ISSN 1881-7815 Online ISSN 1881-7823

BST BioScience Trends

Volume 17, Number 5
October, 2023

IRCA BSSA

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BST
BioScience Trends

ISSN: 1881-7815
Online ISSN: 1881-7823
CODEN: BTIRCZ
Issues/Year: 6
Language: English
Publisher: IACMHR Co., Ltd.

BioScience Trends is one of a series of peer-reviewed journals of the International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) Group. It is published bimonthly by the International Advancement Center for Medicine & Health Research Co., Ltd. (IACMHR Co., Ltd.) and supported by the IRCA-BSSA.

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Surgical indications for solid hepatic benign tumors: An updated literature review

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SUMMARY Hepatic hemangioma, focal nodular hyperplasia, and hepatic adenoma are the most common benign solid liver tumors. However, their surgical indications have been the subject of debate. Minimally invasive liver resection reduces the cost of surgery and may lead to overtreatment of benign liver tumors. Recently, there has been a growing understanding of the etiology, pathogenesis, and natural history of these tumors. Great progress has also been made in imaging. The use of MRI and contrast agents has improved the accuracy of non-invasive diagnosis of these tumors, and especially in the identification of specific molecular subtypes of liver adenoma. These factors have resulted in alterations of surgical indications for these tumors. This article examines recent literature and it discusses the surgical indications for hepatic hemangioma, focal nodular hyperplasia, and hepatic adenoma while summarizing modifications in clinical management.

Keywords benign liver tumors, surgery, management

1. Introduction

There are many types of benign liver tumors with different histomorphology, clinical biological behavior, and imaging findings. Based on the molecular phenotype of tumors, their histological and imaging features, as well as their histopathological classification, the World Health Organization classifies these tumors into three major categories. The first category includes hepatocellular adenoma (HCA), focal nodular hyperplasia (FNH), intrahepatic bile duct adenoma, bile duct hamartoma, intrahepatic bile duct cystadenoma, and biliary papillomatosis; the second category includes hepatic hemangioma (HH), angiomyolipoma, lymphangioma and lymphangiomatosis; and the third category includes teratomas and solitary fibrous tumors (*1*). The precise prevalence of these lesions in the population is unknown, but autopsy series have reported an incidence of up to 50% for these tumors (*2*).

With the widespread availability of imaging techniques such as ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI), the likelihood of detecting a liver mass in asymptomatic patients has increased (*3*). At the same time, the accuracy of non-invasive diagnosis of benign liver tumors is increasing, reducing the need for a histological examination to distinguish between benign and malignant tumors. In recent years, MRI with specific contrast agents has been proven to be the most accurate and specific radiological tool for diagnosing benign liver tumors. More importantly, imaging features can indicate molecular subtypes of HCA (*4-6*). Imaging follow-up for benign liver tumors appears to be increasingly feasible and reasonable (*2,7*).

Surgical indications for benign liver tumors have been controversial, but generally observation is preferred (*8*). However, the development of minimally invasive liver resection techniques has the potential to reduce surgical costs and increase postoperative benefits for treatment of benign liver tumors compared to open hepatectomy. This may lead to overtreatment of some benign liver tumors (*9*). The technical training required of the surgeon may also potentially influence surgical indications. In addition, as the etiology, pathogenesis, and natural history of HH, FNH, and HCA continue to be researched, they are classified in more detailed and disease progression can be predicted more accurately (*10*). New clinical, biological, and molecular tools have gradually been incorporated into diagnostic and therapeutic algorithms for the classification of benign liver tumors and improvement of patient management, resulting in changes to surgical indications.

This article reviews the latest relevant literature and discusses surgical indications for HH, FNH, and HCA.

2. Hepatic Hemangioma (HH)

HH is the most common solid benign liver tumor, with an incidence of 1-20% and a rate of detection of 7% in autopsy studies. It is more common in females (female: male ratio = 5:1), and the average age at diagnosis is around 50 years (*11*). This lesion originates from the proliferation of vascular endothelial cells and is usually a hypervascular lesion with well-defined boundaries. Most hemangiomas are cavernous, liver function tests are normal, and there is no possibility of malignant transformation (*12*). The risk factors contributing to the development of HH are currently unknown, as is the pathophysiology of these hypervascular lesions. The incidence of HH is higher in female patients, raising suspicions about the association between estrogen and HH. Moreover, angiogenesis has been clearly linked to estrogen. However, subsequent studies have confirmed that HHes do not express estrogen or progesterone receptors and there is no significant difference in the growth pattern of hemangiomas between males and females, thus ruling out a relationship between estrogen and HH (*13*). Therefore, oral estrogen contraception can be used safely and pregnancy does not pose a risk (*10*).

2.1. Imaging

HH is usually diagnosed by chance. Most cases are detected and diagnosed by ultrasound, while other suspected cases can also be diagnosed by CT and MRI (*10,14*). A study of 151 HH patients found that only four patients had a radiographically inconclusive diagnosis related to hemangioma over the ten-year study period, and no patient had a preoperative diagnosis of hemangioma that was ultimately inconsistent with postoperative pathology (*2*). Hepatectomy to rule out malignancy is very rare in the treatment of HH. Generally, HH can be easily diagnosed through imaging.

2.2. Natural history

Most hemangiomas increase in size at a slow rate of about 2 mm per year, with an increase in volume of approximately 17.4% per year (*15*). A study indicated that the growth peak for hemangiomas (<30 years old) was 0.46±0.41 cm per year, and the growth rate decreased significantly after age 50 to 0.21±0.40 cm per year. When the size of the hemangioma reaches 8-10 cm, there is another peak in its growth rate of 0.80±0.62 cm per year. However, when the size exceeds this range, the growth rate rapidly decreases to 0.47±0.91 cm per year (*13*). HHes typically exhibit a slow growth pattern, with minimal risk of complications during the progression phase, thus resulting in a non-surgical intervention as the prevailing approach for most patients.

2.3. Surgical indications

In the case of incidentally detected asymptomatic HHes, neither treatment nor imaging follow-up is warranted until clinical symptoms suggestive of hemangioma manifest (*8*). Hepatectomy is necessary for patients with significant hemangioma-related clinical symptoms, but the potential surgical risk and extent of postoperative alleviation of symptoms need to be carefully considered.

Large hemangiomas (giant: ≥ 5 cm, super large: ≥ 10 cm) are believed to be associated with clinical symptoms mainly due to compression, such as abdominal pain, obstructive jaundice, and decreased appetite (*16*). A tumor size greater than 5 cm is considered to be a predictor of clinical symptoms associated with HH (*17*). Therefore, hemangioma equal to or larger than 5 cm has long been regarded as a surgical indication. However, a point worth noting is that caution should be exercised when associating clinical symptoms with HH. Other causes such as gallstones and gastroduodenal diseases should be ruled out. Studies have indicated that about 25% of patients experience persistent symptoms after liver resection (*11,18*). Another study also suggested that surgery for 5-10 cm asymptomatic hemangiomas should be limited (*19*). Comprehensive considerations must be made before making surgical decisions. Table 1 shows the main surgical indications cited in recent studies.

Rupture of a growing hemangioma is also a concern that can cause anxiety. Although hemangioma rupture is a mandatory indication for surgery, a study has indicated that the risk of spontaneous rupture and bleeding is extremely low even for large hemangiomas, and especially for those deep in the liver (*20*). Kasabach-Merritt syndrome is a rare disorder caused by large hemangiomas (≥ 10 cm) that results in thrombocytopenia and consumptive coagulopathy (*21*). It is also one of the mandatory surgical indications, and orthotopic liver transplantation has been reported in severe cases (*22*). These rare conditions should not affect the routine management of HH patients. A multicenter retrospective study also noted that serious complications associated with the observation period were very rare, and surgical treatment of hemangiomas should be carefully considered (*23*). Surgery is inevitable in cases involving HH and hepatocellular carcinoma (HCC) that pose challenges in imaging-based differentiation or that exhibit significantly elevated oncologic indicators (*19*).

2.4. Surgery

Both anatomical hepatectomy and enucleation are effective surgical approaches that should be decided based on the specific location of the lesion. Enucleation is appropriate for a superficial hemangioma with a clear border with the liver, which allows for more preservation of liver parenchyma. Enucleation does not lead to bile leakage and reduces the risk of bleeding because there is no Glisson system crossing the HH and parenchymal

Table 1. Surgical indications for hepatic hemangioma cited in recent studies

Literature	Type of literature	Country or region	Surgical indications
Miura et al. (2014) (*23*)	Multicenter retrospective study	United States	Abdominal symptoms; anxiety (patient's willingness to undergo surgery); tumor enlargement; life-threatening complications (such as traumatic rupture)
Practice Parameters Committee of the American College of Gastroenterology (2014) (*33*)	Clinical guideline	United States	Tumor size >10 cm; symptoms of compression; recurrent abdominal pain
Brazilian Society of Hepatology (2015) (*21*)	Clinical recommendations	Brazil	Large tumors with compression symptoms; rare complications (such as tumor rupture); Kasabach-Merritt syndrome
European Association for the Study of the Liver (EASL) (2016) (*8*)	Clinical guideline	Europe	Tumor enlargement; symptoms of compression; Kasabach-Merritt syndrome
Yuan *et al*. (2022) (*13*)	Observational study	China	Only severe complications (such as Kasabach-Merritt syndrome, spontaneous rupture, obstructive jaundice, gastric outlet obstruction, Budd-Chiari syndrome)
Aziz *et al.* (2022) (*18*)	Review	United States	Uncertain diagnosis; tumor enlargement; certain occupation or hobbies are associated with the risk of abdominal trauma; compression of organs or blood vessels (gastric outlet obstruction, Budd-Chiari syndrome); Kasabach-Merritt syndrome

boundary of the liver. If this condition is not met, anatomical hepatectomy should be selected.

Transarterial embolization (TAE) has also been used in the treatment of HH, initially mainly for high-risk patients who are not candidates for hepatectomy or to temporarily stop bleeding in patients with ruptured HH. More recently, superselective transarterial chemoembolization (TACE) with bleomycin has been used for the safe and effective treatment of giant HHes. Clinical remission was achieved in all patients, with a mid-term (≥ 3 years) and long-term (≥ 5 years) tumor reduction of 85.2% and 86.5%, respectively (*24*). However, considering the benign characteristics of HH and especially its amenability to conservative treatment, a more careful discussion is needed to determine whether the use of chemotherapy drugs for benign diseases is reasonable. In addition, limitations of this technique include other complications associated with embolization, such as migration of the embolic agent to other organs, pain, nausea, fever, liver abscesses, and sepsis. Despite complete ablation of the lesion, radiofrequency ablation (RFA) has been reported to have a high complication rate for the treatment of HH (*25,26*). Insufficient data on the rationale and efficacy of TACE and RFA in treating HH do not support recommending them as first-line treatment.

HH in children is a special condition. Infantile HH grows rapidly in the first year, followed by spontaneous recurrence in most cases, which can lead to elevated AFP levels, abdominal pain, congestive heart failure, Kasabach-Merritt syndrome, or hypothyroidism (*27,28*). For symptomatic children, glucocorticoids and/or propranolol can be used as first-line medical therapy while TACE and hepatectomy are second-line options (*29-31*).

3. Focal Nodular Hyperplasia (FNH)

FNH is the second most common solid benign liver tumor, with a reported rate of detection of 3% in autopsy series (*6*) and an estimated prevalence in the global population of approximately 1%. FNH is highly prevalent among women (female: male ratio = 8:1), and it typically occurs between the ages of 30 and 50 years (*32*). FNH is thought to be caused by portal vein injury, which leads to the formation and enlargement of arterial-to-venous shunts. This causes hyperperfusion and oxidative stress in local arteries, triggering a hepatic stellate cell response that produces a central scar (*33*). The development of hyperplasia is restricted to the vascular region. In most cases, FNH is isolated and smaller than 5 cm; only about 20% are multifocal (*8*). In the vast majority of cases, FNH has a consistent size and little chance of becoming malignant (*14*). Estrogen is highly unlikely to be associated with FNH, so pregnancy, oral contraceptives, or anabolic steroids are not contraindicated (*32*).

3.1. Imaging

FNH is usually asymptomatic and is incidentally diagnosed by imaging. The tumor is well-circumscribed and non-encapsulated, characterized by a central fibrous scar with a fibrous septum radiating from the center in a "spoke-wheel" pattern, surrounded by normal hepatocytes (*6*). The imaging features of FNH are very similar to its histological manifestations, and central scarring is present in about 50% of cases regardless of whether imaging is by ultrasound, CT, or MRI (*14,33*). The use of hepatobiliary contrast agents, such as Eovist and Gd-EOB-DTPA, enables MRI to distinguish between FNH and hepatic adenomas due to the presence of bile ducts within the FNH that absorb contrast medium in the delayed phase, while hepatic adenomas do not (*34*). Studies have reported that hepatobiliary contrast-enhanced MRI has a sensitivity

of 90 to 96.9% and a specificity of 91 to 100% for differentiating FNH from hepatic adenoma (*6,14*). The combination of typical imaging features with contrast-enhanced ultrasound (CEUS), CT, or MRI has close to 100% specificity in diagnosing FNH, and CEUS performs better than MRI in detecting small (< 3 cm) FNH without central scars (*8*).

3.2. Natural history

The natural history of FNH is unremarkable, acute complications are rare, changes in tumor size over time are not significant, and there is no evidence that FNH undergoes malignant transformation (*11*). Only a very small number of patients with FNH present with clinical symptoms, and assessing the relationship between clinical symptoms and FNH is difficult. A higher proportion of liver tumors in children is malignant, making a definitive diagnosis even more important. Although FNH in children is more likely to cause symptoms, management protocols are generally consistent with those in adults (*35*).

3.3. Surgical indications

Surgical resection can significantly improve the quality of life in patients with definite symptoms (*36*). Symptoms are specific to the tumor's aspects, such as stomach compression caused by a large FNH in the left lobe of the liver or abdominal pain from acute torsion of a pedicled tumor. Asymptomatic FNHs do not require treatment or follow-up regardless of size or number (*33*). Malignant transformation of FNH or acute complications such as tumor bleeding and rupture are also extremely rare (*11*), so prophylactic treatment is unnecessary (*37*). When imaging studies cannot distinguish between FNH and HCC, surgical resection and pathological examination are preferred. Liver biopsy may be required if surgical resection is difficult, but it is not routinely recommended due to its high false negative rate of 30% (*14,33*). Table 2 shows the main surgical indications cited in recent studies.

3.4. Surgery

Hepatectomy is preferred over enucleation due to the frequent presence of large blood vessels around the lesion. Methods that preserve more liver parenchyma are chosen during hepatectomy unless malignancy is suspected (*14*). Two small case series have reported that TACE can reduce the size of FNH in adults and children and relieve symptoms (*38,39*). Currently, there is no consensus on the choice of embolization material for TACE (*40*). RFA has also been reported to be an effective treatment for symptomatic FNH (*41,42*). Despite the increasing use of TACE and RFA, however, there is currently insufficient evidence to support their use as first-line treatment options.

4. Hepatocellular Adenoma (HCA)

HCA is the third most common solid benign liver tumor, with a prevalence of less than 0.05% in the general population and a higher incidence in women (female: male = 9:1) (*6*). The presence of more than 10 adenomas is referred to as hepatic adenomatosis. Unlike HH and FNH, hormones are closely related to the development and progression of HCA. Recently, the major risk factors for HCA have shifted from oral contraceptives to obesity and metabolic syndrome (*43*). Elevated androgen levels, steroid abuse, and obesity are also associated with HCA (*44*). In addition, several rare genetic syndromes, such as glycogen storage disorders type I and type III, maturity-onset diabetes of the young type 3 (MODY3), and McCune-Albright syndrome, have been significantly associated with the development of HCA (*45*).

4.1. Pathological molecular subtypes

At present, at least eight different HCA subtypes have been identified based on molecular pathology, each with distinct histopathological features, clinical characteristics, complications and risks of malignant transformation, as well as unique management recommendations. The most common subtypes are inflammatory HCA

Table 2. Surgical indications for focal nodular hyperplasia cited in recent studies

Literature	Type of literature	Country or region	Surgical indications
Practice Parameters Committee of the American College of Gastroenterology (2014) (*33*)	Clinical guideline	United States	Definite tumor-related symptoms
Margonis *et al*. (2015) (*14*)	Review	United States	Definite tumor-related symptoms (such as pain, rupture, and bleeding); suspected malignant tumor or hepatic adenoma
European Association for the Study of the Liver (EASL) (2016) (*8*)	Clinical guideline	Europe	Definite tumor-related symptoms; pedunculated, enlarged, or exogenous
Perrakis *et al*. (2017) (*37*)	Review	Germany	An uncertain diagnosis and a history of malignancy; clinical symptoms; tumor enlarged during follow-up
Fodor *et al*. (2018) (*11*)	Review	Austria	Definite tumor-related symptoms; imaging and biopsy could not rule out malignancy
Nault *et al*. (2022) (*10*)	Review	France	Abdominal pain, compression of surrounding organs

(IHCA), *HNF1α* inactivated HCA (HHCA), β-*catenin* exon 3-mutated HCA (β^{ex3}-HCA), β-*catenin* exon 7 or 8-mutated HCA ($\beta^{ex7,8}$-HCA), sonic hedgehog HCA (shHCA), and unclassified HCA (*10, 44, 46*). The characteristics, risks of complications, and management strategies of specific subtypes of HCA are shown in Table 3.

4.2. Imaging

Approximately 35% of HCAs are incidentally diagnosed by imaging (*44*) , and MRI is the best choice for diagnosis and classification. HCA is sometimes difficult to distinguish from other hypervascular tumors due to the pseudocapsule surrounding it, and the imaging findings of HCA vary greatly between subtypes. The use of a contrast agent in MRI can better distinguish the subtypes of HCA based on the two pathological features of fat and telangiectasia. For example, the atoll sign and hyperintensity are used to distinguish IHCA; scarring and hyperintensity are used to distinguish β-HCA; and steatosis and hypointensity are used to distinguish HHCA (*47*). Contrast-enhanced MRI is reported to have a sensitivity of 87-91% in diagnosing HHCA and a specificity of 89-100%. Contrast-enhanced MRI has a sensitivity of 85-88% in diagnosing IHCA and a specificity of 88-100% (*48*). The diagnostic accuracy of contrast-enhanced MRI is increasing. A recent study found that gadoxetate disodium-enhanced MRI had an accuracy rate of 98% for diagnosing HHCA, 83% for IHCA, and 95% for β-HCA or β-IHCA (*49*).

β-HCA and unclassified HCA can sometimes appear atypical on imaging, making them difficult to distinguish from HCC. A recent study indicated that the uptake of hepatobiliary contrast in gadobenate dimeglumine-enhanced MRI is closely related to the activation of the β-*catenin* signaling pathway, which enables better identification of β-HCA (*50*). A case report also suggested that the degree of β-*catenin* activation in ethoxybenzyl diethylenetriamine pentaacetic acid-enhanced MRI may be correlated with tumor signal intensity in the hepatobiliary phase (*51*). Overall, the advantages of MRI will continue to be exploited, and its non-invasive diagnosis of HCA subtypes will remain the focus of future attention. Although CEUS also has some value in identifying HCA subtypes, its sensitivity and specificity are not as good as those of MRI.

4.3. Natural history

Since only 15 to 20% of HCAs are at risk of complications and malignant transformation, most HCAs tend to stabilize in their natural course. A study of 118 patients indicated that 78% of HCAs remained stable or resolved with long-term MRI follow-up (*52*).

The natural history of different molecular subtypes of HCA varies. Studies have long recommended discontinuing hormone use in female patients. However, tumor regression and the risk of malignant transformation may persist even after discontinuation of hormone therapy (*53*). Estrogen is mainly associated with IHCA, HHCA, and shHCA. Therefore, recommending that patients with these conditions discontinue estrogen use and undergo imaging follow-up is reasonable. Special considerations must be made for the possibility of hormone-induced adenoma growth and HCA rupture during pregnancy. There are no clear recommendations for treating HCA tumors that consistently grow to more

Table 3. The characteristics, risk of complications, and management strategies for major subtypes of HCA

Subtypes	Proportion	Pathology	Clinical features	Risk of complications	Management
Inflammatory HCA (IHCA) 40-50% were mixed β^{ex3}-IHCA and mixed $\beta^{ex7,8}$-IHCA	34-50%	Inflammatory infiltrate; sinusoidal dilatation; dystrophic arteries	Estrogen; obesity; alcohol intake; glycogen storage disease	HCC: low; Bleeding: low; Inflammatory paraneoplastic syndrome: high	Follow-up
HNF1α inactivated HCA (HHCA)	30-40%	Pronounced steatosis	Estrogen; *HNF1α*-associated hepatic adenomatosis MODY3	HCC: low; Bleeding: low	Follow-up
β-*catenin* exon 3 mutated HCA (β^{ex3}-HCA) 15% were mixed β^{ex3}-IHCA	7-15%	Cytological atypia; pseudoglandular formation; cholestasis; Expression of GS (IHC)	Male; androgen	HCC: high; Bleeding: low	Surgery
β-*catenin* exon 7 or 8-mutated HCA ($\beta^{ex7,8}$-HCA) 10% were mixed $\beta^{ex7,8}$-IHCA	4-10%	No or faint expression of GS (IHC)	No specific	HCC: low; Bleeding: low	Follow-up
Sonic hedgehog HCA (shHCA)	4%	Histological hemorrhage	Obesity; estrogen	HCC: low; Symptomatic bleeding: high	Surgery
Unclassified HCA	7-10%	No specific pathology	No specific features	No specific risk	Follow-up

β^{ex3}-IHCA, HCA with β-*catenin* mutations in exon 3 and an inflammatory phenotype; $\beta^{ex7,8}$-IHCA, HCA with β-*catenin* mutations in exon 7 or 8 and an inflammatory phenotype; HCC, hepatocellular carcinoma; MODY3, maturity-onset diabetes of the young type 3; IHC, immunohistochemistry; GS, glutamine synthetase.

than 5 cm during pregnancy.

The overall risk of malignant transformation in HCA is approximately 5-10%, but this risk can vary greatly among different molecular subtypes, ranging from almost 0% to nearly 50% (up to 50% for β^{ex3}-HCA). Male patients have a higher risk of malignant transformation regardless of tumor size, with a risk that is six to 10 times greater than that for female patients. This increased risk is clearly associated with the previously described molecular subtypes. Considering β^{ex3}-HCA as a true precancerous lesion is reasonable. In addition, up to 42% of HCAs present with spontaneous intratumoral hemorrhage, peritoneal hemorrhage, and shock. Risk factors for bleeding include tumor > 5 cm, IHCA, a visibly diseased artery, a left hepatic tumor, and exophytic growth (*54*). Hepatic adenomatosis is associated with a higher risk of hemorrhage, necrosis, and malignant transformation (*52*). Although the proportion of male and female patients with *HNF1α*-related hepatic adenomatosis is equal, males have a higher incidence of bleeding (*55*).

4.4. Surgical indications

Previous studies have recommended surgery and lifelong observation for all patients with HCA (*56*), but the broad surgical indications need to be reconsidered as our understanding of the biological behavior of different molecular subtypes of HCA continues to improve. Overall, only about 15-20% of patients require surgery (*10*). A multidisciplinary team (MDT) discussion of a benign tumor is recommended for all patients requiring surgery. Previous studies have also emphasized the importance of MDT in the treatment of both benign and malignant diseases (*57-59*). Follow-up is necessary for all patients, typically with imaging every 6 months. After 12 months, the frequency of follow-up can be reduced if the tumor remains stable. Patients suspected of having HCC require more frequent MRI scans or surgical resection and biopsy.

Regardless of the subtype, HCAs larger than 5 cm carry a higher risk of bleeding and malignant transformation. Studies have recommended surgical removal for those larger than 5 cm (*60*). However, a study has pointed out that tumor size should not be an independent indication for surgery because surgical or non-surgical weight loss can shrink the tumor to less than 5 cm in some patients (*61*). Whether the surgical decision is based on the risk of bleeding or malignant transformation, combining individualized treatment decisions with molecular subpopulations is more reasonable.

Female patients who require continued oral contraceptives may need more frequent imaging, as well as surgery when the tumor is larger than 5 cm (*44*). All β^{ex3}-HCAs require surgical resection due to the significantly increased risk of malignant transformation. shHCA requires an MDT evaluation, but surgical resection is preferred. Surgical resection is required for all male patients with HCA. Table 4 shows the main surgical indications cited in recent studies.

Liver transplantation is recommended for hepatic adenomatosis in patients with large symptomatic tumors, tumors occupying almost the entire liver, significantly elevated alpha-fetoprotein levels, confirmed malignancy, and tumor progression after hepatectomy, due to the difficulty of achieving complete resection of all tumors (*62*). However, a study has pointed out that the risk of complications is not related to the number of tumors. Liver transplantation is unnecessary for patients with hepatic adenomatosis, and two-stage resection can be performed for large bilateral liver tumors (*63*). The risks of liver transplantation itself need to be weighed against those of the disease (*64*). In addition, studies have indicated that 71% of tumors in patients with multiple HCAs belong to the same subtype. In the remaining cases, β^{ex3}-HCA is often the largest tumor associated with the risk of malignant transformation. Therefore, biopsy of the largest nodule in hepatic adenomatosis to determine whether surgical resection is necessary may be

Table 4. Surgical indications for hepatic adenomas cited in recent studies

Literature	Type of literature	Country or region	Surgical indications
Belghiti *et al*. (2014) (*6*)	Review	France	Tumor > 5 cm (unless HHCA); male patients
Practice Parameters Committee of the American College of Gastroenterology (2014) (*33*)	Clinical guideline	United States	Tumor > 5 cm; β-HCA
Brazilian Society of Hepatology (2015) (*21*)	Clinical recommendations	Brazil	Tumor > 5 cm in women of reproductive age; male patients
European Association for the Study of the Liver (EASL) (2016) (*8*)	Clinical guideline	Europe	Male patients; β-HCA; tumor > 5 cm in female patients; residual tumor after embolization
Haring *et al*. (2023) (*60*)	Original article	Netherlands	Suspected malignancy; male patients; tumor > 5 cm; previous bleeding; symptoms leading to impaired quality of life
Tse *et al*. (2023) (*46*)	Review	United States	Male patients; β^{ex3}-HCA; female patients whose tumors progressed or remained > 5 cm after weight loss and discontinuation of oral contraceptives

a valuable option (*44*).

4.5. Surgery

In most cases, anatomical hepatectomy or segmental resection is preferable due to the risk of malignant transformation and the need to ensure resection margins. The prevalence of HCA is higher in obese patients, indicating a possible role of obesity in the development and progression of HCA. One case series reported complete resolution of HCA in two patients within 1-2 years after bariatric surgery, as well as a >50% reduction in the diameter of the largest HCA and complete resolution of smaller HCAs in another patient within 2.5 years after surgery (*61*). Clearly, the IHCA and shHCA subtypes are associated with obesity. Therefore, patients with these subtypes need to lose weight if they are overweight (*65*). However, using bariatric surgery alone to treat these subtypes of HCA presents a challenge.

TAE is used more frequently in HCA because patients with bleeding tumors may require arterial embolization as initial treatment. TACE has been found to result in partial or complete regression of HCA, allowing 45% of patients to avoid surgery. In addition, TACE can reduce the size of large, bilateral, or multiple liver tumors before surgical resection (*66*). However, whether TACE reduces symptoms and averts the risk of malignant transformation remains unknown.

RFA was initially recommended for residual or progressive tumors after resection, or as the initial treatment of tumors < 3 cm (*67*). Recently, RFA has been increasingly used in patients with HCA and appears to be a potential alternative to lifelong imaging follow-up or elective surgery. This approach seems to offer the best quality-adjusted life expectancy, lifetime costs, and net health benefits compared to hepatectomy, TACE, or no treatment (*68*). However, RFA does not produce specimens for pathological analysis, and ablation is difficult to accept in cases of diagnostic uncertainty (*53*). Its overall use remains limited, and there is no consensus on the indications for RFA, particularly regarding the number, size, and localization of treatable lesions. Therefore, the precise role of this technique remains to be determined.

5. Management

The management algorithms for HH, FNH, and HCA are shown in Figure 1.

6. Role of minimally invasive hepatectomy

The widespread use of laparoscopic surgical equipment and improvements in surgeons' laparoscopic liver resection techniques have led to a continuous reduction in surgical costs (*69-71*). Two studies indicated that quality of life scores were significantly better after laparoscopic surgery for benign liver tumors than after open surgery (*36,72*). A third study indicated that postoperative scarring of benign liver tumors is a common cause of residual symptoms, and laparoscopic surgery has an advantage in this regard (*73*). Laparoscopy is constantly developing towards robot-assisted laparoscopy, which offers more technical advantages (*74*). These benefits of minimally invasive hepatectomy will continue to impact the clinical management of benign liver tumors. However, whether surgical treatment is necessary for benign liver tumors remains a problem that requires continued attention in the field of liver surgery. After all, no matter how minimally invasive the surgery may be, it cannot be as inexpensive as reasonable observation.

7. Conclusion

In conclusion, surgical indications for HH and FNH focus solely on clinical symptoms clearly associated with the tumor and rare complications, while tumor size is not critical. The surgical indications for HCA are closely related to the molecular subtype. Male patients and those with β^{ex3}-HCA require surgical resection. shHCA requires an MDT discussion, but surgery is preferable. Attention should be paid to obesity in IHCA and shHCA, as surgical or non-surgical weight loss may control or even reduce the tumor. Female patients with tumor progression or those whose tumors are larger than 5 cm

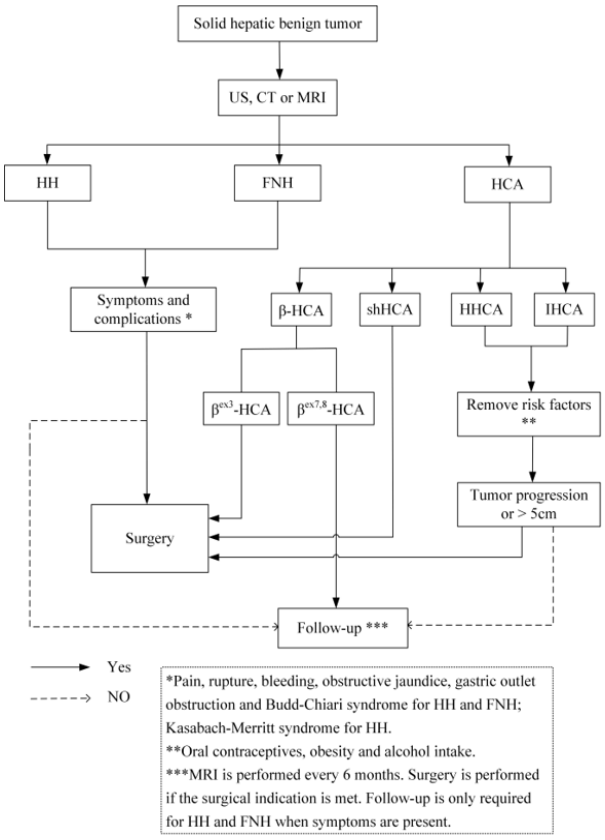

Figure 1. Management algorithms for hepatic hemangioma, focal nodular hyperplasia, and hepatic adenoma.

after cessation of oral contraceptives and weight loss are eligible for surgery. Accurate identification of molecular subtypes of HCA through contrast-enhanced MRI will be crucial to personalized clinical management in the future.

Funding: This work was supported by a grant from the Natural Science Foundation of China (no. 82170621).

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received June 30, 2023; Revised August 11, 2023; Accepted August 15, 2023.

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Released online in J-STAGE as advance publication August 19, 2023.

Review

DOI: 10.5582/bst.2023.01207

Influence of intermittent fasting on autophagy in the liver

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SUMMARY Studies have found that intermittent fasting (IF) can prevent diabetes, cancer, heart disease, and neuropathy, while in humans it has helped to alleviate metabolic syndrome, asthma, rheumatoid arthritis, Alzheimer's disease, and many other disorders. IF involves a series of coordinated metabolic and hormonal changes to maintain the organism's metabolic balance and cellular homeostasis. More importantly, IF can activate hepatic autophagy, which is important for maintaining cellular homeostasis and energy balance, quality control, cell and tissue remodeling, and defense against extracellular damage and pathogens. IF affects hepatic autophagy through multiple interacting pathways and molecular mechanisms, including adenosine monophosphate (AMP)-activated protein kinase (AMPK), mammalian target of rapamycin (mTOR), silent mating-type information regulatory 2 homolog-1 (SIRT1), peroxisomal proliferator-activated receptor alpha (PPARα) and farnesoid X receptor (FXR), as well as signaling pathways and molecular mechanisms such as glucagon and fibroblast growth factor 21 (FGF21). These pathways can stimulate the pro-inflammatory cytokines interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α), play a cytoprotective role, downregulate the expression of aging-related molecules, and prevent the development of steatosis-associated liver tumors. By influencing the metabolism of energy and oxygen radicals as well as cellular stress response systems, IF protects hepatocytes from genetic and environmental factors. By activating hepatic autophagy, IF has a potential role in treating a variety of liver diseases, including non-alcoholic fatty liver disease, drug-induced liver injury, viral hepatitis, hepatic fibrosis, and hepatocellular carcinoma. A better understanding of the effects of IF on liver autophagy may lead to new approaches for the prevention and treatment of liver disease.

Keywords metabolism, diet, nutrient, NAFLD, HCC, liver disease

1. Introduction

Autophagy is a lysosomal degradation pathway by which cells "self-digest" their own components to provide nutrition under challenging conditions as well as to remove excess and damaged organelles, misfolded proteins, or invasive microorganisms from the cell (*1*). In addition to its role in cellular homeostasis, autophagy also plays an important role in embryonic development, cell differentiation, and regeneration (*2*). The liver is the organ in which autophagy was first studied and has benefited from this deeper understanding of autophagy, a process closely linked to liver pathophysiology (*3-5*). Hepatic autophagy helps to maintain essential liver functions such as lipid, glycogen, and protein regeneration, whereas dysregulated hepatic autophagy is associated with a variety of liver diseases, including alcoholic and non-alcoholic fatty liver disease, pharmacological liver injury, viral hepatitis, hepatic fibrosis, and hepatocellular carcinoma (*5-7*).

The term intermittent fasting (IF) encompasses several different dietary regimens. Current research suggests that IF may reduce the inflammatory response and improve the outcome of diseases such as asthma, rheumatoid arthritis, inflammatory bowel disease (IBD), and allergic contact dermatitis (*8-10*). Studies in animals have demonstrated that IF has anti-aging benefits, and they have revealed its potential applications in neurodegenerative diseases in particular. For example, by attenuating the levels of oxidative stress proteins such as NOX2, NOX4, 8-OHdG, and 4-HNE and inhibiting the levels of the pro-apoptotic factor Bax and cleaved cysteine asparaginase-3, IF had a protective effect against cognitive dysfunction in type 1 diabetic mice (*11*). In addition, IF has the potential to optimize gut microbiota and enhance immune

memory (*12*). A study on the treatment of certain tumors has shown that IF can synergistically enhance the anticancer action of conventional treatments and increase the sensitivity to radiotherapy/chemotherapy (*13*). More importantly, IF is associated with autophagy. Through the regulation of hepatic autophagy, IF has a potentially beneficial role in liver pathophysiology (*14*). The current review summarizes the available evidence on the effects of IF on hepatic autophagy and how this may impact physiopathological changes in the liver.

2. Liver autophagy

2.1. Discovery of autophagy

Autophagy was identified as a mechanism for degrading cytoplasmic components in the late 1950s and early 1960s, following the discovery of lysosomes by Christian de Duve (*3*). Autophagosomes were subsequently identified through electron microscopy of isolated liver tissue under stress conditions, and these structures were found to play an important role in the starvation response (*4*). Autophagy is evolutionarily conserved as a ubiquitous, self-degrading catabolic process. It is necessary to meet the metabolic demands of the cell, maintain genome integrity, regulate innate and adaptive immune processes, modulate pro-inflammatory mediators, and promote cell survival. Constitutive autophagy occurs continuously in nutrient-rich environments to maintain the turnover of cellular components and is a fundamental survival mechanism. However, induced or reactive autophagy occurs in response to stress, including starvation, low amino acid levels, lack of trophic factors or hormones, endoplasmic reticulum (ER) stress, hypoxia, irradiation, drugs, and intracellular pathogens (*15*).

2.2. Diverse forms of autophagy

Autophagy can be classified into three main types based on mechanism of cargo delivery to lysosomes, *i.e.,* macroautophagy, microautophagy and chaperone-mediated autophagy (CMA) of proteins containing lysine-phenylalanine-glutamic acid-arginine-glutamine (KFERQ) -like motifs (Figure 1). Moreover, autophagy can be classified into selective and non-selective autophagy based on the selective and non-selective phagocytosis of substrates. Selective autophagy plays an essential role in maintaining cellular homeostasis by ensuring organelle cycling and degrading bound substrates.

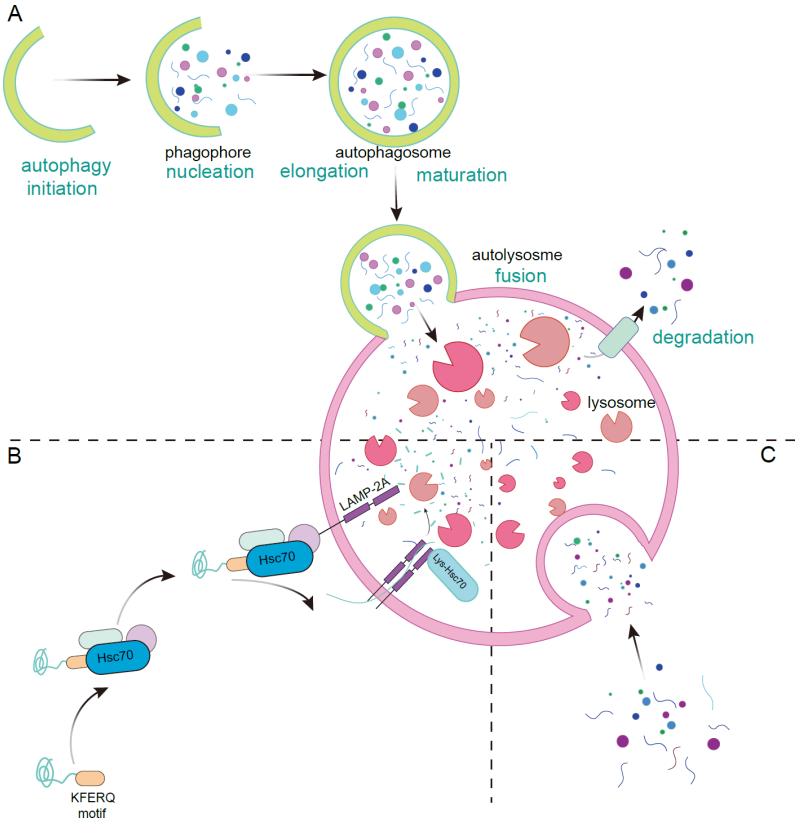

Figure 1. Three main types of autophagy: macroautophagy, CMA, and microautophagy. (A) Macroautophagy is a multistep process involving initiation, nucleation, elongation, maturation, fusion, and finally degradation. **(B)** CMA specializes in chaperone-mediated degradation of cytoplasmic proteins, delivering them to the lysosomal surface where substrate proteins unfold and cross the lysosomal membrane. These substrates, which contain the KFERQ motif, are specifically recognized by Hsc70. Chaperone-targeted proteins bind to LAMP-2A. **(C)** In microautophagy, targeted cargo is directly isolated and subsequently engulfed by lysosomes. Abbreviations: CMA, chaperone-mediated autophagy; Hsc70, heat shock cognate protein of 70 kDa; LAMP-2A, lysosome-associated membrane protein 2A.

2.2.1. Macroautophagy

Macroautophagy is divided into several phases, including autophagy initiation, phagosome nucleation and elongation, autophagosome maturation and fusion with lysosomes, and degradation. The process of autophagy has been extensively described in a number of other reviews and will only be briefly mentioned here. Autophagosomes originate from a membrane called the phagolysosome, a restriction membrane or de novo phagolysosome formed by the assembly of proteins and lipids from various organelles, such as the ER, Golgi apparatus, mitochondria, endocytosis system, or plasma membrane, whose nucleation and elongation are dependent on autophagy-related genes (ATGs) (*16*). More than 40 ATGs have been reported to be involved in autophagy. Once the membrane of the autophagosome is sealed, the cargo-containing vesicle moves along the microtubule to fuse with the lysosome and deliver the loaded cargo to the organelle for further degradation. After degradation, the molecules, amino acids, lipids, and carbohydrates produced are transported to the cytoplasm for recycling, in part by transporter proteins and permeases. Following degradation within autolysosomes, nutrient-supplied reactivation of mammalian target of rapamycin (mTOR) suppresses autophagy initiation and simultaneously initiates autophagic lysosome reformation (ALR), thereby terminating autophagy (*17*).

2.2.2. Chaperone-mediated autophagy (CMA)

Neither CMA nor microautophagy needs autophagosomes. CMA is induced after long-term starvation, and proteins containing the pentapeptide KFERQ motif are recognized by the cytosolic heat shock cognate protein of 70 kDa (Hsc70), which facilitates the translocation of CMA substrates into the lysosomal lumen by binding to lysosome-associated membrane protein 2A (LAMP-2A). Substrate proteins are then rapidly degraded in the lysosomal lumen (*18*). LAMP-2A levels, which limit CMA activity, are controlled both by transcriptional activation and, more commonly, by direct changes in the stability of LAMP-2A at the lysosomal membrane (*19*). CMA was originally described as part of the hepatocyte response to nutritional changes in the liver by replenishing chronically starved cells with amino acids and ATP (*20*). In addition, CMA can act as a defense mechanism against cellular damage, that is by removing damaged proteins and thus maintaining protein homeostasis, maintaining lipid metabolism homeostasis, reprogramming gene transcription, activating the immune response, modulating the cell cycle, and being involved in the regulation of senescence (*18*).

2.2.3. Microautophagy

Microautophagy is the direct phagocytosis of cytoplasmic material by the lysosomal membrane. Based on morphological changes examined using electron microscopy, microautophagy is the major autophagic response in the mouse liver under starvation and refeeding conditions (*21*). Although the first studies of microautophagy were carried out in mammals soon after the identification of the lysosome, many of our previous insights into microautophagy have been gained in yeast, probably because of the relatively large size of the yeast vacuole (analogous to the mammalian lysosome) and the accessibility of the yeast cell (*22*). Most studies of microautophagy have focused on changes associated with the lysosome, vacuole, or endosome. During microautophagy, autophagic cargo is directly engulfed by lysosomes and late endosomes through membrane protrusion and invagination, and the autophagic cargo is then degraded in the lysosomal lumen.

2.2.4. Selective autophagy

Most of the three types of autophagy described above are thought to process, segregate, and degrade some cytoplasmic contents non-selectively. Selective autophagy differs from non-selective autophagy in that it plays a more selective role in response to a variety of physiological stimuli. Depending on the substrate that is being degraded, selective autophagy has been further categorized into mitophagy, ER-phagy, pexophagy, glycophagy, lipophagy, RNautophagy, DNautophagy, ferritinophagy, crinophagy, xenophagy, and aggrephagy. The best described and widely studied types of selective autophagy in the liver are mitochondrial autophagy and lipophagy. Selective autophagy has multiple functions, including protecting mammalian cells from organelle damage by removing dysfunctional organelles (*5*).

2.3. Role of autophagy in liver physiology and metabolism

The liver is the largest solid organ in the human body and plays an important role in various biological activities. The major functions of the liver include bile production, bilirubin metabolism, synthesis of anticoagulant factors and plasma proteins, amino acid and lipid metabolism, vitamin and mineral storage, hormone production, detoxification, and immune response. A point worth noting is that autophagy plays a crucial role in maintaining liver function. Three different types of autophagy coexist in the liver: macroautophagy, microautophagy, and CMA. Autophagy plays an important role in the maintenance of cellular and metabolic homeostasis in the liver.

Hepatic autophagy fluctuates in response to hormonal changes and nutrient availability in the fed and fasted state, as well as circadian rhythmic activity. At the organismal level, liver autophagy varies in response to

pancreatic hormones such as insulin and glucagon and gastric hormones such as growth hormone peptide (GHP) and glucagon-like peptide-1 (GLP-1) analogs, which positively or negatively regulate autophagy in response to feeding and fasting conditions (*23*). Liver autophagy is also regulated by the central nervous system (CNS). Glial cell-derived neurotrophic factor (GDNF) increases hepatic autophagy by inhibiting mTOR (*24*). During nutrient deprivation, adrenaline secretion increases, which ultimately increases hepatic autophagy in response to starvation (*25*). In addition, thyroid hormones (THs) can activate hepatic autophagy to promote fatty acid β-oxidation (*26*).

Nutrient deprivation triggers liver autophagy, which leads to glycogen breakdown, lipolysis, and proteolysis that provide glucose, fatty acids, and amino acids to cells and fuel to other organs. Studies in neonatal and adult mice have shown that autophagy is necessary for the maintenance of amino acid and glucose levels in blood and tissues during fasting (*27*). By converting amino acids to glucose *via* gluconeogenesis, liver-specific autophagy plays an important role in the regulation of blood glucose levels. During starvation, protein homeostasis is maintained by supplementing the intracellular amino acid pool through increased protein degradation in the cytoplasm. Amino acids produced by lysosomal degradation can also participate in the Krebs cycle to promote ATP production or gluconeogenesis (*28*). Moreover, autophagy is involved in lipid metabolism, including lipogenesis, lipolysis, fatty acid oxidation, ketogenesis, and cholesterol efflux. Loss of autophagy in the liver impairs the breakdown of triglycerides into fatty acids and results in steatosis and insulin resistance (*29*). In addition, ketone body production during fasting was significantly impaired by the specific deletion of hepatic *ATG7* or *ATG5* (*20*). Conversely, elevated circulating levels of inulin, glucose, adipokines, regulatory amino acids, and bile acids during feeding inhibit hepatic autophagy (*30*).

Interestingly, autophagy undergoes rhythmic changes that are consistent with circadian patterns in adult mammals. In the liver, cyclic activation of autophagic flux is associated with rhythmic expression of autophagy genes (*31*). The rate of conversion of light chain 3 (LC3)-I to LC3-II in the liver reaches a significant peak during the midday phase and then declines until the dark phase (*32*). A study has shown that the basic helix-loop-helix-PAS transcription factor families BMAL1 and CLOCK, which belong to the clock gene family, activate the transcription of genes involved in the regulation of autophagy (*33*).

In addition, autophagy is essential for the maintenance of protein and organelle homeostasis as well as quality control in hepatocytes. Selective autophagy ensures the removal of specific soluble proteins, protein aggregates, damaged mitochondria, and invasive bacteria from the cell, and defects in autophagy are directly associated with metabolic disorders. For example, mice with liver-specific deletion of *ATG5* or *ATG7* exhibit accumulation of ubiquitinated proteins, abnormal ER, excess peroxisomes, and mitochondrial dysfunction (*34,35*). Autophagy ultimately maintains homeostasis at the subcellular level of the tissue by regulating the renewal of intracellular organelles, including the selective removal of mitochondria (mitophagy), peroxisomes (pexophagy) and the ER (ER-phagy) (*36*). Through these processes, autophagy also has an indirect effect on metabolism and the maintenance of energy homeostasis. In addition, the liver catabolizes organelles and other intracellular components (*e.g.,* specific proteins and invading pathogens) through selective autophagy to maintain cellular homeostasis and protect cells from damage (Figure 2). Mitophagy occurs through many distinct but interrelated mechanisms that can usually be divided into Ub-dependent and Ub-independent pathways. The purpose of mitophagy is to isolate and remove dysfunctional, potentially cytotoxic mitochondria so that they do not cause harm to the host cells. Mitochondrial autophagy plays a key role in maintaining liver homeostasis or in the pathogenesis of liver diseases by selectively targeting damaged/excess mitochondria. Pexophagy removes excess and damaged peroxisomes through P62-dependent or P62-independent pathways to maintain healthy peroxisome homeostasis and it plays a key role in peroxisome biogenesis, reactive oxygen species (ROS) metabolism, fatty acid oxidation, polyamine and D-amino acid oxidation, and synthesis of lipid metabolites, bile acids, and DHA. ER-phagy occurs under normal conditions and is enhanced during starvation. There are two types of ER-phagy: macro-ER-phagy and micro-ER-phagy. In macro-ER-phagy, the autophagic bilayer membrane extends and wraps around the ER fragment, which eventually binds to the lysosome and is degraded. Micro-ER-phagy is a process in which the lysosomal membrane undergoes invagination and extrudes a portion of the ER into the lysosome. ER-phagy plays an important role in the elimination of excess ER, selective removal of misfolded aggregates in the ER, degradation of the nuclear membrane, nutrient supply, quality control, and antioxidant metabolism. The activation of hepatic autophagy protects the liver from oxidative stress, organelle stress, or damage induced by certain xenobiotics (*20*).

2.4. Dysregulation of autophagy in liver pathology

Hepatic autophagy plays a role in the prevention of liver diseases, and disorders or dysfunctions of autophagy play an important role in the pathogenesis of liver diseases such as non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), viral hepatitis, and hepatocellular carcinoma (HCC).

2.4.1. Autophagy in hepatic metabolic diseases

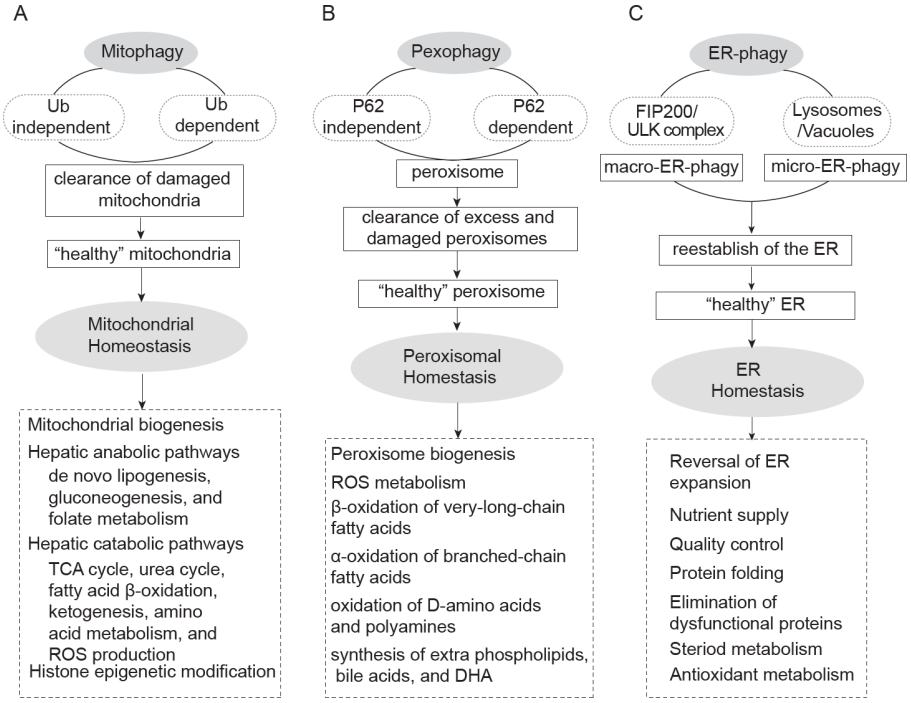

Figure 2. Hepatic autophagy maintains homeostasis through quality control. (A) Mitophagy plays an important role in mitochondrial biogenesis, hepatic anabolism/catabolism, and epigenetic modification of histones by maintaining healthy mitochondrial homeostasis through the clearance of damaged mitochondria, primarily through the Ub-dependent or Ub-independent pathways. **(B)** Pexophagy removes excess and damaged peroxisomes through P62-dependent or P62-independent pathways to maintain healthy peroxisome homeostasis and plays a key role in peroxisome biogenesis, ROS metabolism, fatty acid oxidation, polyamine and D-amino acid oxidation, and synthesis of lipid metabolites, bile acids, and DHA. **(C)** There are two types of ER-phagy: macro-ER-phagy and micro-ER-phagy. By reestablishing the ER through these pathways, ER-phagy plays an important role in the reversal of ER expansion, nutrient supply, quality control, protein folding, elimination of dysfunctional proteins, steroid metabolism, and antioxidant metabolism. Abbreviations: ER, endoplasmic reticulum; ROS, reactive oxygen species; TCA, tricarboxylic acid; ULK complex, unc-51 like autophagy activating kinase complex.

Due to its important role in the pathogenesis of NAFLD, autophagy is gaining attention as a novel therapeutic target. Induction of increased autophagy contributes to lipid degradation in the liver and alleviates disease progression in the early stages of NAFLD. The pathogenesis of NAFLD is due to abnormalities in hepatic lipid metabolism, including increased lipogenesis, increased uptake of free fatty acids (FFAs), and accumulation of hepatocellular lipids. NAFLD comprises a spectrum of liver pathologies, beginning with hepatic steatosis, through inflammatory hepatocellular injury called NASH, to hepatic fibrosis, cirrhosis, and HCC. Autophagy plays a key role in the pathogenesis of NAFLD (*37*). Autophagy facilitates the balance of lipid metabolism through the degradation of metabolic lipid droplets (LDs) and the regulation of LD biogenesis. However, LC3-II and p62 have been observed to accumulate in patients with NASH and correlate with disease severity (*38*). Hepatic metabolic diseases are often associated with metabolic syndrome, and metabolic syndrome has a significant negative impact on autophagy. In the presence of insulin resistance and hyperinsulinemia, there is a loss of regulation of the expression of several ATGs by forkhead box O1 (FoxO1), leading to autophagy dysfunction (*39*). In addition, the expression of tissue proteases has been found to be inhibited in obese mice and NAFLD patients, leading to a reduction in lysosomal activity and ultimately a reduction in lysosomal degradation (*40*). However, the homeostasis of proteins (increased levels of polyubiquitin proteins) and organelles is disturbed when autophagy is blocked. The LD-associated proteins perilipin 2 (PLIN2) and perilipin 3 (PLIN3) are CMA substrates, and CMA can directly degrade LD-associated proteins while increasing LD cytosolic adipose triglyceride lipase (ATGL) and autophagy protein levels, thereby promoting LD lipolysis (*41,42*). However, high-fat diets alter the lysosomal stability of the CMA receptor and reduce the activity of this pathway (*43*). Persistent impairment of hepatic CMA then alters protein homeostasis and metabolic dysregulation, promotes the accumulation of oxidative protein aggregates, and progressively reduces hepatic resistance to stress (*44*). At the same time, changes in intracellular lipid content (*i.e.,* metabolic dysfunction) can have a significant impact on the fusion step of macroautophagy, which in turn affects the overall activity of the protein hydrolysis pathway in that cell, with impaired autophagic flux (*45*). In contrast, impaired hepatic autophagic flux is associated with increased ER stress during the development of NAFLD (*38*). ER stress and ER dysfunction contribute to impaired glucose metabolism in NASH. Healthy mitochondria are critical

for lipid metabolism, and liver lipotoxicity activates a series of mitochondrial dysfunction events, while impaired mitochondrial autophagy has been shown to activate the NOD-like receptor thermal protein domain associated protein 3 (NLRP3) inflammatory vesicle, facilitating progression from NAFLD to NASH (*46,47*). Lipotoxicity, oxidative stress, and chronic activation of the inflammatory response following autophagy failure usually lead to liver cell death and the features of NASH (*i.e.,* inflammation, oxidative stress, cell death, and fibrosis). Apolipoprotein B (APOB)-directed LDs and bortezomib-induced hepatocyte Mallory-Dunn bodies can be eliminated by lipophagy (*48*). These results further indicate that lipophagy may prevent the development of NAFLD. Moreover, re-establishing mitochondrial autophagy may alleviate NAFLD progression (*49*). Therefore, autophagy has been shown to be a protective factor in the development of fatty liver disease, and boosting autophagy activity has been suggested as a strategy for developing therapeutic approaches to treat fatty liver disease.

2.4.2. Autophagy in HCC

There is a complex relationship between autophagy and the development of HCC (*50*). Autophagy, mitophagy, and lipophagy have ambiguous functions in cancer, acting to inhibit tumor growth in the early phase, but they may contribute to tumor progression by meeting the metabolic needs of tumor cells in the late phase (*51*).

In the liver, changes in autophagic activity are closely linked to tumorigenesis, and one of the ultimate outcomes of metabolic dysfunction is the development of HCC. Liver autophagy can serve as a tumor suppressor, while deletion of *ATG5* or *ATG7* can lead to liver tumor formation (*52*). In addition, reduced expression of autophagy proteins and impaired autophagy have been shown to be associated with tumor malignancy and poor prognosis in HCC (*53*). p62 has been reported to serve as a target of rapamycin complex-1 (mTORC1) on mammalian lysosomes and as a signaling hub for the Keap1 (Kelch-like ECH associated protein 1)-Nrf2 (nuclear factor, erythroid 2 like 2) pathway on autophagic cargo, as well as a receptor for selective autophagy (*54*). Failure of selective autophagy leads to accumulation of SQTSM1/p62, mitochondrial dysfunction, ROS generation, and DNA repair, which ultimately affects tumor susceptibility, as well as sustained activation of downstream Nrf2 and NF-κB pathways (*54*). Activation of Nrf2 can divert glucose and glutamine to anabolic pathways such as the pentose phosphate pathway and purine nucleotide synthesis, thereby facilitating tumor cell proliferation (*55*). In addition, Nrf2-activated cells exhibit a constitutive induction of cytoprotective enzymes and drug efflux pumps, which induce resistance to chemotherapeutic agents in HCC (*56*). There is emerging evidence that HCC cells may also harness autophagy to promote tumor progression; for example, elevated levels of autophagy markers (*e.g.,* LC3) in HCC have been associated with a poor prognosis and higher rates of post-operative recurrence (*57*). Moreover, blocking CMA in cancer cells suppresses the uniquely high rate of aerobic glycolysis in tumors in a p53-dependent manner, leading to a reduction in tumorigenicity and metastatic capacity (*58*).

3. IF

3.1. Overview

IF is an increasingly popular dietary strategy that consists of alternating periods of fasting: periods of complete calorie deprivation alternating with periods of ad libitum consumption. There is growing interest in this type of dietary strategy because several studies have shown that it improves body composition, muscle performance, and clinical parameters (*e.g.,* blood pressure, blood glucose, and cholesterol levels), especially in the absence of weight loss (*59*). IF is a general concept that encompasses a wide range of fasting methods. In general, we can divide IF protocols into three main categories: alternate day fasting (ADF), period fasting (PF) and time-restricted fasting (TRF). There are also reports of certain clinical benefits of religious fasting, such as Ramadan IF (RIF). However, comparing RIF studies to other types of fasting studies is not easy, and we will not discuss them further in this review. All IF protocols include regular fasting, but there are differences in the frequency and duration of fasting in different protocols (Table 1). Complete alternate day fasting is the practice of alternating a whole day of fasting with a whole day of unrestricted feeding, meaning that no energy-containing food or drink is consumed on fasting days and unrestricted food is consumed on feeding days. This is more commonly known as a modified ADF (mADF). On modified fasting days, mADF adherents typically eat a light meal at noon. This meal provides about 20–40% of the daily calorie requirement, or about 400–600 kcal. PF protocols include fasting periods of varying frequency and duration, but each fasting period is usually ≥ 24 hours in length. Other implementations of PF include fasting for 24 hours on consecutive days, and some PF regimens include a complete absence of caloric intake for 1 or 2 consecutive or non-consecutive days per week. Similar to mADF, a popular example of a modified PF protocol is the 5:2 diet, in which participants restrict their calorie intake to 25% of their maintenance requirement on two days of the week, and then maintain their normal eating habits for the remaining five days. Other PF protocols involve not eating for 24 hours once or twice a week. TRF involves fasting for the same period each day, consuming free energy for 6–12 hours, and avoiding energy-dense foods for the remaining 12–18 hours of the 24-hour period.

Table 1. Protocols for intermittent fasting

Category / Intermittent fasting	Description
Complete alternate day fasting	No energy-containing foods or beverages on fasting days, alternating with unrestricted food intake on feeding days.
Modified alternate day fasting	20–40% of energy requirements consumed on fasting days, alternating with unrestricted food intake on feeding days.
Period fasting (5:2)	Intake restricted to 25% or less of caloric needs 2 days per week (consecutive or non-consecutive days), with unrestricted food intake the other 5 days.
Time-restricted fasting	Unlimited energy consumption within 6–12 hours, no energy-containing foods for the remaining 12–18 hours of the day.

3.2. Metabolic effects of IF

3.2.1. Lipid metabolism

IF reduces blood lipids. Results from 2–3 months of ADF trials show that IF deceases low density lipoprotein (LDL) levels (20–25%) and triacylglycerol levels (15–30%) (*60*). Similarly, in normal-weight, overweight, and obese populations, trials of ADF for 3 to 12 weeks appear to be effective in reducing total cholesterol (10–21%) and triglycerides (14–42%). Moreover, results from whole-day fasting trials for 3–6 months show that IF lowers total cholesterol (5–20%) and triglycerides (17–50%) (*59*). The lipid changes that occur during IF are designed to meet metabolic needs and preserve protein reserves (*61*). White adipose tissue serves as an energy reserve and releases long-chain fatty acids into the circulation during fasting. Triglycerides are the main source of fuel for the body during the fasting period (*61*). Previous studies have shown that the rate of lipolysis is almost doubled during 3–4 days of fasting, while gluconeogenesis is reduced by about one-third (*62,63*). In addition, changes in lipid metabolism may occur independently of changes in plasma glucose levels (*64*). The increase in lipid metabolism occurred mainly between 18 and 24 hours after fasting. After 24 hours of fasting, lipid oxidation increased by half, and there was a significant increase in plasma FFAs (*65*). Reduced circulating insulin levels, together with plasma glucose, are thought to play an important role in the regulation of lipolysis (*66*). Insulin is thought to inhibit lipolysis *via* the cyclic AMP (cAMP)/protein kinase A (PKA) pathway, ultimately leading to a reduction in lipids and the activity of hormone-sensitive lipase (HSL) (*67*). Nevertheless, changes in lipid metabolism in response to fasting do not appear to be consistent across populations, such as gender, exercise training status, and body composition.

3.2.2. Glucose metabolism

A study has confirmed that fasting lowers blood glucose. The reduction in glucose production in the early stages of fasting appears to be driven by changes in glycogenolysis and gluconeogenesis and the slow decline in endogenous glucose production (*68*). However, the findings are ambiguous. Most of the reduction in glucose production during the first 48 hours of fasting is due to reduced gluconeogenesis (*65*). In subjects fasted for 60 hours, more than 80% of glucose was produced *via* gluconeogenesis (*69*). Liver biopsy confirmed that only 15% of liver glycogen was preserved after 24 hours of fasting (*70*). Glucose production from gluconeogenesis increased from 67% of total glucose production after 22 hours of fasting to 93% after 42 hours of fasting, with no change in total production, implying a reduced role for glycogenolysis (*71*). During fasting, expression of SIRT1 was found to be upregulated, and SIRT1 inhibited glucose production by inhibiting gluconeogenesis mediated by cAMP response element binding protein (CREB)-regulated transcription coactivator 2 (CRCT2) (*72*). Glucose oxidation was reduced by a factor of 10 between 16 and 22 hours after fasting (*73*). In addition to reduced endogenous glucose production and oxidation, studies have shown that the insulin-stimulated uptake of glucose is reduced by as much as 46% after two days of fasting (*68*). Reduced insulin increases the rate of glycogenolysis and gluconeogenesis mainly by inhibiting the insulin receptor substrate-1 (IRS1)/Akt pathway (*74*).

3.2.3. Ketone metabolism

Fasting is characterized by a markedly increased level of ketones in the blood. The production of ketone bodies in the liver can be stimulated by a number of factors during the fasting period. These include a decrease in the hepatic glycogen level, an increase in the glucagon/insulin ratio, and an increase in plasma FFAs. A study has revealed that levels of blood ketone bodies begin to rise within 8–12 hours after fasting. They can reach to 2–5 mmol/L within 24 hours of fasting and continue to rise until reach a steady state after about 2.5 to 5 days of fasting (*75*). Typically, levels of blood ketone bodies rise between 8 and 24 hours after fasting, and particularly after 20 hours of fasting, when the detectable level of residual liver glycogen is minimal (*65*). Providing available glucose to the CNS may be the main role of increased ketone bodies in the early stages of fasting. In contrast, increased

ketone production may provide an alternative energy source for protein conservation during fasting (*76*).

3.2.4. Protein metabolism

During fasting, proteins are oxidized and broken down for energy. Fasting can alter the levels and types of amino acids. Arginine, alanine, serine, threonine, aspartic acid, and proline decreased significantly during fasting, while levels of other amino acids remained almost unchanged (*77*). Total amino acids and total essential amino acids decreased significantly after 3 hours of fasting (*41*). Moreover, the decrease in the content of essential amino acids was greater than that of the non-essential amino acids (*78*). However, levels of lysine, leucine, isoleucine, and taurine changed in two phases. They were found to be at their lowest level 6 hours after fasting, with a brief recovery occurring 12 hours after fasting.

3.3. The hormonal impact of fasting

3.3.1. Insulin

IF reduces plasma insulin levels and improves insulin sensitivity. Insulin is produced and secreted by the beta cells of the pancreas and is an important metabolic hormone. By inhibiting glucose production in the liver, insulin lowers blood glucose after meals to maintain normal blood glucose levels. During acute fasting, reduced plasma insulin levels and improved insulin sensitivity have been observed in humans. Plasma insulin was reduced by about 35% in the first 24 hours after fasting and by as much as half of the initial level after three days of fasting (*64*). The 22 days of ADF significantly inhibited insulin secretion by 50% (*79*). In a trial of an 8-week TRF, there was also a significant reduction in blood insulin levels and insulin resistance (*80*).

3.3.2. Glucagon

The production of glucagon increases during fasting. As an insulin precursor, glucagon plays a key role in glucose metabolism. Glucagon promotes glucose production in the liver by increasing glycogenolysis and gluconeogenesis. Another important role of glucagon in the liver is to inhibit glucose catabolism by promoting fatty acid oxidation, which is a switch in the coordination of energy demand and energy from glucose production (*81*). Moreover, glucagon is involved in the metabolism of lipids and glucose *via* the cAMP pathway in liver cells (*82*).

3.3.3. Thyroid hormone

Human T3 levels fall rapidly after fasting. Monitoring of thyroid hormones in healthy volunteers who fasted for 80 hours revealed a reduction in T3 and thyroid stimulating hormone (TSH) levels within 48 hours of fasting (*83*). In another study, a decrease in serum T3 of up to 55% was reported after an overnight fasting. Unlike serum T3, TSH remained unchanged after fasting (*84*). Short-term (4 weeks) and long-term (more than 6 months) ADF diets reduced the circulating level of T3 without any change in the level of TSH (*85*). Similar results were also observed in another 8-week TRF study (*86*).

3.3.4. Glucocorticoids

IF increases the level and frequency of cortisol secretion and influences the rhythm of cortisol secretion. Typically, the level of plasma cortisol secretion peaks between 7 and 8 AM and gradually declines until midnight. In humans, cortisol increases immediately after the start of fasting (*87*). Studies have shown that fasting for five days increases cortisol levels and shifts the peak of cortisol secretion from the morning to the afternoon (*88*). Other studies have also noted significant improvements in plasma cortisol after fasting for 2.5 or 6 days (*89*). Consistent with these findings, significantly elevated morning changes in serum cortisol levels were observed after 4 days of early TRF (feeding from 8:00 AM to 2:00 PM) (*90*).

3.3.5. Leptin

Leptin levels significantly decrease after fasting. Leptin is an adipokine secreted by adipose tissue and plays an important role in the regulation of food intake and energy expenditure. After overnight fasting, levels of plasma leptin decreased by more than 54% (*91*). Mechanistic studies suggest that interactions between insulin and catecholamine levels may explain the reduction in leptin during fasting, with insulin secretion stimulating leptin production, whereas increased catecholamines and ketones can inhibit leptin production (*92*).

3.3.6. Growth hormone (GH) and insulin-like growth factor 1 (IGF-1)

Levels of human GH increased during fasting. GH can increase by a multiple of 5-fold after fasting for two days (*93*). A point worth noting is that a shorter period of fasting (one day) increased GH more than a longer period of fasting (two days) (*94*). Moreover, after continuous fasting for three days, the total GH level gradually decreased to the pre-fasting level (*95*). Changes in the response of GH to fasting can be attributed to a number of regulators, such as IGF-1 and IGF-1 binding protein (IGFBP-1). In the rapid response to acute fasting, the regulation of GH and IGF-1 displayed an opposite trend (*96*). In the early stage of fasting, IGF-1 is thought to be inhibited by an increase in the production of IGFBP-1. In fact, the biological activity of IGF-1 decreased after

40 hours of continuous fasting, which was paralleled by an increase in IGFBP-1. In addition, there was little change in total IGF-1 levels within 24 hours of fasting, but free IGF-1 levels were suppressed by up to 50%, demonstrating that circulating free IGF-1 rather than total IGF-1 has a greater effect on the production and action of GH during fasting (*96*).

3.3.7. Fibroblast growth factor 21 (FGF21)

FGF21, originally known as the hunger hormone, is a hormone-like FGF, and circulating FGF21 mainly originates from the liver. FGF21 is directly induced by peroxisomal proliferator-activated receptor alpha (PPARα) in the liver in response to fasting (*97*). Protein restriction for one day can increase blood levels of FGF21 (*98*). In addition, long-term protein restriction can lead to a sustained increase in FGF21 (*99*). FGF21 promotes gluconeogenesis and ketogenesis during prolonged fasting. FGF21 induces the expression of peroxisome proliferator-activated receptor-γ-coactivator-1α (PGC-1α), a transcriptional coactivator protein that interacts with several different DNA-binding proteins to regulate metabolism in response to changes in nutrient status. In the liver, fasting induced PGC-1α activation that was involved in gluconeogenesis, fatty acid oxidation, and ketogenic gene transcription (*97*). An important point worth noting is that FGF21 was unable to induce the expression of genes associated with the gluconeogenic pathway in mice lacking PGC-1α. In addition, FGF21 deficiency failed to fully induce PGC-1α expression in mice in response to prolonged fasting, and gluconeogenesis and ketogenesis were also found to be impaired. FGF21 induces several pancreatic lipases, and the induction of these lipases may contribute to the effect of FGF21 on increasing fatty acid oxidation in the liver. By increasing the expression of glucose transporter 3 (GLUT3) through an insulin-independent pathway, FGF21 can also increase glucose uptake by adipocytes (*100*). FGF21 increased the expression of thermogenic genes in white adipose tissue and decreased the expression of lipogenic genes in the liver (*101*). In addition, FGF21 helps to improve insulin sensitivity by increasing insulin-dependent glucose uptake in adipose tissue and reducing hepatic glucose production (*102*).

3.3.8. Sex hormones

The production and secretion of hormones is partly controlled by circadian rhythms, which are also influenced by the daily pattern of feeding and fasting (*103*). IF may affect the levels of sex hormones in the body by affecting the body's circadian rhythm. TRF can shorten the time window during the day when people eat to better match their circadian biology, which can have a beneficial effect on sex hormone levels. FGF21 increases during TRF and stimulates gonadotropin-releasing hormone (GnRH) neurons to secrete GnRH (*104*). For example, 8-hour restricted feeding has the potential to alleviate hyperandrogenemia by increasing levels of sex hormone-binding globulin (SHBG), which may improve the endocrine and metabolic status of women with anovulatory polycystic ovary syndrome (PCOS) (*105*).

4. Hepatic autophagy mediated by IF: Key events

A study noted the maximum number of autophagic vacuoles before feeding and the minimum number of autophagic vacuoles after feeding (*106,107*). Autophagy, which is influenced by a number of factors including circulating hormones, can be inhibited under energy-rich conditions and can be induced by starvation (*108*). The activation of autophagy in male rats exposed to ADF was studied by Donati *et al.* They assessed the rate of autophagic proteolysis in isolated rat hepatocytes and found that the rate of autophagy was higher in the fasting group than in the control group at all ages (*109*). Krustew *et al.* studied the effects of 1–8 days of fasting on the size and number of lysosomes in rat hepatocytes and noted a significant increase in the number of lysosomes, and particularly secondary lysosomes (*110*). After fasting for 12, 24, 48, and 72 hours, the cytoplasmic volume fraction of autophagic vacuoles in liver cells increased significantly compared to feeding for 3 hours. Moreover, serum insulin levels gradually decreased throughout the fasting period (*107*). Fasting may induce anti-stress mechanisms that influence cellular proliferation and energy metabolism and prevent inflammatory and oxidative stress (*10*). Fasting has also been proven to prevent liver ischemia-reperfusion injury in mice by inducing autophagy (*111*).

4.1. IF mediates autophagy in the liver *via* nutrient-sensing pathways

During fasting, a high AMP/ATP ratio, nutrient deprivation, and/or reduced levels of growth factors combine to activate autophagy in the liver (*112*). The key nutrient-sensing pathways involved in fasting-regulated autophagy include mTOR, AMPK, and SIRT1, where the activation of SIRT1 and AMPK is a positive regulator of autophagy while the activation of mTOR is a negative regulator (Figure 3). Moreover, PPARα and farnesoid X receptor (FXR) play a key integrative role in the induction of liver autophagy by IF.

4.1.1. AMPK

A cellular energy sensor, AMPK is a serine/threonine kinase consisting of a functional complex with a catalytic protein subunit (α) and two regulatory protein subunits (β and γ) (*113*). AMPKβ1 is essential for the maintenance of AMPK activity in tissues such as the liver. AMPK activation is triggered by a variety of metabolic

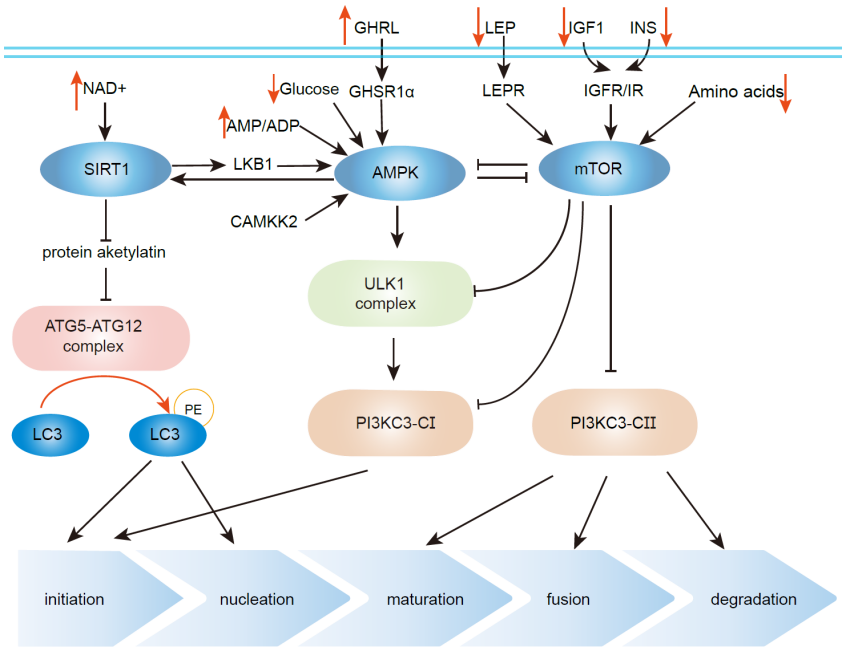

Figure 3. Intermittent fasting regulates autophagy in the liver. The key nutrient-sensing pathways involved include mTOR, AMPK and SIRT1, where the activation of SIRT1 and AMPK is a positive regulator of autophagy while the activation of mTOR is a negative regulator. Intermittent fasting may also affect hepatic autophagy through hormonal changes in the body. Abbreviations: AMPK, AMP-activated protein kinase; CaMKK2, calcium/calmodulin-dependent protein kinase 2; GHRL, growth hormone releasing peptide (ghrelin); GHSR1α, growth hormone secretagogue receptor subtype 1α; IGF1, insulin-like growth factor-1; INS, insulin; IGFR/IR, IGF-I receptor/ insulin receptor; LEP, leptin; LEPR, leptin receptor; LKB1, liver kinase B1; mTOR, mammalian target of rapamycin; PI3KC3-CI, class III phosphatidylinositol-3 kinase complex I; PI3KC3-CII, class III phosphatidylinositol-3 kinase complex II; SIRT1, silent mating-type information regulatory 2 homolog-1; ULK1, unc-51 like autophagy activating kinase 1.

stresses, including amino acid deprivation, hypoxia or hypoglycemia, extracellular matrix (ECM) depletion, physical exertion, and mitochondrial damage. AMP and adenosine diphosphate (ADP) are the primary signals for activation of AMPK. Under low energy conditions, AMP/ADP binds to the cystathionine-β-synthase domains (CBS domains) present in the γ subunit. This induces conformational changes in the catalytic domain of the subunit-α, which is then phosphorylated at threonine 172 by upstream kinases such as liver kinase B1 (LKB1), calcium/calmodulin-dependent protein kinase 2 (CaMKK2), and TGFβ-activated kinase 1 (TAK1) (*113*).

4.1.2. mTOR

Numerous studies have shown that mTORC1 is a key regulator of autophagy, regulating different steps in the autophagy process, such as nucleation, autophagic extension, autophagic maturation, and autophagic termination. Under nutrient-rich conditions, mTORC1 is recruited to the lysosomal surface by amino acid-regulated heterodimers of the Rag family of small GTPases (*114*). mTORC1 subsequently phosphorylates unc-51 like autophagy activating kinase 1 (ULK1) at Ser758 to inhibit its interaction with AMPK, thereby preventing autophagy. During fasting, however, AMPK inhibits mTORC1 and thus ULK1 phosphorylation at Ser758, allowing ULK1 to interact with AMPK. In turn, AMPK promotes the phosphorylation of ULK1 at Ser317 and Ser777 to activate it. Activated ULK1 is then recruited to the vacuolar protein sorting 34 (VPS34) complex. There, it phosphorylates Bcl-2-interacting protein (Beclin-1) at Ser15, which is essential for the initiation of the formation of the autophagosome in autophagy (*115*). Moreover, mTORC1 can indirectly regulate autophagy by repressing the transcription of genes required for lysosome biogenesis, such as transcription factor EB (TFEB) (*116*). As a key transcriptional regulator of lysosomal biogenesis and autophagy genes, TFEB upregulates a number of genes associated with autophagosome formation, those associated with fusion of autophagosomes with lysosomes, and genes required for lysosome biogenesis, such as *UVRAG*, *WIPI*, *MAPLC3B*, *SQSTM1*, *VPS11*, *VPS19*, and *ATG9B*. TFEB is phosphorylated at multiple sites, promoting its nuclear export and switching off the transcription of autophagy genes; this is how mTORC1 negatively regulates autophagy at the transcriptional level (*117*).

4.1.3. SIRT1

Sirtuins are nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylases whose activity is regulated by nutrient availability (*118*). Nicotinamide phosphoribosyltransferase (NAMPT) catalyses the conversion of nicotinamide (NAM) to NAD, activating SIRT-1 through the elevation of cellular NAD and the reduction of NAM. Fasting enhances SIRT-1 activity

by increasing the levels of NAD and reducing the levels of NAM and NADH, thereby increasing SIRT-1 activity. The activity of AMPK is linked to SIRT1 since SIRT1 deacetylates and activates LKB1, the upstream factor of AMPK, which ultimately activates AMPK (*119*). A recent study has shown that SIRT1 affects the formation of autophagosomes through its interactions with ATG5 and ATG7 and through the lipidation of the microtubule-associated protein LC3 (*120*). SIRT1 is known to be activated in response to nuclear perilipin5 (PLIN5) under fasting conditions; in male mouse hepatocytes, SIRT1 promotes the transcriptional network (PGC1α/PPARα), induces autophagy, and alleviates the inflammatory response of the liver (*121*). SIRT3 is located in mitochondria, which are particularly critical in the liver, and it regulates fatty acid oxidation during fasting by deactivating mitochondrial enzymes (*122*). SIRT1 is found to deacetylate forkhead box O3a (FoxO3a), thereby increasing transcription of the *BNIP3* gene and initiating autophagy through Beclin-1 (*123*). Glutamine synthase is a target of FoxO3 and when FoxO3 is activated, the levels of cellular glutamine increase and thus inhibit mTORC1 signaling activity and reduce its negative modulation of autophagy (*123*). In the context of fasting, nuclear FoxOs transactivate genes that determine the formation of autophagosomes and their fusion with lysosomes, and cell membrane FoxO proteins can induce autophagy through direct interaction with autophagy proteins. In addition, cytosolic FoxO1 has been reported to induce autophagy through a transcriptionally independent pathway (*124*).

4.1.4. PPARα and FXR

The nuclear receptor PPARα is activated during fasting while the nuclear receptor FXR is activated during feeding; both are involved in regulating hepatic autophagy. Activated by fatty acids during fasting, PPARα is a nuclear receptor that plays a critical role in lipid metabolism and glucose homeostasis and that can activate or repress genes involved in inflammation, adipogenesis, and energy homeostasis (*125*). FXR and PPARα counter-regulate autophagy to maintain energy homeostasis. FXR has four distinct isoforms (FXRα1-4) that originate from differential promoter usage and alternative splicing of the same gene (*126*). These isoforms are species-specific. They are differentially expressed in different organs depending on the bile acid composition. In the human liver, FXRα1/2 predominates, with FXRα2 being the most transcriptionally active form (*127*). Feeding and fasting cycles dynamically regulate FXR splicing in mice. Fasting can increase the FXRα2 isoform in the liver, facilitating fatty acid β-oxidation, decreased hepatic lipogenesis, enhanced glycerate metabolism, and ammonia clearance (*128*). FXR and the fasting transcriptional activator CREB work in a coordinated manner to regulate the hepatic autophagy gene network (*128*). G protein bile acid receptor 1 (GPBAR1) is a receptor for secondary bile acids. It is mainly expressed in non-parenchymal cells, including macrophages and sinusoidal cells, and in biliary cells. During fasting, GPBAR1 acts genomically *via* the cAMP-CREB pathway and it functions as a positive modulator of liver autophagy; its ligands reverse the repressive effects of FXR on liver autophagy flux during feeding (*129*).

4.2. IF mediates liver autophagy *via* an endocrine pathway

Somatically, hepatic autophagy fluctuates in response to hormonal signals, negatively or positively regulating feeding and fasting autophagy. Activation of the hypothalamic-pituitary-adrenal (HPA) axis is essential for the metabolic adaptation that occurs in response to fasting. Chen *et al.* found that fasting in mice activates autophagy in the liver in parallel with activation of hypothalamic agouti-related protein (AgRP) neurons (*130*). Adrenaline secretion promotes hepatic autophagy in response to starvation (*37*). In contrast, during feeding, hepatic autophagy is inhibited in favor of anabolism (*46*).

4.2.1. Glucagon

Increased circulating glucagon during fasting, together with decreased insulin and amino acids, activates hepatic autophagy. Glucagon and insulin are important regulators of autophagy. The role of glucagon in the induction of hepatic autophagy has been well established for over 60 years (*4*). Subsequent *in vivo* studies further confirmed the role of glucagon in the induction of hepatic autophagy. The glucagon receptor (G protein-coupled receptor) on hepatocytes recognizes glucagon and subsequently inhibits salt-inducible kinase (SIK) *via* the cAMP/PKA pathway. PKA then phosphorylates CREB and SIK dephosphorylates CREB-regulated transcription co-activator (CRTC). Phosphorylated CREB and CRTC act synergistically to upregulate the expression of TFEB, which in turn regulates the expression of autophagy protein genes (*131*). Glucagon may also induce autophagy by increasing the size and number of autophagic vesicles (*132*). During fasting, glucagon stimulates hepatic autophagy, providing substrates for gluconeogenesis/ketogenesis to maintain systemic glucose homeostasis. Glycogen particles are selectively encapsulated by autophagosomes for degradation and metabolism in a process known as "glycophagy". Glucagon induces calcium signaling with subsequent phosphorylation of O-linked β-N-acetylglucosamine (O-GlcNAc) transferase (OGT) by CaMKK2 and it further promotes O-GlcNAc modification and activation of ULK proteins by enhancing AMPK-dependent phosphorylation to promote autophagy (*133*).

4.2.2. FGF21

Liver-derived FGF21 is highly inducible by fasting and is linked to many critical metabolic pathways that are altered under nutrient stress. FGF21 can promote autophagy in liver cells through the AMPK/mTOR signaling pathway, accelerating regeneration of the damaged liver (*134*). Mechanistically, this is closely related to increased FGF21 expression, enhanced AMPKα phosphorylation, and stimulation of autophagy. FGF21 can also induce autophagy by promoting SIRT1 expression (*135*). Epigenetic studies have revealed that the FGF21-JMJD3 signaling pathway links nutrient deprivation to hepatic autophagy (*136*). The impairment of autophagy in *FGF21*-KO mice strongly suggests that physiological levels of hepatic FGF21 during fasting may activate autophagy in an autocrine/paracrine manner.

5. Importance of IF-mediated liver autophagy

By activating hepatic autophagy, IF plays a role in both the physiology and pathology of the liver. The activation of hepatic autophagy mediated by IF may help to maintain the energy balance, improve mitochondrial function, control the quality of the liver, maintain cell homeostasis, and protect cells from harmful factors. IF is a promising intervention for improving liver function (Figure 4).

5.1. IF activates liver autophagy to recycle nutrients

Autophagy is an important regulator of metabolism at the cellular and organismal levels. During fasting, autophagy provides an important source of internal nutrients to maintain cell integrity and survival (Figure 5). Autophagy, which has been shown to be critical for survival in adult mice, is required to maintain circulating nutrients during fasting. Autophagosome membranes engulf glycogen, LDs, or proteins, which are then

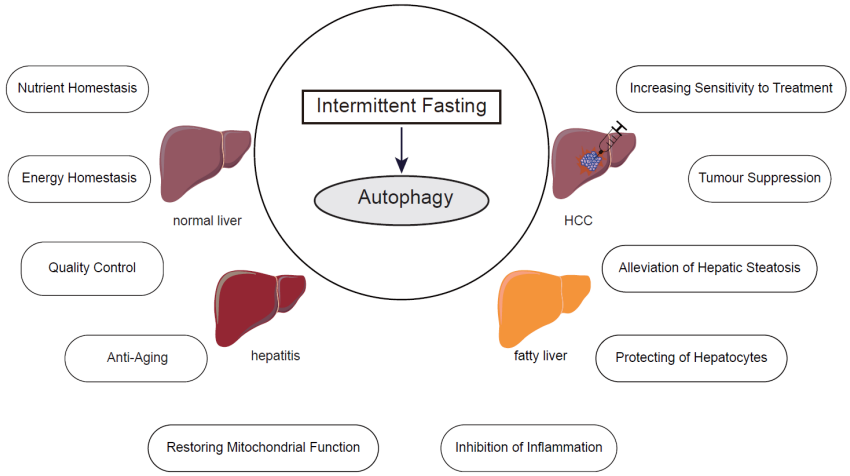

Figure 4. Importance of intermittent fasting in hepatic autophagy. By activating hepatic autophagy, intermittent fasting plays a role in liver physiology and pathology. Abbreviations: HCC, hepatocellular carcinoma.

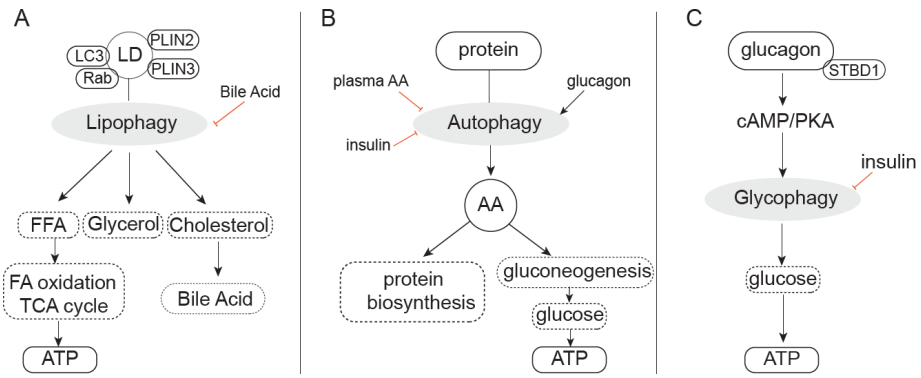

Figure 5. Intermittent fasting activates hepatic autophagy to maintain homeostasis of nutrient and energy metabolism. (A) LDs are selectively removed by lipophagy to produce FFAs, which are continuously released to undergo mitochondrial β-oxidation, supplying ATP. **(B)** Under the influence of hormonal and nutrient signals, hepatic autophagy is activated to provide amino acids to the body through protein catabolism and to maintain glucose homeostasis and energy balance through gluconeogenesis. **(C)** Glycophagy plays an important role in maintaining blood glucose and energy homeostasis. During glycophagy, STBD1 anchors glycogen to the lysosome and ultimately produces glucose through the cAMP/PKA signaling pathway to maintain glucose and energy homeostasis. Abbreviations: AA, amino acid; FA, fatty acid; FFA, free fatty acid; LC3, light chain 3; LD, lipid droplet; PLIN2, perilipin 2; PLIN3, perilipin 3; STBD1, starch-binding domain-containing protein 1; TCA cycle, tricarboxylic acid cycle.

degraded in lysosomes to maintain nutrient cycling and key metabolic pathways. Glycophagy and protein autophagy promote nutrient mobilization during early starvation. After 8 hours of fasting, however, there is a gradual switch from these autophagy substrates to LD degradation. This sequence of selective degradation of autophagy substrates during starvation is somewhat consistent with the progression of conventional metabolic degradation processes in the liver in response to prolonged nutrient deprivation: first glycogenolysis and gluconeogenesis and then mitochondrial β-oxidation, ketogenesis, and gluconeogenesis. Glucose homeostasis during fasting, maintained by autophagy induced by fasting, is an important contribution of liver-specific metabolism to energy homeostasis. In addition, autophagy indirectly affects the metabolic activity of cells by regulating the degradation of intracellular organelles.

5.1.1. Maintenance of balanced glucose metabolism in the liver

Autophagy in the liver plays an important role in blood glucose homeostasis. In addition to the classic process of glycogen degradation, glycophagy plays an important role in maintaining energy homeostasis in the liver. During glycophagy, starch-binding domain-containing protein 1 (STBD1) is thought to anchor glycogen to lysosomes. STBD1 contains a C-terminal CBM20 glycan-binding domain that binds glycogen and an ATG8 interaction motif (AIM) that can bind GABA type A receptor-associated protein-like 1 (GABARAPL1, an Atg8 family member) (*137*). By interacting with GABARAPL1, STBD1 recruits glycogen to the forming phagosome. Following autophagosome-lysosome fusion, acid α-glucosidase mediates lysosomal glycogen degradation. The cAMP/PKA signaling pathway has been found to play a key role in glycophagy. In contrast, insulin is involved in the regulation of glycophagy by indirectly activating mTOR and inhibiting autophagy through the I-PI3-kinase (PI3K-Akt)/protein kinase B (PKB)-mTOR signaling pathway (*138*). By inhibiting protein phosphatase 2a (PP2A), activated mTOR can inhibit glycophagy.

5.1.2. Maintenance of homeostasis in amino acid and protein metabolism

The liver is the main source of serum protein and provides the body with amino acids for energy in response to prolonged fasting by catabolizing protein. Under nutrient-rich conditions, constitutive autophagy degrades about 1.5% of total liver protein per hour, but during fasting, the rate of induced autophagy increases to three times the basic rate (*139*). After one day of fasting, there was an increase in amino acid levels in liver tissue and circulation, and blood glucose levels were within the normal range. Amino acids released by autophagy may be responsible for blood glucose levels (*77*). Further analysis showed that 11 of 18 amino acids (valine, leucine, isoleucine, serine, threonine, methionine, asparagine, phenylalanine, tyrosine, lysine, and arginine) increased in mice fasting for one day (*27*). The glycogenic amino acids released by hepatic autophagy are partially converted to glucose by gluconeogenesis and secreted into the circulation, while the amino acids generated by hepatic autophagy are also used for protein biosynthesis (*77*). After two days of fasting, approximately 40% of total liver protein was catabolized by activated autophagy (*140*). Both hormones and plasma amino acid levels are involved in the regulation of protein autophagy in the liver. Compared to the influence of changes in plasma amino acid levels, insulin and glucagon may be more important in regulating protein autophagy. Insulin has been reported to inhibit liver autophagy by activating the Akt/mTOR pathway (*27*). Studies have found that inhibition of serum insulin in rats increases protein autophagy by approximately 70%. Glucagon remained at high and stable levels during 24-hour fasting. Glucagon not only induces the formation of autophagosomes but also upregulates acid phosphatase and cathepsin D in lysosomes and increases lysosomal susceptibility in the liver (*141*). Plasma amino acid levels could be a regulator of hepatic autophagy independent of hormonal influence.

Hepatic autophagy breaks down not only protein aggregates but also metabolic enzymes involved in lipid, carbohydrate, and amino acid metabolism. During nutrient deprivation, CMA removes glycolytic enzymes to reduce the use of hepatic glucose, allowing it to be recruited to other organs (*29*). For example, the degradation of hexokinase 2 (HK2) may have a direct effect on glycolysis in the liver (*142*). Protein is degraded by autophagy to maintain the intracellular amino acid pool and plasma amino acid levels. In addition to providing metabolic substrates, protein autophagy may also contribute to protein quality control by removing misfolded and aggregated proteins and alleviating protein toxicity caused by abnormal protein levels. Degradation of p62/SQSTM1, a receptor for selective autophagy, is not only part of the regular autophagy process but is also key to preventing inappropriate activation of Nrf2, which would otherwise be the underlying mechanism for severe liver pathology (*143*).

5.1.3. Lipophagy promotes lipid metabolism in the liver

The liver is crucial to the processing and storage of lipids, and hepatic lipid homeostasis is maintained by coordinating lipid uptake, de novo synthesis, storage, and catabolism. The uptake of LDs by autophagy is an alternative pathway to mobilize lipid storage and intracellular LD degradation, a process known as lipophagy that is a subtype of macroautophagy. Portions

of cytosolic LDs are engulfed by lipo-autophagosomes and transported to lysosomes, where triacylglycerols and other lipids are subjected to acid lipolysis by lysosomal acid lipase. Lipophagy is essential for the maintenance of lipid homeostasis (*42*). The lipid content of hepatocyte double-membrane autophagosomes, a hallmark of macroautophagy, was evident in mice fasted for as short as 6 hours (*42*). In addition, inhibition of autophagy increased triglyceride storage in LDs both *in vivo* and *in vitro*. CMA and lipophagy play a synergistic role in the clearance of LDs. CMA-mediated PLIN degradation appears to be a key event in the initiation of lipophagy. CMA targets and degrades the LD-associated proteins PLIN2 and PLIN3, which increase during fasting. The degradation of PLIN proteins appears to be essential for promoting LD degradation by allowing the entry of adipose triglyceride lipase (ATGL) and autophagy proteins to the LD surface. Moreover, inhibition of the CMA process leads to a reduction in both lipase-mediated lipolysis and lipophagy. As the major Rab protein on the surface of LDs, Rab7 helps to regulate lysosome-autophagosome interaction. During fasting, the Rab7 GTP enzyme on the surface of LDs is activated and promotes lipophagy. Rab10, another Rab family protein, co-localizes with autophagy membrane markers (such as LC3 and Atg16) and is involved in lipid degradation during fasting. The results of LD proteomic screening suggest that several other Rab proteins, such as Rab32, Rab18, and Rab25, may also play an important role in lipophagy (*42*).

5.2. Potential impact of IF-mediated liver autophagy on liver pathophysiology

Besides regulating energy and nutrient homeostasis in response to fasting, IF-mediated hepatic autophagy is essential for maintaining organelle homeostasis and quality control in hepatocytes. Autophagy plays a critical role in cellular quality control by regulating the regeneration of intracellular organelles. Misfolded proteins can be toxic to cells, a phenomenon known as proteotoxicity. Autophagy can prevent the degradation of normal long-lived proteins and misfolded proteins, so both correctly folded and misfolded proteins increase significantly in cells lacking autophagy. In addition, many target molecules are overproduced as a result of sustained Nrf2 activation (*56*). Proteomic analysis of autophagy-deficient hepatocytes revealed an increase in the total amount of protein without specific changes in protein composition. Excess proteins, even when properly folded, can be detrimental to cells and this adverse effect can manifest in hepatocyte hypertrophy, hepatomegaly, and liver injury.

5.2.1. Restoring mitochondrial function

Mitophagy is an evolutionarily conserved degradation mechanism responsible for the selective removal of damaged or excess mitochondria to maintain liver function and protect tissues from damage (*144*). Conversely, dysregulation of mitophagy has been shown to contribute to the development of liver diseases such as alcoholic liver disease (ALD), NAFLD, viral hepatitis, drug-induced liver injury (DILI), liver fibrosis, and HCC, suggesting that it plays an important role in maintaining liver homeostasis. After alcohol ingestion, autophagy decreases in hepatocytes. This may be due to reduced intracellular AMPK and altered vesicle transport in hepatocytes (*145*). Alcohol can also damage mitochondria, sometimes by depolarizing the inner mitochondrial membrane and changing mitochondrial permeability (*146*). IF may prevent alcohol-induced acute liver injury by activating AMPK activity, restoring mitochondrial function, and stimulating autophagy, offering a potential therapeutic benefit to patients with ALD.

5.2.2. Alleviation of hepatic steatosis

In mice fed a high-fat diet, TRF reversed the expression of genes involved in fatty acid metabolism, β-oxidation, and antioxidant defense in the liver (*147*). TRF also enhanced the circadian rhythm of expression of a number of different genes in liver cells, including genes encoding PER2, BMAL1, and CRY1, and it prevented fat accumulation in the livers of mice fed a high-fat diet. Tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and interleukin 1β (IL-1β) were found to decrease in the livers of mice treated with TRF. Moreover, metabolomic analysis revealed that changes in liver metabolites (oleates, palmitates and palmitoleates), bioenergy pathway molecules (citrate, glucose-6-phosphate, and phthalates) and an antioxidant (glutathione) induced by a high-fat diet were reversed by TRF (*38*). Mounting evidence has revealed that autophagy is impaired in NAFLD (*148*). Upregulation of autophagy transcription factors such as FoxO1 and TFEB can prevent liver steatosis and reduce apoptosis. By upregulating liver-specific *ATG7* expression in ob/ob mice (genetic models of murine obesity), liver autophagy can help to alleviate metabolic stress and reduce hepatic steatosis (*148*). Due to increased autophagy, ADF can help to ameliorate elevated serum lactate dehydrogenase (LDH) and liver histological changes in the mouse model of NASH induced with a high-fat-fructose (HFF) diet (*149*). A study has reported that IF reduced lipid accumulation, activated AMPK/ULK1 signaling, inhibited mTOR phosphorylation, and prevented NAFLD progression in the liver of mice with diet-induced obesity (DIO) *via* autophagy (*150,151*). In short, findings suggested that IF may play an active role in liver steatosis.

5.2.3. Inhibition of liver inflammation

A recent study has shown that fasting can reduce the number of circulating inflammatory monocytes and inhibit their pro-inflammatory activity by activating liver AMPK and inhibiting chemokine ligand 2 (CCL2) production (*152*). SIRT1 is known to be activated in response to nuclear PLIN5 under fasting conditions. In male mouse liver cells, it can promote transcription of PGC1α/PPARα, induce autophagy, and alleviate liver inflammation (*121*). A study has shown that FGF-21 supplementation can effectively increase the expression of LC3-II and Beclin-1, a key molecule in autophagy, while reducing liver toxicity in wild-type mice exposed to carbon tetrachloride (CCl4) (*135*). FGF21 significantly reduced levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), IL-6, and TNF-α in acute liver injury.

5.2.4. Protecting hepatocytes

In models of liver ischemia-reperfusion injury, IF may have a beneficial protective effect on hepatocytes *via* autophagy mediated by the SIRT1/FoxO3a pathway (*153*). Moreover, in models of hepatic ischemia-reperfusion injury, IF may also protect hepatocytes from damage *via* the AMPK/mTOR autophagy pathway (*154*). In addition, the FoxO-autophagy axis has been identified as playing a cytoprotective role in both alcohol-induced hepatotoxicity and hepatic ischemia-reperfusion injury (*155,156*).

5.2.5. Tumor suppression

Mitochondrial dysfunction, oxidative stress, and DNA damage, all of which are key factors in tumorigenesis, are present in autophagy-deficient hepatocytes. Autophagy has been implicated as a tumor suppressor, and impaired autophagy may promote tumorigenesis in the liver. A study has shown that the AMPK/mTOR pathway is involved in the metabolism and tumorigenesis of HCC (*157*). Activation of AMPK is related to inhibition of HCC cell migration and invasion. By activating the transcription factor CCAAT/enhancer binding protein δ (CEBPD) and increasing LC3B expression, AMPK can induce autophagy and apoptosis in HCC (*158*). Conversely, loss of AMPK in HCC cells can promote cell progression, survival, migration, and invasion through various oncogenic molecules and pathways (*159*). A study has reported that fasting can promote autophagy and p62 degradation, increase AMPKα phosphorylation, upregulate FGF21 expression, downregulate the expression of aging-related molecules, and prevent the development of steatosis-associated liver tumors in hepatitis C virus core gene transgenic (HCVcpTg) mice (*160*).

5.2.6. Improving the sensitivity of tumor therapy

IF has also been proven to improve the sensitivity of radiotherapy for liver cancer. In liver cancer cells, mTORC1 is activated during fasting, thereby increasing their sensitivity to radiation (*161*). Moreover, sorafenib resistance of liver cancer cells is associated with a low level of activity of AMPK and CCAAT/CEBPD and insufficient activation of autophagy (*162*). Fasting is beneficial in improving the efficacy of sorafenib and has a synergistic effect in sensitizing sorafenib-resistant HCC (*163,164*).

6. Conclusion

IF is emerging as a simple but attractive strategy to combat human diseases, including aging, cancer, neurodegenerative diseases, and metabolic disorders. Fasting induces a number of adaptive changes, such as lowering the basal metabolic rate, inducing lipolysis and ketogenesis, regulating hormone levels, and reducing oxidative stress and inflammation. Numerous preclinical and clinical studies have shown that fasting plays a positive role in preventing a wide range of diseases. In particular, preclinical and clinical studies have demonstrated the clinical benefits of this dietary strategy in liver metabolic disorders (*165*).

IF regulates hepatic autophagy primarily *via* the nutritional and hormonal pathways. The regulation of liver autophagy by IF is crucial for liver physiology and pathology. The activation of liver autophagy mediated by IF can maintain the energy balance, improve mitochondrial function, control liver quality, maintain cell homeostasis, and protect cells from harmful factors. In addition, IF-mediated liver autophagy may also help to alleviate liver metabolic diseases, ameliorate liver inflammation, inhibit the development of liver cancer to some extent, and improve efficacy and reduce tumor resistance in combination with chemotherapy/radiotherapy.

IF is a promising intervention to improve liver function. However, research on its effectiveness and safety in people with liver disease is still limited. To determine whether IF can prevent liver diseases or improve their outcomes, randomized controlled trials need to be conducted in people at high risk or in the early stages of liver diseases. Moreover, the mechanisms by which IF works involve multiple pathways and are not well understood. Currently, there are technical barriers to measuring autophagic flux in the human body. As a result, the effects and mechanisms of IF on autophagy in the liver are not well understood at a systemic level. Further research also needs to be conducted to determine whether there is a link between the benefits of IF and refeeding. The safety of IF protocols in clinical practice is a major concern. At present, one of the main problems with the use of IF in clinical practice is the lack of guidelines. Wherever possible, guidance should be provided on how to select an IF regimen that is more appropriate for a particular goal. This should include the

types of foods that should be consumed, how physical activity should be performed, and how long such a regimen should last. The efficacy and safety of different IF protocols in different populations also need to be continually evaluated. More attention should be paid to advances in the research and use of IF in the near future.

Funding: This work was supported by a grant from the National Natural Science Foundation of China (No. 81960446) and the 2023 Foreign Experts Program in Hainan Province (SQ2023WGZJ0002).

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received May 25, 2023; Revised July 23, 2023; Accepted August 28, 2023.

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Released online in J-STAGE as advance publication September 1, 2023.

Review

DOI: 10.5582/bst.2023.01221

A circadian rhythm-restricted diet regulates autophagy to improve cognitive function and prolong lifespan

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SUMMARY Diet and circadian rhythms have been found to have a profound impact on health, disease, and aging. Skipping breakfast, eating late, and overeating have adverse effects on the body's metabolism and increase the risk of cardiovascular and metabolic diseases. Disturbance of circadian rhythms has been associated with increased risk of atherosclerosis, Alzheimer's disease, Parkinson's disease, and other diseases. Abnormal deposition of amyloid β (Aβ) and tau proteins in the brain and impaired synaptic function are linked to cognitive dysfunction. A restrictive diet following the circadian rhythm can affect the metabolism of lipids, glucose, and amino acids such as branched chain amino acids and cysteine. These metabolic changes contribute to autophagy through molecular mechanisms such as adenosine monophosphate-activated protein kinase (AMPK), rapamycin (mTOR), D-β-hydroxybutyrate (D-BHB), and neuropeptide Y (NPY). Autophagy, in turn, promotes the removal of abnormally deposited proteins and damaged organelles and improves cognitive function, ultimately prolonging lifespan. In addition, a diet restricted to the circadian rhythm induces increased expression of brain-derived neurotrophic factor (BDNF) in the forebrain region, regulating autophagy and increasing synaptic plasticity, thus enhancing cognitive function. Consequently, circadian rhythm-restricted diets could serve as a promising non-pharmacological treatment for preventing and improving cognitive dysfunction and prolonging lifespan.

Keywords biological clock, intermittent fasting, metabolism, quality control, protein aggregation, sleep

1. Introduction

Cognitive impairment is a syndrome. Studies show that mild cognitive impairment affects 3–19% of adults over 65, and its prevalence increases with age. More than half of patients progress to dementia within five years (*1,2*). Reduced synaptic function, extracellular aggregation of amyloid β (Aβ), and intracellular tau protein aggregation are closely associated with cognitive impairment (*1,2*). Patients with cognitive dysfunction experience a gradual decline in memory, disorientation, and an inability to lead a regular life (*3*). While there are no specific medications available, most current clinical treatments serve to delay cognitive decline (*3*). Dietary habits strongly correlate with metabolic diseases, immunity, cognitive performance (attention, memory, executive function, *etc.*), and longevity (*4-6*). Disturbance of eating habits is a leading factor in endangering human health. Overeating increases the risk of obesity and metabolic diseases, while long-term restrictive diets can cause malnutrition and compromise the organism's immune system (*6-9*). Maintaining a balance in one's eating habits is essential to ensuring optimal health. Moreover, a rational and healthy diet decreases the risk of death from cardiovascular and cerebrovascular diseases, tumors, neurodegenerative diseases, respiratory diseases, and all-cause mortality (*10*). An intermittent restrictive diet is a cost-effective and widely applicable non-pharmacological therapy. This approach can help develop new healthy eating habits. The literature suggests that an intermittent restrictive diet regimen improves 24-hour glucose levels, modifies lipid metabolism and circadian gene expression, up-regulates autophagy, and has anti-aging action (*11*).

During a restricted diet, autophagy is stimulated by changes in the metabolism of glucose, amino acids, and fatty acids (*12-15*). Autophagy generates new energy sources through lysosomal degradation that contribute to the replenishment and maintenance of the organism and protection against external stressors (*16-19*). By eliminating abnormally accumulating proteins (such as Aβ and tau proteins) and damaged organelles, autophagy can positively influence health, cognitive

function, and disease recovery (*20-23*). The circadian rhythm is a natural phenomenon that regulates the process of autophagy in organisms when subjected to restrictive diets. Research has shown that following circadian rhythms with intermittent fasting can increase the expression of autophagy-related 1 (*ATG1*) and autophagy-related 8a (*ATG8a*), promote autophagy, and ultimately prolong lifespan, whereas disregarding circadian rhythms can negate these benefits (*22*). Implementing restrictive diets according to circadian rhythms can optimize health and increase longevity (*24*). The current work reviews the potential mechanisms by which a diet restricted to the circadian rhythm may affect autophagy and improve cognitive function. The mechanisms involved are outlined in Figure 1.

2. A circadian rhythm-restricted diet

A circadian rhythm-restricted diet is defined as: restriction of food intake to a period of 4–12 hours from the beginning of breakfast to the end of the last meal of the day; fasting for more than 12 hours, with the fasting period coinciding with the circadian rhythm (*24-27*) (Figure 2). An article in Science by Francesco *et al.* classified fasting into four categories: caloric restriction, which entails consuming less than 15–40% of the usual daily intake; time-restricted feeding, in which food intake is limited to a specific 4–12 hour period; intermittent and periodic fasting, in which food intake is periodically reduced; and fasting-mimicking diets (FMD) (*25*). Current research on fasting has mainly focused on different forms of time-limited, intermittent, and periodic fasting, such as alternate-day fasting and the 5:2 diet (*28,29*). These types of fasting have been shown to provide significant health benefits by restricting either caloric intake or the timing of meals, which can enhance repair mechanisms and optimize cellular and organismal health. However, an important point worth noting is that skipping breakfast, having a late dinner, and fasting out of sync with the circadian rhythm can negatively impact the body to varying degrees. Breakfast is often considered the most important meal of the day (*30,31*). Studies suggest that consuming breakfast enhances cognitive abilities and academic performance in school-age children (*31,32*). However, skipping breakfast may increase the risk of atherosclerosis, cardiovascular disease, and mortality (*27,33*). Research also indicates that eating late at night increases the likelihood of obesity by inducing hunger and disrupting crucial pathways linked to lipid metabolism, such as p38 mitogen-activated protein kinase (MAPK) signaling, transforming growth factor-β (TGF-β) signaling, regulation of receptor tyrosine kinases, and autophagy. Consequently, this leads to lower lipolysis and elevated lipogenesis (*34*).

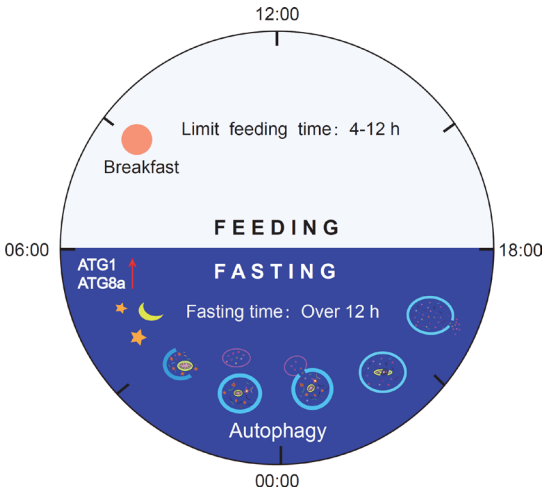

Figure 1. Diagram of a circadian rhythm-restricted dietary intervention. FEEDING: Breakfast within 2 hours of waking up; the day's dietary intake is completed within 4–12 hours after breakfast; FASTING: Fasting time > 12 hours, feeding and fasting with changes in one's circadian rhythm, and ensuring sleep at night to promote enhanced autophagy gene expression.

Circadian rhythms are an evolutionarily conserved timing system that coordinate behavioral control, hormonal fluctuations, physiological homeostasis, metabolism, and energy metabolism across the entire organism. This system includes sleep-wake cycles, feeding-fasting cycles, and activity-rest cycles. Long-term irregular circadian rhythms can lead to organismal dysfunction, resulting in an increased risk of developing many diseases (*35-37*). A clinical report examining the health effects of Ramadan fasting in Saudi Arabia indicated that evening hypercortisolism is associated with fasting during Ramadan and that disturbances to circadian rhythms result in reductions in liver enzymes, total bilirubin, total protein, and albumin as well as altered adipokine patterns, thereby increasing cardiometabolic risk (*38*). A clinical trial on a rhythmic time-restricted eating intervention in patients suffering from metabolic syndrome confirmed that limiting daily eating to 10 hours decreases body weight, blood pressure, and atherogenic lipid levels (*39*). In conclusion, a circadian rhythm of eating and fasting benefits an organism's health by inducing a "fasting physiology" during the fasting period. This process promotes repair, improved metabolism, and rejuvenation, consequently increasing resilience to the effects of undesirable factors. Conversely, an irregular eating pattern appears to be harmful to achieving a healthy metabolism (*24,40*).

3. Autophagy and a circadian rhythm-restricted diet

As a degradative system, autophagy is a crucial protective process of the cell that transports substances from the cytoplasm into lysosomes for degradation. It produces new building blocks and energy for cell renewal and homeostasis (*21*). There are three primary forms of autophagy: macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy

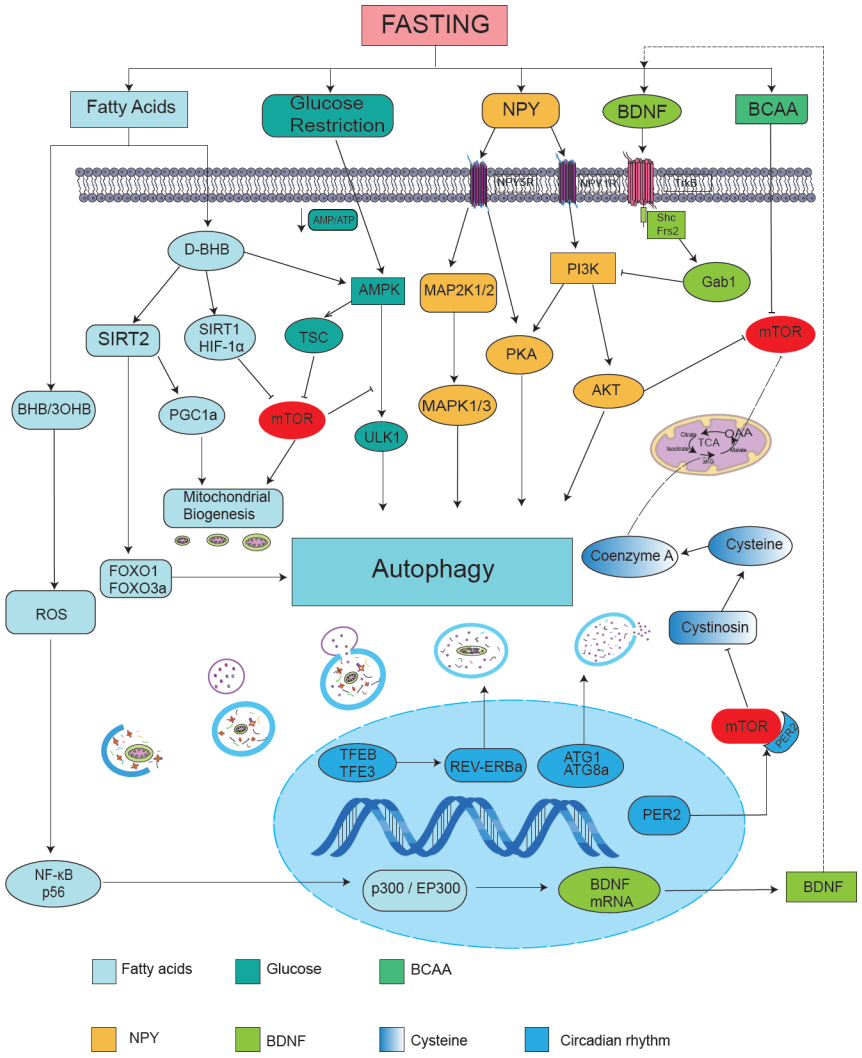

Figure 2. Diagram of the mechanistic role of fasting-mediated autophagy. Abbreviations: AMPK, adenosine monophosphate-activated protein kinase; ATP, adenosine triphosphate; BDNF, brain-derived neurotrophic factor; BHB/3OHB, β-hydroxybutyrate; FOXO3a, forkhead box O3; Gab1, Grb2-associated binder 1; HIF-1α, hypoxia-inducible factor-1α; MAPK, mitogen-activated protein kinase; NF-κB, Nuclear factor-κB; NPY, Neuropeptide Y; PGC1α, peroxisome proliferator-activated receptor γ coactivator 1α; PI3K, Phosphatidylinositol 3-kinase; ROS, reactive oxygen species; SIRT, sirtuin; mTOR, rapamycin; TCA, tricarboxylic acid cycle; TFs, transcription factors; TRKB, tropomyosin receptor kinase B ; TSC, tuberous sclerosis complex ; ULK1, uncoordinated 51-like kinase 1.

involves the formation of an autophagosome, which isolates a segment of cytoplasm and fuses with the lysosome for subsequent degradation of its contents. Microautophagy involves direct phagocytosis of a small portion of cytoplasm by the lysosome. Chaperone-mediated autophagy (CMA) is a degradation process where substrate proteins containing KFERQ-like pentapeptide sequences are recognized by heat shock cognate protein 70 (Hsc70) and auxiliary chaperone proteins in the cytoplasm. The proteins are then transported and bound to lysosomal lysosome-associated membrane protein-2 isoform A (Lamp-2A), translocated into the lysosomal lumen, and finally degraded (*21,41*). CMA degrades damaged or oxidized proteins during starvation to provide amino acids and aid in maintaining cellular quality control (*41*). Most current studies have focused on macroautophagy. Research has shown that during periods of starvation, the organism rapidly and vigorously induces the autophagy process in multiple tissues, leading to the degradation of its own components and providing new energy to sustain survival (*42-44*). Autophagy plays a critical role in intracellular quality control, including that of mitochondria and the endoplasmic reticulum, as well as in maintaining cellular homeostasis by degrading select proteins, organelles, and bacteria (*21,45*). A circadian rhythm-restricted diet can contribute to the induction of autophagy, thereby alleviating cognitive deficits by regulating organelle quality and degrading abnormally deposited proteins.

Mounting evidence suggests that autophagy is influenced by circadian rhythms. Transcription factor EB (TFEB) and transcription factor E3 (TFE3) serve as significant transcriptional regulators of lysosomal biogenesis and autophagy. Both TFEB and TFE3 experience circadian stimulation throughout the day.

During times of nutrient deprivation (in the light phase), these proteins translocate to the nucleus and bind to promoters at the E-Boxes/CLEAR locus, thus regulating the expression of autophagy-related genes (*46*). As a central inhibitory component of the cellular autonomic clock, Rev-erbα is also involved in regulating autophagy *via* its inhibitory action. Research has revealed that the dynamic balance between TFEB/TFE3 and REV-ERBα is responsible for regulating autophagy (*46-48*). The autophagy-related genes *ATG1* and *ATG8a* are regulated by circadian rhythms, and their expression increases at night during fasting. This increases the level of autophagic activity and can prolong lifespan. Knockdown of the genes *ATG1* and *ATG8a* counteracts this benefit of a prolonged lifespan (*22,49*). Research has demonstrated that the core clock protein, period 2, can suppress rapamycin (mTOR) complex activity through tuberous sclerosis complex 1 (Tsc1), which ultimately leads to the stimulation of autophagy (*50*). CMA interacts with the biological clock, facilitating the controlled degradation of clock mechanism proteins (selective temporal phagocytosis) and circadian reshaping of portions of the cellular proteome. However, the absence of a circadian clock eliminates the rhythmicity of CMA, resulting in notable alterations in the proteomes of CMA-dependent cells (*51*). In summary, autophagy is closely related to restrictive diets and circadian rhythms. Under a restrictive diet, starvation induces cell autophagy to promote the degradation of its own components and provide new energy. In addition, circadian rhythms regulate the expression of autophagy-related genes to control autophagy rhythms, as shown in Figure 1.

4. Mechanisms of autophagy activated by a circadian rhythm-restricted diet

Targeting autophagy could be an effective intervention for improving cognition, slowing aging, and extending lifespan (*52-54*). According to one study, a restricted diet induces astrocyte autophagy, reducing amyloid buildup and memory deficits in mice with Alzheimer's disease (AD) (*53*). A limited diet can affect the metabolism of lipids, glucose, and amino acids (including branched-chain amino acids (BCAAs) and cysteine) and facilitate autophagy through molecular mechanisms such as adenosine monophosphate-activated protein kinase (AMPK), D-β-hydroxybutyrate (D-BHB), mTOR, and neuropeptide Y (NPY). These mechanisms function to eliminate abnormally accumulating proteins and damaged organelles, resulting in enhanced cognitive performance. However, a restricted diet results in augmented brain-derived neurotrophic factor (BDNF) expression in the forebrain area and hinders autophagy by impacting the PI3K/AKT pathway. As a consequence, this results in enhanced synaptic plasticity and improved cognitive functionality.

4.1. AMPK / mTOR

During a restricted diet, autophagy is activated by AMPK and inhibited mTOR activity, leading to the degradation of misfolded proteins and damaged cellular organelles, which in turn reduces cognitive dysfunction. AMPK serves as an objective sensor of cellular energy levels and is activated by a decrease in the AMP:ATP ratio in response to energy depletion. The activated AMPK in turn activates the uncoordinated 51-like kinase 1 (ULK1) complex, initiating autophagy as well as phagocytosis of damaged organelles and protein degradation *via* lysosomal fusions (*55,56*). mTOR serves as a regulator of cell growth by integrating signals from growth factors and nutrients. It can detect and integrate various signals, including amino acids, glucose, growth factors, and energy, while participating in the regulation of cellular metabolism, mitochondrial function, and cellular growth through the autophagy pathway (*57*). Studies have indicated that during glucose starvation, AMPK activates UIK1 by phosphorylating Ser317 and Ser777 to encourage autophagy. In addition, AMPK inhibits the mTOR complex by phosphorylating tuberous sclerosis complex 2 (TSC2) and Raptor. In nutrient-limited settings, mTOR complex activity is curtailed, resulting in decreased translation, a reduced growth rate, and enhanced autophagy. However, minimal mTOR complex activity is critical to encouraging lysosomal biogenesis, which is necessary to sustain autophagic degradation required for survival. Dietary restrictions might impede mTOR complex activity and boost autophagy, which sustains basic survival by recycling nutrients from organelles and cytoplasm to provide internal nutrient storage (*58*). Fasting has been shown to boost AMPK activity in agouti-related peptide (AgRP) neurons, inducing spinogenesis and synaptic plasticity and ultimately enhancing cognitive performance (*59*). A study on AD found that downregulation of the mTOR signaling pathway can activate autophagy. Autophagy degrades misfolded proteins and damaged cell organelles, which inhibits the progression of AD and ameliorates cognitive dysfunction (*60*). AMPK and mTOR complex function as controllers of energy and nutrition. Their interaction regulates autophagy, which contributes to enhanced cognitive function (Figure 1).

4.2. Ketone bodies

Ketone bodies can increase autophagy, facilitate the expression of BNDF, increase neuronal synaptogenesis, and enhance cognitive brain function while serving as an alternative source of energy. They act as a crucial metabolic fuel option and primary energy source for many tissues, including the brain, during restricted energy intake. They participate in cellular metabolism, homeostasis, and signaling in various physiological and pathological conditions (*13,61*). When the diet is

restricted, the liver transforms fatty acids into ketone bodies. The brain mitochondria then metabolize these ketone bodies into acetyl-CoA, an energy source that supplants glucose (*61*). Studies have revealed that D-BHB, a ketone body, stimulates autophagy by increasing FOXO1 and FOXO3a expression through SIRT2. In addition, it promotes mitochondrial biogenesis through PGC-1α (*62*). Moreover, research suggests that D-BHB activates the autophagy-lysosome pathway by activating AMPK and TFEB-mediated lysosomal biogenesis (*62*). Ketone bodies have displayed the potential to activate SIRT1 and HIF-1α, hence inhibiting the mTOR complex and leading to the promotion of autophagy in brain neurons. This process facilitates the breakdown of damaged mitochondria and protein aggregates, playing an important role in the improvement of cognitive function (*63-65*). Moreover, 3-hydroxybutyrate (3OHB), a ketone body, actively stimulates the production of reactive oxygen species. This process further activates the transcription factor NF-κB and the histone acetyltransferase p300/EP300, leading to an induced expression of the BNDF gene. This process promotes neurogenesis, synapse growth, and synaptogenesis (*66*). When energy intake is restricted, fatty acids are transformed into ketone bodies, which stimulates brain-derived neurotrophic factor expression and induces autophagy. This is beneficial to brain health by improving cortical neuron function, helping to restore brain function, and helping to alleviate cognitive dysfunction and neurodegenerative diseases (*56*,*67-70*).

4.3. Cysteine

When the diet is restricted, cysteine intake is restricted. Hence, the body undergoes autophagic degradation of lysosomes to release cysteine. This results in heightened cysteine levels, assists in acetyl-CoA metabolism, and constrains the activation of mTOR. Consequently, autophagy is sustained and the life of the organism is prolonged. Cysteine plays a crucial role in regulating hypoxia-inducible factor (HIF), promoting neurogenesis and tRNA thiolation, and providing anti-inflammatory and antioxidant benefits (*71-73*). Research has revealed that taking supplements of cysteine or its modified molecules can help buffer cellular oxidative stress and inhibit inflammatory reactions (*74*). In contrast to many amino acids that promote protein synthesis by increasing mTOR complex activity, cysteine can actually inhibit mTOR complex activity, which can delay the aging process (*14,75*). During starvation, the autophagy mechanism releases cystine in the cell lysosomes, which then increases cytosolic cysteine levels, ultimately inhibiting the activation of mTOR complex signaling and continuously inducing autophagy (*76,77*). If, however, long-term fasting surpasses the threshold for mTOR complex activation, it may harm the body's metabolic balance and pose a risk to health (*76*). A study found that restricting sulfur-containing amino acids in the diet, like cysteine, may boost the expression of cystathionine γ-lyase (CGL) in the transsulfuration pathway (TSP). This can lead to the production of hydrogen sulfide in the body, which provides protection against ischemia/reperfusion injury (IRI) and extends an animal's lifespan (*78*).

4.4. BCAAs

The physiological and molecular mechanisms through which BCAAs maintain metabolic balance are intricate. Studies have shown that restricting the consumption of BCAAs during a restricted dietary reduces mTORC1 activity *in vivo*, leading to improved cognition, a prolonged lifespan, and other benefits attributed to the promotion of autophagy (*57,79,80*). BCAAs are leucine, isoleucine, and valine, which are essential amino acids and the most prevalent amino acids in protein. They have been linked to cognitive decline, aging, frailty, obesity, and diabetes (*81,82*). Studies have shown that BCAAs suppress hepatic autophagy induced by lipids, boost hepatocyte apoptosis, prevent hepatic FFA/triglyceride conversion, and worsen hepatic lipotoxicity by activating the mTOR pathway in hepatocytes (*83*). Prolonged exposure to a diet high in BCAAs could result in hyperphagia, obesity, and a shorter lifespan (*84*). Notably, astrocytic biotinylation and increased BCAAs accumulate in the aging cerebral cortex, which may be related to the inhibition of autophagy and overactivation of the mTOR complex (*85*). However, restricting BCAAs intake has been shown to promote metabolism, delay aging, and prevent disease (*86*). Moreover, Weaver *et al.* suggested that limiting consumption of BCAAs, and particularly isoleucine, may induce starvation and result in a prolonged lifespan by influencing histone acetylation in the brain (*87*). To sum up, restricting BCAAs as part of a diet promotes autophagy, enhances cognition, and delays aging by regulating mTOR.

4.5. NPY

NPY plays a crucial role in maintaining bodily homeostasis. The increased release of NPY during a restricted diet promotes autophagy in hypothalamic neurons, resulting in improved memory and delayed aging by protecting synapses. NPY is a potent biologically active peptide primarily produced by the hypothalamus, and it is involved in diverse physiological and pathological processes (*88*), including learning, memory, feeding behavior, and anxiety (*89,90*). Moreover, NPY can regulate both innate and adaptive immune responses by altering cytokine secretion and megakaryocyte chemotaxis. Studies have confirmed that NPY boosts p62/SQSTM1-mediated autophagy and NRF2 antioxidant signaling pathways in giant cells, which are crucial for the host's inflammatory

response (*91*). Research has suggested that NPY plays a significant role in maintaining energy homeostasis as the primary regulator of feeding (*92*). NPY levels in the arcuate nucleus (ARC) decrease mainly when energy is overconsumed (*93*). A study has found that AGRP neurons secrete the neurotransmitter NPY during fasting, which enhances an organism's attraction to food odors and which contributes to the hunger drive (*94*). Another study has shown that levels of NPY in the ARC increase significantly in response to fasting while energy balance is maintained by increasing food intake (*95*). When energy intake is restricted, increased NPY release in the paraventricular hypothalamic nucleus (PVH) induces hepatic autophagy (*12*). An increase in hypothalamic NPY by caloric restrictions can further induce autophagy in hypothalamic neurons by inducing the activation of neuropeptide Y receptor Y1 (NPY1R) or neuropeptide Y receptor Y5 (NPY5R) intracellular pathways (*96*). AKT and protein kinase A (PKA) signaling pathways are activated by NPY1R in a PI3K-dependent manner, while NPY5R activation increases MAPK/extracellular regulated kinase (ERK) and PKA phosphorylation (*96*). Research in a cell culture medium mimicking heat restriction has shown that autophagy can be stimulated in rat cortical neurons and that it is blocked by NPY or ghrelin receptor antagonists (*97*). Research has indicated that a restricted diet is associated with stimulation of autophagy through inhibition of PI3K/AKT/mTOR and activation of ERK1/2-MAPK by NPY and ghrelin, leading to alleviation of age-related disease (*98*). Impaired autophagy is a key aspect of aging. NPY protects against age-related hypothalamic damage and slows aging by activating NPY, which synergistically stimulates the PI3K, MEK/ERK, and PKA signaling pathways (*99*). There is mounting evidence that NPY plays a significant role in the aging process and an extended lifespan (*96,100*). Aging is associated with a decrease in both autophagy and NPY levels in the hypothalamus (*99,101*). Replenishing NPY can lessen age-related brain alterations by impacting six of the nine cellular aging criteria: mitochondrial dysfunction, dysregulated nutrient sensing, cellular senescence, loss of protein homeostasis, stem cell failure, and altered intercellular communication (*100*). On the whole, NPY protects against neurodegenerative diseases (*102*). In addition, a study found that NPY enhances hypothalamic autophagy, resulting in increased progerin clearance, decreased DNA damage, mitigation of cellular senescence, and other benefits (*103*). Thus, it slows down aging and alleviates cognitive dysfunction (*96*). Impaired neuron autophagy results in decreased memory and learning abilities, particularly during aging. However, neuropeptides can inhibit synaptic degeneration and alleviate memory impairment. Levels of transcription of the NPY family members (sNPF) are regulated by autophagy, and in turn, sNPF can prevent synaptic aging through autophagy (*104*). Metabolism can affect neuronal function and plasticity through autophagy. Restrictions on diet trigger the production of endogenous neuropeptides in the hypothalamus, stimulating autophagy *via* activation of the downstream pathways NPY1R or NPY5R (Figure 1). This protective mechanism preserves synapses, improves cognitive function, and prolongs lifespan.

4.6. BDNF

During nutrient deprivation, BDNF is reported to promote autophagy, but its inhibition of autophagy has also been documented. Moreover, fasting has been shown to upregulate the expression of BDNF, resulting in the activation of autophagy in various regions of the brain, such as the hypothalamus, cortex, and hippocampus. In mice that were older than three months, however, the expression of BDNF induced by fasting produced inconsistent results in the cortex and hippocampus, while neuronal autophagy was suppressed in these areas (*105*). As a member of the neurotrophic factor family, BDNF is essential for neuronal survival and differentiation during development (*106*), and it plays a critical role in regulating learning and memory formation (*107*). BDNF can influence synapse formation in three major ways: increasing the sprouting of axons and dendrites, initiating the formation of axonal and dendritic branches, and consolidating existing synapses (*108*). Nikoletopoulou *et al.* found that BDNF inhibits autophagy *in vivo* through the mediation of the myosin receptor kinase B (TrkB) and phosphatidylinositol-3'kinase (PI3K)/AKT pathways. Moreover, the prevention of autophagy by BDNF during fasting is necessary for improved synaptic plasticity and enhanced memory (*105,109*). BDNF regulates structural plasticity in the suprachiasmatic nucleus of the hypothalamus (SCN) through the BDNF/TrkB signaling pathway in a circadian-dependent manner (*110,111*). Restricted diets trigger BDNF signaling, which boosts peripheral energy metabolism, neuronal bioenergetics, and overall brain health (*111,112*).

5. Associations among circadian rhythm, a restricted diet, autophagy, and cognitive dysfunction

Autophagy is closely correlated with cognitive impairment. A restricted diet can activate autophagy, increasing the expression of BNDF and NPY, clearing Aβ and tau protein plaques, and ameliorating cognitive dysfunction. Mounting evidence indicates a link between cognitive impairment and autophagy-lysosomal pathway damage, which results in misfolded proteins and abnormal intracellular aggregation of dysfunctional mitochondria (*45,113-116*). Research has demonstrated that mutations in *PSEN1* and *PSEN2* disrupt the autophagy-lysosomal pathway, resulting in protein aggregation and neuronal death that significantly contribute to the development of early-onset familial

AD (*114*). Mouse models of AD have shown that abnormal autolysosomal acidification causes autophagic accumulation of Aβ, leading to the production of senile plaques (*117*). Dysregulated mitochondrial autophagy is a prominent neuronal hallmark of AD and Parkinson's disease (PD) (*118,119*), leading to cognitive dysfunction. Autophagy has the ability to improve cognitive function by modifying neuronal metabolism and eliminating damaged organelles and harmful substances. A model of AD revealed that enhanced autophagy decreased Aβ plaque formation and alleviated cognitive impairment (*120,121*). The activation of autophagy by peroxisome proliferator activated receptor alpha (PPARA) lessened Aβ deposition and alleviated cognitive decline in AD (*122*). A restricted diet has been reported to alleviate cognitive impairment by increasing astrocyte autophagic flux and attenuating amyloid pathology in transgenic mice (*53*). A circadian rhythm-restricted diet reduces mitochondrial oxidative stress, promotes mitochondrial biogenesis, enhances autophagy, promotes neuroplasticity, and aids cognition and memory (*29,123,124*). A model in older mouse demonstrated that a regular diet mimicking fasting promoted multisystem regeneration, hippocampal neurogenesis, and improvement in cognitive performance while reducing insulin-like growth factor 1 (IGF-1) levels and PKA activity with an increase in NeuroD1 (*54*). A clinical trial examining the dietary habits of adults in southern Italy indicated that consuming a limited diet was correlated with cognitive status and had a likely impact on brain health (*125*). A clinical study involving obese adults showed that a limiting feeding schedule within a 24-hour window improved glucose control, induced autophagy, increased the level of BNDF, and delayed aging (*11*). Another study of elderly obese patients with mild cognitive impairment (MCI) showed that intentional dietary restrictions significantly improved cognition (*126*). In Huntington's disease, increased autophagy induced by fasting facilitates the elimination of Huntington's protein (mHTT) (*127*). Adopting a circadian rhythm-restricted diet with augmented mitochondrial autophagy gene expression retards the progression of PD (*128*). Preclinical and clinical studies have shown that a circadian rhythm-restricted diets affect amino acid, glucose, and lipid metabolism as well as NPY and BDNF, which regulate intracellular autophagy. This process eliminates abnormal proteins and defective mitochondria, enhances synaptic function, and improves cognitive function, as listed in Table 1.

6. Limitations of restricted diets

There are, however, limitations to restrictive diets due to their potential adverse effects on blood glucose levels, reproductive function, and immune system. A clinical trial on fasting in adults revealed that all types of fasting increased the incidence of hypoglycemic reactions in patients with type 2 diabetes while they were receiving glucose-lowering medications (*135*). Moreover, research has indicated that restrictive diets may disrupt reproduction in young rats *via* the hypothalamic-pituitary-gonadal axis (*136*). Research has demonstrated that 72 hours of intense fasting upregulates signalling upstream of autophagy and it activates essential pathways, thereby promoting autophagy. That said, fasting can inhibit apoptosis by decreasing the expression of pro-apoptotic genes and increasing leukocyte viability, leading to the restructuring of human immune function. Fasting has been found to significantly enhance immune function, and particularly innate immunity, by increasing peripheral neutrophil production and cytokine secretion (*137*). Fasting induces a change in leukocyte migration that extends the lifespan of monocytes and alters disease susceptibility. When the diet is restricted, T cells are recruited from secondary lymphoid organs to the bone marrow, B cells leave Peyer's patches, and the number of circulating monocytes decreases in mice and humans as their mobilization from the bone marrow is prevented (*9*). The effects of fasting on the immune system depend on its duration and form, as well as the purpose of intermittent fasting, so additional research is required. Rough intermittent fasting can lead to lower blood pressure and low levels of cholesterol and triglycerides, but these effects gradually diminish over several weeks following the resumption of a normal diet (*138*). Long-term fasting may cause weakness, hunger, dehydration, headaches, difficulty concentrating, low blood pressure, or fainting, so it is not advisable for pregnant or nursing women, frail elderly individuals, people with immune deficiencies, or people with or at risk for eating disorders to engage in intermittent fasting. The impact of the body's blood glucose, the timing of fasting, and the body's state during a restricted diet or fasting should be considered.

7. Importance of the circadian rhythm

The circadian rhythm plays an essential role in regulating numerous physiological and cognitive functions in the body. The suprachiasmatic nucleus (SCN) in the hypothalamus controls this inherent 24-hour cycle, which is calibrated by external stimuli such as exposure to light. Disrupting the circadian rhythm may result in detrimental consequences for human health, impairing both cognitive and physical performance and elevating the risk for illnesses such as sleep disorders, metabolic disorders, atherosclerosis, AD, PD, and cancer. Therefore, maintaining a stable circadian rhythm is fundamental to maintaining overall health and well-being. A frequent cause of circadian disturbance is shift work, resulting in a desynchronization between one's internal clock and external cues. Other factors that may adversely affect the circadian rhythm are exposure to artificial light at night, inconsistent sleep patterns, and

Table 1. Different types of fasting to modulate autophagy for cognitive function, lifespan

Authors, Year *(Ref.)*	Study subjects	Cytokines	Type of fasting	Circadian rhythm	Autophagy	Results
Whittaker *et al*. 2023 (*129*)	APP23 TG mice	N/A	Fasting for 18 hours, feeding for 6 hours	Circadian modulation	N/A	Reduces amyloid deposition, increases Aβ42 clearance, and improves sleep and memory
Ulgherait *et al.* 2021 (*22*)	Flies	*ATG1, ATG8a*	Fasting for 20 hours, feeding for 28 hours	Constant circadian rhythm	Induces the autophagy process	Delays aging and extends life span
Currenti *et al.* 2021 (*125*)	Humans	N/A	Time-restricted feeding for 10 hours	Constant circadian rhythm	N/A	Improves cognition and has beneficial effects on brain health
Ferreira-Marques *et al.* 2021 (*98*)	Rat cortical neurons	NPY	Caloric restriction	N/A	Stimulates autophagy	N/A
Leclerc *et al.* 2020 (*130*)	Humans	N/A	25% caloric restriction	N/A	N/A	Improves working memory
Wilkinson *et al.* 2020 (*39*)	Humans	N/A	Time-restricted feeding for 10 hours	Constant circadian rhythm	N/A	Weight loss, improves body metabolism and sleep
Jamshed *et al.* 2019 (*11*)	Humans	N/A	Time-restricted feeding at 8 AM and 2 PM	Constant circadian rhythm	Increases autophagy	Enhances circadian clock gene expression and has anti-aging action
Gregosa *et al.* 2019 (*53*)	Mice	N/A	Restricted feeding for 5 days (60% of intake), then ad libitum for 9 ays	N/A	Induced astroglial autophagy	Mitigates cognitive deficits, amyloid pathology, and microglial reactivity
Nikoletopoulou *et al.* 2017 (*105*)	Mice (3–4 month-old male)	BDNF	Fasting for 12, 24, or 48 hours	N/A	Suppresses autophagy in the forebrain	Synaptic plasticity
Kong *et al.* 2016 (*59*)	Mice	AMPK	Fasting for 24 hours	N/A	N/A	Induces spinogenesis and excitatory synaptic activity
Alirezaei *et al*. 2010 (*131*)	Mice (6–7 week-old male)	mTOR	Fasting for 24 or 48 hours	N/A	Increased neuronal autophagy	Neuroprotective effect
Witte *et al.* 2009 (*132*)	Humans	N/A	30% caloric restriction	N/A	N/A	Improves memory function
Davis *et al.* 2008 (*133*)	SD rats	D-BHB	Fasting for 24 hours	N/A	N/A	Improves cognitive function
Lee *et al.* 2002 (*134*)	Mice (two-month-old male)	BDNF	Fasting on alternate days	N/A	N/A	Promotes the survival of newly generated neurons

Abbreviations: Aβ, amyloid β; AMPK, adenosine monophosphate-activated protein kinase; ATG1, autophagy-related 1; ATG8a, autophagy-related 8a; BDNF, brain-derived neurotrophic factor; D-BHB, D-β-hydroxybutyrate; mTOR, rapamycin; N/A, not accessible; TG, transgenic.

lifestyle choices such as drinking and smoking (*139,140*). Prolonged disruption of circadian rhythms increases the likelihood of cardiovascular disease, dementia, and type 2 diabetes; these conditions in turn interfere with sleep, further exacerbating circadian disruption (*141-143*). A study investigating the link between sleep parameters and subclinical atherosclerosis in asymptomatic middle-aged individuals found that reduced sleep duration and fragmented sleep were independently associated with an increased risk of subclinical multiregional atherosclerosis (*141*). Shokri-Kojori *et al.* found that one night of sleep deprivation resulted in a significant increase in Aβ accumulation in the right hippocampus and thalamus (*144*). Elevated norepinephrine levels related to deprivation of rapid eye movement (REM) sleep may impact neuronal autophagy, destabilizing neuronal integrity and homeostasis and leading to altered brain function and associated diseases such as AD and PD (*145*).

8. Conclusion

Currently, preclinical and clinical studies have demonstrated that adhering to a circadian rhythm-restricted diet can modify body metabolism, improve cognitive function, and increase life expectancy. Although the mechanisms are not entirely understood, autophagy is vital to this process. A circadian rhythm-restricted diet triggers autophagy, which clears anomalous protein deposits, engulfs impaired organelles, and improves cognitive performance through its effects on energy, lipid, and amino acid metabolism. In the forebrain, BDNF helps increase synaptic plasticity and improve cognitive function. A circadian rhythm-restricted diet has been found to be critical to maintaining and improving mental health and cognitive function in older adults. Therefore, a circadian rhythm-restricted diet may offer a novel approach to prevent and alleviate cognitive impairment.

Funding: This work was supported by a grant from the 2023 Foreign Experts Program in Hainan Province (SQ2023WGZJ0002) and the Hainan Provincial Center for Clinical Medical Research on Cerebrovascular Disease (no. LCYX202107).

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received July 28, 2023; Revised September 5, 2023; Accepted September 13, 2023.

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Released online in J-STAGE as advance publication September 17, 2023.

Original Article

DOI: 10.5582/bst.2023.01212

The prognostic nutritional index and tumor pathological characteristics predict the prognosis of elderly patients with early-stage hepatocellular carcinoma after surgery

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SUMMARY The elderly comprises over one-third of hepatocellular carcinoma (HCC) patients, however, they are not adequately represented in prognostic studies. The study aims to determine the prognostic significance of the preoperative prognostic nutritional index (PNI) and develop nomograms for predicting their recurrence-free and overall survival (RFS and OS). The study consisted of 282 elderly patients (aged ≥ 65 years) with early-stage HCC (China Liver Cancer Staging System: I-IIA) after curative resection (R0). They were randomly divided into a training (n = 197) and a test cohort (n = 85). The patients were stratified into two groups: PNI-low (PNI ≤ 49.05) and PNI-high (PNI > 49.05) based on a cut-off value. Most patients' demographics and perioperative outcomes were comparable, while patients in the PNI-high group were younger (P = 0.002), heavier (P < 0.001), and had lower comorbidity rates (P = 0.003). Although the tumor stages were earlier in the PNI-low group (P < 0.001), patients' OS (5-year OS: 48.9% *vs*. 93.1%) and RFS (5-year RFS: 27.3% *vs*. 75.7%) were significantly worse compared to the PNI-high group (both P < 0.0001). Patients' OS and RFS nomograms were developed by incorporating independent survival predictors including chronic obstructive pulmonary disease (COPD), age ≥ 75 years, PNI-low, tumor presence of satellite nodules, capsule, and microvascular invasion. The nomograms showed good calibration and discrimination, with all C-indexes ≥ 0.75 and calibration plots essentially coinciding with the diagonal. In conclusion, for elderly HCC patients, COPD, age ≥ 75 years, PNI-low, and tumor presence of satellite nodules, capsule, and microvascular invasion were independent prognostic factors. The nomogram could accurately predict the prognosis of these patients.

Keywords prognostic nutritional index, hepatocellular carcinoma, Recurrence-free survival, overall survival

1. Introduction

As the world's population ages, older hepatocellular carcinoma (HCC) patients will become a growing group (*1-4*). Current studies found surgery resection is still the first choice for elderly patients with HCC. Although current staging systems have identified important prognostic factors in HCC patients, most are based on radiological findings and do not sufficiently consider the basic demographic characteristics of the HCC patients, including age or comorbidities. These ignored factors may significantly influence HCC patients' long-term prognosis, especially in the elderly (*5-7*). As they usually tend to have more severe liver cirrhosis and higher co-morbidities rates such as hypertension (HBP), diabetes mellitus (DB), and chronic obstructive pulmonary disease (COPD), in comparison to younger HCC patients (*8-16*). Thus, the current clinical management and prognostic model for elderly HCC patients may be more complicated and different.

Nutrition and immune biomarkers have shown promising prognostic value for HCC patients. These factors may be of particular concern in elderly HCC patients, given that malnutrition and weakened immunity are more common in the elderly population. The prognostic nutritional index (PNI) based on patients' total lymphocyte count and serum albumin concentration integrates immune and nutrition indicators and has shown great research value (*9-12*). Pinato *et al*. (*13*) and Wang *et al*. (*14*) reported a low PNI was an independent predictor of poor survival in patients with HCC. Unfortunately, not all patients underwent surgical resection in the study by Pinato *et al*. (*13*). In the study by Wang *et al*. (*14*), the mean age of patients included in the study was only 50.41 years. Thus, the prognostic value of PNI in the elderly HCC population after surgical

resection is not well established.

Therefore, in this study, we aimed to determine the value of the preoperative prognostic nutritional index (PNI) and establish nomograms to help predict recurrence-free and overall survival (RFS and OS) for elderly patients (≥ 65 years) with early-stage (China Liver Cancer Staging System, CNLC: I-IIA) after curative resection (R0).

2. Patients and Methods

2.1. Patients

Consecutive HCC patients treated with curative surgical resection from 1st January 2010 to 2022 were retrospectively identified from the databases of West China Hospital, Sichuan University. All included patients were histologically confirmed HCC and obtained negative resection margins. We selected 65 years old at diagnosis as the cut-off value for elderly HCC patients (≥ 65 years old) (*5*). Pretreatment demographics and clinical laboratory data were retrieved through electronic medical records review. Patients excluded from the present study fit the following criteria: *i)* with cancers metastasis to the liver such as colorectal carcinoma liver metastasis or with other types of liver cancer including mixed hepatocellular-cholangiocarcinoma and intrahepatic cholangiocarcinoma; *ii)* diagnosed below 65 years (< 65y), with advanced stages of HCC (CNLC ≥ IIB) or after non-R0 resection (n =338); *iii)* with missing or uncomprehensive demographics, pathological, or survival data were also excluded from the study (n = 15). Finally, the cohort comprised 282 elderly patients (≥ 65y) with early-stage HCC (CNLC I-IIA) after R0 resection for further analysis.

The detailed CNLC criteria are presented in supplementary material Figure S1 (*http://www.biosciencetrends.com/action/getSupplementalData.php?ID=169*). This study was approved by the institutional review committee of West China Hospital, Sichuan University. Since our analysis was retrospective, the need for informed consent was waived by our ethics committee.

2.2. Data Collection

Medical records were reviewed for smoking, alcohol history, body mass index (BMI, kg/m^2), comorbidity (DB, HBP, and COPD), HBV, and HCV infection history. Other clinicopathological factors were also collected: Sex, age at diagnosis, tumor size, number, differentiation grade (poor, moderate, or high differentiation), microvascular invasion (MVI), tumor satellite formation, tumor capsule invasion, liver fibrosis stage (ISHAK) and perioperative outcomes (operative blood loss, operative time, postoperative complication rate, ICU admission, and hospital stay). There are various staging systems for HCC, such as the Barcelona Clinic Liver Cancer (BCLC) system, the Japan Society of Hepatology (JSH) consensus statements and recommendations, and the Asian Pacific Association for the Study of the Liver (APASL). The China Liver Cancer Staging System (CNLC), which is the most used staging system for HCC patients in China, was adopted in our study. All patients were treated according to CNLC staging and after comprehensive multidisciplinary consultation.

Postoperative follow-up included physical examination, laboratory tests, and abdominal CT or MRI to assess the surgical effect and check for recurrence every 3 months in the first 2 years and every 6 months in the subsequent years. Telephone surveys were employed for patients who could not attend follow-up appointments.

2.3. Definitions

All patients' follow-up duration was defined from the diagnosis to the last examination date or lost follow-up. RFS was the duration between curative surgical resection and the first recurrence or death from any other cause. OS was defined as being from the time of surgery to the date of death or most recent follow-up. Obstructive jaundice was defined as serum total bilirubin (TB) concentration above 34.1 umol/L (TB ≥ 34.1 umol/L). The liver fibrosis diagnosis is based on an invasive pathological biopsy approach. The ISHAK classification (ISHAK0–6) was used to classify the severity of liver fibrosis. We classify fibrosis according to scores defined by the American Joint Committee on Cancer (AJCC), the Ishak score ranging from 0 to 4 (undetectable to moderate fibrosis), defined as "F0", and 5 to 6 (severe fibrosis or cirrhosis), defined as "F1". The Clavien–Dindo classification system was used to categorize all postoperative complications. Pre-operative baseline alpha-fetoprotein (AFP) was confirmed as continuous and dichotomous. Patients were grouped into AFP ≥ 400 ng/mL *vs.* < 400 ng/mL. Cancer antigen 19-9 (CA 19-9; also known as carbohydrate antigen 19-9) is used to help differentiate between cancer of the pancreas and other conditions, as well as to monitor treatment response and recurrence. The normal range of CA 19-9 is between 0 and 37 U/mL (units/milliliter). In our study, patients were grouped into CA 19-9 ≥ 37.0 U/mL *vs.* <37.0 U/mL. Infection with hepatitis B and C virus (HBV and HCV) was diagnosed according to blood test outcomes, liver biopsy, or medical records. Clavien-Dindo classification is used for postoperative complication evaluation. Anatomical resection (AR) was defined as the complete removal of a hepatic segment/section. Histopathologic findings of resected HCC specimens included the presence of a capsule and tumor invasion onto the capsule and the presence of microvascular emboli in the surrounding liver parenchyma, these factors were defined as tumor invasion onto the capsule and MVI.

2.4. Statistical analysis

Data on the tumor parameters and patients' demographics are expressed as mean (SD) values for parametric continuous data and as median (range) values for data with the nonparametric distribution. Categorical data are expressed as percentage frequencies (N, %). The distribution of variables was analyzed using the Kolmogorov– Smirnov test. Chi-square, Mann–Whitney *U* test or Fisher's exact tests were used to make comparisons between groups as appropriate. Serum albumin and lymphocyte counts were measured and calculated before surgery and were used to calculate the pre-operative PNI. The index was calculated with the following formula: 10 × serum-albumin (g/dL) + 0.005 × total lymphocyte count in peripheral blood/mm^3. Patients were classified into 'PNI-low' and 'PNI-high' groups according to the optimal cut-point outcomes after maximally selected rank statistics for OS. The optimal cut point for PNI was determined using the maximally selected rank statistics from the 'maxstat' R package. This outcome-oriented method provides a value of a cut point that corresponds to the most significant relationship with the outcome. We also performed univariable, and multivariable Cox regression models to investigate variables associated with survival. The receiver operating characteristic (ROC) curve, area under the ROC curve (AUC), and Harrell's concordance index (C-index) were used to assess discrimination of the model, while the calibration plot was used to graphically evaluate the calibration of the nomogram in both training and validation cohorts.

All analyses were conducted using R software (version 3.6.3; R Foundation for Statistical Computing), and a two-tailed *p*-value < 0.05 was set for statistical significance.

3. Results

3.1. Baseline features of the study population

A total of 282 elderly HCC patients after R0 resection and met all inclusion criteria were included. The study flow diagram is shown in Figure 1. Among them, 197 (70%) and 85 (30%) patients were randomly segregated into the training and or test cohorts. The optimal PNI cutoff values were analyzed according to the survival data of the included HCC patients. The ideal cutoff value of preoperative PNI in our study was 49.05 (supplementary material Figure S2, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=169*), then we divided patients into the PNI-low (PNI ≤ 49.05) and PNI-high (PNI > 49.05) groups according to the canulated cutoff values. We also searched published studies and found comparable cut-off values of PNI in other types of cancers.

3.2. Prognostic value of PNI

Most of the clinicopathologic features of the patients in the PNI-low (n = 180) and PNI-high (n = 102) cohorts were comparable except for patients in the PNI-high group were younger (P = 0.002) and had higher BMI (P < 0.001), and leukocyte count (P < 0.001), meanwhile, shorter smoking years (P = 0.046) and less COPD rates (P = 0.003) were also noticed. While patients in the PNI-

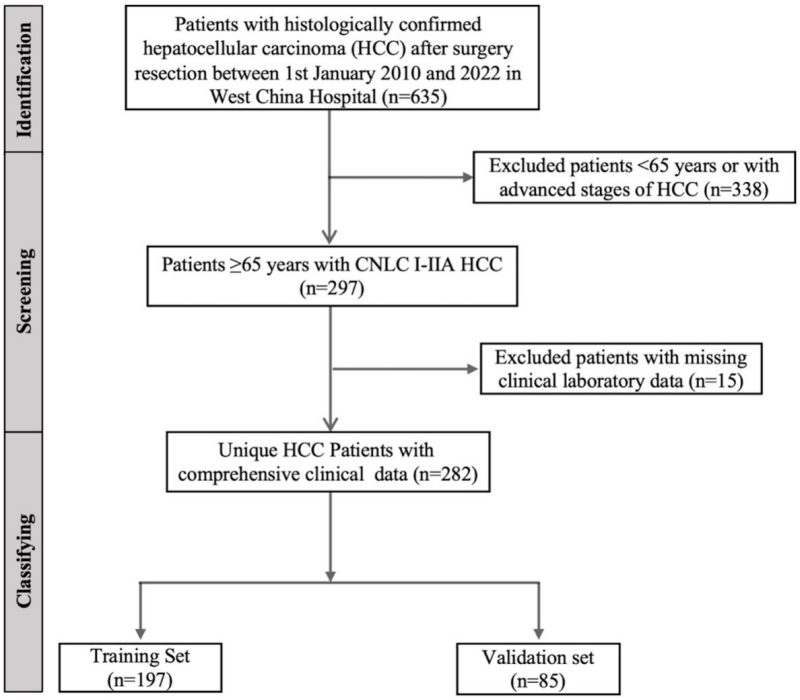

Figure 1. Flow chart of patient inclusion.

high group had higher Cancer Antigen 19-9 (CA19-9) levels compared to patients in the PNI-low group ($P = 0.019$). For patients in the PNI-low group, the prothrombin time (PT) was longer ($P < 0.01$), and the alanine aminotransferase and aspartate aminotransferase level (ALT and AST) was higher ($P = 0.005$ and $P = 0.018$, respectively) when compared with PNI-high group. Although, in the PNI-low group, more patients had a single tumor ($P < 0.001$), and the CNLC stages of HCC were earlier ($P < 0.001$), improved tumor histopathological features resulted in the PNI-high group. In the PNI-high group, tumor of high/well differentiation degree was more common ($P = 0.011$), while in the PNI-low group, more patients had satellite nodules ($P = 0.002$). When we compared the PNI-high and PNI-low groups according to their perioperative outcomes, we found that there was a higher rate of anatomical resection (AR) and hemi-hepatectomy ($P = 0.016$) for the PNI-high group, in addition, the hospital stay ($P =0.002$) was also longer for patients in the PNI-high group (Table 1).

In the Kaplan–Meier analysis, the 1-, 3- and 5-year OS and 1, 3 and 5-year RFS of the entire cohort were 99.6%, 94.1 %, 69.1 %, and 99.3%, 65.8%, 48.7%, respectively (Figure 2A-B). Patients in the PNI-low group had a significantly shorter OS ($P <0.0001$) and RFS ($P < 0.0001$) than the PNI-high group. The 1-, 3- and 5-year OS rate in the PNI-low (PNI ≤ 49.05) and PNI-high (PNI > 49.05) group groups was 99.4%, 91.7%, 48.9% versus 100%, 98.0% and 93.1%, respectively. The 1-, 3- and 5-year RFS rate in the PNI-low group (PNI ≤ 49.05) was 98.9%, 54.3%, 27.3% versus 100%, 83.3 %, and 75.7%, respectively in the PNI-high (PNI > 49.5) group (Figure 2C-D).

3.3. Independent Predictors of OS and RFS

The baseline characteristics of the two cohorts (training and/or test cohorts) are shown in Table 2. Most of the variables were found to be similar between the two cohorts at baseline. To further identify the clinically significant factors for OS and RFS, univariate and multivariate analysis was performed in the training cohort. A multivariate Cox proportional hazards model was entered for all factors with a p-value < 0.05 in the univariate analysis. The outcome is presented in Table 3.

The univariate Cox regression analysis showed that PNI-high was a significant prognostic factor associated with both longer OS (hazard ratio (HR) = 0.16, 95% confidence interval (CI), (0.06-0.44), $P < 0.01$) and RFS (HR = 0.22, 95%CI (0.12-0.41), $P < 0.001$) in elderly patients with HCC. In multivariate analysis, we found that age ≥ 75 years (HR = 2.46, 95%CI (1.16-5.24), $P = 0.02$), PNI-high (HR = 0.16, 95%CI (0.06-0.44), $P < 0.01$), COPD (HR = 2.83, 95%CI (1.39-5.75), $P = 0.004$), tumor of moderate /high differentiation (HR = 0. 38, 95% CI (0.17-0.86), $P = 0.02$), capsular invasion (HR=2.68, 95% CI (1.17-6.12), $P = 0.02$) and satellite nodules (HR = 4.52, 95% CI (1.95-10.45), $P < 0.01$) were independent prognostic markers for OS. Apart from PNI (HR = 0.22, 95% CI (0.12-0.41), $P < 0.001$), we also found that tumor differentiation (moderate/high vs low) (HR = 0.22, 95% CI (0.12-0.41), $P = 0.025$) or microvascular invasion (HR = 0.22, 95% CI (0.12-0.41), $P < 0.001$) were independent prognostic factors for patients' postoperative RFS (Table 3).

3.4. Development and Validation of the prediction models

The nomogram prediction models for patients' OS (Figure 3A) and RFS (Figure 3B) were built by incorporating independent OS and RFS predictors, which included patients' age (≥ 75 years vs < 75 years), PNI (PNI-high *vs*. low), COPD diagnosis, tumor differentiation degree (moderate/high *vs*. low), presence of satellite nodules, tumor capsule invasion, and MVI. As we can see from the nomogram, PNI (PNI-high *vs*. low) had the greatest impact on OS, followed by MVI on RFS.

The C-index of OS in the training cohort and test cohort were 0.875 (95% CI: 0.842–0. 908) and 0.820 (95% CI: 0.714–0. 927), respectively, indicating that the model had good discriminatory power. The C-index of RFS in the training cohort and test cohort were 0.803 (95% CI: 0.765–0.842) and 0.800 (95% CI: 0.768–0.832), respectively, indicating that the model had good discriminatory ability. In the training cohort, the AUC of the predicted nomogram for OS and RFS was 0.851 and 0.877 (Figure 4A-B). In the test cohort, the AUC of the predicted nomogram for OS and RFS was 0.877 and 0.909 (Figure 4 C-D). The ROC curve and the AUC of the PNI of OS and RFS are additionally presented in Figure S3 (*http://www.biosciencetrends.com/action/getSupplementalData.php?ID=169*). The calibration plots for training and test cohorts used to predict 3-year, 5-year OS, and 3-year, 5-year RFS showed good agreement between the actual observations and the model predictions (Figure 5A-H).

4. Discussion

We found significant differences in postoperative recurrence and long-term survival between patients in the PNI-low and PNI-high groups. More importantly, this difference persisted after univariate and multivariate analysis. Therefore, we found that the PNI has a role in predicting the prognosis of elderly HCC patients after surgical resection. In our analysis, we also found that the patient's age, diagnosis of COPD, whether the tumor infiltrated blood vessels, had satellite nodules, and whether it was a poorly differentiated type of tumor also had a significant impact on patient prognosis. Based on these results, we created a nomogram chart that can predict a patient's three-year and five-year survival prognosis. These nomograms are easy to calculate and

Table 1. Patients' demographics grouped by the cutoff value for PNI

Variable	PNI low (≤ 49.05) (*n* = 180)	PNI high (> 49.05) (*n* = 102)	*P* Value
AGE, years, (Median (IQR)	70.00 (67.00 to 75.00)	69.00 (67.00 to 71.00)	0.002
AGE group, *n* (%)			
< 75 years	130 (72.2%)	91 (89.2%)	0.001
≥ 75years	50 (27.8%)	11 (10.8%)	
SEX, *n* (%)			
Female	21 (11.7%)	18 (17.6%)	0.223
Male	159 (88.3%)	84 (82.4%)	
BMI, kg/m^2, median (IQR)	22.20 (20.31 to 24.67)	24.26 (21.67 to 25.95)	< 0.001
BMI group, *n* (%)			
< 18.5 (underweight)	9 (5%)	0 (0%)	< 0.001
≥ 18.5 ≤ 23.9 (normal)	114 (63.3%)	48 (47.1%)	
≥ 24 ≤ 27.9 (overweight)	45 (25%)	39 (38.2%)	
≥ 28 (obesity)	12 (6.7%)	15 (14.7%)	
Comorbidity, *n* (%)			
DB	75 (41.7%)	42 (41.2%)	1.000
HBP	90 (50%)	45 (44.1%)	0.409
COPD	57 (31.7%)	15 (14.7%)	0.003
Cardiovascular disease	63 (35%)	42 (41.2%)	0.367
Smoking and drinking history, *n* (%)			
Smoking ≥ 20 years	87 (48.3%)	36 (35.3%)	0.046
Drinking ≥ 20 years	45 (25%)	27 (26.5%)	0.897
Abdornimal surgery history, *n* (%)	18 (10%)	6 (5.9%)	0.333
HBV infection history, *n* (%)			
Presence	174 (96.7%)	99 (97.1%)	1.000
HCV infection history			
Presence	3 (1.7%)	0 (0%)	0.480
Ishak group, *n* (%)			
F0	63 (35%)	42 (41.2%)	0.367
F1	117 (65%)	60 (58.8%)	
PS score, *n* (%)			
0	33 (18.3%)	24 (23.5%)	0.195
1	81 (45%)	51 (50%)	
2	66 (36.7%)	27 (26.5%)	
Laboratory tests			
PLT,$10*^9$/L, Median (IQR)	112.50 (83.50 to 160.00)	128.00 (93.00 to 169.00)	0.283
WBC, $10*^9$/L, Median (IQR)	4.70 (3.92 to 5.94)	5.27 (4.81 to 6.40)	< 0.001
TB, umol/L, Median (IQR)	13.20 (10.35 to 17.15)	14.10 (10.30 to 18.00)	0.785
ALT, U/L, Median (IQR)	40.50 (26.00 to 59.00)	28.50 (24.00 to 48.00)	0.005
AST, U/L, Median (IQR)	47.00 (29.50 to 60.50)	36.50 (31.00 to 48.00)	0.018
ALP, U/L, Median (IQR)	99.50 (74.50 to 133.00)	99.50 (75.00 to 117.00)	0.662
PT, Seconds, Median (IQR)	12.00 (11.40 to 12.75)	11.30 (10.90 to 11.90)	< 0.001
AFP, ng/mL, Median (IQR)	22.72 (3.40 to 312.80)	12.21 (4.82 to 180.20)	0.601
AFP ≥ 400 ng/mL	51 (28.3%)	30 (29.4%)	0.956
CA19-9 (median [IQR])	21.52 (9.86 to 40.32)	26.74 (16.30 to 60.32)	0.019
CA19-9 ≥ 37.0 U/mL	45 (25%)	33 (32.4%)	0.235
CNLC stage, *n* (%)			
IA	84 (46.7%)	33 (32.4%)	< 0.001
IB	93 (51.7%)	48 (47.1%)	
IIA	3 (1.7%)	21 (20.6%)	
Differentiation, *n* (%)			
H	27 (15%)	24 (23.5%)	0.011
L	60 (33.3%)	18 (17.6%)	
M	93 (51.7%)	60 (58.8%)	
Tumor size (median [IQR])	5.00 (3.50 to 7.75)	5.25 (3.50 to 7.00)	0.259
Tumor number, *n* (%)			
1	171 (95%)	72 (70.6%)	< 0.001
2	8 (4.4%)	19 (18.6%)	
3	1 (0.6%)	11 (10.8%)	
Microvascular invasion, *n* (%)			
Presence	75 (41.7%)	36 (35.3%)	0.355
Tumor Invasion onto The Capsule, *n* (%)			
Presence	90 (50%)	51 (50%)	1.000

HBP: Hypertension, DB: Diabetes Mellitus, COPD: Chronic Obstructive Pulmonary Disease, PNI: Prognostic Nutritional Index, MVI: Microvascular Invasion, BMI: Body Mass Index, CNLC: China Liver Cancer Staging System, TB: Total Bilirubin, Cancer Antigen 19-9, AFP: Alpha-Fetoprotein, HBV And HCV: Hepatitis B And C Virus, CV: Clavien-Dindo Classification, ALT: Alanine Aminotransferase, AST: Aspartate Aminotransferase, ALP: Alkaline Phosphatase, PT: Prothrombin Time, AR: Anatomical Resection, PLT: Blood Platelet Count, WBC: White Blood Cell, N:No, Y: Yes, MVI: Microvascular Invasion.

Table 1. Patients' demographics grouped by the cutoff value for PNI (continued)

Variable	PNI low (≤ 49.05) (*n* = 180)	PNI high (> 49.05) (*n* = 102)	*P* Value
Satellite Nodules, *n* (%)			
Presence	45 (25%)	9 (8.8%)	0.002
Operation time, min, (median [IQR])	185.00 (145.00 to 240.00)	220.00 (135.00 to 260.00)	0.192
Hepatectomy Methods, *n* (%)			
Non-AR	81 (45%)	51 (50%)	0.016
Right hemi-hepatectomy	18 (10%)	9 (8.8%)	
Left hemi-hepatectomy	24 (13.3%)	3 (2.9%)	
AR	57 (31.7%)	39 (38.3%)	
Blood loss (ml) (median [IQR])	200.00 (200.00 to 400.00)	200.00 (150.00 to 400.00)	0.330
Post operative complication, *n* (%)			
Clavien–Dindo classification I	30 (1.7%)	24 (23.5%)	0.211
Clavien–Dindo classification II	150 (83.3%)	78 (76.5%)	
Hospital stays, day (median [IQR])	12.00 (10.00 to 15.00)	13.00 (12.00 to 15.00)	0.002

HBP: Hypertension, DB: Diabetes Mellitus, COPD: Chronic Obstructive Pulmonary Disease, PNI: Prognostic Nutritional Index, MVI: Microvascular Invasion, BMI: Body Mass Index, CNLC: China Liver Cancer Staging System, TB: Total Bilirubin, Cancer Antigen 19-9, AFP: Alpha-Fetoprotein, HBV And HCV: Hepatitis B And C Virus, CV: Clavien-Dindo Classification, ALT: Alanine Aminotransferase, AST: Aspartate Aminotransferase, ALP: Alkaline Phosphatase, PT: Prothrombin Time, AR: Anatomical Resection, PLT: Blood Platelet Count, WBC: White Blood Cell, N:No, Y: Yes, MVI: Microvascular Invasion.

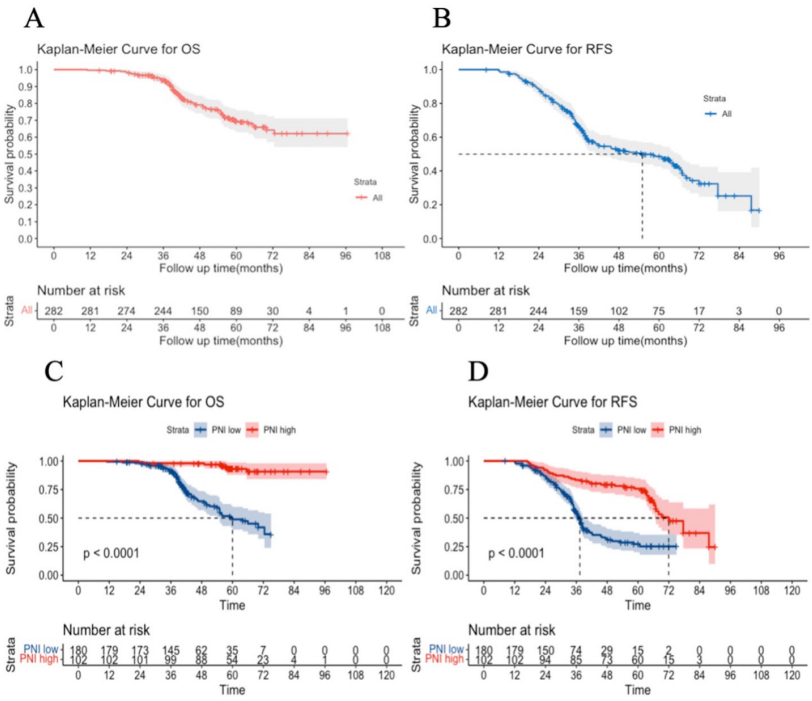

Figure 2. Kaplan-Meier curves of overall survival (OS) and recurrence-free survival (RFS) for different cohorts. (**A**) Kaplan-Meier curves of OS for the entire cohort; (**B**) Kaplan-Meier curves of RFS for the entire cohort; (**C**) Kaplan-Meier curves of OS for patients with high and low prognostic nutrition index (PNI) (log-rank test, $P < 0.001$); and (**D**) Kaplan-Meier curves of RFS for patients with high and low PNI (log-rank test, $P < 0.001$).

have a good calibration and discrimination value.

As life expectancy increases worldwide, the proportion of elderly patients with HCC in need of oncological treatment is likely to increase (*5,6,17-19*). However, even today there is no clinical guideline for this population. Thus, clinical management investigation for elderly HCC patients is important. In our study, we introduced an easily accessible clinical variable, the PNI, which is calculated using the formula: [10 × serum albumin level (gr/dL)] + [0.005 × total lymphocyte count (per mm^3)] by peripheral blood to devalue nutrition and inflammation status.

Nutrition and inflammation status are parts of tumor microcirculation and affect tumor prognosis (*20-22*). This relation in elderly HCC patients has not been explored yet. The PNI measured by laboratory tests is a valuable and convenient tool to evaluate the patient's inflammation and nutritional status. Interestingly, many studies did report that PNI has great clinical significance in evaluating the prognosis of many solid neoplasms (*9,23,24*). It is well known that HCC arises because of hepatocellular carcinoma exposure to proinflammatory

Table 2. Baseline Characteristics of Patients in The Developing and Validation Cohorts

Variable Name	Validation Cohort (*n* = 85)	Development Cohort (*n* = 197)	*P* Value
AGE, Median (IQR)	70.00 (67.00 To 73.00)	69.00 (67.00 To 73.00)	0.414
SEX			
Female	15 (17.6%)	24 (12.2%)	0.302
Male	70 (82.4%)	173 (87.8%)	
PNI, Median (IQR)	48.50 (45.15 To 52.85)	47.85 (45.10 To 51.30)	0.360
PNI GROUP			
> 49.05 (High)	49 (57.6%)	131 (66.5%)	0.199
≤ 49.05 (Low)	36 (42.4%)	66 (33.5%)	
BMI, kg/m^2, Median (IQR)	22.67 (20.20 To 25.95)	22.67 (20.94 To 24.74)	0.500
BMI Group, Median (IQR)			
< 18.5 (Underweight)	3 (3.5%)	6 (3%)	0.069
≥ 18.5 ≤ 23.9(Healthy Weight)	43 (50.6%)	119 (60.4%)	
≥ 24 ≤ 27.9(Overweight)	25 (29.4%)	59 (29.9%)	
≥ 28(Obesity)	14 (16.5%)	13 (6.6%)	
DB Presence, Y	36 (42.4%)	81 (41.1%)	0.951
HBP Presence, Y	40 (47.1%)	95 (48.2%)	0.960
COPD Presence, Y	21 (24.7%)	51 (25.9%)	0.952
Cardiovascular disease	29 (34.1%)	76 (38.6%)	0.564
Abdornimal Surgery History,Y	9 (10.6%)	15 (7.6%)	0.556
HBV Infection History, Y	80 (94.1%)	193 (98%)	0.187
HCV Infection History, Y	0 (0%)	3 (1.5%)	0.609
Smoking History ≥ 20 Years, Y	42 (49.4%)	105 (53.3%)	0.638
Drinking History ≥ 20 Years, Y	32 (37.6%)	79 (40.1%)	0.799
ISHAK			
F0	29 (34.1%)	76 (38.6%)	0.564
F1	56 (65.9%)	121 (61.4%)	
Tumor Number			
1	73 (85.9%)	170 (86.3%)	0.969
2	8 (9.4%)	19 (9.6%)	
3	4 (4.7%)	8 (4.1%)	
Tumor Size, Median (IQR)	5.50 (4.00 To 8.00)	5.00 (3.20 To 7.50)	0.198
CNLC			
IA	32 (37.6%)	85 (43.1%)	0.684
IB	45 (52.9%)	96 (48.7%)	
IIA	8 (9.4%)	16 (8.1%)	
Differentiation Degree			
High	14 (16.5%)	37 (18.8%)	0.281
Moderate	42 (49.4%)	111 (56.3%)	
Poor	29 (34.1%)	49 (24.9%)	
Tumor Invasion onto The Capsule			
N	41 (48.2%)	100 (50.8%)	0.795
Y	44 (51.8%)	97 (49.2%)	
Satellite Nodules			
N	69 (81.2%)	159 (80.7%)	1.000
Y	16 (18.8%)	38 (19.3%)	
MVI			
N	50 (58.8%)	121 (61.4%)	0.782
Y	35 (41.2%)	76 (38.6%)	
Surgery Methods			
Non-AR	36 (42.3%)	96 (48.7%)	0.399
Right hemi-hepatectomy	7 (8.2%)	20 (10.2%)	
Left hemi-hepatectomy	9 (10.6%)	18 (9.1%)	
AR	33 (38.8%)	63(40.0%)	
Blood Loss,ml, Median (IQR)	200.00 (200.00 To 400.00)	200.00 (200.00 To 400.00)	0.394
Operation Time,min, Median (IQR)	200.00 (145.00 To 255.00)	195.00 (140.00 To 250.00)	0.814
CV			
I	17 (20%)	37 (18.8%)	0.941
II	68 (80%)	160 (81.2%)	
Hospital Stay,days, Median (IQR)	12.00 (11.00 To 15.00)	12.00 (11.00 To 15.00)	0.747
PLT,10*^{9}/L, Median (IQR)	109.00 (80.00 To 155.00)	119.00 (85.00 To 164.00)	0.428
WBC, 10*^{9}/L, Median (IQR)	5.41 (4.66 To 6.40)	4.91 (4.10 To 5.98)	0.002
TB, umol/L, Median (IQR)	14.60 (10.90 To 18.00)	13.30 (10.20 To 17.10)	0.141

HBP: Hypertension, DB: Diabetes Mellitus, COPD: Chronic Obstructive Pulmonary Disease, PNI: Prognostic Nutritional Index, MVI: Microvascular Invasion, BMI: Body Mass Index, CNLC: China Liver Cancer Staging System, TB: Total Bilirubin, Cancer Antigen 19-9, AFP: Alpha-Fetoprotein, HBV And HCV: Hepatitis B And C Virus, CV: Clavien-Dindo Classification, ALT: Alanine Aminotransferase, AST: Aspartate Aminotransferase, ALP: Alkaline Phosphatase, PT: Prothrombin Time, AR: Anatomical Resection, PLT: Blood Platelet Count, WBC: White Blood Cell, N: No, Y: Yes, MVI: Microvascular Invasion.

Table 2. Baseline Characteristics of Patients in The Developing and Validation Cohorts (continued)

Variable Name	Validation Cohort (*n* = 85)	Development Cohort (*n* = 197)	*P* Value
ALT, U/L, Median (IQR)	36.00 (26.00 To 55.00)	36.00 (24.00 To 56.00)	0.866
AST, U/L, Median (IQR)	39.00 (31.00 To 53.00)	38.00 (29.00 To 53.00)	0.875
ALP, U/L, Median (IQR)	101.00 (75.00 To 128.00)	99.00 (75.00 To 128.00)	0.926
PT, Seconds, Median (IQR)	11.80 (11.20 To 12.60)	11.80 (11.10 To 12.50)	0.431
AFP, ng/ml, Median (IQR)	42.63 (5.02 To 294.50)	12.50 (3.38 To 301.85)	0.183
AFP ≥ 400 ng/mL	27 (31.8%)	54 (27.4%)	0.550
CA19-9, U/mL, Median (IQR)	26.49 (16.02 To 38.06)	21.88 (11.24 To 48.31)	0.390
CA19-9 ≥ 37.0 U/mL	21 (24.7%)	57 (28.9%)	0.560

HBP: Hypertension, DB: Diabetes Mellitus, COPD: Chronic Obstructive Pulmonary Disease, PNI: Prognostic Nutritional Index, MVI: Microvascular Invasion, BMI: Body Mass Index, CNLC: China Liver Cancer Staging System, TB: Total Bilirubin, Cancer Antigen 19-9, AFP: Alpha-Fetoprotein, HBV And HCV: Hepatitis B And C Virus, CV: Clavien-Dindo Classification, ALT: Alanine Aminotransferase, AST: Aspartate Aminotransferase, ALP: Alkaline Phosphatase, PT: Prothrombin Time, AR: Anatomical Resection, PLT: Blood Platelet Count, WBC: White Blood Cell, N: No, Y: Yes, MVI: Microvascular Invasion.

stimuli, such as hepatotropic virus infection, or ethanol consumption (*2*). Patients with hepatitis have impaired liver function, which then leads to a deterioration in their nutritional status. Thus, the PNI may also be associated with HCC patients' survival. Pinato (*13*) *et al*. and Wang (*14*) *et al*. reported that PNI-low is an independent predictor of poor prognosis for HCC patients. However, in their study, elderly HCC patients were not separately considered. Previous studies have identified age might not be a precise risk factor for mortality or morbidity (*8*), but it is a surrogate marker for comorbidities, in other words, older patients are more likely to have comorbid conditions than younger patients. Since elderly HCC patients more commonly had comorbidities, many researchers such as Rocio I.R. Macias (*15*) *et al*. noticed that elderly HCC patients were more likely to present with chronic inflammatory conditions and nutrition impairment status. Thus, the value of PNI for elderly HCC patients may not be comparable to the younger populations.

Based on the published literature for other tumors, the predictive value of the PNI in older patients may be more representative than in other age groups. In the study by Yan (*25*) *et al.*, the authors found that compared with other variables such as liver invasion and central nervous system invasion, PNI was a stronger predictor of prognosis in elderly patients with diffuse large B-cell lymphoma. Zhang *et al*. (*26*) studied 454 patients with a diagnosis of gastric cancer who were over 60 years of age. A more sensitive prognostic value of PNI was observed in the subgroup aged ≥ 75 years compared to those aged 60 -74 years. Zhu *et al*. (*27*) evaluated the stratified effect of age on gastric cancer and demonstrated the association of low PNI with short disease-free survival and OS in older patients (≥ 65 years). Unfortunately, the predictive value of PNI in older HCC patients has not been reported in the literature. In fact, to our knowledge, a limited number of studies have focused on the effect of age on the prognostic role of PNI. Our study is the first to report the predicted value of PNI for elderly HCC patients. Based on our results, the relationship between nutritional status and prognosis of elderly HCC patients could be accurately interpreted, and precise stratification of patients in the low-risk and high-risk groups could be achieved.

Although elderly HCC patients constitute over one-third of all HCC patients, they were not adequately represented in prognostic and treatment studies. Currently, there is no consensus in the literature as to which system is the most reliable to predict the prognosis for HCC especially in elderly HCC patients. Thus, there is also not enough clinical evidence to guide the treatment for these patients. Unlike other malignancies, the survival of HCC patients is significantly influenced by both primary tumor stages and underlying liver function. These factors may be more important for elderly HCC patients (*28-31*). Considering the specificity of elderly HCC patients when compared with the younger population, such as the higher rates of comorbidities and more severe liver cirrhosis. It was impossible to accurately evaluate the prognosis of elderly patients with HCC by constructing a full age spectrum prognosis model of patients with HCC. However, most studies have failed to distinguish this specific group, making it even more rare to have prognostic models for older people with HCC. Published medical literature supported that advanced tumor stages, tumor presence of vascular invasion, and poorer differentiation were risk factors for a poorer prognosis of HCC (*17,19,29*). We hypothesize the prognostic model for elderly HCC patients should also include the above variables. However, considering the specificity of the elderly population, other indicators should also be considered together. Thus, a prognostic model combining PNI, and other indicators was therefore developed. Based on univariate and multivariate Cox analyses, we identified other independent risk factors besides PNI that affect the prognosis of elderly HCC patients. These included patient age, diagnosis of COPD, tumor microvascular invasion, capsule invasion, low differentiation, and satellite nodules. Most of our independent factors were in line with previous studies. The parameters of the nomogram model constructed in this study include PNI, patient age, diagnosis of COPD, whether the tumor infiltrated blood vessels or invaded the capsule, whether it had satellite nodules, and whether it was a poorly differentiated type of

Table 3. Univariate and Multivariate Cox Proportional Hazards Regression Analyses of Prognostic Factors for OS and RFS in the Training Cohorts.

Variable Names	Univariate Analyses of OS		Multivariate Analyses of OS		Univariate Analyses of RFS		Multivariate Analyses of RFS	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Age, years	1.08 (1.03-1.15)	0.005			1.02 (0.98-1.06)	0.438		
Age (≥ 75 years *vs.* 75 Years)	2.61 (1.42-4.81)	0.002	2.46 (1.16-5.24)	0.02	1.37 (0.86-2.18)	0.185		
SEX (Male *vs.* Female)	1.3 (0.55-3.03)	0.550			2.92 (1.34-6.38)	0.007	2.14 (0.92-4.99)	0.078
PNI (High *vs.* Low)	0.1 (0.04-0.24)	< 0.001	0.16 (0.06-0.44)	< 0.01	0.24 (0.14-0.39)	< 0.001	0.22 (0.12-0.41)	< 0.001
BMI (Healthy Weight *vs*. Underweight)	0.43 (0.1-1.8)	0.246	0.49 (0.1-2.37)	0.372	0.77 (0.24-2.47)	0.662		
BMI (Overweight *vs.* Underweight)	0.21 (0.05-0.96)	0.045	0.31 (0.05-1.82)	0.196	0.79 (0.24-2.6)	0.704		
BMI (Obesity *vs*. Underweight)	0.17 (0.02-1.22)	0.077	0.19 (0.02-1.63)	0.129	0.88 (0.23-3.4)	0.848		
DB (N *vs.* Y)	0.7 (0.4-1.23)	0.213			0.61 (0.4-0.93)	0.02	0.22 (0.12-0.41)	0.169
HBP (N *vs.* Y)	0.89 (0.52-1.53)	0.678			0.81 (0.55-1.19)	0.285		
COPD (N *vs.* Y)	2.53 (1.44-4.45)	0.001	2.83 (1.39-5.75)	0.004	1.74 (1.14-2.66)	0.011	0.22 (0.12-0.41)	0.639
CNLC (IIA vs IA *vs.* IB)	1.12 (0.5-2.54)	0.777			1.04 (0.59-1.83)	0.902		
Differentiation Degree (M/H *vs.* L)	0.19 (0.11-0.33)	< 0.001	0.38 (0.17-0.86)	0.02	0.23 (0.15-0.35)	< 0.001	0.22 (0.12-0.41)	0.025
Tumor Number (Single *vs.* Multiple)	1.49 (0.64-3.48)	0.359			1.81 (0.97-3.39)	0.064		
Tumor Invasion onto The Capsule (Y *vs.* N)	3.41 (1.79-6.48)	< 0.001	2.68 (1.17-6.12)	0.02	1.8 (1.21-2.68)	0.003	1.26 (0.77-2.08)	0.361
Satellite Nodules (Y *vs.* N)	8.53 (4.85-15)	< 0.001	4.52 (1.95-10.45)	< 0.01	2.83 (1.82-4.39)	< 0.001	1.28 (0.78-2.1)	0.33
MVI (Y *vs.* N)	2.67 (1.56-4.58)	< 0.001	1.12 (0.49-2.53)	0.789	6.25 (4.07-9.61)	< 0.001	0.22 (0.12-0.41)	< 0.001
Surgery (Non-AR *vs.* AR vs hemi-hepatectomy)	1.51 (0.82-2.77)	0.188			1.92 (1.24-2.97)	0.004	0.22 (0.12-0.41)	0.433
Surgery (hemi-hepatectomy *vs.* non-hemi-hepatectomy)	1.02 (0.91-1.14)	0.707			1 (0.92-1.08)	0.96		
HBV Infection History (Y *vs.* N)	0.32 (0.08-1.34)	0.120			0.54 (0.17-1.7)	0.289		
AFP (≥ 400 ng/mL *vs.* < 400 ng/mL)	1.33 (0.75-2.34)	0.330			1.36 (0.89-2.06)	0.155		
CA19-9 (≥ 37.0 U/mL *vs.* < 37.0 U/mL)	0.85 (0.47-1.55)	0.606			0.74 (0.47-1.15)	0.184		

HBP: hypertension, DB: diabetes mellitus, PNI: prognostic nutritional index, MVI: microvascular invasion, BMI: body mass index, CNLC: China Liver Cancer Staging System, AR: anatomical resection, HBV and HCV: hepatitis B virus infection history: no, Y: yes;COPD:Chronic Obstructive Pulmonary Disease, AFP: Alpha-Fetoprotein.

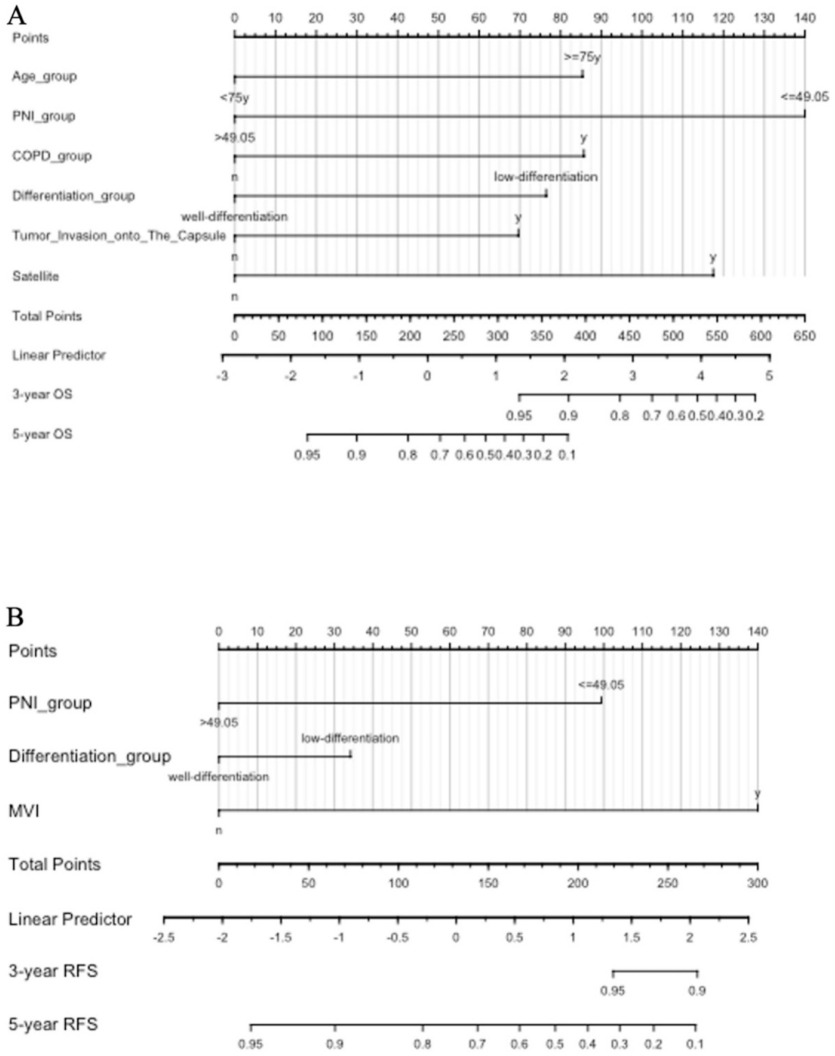

Figure 3. Nomograms to calculate the expected patients' (A) overall survival (OS) and (B) recurrence-free survival (RFS).

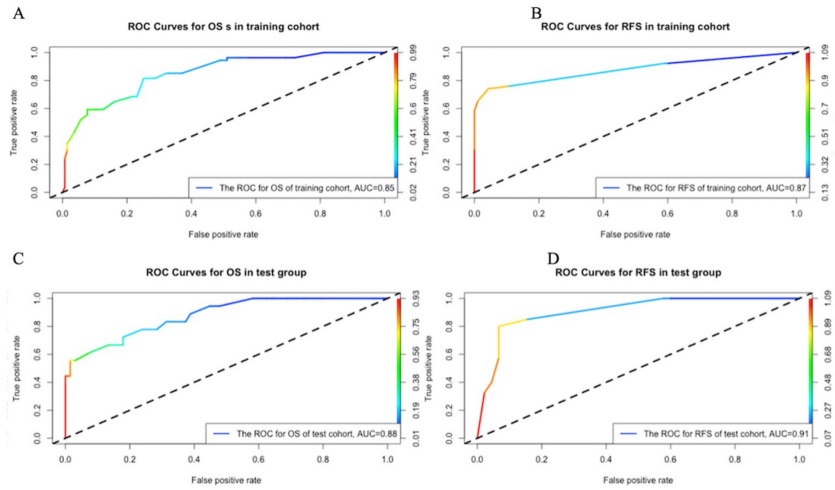

Figure 4. Receiver-operating characteristic (ROC) curve and the corresponding area (AUC) of the predictive models of overall survival (OS) and recurrence-free survival (RFS) for different cohorts. (A) ROC curve of OS and **(B)** RFS in the training cohort. **(C)** ROC curve of OS and **(D)** RFS in the test cohort.

tumor. These can be easily collected in clinical practice. We validated the accuracy and predictive ability of the nomograms for older HCC patients using the calibration curve. In summary, the nomogram was found to be able to accurately predict the OS and RFS of elderly HCC patients at 3 and 5 years. It has good potential for clinical application.

Our study had some limitations. First, this was a retrospective analysis performed in a single medical center. The sample size was not large. External validation could not be performed. There was also a selection bias in the patient population. Second, we could not evaluate the supportive nutritional care methods or the dynamic nutritional status of patients after surgical resection during the postoperative treatment period. This was due to missing data in most of the patient files. Third,

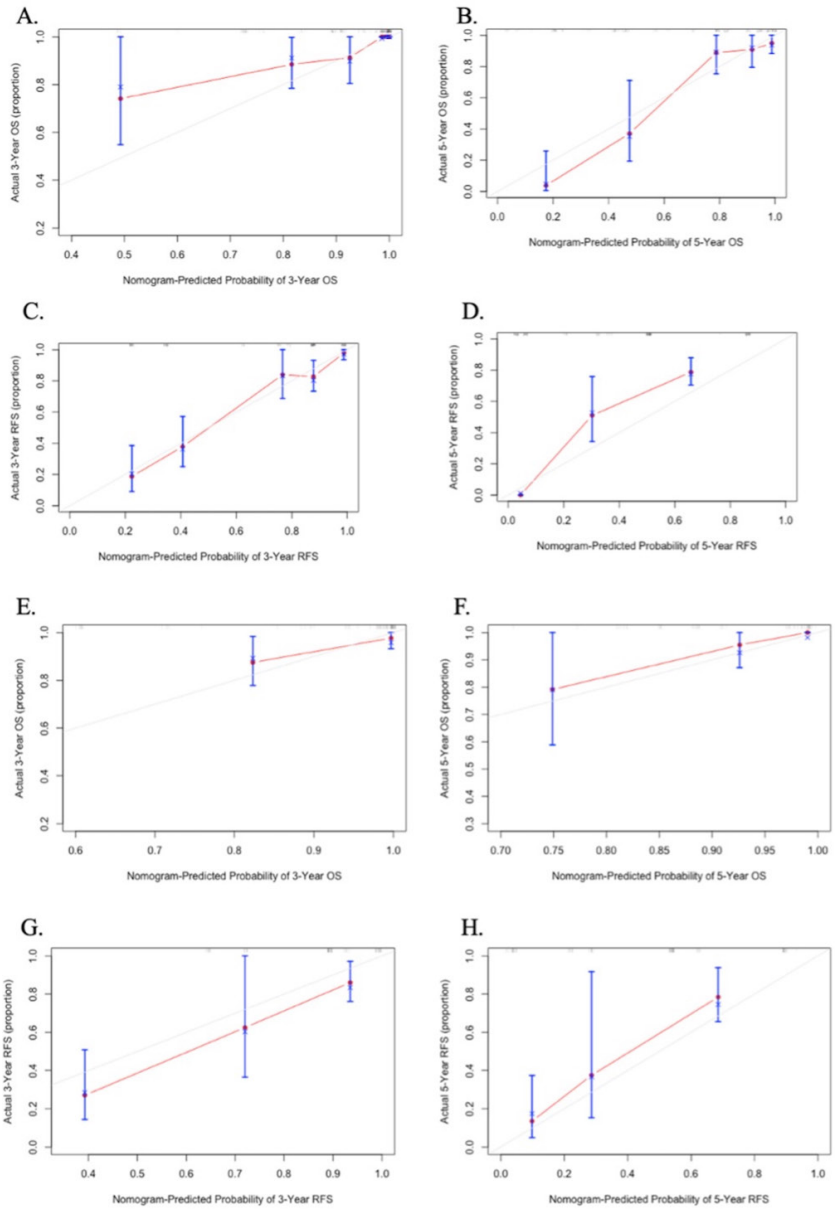

Figure 5. Calibration plots of the models for predicting **(A)** 3-year overall survival (OS) **(B)** 5-year OS. **(C)** 3-year recurrence-free survival (RFS) and **(D)** 5-year RFS for patients in the developing cohort **(E)** 3-year OS **(F)** 5-year OS **(G)** 3-year RFS and **(H)** 5-year RFS in the validation cohort, respectively.

the optimal PNI cut-off values are different in different studies due to different sample sizes and patient inclusion criteria, resulting in a bias in the values. Therefore, to validate the prognostic impact of PNI and its dynamics in elderly HCC patients after surgery, a further large-scale multicenter prospective study is needed.

In conclusion, the results of the present study suggest that the presence of systemic inflammatory response and nutritional status, as measured by PNI, is a useful tool for assessing prognosis in elderly HCC patients following surgery. Two nomogram models with high predictive value were developed after performing univariate and multivariate Cox screening, which could provide a reference for future evaluation of elderly patients with primary HCC. In view of the limited sample size, multicenter, large-sample clinical studies are necessary to investigate the accurate value of our preoperative PNI in the prediction of prognosis for elderly HCC patients after resection.

Funding: This work was supported by 1.3.5 project for disciplines of excellence, West China Hospital, Sichuan University (ZYJC21046); 1.3.5 project for disciplines of excellence-Clinical Research Incubation Project, West China Hospital, Sichuan University (2021HXFH001)

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received August 23, 2023; Revised September 25, 2023; Accepted September 29, 2023.

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Released online in J-STAGE as advance publication October 10, 2023.

Original Article

DOI: 10.5582/bst.2023.01164

The function and immune role of cuproptosis associated hub gene in Barrett's esophagus and esophageal adenocarcinoma

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SUMMARY Barrett's esophagus (BE) is a precancerous lesion of esophageal adenocarcinoma (EAC), with approximately 3-5% of patients developing EAC. Cuproptosis is a kind of programmed cell death phenomenon discovered in recent years, which is related to the occurrence and development of many diseases. However, its role in BE and EAC is not fully understood. We used single sample Gene Set Enrichment Analysis (ssGSEA) for differential analysis of BE in the database, followed by enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO) and GSEA, Protein-Protein Interaction (PPI), Weighted Gene Co-expression Network Analysis (WGCNA), Receiver Operating Characteristic Curve (ROC) and finally Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) and immunohistochemistry (IHC) of clinical tissues. Two hub genes can be obtained by intersection of the results obtained from the cuproptosis signal analysis based on BE. The ROC curves of these two genes predicted EAC, and the Area Under the Curve (AUC) values could reach 0.950 and 0.946, respectively. The mRNA and protein levels of Centrosome associated protein E (CENPE) and Shc SH2 domain binding protein 1 (SHCBP1) were significantly increased in clinical EAC tissues. When they were grouped by protein expression levels, high expression of CENPE or SHCBP1 had a poor prognosis. The CENPE and SHCBP1 associated with cuproptosis may be a factor promoting the development of BE into EAC which associated with the regulation of NK cells and T cells.

Keywords Barrett's esophagus, cuproptosis, hub gene, esophageal adenocarcinoma, immunoinfiltration

1. Introduction

Barrett's esophagus (BE) is characterized by the replacement of normal esophageal squamous cell epithelium with columnar metaplasia and affects approximately 1% worldwide (*1,2*). The incidence of BE associated esophageal adenocarcinoma (EAC) is on the increase. Approximately 3% to 5% of patients with BE will be diagnosed with EAC during their lifetime (*3,4*). However, the specific mechanism of BE that leads to EAC is not yet fully understood.

Cuproptosis is a novel copper ion-dependent cell death type being regulated in cells, and this is quite different from the common cell death patterns such as apoptosis, necroptosis, ferroptosis and pyroptosis (*5,6*). Recently, cuproptosis-related genes have recently been reported to regulate the occurrence and progression of various tumors (*7-10*). The role of cuproptosis in BE in the development of EAC has not been reported yet.

Centrosome associated protein E (CENPE) is a positive end-directed kinetoprotein belonging to the kinase-7 subfamily and plays a key role in mitosis (*11-13*). Studies have reported that the expression of CENPE in EAC is significantly higher than that of pan-cancer, which is related to DNA methylation in EAC and is a negative prognostic factor for patients (*11*). Shc SH2 domain binding protein 1 (SHCBP1) is a SH2 domain specific binding protein (Shc) of Src homologues and collagen homologues, which is mainly involved in the regulation of various signal transduction pathways and plays an important role in cell signal transduction (*14*). SHCBP1 can be used as an immune marker for pan-cancer diagnosis, which is negatively correlated with patient prognosis and can be used as a target for immunotherapy (*14,15*). Recent studies suggest that the high expression of SHCBP1 may be

related to the immunosuppressive microenvironment of tumors, providing a potential new target for tumor immunotherapy (*16,17*). However, the role of CENPE or SHCBP1 in BE, EAC, and BE transformation into EAC has not been reported yet.

In this study, by mining BE cuprotosis related gene modules, CENPE and SHCBP1, markers of BE progression into EAC, were screened out, and verified by gene expression difference, survival analysis and ROC curve. Then, we further verified the clinical samples of our center and analyzed the prognosis, and the results were consistent with previous research reports. By single cell analysis, it was found that CENPE and SHCBP1 were co-expressed on NK cells and T cells, which may relate to the immune function of NK cells and T cells. Therefore, this study deeply analyzed the mechanism of BE developing into EAC through cuprotosis.

2. Materials and Methods

2.1. Data collection

The mRNA expression data of BE (GSE26886) and EAC were downloaded from Gene Expression Omnibus (GEO) database (*https://www.ncbi.nlm.nih.gov/geo/*) and The Cancer Genome Atlas (TCGA) (*https://portal.gdc.cancer.gov/*) database (*18*). All cuproptosis-related marker genes were obtained from the study of Tsvetkov *et al*. (*19*).

2.2. Single sample Gene Set Enrichment Analysis (ssGSEA)

The ssGSEA analysis was commonly performed using Gene Set Variation Analysis (GSVA) package (*20*). Cuproptosis-related genes were considered as a reference gene set, and normalized gene expression data were inputted. Finally, the cuproptosis-related score of each sample was calculated.

2.3. Differential analysis and Weighted Gene Co-expression Network Analysis (WGCNA)

We conducted differential analysis by the limma package for cuproptosis-related score. The screening conditions for the differentially expressed genes (DEG) were: $|\log^2$ (fold change) $| > 1$ and adjusted $P < 0.05$. Then, we drew the volcano maps and heatmaps with the R package "ggplot2" and "pheatmap" to visualize DEGs (*21*). Moreover, we used the WGCNA package to create a gene co-expression network based on the cuproptosis-related score (*22*). Firstly, we built the adjacency matrix using soft-threshold 7 and the topological overlap matrix (TOM). Then the model eigengene was calculated, as well as the correlation between models and clinical traits. Finally, the positively related gene models were selected.

2.4. Functional enrichment analysis

Gene Ontology (GO) (*23*), the Kyoto Encyclopedia of Genes and Genomes (KEGG) (*24*) and Gene Set Enrichment Analysis (GSEA) (*25*) were performed using "clusterProfiler" package. $*P < 0.05$ as a threshold for significantly enriched GO terms and KEGG pathways and adjusted $*P < 0.05$ for GSEA. cBio Cancer Genomics Portal (cBioPortal) (*http://cbioportal.org*) have collected a multidimensional cancer genomics data set. The type and frequency of target genes mutation in tumors were analyzed in "OncoPrint" and "Cancer Types Summary." "OncoPrint" shows the mutation, copy number, and expression of the target gene in the samples in the heatmap. In addition, "Cancer Types Summary" shows the mutation rate of the target genes in a bar chart.

2.5. Patient cohort

All patients for whom samples were collected obtained informed consent from the patient prior to collection. All operations and procedures are in accordance with the rules of the Institutional Ethics Review Committee of the World Health Organization (WHO) Collaborating Centre for Human Production Research and the authorization of the Human Ethics Committee of Fudan University Shanghai Cancer Center (FUSCC).

All EAC sample and benign esophageal were obtained from patients who underwent Esophagostomy without chemotherapy or radiotherapy before surgery between 2012 and 2018, all tissues were surgically removed, and pathological results were reviewed, scored and recorded by experienced pathologists in our center. All clinical records were retrospectively studied.

2.6. qRT-PCR

The total RNA extraction of tissues was performed using RNA extraction kits (QIAGEN, Germany) according to the manufacturer's protocol. qRT-PCR was performed using the Bio-Rad CFX96 Real-Time PCR operating instrument with SYBR qPCR Mix (Takara, Dalian, China). The original Ct values of the target genes CENPE and SHCBP1 were obtained according to the PCR standard reaction curve, and the $2^{-\Delta\Delta Ct}$ method was applied for semi-quantitative analysis. The relative expression of CENPE and SHCBP1 was normalized with GAPDH. The primer sequences are as follows: CENPE-F: 5' -GCTACCGTGATAAACCAGGTTC-3', CENPE-R: 5' -AGGCTCTGAATCGCTCATAGA-3', SHCBP1-F: 5' -GATTCTGCCATACAAGGCTACAA-3', SHCBP1-R: 5' -TGCCCTGGGTATAACTCCCAA-3', SLC31A1-F: GGGGATGAGCTATATGGACTCC, SLC31A1-R: TCACCAAACCGGAAAACAGTAG, GAPDH-F: 5' -GGAGCGAGATCCCTCCAAAAT-3', GAPDH-R: 5' -GGCTGTTGTCATACTTCTCATGG-3'.

2.7. Immunohistochemical (IHC) and immunoreactivity score (IRS)

IHC was used to detect the expression of hub genes (CENPE and SHCBP1) in esophageal cancer tissues. The slice thickness was set at 5 μm, and 3 sections were selected from each specimen. Slides were rinsed and incubated with primary antibodies against CENPE and SHCBP1 (CENPE: 1:1000, abcam, ab5093; SHCBP1: 1:1000, abcam, ab184467). Subsequent antibody detection was carried out with a Cy3-conjugated goat anti-rabbit/mouse secondary antibody (1:300; Invitrogen) (26-28).

The tissue chip (TMA) included 148 normal tissue samples and 164 esophageal cancer tissue samples. TMA was scanned and analyzed by immunoreactivity score (IRS) scoring method. The expression of Hub protein was divided into four levels: 0 (negative), 1 (weakly positive), 2 (moderately positive), and 3 (strongly positive) (*29,30*).

2.8. Survival analysis

Patients with negative (N) and weak positive (W) expression of Hub were included in the low Hub expression group, and patients with moderate positive (M) and strong positive (S) expression were included in the high Hub expression group. The primary outcome of overall survival (OS) was defined as the time from initial treatment to death or the last follow-up, and the secondary end point of progression-free survival (PFS) was defined as the time from initial treatment to disease progression or death (*31-33*). OS and PFS were compared between high-low expression groups.

2.9. Cell lines, culture conditions and treatments

Eca109 and KYSE450 cells were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA) and authenticated by short tandem repeat profiling. All cell lines were cultured at 5% CO_2 and 37°C in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% foetal bovine serum (FBS; Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA).

Copper ionophores: Elesclomol (S1052, Selleck, Shanghai, China) was purchased from Selleck (Shanghai, China). Metal ion chloride: Copper (II) chloride ($CuCl_2$, 751944, Sigma-Aldrich, Darmstadt, Germany), were purchased from Sigma-Aldrich Technology (Darmstadt, Germany).

2.10. Stable and transient transfections

For lentivirus production, the packaging vectors psPAX2 and pMD2.G were cotransfected with lentiviral vectors into HEK293T cells using Lipofectamine 3000 (Invitrogen, CA, USA). The virus particles were harvested after transfection for 48 h. Lentiviruses were then transduced into cells with polybrene (2 μg/mL) to increase the infection efficiency. Finally, the positive cells were selected with 2 μg/mL puromycin for 1 week to establish stable cell lines. Synthetic siRNA oligonucleotides were synthesized by Shanghai Genomeditech Co., Ltd. (Shanghai, China). Lipofectamine RNAiMAX (Invitrogen, CA, USA) was used for siRNA transfection. Transient transfection was performed using Lipofectamine 3000 (Invitrogen, CA, USA) according to the manufacturer's instructions. The transfection efficiencies were verified by qRT-PCR and WB.

2.11. Cell viability assays

For the cell proliferation assay, cells were seeded and cultured in 96-well flat-bottom plates, with each well containing 5000 cells in 100 μL. 10 μL Cell Counting Kit-8 (CCK-8) reagent (MedChemExpress, Monmouth Junction, NJ, USA) was added to 90 μl complete culture substrate, and the cell proliferation ability was measured at 450 nm absorption wavelength after 1 hour.

2.12. Intracellular Content of Copper

Intracellular content of copper was measured by copper assay kit (ab272528, Abcam, Boston, MA, USA). Then, 1×10^7 Eca109 cells after elesclomol-$CuCl_2$, shCENPE, shSHCBP1, shSHCBP1-$CuCl_2$, or shSHCBP1-$CuCl_2$ treatment were collected, and they were homogenized after adding 1 ml distilled water. Next, 100 μL samples per well were transferred to a flat-bottom 96-well UV plate. For each assay well, 35 μl Reagent, 5 μL Reagent B and 150 μL Reagent C were thoroughly mixed with samples, incubated for 5 min at room temperature and the optical density read at 359 nm using a microplate reader (Synergy 2, Bio-Tek Instruments, Winooski, VT, USA). Copper content was normalized by protein concentration.

2.13. Western blotting (WB)

Cells were lysed in 10% NP40 (ratio: 890 μL NP40 buffer + 100 μL NP40 + 10 μL protease inhibitor cocktail) (Shanghai Shenger Biotechnology) and lysed in a 4°C centrifuge for 30 minutes (min). Then, the lysates were centrifuged (12,000 g, 4°C, 15 min) to collect the supernatants. The Bio-Rad Protein Assay kit (Hercules, CA, USA) was used to quantify the protein concentration. Approximately 25-30 μg of protein from each sample was separated by 10% SDS–PAGE and electroblotted onto a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% nonfat milk, the membranes were incubated individually overnight at 4°C with the corresponding primary antibodies (SLC31A1

(Abmart Technology, Shanghai, A13469) and β-actin (Sigma-aldrich, #A3854).) and incubated with the secondary antibody, either horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit, for 1 h at room temperature (RT).

2.14. Immune cell infiltration analysis

Immune cell infiltration analysis was evaluated between high and low cuproptosis-related score using Immuno-Oncology Biological Research (IOBR) package (*34*). In this part, a total of seven algorithms (CIBERSORT, EPIC, MCP, XCELL, ESTIMATE, TIMER, QUANTISEQ) were used. Then, the differences of all immune cell infiltration were visualized by boxplots. Subsequently, we drew a heatmap to show the level of immune cell infiltration.

2.15. Single cell-seq analysis

Single cell-seq data of EAC (GSE173950; Platform: Drop-seq) was downloaded and loaded in R (*35*). Seurat package was used to process the single cell data (*36*). Quality control was performed by calculating the percentage of mitochondria. The threshold of mitochondria was 10%. Then top 2000 variable genes were filtered and normalized. Principal component analysis (PCA) was performed on the scaled data and t-distributed stochastic neighbor embedding (t-SNE) algorithm was performed for cluster identification. The SingleR package was used to annotate all cell types and FindMarkers was used to performed differential analysis (*37-39*).

2.16. Statistical analysis

Statistical analysis was performed with IBM SPSS 23.0 (Chicago, IL, USA), GraphPad Prism 8, and R language. Comparisons between two conditions were based on a two-sided Student's test. OS and PFS curves were plotted with the Kaplan-Meier (KM) method and log-rank test. All the data are shown as the mean ± SD. The results of all statistical analyses were reported as *P* values from two-tailed tests, and $P < 0.05$ was considered to indicate statistical significance (*$P<0.05$, ** $P<0.01$, and *** $P<0.001$).

3. Results

3.1. The results of differential analysis

After data normalization (Figure S1A, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=171*), we performed differential analysis between high cuproptosis-related score group and low cuproptosis-related score group and finally identified 236 differently expressed genes (57 down regulated and 179 up regulated). Volcano plot showed all the DEGs and top 10 were marked, while the heatmap showed the top 30 DEGs (Figures 1A-B). Then, functional enrichment analyses were performed. We found cell cycle-related biological processes were significantly enriched, such as cell cycle and G2M checkpoint (Figures 1C, 1E, and Figures S1B-D, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=171*). Moreover, some tumor-associated biological processes were also enriched, for instance E2F targets and MYC targets (Figure 1E). These indicated that cuproptosis-related score was associated with the tumor malignancy. Finally, Protein-Protein Interaction (PPI) network analysis was performed and ten hub genes were identified by cytoHubba (Figure 1D).

3.2. Cuproptosis-related Gene Models of Barrett's Esophagus

A dendrogram of all BE samples with cuproptosis-related score was clustered using average linkage (Figure S2A, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=171*). Then co-expression analysis was performed to identify gene models and 7 was selected as the soft-threshold (Figure 2A). Based on Spearman correlation coefficient, the heatmap of gene module-trait relationship was drawn to show the correlation between them (Figure 2B). In this study, three gene models (MEsalmon, MEgreen, MEcyan) were positively corrected with the cuproptosis-related score (Figure 2C). Then we combined all the genes and performed functional enrichment analyses and PPI network analysis (Figures 2D-E; Figures S2B-D, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=171*). We found that cell cycle and metabolism-related pathways were significantly enriched. We surmised that copper ions would intervene tricarboxylic acid cycle and further influence other metabolic processes.

Finally, we took an intersection of the results from DEGs and WGCNA to obtain two hub genes (Fig. 2F).

3.3. Hub genes in EAC

After getting two hub genes, differential analysis, ROC curve and GSEA were performed. Both the CENPE and SHCBP1 were expressed higher in tumor and could be effective biomarkers (Area under curve (AUC) for CENPE: 0.95; AUC for SHCBP1: 0.946; Figures 3A-D). Moreover, the results of single gene GSEA showed that cell proliferation related pathways were significantly enriched, such as cell cycle, DNA replication and p53 signaling pathway (Figures 3E-F).

By analyzing the correlation between CENPE and SHCBP1 in EAC, we found a strong correlation between CENPE and SHCBP1 (Figure 4A). Moreover,

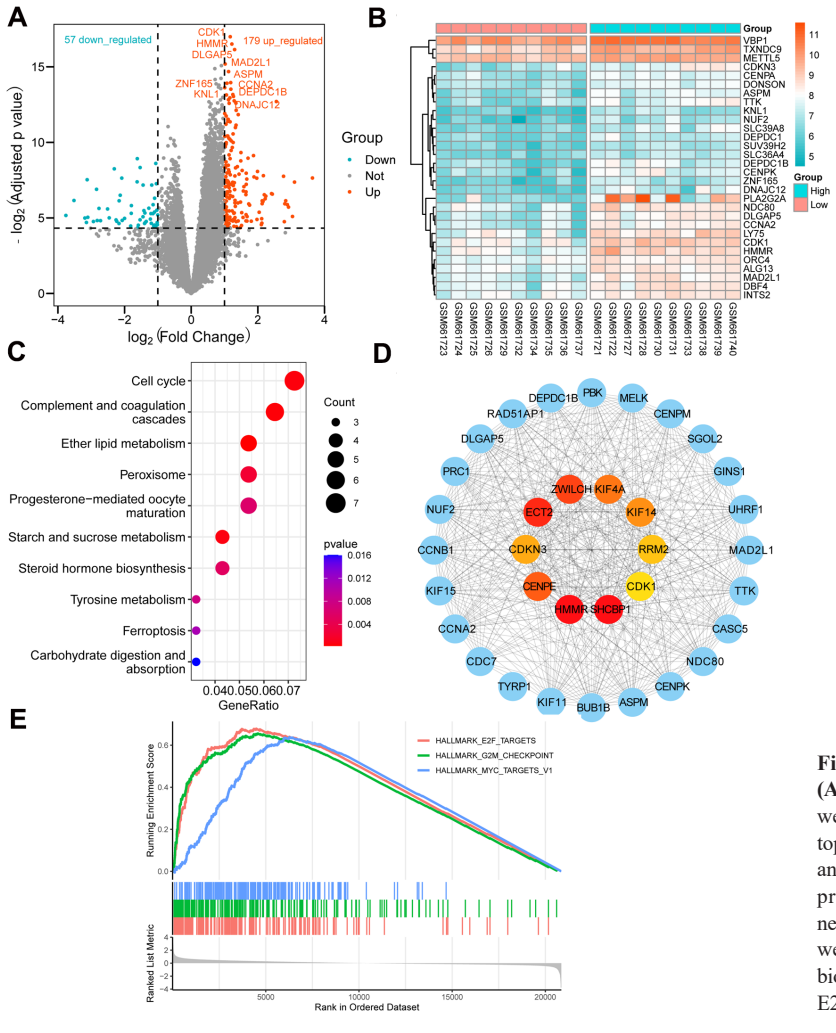

Figure 1. The results of differential analysis. **(A)** Volcano plot showed all the DEGs and top 10 were marked, while **(B)** the heatmap showed the top 30 DEGs. **(C)** Through functional enrichment analysis, it was found that cycle related biological processes were significantly enriched. **(D)** PPI network analysis was performed and ten hub genes were identified by cytoHubba. **(E)** Tumor related biological processes were significantly enriched in E2F targets and MYC targets.

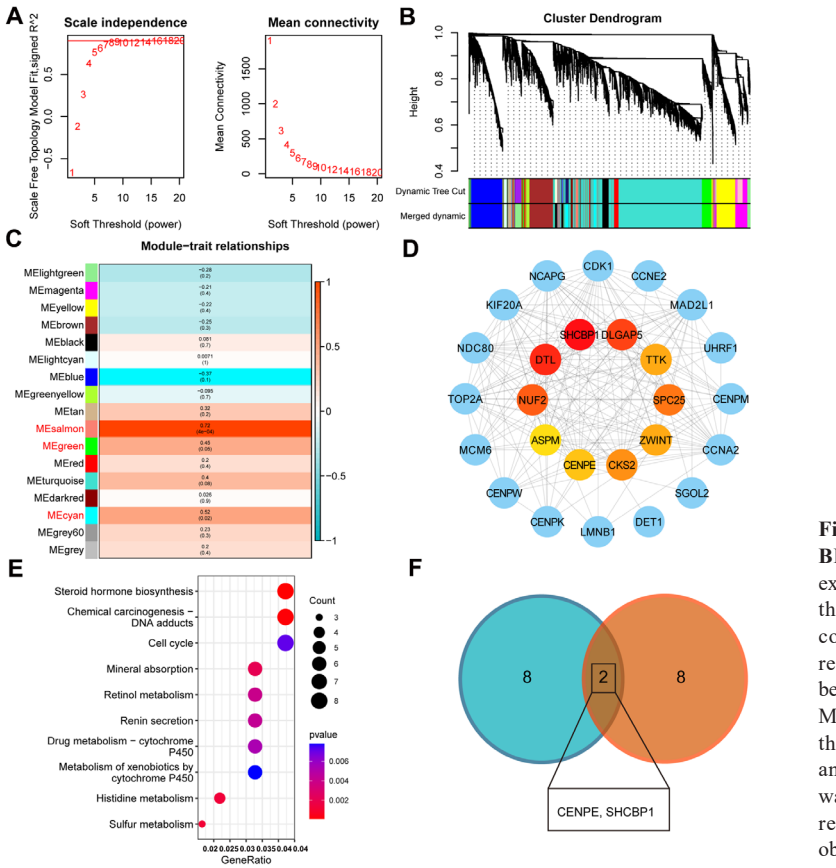

Figure 2. Cuproptosis-related gene models of BE. **(A)** The gene model was explored by co-expression analysis, and 7 was selected as the soft threshold. **(B)** Based on Spearman correlation coefficient, the heatmap of gene module-trait relationship was drawn to show the correlation between them. **(C)** Three gene models (MEsalmon, MEgreen, MEcyan) were positively corrected with the cuproptosis-related score. **(D)** PPI network analysis and **(E)** functional enrichment analysis was performed by combining all genes. **(F)** The results of DEGs and WGCNA were crossed to obtain two hub genes.

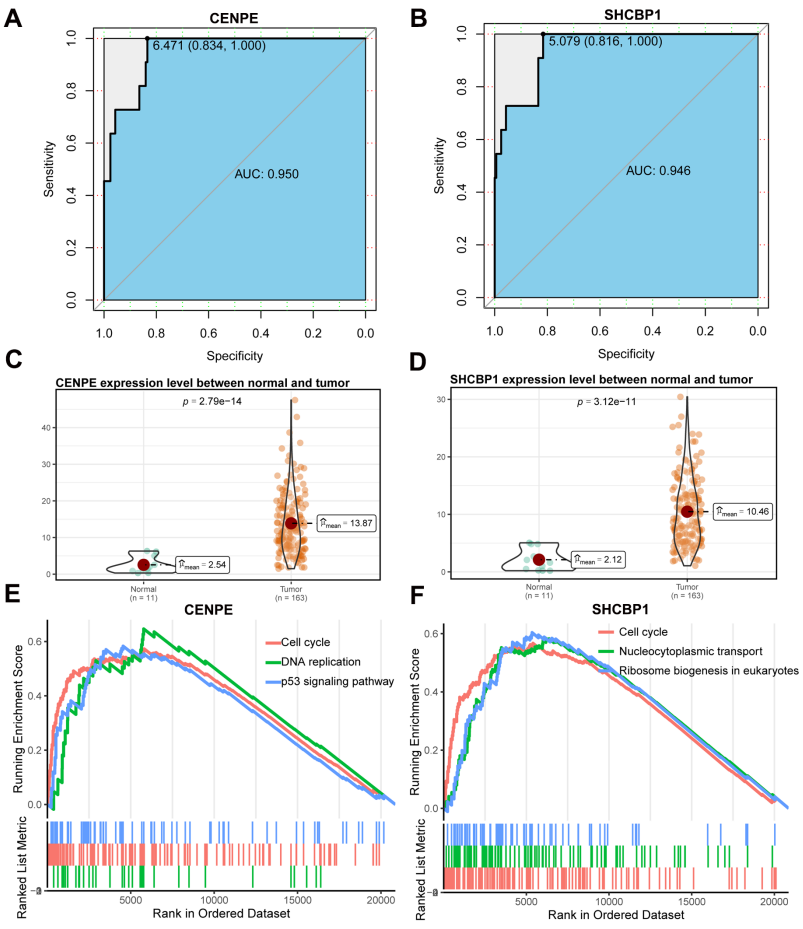

Figure 3. ROC curve and GSEA analysis based on hub gene. (A) The ROC curve AUC of CENPE could reach 0.950. **(B)** The ROC curve AUC of SHCBP1 can reach 0.946. **(C)** Both the CENPE and **(D)** SHCBP1 were expressed higher in tumor. **(E)** GSEA of CENPE and **(F)** SHCBP1 showed that cell proliferation related pathways were significantly enriched, such as cell cycle, DNA replication and p53 signaling pathway.

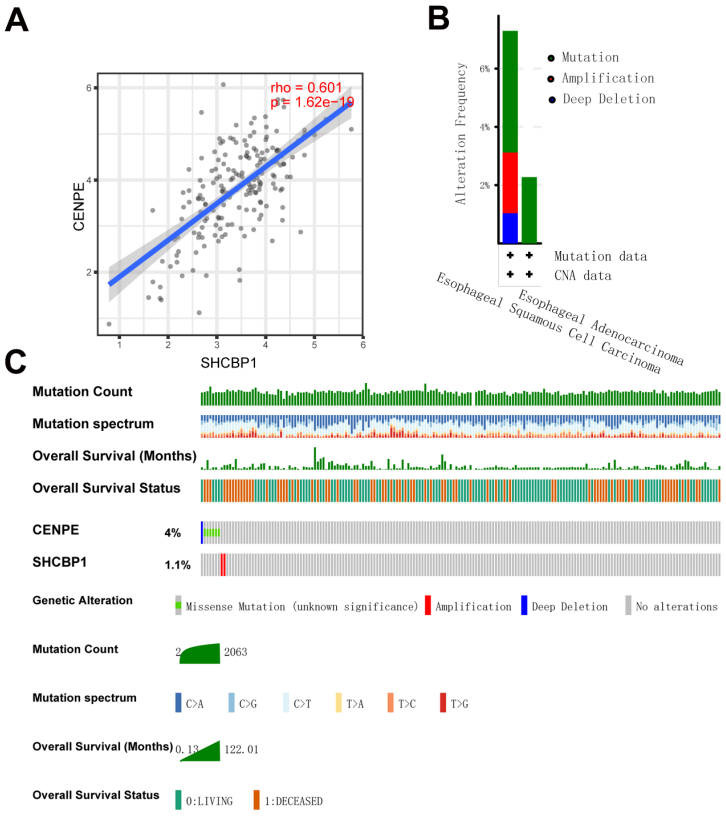

Figure 4. Mutations of CENPE and SHCBP1 in EAC. (A) CENPE was positively correlated with SHCBP1. **(B)** Genetic variants of two hub genes were present in 7% of ESC patients and 2% of EAC patients. **(C)** CENPE mutations were the most common (4%), and most were missense mutations.

we investigated the mutational landscape of two hub genes. By analyzing the cBioPortal data, seven percent of esophageal squamous cell carcinoma (ESC) patients and two percent of EAC patients had genetic alternations (Figure 4B). More specifically, mutations were most frequently identified for CENPE (4%), most of which were missense mutation (Figure 4C).

3.4. Validation of mRNA and protein levels in clinical esophageal adenocarcinoma tissues

The correlation between hub gene (*CENPE* or *SHCBP1*) expression level and clinicopathological parameters of patients was analyzed, and the results showed that CENPE was correlated with tumor stage (*P = 0.045, Table 1). Then, mRNA comparison was conducted between EAC tissues and normal tissues, and the results showed that the expressions of CENPE and SHCBP1 were significantly increased in the EAC group (*P-CENPE*<0.01, *P-SHCBP1*<0.001; Figures 5A-B). IHC results showed that the expressions of CENPE and

Table 1. Correlation between hub gene (*CENPE* or *SHCBP1*) and clinicopathological parameters in patients with EAC.

Variables	*n*	Expression of CENPE		*P* value (CENPE)	Expression of SHCBP1		*P* value (SHCBP1)
		Low	High		Low	High	
Tumors	164	73	91		69	95	
Age (years)				0.494			0.757
≤ 65	76	36	40		31	45	
> 65	88	37	51		38	50	
Sex				0.627			0.809
Male	95	35	60		45	50	
Femal	69	28	41		34	35	
Tumor stage				0.045			0.756
pT1-pT2	69	37	32		30	39	
pT3-pT4	95	36	59		39	56	
Grading				0.473			0.345
G1-G2	78	37	41		40	48	
G3-G4	86	36	50		29	47	
pN category				0.763			0.680
N0-N1	92	40	52		40	52	
N2-N3	72	33	39		29	43	
M status				0.318			0.568
M0	88	36	52		43	55	
M1	76	37	39		26	40	

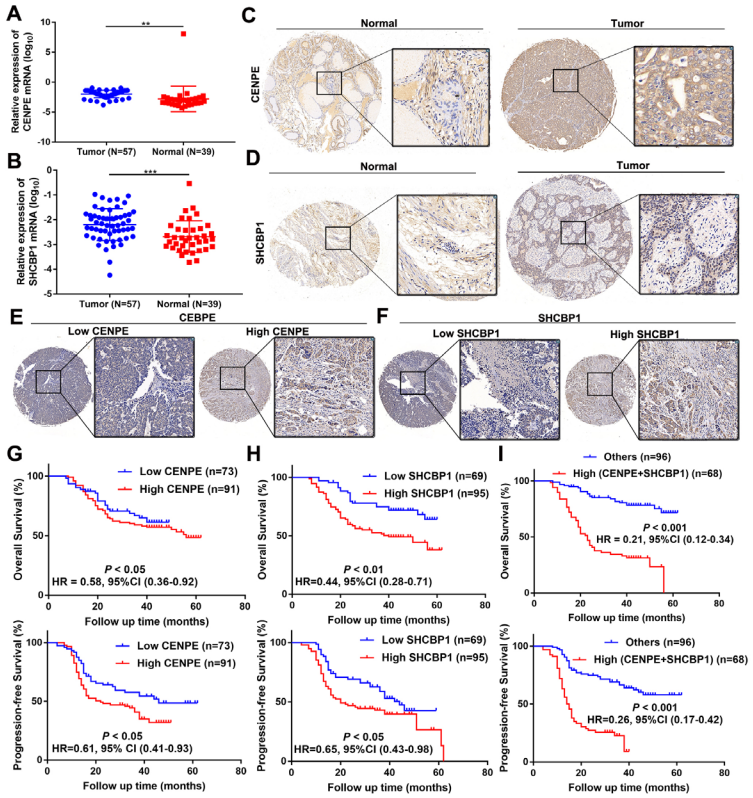

Figure 5. CENPE and SHCBP1 are overexpressed in EAC and are clinically associated with patient prognosis. (A-B) The expressions of CENPE and SHCBP1 mRNA were significantly increased in EAC tissues compared with normal tissues. **(C-D)** Immunohistochemical staining of CENPE and SHCBP1 in normal esophageal tissue and EAC. **(E-F)** Representative pictures of CENPE and SHCBP1 high and low IHC groups. **(G)** CENPE was divided into two groups according to the level of CENPE and it was found that the high expression group had a poor prognosis (*P-OS* < 0.05, HR = 0.58, 95% CI (0.36 - 0.92); *P-PFS* < 0.05, HR = 0.61, 95% CI (0.41 - 0.93). **(H)** SHCBP1 was divided into two groups according to the level of SHCBP1 and it was found that the high expression group had a poor prognosis (*P-OS* < 0.01, HR = 0.44, 95% CI (0.28 - 0.71); *P-PFS* < 0.05, HR = 0.65, 95% CI (0.43 - 0.98). **(I)** The results showed that the high CENPE and SHCBP1 protein expression group had lower OS and PFS (***P-OS* < 0.001, HR = 0.21, 95% CI (0.12 - 0.34); ***P-PFS* < 0.001, HR = 0.26, 95% CI (0.17 - 0.42)).

SHCBP1 were significantly increased in EAC (Figures 5C-D).

3.5. The expression of hub gene was negatively correlated with the prognosis of patients with EAC

EAC TMA were stained with CENPE or SHCBP1, respectively, and scored by IRS. After scoring, they were divided into high and low groups. The representative IHC pictures of CENPE and SHCBP1 are shown in Figures 5E-F. The prognostic results showed that the high expression group of CENPE had poor OS (P<0.05, HR = 0.58, 95% CI (0.36-0.92)) and PFS (P<0.05, HR = 0.61, 95% CI (0.41-0.93)), the high expression group of SHCBP1 had poor OS (P<0.01, HR = 0.44, 95% CI (0.28-0.71)) and PFS (P<0.05, HR = 0.65, 95% CI (0.43-0.98)), and the OS (P<0.001, HR = 0.21, 95% CI (0.12-0.34))and PFS (P<0.001, HR = 0.26, 95% CI (0.17-0.42)) of patients with high expression of CENPE and SHCBP1 was worse than that of other patients (Figure 5G-I).

3.6. CENPE and SHCBP1 can induce cuproptosis in EAC

Knocking down CENPE or SHCBP1 significantly inhibited the growth of Eca109 and KYSE450 cells (Figure 6A). Knocking down CENPE, SHCBP1, $CuCl_2$ treatment or elesclomol-$CuCl_2$ promoted Eca109 cells death alone, and combined with CuCl2 or elesclomol-$CuCl_2$ aggravated the cell death in Eca109 cells (Figure 6B). The copper accumulation increased after Knocking down CENPE or SHCBP1 in Eca109 cells, also suggesting that copper is involved these two genes induced cell death (Figure 6C). Next, the mRNA and protein expression of the copper import gene SLC31A1 was increased after knocking down CENPE or SHCBP1 with or without $Cucl_2$ (Figure 6D).

3.7. Association between cuproptosis-related score and immune infiltration

Firstly, seven different immune infiltration algorithms were utilized to calculate the immune infiltration landscape (Figure S3, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=171*). As shown in Figure 7A, there were more immune cell infiltrates in the low cuproptosis-related score group, especially the T cells. Then relationship between the level of immune infiltration and two hub genes (CENPE, SHCBP1) were estimated. It was found that both two hub genes positively correlated with Neutrophil and Myeloid dendritic cell (Figure 7B-C).

3.8. Single cell sequencing analysis

In order to confirm the functions of CENPE and SHCBP1, single cell-seq data were analyzed. After quality control (Figures S4A-E, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=171*), all 34,706 cells were retained for t-SNE analysis. Based on the signature gene expression, 19 cell clusters were annotated (Figure 8A). According to the well-known marker genes (Figure S4F, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=171*), we designated all clusters as pre-myeloid cells, epithelial, fibroblasts, NK and T cells, endothelial, neutrophils and B cells (Figure 8B). Interestingly, we found CENPE and SHCBP1 were co-expressed in a special NK and T sub cell type (Figures 8C-D). Next,

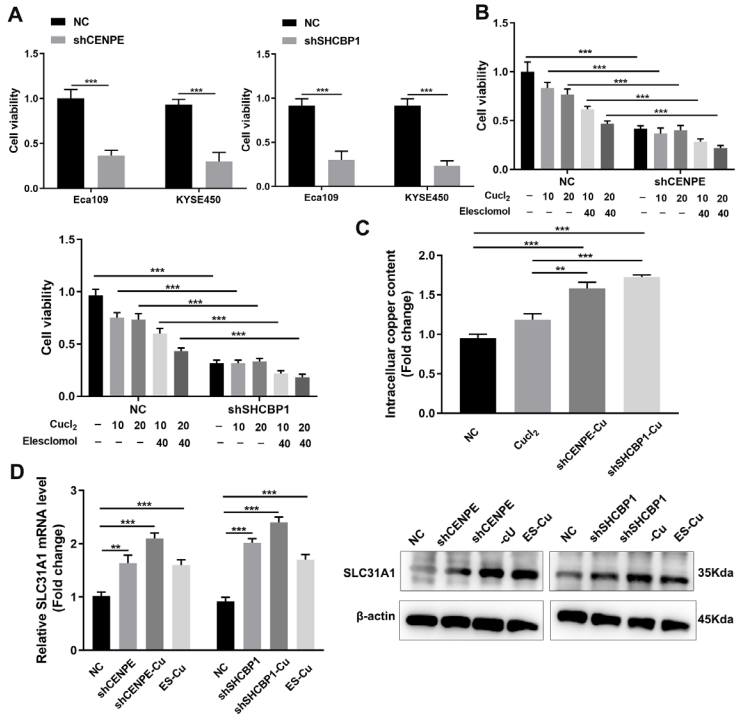

Figure 6. CENPE and SHCBP1 can induce cuproptosis in EAC. (A) Knocking down CENPE or SHCBP1 significantly inhibited the growth of Eca109 and KYSE450 cells. **(B)** Viability of Eca109 cells after knocking down CENPE or SHCBP1, or indicated elesclomol-Cu treatment. **(C)** Intracellular copper content in EAC cells after knocking down CENPE or SHCBP1, or $Cucl_2$ (10μM) treatment. **(D)** mRNA levels and protein levels of copper ion transporter in Eca109 cells after knocking down CENPE or SHCBP1, or ES-Cu treatment.

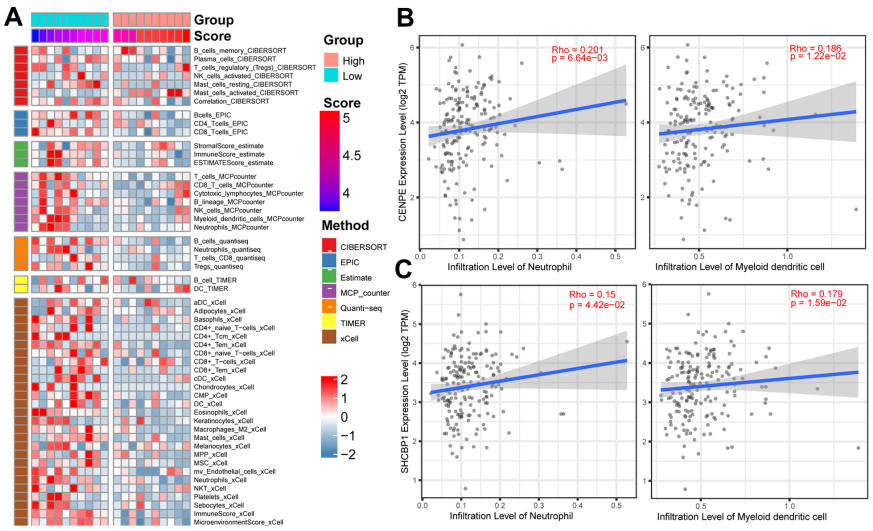

Figure 7. Association between cuproptosis-related score and immune infiltration. (A) There were more immune cell infiltrates in the low cuproptosis-related score group, especially the T cells. **(B-C)** By assessing the relationship between immune infiltration levels and two central genes (CENPE, SHCBP1), we found that both neutrophils and myeloid dendritic cells were able to positively correct both hub genes.

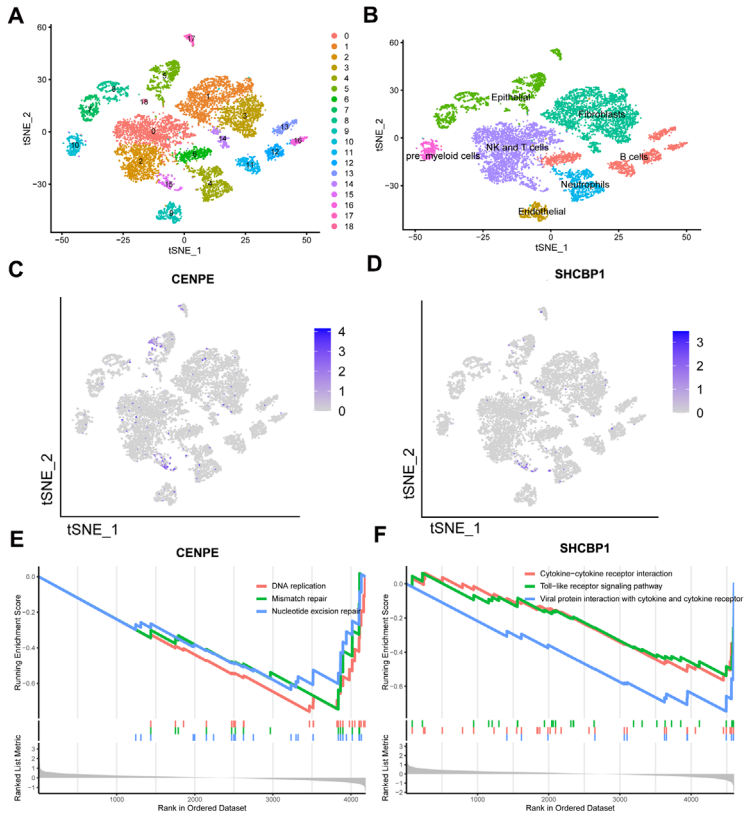

Figure 8. The function of CENPE and SHCBP1. (A) Single cell sequence data sequencing revealed that 34,706 cells were used for t-SNE analysis and 19 cell clusters were annotated based on characteristic gene expression. **(B)** According to the well-known marker genes, we designated all clusters as pre-myeloid cells, epithelial, fibroblasts, NK and T cells, endothelial, neutrophils and B cells. **(C-D)** CENPE and SHCBP1 can be co-expressed in a specific NK and T subcell type**. (E)** CENPE is associated with biological processes related to DNA repair and **(F)** SHCBP1 is associated with biological processes related to inflammation or immunity.

GSEA analysis was performed to uncover the function of two hub genes in this sub cell type. It was showed that CENPE corrected with DNA repair-related biological processes and SHCBP1 corrected with inflammation or immune related biological processes (Figures 8E-F). We considered that CENPE and SHCBP1 might be related to the immune function of NK cells and T cells.

4. Discussion

BE is one of the most common premalignant lesions in which normal squamous epithelium of the esophagus is replaced by metaplastic columnar epithelium. EAC develops through progression from BE to low-grade and high-grade dysplasia (LGD/HGD) and to adenocarcinoma (*40,41*). Previous studies found that the incidence of EAC in patients with BE was 11.34 times higher than that in the general population (*42*). Cuproptosis is a recently discovered form of cell death that occurs due to an excess of copper ions in cells, resulting in the generation of reactive oxygen species and oxidative stress. This process can lead to various types of cellular damage and ultimately cell death (*19*). Targeting copper cytotoxicity and cuproptosis are considered potential options for treating oncological diseases.

In this study, we used ssGSEA to calculate

cuproptosis-related scores of BE patients. Based on the score, two hub genes (CENPE and SHCBP1) were identified by differential gene analysis and WGCNA. We further targeted at these two hub genes to predict EAC by ROC curve, and the AUC could reach 0.950 and 0.946 respectively. Bioinformatics analysis showed that the expression of these two genes was significantly increased in EAC. In addition, mutations of these two genes in esophageal cancer were analyzed. Genetic variants were found in 7% of patients with ESC and 2% of patients with EAC.

We further verified the expression of these two genes in clinical tissues of EAC, and found that the mRNA and protein levels of CENPE and SHCBP1 were significantly increased. The two hub genes were grouped by protein expression level, and the results showed that patients with high CENPE expression, high SHCBP1 expression or both high expression groups had poor prognosis. Previous studies have reported that CENPE was increased in EAC, but only at the mRNA level, with a very limited sample size. At the same time, CENPE was negatively correlated with prognostic OS, while other prognostic PFS were not involved, and the sample follow-up data was poor (*11*). The reports on SHCBP1 are only database-based results, and mRNA and protein levels have not been verified in clinical samples (*14*). In this study, we made full use of our tumor center's powerful clinical sample bank and patient follow-up data to analyze mRNA and protein levels and prognosis. These findings suggest that cuproptosis-related CENPE and SHCBP1 genes may promote the development of BE into EAC, and may be a poor prognostic factor for patients. In addition, we further verified in esophageal cancer cells that CENPE and SHCBP1 can cause cuproptosis and are hub genes associated with cuproptosis. At present, the study of CENPE and SHCBP1 genes in BE and the transformation of BE to EAC has not been reported.

DNA damage, genetic changes and chronic inflammation have also been reported to be important factors in the development of epigenetic changes in BE (*43*). The process of cuproptosis is also accompanied by an inflammatory response (*7*). In order to confirm the function of CENPE and SHCBP1 in EAC, we analyzed the relationship between CENPE and SHCBP1 genes and inflammatory immunity. The results showed that the infiltration of immune cells, especially T cells, was more in the group with low copper death score. Previous studies have reported that SHCBP1 is significantly associated with CD8+ T cell infiltration, cytokines, cytokine receptors, MHC genes and multiple immune antigens in pancarcinoma (*14*). In addition, CENPE was strongly associated with SHCBP1, and they were strongly associated with neutrophil or myeloid dendritic cell infiltration.

Epigenetically inactivated genes in BE were found to be involved in important molecular pathways of tumorigenesis, including cell cycle regulation, apoptosis, DNA repair, antioxidative defense, or cell adhesion (*44-49*). Single-cell RNA sequencing has become a powerful tool to characterize different functional states at single-cell resolution and is widely used in cancer research (*50,51*). By single cell sequencing, CENPE and SHCBP1 were co-expressed in NK and T cells. GSEA analysis showed that CENPE was negatively correlated with DNA repair and SHCBP1 was negatively correlated with biological

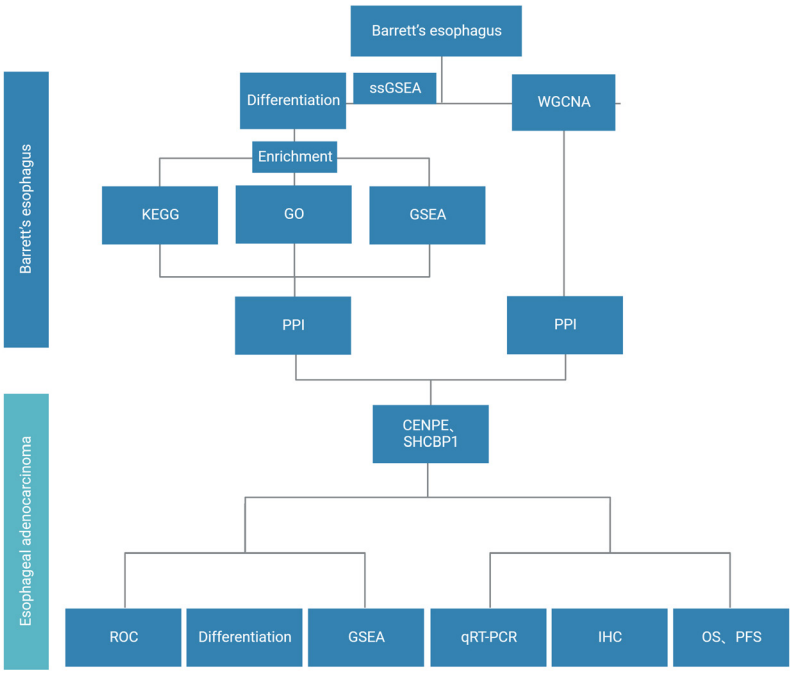

Figure 9. Flow diagram of full text.

processes related to inflammation and immunity. Therefore, CENPE and SHCBP1 may associated with the immune function of NK cells and T cells.

The main line of this paper is original, which starts from the clinical BE, which excavates the gene module related to cuproptosis. Through difference analysis, survival analysis, and ROC analysis, BE and EAC are connected through the copper death gene, which indicates which BE may develop into EAC (Figure 9). Therefore, early screening and biopsy analysis of BE can avoid the occurrence of EAC, which is of great clinical transformation value.

In conclusion, the hub gene (CENPE and SHCBP1) associated with cuproptosis may be a factor promoting the development of BE into EAC which associated with the regulation of NK cells and T cells.

Acknowledgements

We are grateful to all those who helped with this project.

Funding: This work was supported, in part, by grants from the National Natural Science Foundation of China (No. 81972431, 82272898); Shanghai Anticancer Association EYAS PROJECT (No. SACA-CY19A07); and the Female Tumour Project of Shanghai Key Clinical Specialty (No. SHSLCZDZK06301).

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received July 12, 2023; Revised October 10, 2023; Accepted October 15, 2023.

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Released online in J-STAGE as advance publication October 21, 2023.

Brief Report

DOI: 10.5582/bst.2023.01214

Genome-wide identification of mammalian cell-cycle invariant and mitotic-specific macroH2A1 domains

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SUMMARY The histone variant macroH2A has been found to play important regulatory roles in genomic processes, especially in regulating transcriptomes. However, whether macroH2A nucleosomes are retained on mitotic chromosomes to enable maintenance of cell-specific transcriptomes is not known. Here, examining mouse embryonic fibroblast cells (NIH-3T3) with native chromatin immunoprecipitation and sequencing (nChIP-seq), we show that the overwhelming majority (~90%) of macroH2A1 domains identified at the G1/S stage are indeed stably retained on mitotic chromosomes. Unexpectedly though, we also find that there are a number of macroH2A domains that are specific for either mitotic or G1/S cells. Notably, more than 7,000 interphase expressed genes flanked by macroH2A1 domains are loaded with macroH2A1 nucleosomes on the mitotic chromosome to form extended domains. Overall, these results reveal that, while the majority of macroH2A1 domains are indeed faithfully transmitted through the mitotic chromosomes, there is a previously unknown cell-cycle dependent exchange of macroH2A1 nucleosomes at numerous genomic loci, indicating the existence of molecular machineries for this dynamically regulated process. We anticipate that these findings will prove to be essential for the integrity of mitotic progression and the maintenance of cellular identity.

Keywords Histone variant macroH2A1, cell cycle, mitotic chromosomes, nChIP-seq

1. Introduction

The most basic structural and regulatory unit of eukaryotic chromatin is the nucleosome, consisting of an octamer of four core histone proteins, H2A, H2B, H3 and H4 or their variants. The locations of nucleosomes within the genome, their composition, as well as the modifications to the core histones are all well-known to play important roles in a wide range of genomic processes (*1,2*). Among the known histone variants, macroH2A is markedly distinguished, both structurally and functionally (*3*). Structurally, macroH2A is roughly three-fold larger than the canonical H2A histone owing to the presence of a unique 30 kDa macro-domain at its C-terminus (*4*). Functionally, previous work has found that macroH2A forms broad domains spanning many kilobases which are distributed over the entire genome (*4,5*) and often associated with transcriptional silencing with colocalization with heterochromatin domains marked by H3K27me3 and H3K9me3 in some cases (*6-8*). As a result, macroH2A is thought to contribute significantly to the means by which the cell produces its characteristic transcriptome, and thus its identity.

As such, it may be expected that the locations of macroH2A within the genome are retained through mitosis in order to ensure the maintenance of the phenotypical properties within the daughter cells, similar to what is believed for the histone modifications (*9-12*). However, to date, direct examinations of the nucleosomal compositions of the mitotic chromosomes, and in particular the macroH2A content, has not been reported. In fact, owing to the significant compaction of the chromatin during mitosis, it may be that macroH2A is removed from the chromatin prior to mitosis, owing to its large macrodomain, to facilitate a maximal extent of packaging of the genome to ensure its faithful transmission to daughter cells (*13,14*). Indeed, there has been recent work describing mechanisms by which macroH2A can be dynamically exchanged within interphase (*15*), although whether these, or other mechanisms, are functional in a cell cycle dependent manner is presently unknown.

To address these questions, we performed genome-wide profiling of macroH2A1 on both mitotic chromosomes and G1/S chromatin in mouse embryonic fibroblast cells (NIH-3T3) using native chromatin immunoprecipitation and sequencing (nChIP-seq). We found that a large fraction of macroH2A1 domains is indeed invariant during the cell cycle. However, mitotic chromosomes also contain substantially more macroH2A1 domains when compared with that of G1/S chromatin, indicating that macroH2A1 must be reloaded before and unloaded after mitosis at specifically defined genomic regions. Many of these mitotic-specific domains overlap with genes that are expressed in the interphase. Thus, while the majority of macroH2A1 domains are preserved in the mitotic chromosomes, there are also numerous specifically defined genomic regions that exhibit changes in macroH2A1 nucleosomes in a cell cycle dependent manner, whose functional consequences may also prove to be critical for the proper progression of mitosis and the maintenance of phenotypic properties.

2. Materials and Methods

2.1. Cell culture and cell cycle synchronization

Mouse embryonic fibroblast NIH-3T3 cells were cultured in DMEM (GIBCO, Carlsbad, CA, USA) supplemented with 10% FBS (GIBCO, Carlsbad, CA, USA) and 1% Pen/Strep (GIBCO, Carlsbad, CA, USA) at 37℃, 5% CO_2.

To obtain G1/S synchronized NIH-3T3 cells, cells were cultured with starvation treatment (DMEM with 0.5% FBS and 1% Pen/Strep) for 48 h, followed with fresh medium containing 1% Aphidicolin (Abcam, Cambridge, MA, USA) for 18 h before collection. With this procedure, about 97% of the collected cells were G1/S cells based on FACS (Fluorescence-Activated Cell Sorting) analysis (Supplementary Figure S1A, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=168*). To obtain mitotic cells, cells in culture reached 70-80% confluence were treated with colcemid (100 ng/mL, Sigma-Aldrich, St. Louis, MO, USA) for 12 h, and mitotic cells were shaken-off and collected for mitotic chromosome purification. The purity of the collected mitotic cells was about 77% by FACS analysis (supplementary Figure S1B, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=168*).

2.2. Mitotic chromosome purification and mononucleosome preparation

To minimize the contamination of interphase cells, highly purified mitotic chromosomes were obtained using the protocol described previously (*16*) with modifications (see SI for details). Briefly, the collected mitotic cells were centrifuged and resuspended in a hypotonic solution (75 mM KCl) for 30 min at 37℃. The cells were then suspended in the polyamine buffer (PA buffer) (*17*) developed to protect the integrity of the chromosomes and were homogenized on ice. Large debris were removed with centrifugation at 190× *g* at 4°C and the supernatant was filtered by 10 μm and 5 μm filter membranes sequentially. The mitotic chromosomes were collected by centrifugation and the pellet was resuspended in the MNase (Micrococcal Nuclease) buffer containing spermidine and spermine, as well as a protease inhibitor cocktail. Under such conditions, the mitotic chromosome morphology remained intact as examined with fluorescence microscopy with DAPI (Vector Laboratories, Burlingame, CA, USA) staining (supplementary Figure S1C, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=168*). These mitotic chromosomes were then digested with MNase (3,000 gel units/mL, NEB, Ipswich, MA, USA) at 4°C overnight and terminated with 20 mM EDTA. The supernatant containing mononucleosomes was collected after centrifugation at 10,000× *g* at 4°C. As shown in Figure S1D (*http://www.biosciencetrends.com/action/getSupplementalData.php?ID=168*), the chromosomes were fully reduced to mononucleosomes with the characteristic 146 bp length DNA. Under our conditions, the nucleosomal DNA was well protected and no further loss of DNA was found with additional MNase digestion beyond this point. These mononucleosomes were used for immunoprecipitation.

2.3. Mononucleosome preparation from G1/S chromatin

G1/S synchronized 3T3 cells were collected after trypsin digestion and washed with ice-cold PBS before resuspended in the MNase buffer supplemented with 0.5% NP-40 for membrane permeabilization. These G1/S cells were digested with MNase (2,000 gel units/mL) at 4°C overnight. After this step, the G1/S chromatin was mostly reduced to mononucleosomes with the characteristic 146 bp length DNA (supplementary Figure S1E, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=168*). These mononucleosomes were ready for immunoprecipitation.

2.4. Native chromatin immunoprecipitation and sequencing

Native ChIP of macroH2A1 was performed with the collected mononucleosomes of either G1/S or mitotic preparations in the immunoprecipitation (IP) buffer (*18*), both with two biological replicates. Rabbit anti-macroH2A1 antibody (ab37624 that recognizes both 1.1 and 1.2 isoforms; Abcam, Cambridge, MA, USA) was first loaded onto protein A+G coated magnetic beads (16-663, Millipore, Billerica, MA, USA) following the

recommended procedure by the supplier. These beads were then mixed with the mononucleosome solution and incubated overnight at 4°C under constant rotation. The recovered magnetic beads were washed and the bound nucleosomes were eluted as recommended. Input mononucleosomes (used for normalization) or the ChIPed macroH2A1 nucleosomes were first incubated with 100 μg/mL RNase A (Invitrogen, Carlsbad, CA, USA) to remove RNA contamination followed with 1% SDS and 200 μg/mL Proteinase K (Invitrogen, Carlsbad, CA, USA) incubation overnight at 56°C. The DNA in these samples were purified with phenol chloroform and ethanol precipitation. Sequencing libraries were prepared from ~1 ng of DNA per sample using the NEBNext® Ultra™ II DNA Library Prep Kit (E7645S, NEB, Ipswich, MA, USA) and sequencing was performed with Illumina® NovaSeq 6000.

2.5. ChIP-seq data processing

Quality control and adapter trimming were performed with TrimGalore. The reads with Q ≥ 20 were retained for further analysis. Qualified reads were aligned to the mouse reference genome (UCSC, mm10) using Bowtie2 (*19*). Samtools (*20*) was used to remove the reads mapped to blacklist regions, unknown chromosome segments, as well as those unmappable reads and the reads mapped to mitochondria genome. The mapped reads were de-duplicated using Sambamba markdup (*21*) and the unique reads were retained. Pearson correlation between the unique reads was calculated using multiBamsummary with 1 kb bins and visualized with the Deeptools (*22*) of plotCorrelation. Well correlated replicates were then combined for macroH2A1 domain analysis after normalization by the control (input nucleosome DNA) by normR (*23*).

To identify macroH2A1 enriched domains, we divided the reference genome into 1 kb bins. In each bin, the midpoint of each mapped fragment was counted, the normalized ratio of macroH2A1 ChIP/Input were calculated as the enrichment-score using normR (*23*). Fisher's exact test was used to identify significantly enriched macroH2A1 bins and adjacent ones were merged into enriched domains. Using exportR (*23*), the coordinates of enriched domains were created and displayed on Integrative Genomics Viewer (*24*).

H3K27me3 and H3K9me3 ChIP-seq data for NIH-3T3 cells were downloaded from Gene Expression Omnibus (GEO) (accession number: GSE73432) and re-analyzed using the same procedure for macroH2A1.

To identify macroH2A1 associated genes, BedTools (*25*) was used to align macroH2A1 enriched domains onto annotated genes including non-coding genes. ComputeMatrix and plotProfile options of the DeepTools (*22*) were used to visualize macroH2A1 coverage on different types of genes.

2.6. RNA data analysis

RNA-seq data of unsynchronized NIH-3T3 cells were downloaded from GEO (accession number: GSE152724). TrimGalore (*26*) was used to perform quality control and adapter trimming, and reads with Q ≥ 20 were retained and mapped onto the UCSC mm10 reference genome using Hisat2 (*27*) and uniquely mapped reads were retained. Count table was generated using featureCounts and calculated into FPKM (Fragments Per Kilobase of transcript per Million fragments mapped). Reads coverage files were generated using Deeptools (*22*).

3. Results and Discussion

3.1. macroH2A1 is broadly distributed across the genome in both G1/S and mitotic cells

To gain insight into the degree to which macroH2A is maintained on the chromatin during the cell cycle, we profiled the genomic distribution of macroH2A1 in mouse embryonic fibroblast cells (NIH-3T3) synchronized at G1/S phase and at metaphase (supplementary Figure S2, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=168*) using magnetic beads-based ChIP-seq of native chromatin. With this method, the DNA that is protected by the macroH2A1-containing nucleosomes is recovered and sequenced. For the mitotic cells, we sequenced two biological replicates with about 113 million and 93 million uniquely mapped pair-end reads after removal of duplicates, while for the G1/S cells, we sequenced two replicates with 28 million and 42 million uniquely mapped reads (supplementary Table S1, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=168*). The replicates were highly reproducible under both conditions (Pearson correlation: $R^2 > 0.97$) (Figure 1A), and so were combined to improve the reliability of the subsequent analysis.

We first calculated the macroH2A1 enrichment using 1 kb bins over the entire genome. Using a threshold FDR (False Discovery Rate) of 0.1, we found that 64.3% of the mappable genome in the G1/S cells was enriched for macroH2A1-containing nucleosomes (supplementary Figure S3A, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=168*), in agreement with previous findings (*6*). By contrast, we found that 75.6% of the genome in the mitotic cells was enriched with macroH2A1 nucleosomes (supplementary Figure S3A, Table S2, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=168*). Figure 1B is a graphic representation of the macroH2A1 domains on chromosome 19 for both G1/S and mitotic cells. These results demonstrate that a substantial fraction of the genome (> 10%) must be selectively loaded

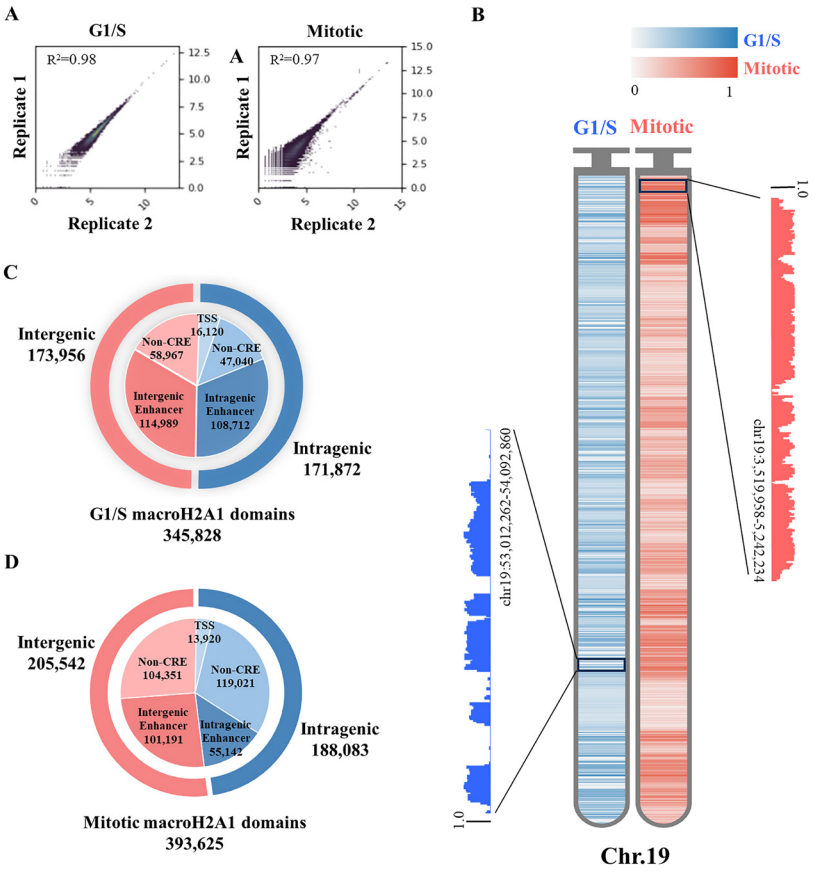

Figure 1. macroH2A1 enriched domains are broadly distributed across the entire genome in both G1/S chromatin and mitotic chromosomes. (**A**) The replicates of the ChIP-seq data are highly consistent for both G1/S and mitotic cells. (**B**) Graphic demonstration of macroH2A1 enrichment distribution on chromosome 19 for G1/S and mitotic cells. The enrichment score is color coded as indicated. Distribution of macroH2A1 domains with known functional elements on the genome: (**C**) G1/S cells; (**D**) mitotic cells. Overall, mitotic cells have more macroH2A1 enriched domains than that of G1/S cells (393,625 vs. 345,828). The median size of these domains is similar under both conditions.

with macroH2A1 nucleosomes before mitosis which must also be removed subsequently in the G1 phase after mitosis. Thus, these data clearly indicate that the nucleosome composition of the chromatin is more dynamic during the cell cycle than presently believed.

Since many of the macroH2A1-enriched bins were neighboring each other, we merged adjacent bins into extended domains for both G1/S and mitotic cells. In this way, we found that there are ~14% more macroH2A domains in mitotic cells over the G1/S cells (393,625 and 345,828, respectively) (Figures 1C and 1D), although both exhibit a median size of 10 kb (supplementary Table S2, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=168*). The normalized average reads density of these enriched domains is 5.8 (± 0.8) and 6.6 (± 1.0) CPM (counts per million) per kb for G1/S and mitotic cells, respectively, indicating that the average density of the macroH2A1 containing nucleosomes in these enriched domains is comparable across the genome under both conditions.

3.2. macroH2A1 domains in G1/S cells are significantly enriched at silenced genes

Since macroH2A1 is known to play critical roles in gene regulation (*4,6,28*), we examined the relationship of the macroH2A1-enriched domains with the expression status of the associated genes in the G1/S cells. We found that about 50% (173,956) of the domains were located in the intergenic regions, with the remaining domains localized within the annotated genes. For the latter, 16,120 domains overlapped putative TSS (Transcription Start Site) regions, while 155,752 were found within the gene bodies (supplementary Table S3, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=168*). Overall, the bodies of 18,679 annotated genes were substantially covered with macroH2A1 domains (Figure 2A). As expected (*28*), 90% of these genes (16,811) were significantly down-regulated or silenced (FPKM < 1), which constitutes about 40% of all silenced genes in this cell. Interestingly, it should be noted that for the remaining 10% of the genes (1,868), nearly half (784) were expressed at levels above the median of all expressed genes (median FPKM = 9.14). Although the molecular basis for these exceptions is not clear, it may be of interest that a fraction of these "abnormally" expressed genes are non-coding RNA genes or pseudogenes. The macroH2A1 domains located at TSS regions corresponded to 7,254 annotated genes that were expressed at a low level (median FPKM = 1.2), similar to previous observations in other cell types (*29*). For the intergenic macroH2A1 domains, about 66% (114,989) were found at annotated enhancers and 99,407 enhancers were fully covered by the macroH2A1 domains. Together with the intragenic

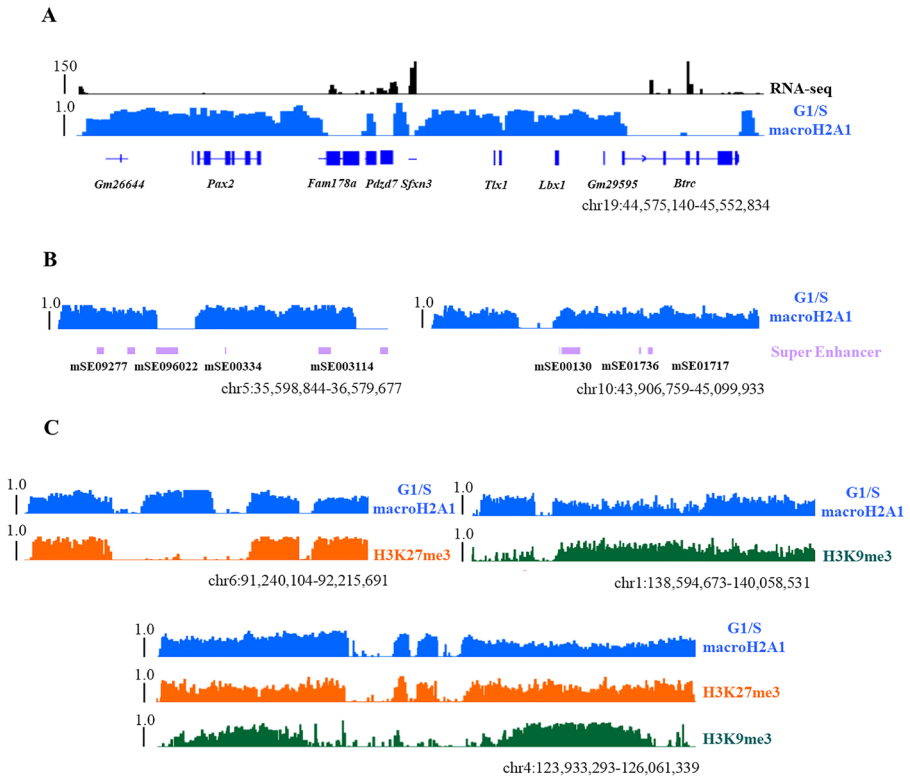

Figure 2. Representative examples to demonstrate the various localization of macroH2A1 domains. (**A**) Gene bodies fully covered by macroH2A1 are sufficient to inhibit expression and the expressed genes are mostly devoid of macroH2A1 occupancy. (**B**) Some (super) enhancers are extensively covered by macroH2A1 domains, presumably also silenced. (**C**) Examples of co-localization of macroH2A1 domains with heterochromatin domains demarcated by either H3K27me3 (upper penal, left) or H3K9me3 (upper panel, right) or both (lower panel).

enhancers that were also fully covered with macroH2A1 domains, we found that 1,276,213 putative enhancers (Figure 2B) were presumably silenced by macroH2A1, corresponding to 59% of all putative enhancers noted in the ENCODE database (based on all cell-types characterized to date) (*30*).

Since it has been reported that macroH2A was also preferentially localized in heterochromatin domains (*4*), we next examined colocalization of the macroH2A1 domains with the domains demarcated by the canonical heterochromatin markers, H3K27me3 and H3K9me3 (*6,7*). Using published data for this cell type with the same criteria for macroH2A1 domain assignment, we found that 41,008 out of 345,828 macroH2A1 domains colocalized with H3K27me3 domains (median size of 2 kb) and 90,336 with H3K9me3 domains (median size of 1 kb). Together, 124,064 macroH2A1 domains (36%) co-localized with either or both of the heterochromatic H3 marked domains (Figure 2C and supplementary Table S3, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=168*). Interestingly, among the silenced genes (16,811) associated with macroH2A1, less than half (47%) were found in these co-localized domains where macroH2A1 and heterochromatin could play redundant functions. It is perhaps more intriguing to note that more macroH2A1 silenced genes (8,937) were not found in the heterochromatin domains, suggesting that macroH2A1 is not simply redundant with heterochromatin as proposed previously (*6*) and could be sufficient for gene silencing.

Thus, overall, our results, as well as those in other studies (*28,30*), unequivocally implicate an important role of macroH2A1 domains in the silencing of a large number of genes and enhancers. Therefore, it follows that their retainment at specific genomic loci, particularly those important to the phenotype, must be reliably regulated, especially during cell proliferation.

3.3 Mitotic chromosome-specific macroH2A1 domains are abundant and loci-specific

To determine the extent to which the macroH2A domains were retained during the cell cycle, we compared the domains identified in the G1/S cells with those present in the mitotic cells. We found that the overwhelming majority of the G1/S macroH2A1 domains, nearly 90% (305,595 out of 345,828), were also present in the mitotic chromosomes in terms of both location and size, demonstrating that macroH2A1 domains are indeed largely conserved through the cell cycle (Supplementary Figure S3B, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=168*). These fully conserved macroH2A1 domains included 58% of the genes with substantial gene body coverage in the G1/S cells, and 70%

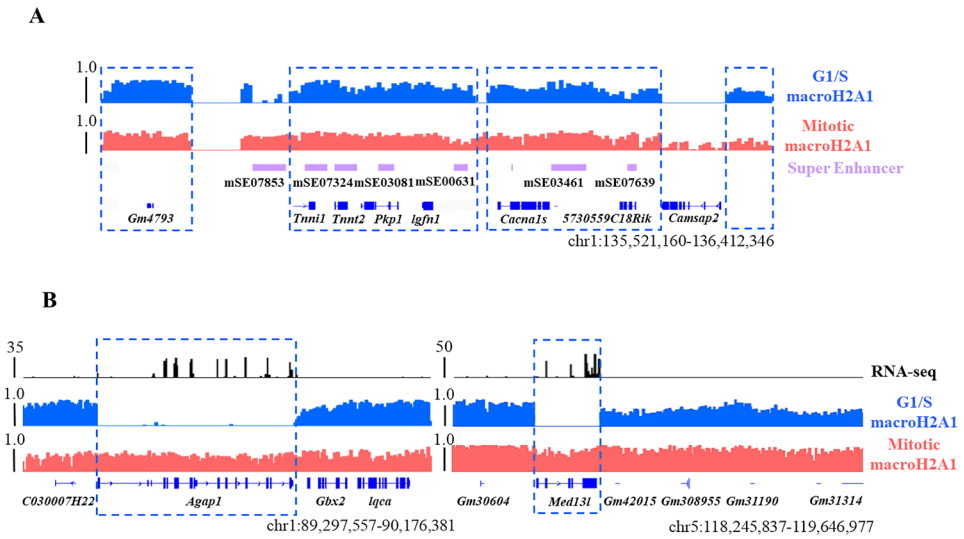

Figure 3. Comparison of macroH2A1 enriched domains in G1/S and mitotic cells. (**A**) Representative example of conserved macroH2A1 domains between G1/S and mitotic cells (highlighted by dashed blue boxes). (**B**) Two examples to demonstrate that some of the highly expressed genes with flanking macroH2A1 domains in G1/S cells are loaded by macroH2A1 nucleosomes before mitosis to form extended and continuous macroH2A1 domains.

of the TSS localized domains, as well as 86% of the enhancers (Figure 3A and supplementary Table S4, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=168*). This high level of transmission of the macroH2A1 domains through mitotic chromosomes suggests that macroH2A1 might also be a constituent of mitotic bookmarkers in order to ensure phenotype identity, similar to histone modifications that are stably transmitted through cell division (*11*). However, we also found that a fraction (40,233) of G1/S macroH2A1 domains was not present on the mitotic chromosomes (Supplementary Figure S3B, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=168*), including 744 out of the 16,811 macroH2A1 silenced genes and 23,422 of the 1,276,213 macroH2A1 covered enhancers (supplementary Table S4, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=168*). The functional significance underlying the removal of these macroH2A1 nucleosomes from the chromatin before entry into mitosis is not understood, although it is clear that they are restored after mitosis.

More unexpectedly though is the finding that there are 88,030 macroH2A1 domains that are specific to the mitotic chromosome (supplementary Figure S3B, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=168*), indicating that these macroH2A1 domains were established before entering mitosis. Among these mitotic-specific macroH2A1 domains, 60% (52,828) were found to overlap substantially with 7,715 gene bodies (supplementary Figure S3C, *http://www.biosciencetrends.com/action/getSupplementalData.php?ID=168*) and the remaining were distributed in the intergenic regions, including 10,873 enhancers. Moreover, the genes that overlap these mitotic-specific domains were all well-expressed in the G1/S cells, and include some that were highly expressed, such as 64 histone genes (such as H2ac8 and H2bc3), 42 housekeeping genes and other important genes such as the ubiquitin gene, *Ubc*. A clearly notable feature of the genes specifically loaded with macroH2A1 in the mitotic cell was that they were flanked on both sides by macroH2A1 domains in the G1/S cells, so that extended macroH2A1 domains were formed on the mitotic chromosome (Figure 3B). We speculate that the formation of these extended domains in mitotic cells is necessary to fully silence the transcription in these regions on the mitotic chromosome (*31*), the failure of which could lead to aneuploidy (*32*), in order to ensure a proper packing of the chromatin, although further studies are certainly required to determine their functioning. Nonetheless, these results clearly indicate that there must be molecular machinery in the cell that not only performs loading and removal of macroH2A1 nucleosomes during the cell cycle but must also targets select loci robustly. In this regard, the ability to exchange macroH2A nucleosomes in the interphase was already shown with modified fibroblasts (*15*), but whether this mechanism also plays a role in the cell cycle-dependent macroH2A1 exchange remains to be examined.

In conclusion, we have performed the first genome-wide profiling of macroH2A1 domains at different stages of the cell cycle. We found that macroH2A1 domains are widely distributed over the entire genome in both G1/S and mitotic cells, with nearly 90% of the domains stably retained. We speculate that this retainment may be a critical means of maintaining proper cellular functioning and thus, *in vivo*, is important especially within stem

cells and progenitor cells (that is, the major cycling cells in any organism) to prevent transformation into pathological phenotypes. However, we also found that for a subset of the expressed genes in G1/S cells that are demarcated by flanking macroH2A1 domains, macroH2A1 nucleosomes are loaded before mitosis to form extended macroH2A1 domains on the mitotic chromosomes. Our data also indicate that macroH2A1 coverage over the gene body alone (that is, without co-localizing heterochromatin) might be sufficient for transcriptional repression in 21% of all silenced genes in the G1/S cells. Together with macroH2A1 domains localized at the enhancers, our results support the notion that macroH2A1 could play important epigenetic functions in phenotype maintenance.

Acknowledgements

We thank Yixin Liu for useful discussions and experimental assistance.

Funding: This work was supported by grants from the National Key R&D Program of China (2020YFA0908100), the National Natural Science Foundation of China (31971151, 81627801, 81972909) and the K.C. Wong Education Foundation (H.K.).

Conflict of Interest: The authors have no conflicts of interest to disclose.

Data accessibility

The data presented in this study are uploaded on the NCBI GEO (*https://www.ncbi.nlm.nih.gov/geo/*) under accession number GSE234016.

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Received August 26, 2023; Revised September 14, 2023; Accepted September 24, 2023.

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Released online in J-STAGE as advance publication September 29, 2023.

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DOI: 10.5582/bst.2023.01220

Improving the accessibility of health care for internal migrants in China: Achieving the aim of equalization

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SUMMARY Although equitable access to healthcare is considered key to the health of internal migrants, more concerted efforts are needed to improve the accessibility of healthcare in low- and middle-income countries. The software CiteSpace was used to analyze scientific literature on healthcare utilization among internal migrants in China since 2000. We focused on factors influencing access to healthcare, including geographical, economic, sociocultural, and institutional aspects. The government is urged to play a role in ensuring equal access to healthcare through policies, resource distribution, and information technology. Improving the accessibility of healthcare for internal migrants and achieving egalitarian goals is of great significance to promoting public health and fostering social equity and inclusivity.

Keywords accessibility, health care, migration, low- and middle-income countries

As a special socio-economic phenomenon arising from social transformation and development, population mobility is an important factor in achieving the miracle of China's rapid economic growth. Migration takes place under China's household registry system and mainly occurs from rural to urban areas, from the undeveloped western regions to the developed eastern regions (*1*). According to The Seventh National Population Census in 2020, internal migrants in China totaled 376 million in 2020, accounting for approximately 26.62% of the total population (*2*). The provision of healthcare to China's internal migrants is a classic and essential topic, especially in the context of current public health emergencies such as the COVID-19 pandemic. Improving healthcare for internal migrants requires considering the characteristics of the new era, including prevention and control of emerging and re-emerging infectious diseases or chronic non-communicable diseases, mental health, and major public health emergencies. In addition, external environmental changes also need to be considered, such as technological developments, construction of transportation infrastructure, digital media, new business models.

With the increasing popularity of mobile Internet technology, various online medical, health management, and appointment reservation services are gradually emerging (*3*). Innovative mobile payment and e-commerce models provide more convenient access to healthcare among internal migrants. While developing these new technologies and business models, however, attention must also be paid to privacy protection and information security. Scientific and technological progress provides more opportunities, but avoiding adverse effects such as information asymmetry and a digital divide is also important (*4*). We need to actively promote the use of medical technology, establish intelligent medical models based on the Internet and big data, and enhance health education for internal migrants in order to improve their access to healthcare.

To comprehensively understand the hotspots of research on healthcare utilization among internal migrants in China since 2000, the software CiteSpace (Chaomei Chen, Drexel University, College of Computing and Informatics, *https://citespace.podia.com/*) was used to perform a quantitative analysis of scientific literature and generate a series of knowledge maps (*5*). Using the keywords "healthcare", "migrant population", "internal migrants", and "China/Chinese" in the Web of Science Core Collection database, we retrieved a total of 207 relevant articles. Figure 1 shows a co-occurrence network of research keywords. The main theme revolves around the accessibility of healthcare for internal migrants. The size of the circles indicates the frequency of the keywords, with larger circles representing higher frequencies. Figure 2 shows all newly emerging keywords for each time period.

For instance, accessibility emerged most often during the period of 2008-2009. The timeline provides a more detailed depiction of the temporal evolution of keywords within a specific cluster. The smaller the cluster number, the more keywords are included in the cluster. Figure 3 showcases topics such as health discrimination against internal migrants and barriers to seeking healthcare. As is shown, research has focused on access to healthcare, the healthcare-seeking behavior of key populations, and mental health.

Equity in access to healthcare is key to the health of internal migrants. The World Health Organization (WHO) pioneered the concept of accessibility, which was used to denote the local population's ease of access to primary care. Accessibility to healthcare is a vague term with various definitions (*6*). In 1968, R.M. Andersen proposed that access to healthcare is the process of accessing the healthcare system by any effective means and continuing to function (*7*). Previous studies have indicated that accessibility can be divided into availability, affordability, acceptability, adequacy, and accessibility (*8*). Ascertaining the factors influencing the accessibility of healthcare for internal migrants is important to improving the quality of their health.

The Andersen health behavior model has been widely used to explore the factors influencing access to healthcare (*9*). This model suggests that individual characteristics, community resources, and the healthcare policy environment interact with each other to collectively influence an individual's healthcare needs, healthcare-seeking behavior, and utilization of services (*10*). We categorized the factors influencing internal migrants' access to healthcare into geographical, economic, sociocultural, and institutional aspects. (*1*) Geographic accessibility refers to the proximity and ease of movement between where internal migrants are located and healthcare facilities. For example, in remote areas or places with limited transportation options, internal migrants may have difficulty accessing timely medical care. (*2*) Economic accessibility refers to the ability of internal migrants to afford healthcare. It is influenced by factors such as income levels, health insurance coverage, and the cost of medical care. (*3*) Sociocultural accessibility encompasses the knowledge, cultural background, and social support that internal migrants possess in terms of healthcare. This includes awareness, attitudes, beliefs, and social support related to healthcare issues. The lack of cultural understanding

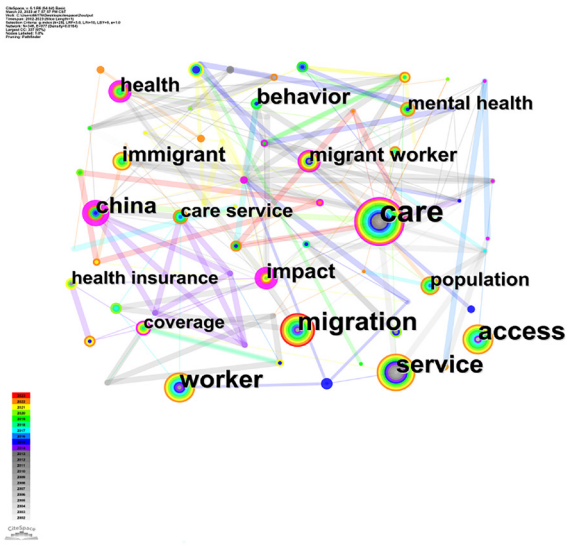

Figure 1. Keyword co-occurrence map for research on healthcare among internal migrants in China since 2000.

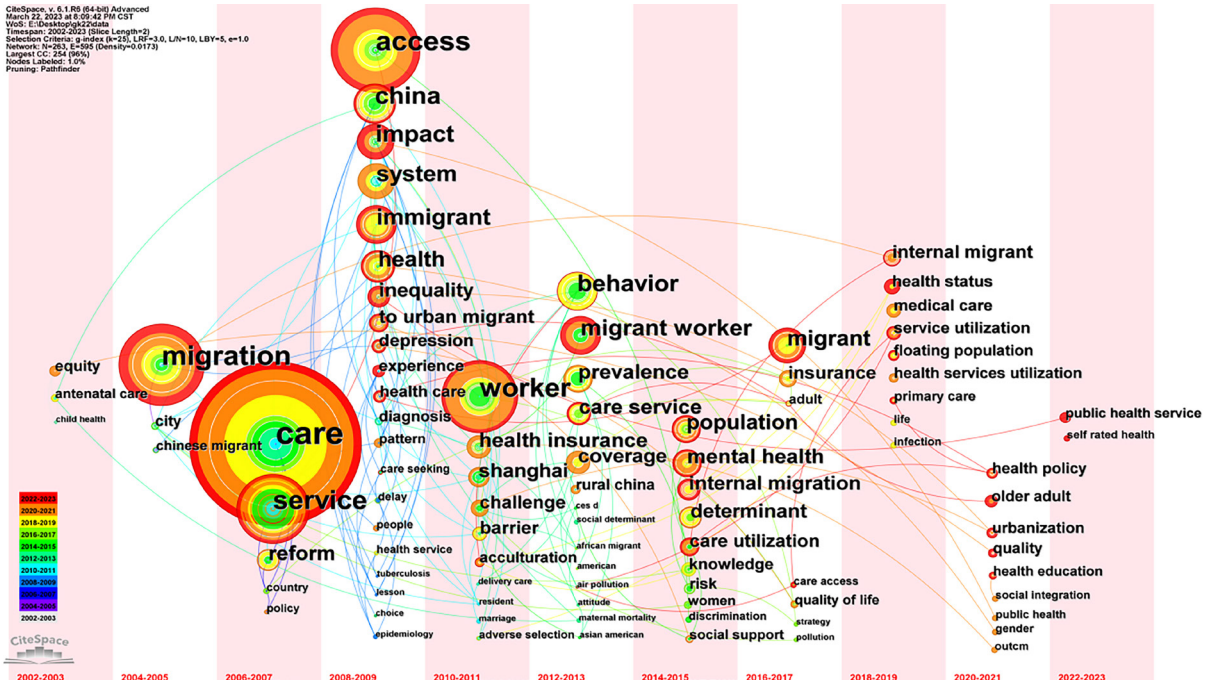

Figure 2. Time zone map for research on healthcare among internal migrants in China since 2000.

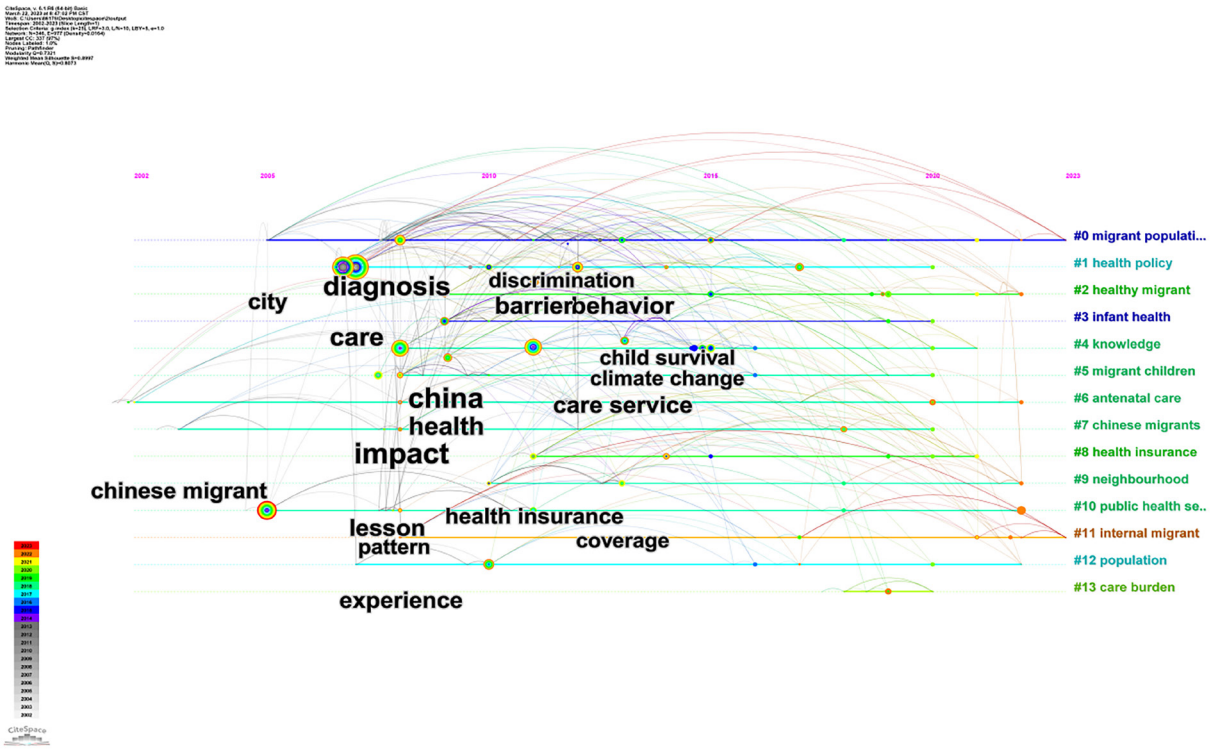

Figure 3. Timeline of research on healthcare for internal migrants in China since 2000.

and social support can reduce the willingness and ability of internal migrants to utilize healthcare. (*4*) Institutional accessibility refers to the inclusive and supportive nature of policies and systems towards internal migrants' utilization of healthcare. This includes aspects such as health insurance coverage, registries for internal migrants, and the availability of community healthcare facilities. Institutional inequities can preclude internal migrants from enjoying the same level of healthcare as local residents.

The government plays a vital role in ensuring equal access to healthcare for internal migrants, including efforts in areas such as systems, healthcare resources, and information technology. First is establishing robust policies and legal frameworks to ensure equal access to healthcare for internal migrants. This may involve implementing specific policies such as providing temporary residency permits to safeguard their right to healthcare. Second is increasing the distribution of healthcare facilities and resources, particularly in areas with high concentrations of internal migrants. Third is utilizing information technology to enhance healthcare for internal migrants. This involves, for example, creating electronic health records that enable the sharing of and access to medical information across regions, improving diagnostic and treatment continuity. In addition, the involvement of social healthcare organizations is a positive aspect to further bridge the gaps in healthcare and meet the diverse needs of internal migrants. These organizations can offer services such as health education, preventive measures, and psychological support to address specific healthcare needs.

Enhancing the accessibility of healthcare for internal migrants in China necessitates the acknowledgement and remediation of various individual and systemic issues, encompassing the provision of healthcare that is not only economically feasible but also culturally congruent, as well as the implementation of policy reforms aimed at removing barriers hindering migrants' access to healthcare. By concurrently targeting both individual and systemic factors, such as the affordability and cultural appropriateness of healthcare, as well as policy restrictions impeding access to healthcare, significant progress can be made in improving the overall accessibility of healthcare for internal migrants in China. In line with 3.8 of the Sustainable Development Goals (SDGs) - namely, to achieve universal healthcare coverage, including access to quality and safe essential healthcare - we are now exploring classic themes by incorporating the new features of the era. We hope to provide a useful shared learning platform for all policymakers and healthcare providers working in migration and health.

Funding: This work was supported by the National Science Foundation of China (grant numbers 72274109, 71774104, 71473152, 71974117), the China Medical Board (grant number 16-257), and the Cheeloo Youth Scholar Grant, Shandong University (grant numbers IFYT1810, IFYT18032, 2012DX006). The study sponsor had no role in study design, data analysis and interpretation of data, the writing of manuscript, or the

decision to submit the paper for publication.

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received September 5, 2023; Revised September 25, 2023; Accepted October 11, 2023.

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Released online in J-STAGE as advance publication October 14, 2023.

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DOI: 10.5582/bst.2023.01243

Call for special attention to the caregiver burden of patients with drug-resistant tuberculosis in low- and middle-income countries

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SUMMARY The tuberculosis (TB)-related caregiver burden (CB), and particularly the multidrug and extensively drug-resistant tuberculosis (M/XDR-TB)-related CB, is not rare in caregivers caring for TB patients, especially when a family member is the caregiver. However, the existing studies on this topic are insufficient. This study briefly summarized the risk factors for the imposition of a TB-related CB and reasons why caregivers for patients with M/XDR-TB are more susceptible to a CB. We propose that special measures should be implemented to alleviate the TB-related CB based on our clinical experience and insights from China. This may improve the situation of caregivers for TB patients and ultimately improve the quality of life of TB patients.

Keywords tuberculosis, caregiver burden, multidrug and extensively drug-resistant tuberculosis, caregiver, quality of life

For a long time, tuberculosis (TB) has been regarded as a global public concern, particularly in low- and middle-income countries. Drug-resistant TB (DR-TB) is highlighted because most cases of DR-TB are refractory and often have a poor clinical outcome. Nonetheless, DR-TB has never been rigorously defined thus far. According to a report from the World Health Organization (WHO) (*1*), such cases are conventionally classified into multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB). They are sometimes grouped together as multidrug and extensively drug-resistant tuberculosis (M/XDR-TB). MDR-TB is a TB infection that is resistant to the most effective anti-TB drugs (such as isoniazid and rifampicin), whereas XDR-TB is MDR-TB plus resistance to fluoroquinolone as well as at least one second-line injectable agent, such as amikacin, kanamycin and/or capreomycin (*1*). According to a later expert consultation issued by the WHO, the definition of XDR-TB was updated, and pre-XDR-TB was suggested. Briefly, pre-XDR-TB is MDR-TB plus resistance to fluoroquinolone, whereas XDR-TB is MDR-TB plus resistance to fluoroquinolone and at least one additional Group A drug (*2*). Here, we have used the conventional definition of M/XDR-TB. Cases of M/XDR-TB are clinically problematic (Figure 1). Patients often undergo long-term anti-TB treatment that does not have satisfactory efficacy; targeted organs deteriorate, TB is a persistent airborne illness, and there are severe adverse reactions to long-term repeated therapy. According to the WHO's 2022 Global Tuberculosis Report, the number of people newly diagnosed with TB totaled approximately 6.4 million worldwide, 450,000 of whom were rifampicin-resistant TB (RR-TB). Among the patients with RR-TB and MDR-TB, only 1/3 underwent regular treatment during the COVID-19 pandemic, resulting in up to 1.6 million people dying from TB (3). Among patients with MDR-TB undergoing regular treatment, the disease was satisfactory controlled in only 59% (3), and the remaining patients may develop XDR-TB. The refractory nature of M/XDR-TB and the long duration of illness may impose a substantial burden on patients, their family, medical insurance, and needless to say, their caregivers.

For those patients with severe illness, caregivers usually have to play a key role in maintaining the patients' activities of daily living (ADL), such as making meals, performing household chores, running errands, and even assisting them financially (*4*). The psychological, physical, and financial burden on caregivers, referred to as the "caregiver burden (CB)", has garnered a great deal of attention in the last decade. The CB is a multidimensional response to perceived

stress and negative assessments involving objective and subjective feelings (*5*). It is reportedly associated with the financial status, social role, and educational level of the caregivers, the severity of the patient's disease, and long-term care (*6-9*). Most of the previous studies on the CB mainly focused on non-communicable diseases such as cancer (*7*), stroke (*9*), and Alzheimer's disease (*6*). Few studies have investigated the CB of M/XDR-TB. However, the M/XDR-TB-related CB cannot be ignored since M/XDR-TB is communicable and often long in duration. China is the second leading country suffering from a high burden of M/XDR-TB. Here, we have briefly summarized our clinical experience and insights regarding the M/XDR-TB-related CB. This information could help to control and prevent the TB-related CB.

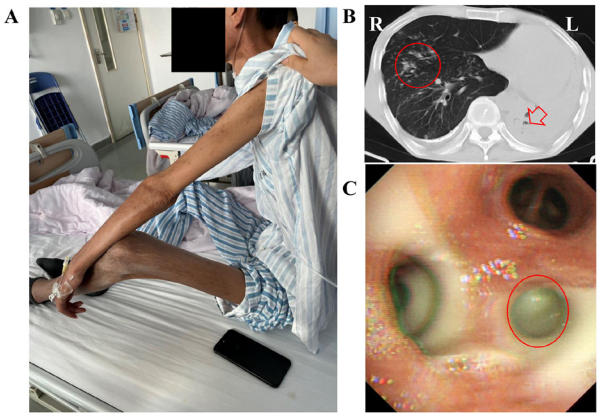

Figure 1. Images from a typical patient with M/XDR-TB. A 49-year-old man with a 10-year history of XDR-TB. Six months later, he died from respiratory failure. (A). The general appearance of the patient. He is suffering from malnutrition, dyscrasia, and chronic respiratory failure. He has almost lost the ability for self-care and he depends on a caregiver for his daily activities. (B). Chest CT findings suggest that the left lung is destroyed (red arrow) and that disseminated TB is present in the right lung (red ring). (C). Findings from tracheobronchoscopy. White purulent exudate and phlegm were visible, even in the bronchus (red circle). CT: computerized tomography; TB: tuberculosis; XDR-TB: extensively drug-resistant tuberculosis.

Risks factors for the imposition of a TB-related CB

In general, risk factors for a CB among caregivers mainly include the physical burden, financial burden, and psychological and social burden. Examples are concerns of being infected due to living with patients, anxiety, depression, social isolation, and a long duration of caring for the patient (Figure 2) (*10*). If these factors are not properly controlled, they may impose a CB on caregivers, influence the quality of care, and ultimately worsen the clinical outcome for TB patients.

i) Physical burden

Patients with M/XDR-TB are commonly suffering from deterioration of pulmonary function caused by airway stenosis, destruction of the pulmonary parenchyma, and other structural damage to the lungs (Figure 1). The decreased tolerance for activity due to a poor vital capacity always causes a marked reduction in ADL and quality of life (QOL) (*11*). Accordingly, patients have to depend more on caregivers for their daily lives. Stressed patients might make exorbitant demands while receiving care. Moreover, the stress in patients might be "transmitted" to caregivers and induce a stressful state in caregivers. Choi *et al*. reported that approximately 43–53% of caregivers developed clinically significant fatigue in the intensive care unit. They had worse scores in terms of a depression assessment, health risk behaviors, and sleep quality that were believed to be caused by the burden of patient care and long-term hospitalization (*12*). In addition, most caregivers for M/XDR-TB patients are family members who were never

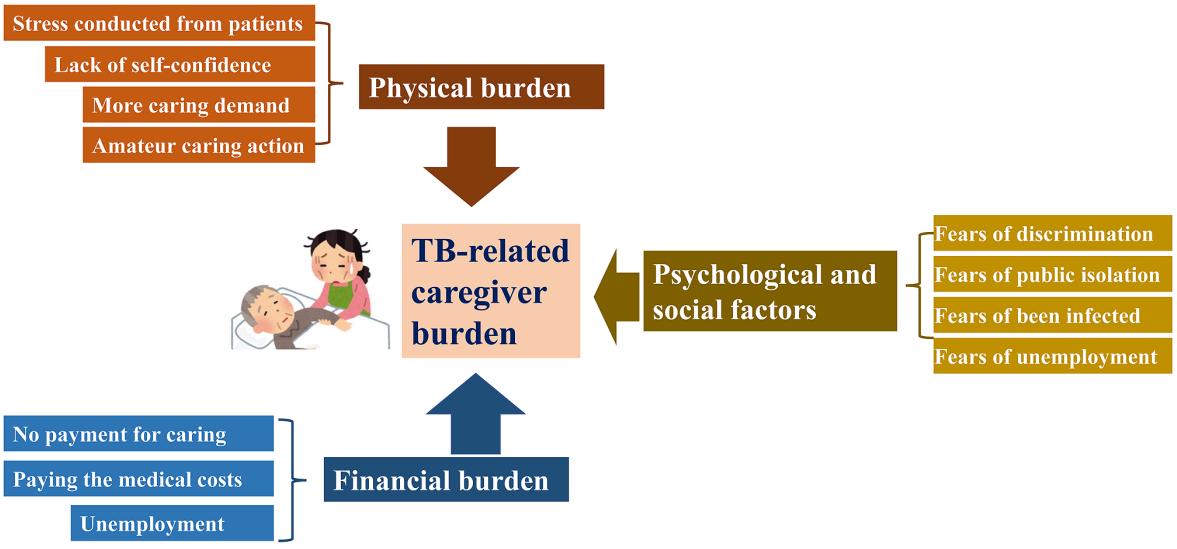

Figure 2. Risk factors for a TB-related caregiver burden.

trained in nursing, let alone nursing in a TB scenario, instead of professional caregivers. Mollica *et al*. reported that approximately 56.6% of caregivers had never been trained for all of the tasks they were performing (*13*). Such a "lack of training" may increase the physical burden (care by a layman). Moreover, lack of self-confidence might also contribute to the development of stress in these untrained caregivers (*13*).

ii) Financial burden

Costs of medical care might be a factor causing stress. According to a WHO report, 47% of TB patients and their families on average have to face TB-related catastrophic costs in 25 countries (*14*). Moreover, patients with M/XDR-TB have to face much-higher medical costs and develop stress more easily, which might be associated with longer hospitalization and more severe illness (*15*). A China-based study found that patients with M/XDR-TB had a 68.3% longer duration of therapy and an 87.0% higher hospitalization rate in comparison to patients with common TB (*16,17*). In addition, patients with M/XDR-TB are more susceptible to income loss and/or unemployment. In China, approximately 48% of TB patients had an average out-of-pocket spending of over 300 USD per month for their M/XDR-TB treatment (*18*). All of these factors worsen the financial situation of patients with M/XDR-TB, preventing caregivers, and particularly caregivers who are family members, from receiving full payment. Conversely, as a family member, the caregiver commonly has to pay the treatment costs together with the patient. In addition, unemployment due to caregiving is not rare. Pucciarelli *et al*. found that more than half (46%) of caregivers for stroke patients experienced at least one change in their employment due to caregiving (*19*). This heavy financial burden may cause stress both in patients and their caregivers.

iii) Psychological and social factors

Psychological problems in caregivers have been gradually recognized and garnered attention. Thana *et al*. found that anxiety and depression in caregivers were closely associated with lower caregiver self-esteem, a higher perceived health burden, and lack of family support (*20*). Hu *et al*. reported that of 117 caregivers, 43.9% had mild depression, 26.5% had moderate depression, and 27.4% had severe depression (*21*). The psychological problems originate from the following misgivings: *i*) Fears of discrimination and public isolation (*22*); *ii*) Worrying about unemployment; and iii) Worrying about been infected. Indeed, caregivers per se also have a higher risk of TB infection. The prevalence of a latent TB infection is reported to potentially increase with prolonged exposure, with an incremental increase in prevalence of 8.2% per 250 hours of exposure (*23*). Although prophylactic treatment has been proved to be effective in preventing caregivers from developing active TB, it cannot completely eliminate the risk of TB developing. This situation differs markedly from caring for patients with non-communicable diseases (cancer, Alzheimer's disease, diabetes, stroke, *etc*.). Hence, worrying about the risk of infection may further exacerbate the psychological burden in caregivers for TB patients.

Solutions: What can we do next?

Other than TB patients per se, the burden on caregivers for TB patients, and particularly the M/XDR-TB-related CB, cannot be ignored. Nevertheless, studies on this topic remain insufficient, so further information is not available. Other than scientific research, several measures should be considered to alleviate the TB-related CB. This may improve the situation of caregivers for TB patients and ultimately improve the QOL of TB patients.

i) In terms of the physical burden, professional training in nursing for family caregivers should be provided to improve the efficiency of care in order to reduce the mental and physical exhaustion and enhance the self-confidence of caregivers for TB patients.

ii) In terms of the financial burden, the government should pay attention to this population. Measures should be taken to alleviate the financial burden on TB patients and their caregivers, such as the establishment of a special insurance system to assist patients with refractory M/XDR-TB and their caregivers, reducing or exempting them from taxes, and providing a special allowance to them.

iii) In terms of the psychological and social factors, regular psychological care such as psychological counseling and therapy are highly recommended to ameliorate the depression and anxiety in patients and their caregivers.

The TB-related CB, and the refractory M/XDR-TB-related CB in particular, should not be ignored especially in low- and middle-income countries. On the basis of our clinical experience, we therefore propose that special attention should be paid to the CB of TB, and particularly the refractory M/XDR-TB-related CB.

Funding: This work was supported by the Shenzhen Science and Technological Foundation (grant nos. JSGG20220301090005007 and JSGG20220606141001003), the Third People's Hospital of Shenzhen Foundation (grant nos. G2021027 and G2022062), and the Shenzhen High-level Hospital Construction Fund (grant no.23274G1001).

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received September 14, 2023; Revised October 4, 2023; Accepted October 10, 2023.

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Released online in J-STAGE as advance publication October 14, 2023.

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DOI: 10.5582/bst.2023.01042

Clinical characteristics of solitary intrahepatic biliary cyst

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SUMMARY Solitary intrahepatic biliary cyst (SIBC) is a rare disease, and due to the lack of adequate understanding of it, SIBC is often misdiagnosed as simple liver cyst (SLC), which in turn affects the therapeutic effect. In order to arouse more attention to SIBC, combined with clinical experience in our center, this study specifically screened 3 representative cases of SIBC, and conducted a comprehensive retrospective analysis of their clinical characteristics, diagnosis and treatment process. Combined with the relevant literature, the diagnosis and treatment process of SIBC is widely discussed.

Keywords solitary intrahepatic biliary cyst, simple hepatic cyst, hepatic cyst, hepatectomy

Congenital hepatic cysts are relatively rare liver lesions that can be divided into simple hepatic cysts (SLC) and solitary intrahepatic biliary cysts (SIBC) depending on whether the cyst communicates with the bile duct (*1,2*). SIBC is a special type of biliary dilatation. Although congenital hepatic cysts are considered a congenital disease, most patients remain asymptomatic for many years and are occasionally detected during physical examinations in adulthood. Only a very small number of patients present with corresponding clinical symptoms in infancy and are diagnosed (*3*).

As a rare type of congenital hepatic cysts, SIBC is often misdiagnosed as SLC because it has not attracted enough concern and attention, which in turn affects the therapeutic effect and the prognosis of patients. In view of this, the authors selected 3 representative cases from SIBC patients diagnosed and treated in our center, and systematically summarized and analyzed their clinical characteristics as well as diagnosis and treatment process (Table 1).

Case 1: A 30-year-old female patient was admitted due to intermittent abdominal pain and discomfort for 3 months. The patient was admitted on June 5, 2017. The patient was diagnosed with "hepatic cyst, duodenal ulcer and pyloric obstruction" 16 months ago, and underwent "fenestrated jejunostomy and internal drainage of hepatic cyst, distal gastrectomy and Roux-en-Y gastrojejunostomy" in a local hospital. The patient had repeated nausea and vomiting after surgery. She was admitted to our department for further treatment. Contrast-enhanced MR of the upper abdomen revealed "biliary cyst involving S4, S5, S8 after biliary cyst surgery and subtotal gastrectomy" (Figure 1 A, 1B). Middle lobectomy was considered. During the surgery, biliary dilatation in the right anterior lobe and middle liver was observed. Middle lobectomy and choledochocystectomy + intraoperative biliary T-tube drainage + intraoperative hilar cholangioplasty + choledochojejunostomy were performed. The surgical specimen was shown in Figure 2A. Postoperative pathology revealed biliary dilatation with biliary cyst formation. After discharge, the patient was followed up regularly at the 25th month, MRCP was performed during the follow-up (Figure 3A, 3B, 3C), no recurrence or complication occurred.

Case 2: A 70-year-old male patient was admitted to our hospital due to jaundice and icteric sclera for 20 days. The patient was admitted on April 16, 2017. One year ago, the patient developed jaundice with abdominal pain without obvious inducement. Abdominal MRI showed hilar cyst with biliary compression, mild intrahepatic biliary dilatation, gallbladder atrophy, and Chilaiditi syndrome. Resection of hilar biliary cyst, gallbladder extirpation and hepaticojejunostomy were performed in a local hospital, and a 4.0*3.5 cm cyst was palpable in hilar region during the surgery. Half a month after surgery, the patient started having intermittent high fever with nausea and vomiting. MRCP/CT in our hospital showed (Figure 1C, 1D): mild intrahepatic biliary dilatation and possbile compensatory changes. In our hospital, left hemihepatectomy + resection of residual biliary cyst + choledochojejunostomy of the right hepatic

Table 1. General information of patients

No.	Gender	Major symptoms	Imaging	Location of biliary cyst	Cyst diameter (cm)	History of surgery at lesion site	Follow-up time (months)	Prognosis
1	Female	Abdominal pain	US/CT/MRCP	Porta hepatis	8.5	3	25	NDNR
2	Male	Abdominal pain	US/MRCP	Porta hepatis	2.8	1	33	NDNR
3	Female	Hyperpyrexia/jaundice	US/CT/MRCP	Porta hepatis	5.0	0	19	NDNR

US: ultrasonography; CT: Computer Tomography; MRCP: Magnetic Resonance Cholangiopancreatography; NDNR, No discomfort/no recurrence.

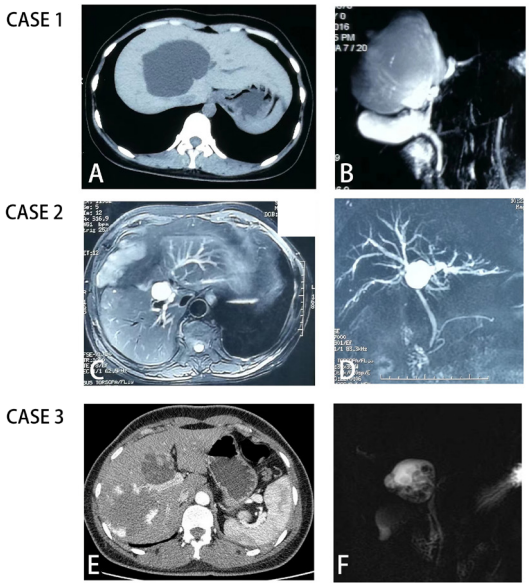

Figure 1. Imaging findings of the 3 cases of solitary intrahepatic biliary cyst. Case 1, abdominal CT **(A)** showed after biliary cyst surgery+subtotal gastrectomy, cyst lesion recurrent in the right liver lobe; MRCP **(B)** revealed single cystic lesion in the hilum, involving the left medial lobe and right anterior lobe. Case 2, MRCP **(C, D)** revealed after resection of biliary cyst+cholecystectomy+choledochoje junostomy, intrahepatic biliary dilatation recurrent. Case 3, abdominal CT **(E)** showed cavernous hemangioma in the right liver lobe and biliary cyst in the left liver lobe. MRCP **(F)** showed biliary cyst in the left liver lobe, and large possibility of cavernous hemangioma in the right liver lobe.

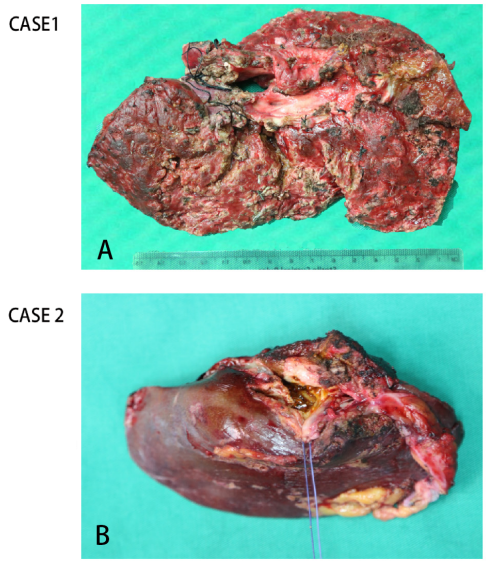

Figure 2. Surgically resected specimens: Case 1 was showed in (A), while case 2 was in (B). Both pathology result were biliary dilatation with biliary cyst formation.

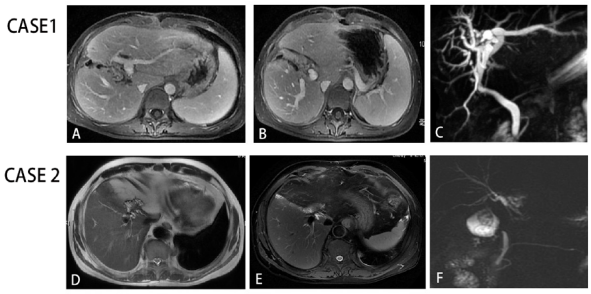

Figure 3. Imaging findings of postoperative reexamination. Case 1, MRCP findings **(A, B, C)** at 25th months after surgery, consistent with s/p partial hepatectomy, and no new abnormal dilated bile ducts were observed. Case 2, upper abdominal MRCP findings **(D, E, F)** 33rd at months after surgery, consistent with s/p left hemihepatectomy, and no new abnormal dilated bile ducts were observed.

duct was performed. The resected specimen is shown in Figure 2B. The patient was discharged safely on Day 6 after surgery. After discharge, the patient was followed up regularly for 33 months, MRCP was performed at the follow-up of the 33rd month (Figure 3D, 3E, 3F). No discomfort or complication occurred. Postoperative pathology revealed biliary dilatation with biliary cyst formation.

Case 3: A 33-year-old female patient was admitted due to intermittent right upper abdominal pain for 10 months. The enhanced CT of the upper abdomen revealed (Figure 1E, 1F): biliary cyst in the left liver lobe complicated with stones. The cyst was located in the left hepatic duct, and the surgical approach was tentatively left hemihepatectomy. During the surgery, dissection of the hilar bile duct showed that the hilar cyst originated from the common hepatic duct and showed a diverticular pattern. It measured about 5 × 5 × 4 cm and closely adhered to the left medial lobe of the liver and gallbladder. After incision from the bottom of the cyst, dark bile outflow was observed. Multiple stones were observed in the cyst, with the larger one about 1.5 cm in diameter, and cauliflower-like tissue was observed in the cyst wall. Specimens were taken during the surgery for pathology. Pathology (frozen): (intracystic polypoid mass) papillary cystadenoma with malignant changes. The patient was diagnosed with congenital biliary dilatation (type V) with cyst carcinogenesis and intracystic stones. Finally, cholecystectomy,

choledochal cyst resection, and hepatoduodenal lymph node dissection were performed. The surgery was successful, and the patient recovered well after surgery and was discharged safely on Day 8 after surgery. The patient was followed up regularly for 19 months after surgery. No discomfort or complication occurred and no tumor recurrence occurred. Postoperative pathology: Biliary cyst (common hepatic duct), with multiple well-differentiated papillary adenocarcinomas.

Biliary dialation can occur in the intrahepatic and extrahepatic bile ducts and has a variety of different morphological manifestations, generally with choledochal cysts being the most common. The most widely used classification method is the protocol proposed by Todani et al. in 1977 (*4*). According to their classification, biliary dilatation is mainly divided into five types. Among them, Caroli disease is currently the only type identified to have a genetic background, which is caused by mutations in the PKHD1 gene and belongs to autosomal recessive diseases (*5*). According to this classification, all the three cases of biliary dilatation in this study can be classified as special Todani type V. According to Dong's classification proposed by Jiahong Dong et al in 2017 (*6*), the three cases of biliary dilatation in this study can be classified as special Dong type B1. but solitary intrahepatic biliary cyst are more accurate. Obstructive jaundice occurs when there is marked dilatation and bile retention at the lesion site, and complications occur. Among them, the common complications are: stones, cholangitis, acute pancreatitis, malignant tumors, portal hypertension, and chronic pancreatitis (*7*).

Since the findings on CT/MRI are very similar, biliary dilatation is often misdiagnosed as SLC (*8*), which has a huge impact on the choice of treatment options for this disease as well as therapeutic effect. In differentiating these two diseases, the most important is to determine whether cystic lesions communicate with the biliary system of the liver. In addition to routine examinations such as CT/MRI, ERCP is more helpful in judging whether cystic lesions communicate with the biliary system, thus helping to differentiate SLC from biliary dilatation (*7*). At the same time, dilated gallbladder diseases also need to be differentiated from intrahepatic and extrahepatic bile duct stones, cholangitis, hepatic hemangioma, primary liver cancer and other liver diseases. Differential diagnosis can be made according to the clinical manifestations of various diseases and typical imaging features.

In this study, Case 1 suggested that the first preoperative diagnosis has a great impact on the choice of surgical approaches, which may be the main cause of serious complications soon after surgery and also leads to the passive condition of the second surgery, and quality of life of the patients after surgery is seriously affected. As a result, the patient experienced obvious biliary dilatation again within 2 years after surgery, accompanied by obvious discomfort, and had to undergo another larger surgery. Fortunately, the disease was completely cured after the surgery, and no discomfort and complication occurred during the follow-up at 2 years after surgery.

Case 2 also had a history of surgery at another hospital. Dilated bile duct not completely removed was the main reason of poor prognosis. According to the Guideline for congenital biliary dilatation issued in Japan in 2017 (*9*), there is no consensus on the extent of resection for this type of lesion. According to Chinese guidelines (*10*), cholecystectomy, resection of the affected hepatic segment, choledochectomy + choledochojejunostomy for extrahepatic lesions are recommended for type V, if the lesion invades the central hepatic duct at grade 3 and above. At the same time, according to previous studies by the author's team, a more aggressive surgical treatment is recommended for patients with type V biliary dilatation (*11*), and the extent of lesion resection needs to be extended to the distal normal bile duct. In terms of stone clearance rate, with or without postoperative discomforts, long-term cancer rate and other aspects, patients may benefit more. This case suggests that in the management of type V biliary dilatation, the extent of lesion involvement in patients should be fully assessed and the most appropriate surgical approach should be selected. This is important for the treatment outcome, as well as for the prevention and control of postoperative complications.

It has been shown that although biliary dilatation is a benign lesion, accounting for approximately 1% of benign biliary diseases (*12*), it increases the probability of malignant transformation at the lesion site, with higher rates of malignant transformation in types I, IV, and V. At the same time, the rate of malignant transformation increases further with age (*13*). The data showed that the probability of malignant transformation in patients with biliary dilatation was less than 1% before the age of 10 years and increased to 6.8% between 10 and 20 years, which further increased to 14.3% after the age of 20 years (*14*). The age of the highest incidence of complicated biliary malignancies is 32 years, which is 20 years earlier than normal people without biliary dilation (*15*). In Case 3 of this study, papillary adenocarcinoma associated with dilated cyst wall resulted in a poor prognosis (*16*), so early diagnosis as well as surgical intervention are very important for the treatment outcome as well as long-term prognosis of patients (*15*). At the same time, the age of the patient in case 3 was 33 years, which was consistent with the high incidence age group reported in the literature, suggesting that we should pay more attention to whether it is associated with malignant lesions and do a good job in surgical planning when diagnosing and treating patients with biliary dilatation in this age group. It is worth noting that in case 3, despite strict collection of medical history, adequate auxiliary examinations, including contrast-enhanced abdominal CT, MRI and other imaging examinations, biochemistry tests such as

tumor markers, were performed. However, we still failed to identify malignant lesions at the site of cystic dilatation before surgery. This suggests that although the malignant lesion rate of biliary dilatation is rare, we should still be fully prepared when performing the surgical plan, which is very important for the standardized treatment of biliary dilatation with malignant lesions.

As far as we know, although there have been previous case reports of SIBC (*17,18*), including cases of SIBC in infants (*13,19*), our series of SIBC cases reported this time are more comprehensive and representative, including multiple surgeries after initial misdiagnosis, the occurrence and treatment of surgical complications, the first diagnosis combined with carcinogenesis, and surgical approach adjustment after accidental detection of malignant lesions during surgery, which is the first comprehensive series of cases and comprehensive analysis of SIBC reports so far. Accordingly, we suggest that SIBC should be considered in the differential diagnosis of patients with solitary cystic mass at the hilum. Aggressive surgical treatment is also recommended in order to achieve radical results.

Funding: This work was supported by National Natural Science Foundation of China (grant number: 82090052); CAMS Innovation Fund for Medical Sciences (grant number: 2019-I2M-5-056); Tsinghua University Initiative Scientific Research Program of Precision Medicine (grant number: 2022ZLA007).

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received April 26, 2023; Revised August 10, 2023; Accepted August 25, 2023.

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Released online in J-STAGE as advance publication August 30, 2023.

Letter to the Editor

DOI: 10.5582/bst.2023.01246

Sarcopenia and risk of cardio-cerebrovascular disease: A two-sample Mendelian randomization study

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SUMMARY Based on the association between sarcopenia and the risk of developing cardio-cerebrovascular disease (CCVD) established by a meta-analysis by Fang *et al*.(*Biosci Trends. 2023; 17:293-301*), we have used Mendelian randomization (MR) analysis to test the authenticity and accuracy of such an association. In this MR study, appendicular lean mass, handgrip strength, and walking pace were used as sarcopenia-related traits, with cardiovascular diseases and stroke set as outcomes of CCVD. MR analysis was performed using inverse-variance weighting, the MR Egger, weighted median, simple mode, and weighted mode. No heterogeneity or horizontal pleiotropy in MR estimates was observed (Cochran's Q *P* value > 0.05, MR-PRESSO global test *P* value > 0.05, and MR-Egger intercept *P* value > 0.05). Results of that analysis proved a causal relationship between sarcopenia-related traits and cardio-cerebrovascular disease, with a causal association between appendicular lean mass and cardiovascular diseases and an inverse causal relationship between appendicular lean mass and stroke. However, such a relationship was absent in the case of handgrip strength and the risk of cardiovascular diseases as well as in the case of walking pace and lacunar/ischemic stroke. Therefore, the effect of sarcopenia on CCVD should be carefully explained.

Keywords causal relationship, Mendelian randomization, sarcopenia, cardio-cerebrovascular disease

To the Editor,

We read with great interest where a systematic review and meta-analysis by Fang *et al*. (*1*) suggested that sarcopenia is significantly associated with an elevated risk of developing cardio-cerebrovascular disease (CCVD). However, the causal relationship between sarcopenia and CCVD needs to be further determined; due to the observational studies that were analyzed, residual confounders are difficult to isolate and the limitations of the race and size of the study population are not known. Mendelian randomization (MR) analysis uses genetic variation as an instrumental variable to estimate causal effects, and results are not affected by confounding factors (*2*). Here, we leveraged data from large-scale genetic association studies in European populations and applied two-sample MR analyses to investigate the causal relationship between sarcopenia-related traits (appendicular lean mass, hand grip strength, and walking pace) (*3*) and CCVD (cardiovascular diseases and stroke).

GWAS data on sarcopenia-related traits from the UK Biobank (UKB) were available for appendicular lean mass, hand grip strength (right), and walking pace. We used summary-level genetic data for cardiovascular diseases from the FinnGen biobank, including 218,792 European participants and 16,380,466 single-nucleotide polymorphisms (SNPs). GWAS data on stroke from the FinnGen biobank were available for 180,862 European participants and 16,380,350 SNPs. GWAS data on hard cardiovascular diseases, lacunar stroke, and ischemic stroke were also analyzed at the same time. All GWAS summary statistics can be downloaded from the IEU OpenGWAS project (*https://gwas.mrcieu.ac.uk/*).

The MR analysis was performed using inverse-variance weighting, the MR Egger, weighted median, simple mode, and weighted mode. Cochran's Q statistic, the MR-Egger intercept test, and MR pleiotropy residual sum and outlier (MR-PRESSO) were performed, allowing estimation of heterogeneity and horizontal pleiotropy. Single-nucleotide polymorphisms at $P < 5 \times 10^{-8}$ were selected as instrumental variables. The linkage disequilibrium threshold was set to $r^2 = 0.001$ within a distance of 10,000 kb.

Results of analysis (Figure 1) revealed a causal relationship between sarcopenia-related traits and cardio-cerebrovascular disease. Findings indicated an inverse causal relationship between appendicular lean mass and stroke as well as a causal association between

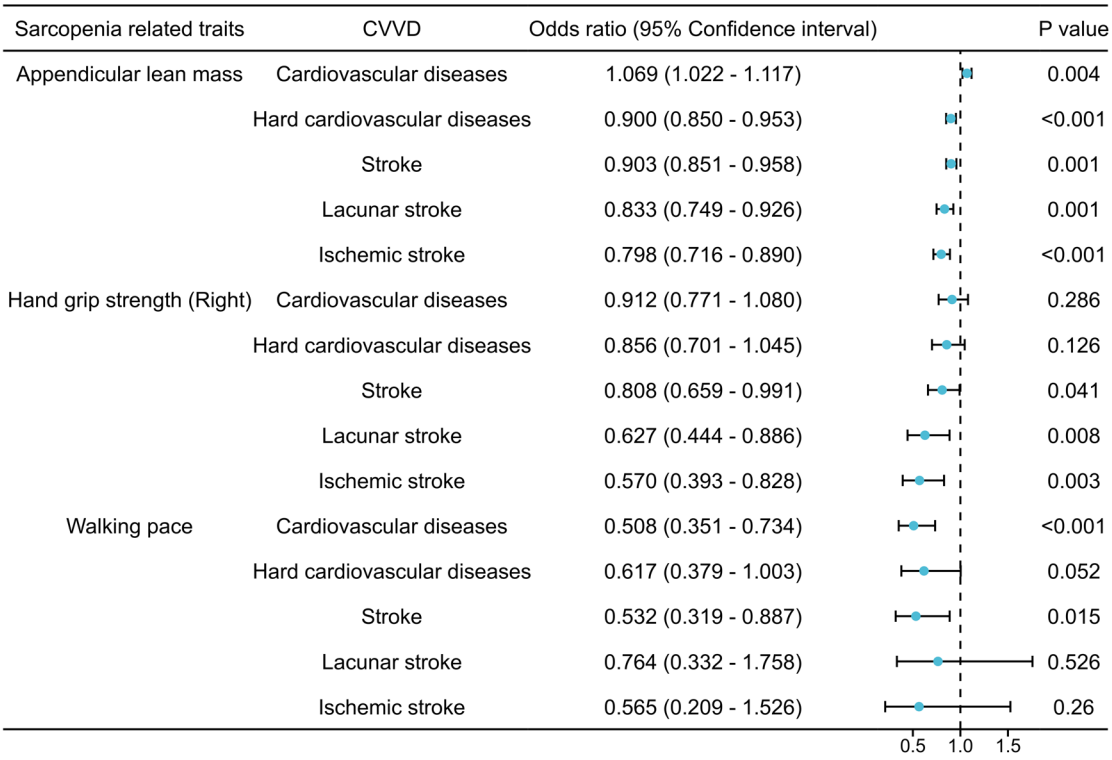

Figure 1. Mendelian randomization analysis of sarcopenia-related traits and CCVD (cardiovascular diseases, hard cardiovascular diseases, stroke, lacunar stroke, and ischemic stroke) with inverse-variance weightingns.

appendicular lean mass and cardiovascular diseases. Notably, hand grip strength (right) was not associated with the risk of cardiovascular diseases but was inversely associated with stroke. Moreover, walking pace was inversely associated with both cardiovascular diseases and stroke but it was not significantly associated with their respective subtypes (lacunar stroke and ischemic stroke). In other words, sarcopenia-related traits, except hand grip strength, have a causal relationship with the risk of cardiovascular diseases. In addition, there was a significant inverse causal relationship between sarcopenia-related traits and stroke. There was neither heterogeneity or horizontal pleiotropy in MR estimates (Cochrane's Q *P* value > 0.05, MR-PRESSO global test *P* value > 0.05, and MR-Egger intercept *P* value > 0.05). Detailed information is presented in Supplemental material (*http://www.biosciencetrends.com/action/getSupplementalData.php?ID=170*).

In conclusion, this MR study provided evidence for the causal relationship between sarcopenia and CCVD even though such a relationship is absent in the case of handgrip strength and the risk of cardiovascular diseases as well as in the case of walking pace and lacunar/ischemic stroke. Therefore, the effect of sarcopenia on CCVD should be carefully explained.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

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Received September 15, 2023; Revised October 12, 2023; Accepted October 16, 2023.

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Released online in J-STAGE as advance publication October 21, 2023.

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(As of December 2022)

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