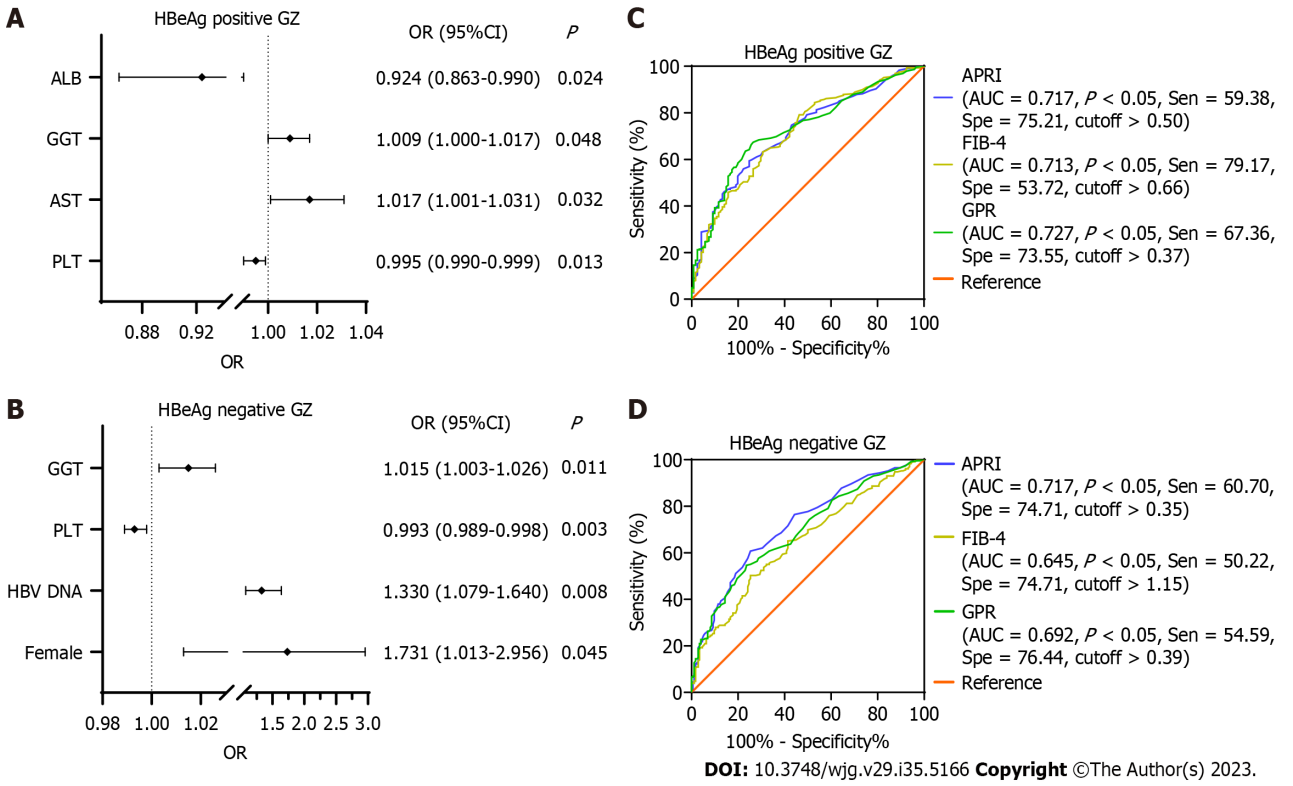

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Figure 2 Multiple logistic regression analysis. A: Multiple logistic regression analysis of clinical parameters of chronic hepatitis B patients in HBeAg-positive grey zones associated with significant hepatic injury; B: Multiple logistic regression analysis of clinical parameters of chronic hepatitis B patients in HBeAg-negative grey zones associated with significant hepatic injury; C: Receiver operating characteristic (ROC) curves of aspartate aminotransferase-to-platelet ratio index (APRI), fibrosis score based on four factors (FIB-4), and gamma-glutamyl transpeptidase-to-platelet ratio (GPR) in the prediction of significant hepatic injury (SHI) in HBeAg-positive grey zones; D: ROC curves of APRI, FIB-4, and GPR in the prediction of SHI in HBeAg-negative grey zones. PLT: Platelet; AST: Aspartate aminotransferase; GGT: Gamma-glutamyl transpeptidase; ALB: Albumin; APRI: Aspartate aminotransferase-to-platelet ratio index; FIB-4: Fibrosis score based on four factors; GPR: Gamma-glutamyl transpeptidase-to-platelet ratio.

"truly" HBeAg-positive chronic HBV infection group due to high HBV DNA levels and low ALT levels. Similarly, GZ-C patients had a significantly higher SHI ratio than the "truly" HBeAg-negative chronic HBV infection patients (62.6% *vs* 48.1%, $P = 0.032$) (Figure 3D).

According to the new recommendations for the treatment threshold of ALT (30 U/L for males and 19 U/L for females), we investigated the rate of SHI in different groups separately. Among the 794 chronic HBV infections, nearly half of them (393/794, 49.5%) were below the new criteria (Figure 4A). Among these 393 patients, 46.0% (74/161) with HBeAg-positive chronic HBV infection, 36.7% (47/128) with GZ-A, 64.9% (111/171) with HBeAg-negative chronic HBV infection and 48.2% (161/334) with GZ-C were below this ALT threshold (Figure 4B). Notably, the proportions of significant liver inflammation (≥ G2) and SHI in patients above the ALT threshold were significantly higher than those in patients below the ALT threshold (50.9% *vs* 41.7%, $P = 0.01$; 57.4% *vs* 50.4%, $P = 0.049$) (Figure 4C). In total, the proportion of patients with ALT ≤ 40 U/L who required antiviral therapy was 75.44% [(401 + 198)/794] according to the "expert opinion on expanding anti-HBV treatment for chronic hepatitis B" in China.

The SHI values in patients with HBeAg-positive chronic HBV infection below the new ALT threshold was 36.4%. However, higher SHI values of 67.9% were seen in the GZ-A patients above the new ALT threshold, and the difference was statistically significant ($P < 0.001$). However, there was no significant difference in the proportion of SHI between patients with HBeAg-negative chronic HBV infection below the new ALT threshold and GZ-C patients above the new ALT threshold (45.0% *vs* 54.9%, $P = 0.105$) (Figure 4D).

An age > 30 years may not be a limitation for initiating antiviral therapy

The median age was 31, 35, 37, and 40 years for HBeAg-positive chronic HBV infection, GZ-A, HBeAg-negative chronic HBV infection, and GZ-C patients, respectively. There was an increasing trend of age in these states (Figure 5A). Among 794 patients, 76.7% (609/794) were > 30 years old, and almost 70% were HBeAg-negative patients, including 77.8% with HBeAg-negative chronic HBV infection and 88.3% with GZ-C (Figure 5B). The ratio of SHI in patients ≤ 30 years old was 49.1%, and it increased to 55.3% for those patients > 30 years old. However, there was no significant difference ($P = 0.136$). A similar result was observed in all states regardless of whether they were older or younger than 30 years old ($P > 0.05$) (Figure 5C).

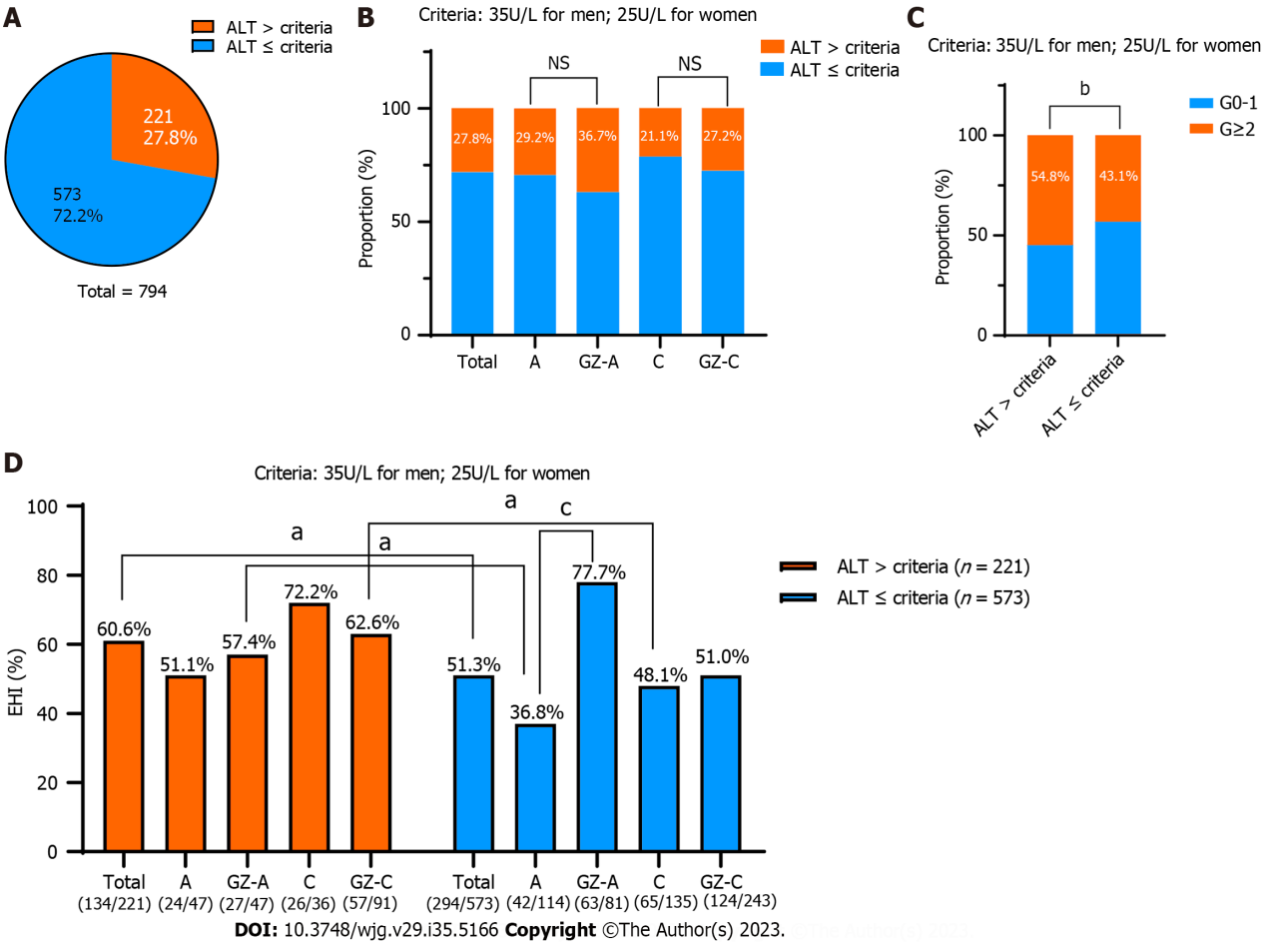

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Figure 3 The alanine aminotransferase treatment threshold was lowered to the American Association for the Study of Liver Diseases 2018 treatment criteria (35 U/L for males and 25 U/L for females). A: Among the 794 chronic hepatitis B virus infections, 27.8% (221/794) of patients were above the American Association for the Study of Liver Diseases criteria; B: Comparison of the proportions of patients exceeding the alanine aminotransferase (ALT) threshold in different groups; C: A total of 43.1% (247/573) of patients below the ALT threshold had significant liver inflammation (≥ G2); D: The proportion of significant hepatic injury (SHI) in patients below the ALT threshold was 51.3%. Comparison of the proportions of SHI in each group. ALT: Alanine aminotransferase; NS: Not significant. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

DISCUSSION

This retrospective cohort study examined a group of CHB patients who underwent liver biopsy at the Third Affiliated Hospital of Sun Yat-Sen University. The study showed that 50.22% of the patients with HBV infection fell into the GZ category, with 56.8% and 53.4% having significant liver inflammation (≥ G2) and fibrosis (≥ S2), respectively. More than half of the patients (63.7%) in the GZ category exhibited SHI, which was less than the proportion observed in HBeAg-positive and HBeAg-negative chronic hepatitis patients but more severe than those in the HBeAg-positive and HBeAg-negative chronic infection categories. While current guidelines do not require urgent antiviral therapy for GZ patients[4-6], the study findings indicated that HBeAg-positive and HBeAg-negative chronic HBV infections had relatively high proportions of SHI. The proportions were higher than those of a meta-analysis, which indicated the prevalence of significant fibrosis for chronic HBV infection as 16.9% (95%CI: 7.8-26.1) for HBeAg-positive and 24.8% (95%CI: 4.5-45.1) for HBeAg-negative chronic HBV infection[16]. This may be because the population included in the study is Asian and the genotypes are mainly B and C. Therefore, noninvasive methods, including liver biopsy, should be considered to evaluate liver inflammation and fibrosis in these individuals[17-19].

To better direct clinical diagnosis and treatment strategies, we analysed the risk factors for SHI in GZ patients. In the HBeAg-positive cohort, multiple logistic regression analysis indicated that PLT, AST, GGT, and ALB were associated with SHI. For the HBeAg-negative cohort, female sex, HBV-DNA, GGT, and PLT were associated with SHI by multiple logistic regression analysis. Based on these risk factors, we compared the diagnostic performance of APRI, FIB-4, and GPR in predicting SHI. The AUROCs of APRI, FIB-4, and GPR were 0.717, 0.713, and 0.727, respectively, in the HBeAg-positive GZ phases and 0.717, 0.645, and 0.692, respectively, in the HBeAg-negative GZ phases. Previous studies have shown that GPR provided a significantly higher AUROC than APRI and FIB-4, implying the superiority of GPR in predicting significant liver fibrosis and cirrhosis. For diagnosing significant fibrosis, the AUROCs of GPR were 0.66-0.86 and the cut-off was 0.32-0.43[20-22]. Thus, for simplicity of use in clinical practice, we advised utilizing a GPR cut-off of 0.37 as the optimal cut-off for predicting SHI in GZ patients. Treatment should be individualized for GZ patients, especially those who are over 40 years of old, HBeAg positive, and exhibit high ALT and HBV DNA levels. The state of

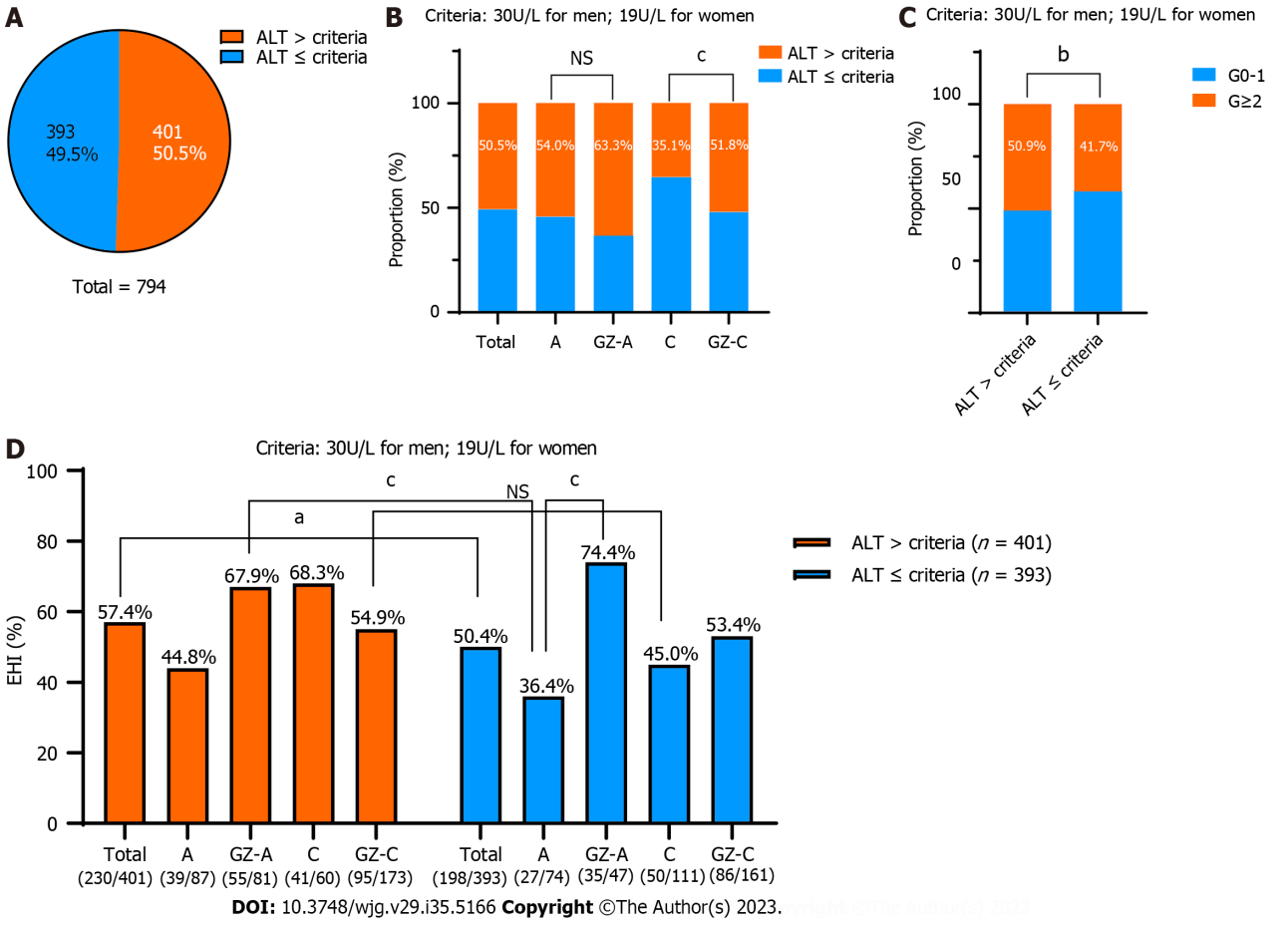

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Figure 4 Lowering the alanine aminotransferase treatment threshold to new criteria (30 U/L for men and 19 U/L for women). A: Among the 794 chronic hepatitis B virus infections, 50.5% (401/794) of patients were above the new criteria; B: Comparison of the proportions of patients exceeding the alanine aminotransferase (ALT) threshold in different groups; C: A total of 41.7% (164/393) of patients below the ALT threshold had significant liver inflammation (≥ G2); D: The proportion of significant hepatic injury (SHI) in patients below the ALT threshold was 50.4%. Comparison of the proportions of SHI in each group. NS: Not significant. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$.

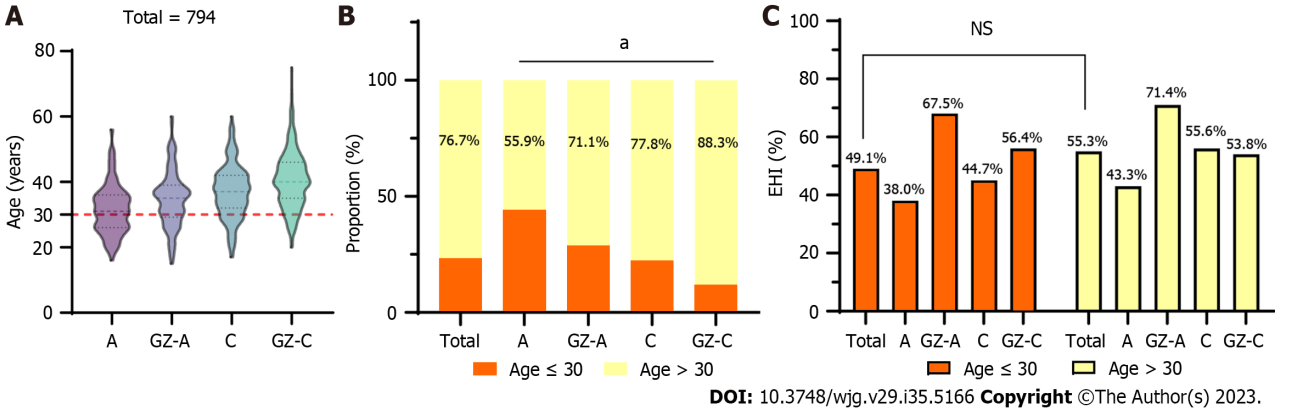

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Figure 5 Comparison in different groups. A: Comparison of age in different groups; B: Comparison of the proportions of patients older than 30 years in different groups; C: The proportion of significant hepatic injury (SHI) in patients ≤ 30 years of age was 49.1%. Comparison of the proportions of SHI in each group. NS: Not significant. $^{a}P < 0.05$.

the GZ is not constant but should be dynamic. Periodic monitoring is particularly important.

The main purpose of this study was to investigate the effect of lowering the treatment threshold of ALT according to different clinical guidelines in identifying SHI patients with CHB virus infection in the GZ. A total of 794 patients with normal ALT levels (ULN ≤ 40 U/L) were selected for further investigation; of these patients, 53.90% (428/794) necessitated antiviral therapy. The proportion of patients with ALT ≤ 40 U/L who required antiviral therapy was 64.86% [(221 + 294)/794] according to the AASLD 2018 Clinical Practice Guidelines. Furthermore, the proportion of patients with

ALT ≤ 40 U/L who required antiviral therapy was 75.44% [(401 + 198)/794] according to the "expert opinion on expanding anti-HBV treatment for chronic hepatitis B" in China.

The current criteria for determining "normal" ALT levels were established based on populations that encompassed individuals with subclinical liver disease. Prati *et al*[23] propose that it is prudent to reconsider the established thresholds for ALT levels in patients diagnosed with chronic HCV infection or nonalcoholic fatty liver disease[23]. Previous studies have shown that even if the ALT level is within the normal range, the ALT level correlates with the degree of liver inflammation and fibrosis. Sonneveld *et al*[24] showed that 52% of 168 patients without liver fibrosis and 82% of 66 patients with significant liver fibrosis with normal ALT levels had mild and moderate inflammation[24]. More importantly, even if the ALT level is within the normal range, higher ALT levels have a higher incidence of decompensated cirrhosis and HCC. Compared to patients with ALT levels < 0.5 × ULN (53 U/L and 31 U/L for males and females, respectively), patients with ALT levels of 0.5-1 × ULN had an increased risk for the development of complications including ascites, spontaneous bacterial peritonitis, oesophageal varices, encephalopathy and HCC[25]. Similarly, REVEAL-HBV research demonstrated that compared to ALT < 15 U/L, patients with ALT 15-44 U/L had an increased risk of cirrhosis (aHR = 1.97, 95%CI: 1.56-2.48) and HCC (aHR = 2.45, 95%CI: 1.74-2.48)[26]. Therefore, lowering the ALT threshold in CHB patients is conducive to early initiation of antiviral therapy, which in turn reduces the incidence of cirrhosis and HCC, especially in the GZ phases.

Current studies and guidelines recommend that age > 30 years old is an independent risk factor for disease progression and can be an indication for initiating antiviral therapy. A linear correlation between age and the mortality risk of primary liver cancer, chronic liver disease and cirrhosis, and viral hepatitis was found in those whose ages ranged from 15 years to 74 years[27]. Among 794 individuals in our study with normal ALT levels (ULN ≤ 40 U/L), 23.3% of the patients were under 30 years old. The ratio of SHI in patients ≤ 30 years old was 49.1%, and it increased to 55.3% for those patients > 30 years old. However, the difference was not significant. Huang *et al*[7] showed that among patients who remained indeterminate, an age ≥ 40 years (aHR = 9.06) and ≥ 45 years (aHR = 18.40) were independently associated with HCC development[7]. This suggested that setting 30 years old as a threshold is not suitable for GZ patients. We noticed that a large proportion of CHB patients ≤ 30 years old with normal ALT levels still had inflammation and fibrosis. This finding was consistent with a previous study that noted that among 432 CHB patients with normal or mildly elevated ALT who underwent liver biopsy, the inflammation and fibrosis scores increased with age. Of these patients < 30 years old, G ≥ 2 accounted for approximately 50%, and S ≥ 2 accounted for approximately 40%[28]. Hence, age may not be a limitation for initiating antiviral therapy in patients with CHB who have normal ALT levels. Instead, more individualized attention should be given to the patient's liver inflammation and fibrosis, with the aim of reducing misdiagnosis and underdiagnosis. Additionally, patients with hepatitis B who need treatment and are at risk of disease progression should be placed on antiviral therapy in a timely manner.

Nevertheless, our study has several limitations. First, selection bias could not be ruled out because this was a retrospective and cross-sectional study. Second, the proportion of patients with SHI in this cohort may be higher than the natural population because the patients were sourced from tertiary care hospitals rather than the community, and there may be a bias in the enrolled patients. Third, because the follow-up of patients after liver biopsy was insufficient, the phase transition, benefits of antiviral therapy, and prognosis of GZ patients could not be assessed. Fourth, the study was unable to obtain information on the genotypes of all hepatitis B patients, and the limited data suggest a predominance of genotype B (65%) and genotype C (33%).

CONCLUSION

In conclusion, this study showed that 50.22% of CHB patients were in the GZ, and over half of GZ patients (63.7%) had SHI. Lowering ALT thresholds can more accurately identify patients with significant liver damage at an earlier stage and reduce the need for some unnecessary liver biopsies. Furthermore, age may not be a limitation for initiating antiviral therapy in patients with CHB who have normal ALT levels. This may have significance for refining the natural history of CHB and providing supporting evidence of lowering the antiviral therapy threshold for ALT.

ARTICLE HIGHLIGHTS

Research background

In clinical practice, a considerable proportion of chronic hepatitis B (CHB) patients (27.8%-55%) fall into the "grey zone (GZ)" or "indeterminate phase". Additionally, there is still debate regarding how best to treat these GZ patients and the advantages of antiviral therapy. Moreover, an issue that complicates the management of CHB is the disagreement regarding the appropriate treatment threshold for alanine aminotransferase (ALT) levels.

Research motivation

To explore the impact of varying the threshold of ALT levels in identifying significant hepatic injury (SHI) among GZ patients.

Research objectives

Our research evaluated the clinical and histological characteristics and additionally explored the impact of adjusting the threshold of ALT in identifying significant liver injury among GZ patients.

Research methods

This retrospective analysis involved a cohort of 1617 adult patients diagnosed with CHB who underwent liver biopsy. Significant hepatic injury was defined as the presence of notable liver inflammation (≥ G2) and/or significant fibrosis (≥ S2). Kruskal-Wallis tests and Pearson's chi-squared tests were applied to compare variables that were significantly different between groups.

Research results

The study showed that 50.22% of the patients with HBV infection fell into the GZ category, and more than half of the patients (63.7%) in the GZ category exhibited SHI. The areas under the receiver operating characteristic curves of Aspartate aminotransferase-to-platelet ratio index, fibrosis score based on four factors, and gamma-glutamyl transpeptidase-to-platelet ratio in predicting SHI were 0.717, 0.713, and 0.727, respectively, in the HBeAg-positive GZ phases and 0.717, 0.645, and 0.692, respectively, in the HBeAg-negative GZ phases. Lowering the ALT treatment thresholds to the American Association for the Study of Liver Diseases 2018 treatment criteria can more accurately identify patients with significant liver damage in the GZ phases. When we lowered the ALT treatment threshold to the new criteria, the same outcome was revealed.

Research conclusions

This study showed that 50.22% of CHB patients were in the GZ, and over half of GZ patients (63.7%) had SHI. Lowering ALT thresholds can more accurately identify patients with significant liver damage at an earlier stage and reduce the need for some unnecessary liver biopsies. Furthermore, age may not be a limitation for initiating antiviral therapy in patients with CHB who have normal ALT levels.

Research perspectives

Further investigation is needed to determine the assessment and treatment strategy for CHB patients in the GZ phases.

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FOOTNOTES

Author contributions: Yu HS and Jiang H have contributed equally to this work; Yu HS and Jiang H designed the study, performed the statistical analysis, and drafted the manuscript; Li MK, Yang BL, Smayi A and Chen JN collected the clinical data and then reviewed the data; Wu B and Yang YD revised the manuscript; Yang YD and Wu B were responsible for the study conception, design, data, analysis; All authors have read and approved the manuscript.

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CORRECTION

Correction to "Role of prebiotics, probiotics, and synbiotics in management of inflammatory bowel disease: Current perspectives"

Supriya Roy, Suneela Dhaneshwar

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Abstract

Correction to "Roy S, Dhaneshwar S. Role of prebiotics, probiotics, and synbiotics in management of inflammatory bowel disease: Current perspectives. *World J Gastroenterol* 2023; 29: 2078-2100 [PMID: 37122604 DOI: 10.3748/wjg.v29.i14.2078]". In this article, a correction note is to be added.

Key Words: Ulcerative colitis; Crohn's disease; Pouchitis; Dysbiosis; Microbiota; Inflammation

Core Tip: This manuscript is to add a "correction note" to "Roy S, Dhaneshwar S. Role of prebiotics, probiotics, and synbiotics in management of inflammatory bowel disease: Current perspectives. *World J Gastroenterol* 2023; 29: 2078-2100 [PMID: 37122604 DOI: 10.3748/wjg.v29.i14.2078]".

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TO THE EDITOR

Correction to: Roy S, Dhaneshwar S. Role of prebiotics, probiotics, and synbiotics in management of inflammatory bowel disease: Current perspectives. *World J Gastroenterol* 2023; 29(14): 2078-2100.

In this article, a correction note is to be added[1]. The corresponding author received a mail from Dr. Claudio De Simone sharing his concern about a probiotic formulation VSL#3 which is mentioned in this review article. Dr. Claudio wishes us to publish a corrigendum. It is an earnest request that please incorporate the following correction note and publish a corrigendum regarding the same.

Correction note: This review article includes references to several studies that investigated a probiotic formulation formerly marketed as VSL#3 which is currently, generically referred to as the "De Simone Formulation". It is important to note that the current product marketed as VSL#3 differs from the De Simone Formulation as also stated by the federal court of Maryland in the Civil Action, No. TDC-15-1356. They are distinct probiotic formulations with different compositions and characteristics. Currently, the De Simone Formulation is accessible under Vivomixx (in Europe) and Visbiome (in the United States) brand names.

FOOTNOTES

Author contributions: Dhaneshwar S conceived the idea and designed the review article protocol, edited the final draft of the manuscript; Roy S collected the data and wrote the paper; and all authors reviewed and approved the manuscript.

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