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REVIEW

Nanotheranostics: A powerful next-generation solution to tackle hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is an epidemic burden and remains highly prevalent worldwide. The significant mortality rates of HCC are largely due to the tendency of late diagnosis and the multifaceted, complex nature of treatment. Meanwhile, current therapeutic modalities such as liver resection and transplantation are only effective for resolving early-stage HCC. Hence, alt-ernative approaches are required to improve detection and enhance the efficacy of current treatment options. Nanotheranostic platforms, which utilize biocompatible nanoparticles to perform both diagnostics and targeted delivery, has been considered a potential approach for cancer management in the past few decades. Advancement of nanomaterials and biomedical engineering techniques has led to rapid expansion of the nanotheranostics field, allowing for more sensitive and specific diagnosis, real-time monitoring of drug delivery, and enhanced treatment efficacies across various malignancies. The focus of this review is on the applications of nanotheranostics for HCC. The review first explores the current epidemiology and the commonly encountered obstacles in HCC diagnosis and treatment. It then presents the current technological and functional advancements in nanotheranostic technology for cancer in general, and then specifically explores the use of nanotheranostic modalities as a promising option to address the key challenges present in HCC management.

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Core Tip: Hepatocellular carcinoma (HCC) is a global epidemic burden. The high mortality rate is mostly due to late diagnosis and complexity of treatment. Nanotheranostics is a potential approach for HCC management. We herein discuss the challenges of HCC management, the advancement of nanotheranostics in cancer, and the potential role of nanotheranostics to address the current challenges in HCC management.

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INTRODUCTION

Epidemic burden and risk factors of hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the most common primary cancer of the liver and remains the second leading cause of cancer-related deaths worldwide[1,2]. It accounts for around 90% of all primary liver cancer cases worldwide[3], and is an epidemic burden in both developing and developed countries[4]. Whilst certain endemic areas such as East Asia has shown a decreasing trend, regions such as Europe, Africa, and the United states display increasing trends in HCC incidence rate with substantial morbidity and mortality[5,6]. Concerningly, cases have doubled in Europe and America as a result of lifestyle factors such as alcohol abuse, smoking, obesity, and metabolic diseases[7-10]. Variations among age and gender are also interesting epidemiologic features of HCC. Men have a higher prevalence of HCC than women with a ratio of 462.4:185.8 new cases per year in developing countries[11]. Respectively, the risk of HCC significantly increases among those who are older than 40 years of age[12].

The risk factors and etiologies of HCC vary depending on geographic region and lifestyle. Hepatitis B and C infections are major etiological factors that significantly contribute to HCC globally[13-15], accounting for 44% and 21% of HCC cases respectively[16], with the highest number of hepatitis B cases occurring in Asia. Other possible risk factors include the increasing number of nonalcoholic fatty liver disease (NAFLD)[17], alcohol addiction[18,19], and aflatoxin consumption[20]. In Western, Central, and Eastern Europe and North America, a majority of HCC cases were attributed to NAFLD/non-alcoholic steatohepatitis, obesity, and excessive alcohol consumption. In contrast, most HCC cases in Asia and Africa were attributed to hepatitis B virus infection[21,22]. The viral and metabolic etiologies described above not only contributes to HCC occurrence, but also implicates a high risk of de novo recurrence, leading to the development of incurable and advanced stage disease that is resistant towards therapeutic efforts such as complete tumor resection or ablation[6].

Pathological complexity of HCC

Due to the multifactorial nature of HCC, several cellular phenomena can be observed, including hypoxia, inflammation, oxidative stress, and tumor microenvironment. Indeed, the molecular mechanism of liver carcinogenesis involves multiple endogenous and exogenous genetic alterations[23]. Hepatocarcinogenesis is a deliberate and complex multistep process associated with somatic genomic alterations, leading to the production of cellular intermediates that progress into hepatocellular carcinoma [24]. The development of HCC involves a combination of continuous inflammatory damage, necrosis, and fibrotic deposition. The pre-neoplastic stage is a long process that typically requires 10 to 30 years of time. During this stage, phenotypically altered hepatocytes are formed as a result of either DNA methylation alterations, pathogenic agent's reaction, and point mutation or loss of heterozygosity, which occur in part

through epigenetic mechanisms that lead to the development of dysplastic hepatocytes in foci and nodules. Aberrant and dysplastic hepatocytes are related to the accumulation of permanent structural alteration and changes in genes and chromosomes[25]. Alterations in the malignant phenotype are often distinct, suggesting heterogeneity at the genomic level[25,26].

Challenges in HCC diagnosis

Early diagnosis is a major challenge in HCC management, and in most cases, the lack of early diagnostic modalities lead to less than optimal treatment outcomes. In developed countries, 30%-60% of HCC cases can be diagnosed early, enabling higher success rates of curative treatment. Contrastingly, HCC cases in developing countries are mostly diagnosed in late stages, leading to substantially lower likelihood of curative treatment[27]. Diagnosis of HCC is in fact, an important and critical phase that relates directly to the survival and prognosis of the patients.

Complexity of HCC treatment

Treatment of HCC itself is also complex and multifaceted, and outcome depends on the time of diagnosis and the presence of additional comorbidities. Prompt diagnosis of HCC is correlated with better outcomes of curative therapies. This is demonstrated by studies that show higher efficacy of local radiofrequency ablation and surgical intervention (liver transplantation and liver resection) in the very early and early-stage HCC as compared to later stages[8,28]. However, most HCC patients are excluded from definitive surgical resection due to late diagnosis. In such cases, liver transplantation can be the best treatment for HCC with a low risk of recurrence, though it is suggested as a second line treatment due to the disparity between limited liver donor resources and the increasing number of patients[29,30]. Based on international guidelines, late stage HCC patients (intermediate and advanced) may receive palliative treatment such as chemoembolization and systemic therapy, while terminal patients can only receive supportive care[27,28,31].

Issues such as tumor recurrence and drug resistance are also major obstacles that frequently complicate HCC management[32,33]. The 5-year recurrence probability of HCC is around 62% after liver ablation[34] and 80% after liver resection[35]. Palliative treatment often have unexpected and poor outcomes related with high refractoriness to systemic therapy that lead to development of multidrug resistance[36-38].

Need for a novel approach in HCC management

The challenges associated with both diagnosis and treatment of HCC has resulted in the high mortality rates across the globe, and calls upon innovative approaches that can improve the prognosis of HCC patients. In the following sections, we describe the rapid advances and implementation of theranostic-based nanomedicine and nanoparticles (nanotheranostic) as a promising option (Figure 1) for the improvement of HCC patient outcomes and quality of life.

POTENTIAL OF NANOTHERANOSTICS FOR PRECISION CANCER MEDICINE

Nanotheranostic modalities present a promising solution to the diagnostic and therapeutic challenges encountered in HCC management, through the use of biocompatible nanoparticles that simultaneously performs both diagnostic and therapeutic functions. This approach potentially provides a more personalized and targeted approach to cancer therapy, wherein the nanoparticles can be designed to detect specific biomarkers of the target malignant region, allow real-time monitoring or visualisation of the target, and finally deliver therapeutic modalities in a more precise manner. In recent years, the nanosensor and nanomedicine technologies have experienced major development, and have paved the way for promising means of nanotheranostics implementation in cancer management.

Nanotheranostic is a real-time combination of novel therapeutic and modern diagnostic tool or imaging into a single agent linked and integrated by nanoparticles [39,40]. Nanoparticles are the key components of the nanotheranostic agent[41] which include aptamer[42], DNA nanostructure[43], lactosome-based nanoparticles[44], metallic nanoparticles[45], gold nanoparticle[46], silver nanoparticle[47], dendrimer and copolymer-based nanoparticles[48], lipid-based nanomaterials[49], magnetic nanoparticles[50] iron oxide nanoparticle[51], mesoporous silica nanoparticle[52] and

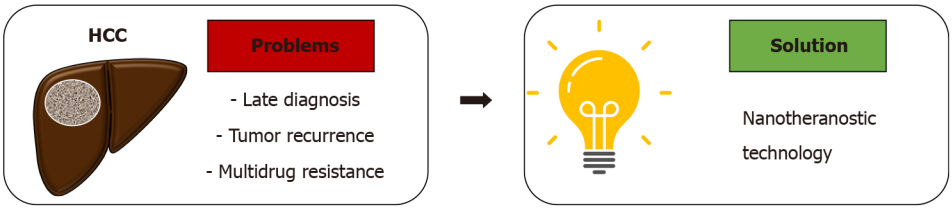

Figure 1 Hepatocellular carcinoma current problems and solution. HCC: Hepatocellular carcinoma.

quantum dots nanoparticle[53].

Nanotheranostic is an ideal choice for cancer treatment in the era of personalized medicine due to its potential to overcome the diagnostic and therapeutic challenges described prior[54]. Nanotheranostic not only provides the means for early diagnostic tools[55], nanoimaging-therapeutic integrated medicine[56], targeted-therapy[57] and tumor-specific nano-delivery agent[58], it also holds potential for real-time monitoring of drug response, and reduce side effects and drug toxicity in patients[59,60] as shown in Figure 2.

Successful demonstration of nanotheranostics for diagnostics and targeted therapy has been shown by Roy *et al*[61] in which highly sensitive, polymer-modified gadolinium-doped iron oxide-based T_1 contrast agents were used for successful methotrexate drug delivery. In the second application, nanotheranostics have been utilized for simultaneous imaging and cancer monitoring[62]. An auto-fluorescent platform, constructed from a positively charged amphiphilic polymer polyethyleneimine-polylactide, was utilized to simultaneously load the antiangiogenesis agent cobretrastatin together with near-infrared (NIR) dye IR825 and heat-shock protein inhibitors. Altogether, the mechanism represents self-monitoring nanotheranostics, which in a mouse model demonstrated inhibitive properties in the tumour site through anti-angiogenesis and gene silencing enhanced photothermal therapy, while allowing real-time fluorescence monitoring.

The final and most widely developed application of nanotheranostics is for simultaneous imaging and targeted therapy, which has been shown to substantially increase the overall efficacy of therapies[63-65]. Theranostic platform choice has expanded rapidly in the past decade, and typically combines imaging modalities such as magnetic resonance imaging (MRI), NIR fluorescence, photoacoustic (PA) or ultrasound imaging, with therapeutic modalities such as chemotherapeutic agents, x-rays, hyperthermia, or free radicals.

Depending on the desired diagnostic and therapeutic modality, the nanoparticle of choice may be composed of metals, polymers, carbons and lipids. Each choice provides its own unique characteristics and physicochemical interactions, and also require different fabrication and functionalization procedures. As an example, successful magnetic-based imaging in a nanotheranostic platform is achievable using iron oxide, which is also desired due to its low toxicity and chemical stability. But many platforms prefer the use of multi-functional semiconducting polymers with hydrophobic properties, which simultaneously allow imaging through easy interactions with aromatic chemotherapeutic agents[66]. Nanoparticles can also be engineered to provide multimodal imaging[67] which utilizes modified ultrasmall Ag_2Se nanodots to allow upconversion luminescence, downshifting luminescence, computed tomography and PA imaging techniques.

An increasingly common approach in cancer theranostics is the use of multimodal therapy. To illustrate, the study by Zhang *et al*[68] utilized Janus-type γ-Fe_2O_3/SiO_2 nanoparticles to combine the glucose oxidase-mediated cancer starvation strategy with hydroxyl radicals as chemodynamic therapy. Interestingly, nanoparticles can also be designed to become responsive towards environmental stimuli in drug-resistant tumours, meaning that it can be developed specifically towards the pathological profile of the tumor microenvironment as well as the organ-specific tissues and compartments, which contribute to the overall specificity of the drug delivery. For highly complex pathologies such as HCC with drug resistance, this provides a myriad of options for exploration, and becomes an interesting approach for future implementation of HCC-specific nanotheranostics.

Finally, it is also worth noting that metastasis remains a major issue in cancers such as HCC where diagnosis tends to be late. An interesting strategy[69] showed the use of immunotherapy-based theranostics to specifically target metastatic tissue. In said study, magnetic-responsive immunostimulatory nanoagents were added with

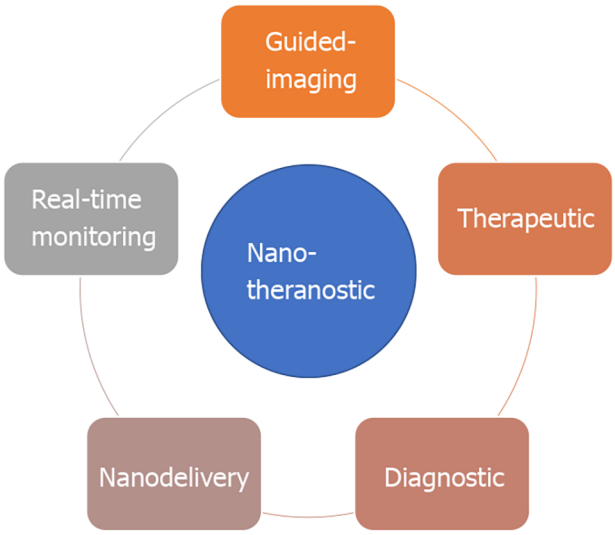

Figure 2 Applications of cancer nanotheranostic.

superparamagnetic iron oxide nanoparticles and cytosine-phosphate-guanine oligodeoxynucleotides. These engineered components allow for PA and MRI in addition to acting as a therapeutic agent for photothermally triggered immunotherapy.

To illustrate these advancements, we present the current modalities of cancer nanotheranostics in Table 1. In general, cancer nanotheranostics has been used for simultaneous diagnostics and therapeutic[70], real-time monitoring of malignancies [71], guided-imaging[72,73], drug-delivery[74] and multimodal-targeted therapy[75-79].

TACKLING HCC WITH NANOTHERANOSTICS

The nanotheranostic platform is a promising approach that is urgently needed to overcome the limitations of conventional therapy and diagnosis for more efficient HCC management. Figure 3 illustrates the multimodality of the nanotheranostic platform, which utilizes multipurpose nanoparticles for targeted nano-delivery, continuously controlled release of anticancer agents, guided imaging and early detection, for superior effectivity of transport[80]. Management of HCC requires powerful theranostic-based nanoparticles for early diagnostics and therapeutics with higher sensitivity and specificity, and to surpass the limitation of tissue penetration [81]. Previous studies have demonstrated the promising potential of silica-based nanomaterial as a potent nanotheranostic platform of HCC targeted-therapy nano-delivery[82-86]. In addition, many advances in HCC-specific nanotheranostics platforms are illustrated in Table 2, which demonstrates the multifunctional role of nanotheranostics as a detector to identify the HCC cell and tumor inhibitor by suppressing proliferation, migration and invasion of HCC[87,88].

One of the most remarkable advancements of the nanotheranostic platform is imaging and nano-delivery integration as an innovative resolution for early HCC diagnosis and *in situ* drug release. *In vivo* and *ex vivo* investigations have observed specific nanoplatform activation by the tumor, with minimized toxicity towards non-target cells[89]. Integration of multifunctional nanoparticles with MRI may provide novel perspectives in tumor imaging technology to enhance HCC management and treatment strategy. High precision quantification and sensitivity of nanoimaging technology is needed for tissue penetration issue in early diagnosis of HCC[90].

Aptamer-based nanotheranostic is also a potential tool for HCC management due to its unique characteristics. This oligonucleotide nanomedicine has high specificity and affinity towards various types of target molecules[91]. In HCC clinical application, aptamer-based nanotheranostic development targeting the epithelial cell adhesion molecule demonstrated an improvement of MRI application and drug-delivery with high efficiency of doxorubicin released specifically towards cancer cells[92].

Improvement of therapeutic success is urgently needed for patients with unresectable and advanced HCC. The combination between nanomedicine as a nano-

Table 1 Cancer nanotheranostics

Applications	Principle	Ref.
Diagnostic and therapeutic	Stimuli responsive nanoparticle and targeted drug delivery	[61]
	Activatable nanotheranostic systems diagnosis and therapy of peritoneal metastasis	[70]
Real-time monitoring and therapeutic	Self-monitoring and triple-collaborative therapy *via* auto-fluorescence nanoparticles	[62]
	Real-time monitoring and tumor targeting *via* dual-fluorescent hydroxyapatite–doxorubicin	[71]
Guided-imaging and nanodelivery	Nanoparticle conjugated with antibody for tumor targeting and guided drug delivery	[63]
	A protein-stabilized multifunctional nanoplatform for multimodal imaging and drug-delivery	[64]
Guided-imaging and therapeutic	Dual-targeting nanotheranostic with chemosensitizing agent for MDR chemotherapy	[65]
	Multifunctional nanocarrier for fluorescence imaging guided chemo-photothermal	[66]
	Dual-modal imaging and synergistic cancer starvation/chemodynamic therapy	[68]
	Tetra-modal imaging guided photothermal therapy	[67]
	Bimodal imaging guided photothermal-triggered immunotherapy	[69]
	Hierarchical tumor acidity-responsive magnetic nanobomb photodynamic therapy	[73]
	Lipid based nanoparticles nanodelivery-anticancer drug and nanoimaging	[74]
	The self-assembly nanoparticles with guided imaging and chemotherapeutic drugs	[76]
	Biocompatible nanoparticles as targeted-nanodelivery of chemotherapeutic agent	[77]
	Dual-modality mapping guided photothermal ablation for metastatic cancer	[78]
	Magnetic nanoparticle-doxorubicin for enhancing nanoimaging and targeted therapy	[79]

MDR: Multidrug resistance.

Table 2 Nanotheranostic development againts hepatocellular carcinoma

Applications	Principle	Ref.
Diagnostic and therapeutic	Conventional SELEX	[87]
	CE-SELEX	[88]
	Magnetic nanoparticle-aptamer	[92]
Enhancing therapeutic	Inducing tumor regression using siRNA-nanoparticle construction	[100]
	Enhancing the anticancer efficacy using siRNA-nanoparticle construction	[101]
	Enhancing chemotherapy using microRNA 375-nanoparticle construction	[102]
	Synergistic antitumor effect of microRNA 375-nanoparticle construction	[103]
Diagnostic and guided-imaging	'Activatable' aptamer-based fluorescence probe	[104]
	Streptavidin-fluorescent silica nanoparticles combination	[105]
	Aptamer- based electrochemical biosensors	[106]
Gene editing	Next-generation CRISPR/Cas technology	[107]

HCC: Hepatocellular carcinoma; SELEX: Systematic Evolution of Ligands by Exponential Enrichment.

delivery system with cancer immunotherapy holds great potential for enhancing the nanotherapeutic outcome for this population. A promising targeted-nano-delivery immunotherapy for advanced HCC that is currently undergoing clinical trial is the 4th generation chimeric antigen receptor (CAR) T cells targeting glypican-3 (GPC3) (ClinicalTrials.gov Identifier: NCT03884751)[93]. This study showed promising phase I results in regard to antitumor activity and safety profile of CAR-GPC3 T-cell immunotherapy. The antitumor activity is positively associated with tumor response with no grade 3/4 neurotoxicity effect in any patients[94]. Several studies have also been done

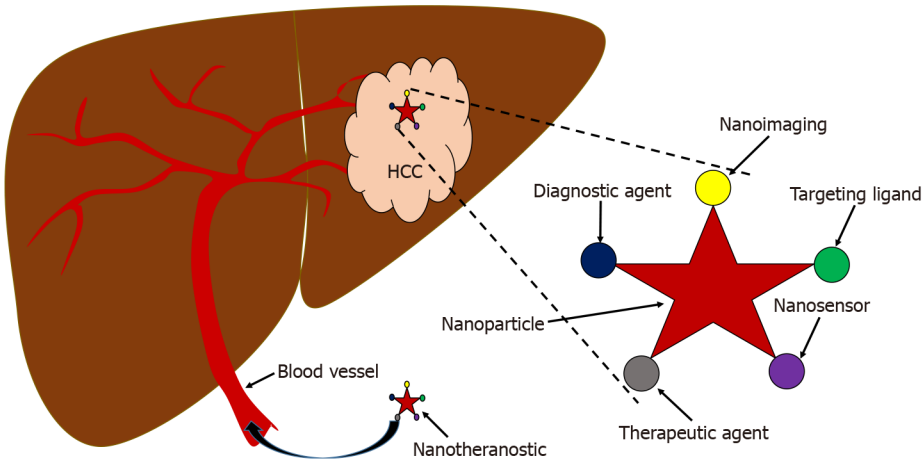

Figure 3 Multimodality of the nanotheranostic platform. HCC: Hepatocellular carcinoma.

to achieve said goal by conjugating anticancer drugs with nanoparticles, rendering the treatment safer with more effective systemic administration due to the platform's capability of controlling and postponing drug release. In the *in vivo* mouse model, tumor specific uptake of the controlled drug release for several weeks was observed, with minimal toxicity[95].

Molecular-targeted nano-therapies have also been constructed for nano-delivery using a modular design of polymeric nanoparticles for selective accumulation of drug pay load within tumor lesions. In *in vivo* mouse models, the intravenous drug injection was more effective for tumor inhibition than oral administration. This has revolutionized anticancer therapy by enhancing the efficacy and potency of therapeutics through inhibition of the angiogenesis pathway, tumor growth, tube formation and metastasis[96]. Targeted drug delivery using mesoporous silica nanoparticle is also promising. Nanoconstruction of silica nanorattle encapsulated docetaxel exhibited low toxicity with high antitumor activity, making it a prospective candidate for nano-delivery system[97]. Moreover, modified silica nanoparticles targeting low density lipoprotein and loaded with two anticancer drugs for liver cancer chemotherapy showed increased delivery efficiency based on *in vitro* and *in vivo* analysis[98].

In addition to anticancer drug nano-delivery for HCC treatment, the nanotheranostic platform is also suitable for targeted nano-delivery of small interfering RNA based therapeutics. This can be used as gene therapy to knock down a specific gene [99-101], and micro RNA for enhancing chemotherapy efficiency[102] to overcome multi-drug resistance in HCC[103].

FUTURE PERSPECTIVES

HCC is an extremely complex and heterogeneous disease with diverse molecular profiles, aetiology and subtypes. Since conventional approaches still fail to overcome limitations in HCC management, nanotheranostic is a promising alternative to overcome the problems. Rapid development in nanotechnology has added a tremendous value on cancer therapy. The future of cancer nanomedicine lies on multimodal nanoplatforms that combine targeting ligands, imaging agents, diagnostic agents and therapeutic components into one unit of functionalized nanoparticles. Thus, multifunctionality is a powerful and unique advantage of nanotheranostic over traditional methods, and evidence has shown its capacity to work efficiently and noninvasively *in vivo* without systemic toxicity. Development of nanotheranostic in the right direction requires improvement of platforms so that it can be optimized simultaneously for proficient performance as the best clinical outcome in HCC.

CONCLUSION

In summary, nanotheranostic is an emerging and promising approach for HCC diagnosis/imaging and therapy in the future. Nanotheranostic is a powerful, unique, and multifunctional tool that yields positive impact both in the basic research and

clinical application of HCC. We predict that in a near future nanotheranostic platform will continue to exponentially grow and progressively implemented in the development of novel and efficacious diagnostic and therapeutic agents towards cancers, including HCC. Further expansion would be needed to assist clinical translation of the promising preclinical studies in HCC.

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MINIREVIEWS

Multiple subcellular localizations and functions of protein kinase Cδ in liver cancer

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Abstract

Protein kinase Cδ (PKCδ) is a member of the PKC family, and its implications have been reported in various biological and cancerous processes, including cell proliferation, cell death, tumor suppression, and tumor progression. In liver cancer cells, accumulating reports show the bi-functional regulation of PKCδ in cell death and survival. PKCδ function is defined by various factors, such as phosphorylation, catalytic domain cleavage, and subcellular localization. PKCδ has multiple intracellular distribution patterns, ranging from the cytosol to the nucleus. We recently found a unique extracellular localization of PKCδ in liver cancer and its growth factor-like function in liver cancer cells. In this review, we first discuss the structural features of PKCδ and then focus on the functional diversity of PKCδ based on its subcellular localization, such as the nucleus, cell surface, and extracellular space. These findings improve our knowledge of PKCδ involvement in the progression of liver cancer.

Key Words: Protein kinase Cδ; Liver cancer; Subcellular localization; Tumor suppression; Tumor progression

Core Tip: Protein kinase Cδ (PKCδ) plays multifunctional roles in various cancers, including liver cancer. PKCδ has been shown to exert pleiotropic functions through various stimuli responsiveness, post-translational modifications, and subcellular localization. Recently, we found that PKCδ is secreted extracellularly and resides on the cell surface of liver cancer cells, which contributes to tumorigenesis. In this review, we focus on the localization of PKCδ to discuss its characteristic localization patterns and functions in liver cancer, and outline the involvement of PKCδ localized intra- and extracellularly with distinct functions in the progression of liver cancer.

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Grade A (Excellent): 0
Grade B (Very good): 0
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Grade D (Fair): D
Grade E (Poor): 0

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INTRODUCTION

The protein kinase C (PKC) family of serine/threonine kinase proteins in mammals, comprising the classical PKC (cPKC), novel PKC (nPKC), and atypical PKC (aPKC) subfamilies, is one of the defining families of AGC kinases[1,2]. To date, 10 isoforms of PKC have been identified in humans, including four cPKCs (PKCα, -βI, -βII, and -γ), four nPKCs (PKCδ, -ε, -η, and -θ), and two aPKCs (PKCζ and -λ/ι)[2,3]. PKC activation depends on the conformational activation of certain intracellular factors. Notably, PCK activation is regulated not only by binding to lipid factors, such as diacylglycerol (DAG) and phorbol esters, but also by protein phosphorylation[4]. PKCδ is often phosphorylated at several Tyr residues by various types of stimulations, including DNA-damaging reagents and oxidative stress[5,6]. Kinases that phosphorylate PKCδ at Tyr include the Src family of tyrosine kinases (*e.g.*, Src, Fyn, Lyn, and Lck) and c-Abl [6].

Among PKC families, PKCδ is a unique non-signal peptide-containing intracellular protein that has been reported to translocate to a diverse range of distributions, including the cytosol, nucleus, endoplasmic reticulum, Golgi, mitochondria, and plasma membrane, in response to different stimuli and cell types[8]. For example, a nuclear localization signal (NLS) was identified in the catalytic domain of PKCδ, which is necessary for the transport of PKCδ across the nuclear pore. Nuclear localization of PKCδ is associated with pro-apoptotic functions. Phosphorylation also affects the subcellular localization of PKCδ and its activation. Our recent study revealed that cytosolic PKCδ translocates to the extracellular space and acts as a growth factor for liver cancer cells or tumors[9]. In this review, we summarize studies reported to date regarding the intracellular function of PKCδ in cancerous phenotypes of liver cancer. We then focus on and discuss the relationship with subcellular localizations, which exist in extracellular and intracellular locations, and the functions of PKCδ. Increased knowledge on where PKCδ protein is localized and how it functions in living cells allows a more profound understanding of the functional diversity of PKCδ.

STRUCTURAL FEATURES OF PKCδ

PKCδ comprises an N-terminal regulatory domain and a C-terminal kinase core domain[5]. The C-terminal catalytic domain of PKC is conserved between isoforms and includes ATP- and substrate-binding sites and a kinase core[10,11]. The N-terminal regulatory domain is much less conserved and contains specific motifs for each isoform that are activated in response to unique signals. The regulatory modules in this N-terminal domain include the pseudosubstrate motif and C1 and C2 domains, which bind to Ca^{2+} and DAG. The affinity of the C1 and C2 domains for Ca^{2+} and DAG determines the cofactor requirements for the activation of specific PKC isoforms. cPKCs have functional C1 and C2 domains that bind to both Ca^{2+} and DAG[12-14], whereas nPKCs have a functional C1 domain that binds to DAG alone and a non-functional C2 domain, rendering these kinases independent of Ca^{2+} for activation[15].

Generally, upon PKC activation, growth factors and G protein-coupled receptors trigger the hydrolysis of membrane lipids by recruiting phospholipase C[16,17]. Phospholipase C generates DAG and inositol-1,4,5-triphosphate through the hydrolysis of membrane phosphoinositol. In response to DAG, PKC is translocated to the lipid membrane *via* the C1 domain, enabling interaction with its substrates and the phosphorylation response[18].

PKCδ confers distinct allosteric regulation *via* protein binding to the C1, C2, and V5 domains, tyrosine phosphorylation, and the removal of the regulatory domain by caspase cleavage (Figure 1). In particular, various reports have identified that a variety of tyrosine phosphorylation sites affect cellular functions. For example, many studies have demonstrated that tyrosine phosphorylation of PKCδ plays a critical role in cell death in response to apoptotic stimuli. Tyrosine residues important in the context of

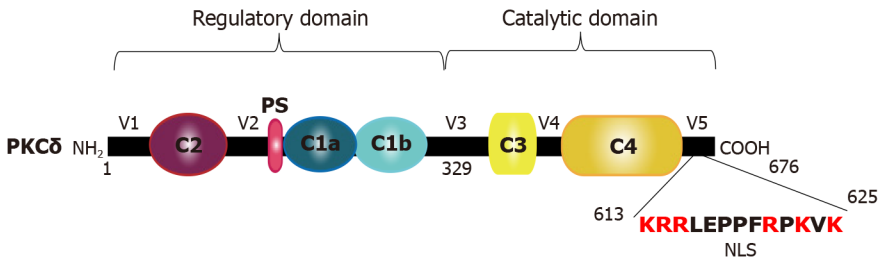

Figure 1 Schematic representation of protein kinase Cδ domains. The N-terminal regulatory domain is composed of 1 to 329 amino acids, non-functional C2, pseudosubstrate, and lipid binding C1 (a and b) domains. The C-terminal catalytic domain is composed of 330 to 676 amino acids, ATP-binding C3, and kinase C4 domains. The 329 amino acids at the V3 region allow the cleavage site by caspase-3 to be constitutively active. The V5 region includes nuclear localization signal necessary for the nuclear transport of protein kinase Cδ. NLS: Nuclear localization signal.

apoptosis include Tyr64, Tyr155, Tyr187, Tyr311, Tyr332, and Tyr512[18-20]. Furthermore, tyrosine phosphorylation of Tyr64 (C2 domain) and Tyr155 (C1a domain) is crucial for the exposure of PKCδ to NLS by disrupting the association between C2 and catalytic domains to enable nuclear transport.

FUNCTIONAL FEATURES OF INTRACELLULAR PKCδ IN LIVER CANCER

Tumor suppressive function

Studies on PKCδ$^{-/-}$ mice have confirmed the pro-apoptotic role of this molecule in response to several stimuli, such as DNA damage. Although these mice developed normally and were fertile, increased B cell proliferation was observed[21]. Smooth muscle cells derived from PKCδ$^{-/-}$ mouse aortas were also shown to be resistant to cell death in response to several stimuli. Hence, these studies with PKCδ$^{-/-}$ mice demonstrated that PKCδ is not required for cell proliferation during development.

PKC also binds to and is activated by tumor-promoting phorbol esters[4]. Therefore, PKC is considered a tumor-promoting protein. However, it has been reported that persistent treatment with phorbol esters causes degradation or downregulation of PKC [3,22-24]. In particular, PKCδ has been reported to enhance ubiquitin proteasomal degradation upon activation of PKCδ by lipids. Furthermore, accumulating evidence on PKCδ in cancer has shown that downregulation, rather than activation, of PKCδ is associated with tumor progression. Therefore, PKCδ is believed to act as a tumor suppressor because its downregulation facilitates tumor promotion and causes cell cycle arrest or induces apoptosis in response to various stimuli, such as H_2O_2, ceramide, tumor necrosis factor-α (TNF-α), ultraviolet radiation, cisplatin, and etoposide[22,25-27]. In fact, the *PKCδ* gene is deleted in many cancers[28]. Ectopic expression of PKCδ has been shown to decrease the anchorage-independent growth of NIH3T3 cells and reverse the transformation of rat fibroblasts and colonic epithelial cells by Src. Low levels of PKCδ have been reported in colon cancer, and overexpression of PKCδ suppresses the neoplastic phenotype of colon cancer cells[25]. A recent report suggested that PKCδ is lost in human squamous cell carcinoma due to transcriptional repression[29]. PKCδ has also been reported to decrease cell migration in breast cancer cells, whereas knockout of the PKCδ gene increases cell migration in mouse embryonic fibroblasts. These studies strongly support the role of PKCδ in tumor suppression.

Multiple reports suggest that PKCδ is responsible for apoptotic signaling in liver cancer cells (Table 1). In sorafenib-resistant hepatocellular carcinoma (HCC) cells, PKCδ activation was shown to induce cellular apoptosis *via* p38 activation[30]. Annexin A3 (ANXA3) interacts with PKCδ and thereafter suppresses PKCδ/p38-associated apoptosis and activates autophagy for cell survival. Thus, inhibition of ANXA3 by a monoclonal antibody is likely to impair cell survival and tumor growth. Although FTY720, a synthetic sphingosine immunosuppressor, has been known to have antitumor effects on HCC cells, PKCδ activation occurs in FYT720-treated HCC cells. FTY720 is thought to activate PKCδ *via* the generation of reactive oxygen species (ROS) and subsequent caspase 3-dependent cleavage to induce apoptosis. The relationship between intracellular activation of PKCδ and apoptosis in HCC cells has also been reported in the antitumor mechanism of an antagonist of FZD7, which is a membrane receptor overexpressed in HCC[31]. These lines of evidence suggest that PKCδ activation is not favorable for malignant transformation in liver cancer and may

Table 1 The relationship between subcellular localizations and functions of protein kinase Cδ in liver cancer

Response	Localization	Function	Mechanisms	Ref.
ANXA3 expression	Cytosol/plasma membrane	Interacts with PKCδ and inhibits apoptosis	p38MAPK activation	[30]
ROS	Nucleus	Activates PKCδ and induces apoptosis	Activates caspase 3 and induces cleavage of PKCδ	[31]
Claudin-1	Cytosol/plasma membrane	Enhances the ability of cell migration/invasion	Induces c-Abl-PKCδ signaling	[33]
mtROS	Plasma membrane	Induces gene expression for cell migration	Triggers oxidation of HSP60 and then induces MAPK activation	[34]
HIF-2α expression	Cytosol/plasma membrane	Induces cell migration	Phosphorylates PKCδ at Tyr311	[35]
HSP27 expression	Cytosol/plasma membrane	Inversely correlates with tumor malignancy	p38MAPK activation by PKCδ induces phosphorylation of HSP27	[36]
No response	Extracellular space/cell surface	Enhances cell proliferation	Activates MAPK signaling	[9]

mtROS: Mitochondrial reactive oxygen species; ANXA3: Annexin A3; PKCδ: Protein kinase Cδ; MAPK: Mitogen-activated protein kinase; HIF: Hypoxia-inducible factor; HSP: Heat shock protein.

be inactivated in these cells.

Tumor promotive function

Many studies have shown that PKCδ promotes the survival of multiple types of cancers, including non-small cell lung cancer, breast cancer, pancreatic cancer, chronic lymphocytic leukemia, and liver cancer.

PKCδ has been reported to induce signal survival. In fact, PKCδ promotes cell survival *via* several well-known pro-survival pathways, including NF-κB, Akt, and extracellular signal-regulated kinase (ERK). It has been reported that PKCδ inhibits apoptosis by inhibiting apoptosis protein-2 and FLICE-like inhibitory protein *via* NF-κB[32].

Numerous publications have reported that PKCδ is actively involved in the promotion of liver cancer, including cell migration, invasion, and tumor stage (Table 1). For example, claudin-1, a member of the tetraspanin family, plays a critical role in the acquisition of invasive capacity in human liver cells, and c-Abl-PKCδ signaling is important for malignant progression induced by claudin-1[33]. This c-Abl-PKCδ signaling pathway was shown to activate MMP-2, a key factor in cell migration and invasion. The cross talk between PKC and ROS may induce mitogen-activated protein kinase (MAPK) activation for cell migration and progression. Mandal *et al*[34] found that activation of PKCδ generated mitochondrial ROS triggers the oxidation of heat shock protein 60 (HSP60), a chaperone protein in the mitochondria, which induces the activation of ERK and c-Jun N-terminal kinase (JNK) in the cytosol, resulting in gene expression leading to migration in liver cancer. PKCδ and hypoxia have also been reported to be associated with cell migration in liver cancer. Hypoxia-inducible factor-2α expression regulates CUB domain-containing protein 1, which stimulates the phosphorylation of PKCδ at Tyr311 to induce malignant migration in various cancer cells, such as liver cancer cells[35]. Furthermore, the levels of HSP27 are inversely correlated with tumor stage, as per the tumor, node and metastasis classification, in patients with HCC. Takai *et al*[36] showed that PKCδ activation regulates the phosphorylation of HSP27 *via* p38 MAPK.

There is supportive evidence that PKCδ acts as a tumor promoter in many types of cancers. For example, the mRNA levels of PKCδ were higher in estrogen receptor (ER)-positive tumors than in ER-negative tumors, and an increase in PKCδ mRNA was associated with reduced overall survival[37]. PKCδ knockdown decreased the survival of MCF-7 and MDA-MB-231 breast cancer cells[38]. Overexpression of PKCδ was also observed in human ductal pancreatic carcinomas compared to its normal counterparts. PKCδ has been reported to be associated with melanoma cell metastasis[39]. A recent study demonstrated that integrin αvβ3-mediated invasion of melanoma cells is mediated *via* PKCα and PKCδ.

SUBCELLULAR LOCALIZATIONS AND FUNCTIONS OF PKCδ

Cytosol and plasma membrane

PKCδ is translated on the ribosome in the cytosol and generates its inactive cytosolic form. Similar to other PKC families, in response to DAG, PKCδ is also translocated to the plasma membrane *via* the C1 domain, which exerts a subsequent phosphorylation response. PKCδ activation is also required for Akt activation by Ras[40] (1-98). Activating mutations with Ras or PI3K increases PKCδ levels and induces Akt activation. PKCδ also induces ERK1/2 activation[41,42]. Akt and ERK1/2 activation have been implicated in the PKCδ-mediated increase in anchorage-independent growth and resistance of pancreatic ductal cancer cells to apoptotic stimuli[43]. Conversely, cytosolic PKCδ reportedly triggers apoptosis by activating p38 MAPK to inhibit Akt[8], indicating that PKCδ activation can behave as both a prosurvival and pro-apoptotic factor. Liver damage has been reported to induce inflammation and PKCδ translocation to the plasma membrane[44,45]. PKCδ activation has been observed in the tissues of patients with non-alcoholic steatohepatitis and non-alcoholic fatty liver disease and in a mouse model of hepatic cirrhosis[46-49].

Nucleus

Importantly, PKCδ is a PKC isoform that has been identified as a substrate for caspase-3[50]. Cleavage of PKCδ by caspase-3 separates the regulatory domain and catalytic fragment to allow constitutive activation of PKCδ even in the absence of any co-factors [22] and then translocates to the nucleus, where the catalytic fragment of PKCδ induces apoptosis[22]. Others and we have shown that full-length or fragmented PKCδ is translocated to the nucleus by transiting the nuclear pore[6,51]. Nuclear PKCδ interacts with and phosphorylates its substrates such as α-Abl, p53, p73, lamin B, Rad9, topoisomerase II, heterogeneous nuclear ribonucleoprotein K (hnRNP-K), and DNA-dependent protein kinase[22,52-54]. Moreover, nuclear PKCδ regulates the transcription of target genes in response to cellular stresses such as DNA damage, which is implicated in pro-apoptotic functions.

Upon oxidative stress, we previously showed that PKCδ associates with and activates IKKα in the nucleus[55]. Although IKKα activates NF-κB by phosphorylating IκB in the cytoplasm, which leads to prosurvival signaling, PKCδ-mediated IKKα activation at the nucleus causes phosphorylation of p53 at Ser20; however, it does not affect NF-κB activation.

The tumor suppressor p53 is a master regulator of cellular processes, such as cell cycle arrest, DNA repair, or apoptosis[56,57]. Several studies have suggested that p53 is located downstream of PKCδ. In response to genotoxic stress, PKCδ phosphorylates p53 at Ser46 to trigger p53-mediated apoptosis.

In the nucleus, PKCδ also regulates p53 expression by increasing *p53* transcription. We previously reported that PKCδ interacts with the death-promoting transcription factor Btf to induce Btf-mediated *p53* gene transcription and apoptosis[58]. In addition, TNF-α treatment induces translocation of PKCδ into the nucleus[59]. PKCδ can bind to the NF-κB RelA subunit and subsequently induce the transactivation of p65/RelA[59]. These findings demonstrate that NF-κB is involved in PKCδ-mediated TNF/TNF-related apoptosis-inducing ligand (TRAIL) resistance. PKCδ inhibition or knockdown decreased NF-κB expression and sensitized MCF7 cells to TNF/TRAIL-induced cell death[60].

Mitochondria

Bax and Bak are pro-apoptotic factors, and the Bcl-2 family regulates mitochondrial membrane permeability to induce apoptosis[61]. Upon exposure to ionizing radiation, Bax and Bak are activated *via* the c-Abl-PKCδ-p38 pathway to trigger mitochondrial cell death[30,62]. Mcl-1, an anti-apoptotic Bcl-2 family member, is a direct target of PKCδ. The catalytic fragment of PKCδ phosphorylates Mcl-1 and degrades it, leading to cell death. During the early stages of hypoxic stress, PKCδ induces autophagy *via* JNK-mediated phosphorylation of Bcl-2 to dissociate the Bcl-2/beclin-1 complex, and prolonged hypoxic stress induces PKCδ cleavage[63].

Cell surface

We recently showed that PKCδ is localized at the cell surface of liver cancer cell lines (Figure 2). Cell surface PKCδ was found to be anchored by other cell surface proteins, such as heparan sulfate proteoglycans (HSPGs). Some growth factors, such as fibroblast growth factors, vascular endothelial growth factor, and hepatocyte growth factor[64,65] have cationic amino acid clusters that can interact with heparan sulfate,

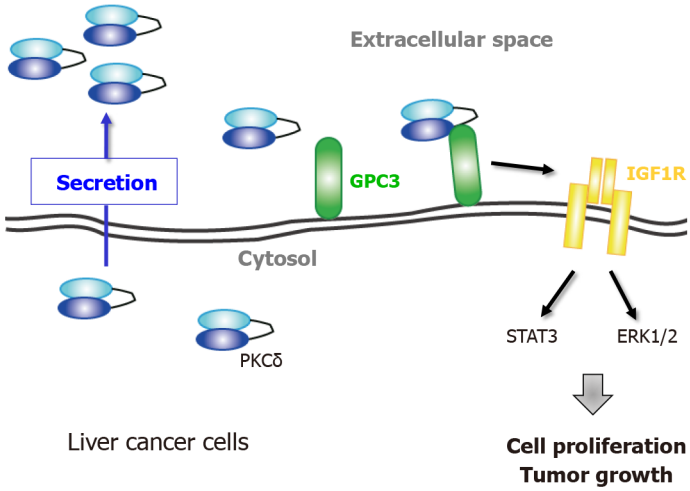

Figure 2 Extracellular protein kinase Cδ shows oncogenic property in liver cancer. Model of the proliferative regulation of extracellular protein kinase Cδ (PKCδ) in liver cancer cells. PKCδ is secreted from living cells and resides at the plasma membrane through its association with glypican 3, leading to an increase in insulin-like growth factor 1 receptor activation and enhancement of subsequent proliferative signaling to increase cell growth. PKCδ: Protein kinase Cδ; GPC3: Glypican 3; ERK: Extracellular signal-regulated kinase; IGF1R: Insulin-like growth factor 1 receptor.

which is composed of one or more unbranched anionic polysaccharide(s) known as glycosaminoglycans[65-67]. The cationic amino acid clusters closely resemble the NLS of intracellular proteins[68]. In fact, extracellular NLS-containing proteins, such as importin α1, huRNP-K, and PKCδ are detected at the cell surface of human cells[9,69,70]. These extracellular NLS-containing proteins are more likely to be located at the cell surface by binding to HSPGs.

Furthermore, glypican3, a liver cancer-specific HSPG, was identified as a receptor for cell surface PKCδ[71]. Both Cheng *et al*[72,73] and we showed that GPC3 regulates the activation of insulin-like growth factor 1 receptor (IGF1R)[9]. In fact, we found that extracellular PKCδ induces activation of IGF1R *via* association with GPC3 and its downstream signaling molecules, such as ERK1/2 and STAT3. Thus, these lines of evidence strongly suggest that cell surface PKCδ acts as a growth factor. In addition, we showed that anti-PKCδ monoclonal antibody (mAb) inhibits the proliferation and tumorigenesis of liver cancer cells, but not PKCδ-CRISPR knockout cells. Thus, cell surface PKCδ may be a potential therapeutic target for liver cancer.

Extracellular space

We also found that PKCδ is secreted into the extracellular space in liver cancer[9]. Extracellular accumulation of PKCδ was detected in different liver cancer cell lines but not in hepatocytes, suggesting that PKCδ secretion may be specific to liver cancer cells. Interestingly, our proteomics study showed that PKC, rather than PKCδ, was not detected in the culture medium of liver cancer cell lines. This means that PKCδ is a unique isoform of the PKC superfamily that is secreted extracellularly. Furthermore, higher levels of PKCδ were detected in the serum of patients with liver cancer, but not in patients with chronic hepatitis, hepatic cirrhosis, or healthy donors. This increase in serum PKCδ levels was also noted in a limited number of AFP- and PIVKA-II-negative liver cancer patients. Based on these clinical data, we propose that serum PKCδ may be a novel biomarker for liver cancer.

Recently, we and other groups have reported the extracellular localization of proteins with no signal peptide-containing proteins, such as FGF1, FGF2, HMGB1, hnRNP-K, importin α1, and IL-1β[69,70,74-77]. Secretion of these proteins is referred to as unconventional secretion[74,78]. Since the *PKCδ* gene does not encode a signal peptide, the extracellular secretion of PKCδ is also categorized as unconventional secretion. PKCδ has been shown to be full-length in the extracellular space and continues to be released from growing cells[9]. Many studies have reported that IL-1β secretion often occurs in immune cells after induction of inflammatory stimulation[77,79,80]. There are some differences in the secretion modes between immune and cancer cells. Unlike immune cells (IL-1β), liver cancer cells constitutively secrete importin α1 and PKCδ even under physiological culture conditions (using 10% FBS medium)[9,69]. Conversely, some features were common between immune and liver cells, including the induction of unconventional secretion by ATP treatment[81] and independent of brefeldin A, an inhibitor in the "conventional" secretion pathway of

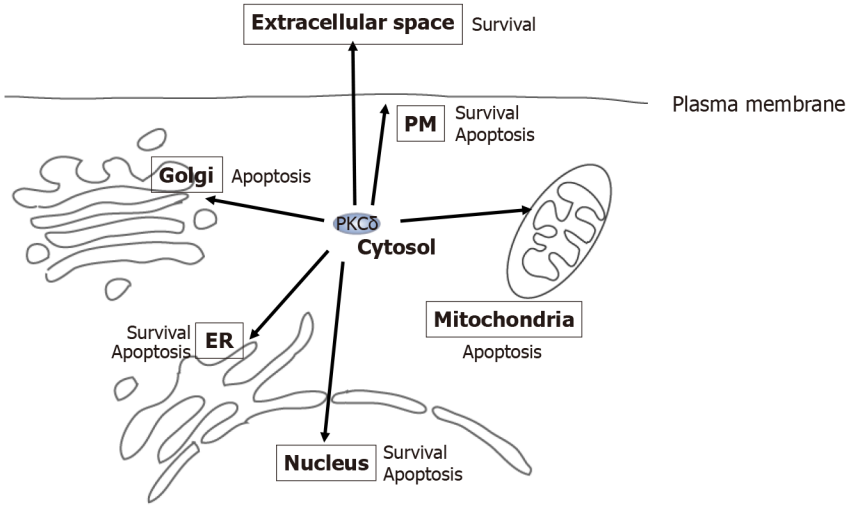

Figure 3 Multi-localization and functional diversity of protein kinase Cδ. Protein kinase Cδ (PKCδ) resides at various locations, including the cytosol, nucleus, estrogen receptor (ER), mitochondria, Golgi, extracellular space, and plasma membrane (inside and outside the cell). At each location, PKCδ acts as an apoptotic or survival factor in response to various stimuli, such as genotoxic stresses, phorbol ester, DNA damage, ER stress, tumor necrosis factor (TNF)-α, and TNF-related apoptosis-inducing ligand. PKCδ: Protein kinase Cδ; ER: Estrogen receptor; PM: Plasma membrane.

signal peptide-containing proteins[82].

We found that PKCδ secretion was initiated in the cytosol. Phorbol ester treatment inhibited PKCδ secretion, and the NLS active mutant was not secreted into the extracellular space. In fact, secreted PKCδ showed a lower level of phosphorylation (Tyr311 and Thr505). These lines of evidence support the possibility that cytosolic PKCδ, as a starting point for extracellular localization, could contribute to tumor progression in liver cancer.

Other organelles

A previous study has shown that PKCδ is translocated to the ER in response to ER stress and interacts with ER-bound c-Abl[83]. This PKCδ-c-Abl complex consequently moves to the mitochondria to trigger apoptosis[83]. It has been reported that tyrosine phosphorylation of PKCδ is associated with this interaction with c-Abl[84]. The chemical inhibitor rottlerin blocks the translocation of the PKCδ-c-Abl complex from the ER to the mitochondria, which confers protection against apoptosis[83]. Another ER protein, p23 (Tmp21), interacts with PKCδ, which enables the retention of PKCδ in the ER[85]. Translocation of PKCδ to the ER has also been reported in cells with Sindbis virus and in glioma cells treated with TRAIL, where PKCδ exerts an anti-apoptotic effect. Furthermore, a small amount of PKCδ has been observed in the Golgi apparatus. Ceramide or IFN-γ stimulation has been shown to translocate PKCδ to the Golgi apparatus, which is associated with ceramide-induced apoptosis in HeLa cells.

CONCLUSION

The apoptotic and survival functions of PKCδ are defined by cell and tissue types and their cellular conditions (Figure 3). In response to cellular stresses, PKCδ may be translocated to different organelles (including the cytosol and extracellular space), where PKCδ executes distinct functions in each location. Among the many types of tissues and cells, liver cancer cells have the most patterns of localization of PKCδ, including conventional intracellular and extracellular localization. Notably, extracellular PKCδ is involved in the tumorigenesis of liver cancer; therefore, it is a promising novel diagnostic and therapeutic target for liver cancer. Additional studies are required to elucidate further the various roles of PKCδ in liver cancer cells, which are dependent on the expression, subcellular distribution, and tumor microenvironment.

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MINIREVIEWS

Therapeutic endoscopy for the treatment of post-bariatric surgery complications

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Abstract

Obesity rates continue to climb worldwide. Obesity often contributes to other comorbidities such as type 2 diabetes, hypertension, heart disease and is a known risk factor for many malignancies. Bariatric surgeries are by far the most invasive treatment options available but are often the most effective and can result in profound, durable weight loss with improvement in or resolution of weight associated comorbidities. Currently performed bariatric surgeries include Roux-en-Y gastric bypass, sleeve gastrectomy, and laparoscopic gastric banding. These surgeries are associated with significant weight loss, but also with significant rates of major complications. The complexity of these patients and surgical anatomies makes management of these complications by a multidisciplinary team critical for optimal outcomes. Minimally invasive treatments for complications are typically preferred because of the high risk associated with repeat operations. Endoscopy plays a large role in both the diagnosis and the management of complications. Endoscopy can provide therapeutic interventions for many bariatric surgical complications including anastomotic strictures, anastomotic leaks, choledocholithiasis, sleeve stenosis, weight regain, and eroded bands. Endoscopists should be familiar with the various surgical anatomies as well as the various therapeutic options available. This review article serves to delineate the current role of endoscopy in the management of complications after bariatric surgery.

Key Words: Therapeutic endoscopy; Bariatric surgery; Complications; Weight regain; Sleeve stenosis; Sleeve leak

Core Tip: Bariatric surgery is the most effective treatment for morbid obesity. While surgical techniques have improved, complications after these surgeries remain

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common. Multidisciplinary management of these complications is important given their complexity. Therapeutic endoscopy provides a minimally invasive option for treatment of complications. This review article serves to delineate the current role of therapeutic endoscopy in the management of complications after bariatric surgery.

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INTRODUCTION

Obesity rates have grown dramatically both in wealthy nations and the developing world; resulting in a worldwide global health problem. According to the WHO, in 2016, 1.9 billion people worldwide were affected by this disease[1]. Obesity often contributes to other comorbidities such as type 2 diabetes, hypertension, heart disease and is a known risk factor for many malignancies[2]. Obesity can dramatically affect a patient's quality of life and significantly reduces life expectancy.

Many treatments for obesity exist including dieting, exercise, weight loss medications, endoscopic bariatric therapies, and bariatric surgery[3]. However, treatment is difficult as most therapies result in, at most, moderate weight loss and once the therapy is withdrawn, significant weight recidivism occurs[4]. Bariatric surgeries are by far the most invasive treatment options available but are often the most effective and can result in profound, durable weight loss with improvement in or resolution of weight associated comorbidities[5]. For this reason, 256000 people underwent bariatric surgery in the United States in 2019, and 696191 underwent surgery worldwide in 2018[6,7]. While bariatric surgery is very effective, it does represent a major operation often being performed in high-risk individuals with multiple comorbidities. While surgical techniques have improved, and these surgeries have become safer over time, they remain associated with significant complications. About 9%-12% of patients undergoing bariatric surgery will experience 1 or more adverse events in the first five years after surgery[8]. While most complications will be minor, they can also be life threatening or associated with significant morbidity. These are often very complex patients such that adverse events are best managed by a multidisciplinary bariatric team which can include bariatric surgeons, endocrinologists, interventional radiologists, dieticians, and gastroenterologists. Minimally invasive, endoscopic or percutaneous treatments are preferred as reoperation is associated with significant additional risk. This review will focus on the large subset of adverse events after bariatric surgery that can be managed through endoscopic techniques (Table 1).

BARIATRIC SURGERY

Effective endoscopic treatment of post-bariatric surgery complications requires a detailed understanding of the gastrointestinal anatomy created by the particular surgical procedure. Currently the most performed surgical procedures are the sleeve gastrectomy and the laparoscopic Roux-en-Y gastric bypass (RYGB). Less commonly performed surgeries include laparoscopic adjustable gastric banding (LAGB) and the duodenal switch. Gastroenterologists will also still encounter patients with complications after vertical banded gastroplasty though this procedure is no longer performed.

RYGB

Despite the fact that it has lost some degree of popularity secondary to the rise of the sleeve gastrectomy, Roux-en-Y gastric bypass remains a commonly chosen bariatric surgery because of its excellent efficacy. Originally an open surgical procedure, gastric

Table 1 Endoscopic management of bariatric complications

Surgery	Complication	Diagnosis	Management options
RYGB	Gastrojejunal anastomotic stricture	Upper GI series; Endoscopy	Endoscopic balloon dilation; Steroid injection; Needle knife radial incisions; Lumen-opposing metal stent
	Gastrogastric fistula	Upper GI series; Endoscopy	Endoscopic suturing; OTSC
	Anastomotic leaks	CT imaging; Upper GI series; Endoscopy	CSEMS; Internal drainage with pigtail stents; OTSC; Endosponge therapy; Endoscopic suturing
	Choledocholithiasis	MRCP; CT imaging; Ultrasound	Overtube-assisted ERCP; Laparoscopic-assisted ERCP; EDGE
	Weight regain	EGD-Dilated gastrojejunostomy	Stoma reduction: Endoscopic suturing; OTSC; Serial APC treatments; Radiofrequency ablation
Sleeve gastrectomy	Staple line leak	CT imaging; Upper GI series; Endoscopy	CSEMS; Internal drainage with pigtail stents; OTSC; Endosponge therapy; Endoscopic suturing
	Sleeve stenosis	Upper GI series; Endoscopy	Radial expanding balloon dilation; Pneumatic balloon dilation
LAGB	Band migration	CT imaging; Endoscopy	Mechanical lithotripter band cutting

RYGB: Roux-en-Y gastric bypass; CSEMS: Covered self-expandable metallic stent; OTSC: Over-the-scope-clip; EDGE: Endoscopic ultrasound-directed transgastric endoscopic retrograde cholangiopancreatography; APC: Argon plasma coagulation; LAGB: Laparoscopic adjustable gastric band.

bypass is now done nearly exclusively laparoscopically. This minimally invasive approach combined with improved surgical techniques have led to decreased morbidity. This surgery involves partitioning a small gastric pouch from the proximal stomach and the creation of a Roux limb which diverts most absorption to the distal small bowel. Weight loss occurs secondary to the restrictive effect of the small gastric pouch, malabsorptive effects, and complex hormonal changes that occur as a result of the bypass. Hormonal changes are likely responsible for the profound effect this surgery has not just on the treatment of obesity but also for diabetes[9]. Surgical anatomies are not uniform and highly operator dependent. Details such as the size of the gastric pouch, diameter of the gastrojejunostomy, length of the Roux limb, and length of the pancreatobiliary limb will vary significantly by surgeon.

Complications of this surgery can manifest at any point after surgery with GI bleeding and leaks typically presenting early while vitamin deficiencies may take years to manifest. Possible adverse events related to this surgery include marginal ulceration, anastomotic strictures, gastrogastric fistulas, anastomotic leaks, choledocholithiasis, dumping syndrome, metabolic abnormalities, vitamin deficiencies, or chronic abdominal pain among others.

Anastomotic strictures

Stricturing at the gastrojejunal anastomosis is a common adverse event after RYGB which can occur as early as the first few weeks post-operatively or many years after the surgery. In some surgical series, the frequency of strictures is in the 20%-30% range [10]. The exact etiology of these strictures has not been completely elucidated-ulceration, ischemia, and minor anastomotic leaks as well as foreign body reactions to staples or suture are likely contributors. Surgical technique likely contributes as it has been shown that operations involving circular staplers are more likely to stricture[11].

Patients with anastomotic strictures typically present with nausea and vomiting symptoms or in the early post-operative period will report inability to advance their diet beyond liquids. Some stricture patients will describe dysphagia while others may endorse significant post-prandial abdominal pain. Symptoms of gastrojejunal stricturing are often very similar to those of marginal ulceration and differentiating between these 2 adverse events prior to endoscopic evaluation can be very difficult. Tight strictures can be diagnosed using a radiologic upper GI series, but more subtle narrowing can be missed as liquid will continue to pass through to the jejunum. Some patients with strictures will have inadvertent weight gain as they will compensate for the stricture by sticking to a high calorie liquid diet.

All patients with symptoms that are suggestive of a possible anastomotic stricture should undergo an upper endoscopy for diagnosis and treatment. Normal anastomotic diameter should be between 10 mm and 15 mm, therefore a stricture can be diagnosed in any patient in which the standard upper endoscope cannot pass easily through the

anastomosis into the jejunum. Treatment for the stricture can then be performed immediately after diagnosis through endoscopic through-the-scope balloon dilation. Fluoroscopy can often be helpful to ensure the correct positioning of the balloon and to avoid trauma to the thin jejunum. Therapy should be aimed at dilating the stricture to achieve a luminal diameter of 10-15 mm. Dilation should be gradual and often requires more than 1 dilation session-particularly in patients with very tight strictures. Care should be taken to avoid over dilating the anastomosis as this is associated with an increased risk of complications and can result in the patient experiencing a decreased sense of restriction and ultimately result in weight gain. Overall, endoscopic balloon dilation is safe, with very few complications and is effective for most patients[12,13] (Figure 1).

Multiple options are available for the treatment of refractory strictures. Increasing balloon diameter combined with intralesional steroid injection can be effective. Our group will typically utilize Triamcinolone 10 mg/mL with 1-2 mL injected in a 4-quadrant fashion. An additional technique which can be added is incisional ablation of the stricture with radial cuts with a needle knife prior to dilation therapy. For patients who continue to be refractory, these strictures can be treated with endoscopic stenting. While treatment with esophageal stents in gastric bypass patients is typically poorly tolerated secondary to associated side-effects (pain, nausea, vomiting, reflux) and frequent stent migration, treatment with new lumen apposing metal stents (LAMS) is well tolerated and effective[14,15]. Our group has had good success with the 10 mm × 15 mm LAMS but in patients whose strictures continue to recur, the 20 mm stent may also be effective. Truly refractory strictures which require a surgical revision are very rare and usually represent narrowing secondary to twisting/torsion of the post-anastomotic jejunum rather than true fibrotic strictures of the anastomosis.

Gastrogastric fistula

A gastrogastric fistula is a communication between the gastric pouch and the gastric remnant. In the traditional open RYGB surgery this was a common occurrence seen in up to 30% of patients. However, in laparoscopic patients this is a much less frequently seen complication secondary to changes in surgical technique[16]. In open surgeries the gastric pouch was partitioned using a staple line but was not completely separated from the defunctionalized stomach as is done in laparoscopic RYGB. While the exact etiology of these fistulas is not known, anastomotic leaks, ulceration, ischemia, and erosions of foreign bodies are thought to contribute[17-19]. The presenting symptoms of gastrogastric fistula patients are highly variable and can range from being completely asymptomatic to refractory or perforating marginal ulcers. Weight regain is a common occurrence with these fistulas as they can allow a significant amount of food to pass into the gastric remnant which functionally reverses the effects of the surgery. Gastric acid can pass through the fistula from the gastric remnant to the pouch and result in gastroesophageal reflux disease, abdominal pain, marginal ulcers, and anastomotic strictures.

Gastrogastric fistulas can be diagnosed *via* upper endoscopy, upper GI series, or sometimes *via* computed tomography (CT) scan. While the radiological tests can be helpful one must be careful not to overinterpret the presence of contrast in the gastric remnant as contrast can often reflux up the pancreatobiliary limb. Endoscopic diagnosis is particularly helpful as it provides the most accurate measure of the size of the fistula which is important in determining treatment.

Patients with symptoms of GERD or other consequences of gastric acid passage through the gastrogastric fistula can often be treated with therapy with proton pump inhibitors or other antacids alone. Indications for closure of a gastrogastric fistula include refractory symptoms or significant weight regain.

Endoscopic therapy is most effective for smaller fistulas-particularly those that are smaller than 1 cm in diameter. Unfortunately, even these smaller fistulas have high rates of reopening after endoscopic closure[20]. The most studied endoscopic therapy is endoscopic suturing which results in a very high initial closure rate, but long-term efficacy may be as low as 20%[21]. The over-the-scope-clip (OTSC) is another option for fistula closure though it has been less studied for this indication[22]. In our experience these clips can be effective for small fistulas and can be used to reinforce an endoscopic suturing closure. One concern about using this device is that the large clip can interfere with surgical revision if that becomes necessary secondary to failure of closure. However, endoscopists now have access to a device specifically designed to help remove these large clips which can be done prior to a surgical closure[23]. Patients with persistent symptoms despite medical therapy, with large fistulas or those that are refractory to endoscopic closure should be evaluated by a bariatric surgeon for surgical revision.

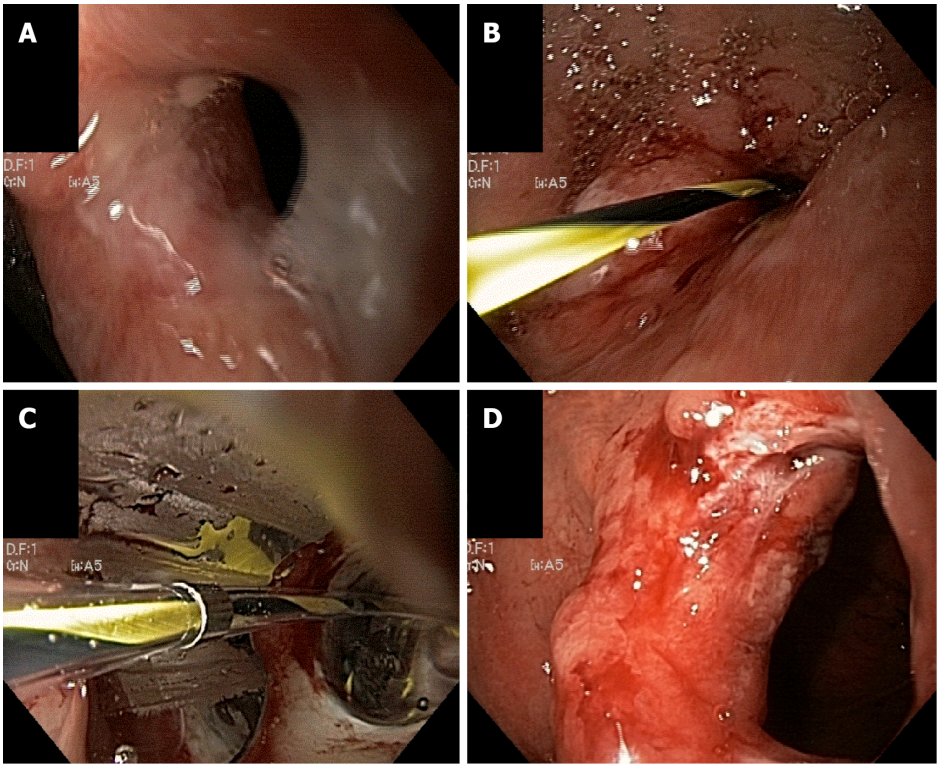

Figure 1 Anastomotic stricture dilation. A: Tight stricture of gastrojejunostomy; B: Wire placement through stenosis; C: Balloon dilation; D: Stricture appearance after dilation.

Anastomotic leaks

Anastomotic leaks can be the most severe and debilitating complications after bariatric surgery and are seen most commonly after sleeve gastrectomy (discussed in sleeve gastrectomy section) but can also be seen typically as a very early complication after RYGB[24]. RYGB involves the creation of multiple different surgical anastomoses, and leaks can occur at any of these sites including the gastric pouch, gastrojejunostomy, blind limb of jejunum, jejunojejunostomy, and the gastric remnant. Leak patients will typically present with abdominal pain, fevers, and potentially sepsis and clinical instability[25]. The initial treatment for all leak patients involves antibiotics, fluid resuscitation, and NPO status[26]. Patients who are clinically unstable will typically need to be taken to the operating room for a wash-out and attempted surgical closure. In more stable patients cross-sectional imaging should be obtained in order to evaluate the location of the leak.

Patients with leaks originating from the gastric pouch or gastrojejunostomy can be treated successfully with endoscopic therapy using covered endoluminal stents[27, 28]. The majority of published research on this treatment involves the use of self-expandable metal esophageal stents which can be fully covered or partially covered with plastic. Unfortunately, these stents are poorly tolerated by patients but can typically be removed in 6 to 8 wk with a leak resolution rate of 87.8%[29]. For leaks at the gastrojejunostomy, the much shorter, dumbbell shaped LAMS stents can be used and are significantly better tolerated, but currently experience with this technique is limited.

Another option for treatment of leaks is internal drainage, which involves the placement of double pigtail stents through the opening of the leak into the associated abscess cavity[30]. This drainage allows the leak to heal around the stents similar to what occurs in drainage of pancreatic fluid collections. The stents are then exchanged every 6 wk until resolution of the abscess cavity and leak are seen. This technique is much better tolerated than esophageal stents and has the benefit of being able to be performed at leak sites throughout the RYGB anatomy-even those that require an enteroscope to reach. Other options for treating leaks include endoscopic suturing, endoscopic clipping, eVAC therapy (transnasal wound vac with endosponge), as well as over-the-scope clips[31]. However, the published data for their use remains very limited.

Choledocholithiasis

The development of cholelithiasis is common after bariatric surgery and particularly so after RYGB. Up to 36% of patients will have gallbladder stones 6 months after surgery, and a large portion of those patients have gallstone-related symptoms[32]. Rapid weight loss is believed to increase the cholesterol saturation of bile, and anatomic changes occurring during the surgery may affect gallbladder emptying, both of which promote.

stone formation. Studies have shown that 2%-7% of symptomatic patients will have choledocholithiasis which is best treated by endoscopic retrograde cholangiopancreatography (ERCP), a procedure that is technically very challenging for post RYGB patients as the major papilla cannot be reached with a standard duodenoscope[33]. Currently there are 3 endoscopic options for performing ERCP in the RYGB patient-overtube-assisted enteroscopy ERCP, lap-assisted transgastric ERCP, or an endoscopic ultrasound-directed transgastric ERCP (EDGE).

Enteroscopy ERCP can be performed with a single- or double-balloon enteroscope, or spirus assisted, or in patients with short Roux limbs, with a colonoscope. Published data suggest a 70% efficacy rate of this technique[34]. Limitations are primarily related to the lack of appropriate length and size of devices and lack of elevator on the scope. Long Roux limbs or pancreatobiliary limbs can also affect the likelihood of success. Use of a clear cap on the end of the enteroscope can improve visualization and positioning of the scope in the descending duodenum.

Laparoscopic-assisted ERCP requires taking the patient to the operating room where a surgeon will create a gastrostomy in order to facilitate percutaneous passage of a standard duodenoscope into the second portion of the duodenum[35]. This access allows the endoscopist the ability to perform ERCP with standard devices and techniques. This technique has been shown to be effective with a technical success rate of 97.9%, but adverse event rates are higher (19%) than that seen with overtube-assisted ERCP (6.5%)[36]. For patients who need multiple ERCPs a gastrostomy tube can be left in place to provide an access tract, however repeat ERCP can be challenging as the duodenoscope will not pass easily through a standard gastrostomy tube tract.

EDGE is a new and still somewhat controversial technique for performing ERCP which involves the creation of a gastrogastric fistula with EUS-guided deployment of an Axios (Boston Scientific, Marlborough, MA) LAMS[37] (Figure 2). The first step in this process is to identify the gastric remnant from the gastric pouch with a linear EUS scope. A 19-gauge needle is then advanced into the gastric remnant and used to infuse water into the gastric remnant to distend it. If not already in the left lateral position, moving the patient onto their left side can facilitate keeping this fluid in the gastric remnant. Once the gastric remnant is distended with fluid a LAMS can be deployed in a similar fashion to draining a pancreatic pseudocyst. As the gastric remnant is typically separated from the gastric remnant the stent does cross the serosa of the gastric pouch and the gastric remnant such that if the stent migrates a perforation will occur. Therefore, this procedure is done most safely if the stent is deployed and then 2-3 wk is allowed for a tract to form along the stent prior to passing the duodenoscope through to perform ERCP[38]. For those patients that cannot wait for their ERCP, the stent should be sutured or clipped in place, and the stent should not be removed immediately after the procedure. Once the LAMS stent is in place a duodenoscope can pass through it and then achieve standard positioning for ERCP. If repeat ERCPs are needed the stent can be left in place, but plans should be made to remove the stent as soon as possible to reduce the risk of a persistent gastrogastric fistula. Data on this technique is limited, though a retrospective multicenter review of 178 patients demonstrated a technical success of 98% and only 4 patients with adverse events[39]. It remains controversial because of the risk of persistent gastrogastric fistula which was 10% in this review.

ERCP for patients after RYGB is challenging and should involve a multidisciplinary team. Bariatric surgery consult should be considered prior to embarking on any of the 3 potential endoscopic treatment options. Balloon-assisted ERCP is our first line therapy for most ERCP indications, but a plan should be in place prior to the procedure as to what second-line option will be utilized in the event that this is not successful. If EDGE is planned after failed balloon-assisted ERCP, then water can be pumped quickly into the gastric remnant through the enteroscope prior to exchanging for the linear EUS scope, and the LAMS is deployed to create the gastrogastric fistula as part of the same procedure.

Weight regain

Patients who undergo Roux-en-Y gastric bypass typically experience a profound

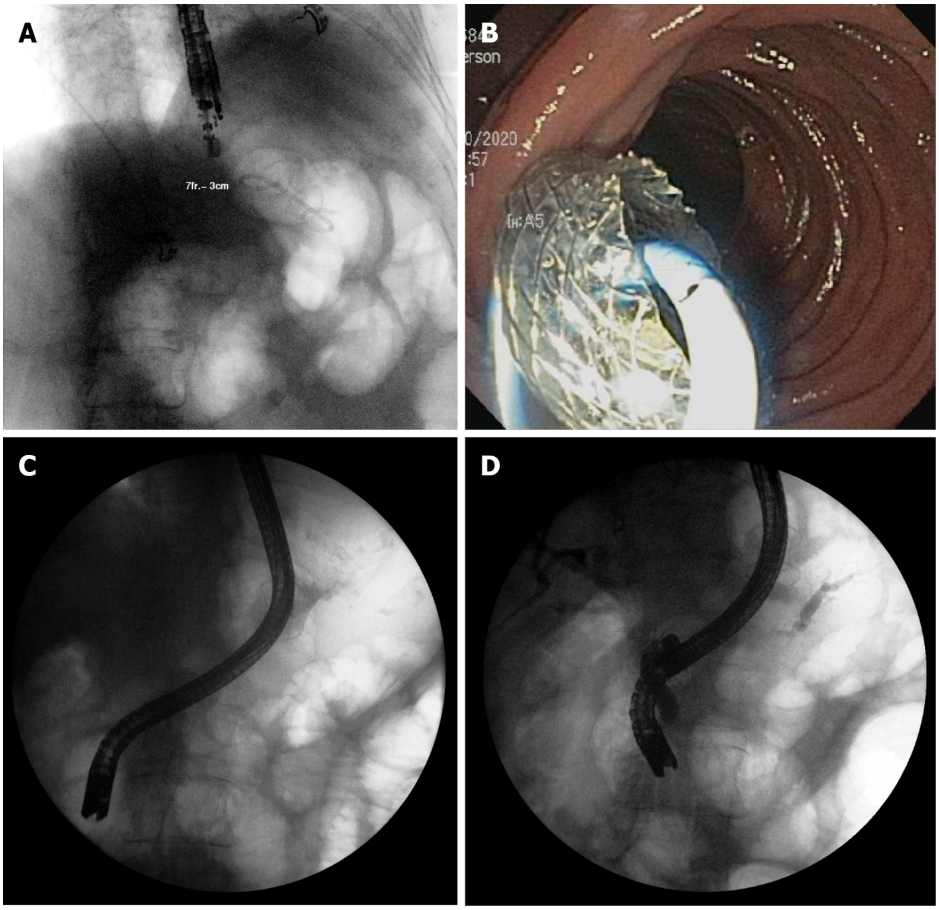

Figure 2 Endoscopic ultrasonography-directed transgastric endoscopic retrograde cholangiopancreatography. A: Endoscopic ultrasonography placement of lumen apposing metal stents (LAMS) gastrogastric fistula; B: Endoscopic view of LAMS; C: Duodenoscope passing through LAMS for endoscopic retrograde cholangiopancreatography; D: Successful cholangiogram and pancreatogram.

weight loss in the first 6 mo after surgery. While the rate of weight loss will slow after this initial period, significant weight loss will continue for the first 12-15 mo after which patients will typically hit a weight loss plateau with expected total weight loss of roughly 30% total weight or 60% excess weight loss. It is common for patients to gain back a small amount (10%) of this significant weight loss over the subsequent 1-2 years. However, about 18%-30% of RYGB patients will suffer from significant "weight regain"-a term which generally denotes regaining more than 50% of the initial weight loss[40-42]. This kind of weight gain can be devastating emotionally for these patients who have undertaken significant surgery only to see their health gains eliminated.

The etiology of weight regain after RYGB is often multifactorial and can involve dietary, hormonal, and behavioral factors but also often involves anatomic changes to the RYGB anatomy. Gastrogastric fistulas can contribute significantly to weight gain and their management was discussed earlier in this review. Dilation of the gastric pouch and/or dilation of the gastrojejunostomy are other anatomic changes that have been shown to be associated with weight gain in RYGB patients[43]. Presumably these changes result in weight gain by removing the sense of restriction or satiety that these patients are used to experiencing after eating. A dilated gastrojejunostomy will allow food to empty the gastric pouch very quickly after eating. These patients will often describe an initial sense of fullness after eating which passes very quickly, resulting in frequent hunger and resultant increase in caloric intake. While a gastrogastric fistula can be diagnosed by upper GI series these anatomical changes are typically best evaluated by endoscopy as an upper GI series cannot measure the size of the gastrojejunostomy.

Surgical revisions for alterations in gastric bypass anatomy are associated with very high rates of adverse events - making minimally invasive endoscopic treatments better options. The most studied endoscopic treatment for a dilated gastrojejunostomy is to narrow the stoma diameter using endoscopic suturing (Figure 3). This has been done with multiple different suturing platforms and has been shown to be effective for weight loss in a sham controlled randomized controlled trial[44]. Data with the newer

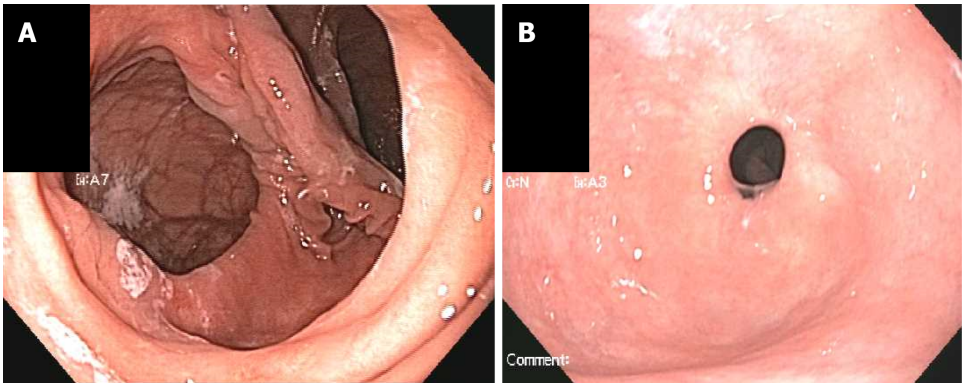

Figure 3 Dilated gastrojejunostomy treated with endoscopic suturing. A: Dilated gastrojejunostomy; B: Gastrojejunostomy several months after suturing for stoma reduction.

Overstitch (Apollo Endosurery, Austin Tx) device which allows full thickness suturing demonstrates that this device is the most effective[45]. Argon plasma coagulation (APC) treatment to the 2 cm of tissue on the gastric side of the gastrojejunostomy to expose the submucosa is typically performed prior to suturing. Multiple different suturing patterns have been used with some published data suggesting that a circumferential running stitch pattern provides a more robust weight loss than interrupted suture patterns[46]. The goal of suturing is typically to reduce the diameter of the gastrojejunostomy to 8 to 10 mm. When this is achieved, patients will typically stop regaining weight and data from the largest published study (n = 331) demonstrates weight loss of 8.5% ± 8.5% at 1 year.

Long-term follow-up has also shown persistent effect of the intervention with patients losing 8.8% ± 12.5% total weight at 5 years[47]. For those patients who are not able to undergo endoscopic suturing there are other endoscopic options to narrow the gastrojejunostomy though data on these alternate methods at this point is limited. Investigators have shown that placement of an OTSC at 1 edge of the gastrojejunostomy or 2 clips at opposite sides of the gastrojejunostomy can be effective at narrowing the stoma diameter[48]. Alternatively, serial sessions of treatment with APC alone to the rim of the gastrojejunostomy without any suturing can be effective [49]. In this technique the APC is applied with some degree of contact with the mucosa in order to achieve a deeper penetration of the thermal effect. This technique is less effective for gastrojejunostomies with a diameter greater than 30 mm. One case series also described the use of serial sessions of radiofrequency ablation to the edges of the gastrojejunostomy with 18.8% excess weight loss at 12 mo[50]. Serial sessions with injection of the sclerosant sodium morrhuate has also been shown to be effective, but unfortunately this product is no longer available for purchase for medical usages.

SLEEVE GASTRECTOMY

The creation of a gastric sleeve was initially just 1 part of the more complex and rarely performed duodenal switch operation. However, since becoming a stand-alone surgery, sleeve gastrectomy has gained popularity over the past 2 decades and is now the most commonly performed bariatric surgery in the U.S. Sleeve gastrectomy now accounts for greater than 50% of all bariatric surgery[51].

The creation of the gastric sleeve involves the surgical excision of a large portion of the greater curve of the stomach. The surgeon then closes the resulting defect with a long staple line. The resulting anatomy is a narrow tubular stomach. The surgery is thought to induce weight loss in multiple different ways. Resection of the greater curve of the stomach results in the removal of the majority of the ghrelin-producing cells which results in decreased hunger[52]. Additionally, the resultant tubular sleeve has much less capacity than a normal stomach and results in greater satiety after eating. Interestingly, the sleeve gastrectomy is thought to result in quicker gastric emptying which results in weight loss through various hormonal effects. Overall, the weight loss seen with this surgery is significant and can be similar to that seen with Roux-en-Y gastric bypass[53].

While typically considered a more straight-forward surgery than RYGB, complications occur with similar frequency with the sleeve gastrectomy[54]. Common complications which can occur and be treated with therapeutic endoscopy include staple line leaks as well as the development of a stenosis of the gastric sleeve.

Sleeve gastrectomy staple line leak

As discussed above with regard to leaks after RYGB, surgical leaks can occur at any surgical anastomosis. Leaks can be a devastating complication which can take a very long time and multiple endoscopic procedures to heal. The creation of a gastric sleeve results in a very long staple line anastomosis which extends from the antrum of the stomach up to the GE junction. Leaks are seen in up to 5% of sleeve gastrectomy patients and can occur anywhere along the staple line[55]. However, most leaks occur near the top of this staple line at the angle of His which is particularly susceptible to leaks because this area has a very thin gastric wall, often experiences relative ischemia secondary to surgical ligation of the short gastric arteries, has relative dysmotility, and is an area of increased intragastric pressure. Downstream obstructions such as a concomitant sleeve stenosis often contribute to the development of leaks and need to be addressed as part of treatment[56]. Sleeve leaks typically present in the early post-operative period, but chronic leaks can present at any point-sometimes even many years after their surgery.

Leak patients' clinical presentation typically varies based on the timing of their leak. Patients with acute leaks (< 7 d after surgery) typically present with signs of sepsis and are often hemodynamically unstable and require urgent operative intervention with abdominal wash-out and placement of abdominal drains. Patients with subacute or chronic leaks are typically less acutely ill and present with signs and symptoms including abdominal pain, fever, and tachycardia. Prompt evaluation is needed to reduce associated complications[57]. Diagnosis of a sleeve leak is typically made by a combination of CT scan and upper GI series. If a leak is suspected but not confirmed on upper GI series, then endoscopy with direct visualization can typically secure a diagnosis. Initial leak treatment involves initiation of broad-spectrum antibiotics, IV fluid resuscitation, NPO status, and surgical consultation.

While some leak patients will require re-operation, the majority can be treated with various endoscopic techniques. Unfortunately, this often involves a prolonged treatment course which can involve long hospital stays and significant impacts to quality of life. Many patients will require multiple different modalities of endoscopic treatment to achieve resolution of the leak[58]. Available endoscopic treatment options include covered esophageal stents, internal drainage, OTSC, endoscopic suturing, eVAC endosponge therapy, and endoscopic septotomy. Many patients will also require supplemental enteral nutrition delivered through a jejunostomy tube during their treatment.

The most commonly used endoscopic treatment for sleeve gastrectomy leaks is the use of covered self-expandable metal stents (CSEMS) (Figure 4). These stents treat sleeve leaks through 2 mechanisms-the stent both covers the leak orifice and treats the distal gastric stenosis and reshapes the stomach. Stents used can include both fully covered and partially covered stents. Our group utilizes more partially covered stents for this purpose because they result in decreased stent migration and can result in a more effective seal at the top of the stent as tissue grows into the partially covered portion of the stent. The downside to using partially covered stents is the increased difficulty of removing these stents once the leak has healed. In order to achieve successful removal of a partially covered stent one must place an overlapping fully covered stent within the stent. The expansile pressure from the second stent results in tissue necrosis of tissue ingrowth of the first stent and allows removal of both stents about 1 week after placement[59]. A recent systematic review and meta-analysis demonstrated that the use of CSEMS for treatment of sleeve leaks was effective in 72.8% of cases[60]. Unfortunately, use of these stents is associated with poor patient tolerance with symptoms of chest pain, gerd, nausea and vomiting, and frequent stent migration when fully covered stents are used. Because of the associated side effects of these stents, patients can rarely maintain adequate nutrition *via* PO intake, and therefore we typically place an endoscopic jejunal feeding tube at the initiation of treatment to provide supplemental enteral nutrition to promote leak healing. Multiple techniques have been tried to prevent stent migration including using hemoclips, over-the-scope-clips, and endoscopic suturing to attempt to secure the proximal aspect of the stent to the esophagus. However, migration can still occur despite these measures and was seen in 15.9% of patients in a large meta-analysis[61].

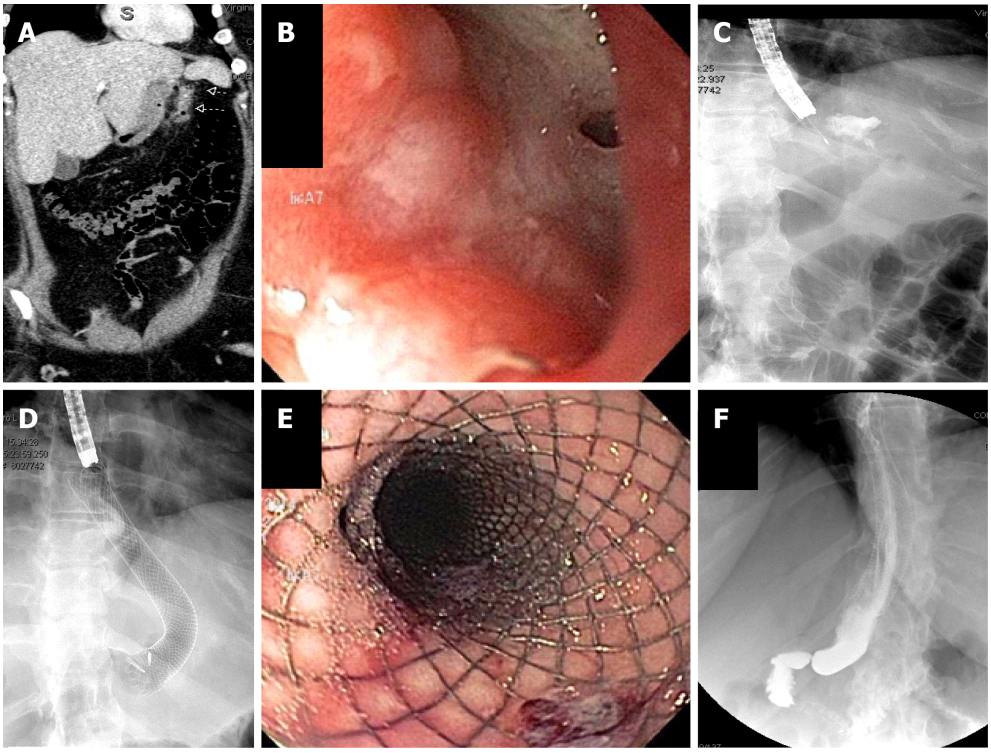

Figure 4 Sleeve leak treated with covered esophageal stent. Computed tomography imaging demonstrating sleeve leak; B: Endoscopic appearance of leak site; C: Contrast injection to confirm leak site; D: Placement of covered self-expandable metal stents; E: Endoscopic appearance of stent; F: Subsequent upper gastrointestinal series showing no residual leak after stent placement.

Internal drainage using transgastric double pigtail stents is a more recently described technique for treating sleeve gastrectomy leaks[30]. This involves the drainage of the associated abscess cavity with transgastric plastic stents (Figure 5). If the leak opening is large enough prior to stenting, the endoscope can be passed through the leak and used to lavage and debride the associated cavity prior to stenting. If there is a stenosis of the distal sleeve, then this should be combined with dilation of the sleeve as well. The stents are then exchanged every 6 wk until the cavity and leak have resolved through healing by secondary intention. Retrospective studies have shown improved decreased morbidity and mortality, and improved technical success when compared with the use of CSEMS. The largest published study included 67 sleeve leak patients and demonstrated a 72.8% clinical success rate and a mean of 3.18 endoscopic procedures[30].

In our experience, this technique is very well tolerated by patients, and PO intake can be started early which obviates the need for PEJ tube, and the internal drainage avoids the need for percutaneous drainage. This method of endoscopic treatment has become our primary method for treatment of sub-acute leaks, and some data has demonstrated effectiveness in acute leaks as well.

Other treatment options for leak closure include devices which attempt to directly close the leak orifice, including the use of through the scope clips, OTSC, and endoscopic suturing. Data on the use of these techniques in sleeve leaks is limited though retrospective case series, have shown that OTSCs and endoscopic suturing can be effective in some leak patients[62]. In our experience the typical location of sleeve leaks at the proximal end of the staple line makes access for suturing or clipping challenging. Furthermore, the leak site tissue is generally of poor quality secondary to associated infection and relative ischemia such that clips and sutures may initially appear to close the defect-only to have it re-open at a later time (similar to what occurs during attempts at operative repair). Use of these closure devices should be limited to very small holes and those that are easily accessed endoscopically.

A novel treatment for gastrointestinal leaks is the use of endoscopic vacuum therapy (EVT) which involves the placement of a wound vac sponge attached to a naso- gastric tube into or adjacent to the leak. Continuous suction from a wound-vac device is then applied through the NG tube. The sponge induces the formation of granulation tissue while the suction removes fluid, pus, and any associated necrotic debris in order to induce leak healing. This treatment requires placement of a new

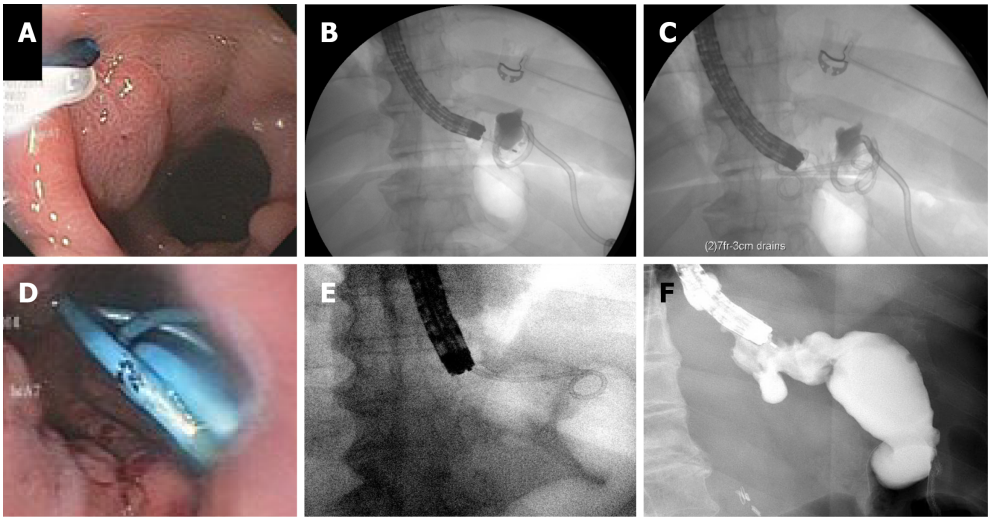

Figure 5 Sleeve gastrectomy leak treated with internal drainage. A: Endoscopic appearance of leak site; B: Contrast injection to confirm leak site; C: Placement of transgastric double pigtail stents; D: Endoscopic appearance of stents; E: Repeat endoscopy for stent removal; F: Contrast injection after stent removal confirming no residual leak.

sponge every 3-7 d, resulting in multiple endoscopic procedures. While the sponge is in place the patient cannot have any PO intake, therefore typically a PEJ tube should be placed for enteral nutrition. Several small retrospective case series have demonstrated the efficacy of this treatment for sleeve gastrectomy leaks. One single center series evaluated 9 patients who all had resolution of their leak after a mean of 50 d of treatment[63]. Another series demonstrated leak resolution in 7 of 8 patients, and a third series of 3 patients demonstrated resolution in all 3 cases with a mean treatment time of 72 d[64]. While EVT appears to be an effective treatment it is significantly uncomfortable for the patient and typically requires many endoscopies for sponge exchanges resulting in this primarily being a therapy for refractory or very large leaks not amenable to other therapies.

Sleeve stenosis

Stenosis of the gastric sleeve occurs in up to 4% of patients and typically occurs at the level of the incisura[65]. Patients typically present with symptoms of nausea and vomiting when advancing their diet beyond liquids. Other patients can present with symptoms of refractory gastroesophageal reflux. The diagnosis can often be made through upper GI series which will typically demonstrate an area of significant narrowing at the level of the incisura within the gastric sleeve. The diagnosis can also be made during upper endoscopy, but the endoscopist must be familiar with the condition - the narrowing is not typically a true mucosal stricture that prevents scope passage but rather an area of relative narrowing often with significant angulation requiring scope manipulation to get through. Contributing factors to the development of the stenosis include forming the sleeve around a small bougie, over-sewing the staple line, and inadvertent rotation of the sleeve during stapling can result in a twisted and narrowed sleeve[66].

Endoscopic treatment of sleeve stenosis involves the use of balloon dilation of the stricture. Dilation can be started at 20 mm with a radial expanding balloon. However, if dilation with the 20 mm balloon does not have much apparent effect on the stenosis then dilation can immediately proceed to pneumatic dilation with a 30 mm achalasia balloon under fluoroscopic guidance (Figure 6). Inflation to the 20 PSI maximum inflation pressure may not be possible during the first session. The patient can then return every 2 wk for repeat dilations with gradually increased inflation pressures and balloon sizes until the 40 mm balloon is used. The addition of dilating the pylorus up to 20 mm can also be helpful in improving symptoms. Dilations can be stopped once the patient is symptomatically improved. Efficacy of this dilation regimen was shown to be 76% effective in a large meta-analysis[67]. Complications of this treatment include bleeding or perforation and occur in about 6 % of patients[68]. Patients who fail endoscopic dilation will require either surgical seromyotomy or conversion to RYGB.

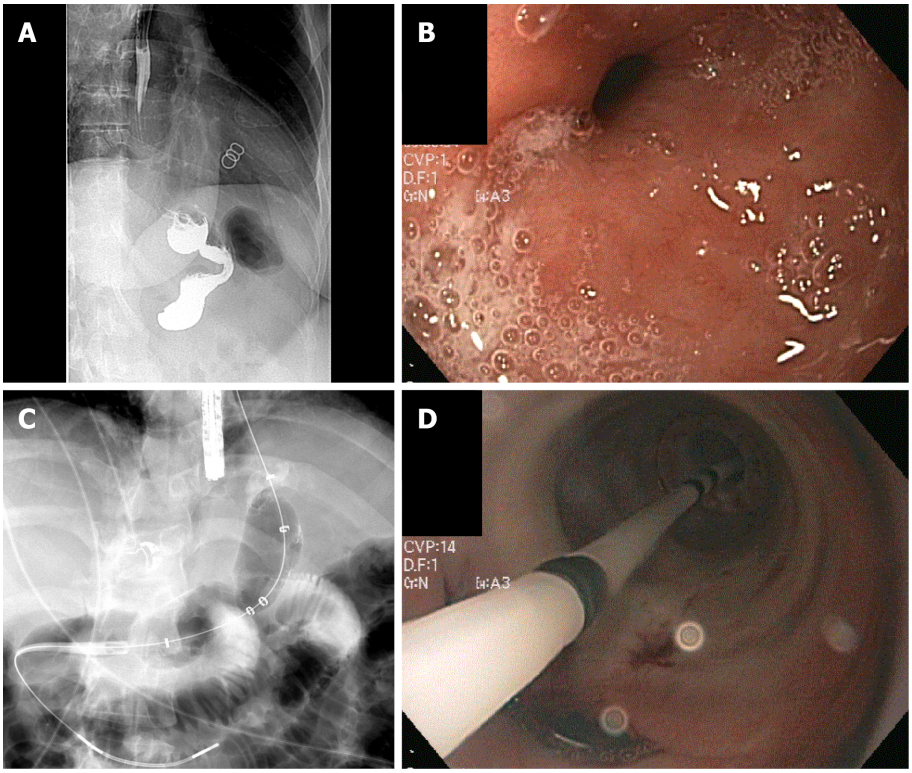

Figure 6 Sleeve stenosis treated with balloon dilation. A: Upper gastrointestinal series demonstrating stenosis at the level of the incisura; B: Endoscopic appearance of the stenosis; C: Pneumatic balloon dilation of stricture; D: Endoscopic appearance of balloon dilation.

LAGB

At the beginning of the century the LAGB was the most popular bariatric surgery in the United States. However, the use of this device has dramatically reduced secondary to poor efficacy and high numbers of complications, and it now represents less than 10% of bariatric surgeries performed nationwide. Many patients still have these bands in place and will continue to have adverse events needing endoscopic therapy. LAGB surgery involves the placement of a silicone band around the cardia of the stomach which creates a small proximal pouch to create a feeling of satiety after eating small amounts of food. The band is attached to a port that is placed in the subcutaneous tissue of the abdomen *via* a thin piece of tubing. The port can be used to inflate or deflate the band with saline to adjust the restrictive effect of the device. Common adverse events that occur with the LAGB include the development of GERD, esophageal dilation, port infection, band slippage and band erosion.

Band erosion

Placement of LAGB can be complicated by erosion of part of or the entire band into the gastric lumen. This can occur in the early post-operative period or many years later. Erosion of the band is thought to occur secondary to an inflammatory reaction between the band and the gastric wall. Precipitating factors include perforations at the time of surgery, bands placed too tightly around the stomach, bands that are sutured to the stomach and port infections[69]. Symptoms vary from patients being completely asymptomatic to the development of GERD, nausea and vomiting, or signs of infection [70]. Bands that have a significant portion visibly eroded into the gastric lumen can be removed endoscopically. If the band buckle is visible the likelihood of successful removal is greater. If the band appears to be insufficiently migrated, then placement of a covered esophageal stent for 2 wk can be considered to promote additional migration of the band[71]. Prior to endoscopic removal the subcutaneous port must be removed surgically- if necessary, this can be done simultaneously in the endoscopy suite.

The technique for endoscopic removal of the LAGB involves the use of the emergency mechanical lithotripter typically used for ERCP[72] (Figure 7). Initially the scope is passed through the center of the band, and a wire is placed into the distal stomach. The scope is then withdrawn, leaving the wire in place, and then re-

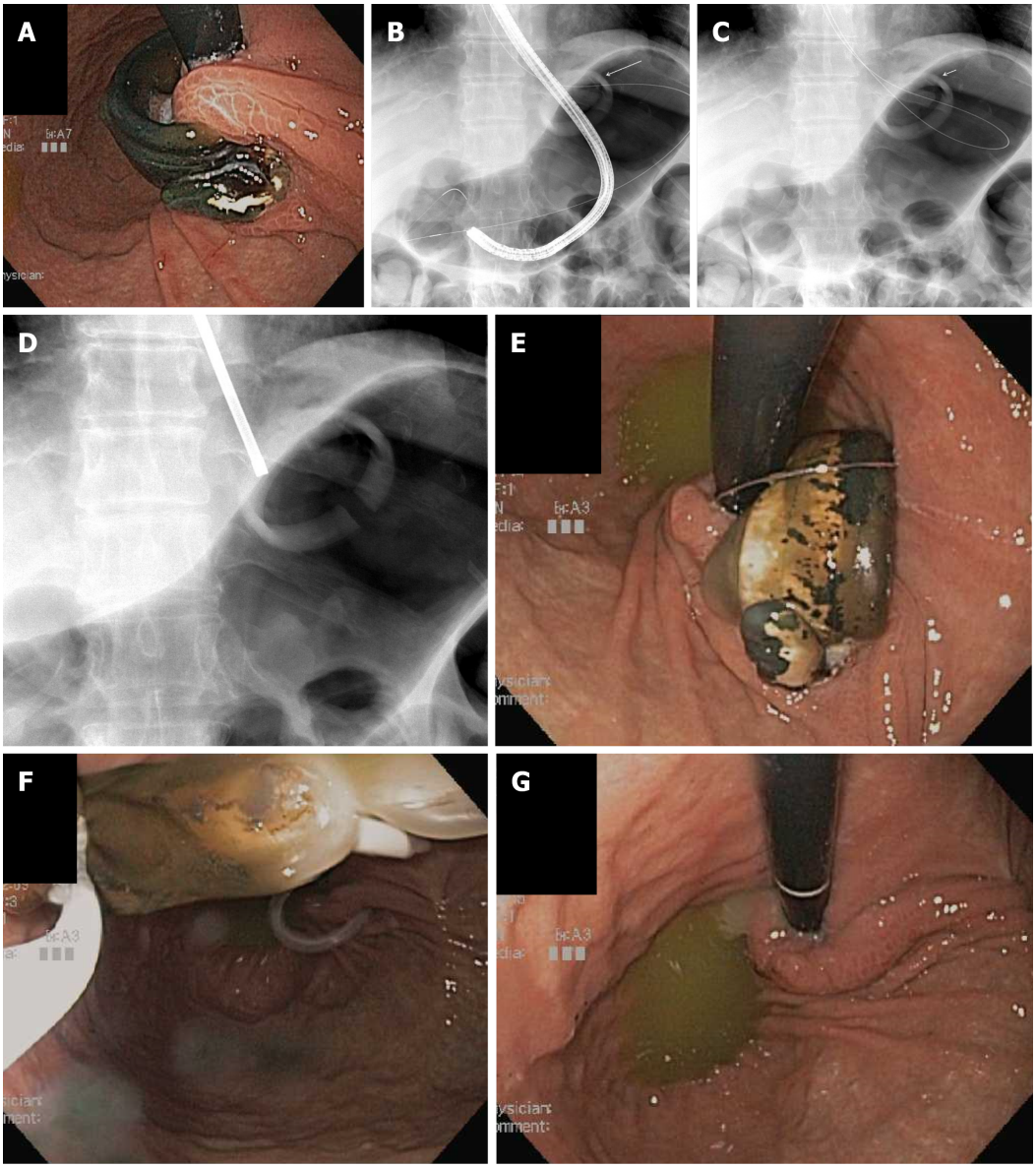

Figure 7 Eroded lap band removed endoscopically. A: Eroded lap band; B: Endoscope passage through the band with distal deployment of wire; C: Wire looped around band; D: Fluoroscopic view of cut lapband; E: Endoscopic view of cut lap band; F: Cut band grasped by snare; G: Endoscopic view after band removal.

introduced alongside the wire. The scope must be driven into the distal stomach without going through the center of the band, and the end of the wire is then grasped with a snare and pulled out through the mouth forming a loop around the band. The sheath of the mechanical lithotripter is then advanced over 2 ends of the wire up to the band. We use a combination of endoscopic and fluoroscopic imaging to ensure that only the band is trapped in the wire to avoid significant tissue trauma. The lithotripter is then cranked until it pulls the wire through the band to transect it. The lithotripter is then removed, and the scope is re-advanced into the stomach. If visible, the cut side with the buckle is grasped and used to pull the band out through the mouth[72]. The body forms a capsule around the band which prevents a perforation from occurring after removal. Adhesions can occur between the band and adjacent structures which prevent successful endoscopic removal. Bands that cannot be removed successfully endoscopically do need surgical removal.

CONCLUSION

The prevalence of obesity continues to rise around the globe, resulting in 1 of the most significant health problems affecting the world. Bariatric surgery rates will continue to climb in order to combat this disease. Despite improved surgical techniques, complic-

ations after these major surgeries are not uncommon. Post bariatric surgery patients are complex, and these complications are best managed with a multidisciplinary team with experience in this field. The endoscopic armamentarium continues to expand and helps serve as a minimally invasive treatment option for many patients with post-bariatric surgery complications.

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MINIREVIEWS

Update on the applications and limitations of alpha-fetoprotein for hepatocellular carcinoma

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Abstract

Alpha-fetoprotein (AFP) is an oncofetal glycoprotein that has been used as a tumor marker for hepatocellular carcinoma (HCC) in combination with ultrasound and other imaging modalities. Its utility is limited because of both low sensitivity and specificity, and discrepancies among the different methods of measurements. Moreover, its accuracy varies according to patient characteristics and the AFP cut-off values used. Combination of AFP with novel biomarkers such as AFP-L3, Golgi specific membrane protein (GP73) and des-gamma-carboxyprothrombin significantly improved its accuracy in detecting HCC. Increased AFP level could also signify severity of hepatic destruction and subsequent regeneration and is commonly observed in patients with acute and chronic liver conditions and cirrhosis. Hereditary and other non-hepatic disorders can also cause AFP elevation.

Key Words: Alpha-fetoprotein; Hepatocellular carcinoma; Alpha-fetoprotein-L3; Cirrhosis; Tumor markers; Hereditary persistence of alpha-fetoprotein

Core Tip: Alpha-fetoprotein has been used commonly as a biomarker for hepatocellular carcinoma (HCC) surveillance. Its sensitivity and specificity can be affected by the assay methods, patient characteristics and severity of the underlying liver conditions.

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Combination with other novel markers has shown promising results. Algorithms integrating these serum markers with noninvasive diagnostic imaging modalities are essential for the accurate and timely diagnosis of HCC.

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INTRODUCTION

During fetal and neonatal life, alpha-fetoprotein (AFP) is produced by yolk sac, fetal liver and gastrointestinal tract. Only a trace amount of AFP can be measured in adults due to its rapid decline in adults[1-4]. The use of AFP as a tumor marker for hepatocellular carcinoma (HCC) was first proposed in 1960s. The utility of AFP as a surveillance and diagnostic test has been criticized due to both low sensitivity and specificity. AFP, however, continues to play a significant role for HCC surveillance in combination with ultrasound (US) and other imaging modalities[5-8]. This review focused on both the clinical roles and limitations of AFP. In addition, the patterns of AFP elevation in various non-HCC etiologies were discussed.

BIOLOGY OF AFP

AFP is an oncofetal glycoprotein consisting of a single polypeptide chain of approximately 600 amino acids and nearly 4% carbohydrate. With molecular weight of 67500 dalton, it is a negatively charged protein with an isoelectric point of pH 4.57 and displays several charge isomers by extended agarose gel electrophoresis[1,2]. During the first trimester, AFP is mainly produced by yolk sac. After the atresia of yolk sac by 11-12 wk, fetal liver becomes the predominant source of AFP. A trace amount is also produced by the gastrointestinal tract of the fetus[1]. AFP in fetal serum can be detected as early as 29 d after conception and reaches its peak value of 3.0×10^6 ng/mL by 14th week of gestation. It declines to 2.0×10^5–3.0×10^5 ng/mL by week 32 and further decreases to 20-120 ng/mL at term[3]. By six months of life, serum AFP level is approximately 30 ng/mL. Thereafter, it is detectable between 3 and 15 ng/mL in adulthood[3,4].

AFP belongs to the family of serum albumin and the genes of which are present on the chromosome 4. It shares structure and physiochemical properties with its family members namely albumin, vitamin D-binding protein and afamin[9]. As both albumin and AFP show significant amino acid homologies, AFP is suggested to be an embryonic analogue of albumin[10]. Furthermore, because of its similarity to albumin, it has been postulated that AFP could be a carrier protein. In addition, its role as an immune-regulator and as a carrier protein for bilirubin has also been suggested[11].

AFP, being a principal serum binding protein in the fetus, plays a vital role in carrying various ligands such as fatty acids, hormones, minerals and bilirubin[12]. Though it performs many physiological functions during fetal development, elevated levels in adults are frequently observed in liver carcinogenesis and various disease processes[13-15]. Many biologic studies support the pro-oncogenic as well as anti-apoptotic effects of AFP. AFP can stimulate cell proliferation, cell motility and invasive properties of HCC cell lines and there is evidence that apoptosis of cancer cells could be achieved by blocking AFP[16,17]. On the other hand, a number of cancerous and non- cancerous causes involving both the liver and other organs can also lead to AFP elevation. AFP, therefore, is a non-specific marker of HCC[7] (Table 1).

HOW IS AFP MEASURED?

AFP was originally measured by immunoelectophoresis, but this method was not very

Table 1 Causes of alpha-fetoprotein elevation[13-15]

Hepatic	Non-hepatic
Neoplastic	**Neoplastic**
Hepatocellular carcinoma	Germ cell tumors (testicular and ovarian malignancies)
Intrahepatic cholangiocarcinoma	Gastric cancer
Non-neoplastic	**Non-neoplastic**
Liver cirrhosis	Normal pregnancy/infancy
Fulminant acute hepatitis	Colitis
Acute and chronic viral hepatitis	Fetal disorders (Gastroschisis, Neural tube defect)
Chronic liver diseases	Ataxia telangiectasia
Biliary obstruction (Intrahepatic and extrahepatic causes)	Hereditary tyrosinemia type 1
Drug induced hepatitis	Hereditary AFP persistence
Alcohol liver disease	Beckwith-Wiedemann syndrome
Non-alcoholic liver disease	Systemic lupus erythematosus
Neonatal hepatitis	Hirschsprung's disease
Massive hepatic necrosis	
Wilson disease	
Hemochromatosis	

AFP: Alpha-fetoprotein.

sensitive. In the 1970s and 1980s, new techniques such as radioimmunoassay and enzyme immunoassays were used. Subsequently, a quantitative automated chemiluminescent enzyme immunoassay was developed, which replaced and refined the previous clinical assays[18,19]. In this method, the serum sample is placed on a magnetic plate which is already bound by an anti-AFP antibody. A second chemiluminescent detection antibody, added to the same magnetic plate, then binds to all the AFP that is present in excess. All the unbound detection antibody is washed off and an organic substrate called as developer is added which emits light and become luminescent (Figure 1A). A chemiluminometer is used to detect the antibody, and quantification of the results is done against the known AFP standards. However, measurement interference may occur. In a single step method of AFP detection, sometimes interfering antibodies would bind to both the capture and detect antibodies leading to a false positive result (Figure 1B). Conversely, the interfering antibodies may blind to the reagents and inhibiting the proper interaction of AFP with the specific anti-AFP antibodies[20] (Figure 1C).

AFP as a diagnostic marker for HCC

The association between AFP and HCC is well recognized; however, the sensitivity and specificity of the AFP assay varies according to patient characteristics, design of the study, and the AFP cut-off values used[21,22] (Table 2). A systematic review of studies using AFP at a threshold value of 20 ng/mL in patients with cirrhosis, the sensitivity and specificity of detecting HCC were 41%-65% and 80%-94%, respectively [22]. Lowering the cut-off can increase the sensitivity of AFP but at the cost of higher false positives. By increasing the AFP value from 20 to 50 ng/mL, the specificity increased to 96% with a positive predictive value of 75% but sensitivity was reduced to 47%[23]. AFP values also vary according to the tumor size. The correlation of AFP to the size of the tumor was evaluated by Saffroy *et al*[24]. The bigger tumors generally had higher AFP values. The sensitivity of AFP, therefore, decreased from 52% for HCC > 3 cm to 25% for those < 3 cm in diameter[24].

For AFP-producing HCC, AFP plays an important role in initiating monitoring and terminating HCC therapy. A complete response of the treatment can be expected if preceding levels of AFP decrease to normal on follow ups. AFP, however, cannot be applied to monitor treatment response if its levels were not elevated prior to treatment [25-27]. Approximately one-third of the patients with HCC have no AFP elevation[28].

Table 2 Sensitivity and specificity of alpha-fetoprotein at different cut-off values[22-24]

AFP cut-off value	Sensitivity	Specificity
20	Approximately 60%	90%
50	47%	96%
100	31.2%	98.8%
400	17%	99.4%

AFP: Alpha-fetoprotein.

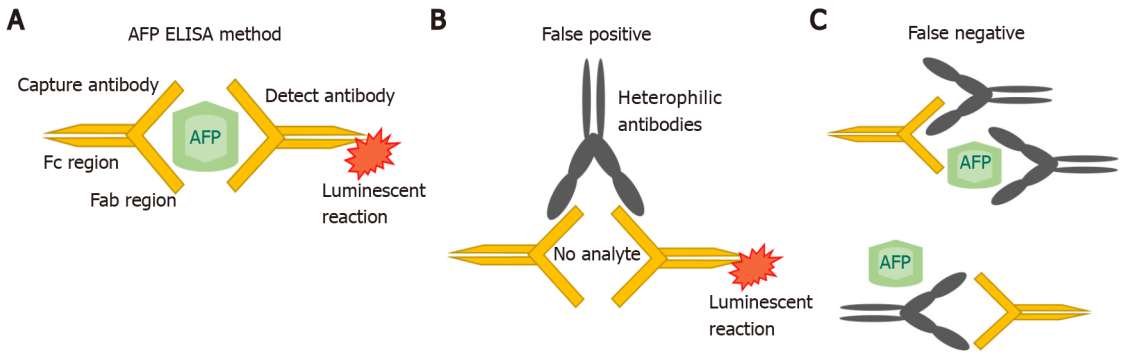

Figure 1 ELISA measures. A: ELISA measures alpha-fetoprotein (AFP) by detecting reactivity with two anti-AFP antibodies; B: Interfering antibodies that bind directly to the capture and detect reagents without the target AFP (no analytes). This binding would emit light and become luminescent causing false positive results and high values; C: Interfering antibodies can inhibit reactivity of the ELISA by binding to the reagents and prevent the detection of AFP by the anti-AFP antibodies; that leads to a false negative result. AFP: Alpha-fetoprotein.

Nomura *et al*[29] characterized those HCCs with low or no elevation of AFP. These patients generally have favorable prognosis with low probability of HCC recurrence and improved survival as compared to those with elevated AFP levels[29,30]. In a large retrospective study including 1800 patients with HCC, 42% had AFP < 20 ng/mL whereas 16% had AFP levels between 20 and 100 ng/mL. Thus, a total of 58% had AFP < 100 ng/mL. The authors observed that 67% of patients with HCC smaller than 5 cm had AFP ≤ 100 ng/mL *vs* only 49% of the patients with HCC larger than 5 cm had AFP ≤ 100 ng/mL. In addition, HCCs without AFP elevation (≤ 100 ng/mL) were less likely to develop portal vein thrombosis[31].

AFP-L3 and other HCC biomarkers

Based on the binding capacity of AFP to lens culinaris agglutinin (LCA), three separated bands of AFP glycoforms can be identified with the Western blotting. These are AFP-L1 (non-binding), AFP-L2 (intermediate binding) and AFP-L3 (LCA-reactive). AFP-L1 generally correlates with hepatic inflammation in chronic hepatitis while AFP-L3 is more specific for HCC. AFP-L2 is derived from yolk sac and is detectable in the maternal serum during pregnancy[32] (Table 3).

AFP-L3 is calculated as a fraction of AFP-L3 to total AFP. The cut-off values most commonly used for a positive AFP-L3 test for patients with HCC is 10% though levels above 15% are more specific[33]. It is secreted in the initial tumor stages and thus can be used as an early HCC diagnostic marker[32,34]. AFP-L3 level can also distinguish the histological differentiation of tumors[25]. In patients with total AFP level ≤ 200 ng/mL, AFP-L3 specificity may reach 100% for HCC if it increases more than 35% of the total AFP[35]. In a large multicenter prospective study of hepatitis C virus related HCC, the specificity of AFP-L3 was observed to be 92% but its sensitivity was fairly low at 37%, irrespective of the tumor stage[36]. On the contrary, a number of recent studies reported a much higher sensitivity of AFP-L3 for diagnosing HCC[37-39]. Ibrahim *et al*[39] evaluated the diagnostic efficacy of AFP-L3 among 20 healthy individuals, 40 patients of chronic active hepatitis and 40 HCC patients with underlying chronic hepatitis B (CHB) or C. Among the HCC patients, 30 (75%) had tumor size > 5 cm. At a cut-off point of > 12.3 ng/mL, AFP-L3 was 100% accurate in diagnosing the HCC[39]. In a study by Ibrahim *et al*[39], 20 healthy individuals, 20 chronic liver disease patients and 40 patients HCV-induced HCC were included in the

Table 3 Overview of the diagnostic parameters for hepatocellular carcinoma

Diagnostic parameter (cut off)	Remark	AUROC
AFP (> 20 ng/mL)	Sensitivity: 40%–65%	0.54–0.80
	Specificity: 76%–96%	
AFP-L3 (> 15%)	Sensitivity: 45%–90%	0.74–0.84
	Specificity: 95%	
AFP-L3 + AFP-P4 + P5 (> 15% for both biomarkers)	Sensitivity: 55.3%–61%	0.76
	Specificity: 82.3%–93.9%	
AFP, AFP-L3 and DCP (3-25 ng/mL, 1%-10%, 0.48 ng/mL or 40 mAu/mL)	Sensitivity: 81%–93%	0.88–0.93
	Specificity: 69%-87%	
AFP and GP73	Sensitivity: 75%-91%	0.91–0.95
	Specificity: 81%-97%	

AFP: Alpha-fetoprotein; DCP: Des-gamma-carboxyprothrombin.

analysis. A large proportion (65%) of the HCC patients had advanced stage of HCC. By using a cut-off value of 23 ng/mL, AFP-L3 had a sensitivity of 97.5% and specificity of 100% in predicting HCC at[37]. Cerban *et al*[38] investigated the diagnostic performance of AFP, AFP-L3, PIVKA-II and GPC-3 for HCC in a cohort including 52 cirrhotic and 101 HCC patients. AFP-L3 at a cut-off point of > 13.5 ng/mL was found to have a higher sensitivity of 84.7%, compared to other biomarkers[38]. The sensitivity of AFP-L3 for HCC detection can be affected by its cut-off values, as well as the tumor size and stage[40]. Data suggested that AFP-L3 could potentially be a valuable prognostic tool. The persistent elevation of AFP-L3 after HCC therapy was found to be associated with shorter survival[25].

Recently, a highly-sensitive AFP-L3 or hs-AFP-L3 was developed. Toyoda el al. determined the performance of hs-AFP-L3 among patients with 44 patients with chronic hepatitis or cirrhosis, and 54 patients with HCC. The sensitivity and specificity of hs-AFP-L3 were 84.9% and 88.6%, respectively[41].

Since both total AFP and AFP-L3 have limitations for the detection of HCC, studies have been conducted to combine AFP and AFP-L3 with other novel immunoassays to improve their performance. Des-gamma-carboxyprothrombin (DCP), also known as PIVKA-II (Protein Induced by vitamin K absence or antagonist-II), is a promising HCC biomarker. PIVKA-II is an abnormal product of liver carboxylation that reflects oncogenesis and HCC progression and is undetectable in healthy patients[25,42].

A systematic review noted that AFP, AFP-L3 combined with DCP enhanced their individual HCC predictive values with pooled sensitivity and specificity of 88% and 79%, respectively[43,44]. Since 2002, Japan has included these 3 biomarkers in their HCC surveillance programs and they noted improved rates of early HCC identification and prognosis[43]. When applying the GALAD score (age, sex, AFP, AFP-L3 and DCP), the HCC diagnostic accuracy was even higher [area under curve (AUC) 0.976][45,46]. A recent meta-analysis reported that AFP combined with GP73 (a Golgi specific membrane protein found in epithelial cells of bile ducts) has favorable sensitivity (84%) and specificity (92%) in diagnosing HCC with an AUROC value of 0.93[47].

Lately, a new HCC diagnostic marker, the long noncoding RNAs (lncRNAs), was developed[48]. LncRNAs are transcribed by RNA polymerase II and play a role in the regulation of transcription and translation of proteins. Small extracellular vesicles (EV) transfer proteins, DNA, and RNA between tumor and nontumor cells. The dysregulated lncRNAs including EV-derived lncRNAs contribute to HCC progression and metastasis. EV-derived lncRNAs are, therefore, potential novel serum biomarkers. Kim *et al*[48] evaluated LINC00853, an EV-derived lncRNA in 90 patients with HCC and 92 without HCC in a cohort study. The AUROC of LINC00853 was 0.935 for identifying all-stage HCC and 0.969 for detecting early-stage HCC. These results were significantly better compared to AFP[48]. In another study by Yu *et al*[49] reported that the combination of AFP with 2-lncRNA had a higher discriminative power than that of AFP alone in the diagnosis of HCC[49].

AFP ELEVATION IN LIVER DISEASES AND CIRRHOSIS

Rise in AFP has been recorded in different chronic liver diseases without HCC and other malignancies, hence, the results of AFP levels should be interpreted with caution [50]. AFP is known to have a role in liver regeneration, inflammation and liver fibrosis. Less pronounced AFP elevation > 10 ng/mL has been reported in 15%-58% of chronic hepatitis and 11%-47% of cirrhosis[51]. The usual cut off for differentiating benign *vs* malignant liver conditions was at 500 ng/mL but can be variable[52]. Studies have reported a progressive rise in serum AFP levels with increased histological severity from inflammation to cirrhosis to HCC[53].

ACUTE HEPATITIS

The degree of AFP elevation in acute hepatitis is related to the severity of hepatic destruction[50,54]. The levels range from 10 ng/mL-1000 ng/mL with occasional higher value from 3000 to 7190 ng/mL has been reported. Among children with acute hepatitis B, AFP was detected within 1 wk of the onset of clinical hepatitis and returned to normal by the time of recovery with loss of HBsAg[55]. The possible mechanisms of AFP elevation include acute phase reaction to the liver injury, hepatocyte regeneration, or viral control or mediated AFP synthesis[56]. AFP elevation after significant alanine aminotransferase (ALT) elevation is most likely due to liver regeneration and there is generally a latent period of 5-16 d[57]. AFP generally peaks at a time when liver destruction is subsiding and hepatic remodeling begins[57]. Hence, the highest level of AFP in acute hepatitis generally occurs during the recovery phase of illness and is an indicator of the liver regenerative process[58].

CHB

Elevated AFP levels are frequently observed during the course of CHB without HCC. The commonest cause of AFP elevation was the exacerbation of the underlying liver disease with or without changes in the status of hepatitis B virus (HBV) replication[41, 52]. High AFP levels associated with hepatitis flare were also found to be predictive of HBeAg to anti-HBe seroconversion[59,60]. AFP levels usually decrease within 12 mo on antiviral therapy[61]. Kim *et al*[61] reported that AFP normalization (< 20 ng/mL) was achieved in 89.5% of treated patients but remained abnormal in 40.6% of antiviral treatment naïve patients[61].

Persistent elevation of AFP > 100 ng/mL without a parallel increase in ALT level was reported to be a predictor of HCC with a sensitivity of 98.7% and specificity of 66.7%[59]. Yuan *et al*[62] conducted a retrospective study of 302 treatment naive CHB patients with AFP positive status. After antiviral therapy, they found that there was a 6.35-fold increased HCC risk among those with persistently elevated AFP compared to those with normalized AFP levels[62].

In a metanalysis, Peng *et al*[63] concluded that among HBV–related HCCs with low AFP levels, circulating miRNAs could be potential valuable biomarkers. For patients with AFP levels < 400 ng/mL, miR-125b and miR-205 demonstrated a sensitivity of 90% while combination of miR-15b and miR-130b had > 90% sensitivity and specificity. For AFP < 20 ng/mL, miR-26a, 27a, 7b and combination of miR-122 and miR-7b exhibited a sensitivity of 80% while combination of mir-29a, 29c, 133a, 143, 145, 192 and 505 produced a specificity of > 80%. Additionally, the combination of miR-15b and miR-130b showed high diagnostic accuracy with sensitivity and specificity exceeding 90%. For differentiating HCC patients with low AFP levels (< 20 ng/mL) from non-HCC patients, the overall sensitivity and specificity of miRNAs were 0.85 and 0.74, respectively with AUC of 0.88 (95%CI: 0.85–0.90). For patients with AFP < 400ng/mL, the overall sensitivity and specificity were 0.84 and 0.76 with AUC of 0.88 (95%CI: 0.84–0.90). Hence, miRNAs are attractive HCC markers to detect HBV-associated HCC with low AFP levels[63].

Dickkopf WNT signaling pathway inhibitor 1 (DKK-1) is a glycoprotein that is expressed in various malignancies. Shen *et al*[64] conducted a study on 424 HCC, 98 CHB, 96 cirrhosis and 213 healthy controls and measured their DKK-1 Levels. The diagnostic cut-off was established at 2.153 ng/mL. DKK-1 proved useful in the detection of AFP-negative HCC with 70.4% sensitivity and 90.0% specificity. These values were validated in another cohort. When combined with AFP, DKK-1 increased detection rate of early-stage HCC with 73.1% sensitivity and 90.0% specificity. Add-

itionally, elevated DKK-1 Levels can help differentiate HCC from other liver diseases such as CHB and cirrhosis with 69.1% sensitivity and 84.7% specificity[64,65].

Hepatitis B flare is defined as an abrupt elevation of serum ALT to 5 times the ULN or greater than 3-fold increase from baseline[66]. During hepatitis flare, peak AFP level was usually observed 1-2 wk after peak ALT rise. Significant AFP elevation to > 2500 ng/mL had been documented with hepatitis flare. The normalization of AFP might take up to 3-12 mo[66].

The presentation varies from asymptomatic to overt hepatitis with decompensation and liver failure. AFP elevation was noted in 25%-30% of the patients with hepatitis flares. A study reported the annual incidence of hepatitis flares to be 27% in HBeAg-positive patients and 10% in HBeAg-negative patients with a mean follow-up period of 2 years. Chang *et al*[67] reported that patients with AFP > 100 ng/mL during flare cleared HBeAg at a rate of 31% in 3 mo, 62% in 12 mo and 72% in 18 mo. In contrast, the corresponding HBeAg clearance rates for those with AFP < 100 ng/mL were only 4%, 15% and 19% in 3, 12 and 18 mo respectively[67]. Patients with bridging hepatic necrosis (BHN) had increased activation of the AFP-producing oval cell. BHN was noted in 80% of the cases with AFP levels > 100 mg/dL[53]. BHN, AFP > 100 ng/mL and decreasing HBV DNA titers were identified as good prognostic indicators of an effective immune control with HBV DNA suppression and/or HBeAg clearance.

Severe and repeated hepatitis flares could also lead to development of cirrhosis or hepatic decompensation. Patients with severe hepatitis flare with BHN or AFP > 100 ng/mL but fail to suppress HBV DNA should be treated promptly with antiviral therapy.

CHRONIC HEPATITIS C

AFP elevation is more greatly associated with HCV-associated HCC than HBV-associated HCC[64]. The incidence of elevated AFP in chronic hepatitis C (CHC) patients range from 10%-43%[61]. Elevated AFP > 20 ng/dL in CHC is associated with female gender, black race, increased age, genotype 1b, low albumin level, elevated aspartate aminotransferase (AST), elevated AST/ALT ratio, low platelet, prolonged PT, and increased ferritin levels[68,69]. Yang *et al*[70] evaluated 279 CHC patients and found no correlation between AFP and the levels of HCV RNA[70]. In the HALT C (Hepatitis C Antiviral Long-term Treatment) study, Di Bisceglie *et al*[71] observed AFP > 20 ng/mL in 16.6% of patients without HCC; it was associated with cirrhosis, female and Black patients[71]. Black patients with CHC tend to have higher AFP elevation compared to other racial groups[71]. Generally, HCV-related HCC does not have significant AFP elevation. Thus, AFP has especially low sensitivity to identify HCC in Black populations[71].

Fouad *et al*[72] reported that sustained virological response with direct-acting antiviral agents (DAA) therapy was associated with a significant reduction in serum AFP and might be used as a predictor of treatment response[72]. In another study, 60% of the DAA treated patients normalized AFP compared to only 23% without therapy [61].

Therapeutic phlebotomy has also been reported to reduce AFP levels in CHC[44]. It has been postulated that iron mediated oxidative stress is associated with hepatic injury; iron depletion decreases the oxidative stress and indirectly lowers the AFP level[73].

NONALCOHOLIC FATTY LIVER DISEASE

Nonalcoholic fatty liver disease (NAFLD) has become the most common liver disease in the Western world with about 10%-30% progress to cirrhosis[74]. It is estimated that 6 million people in the United States have NASH with 1%-2% incidence of HCC annually. Published reports estimated that over 25% of NASH-related HCC occurred in patients without cirrhosis. With the high prevalence of NAFLD, risk stratification is essential to implement HCC surveillance programs[75]. Currently, no surveillance of NALFD patients for HCC is recommended by AASLD[76]. However, it has been suggested that NAFLD patients with cirrhosis or possible fibrosis or diabetes mellitus should have 6 monthly surveillances *via* ultrasound (US) and tumor markers (AFP, AFP-L3 and DCP). Abdominal fat can be a hindrance, hence, patients with obesity can be alternatively surveilled using CT-Scan or magnetic resonance imaging[77].

An association between AFP elevation and NAFLD was first established by Babalı *et al*[78] in 2009. This study demonstrated a significant increase in AFP levels in patients suffering from NAFLD (4.09 ± 1.68) in comparison to the control group (2.95 ± 0.41) with P value < 0.05.Additionally, three subgroups of NAFLD were created based on the liver ultrasonography findings with presence of hepatorenal contrast or bright liver corresponding to grade 1, both hepatorenal contrast and bright liver signifying grade 2, and both pre-mentioned findings with bright liver being severe representing grade 3. The results showed a significant increase in AFP in grade 3 NAFLD (5.43 ± 1.51) in comparison to grade 2 (3.97 ± 1.45) and grade 1 (2.92 ± 1.06) with a P value of 0.001. Triglyceride, cholesterol, low density lipoprotein, high-density lipoprotein, glucose and ALT levels were not significantly different in these groups[78].

Best *et al*[79] published a case control study on German centers in 2020 describing the relationship between HCC and NASH using AFP, AFP-L3, DCP and the GALAD score for assessment. 125 NASH patients with HCC and 231 NASH control patients without HCC were taken. The GALAD score recognized patients with HCC at any stage with an AUC of 0.96 which was superior to AUC of AFP (AUC 0.88), AFP-L3 (AUC 0.86) and DCP (AUC 0.87) levels with P < 0.001 at a cut off about -0.63. The sensitivity was 84.8% and specificity was 95.2% at this cut off. They also conducted Japanese cohort study on 392 patients with NAFLD out of which 28 patients developed HCC. The mean GALAD score in these 28 patients predicted occurrence of HCC 1.5 years before its diagnosis. GALAD score was considerably higher in these patients compared to NASH patients who did not develop HCC[79].

EARLY *VS* ADVANCED LIVER CIRRHOSIS WITH DECOMPENSATION

Arrieta *et al*[80] reported that AFP ≥ 200 and 400 ng/mL had a sensitivity of 36.3% and 20.2% in determining HCC with 100% specificity. Improved method of analysis using progressive elevation of AFP ≥ 7 ng/mL/mo among cirrhotic patients increased the sensitivity to 71.4% with 100% specificity. This method was successful even when AFP values were below 200 ng/mL[80].

AFP can be elevated in patients with CHC or cirrhosis without any evidence of HCC [81]. A persistent AFP value of 17.8 ng/mL was noted to have a 98.6% specificity, 35% sensitivity and a positive predictive value of 97.7% in identifying patients with cirrhosis[82]. Sudden fluctuations in AFP levels in patients with cirrhosis can be indicative of hepatitis flare, deterioration of liver disease and development of HCC [83]. AFP has been applied routinely for HCC surveillance in cirrhotic patients. Harada *et al*[83] showed that 40% of cirrhotic patients had AFP levels > 20 mg/dL[83]. Manuc *et al*[84] reported in a study of 2068 CHC patients with cirrhosis, 30.1% without HCC had AFP > 15 ng/mL due to advanced age, severe liver injury with raised AST and ALT levels and low platelets[84].

In patients with cirrhosis, both the sensitivity and specificity of AFP for detection of early-stage HCC diagnosis can be enhanced by combining it with US. In a meta-analysis, the pooled sensitivity of AFP and US for any stage and early-stage HCC were 95% (83%-100%) and 60% (45%–74%) respectively, compared to only 72% (56%–86%) and 40% (22%–58%) for US only[85]. Surveillance with AFP and ultrasonography in cirrhotic patients having an annual risk of HCC > 0.4% is cost-effective[86].

There are many risk scores that have been developed to predict HCC development among patients with chronic hepatitis and cirrhosis. However, many of them have not been standardized or carefully validated in different patient populations. Ideally, HCC prediction models should be developed for treated and untreated patients taking into account the patient ethnicity, significant comorbid conditions and ideally the molecular biomarkers[87].

PERSISTENT AFP ELEVATION IN THE ABSENCE OF LIVER DISEASE

AFP is an important screening test for HCC and is also used in the diagnostic evaluation of other hepatic and non-hepatic conditions. Persistent AFP elevation has also been reported in patients without malignant or nonmalignant conditions. Hereditary persistence of AFP (HPAFP), a benign autosomal dominant disorder with no apparent disease or abnormality, should be considered as one of the differential diagnoses in patients with unclear etiology of persistent AFP elevation[88]. HPAFP is a rare condition with only 20 reported cases in the literature[89]. In contrast to malignant tumors with AFP usually > 500 ng/mL, the AFP concentration in HPAFP is mostly

below 200 ng/mL, but levels up to 1500 ng/mL have been reported in some cases[88,90,91]. The molecular mechanism of HPAFP can differ in unrelated families. Specific heterozygous point mutations are frequently found in the promoter region of the AFP gene related to hepatocyte nuclear factor 1 (HNF1) binding sites[92,93]. These mutations usually result in an increased affinity for HNF1 and subsequently lead to increased AFP promoter activity and AFP gene transcription[94]. Two-point mutations have been identified; the upstream substitution of cytosine (C) with adenosine (A) at amino acid position of 55 (-55 C > A) in the proximal HNF-1 binding site and upstream mutation caused by the substitution of Guanine (G) by Adenosine (A) at position 119 (-119 G > A) in the distal HNF-1 binding site[95,96]. The first case of HPAFP was reported in 1984 by Greenberg *et al*[91] in a Scottish family where a 38-year-old woman was noted to have persistently elevated AFP during post-partum[91]. The AFP concentration of her amniotic fluid was normal and HPAFP was later confirmed by evaluating serum AFP levels and molecular testing in family members. In another report of 20 HPAFP cases, 2 patients underwent unnecessary surgery and 3 had unnecessary chemotherapy due to their persistently elevated AFP[96]. Though rare, hereditary causes should be considered in patients with unexplained and persistent AFP elevation. In a recent study by Jeon *et al*[89] on 4 Korean patients with persistently elevated AFP levels from 12.1 to 186.1 ng/mL for > 1 year, 1 patient was found to have a hereditary cause by pedigree analysis even though the typical mutation of the AFP gene in the promoter region was absent. This case elucidated the heterogeneous nature of persistent AFP elevation and HPAFP is not always the result of mutation in the AFP transcription regulatory regions[89].

CONCLUSION

AFP has been used for HCC surveillance widely in clinical practice. It, however, has limited sensitivity and specificity for HCC detection, and a proportion of patients with advanced HCC do not have AFP secretion. Moreover, patients with chronic liver diseases, especially those with cirrhosis are commonly identified with persistent AFP elevation without radiological evidence of HCC. For all these reasons, AFP is not recommended to be used as a sole marker alone for HCC surveillance. The diagnostic potential of AFP for early diagnosis of HCC can be enhanced by combining it with other novel diagnostic markers such as AFP-L3, PIVKA-II, and GP73. Although many have already endorsed diagnostic value, large number of multicenter studies encompassing larger cohorts and long-term assessment are required to confirm clinical utility. Additionally, algorithms integrating these serum markers with noninvasive diagnostic imaging modalities are needed to be developed for the early and accurate diagnosis of HCC.

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ORIGINAL ARTICLE

Case Control Study

Obesity is associated with decreased risk of microscopic colitis in women

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Abstract

BACKGROUND

Microscopic colitis is a leading cause of diarrhea in the older adults. There is limited information about risk factors. We hypothesized that obesity would be associated with microscopic colitis.

AIM

To examine the association between obesity and microscopic colitis in men and women undergoing colonoscopy.

METHODS

We conducted a case-control study at the University of North Carolina Hospitals. We identified and enrolled men and women referred for elective, outpatient colonoscopy for chronic diarrhea. We excluded patients with a past diagnosis of Crohn's disease or ulcerative colitis. A research pathologist reviewed biopsies on every patient and classified them as microscopic colitis cases or non-microscopic colitis controls. Patients provided information on body weight, height and exposure to medications *via* structured interviews or Internet based forms. The analysis included 110 patients with microscopic colitis (cases) and 252 non-microscopic colitis controls. Multivariable analyses were performed using logistic regression to estimate odds ratios and 95% confidence intervals.

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RESULTS

Cases were older and more likely than controls to be white race. Study subjects were well educated, but cases were better educated than controls. Cases with microscopic colitis had lower body mass index than controls and reported more weight loss after the onset of diarrhea. Compared to patients who were normal or under-weight, obese (BMI > 30 kg/m^2) patients were substantially less likely to have microscopic colitis after adjusting for age and education, adjusted OR (aOR) 0.35, 95% confidence interval (CI) 0.18-0.66). When stratified by sex, the association was limited to obese women, aOR 0.21, 95%CI: 0.10-0.45. Patients with microscopic colitis were more likely to report weight loss after the onset of diarrhea. After stratifying by weight loss, there remained a strong inverse association between obesity and microscopic colitis, aOR 0.33, 95%CI: 0.10 – 1.11 among the patients who did not lose weight. Ever use of birth control pills was associated with lower risk of microscopic colitis after adjusting for age, education and BMI, aOR 0.38, 95%CI: 0.17-0.84.

CONCLUSION

Compared to controls also seen for diarrhea, microscopic colitis cases were less likely to be obese. Mechanisms are unknown but could involve hormonal effects of obesity or the gut microbiome.

Key Words: Colitis; Microscopic/epidemiology; Humans; Diarrhea/epidemiology; Obesity

Core Tip: We conducted a case control study among patients undergoing colonoscopy for diarrhea. The analysis included 110 patients with microscopic colitis and 252 controls. Obesity was associated with a substantially lower risk of microscopic colitis among women that was not explained by weight loss following the onset of diarrhea. Ever use of birth control pills was associated with lower risk of microscopic colitis after adjusting for age, education and BMI. The mechanism could involve hormonal effects of obesity or the gut microbiome.

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INTRODUCTION

Microscopic colitis is a chronic inflammatory disease of the colon characterized by a normal or near normal endoscopic appearance but abnormal pathology. It is increasingly recognized that endoscopically visible lesions can be recognized in nearly 40% of patients although they are non-specific[1]. Histologically, there is a thickened collagen band (collagenous colitis) or an increase in intraepithelial lymphocytes (lymphocytic colitis)[1]. Microscopic colitis was first described in 1976 by Lindstrom [2]. The term microscopic colitis was used by Read *et al*[3] in a 1980 publication describing a series of patients with chronic diarrhea of unknown origin. While initially considered uncommon, population-based studies have shown a rising incidence that may have started to plateau[4-6].

Microscopic colitis is now a frequent cause of chronic diarrhea, particularly in older adults. In some regions, the incidence of microscopic colitis exceeds Crohn's disease and ulcerative colitis[7]. Despite an increasing burden of disease, the etiology of microscopic colitis is not known. Prior studies implicated a range of medications including proton pump inhibitors (PPIs), nonsteroidal anti-inflammatory drugs (NSAIDs), statins, and beta blockers[8]. Cigarette smoking has also been implicated[9, 10]. The condition has been reported to be more common in patients with a number of auto-immune diseases[11].

There is currently limited information that obesity might be inversely associated with microscopic colitis[12,13]. We conducted a case-control study among a population of patients scheduled for colonoscopy due to chronic diarrhea in order to assess whether obesity and hormonal factors might be associated with microscopic colitis.

MATERIALS AND METHODS

Briefly, we identified male and female patients between April 1, 2015 and December 22, 2020 who were referred for outpatient colonoscopy for diarrhea. We excluded patients with a prior history of Crohn's disease and ulcerative colitis. Potential participants were mailed information about the study in advance of their procedure. On the day of their exam, eligibility was verified by a research assistant who obtained informed consent for participation. To be eligible for the study, patients had to report a Bristol Stool Form type 5, 6, or 7 (mushy, loose, watery) during the week prior to their colonoscopy regardless of the number of stools[14] . We recruited patients from each of the 3 endoscopy units at the University of North Carolina Chapel Hill. If the research assistant was not available for consent at the time of the procedure, the patient was later contacted to verify eligibility and obtain consent. We also queried the hospital pathology database every month and contacted patients with biopsy proven microscopic colitis who were not identified prior to their colonoscopy. This group included patients who were referred for colonoscopy for other reasons (generally screening) but reported diarrhea and had colon biopsies taken during the colonoscopy to assess for microscopic colitis. In sensitivity analyses, the patients identified retrospectively from pathology were excluded.

Patients with signs of gross inflammation on colonoscopy were excluded. Patients with subtle or isolated mucosal abnormalities were not excluded. Patients with non-lymphocytic colitis on biopsies were also excluded. A research pathologist (JTW) who was not aware of the clinical diagnosis reviewed the slides for all enrolled study subjects. Cases were patients with microscopic colitis on biopsy defined by increased number of intraepithelial lymphocytes. Additional features included increased lamina propria chronic inflammation, with minimal crypt distortion or active cryptitis. Collagenous colitis was defined by a thickened subepithelial collagen band. Slides were initially reviewed by a clinical pathologist. The slides were then re-read by the study pathologist. When there was a disagreement between the clinical pathologist and the research pathologist, the research pathologist re-read the slides. In addition, a 20% sample of slides were resubmitted to the research pathologist. After excluding indeterminate colitis, there was a 100% match between the initial and final reading by the research pathologist. Controls were patients with normal pathology. Patients with indeterminate microscopic colitis based on a sparse number of lymphocytes were excluded to avoid possible misclassification and because there were too few for separate analysis.

After the colonoscopy, all participants completed a 30 to 40-minute structured telephone interview or self-completed the same questionnaire using an internet-based form. The telephone interviewer verified eligibility, including the diarrhea criteria, for patients who were identified from pathology or who were missed in the endoscopy unit. Participants self-reported demographics, body weight, height, medical history including over-the-counter and prescription medications, reproductive history, bowel habits, and gastrointestinal symptoms. The reproductive history for women included questions about age at first menses, oral contraceptive use, gravidity, parity, and if postmenopausal, age at and type of menopause, and menopause hormone therapy use. All exogenous hormone questions included age at initiation, duration of use, and use in the last year, but not type of hormone or dose. BMI was calculated from self-reported weight in kilograms divided by height in meters squared (kg/m^2). BMI was categorized using Centers for Disease Control criteria: BMI < 25 kg/m^2 (under- or healthy weight), BMI 25.0 < 30 kg/m^2 (overweight), BMI > 30 kg/m^2 (obese).

Data analysis was conducted using Stata 17.0 (Stata Corp. College Station, TX). The analysis was reviewed by a biostatistician (JAG). Variables were examined one-by-one in bivariate analyses using chi square tests for categorical variables and Student t-tests for continuous variables. Multivariable logistic regression models were used to calculate adjusted odds ratios and 95% confidence intervals adjusted for age, educational status and BMI. Smoking was not included in models because smoking was not independently associated with microscopic after adjusting for education. We have previously examined the association between medications thought to be

associated with microscopic colitis (PPI, statins, SSRI, NSAIDs)[15]. There was no association in our study so those drugs were not included in models. Multivariable logistic models also included terms for reproductive factors including age at first live birth, number of live births and age of menarche. The model terms were informed by review of the relevant literature and directed acyclic graphs (DAGs)[16]. For analyses of menopause, the reference group was the most common age of natural menopause, age 45-54. Missing data were not imputed.

The study was approved by the University of North Carolina Office of Human Research Ethics. All patients gave informed consent.

RESULTS

Patients were enrolled in the study between April 1, 2015 and December 22, 2020. Among the 1008 patients who were referred for colonoscopy for diarrhea, 176 cancelled their colonoscopy, 161 were ineligible, 99 were missed in the endoscopy unit and not subsequently recruited, and 196 refused. After excluding indeterminate colitis (n = 14), there were 362 who serve as the basis of this report. There were 110 microscopic colitis cases (including 34 identified from pathology reports) and 252 controls. Interviews were completed by phone by 84% of subjects and Internet for the remainder.

Table 1 shows characteristics of the study population. The cases were older than controls: case mean age 63.2 (standard deviation (SD) 12.7, interquartile range 53-73) *vs* control mean age 54.5 (SD 11.8, interquartile range 45-64). Cases were more likely than controls to be white (96.2% *vs* 85.7%). As a group, the study subjects were very well educated, but cases were more likely to have a college degree or have attended graduate school, 66.0% *vs* 44.3%. Cases were less likely to be current smokers, 11.3% *vs* 21.0%. There was a strong correlation between smoking and education, with better educated patients substantially less likely to smoke (not shown). There was no difference between cases and controls for marital status, race, or smoking after adjustment.

Overall, mean BMI was lower in cases (BMI 25.7 kg/m^2 (SD 6.4)) than controls (BMI 29.5 kg/m^2 (SD 7.2)). As shown in Table 2, the risk for microscopic colitis was lower for BMI 25-30 kg/m^2 (OR 0.71, 95%CI: 0.40-1.25) and BMI > 30 kg/m^2 (OR 0.31, 95%CI: 0.17-0.55) compared to under- or healthy weight (BMI < 25 kg/m^2) as the reference. Similar results were seen in analyses adjusted for age and education. To determine whether the results were comparable for men and women, we stratified by sex. The results for women were similar to the overall results with lower risk for obese women. The results for men were null, but the number of men was small making estimates unstable with wide confidence intervals around risk estimates. Patients with microscopic colitis were more likely to report weight loss following the onset of diarrhea than controls: 65.3% cases *vs* 42.5% controls, p < 0.001. Because current BMI could be misclassified due to weight loss following the onset of diarrhea, we stratified on weight loss. The results were similar in the weight loss and the no weight loss strata. We conducted analyses separately for lymphocytic colitis and collagenous colitis and the results were similar to the overall.

All of the patients in our study had diarrhea. We asked patients if they had ever been told by a physician that they had irritable bowel syndrome (IBS). Not surprisingly the cases were less likely to have a history of IBS, odds ratio 0.40, 95%CI: 0.20 – 0.78). When we stratified by IBS, patients in the highest BMI category were less likely to have microscopic colitis in the non-IBS stratum, aOR 0.38 (95%CI: 0.18-0.79) an estimate similar to the overall estimate in cases and controls. We compared the BMI in patients with IBS stratified by microscopic colitis status. In the microscopic colitis cases, the mean BMI was not different in the IBS group (24.6 kg/m^2 (SD 7.2)) and the non-IBS group (25.7 kg/m^2 (SD 6.4), P = 0.55). Similarly, the mean BMI was not different among the controls with IBS (30.3 kg/m^2 (SD 6.6)) compared to non-IBS controls (29.1 kg/m^2 (SD 7.2), P = -0.27)).

Because the risk for microscopic colitis was lower in obese women than men (recognizing small numbers of men), we examined potential hormonal risk factors in women. Reproductive factors are shown in Table 3. The table shows crude odds ratios and odds ratios adjusted for age, education and BMI (model 1) and age, education, BMI, number of live births and age at menarche (model 2). Age at menarche, parity, number of live births and age at first live birth were not different in cases and controls. Use of oral contraceptive pills was inversely associated with microscopic colitis in crude (OR 0.41, 95%CI: 022-0.79), adjusted (aOR 0.38, 95%CI: 0.17-0.84), and multiply

Table 1 Characteristics of the study population

	Cases, *n* = 110		Controls, *n* = 252	
	n	Percent	*n*	Percent
Age[a]				
mean	63.2	12.7	54.5	11.8
Race[b]				
White	102	96.2	186	85.7
Non-White	4	3.8	31	14.3
Sex[a]				
Female	94	86.2	176	69.8
Male	15	13.8	76	30.2
Marital status				
Married	75	70.8	146	66.7
Not married	31	29.3	73	33.3
Education[a]				
Less than college	36	34.0	122	55.7
College or postgrad	70	66.0	97	44.3
Cigarette smoking[c]				
Never smoker	48	45.3	107	48.9
Former smoker	46	43.4	66	30.1
Current smoker	12	11.3	46	21.0
Irritable bowel syndrome[b]				
Yes	14	13.6	60	28.0
No	89	86.4	154	72.0

[a]$P < 0.001$.
[b]$P < 0.005$.
[c]$P < 0.05$.

adjusted analyses (aOR 0.20, 95%CI: 0.08-0.52). The results were the same when cases identified by pathology were excluded. The results were also similar for lymphocytic and collagenous colitis.

Menopausal factors are shown in Table 4. The percent of women who were postmenopausal was higher in the cases in crude analyses, but that is because they were older. The difference was absent in the adjusted model. Cases were more likely to have ever-used menopausal hormone hormones in the crude analysis OR 2.79, 95%CI: 1.44-5.41). After adjusting for age, education and BMI the risk estimate was lower (aOR 1.63, 95%CI: 0.73-3.62).

DISCUSSION

We found a striking difference in the risk for microscopic colitis with BMI, with a strong inverse association with obesity in women. Women with microscopic colitis were substantially less likely to be obese. There was no apparent effect of BMI on risk for microscopic colitis in men, although the number of men in our study was small. Given the sex differences, we also looked for possible reproductive or hormonal associations. We found a strong inverse association of microscopic colitis with ever use of oral contraceptives. In contrast to most prior studies, we enrolled patients who were referred for colonoscopy for diarrhea. At the time of the referral, the status as a microscopic colitis case or control was not known. All participants had similar symptoms, access to care, colonoscopy and biopsies.

Table 2 Body mass index and risk of microscopic colitis

	Cases		Controls			
	n = 101		*n* = 238		Crude	Adjusted[1]
BMI	*n*	%	*n*	%	OR (95%CI)	aOR (95%CI)
Overall						
< 25	50	49.5	73	30.7	Ref.	Ref.
25 < 30	29	28.7	60	25.2	0.71 (0.40-1.25)	0.70 (0.37-1.31)
≥ 30	22	21.8	105	44.1	0.31 (0.17-0.55)	0.35 (0.18-0.66)
Women						
< 25	47	53.4	47	28.7	Ref.	Ref.
25 -30	24	27.3	34	20.7	0.71 (0.36-1.37)	0.67 (0.32-1.40)
≥ 30	17	19.3	83	50.6	0.20 (0.10-0.40)	0.21 (0.10-0.45)
Men						
< 25	3	23.1	26	35.1	Ref.	Ref.
25 -30	5	38.5	26	35.1	1.67 (0.36-7.71)	2.22 (0.41-12.05)
≥ 30	5	38.5	22	29.7	1.97 (0.42-9.19)	2.92 (0.52-16.22)
Lost weight						
< 25	34	60.7	32	44.4	Ref.	Ref.
25 -30	13	23.2	17	23.6	0.72 (0.30-1.71)	0.89 (0.35-2.22)
≥ 30	9	16.1	23	31.9	0.37 (0.15-0.91)	0.49 (0.19-1.28)
No weight loss						
< 25	10	31.3	20	19.4	Ref.	Ref.
25 -30	13	40.6	30	29.1	0.87 (0.31-2.36)	0.64 (0.20-2.04)
≥ 30	9	28.1	53	51.5	0.33 (0.12-0.96)	0.33 (0.10-1.11)
IBS						
< 25	9	69.2	15	25.4	Ref.	Ref.
25-30	2	15.4	15	25.4	0.22 (0.04-1.21)	0.26 (0.04-1.50)
≥ 30	2	15.4	29	49.2	0.11 (0.02-0.60)	0.13 (0.02-0.76)
No IBS						
< 25	39	47.6	47	32.6	Ref.	Ref.
25 -30	26	31.7	37	25.7	0.85 (0.44-1.63)	0.78 (0.39-1.58)
≥ 30	17	20.7	60	41.7	0.34 (0.17-0.68)	0.38 (0.18-0.79)

[1]Adjusted for age and education. BMI: Body mass index; IBS: Irritable bowel syndrome.

Although BMI is an important risk factor for a number of diseases, there is surprisingly little information on the association between BMI and microscopic colitis. In a study using population controls, Larsen *et al*[17] reported data on 135 microscopic colitis who were compared to 27960 participants in the Malmo Diet and Cancer Study. There was no difference between the groups with respect to BMI. Similar to our study, Pascua *et al*[18] included 259 diarrhea controls in a small study with 26 microscopic colitis patients. There was no difference in BMI. Another small study was designed to examine the microbiome in 20 patients with microscopic colitis, 20 age- and sex-matched healthy controls, and 20 patients with functional diarrhea according to Rome IV criteria[19]. The BMI was 24.7 (SD 3.5) in microscopic colitis patients, 28.2 (SD 6.9) in healthy controls, and 27.9 (5.5) in the patients with chronic diarrhea. No statistics were reported in the paper, but based on the sample size and the estimates, the difference was significant for healthy controls (P = 0.05) and for diarrhea controls (P =

Table 3 Reproductive factors and risk of microscopic colitis in women

	Cases		Controls				
	n = 94		*n* = 176		Crude	Model 1[1]	Model 2[2]
	n	%	*n*	%	OR (95%CI)	aOR (95%CI)	aOR (95%CI)
Age of menarche							
≤ 11	18	20.5	46	30.3	Ref.	Ref.	Ref.
12	24	27.3	36	23.7	1.70 (0.80-3.61)	1.30 (0.53-3.17)	1.41 0.52-3.83)
13	23	26.1	38	25.0	1.55 (0.73-3.28)	0.76 (0.31-1.85)	0.54 (0.20-1.43)
≥ 14	23	26.1	32	21.1	1.84 (0.86-3.94)	0.90 (0.36-2.28)	0.85 (0.30-2.42)
Parity							
Nulliparous	15	16.5	29	19.0	Ref.	Ref.	Ref.[3]
Parous	76	83.5	124	81.1	1.04 (0.88-1.24)	1.02 (0.83-1.25)	1.00 (0.81-1.23)
Number of live births							
None	15	16.5	29	19.0	Ref.	Ref.	Ref.[3]
1	13	14.3	20	13.1	1.26 (0.49-3.20)	1.16 (0.39-3.48)	1.19 (0.40-3.60)
2	42	46.2	60	39.2	1.35 (0.65-2.83)	1.20 (0.50-2.89)	1.15 (0.47-2.81)
3 or more	21	23.1	44	28.8	0.92 (0.41-2.08)	0.78 (0.28-2.16)	0.83-0.30-2.35)
Age of first live birth (among parous women)							
≤ 19	13	17.1	32	25.8	Ref.	Ref.	Ref.
20-23	12	15.8	42	33.9	0.70 (0.28-1.75)	0.24 (0.07-0.82)	0.23 (0.07-0.85)
24-29	26	34.2	27	21.8	2.37 (1.02-5.49)	0.76 (0.24-2.43)	0.78 (0.23-2.63)
30+	25	32.9	23	18.6	2.68 (1.13-6.31)	1.07 (0.32-3.58)	1.02 (0.28-3.68)
Oral contraceptives							
No	27	29.7	23	15.0	Ref.	Ref.	Ref.
Yes	64	70.3	130	85.0	0.42 (0.22-0.79)	0.38 (0.17-0.84)	0.20 (0.08-0.52)

[1]Model 1 Adjusted for age, education and body mass index (BMI).
[2]Model 2 adjusted for age, education, BMI, age first live birth, number of live births, age of menarche.
[3]Omitted age of first live birth from model because of collinearity.

0.03). Roth *et al*[20] identified microscopic colitis cases from pathology records from 2002 – 2010 from the Skåne University Hospital, Malmö, with controls selected from a population-based study of breast cancer. The BMI in cases was 24.84 kg/m^2 and controls 24.88 kg/m^2, P = 0.451. The cases and controls were not recruited contemporaneously.

Cotter *et al*[13] sought to develop a scoring system to predict microscopic colitis among patients presenting with diarrhea. In a derivation cohort of 617 patients, BMI < 30 kg/m^2 was associated with an increased risk of microscopic colitis, OR 2.15 (95%CI: 1.19-3.88). Weight loss has been shown to be associated with MC in publications by the same authors[21,22], and it is not clear whether weight loss might have led to misclassification of BMI category. Liu *et al*[12] used the two Nurses' Health Study cohorts to identify 244 cases of self-reported microscopic colitis with 4.2 million person-years of observation. Compared to the women in the lowest BMI category, BMI < 18.5 kg/m^2, those with a BMI > 30 had an adjusted hazard ratio of 0.50 (95%CI: 0.32-0.79). The p for trend was < 0.001. Weight gain since early adulthood was also associated with reduced risk of microscopic colitis. The results were seen in both Nurses cohorts.

Cigarette smokers weigh, on average, 4–5 kg less than nonsmokers and are less likely to be overweight or obese[23]. Cigarette smoking has been associated linked with microscopic colitis in a meta-analysis[9]. In our study, however, current smoking was more common in the controls (who were heavier) and smoking was not associated with microscopic colitis after controlling for education.

Table 4 Menopausal factors and risk of microscopic colitis in women

	Cases		Controls			
	n = 94		*n* = 176		Crude	Adjusted
	n	%	*n*	%	OR (95%CI)	OR (95%CI)[1]
Menopausal status						
Pre-menopausal	13	14.4	40	26.1	Ref.	Ref.
Post-menopausal	77	85.6	113	73.9	2.10 (1.05-4.18)	1.20 (0.45-3.20)
Age at menopause						
< 45	17	24.3	49	45.8	0.37 (0.19-0.74)	0.64 (0.28-1.46)
45-54	43	61.4	46	43.0	Ref.	Ref.
55+	10	14.3	12	11.2	0.89 (0.35-2.27)	079 (0.28-2.22)
Menopausal type						
Surgical	31	40.3	70	62.0	Ref.	Ref.
Natural menopause	46	59.7	43	38.1	0.41 (0.23-0.75)	0.76 (0.37-1.54)
Menopausal hormones[2]						
No	46	60.5	90	81.1	Ref.	Ref.
Ever	30	39.5	21	18.9	2.80 (1.44-5.41)	1.63 (0.73-3.62)
Years Postmenopausal hormones[2]						
None	46	60.5	90	81.8	Ref.	Ref.
1-7 yr	15	19.7	11	10	2.67 (1.13-6.28)	1.65 (0.59-4.65)
9+ yr	15	19.7	9	8.2	3.26 (1.33-8.02)	1.68 (0.60-4.73)

[1]Adjusted for age, education and body mass index.
[2]Among women after menopause.

The controls in our study had diarrhea. If the diarrhea controls had a condition associated with obesity, that could potentially explain the findings. Many of the diarrhea patients who did not have microscopic colitis probably had irritable bowel syndrome. In a stratified analysis, the inverse association with obesity was particularly strong in the IBS strata (both cases and controls had a history of IBS). It is not clear that patients with IBS are more likely to be obese. A comprehensive review has not shown BMI differences in patients with irritable bowel syndrome[24]. In our study the controls with IBS did not have a higher BMI than the controls without IBS.

The mechanism by which increased body weight might be inversely associated with microscopic colitis in our study and others is uncertain. Men are at substantially reduced risk of microscopic colitis than women[4]. Perhaps the reduced risk is due to a protective effect of androgens. Obesity has been associated with increased levels of androgens[25,26]. Obesity has been linked with change in the gut microbiome[27] which has, in turn, been linked with microscopic colitis[19,28,29]. The gut microbiota can metabolize androgens and estrogens[30].

Despite the marked sex discrepancy in microscopic colitis, the literature on reproductive and hormonal factors is very limited. Burke *et al* used data from the Nurses' Health Study cohorts[31]. Similar to our results, the authors found no association with age of menarche, parity, age of first live birth or age of menopause. They found an increased risk for postmenopausal hormones. Verhaegh *et al*[32] reported that hormonal factors were not associated with microscopic colitis in multivariable analysis, although number of cases might not have been large enough to detect small differences between populations.

We found that ever-use of oral contraceptive pills was associated with a reduced risk of microscopic colitis in crude and adjusted analyses. In contrast to our study, Burke *et al*[31] reported that ever-use of oral contraceptives was associated with an increased risk of microscopic colitis based on data from the two Nurses' Health Study cohorts. There was a large difference in oral contraceptive use in the two cohorts - 45% in the Nurses' Health Study (NHS) and 85% in Nurses' Health Study II (NHSII). Oral

contraceptive use was not queried after 1984 in NHS (almost 40 years ago), or after 2009 in NHSII. The elevated risk was only statistically significant in the earlier cohort. There has been a temporal change in the hormone concentration of oral contraceptive pills, and the different results between our study and the Nurses' cohort study might be due to different times of enrollment. OCPs generally contain a mix of estrogen (usually estradiol) and a progestin. The estrogen dose has decreased from over 100 μg when first introduced in the 1960 to 20-30 μg or less now. Progestin-only contraceptives are also now available. These changes mean studies may not be comparable if they were performed at different times.

The mechanism behind the observed associations of menopausal hormones and oral contraceptives with microscopic colitis are unknown although there are some possibilities to consider. Estrogen and progesterone receptors are expressed in the gut, and sex steroids have been shown to influence colonic transit time, chloride secretion and intestinal permeability[33]. Autoimmune diseases are more common in women than men, possibly due to hormonal factors[34]. Hormonal contraceptives have effects on the immune system and have been linked with a number of different autoimmune diseases[35]. Microscopic colitis has been regarded as an autoimmune disease, perhaps because of similarities to celiac disease[36]. In a genome-wide association study using the UK Biobank, there was an association with single nucleotide polymorphisms on the MHC 8.1 haplotype, supporting an immune component to the pathogenesis of microscopic colitis[37]. We did not find autoimmune disease was over-represented in our microscopic colitis cases (unpublished data). The gut microbiome is involved in the metabolism, excretion and circulation of sex hormones[30]. The effect of menopausal hormones and oral contraceptives on microscopic colitis could be mediated by gut microbes. Gut microbes could metabolize sex steroids or exogenous steroids and alter the gut environment.

Our study had some important strengths. The patients were drawn from the same referral area and had similar access to and receipt of care. All of the patients had diarrhea. Other studies have used community[38], population[39], or disease controls [40]. A single experienced gastrointestinal pathologist reviewed all of the slides to classify patients as microscopic colitis cases or normal controls. The study included men and women. Detailed information was obtained from study participants using structured interviews.

A limitation of the study was the small size, particularly for men. Microscopic colitis is an uncommon disease and most reports in the literature are hampered by small numbers. Many of the patients referred for colonoscopy were either not eligible, cancelled their appointments or refused to participate. Nonresponse, along with the selected nature of the study population, may affect generalizability but should not lead to bias. Exposures were determined by self-report which is common in case-control studies. Recall of past exposures may be inaccurate, but we would not expect the recall for cases and controls to be differential as all of the patients were enrolled in the study because of diarrhea. . Cases were older than controls. We adjusted for age in all models and we performed sensitivity analyses with similar results. Lymphocytic colitis and collagenous colitis are considered to be histologic subtypes of the same disease[41] We combined the two entities to improve study power. In exploratory analyses we found similar results when we examine each type separately. The study was conducted in a developed country. Geographic variations in the incidence of microscopic colitis have been reported but there have been a limited number of direct comparative studies[1]. There are few studies from developing countries[42].

Microscopic colitis, first described in 1976, is a relatively new disease. With any new disease there is the presumption that an environmental factor, as opposed to a genetic factor, is responsible. Obesity has increased in the US since the 1980[43].The incidence of microscopic colitis has also been increasing in the US [44]. The fact that we and others have found lower risk of microscopic colitis with obesity suggests that obesity must interact with some other factor such as the microbiome to mediate risk. Given the difference in distribution in microscopic colitis by sex, exogenous hormones in the form of birth control pills and postmenopausal hormones are naturally of interest. In this study we found that postmenopausal hormones were modestly associated with a increased the risk of microscopic colitis and oral contraceptives with decreased risk.

As the population ages, the number of patients with microscopic colitis is likely to increase. Identifying factors associated with risk for microscopic colitis is an important first step developing hypotheses about etiology.

CONCLUSION

Compared to controls also seen for diarrhea, microscopic colitis cases were less likely to be obese. Mechanisms are unknown but could involve hormonal effects of obesity or the gut microbiome.

ARTICLE HIGHLIGHTS

Research background

There is limited information about risk factors for microscopic colitis, a leading cause of chronic watery diarrhea.

Research motivation

We hypothesized that obesity might be associated with microscopic colitis.

Research objectives

To compare patients with microscopic colitis to patients with chronic diarrhea to learn more about associations with obesity and hormones.

Research methods

We conducted a case-control study among patients who were referred to a single academic medical center for chronic diarrhea. The biopsies were reviewed by a research pathologist and classified as microscopic colitis cases or diarrhea controls. We used logistic regression to estimate odds ratios and 95% confidence intervals.

Research results

Cases with microscopic colitis had a lower body mass index than controls in adjusted models. Although patients with microscopic colitis reported that they lost more weight following the onset of diarrhea, the associations with BMI persisted in analyses stratified by weight loss. Oral contraceptives were inversely associated with microscopic colitis.

Research conclusions

Microscopic colitis cases were less likely to be obese than diarrhea controls. While the mechanism behind the association is not known, it could involve hormonal effects of obesity or the gut microbiome.

Research perspectives

Additional research is needed to understand the association between obesity and microscopic colitis.

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ORIGINAL ARTICLE

Observational Study

Identification of functional tumor necrosis factor-alpha promoter variants associated with *Helicobacter pylori* infection in the Sudanese population: Computational approach

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Author contributions: Idris AB and Hassan MA conceptualized and designed the study; Idris AB, Idris A, Gumaa MA, Idris MB, Mansour M, Allam D and Arbab MOB participated in sample collection and analysis of the data; Idris AB and Elgoraish A statistically analyzed the data; Idris AB performed the bioinformatics analysis, interpretation of the data and drafting the article; Idris AB, Beirag N, Mansour M, Hassan MA and Ibrahim EM revised the manuscript's language and critically assessed the intellectual content; all authors approved the final version of the article to be published.

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statement: The study was reviewed and approved by the Khartoum Ministry of Health research department, University of Khartoum, Faculty of Medical Laboratory Sciences review board, and Research Ethics Committees of hospitals.

Informed consent statement: Written informed consent was taken from participants before they enrolled in the study.

Conflict-of-interest statement: The authors declare that there are no conflicts of interest.

Data sharing statement: The data regarding TNF-A-1030 T>C genotypes and alleles distributions among participants and the in silico results of the software that used to support the findings of this study are available from the corresponding author at abeer.babiker89@gmail.com on a reasonable request.

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Abstract

BACKGROUND

Helicobacter pylori (*H. pylori*) is a ubiquitous bacterium that affects nearly half of the world's population with a high morbidity and mortality rate. Polymorphisms within the tumor necrosis factor-alpha (*TNF-A*) promoter region are considered a possible genetic basis for this disease.

AIM

To functionally characterize the genetic variations in the *TNF-A* 5′-region (-584 to +107) of Sudanese patients infected with *H. pylori* using in silico tools.

METHODS

An observational study was carried out in major public and private hospitals in Khartoum state. A total of 122 gastric biopsies were taken from patients who had been referred for endoscopy. Genomic DNA was extracted. Genotyping of the *TNF-A*-1030 polymorphism was performed using PCR with confronting two-pair primer to investigate its association with the susceptibility to *H. pylori* infection in the Sudanese population. Furthermore, Sanger sequencing was applied to detect single nucleotide polymorphisms in the 5′-region (-584 to +107) of *TNF-A* in *H. pylori*-infected patients. Bioinformatics analyses were used to predict whether these mutations would alter transcription factor binding sites or composite regulatory elements in this region. A comparative profiling analysis was conducted in 11 species using the ECR browser and multiple-sequence local alignment and visualization search engine to investigate the possible conservation. Also, a multivariate logistic regression model was constructed to estimate odds ratios and their 95% confidence intervals for the association between *TNF-A* -1030, sociodemographic characteristics and *H. pylori* infection. Differences were statistically significant if $P < 0.05$. Statistical analyses were performed using Stata version 11 software.

RESULTS

A total of seven single nucleotide polymorphisms were observed in the *TNF-A* 5′-region of Sudanese patients infected with *H. pylori*. Only one of them (T > A, -76) was located at the in silico-predicted promoter region (-146 to +10), and it was predicted to alter transcription factor binding sites and composite regulatory elements. A novel mutation (A > T, +27) was detected in the 5′ untranslated region, and it could affect the post-transcriptional regulatory pathways. Genotyping of *TNF-A*-1030 showed a lack of significant association between -1030T and susceptibility to *H. pylori* and gastric cancer in the studied population ($P = 0.1756$) and ($P = 0.8116$), respectively. However, a significant association was detected between T/C genotype and *H. pylori* infection (39.34% *vs* 19.67%, odds ratio = 2.69, 95% confidence interval: 1.17-6.17, $P = 0.020$). Mammalian conservation was observed for the (-146 to +10) region in chimpanzee (99.4%), rhesus monkey (95.6%), cow (91.8%), domesticated dog (89.3%), mouse (84.3%), rat (82.4%) and opossum (78%).

CONCLUSION

Computational analysis was a valuable method for understanding *TNF-A* gene expression patterns and guiding further *in vitro* and *in vivo* experimental validation.

Key Words: 5'-region; Promoter; TNF-A; *Helicobacter pylori*; In silico analysis; Sudan

Core Tip: According to the literature, a considerable number of polymorphisms have

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been discovered in the tumor necrosis factor-alpha (*TNF-A*) promoter. A crucial question is whether these polymorphisms have any significant functional impact on disease incidence or severity. Seven single nucleotide polymorphisms were detected in the *TNF-A* 5'-region; only one of them, *TNF-A*-76, was located at the in silico-predicted promoter region (-146 to +10). This single nucleotide polymorphism may lead to the modification of the transcriptional regulation of *TNF-A* in *Helicobacter pylori* infection. Nevertheless, this conclusion cannot substitute for the experimental proofs, but it can provide direction or insight for them.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a widespread bacterium that affects nearly half of the world's population with a high morbidity and mortality rate[1]. Generally, the decreasing prevalence of *H. pylori* infection is continuing in many parts of North America and Europe. Still, no such decline has been noted in most developing countries, including Sudan, in which the *H. pylori* infection represents a critical public health challenge[2-4]. Although there is a common consensus that the risk of acquisition and transmission of *H. pylori* can be minimized and prevented to a large extent, this drop in those countries, whether it is a local phenomenon or a national trend, has to be investigated[2].

Infection with *H. pylori* is usually acquired during childhood. However, it may persist if not treated in the host's stomach throughout life due to the synchronized balance between the bacteria and its host[5,6]. The long duration of *H. pylori* in the gastric environment will develop chronic gastritis in *H. pylori*-infected persons. At the same time, a few may be complicated to more severe diseases such as peptic ulcer, atrophic gastritis, mucosa-associated lymphoid tissue lymphoma or gastric adenocarcinoma[7,8]. The variability in *H. pylori* infection outcomes is determined by bacterial factors, genetic characteristics of the host, especially those regarding polymorphisms in specific cytokines, and the environmental factors[7,9,10]. However, the crucial role of cytokine-related polymorphisms in the susceptibility or severity of *H. pylori* infection has been attracting considerable attention after El-Omar *et al*[11] proposed that functional polymorphisms in proinflammatory cytokine genes in *H. pylori*-infected patients are associated with a two- to three-fold increased gastric cancer risk. Because tumor necrosis factor-alpha (TNF-a) production has been implicated as an essential factor in immune regulations and inflammatory responses against *H. pylori* infection[12], polymorphisms within the *TNF-A* promoter are considered a possible genetic basis for this disease[13].

TNF-a is a potent pleiotropic proinflammatory cytokine that mediates diverse biological and pathological processes, in addition to its responsibility for the regulation of two opposite processes: proliferation and apoptosis (reviewed in[14,15]). *TNF* gene transcription is regulated by nucleoprotein complexes known as enhanceosomes that involve distinct sets of transcription factors and coactivators at the proximal *TNF* promoter and in the pattern of chromosomal organization of the *TNF/lymphotoxin* locus that function in synergy to drive transcription[16]. *TNF* enhanceosome assembly is cell type- and stimulus-specific, partially because of differential occupancy of overlapping DNA motifs in the *TNF* promoter. For example, after lipopolysaccharide stimulation of monocytes where no inducible translocation of NFATp can be detected, Sp1 and Ets/Elk proteins can successfully bind to sites that NFATp occupies in T cells[13,14]. Furthermore, many *TNF* activator binding motifs can be recognized by more than one class of transcription factor depending on the ambient concentration of different elements in the nucleus and the stimulus and cell type[16]. These features provide the *TNF* promoter with a remarkable degree of flexibility to respond to multiple stimuli in a specific manner through a short cis-

regulatory region[13].

The *TNF* locus lies within the major histocompatibility complex region on the short arm of human chromosome 6p21.3, a highly polymorphic region[17]. Many bi-allelic polymorphisms and microsatellites are found at or around the *TNF* locus[14,18]. Several have been suggested to be implicated in diseases susceptibility/resistance or severity by modifying the transcriptional regulation of *TNF*[13,18]. There are different transition variants in the *TNF-A* promoter region at the positions of -237, -307, -375, -856, -862 and -1030 in which the positions -307 and -237 have been most frequently evaluated for the association with *H. pylori* infection (susceptibility or progression)[13]. It has been reported that the presence of the adenine allele at -237 or -307 positions causes higher constitutive and inducible transcriptional levels than the guanine allele, which results in enhanced production of TNF-a up to five-fold *in vitro*[19,20]. In addition, the single nucleotide polymorphism (SNP) at the -1030 position, which is frequently observed in African populations[21,22], has been suggested to be related to high TNF-α production[23]. However, the influence of these SNPs on TNF-α production is not fully known and still a contradictory topic of debate, which is explained, in part, by ethnic differences and genetic variations between populations[19,20].

To our knowledge, there are no previous studies in Sudan that have addressed the association between *TNF-A* and *H. pylori* infection or *H. pylori*-associated diseases. Therefore, in this study, we applied DNA Sanger sequencing to detect genetic variations in the *TNF-A* 5′-region (-584 to +107) of Sudanese patients infected with *H. pylori* and predicted if these SNPs could alter transcription factor binding sites (TFBSs) or composite regulatory elements (CEs) using a computational approach. In addition, a comparative profiling analysis was performed to investigate the conservation of these SNPs in 11 species. Regulation mechanisms of TNF-α production may be linked to the difference in responses to *H. pylori* infection. This emphasizes the importance of studying the regulation of *TNF-A* gene expression.

MATERIALS AND METHODS

Research methodology

In the present study, in silico analysis was employed for the *TNF-A* 5′-region (-584 to +107) of the Sudanese patients infected with *H. pylori* in two steps: (1) In silico prediction of the promoter region; and (2) In silico analysis of the predicted promoter region (-146 to +10). To obtain accurate results, we applied various software/servers to predict the promoter region, CpG island and regulatory motifs because promoters have complex and specific architecture and contain multiple transcription factors involved in particular transcription regulation[24]. Therefore, the unique composition of TFBSs and different features of each promoter may lead to different powers and algorithms for promoter identification[25]. In addition, genotyping of the *TNF-A*-1030 T>C polymorphism was performed to assess its association with *H. pylori* infection in the Sudanese population using PCR with confronting two-pair primer method.

However, the initial studies have numbered the positions of SNPs relative to the *TNF* mRNA cap site incorrectly (*e.g.*, -308 instead of -307 and -238 instead of -237)[13]. In contrast, the corrected numbering should have proceeded 5′ from the adenine at the +1 position[26] as in the Cytokine Gene Polymorphism In The Human Disease Database[27], and we used it throughout this study. The methodology followed in this study is represented in Figure 1.

Study population and study setting

An observational study was carried out in major public and private hospitals in Khartoum state in 2019-2020. These hospitals included Soba teaching hospital, Ibin Sina specialized hospital, Modern Medical Centre, Al Faisal Specialized Hospital and Police hospital. In addition, the molecular laboratory processes were conducted in the Molecular Biological Research laboratory at the Faculty of Medical Laboratory Sciences at the University of Khartoum.

The matched case-control formula was selected to calculate the sample size using Epi Info software version 7[28,29], assuming 95% confidence interval (CI), 80% power of the study, one ratio of control to the case, 15% of controls exposed, 3.36 odds ratio (OR) and 37.2% of cases exposed. According to the sample size calculation, the present study included 122 patients referred for upper gastrointestinal tract endoscopy, and most of them were because of dyspepsia. Out of that, 61 were *H. pylori*-negative patients and regarded as controls. The demographic characteristics of the study

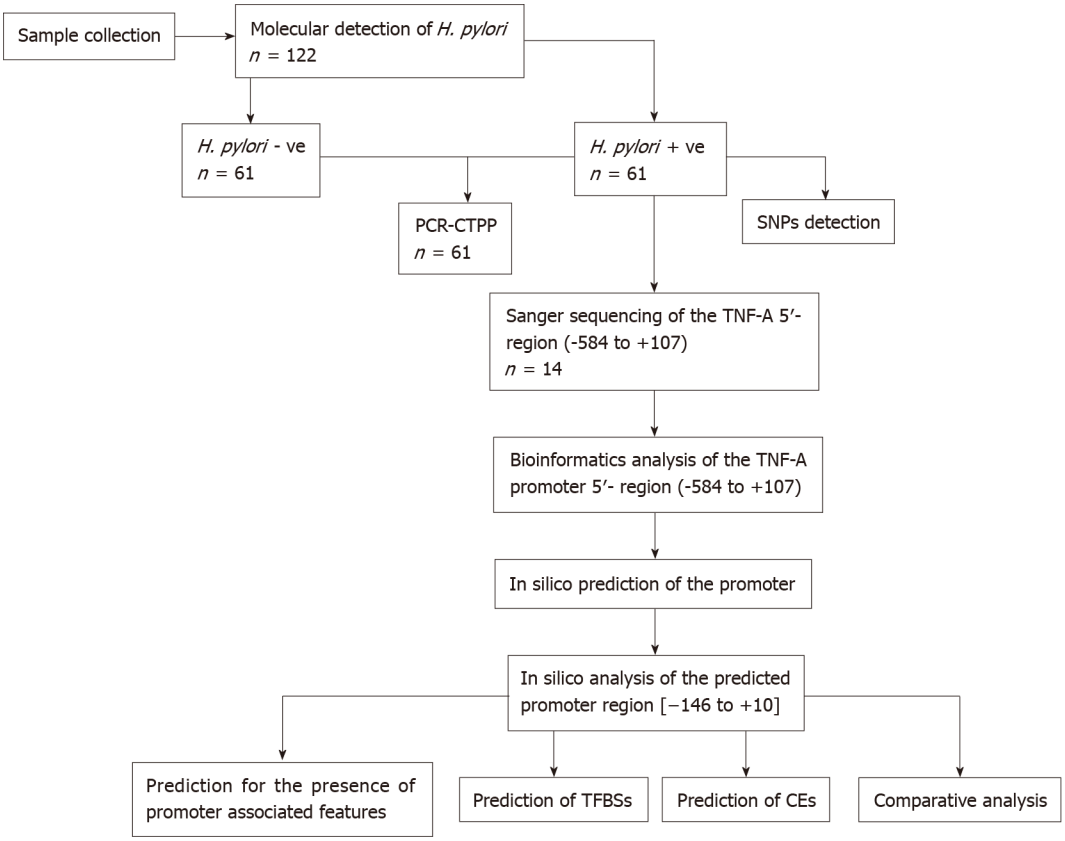

Figure 1 Schematic representation of the study methodology. *H. pylori*: *Helicobacter pylori*; -ve: Negative; +ve: Positive; CTPP: Confronting two-pair primer; SNP: Single nucleotide polymorphism; TNF-A: Tumor necrosis factor-alpha; TFBS: Transcription factor binding site; CE: Composite regulatory elements.

population are shown in Table 1.

The diagnosis of gastroduodenal diseases was based on the assessment of an experienced gastroenterologist, and in cancerous cases, histological examinations were also performed to confirm the diagnosis. The gastric pathologies of the study population are presented in Table 2.

The study population's demographic and clinical data were obtained by personal interviews, a structured questionnaire and a review of case records. The selection criteria included the Sudanese population from both sexes who were not taking antibiotics or nonsteroidal anti-inflammatory drugs. Written informed consent was granted by the participants after they were informed of the objectives and purposes of the study.

DNA extraction and PCR amplification of specific 16S rRNA gene of H. pylori

For DNA extraction purposes, gastric biopsies were collected in 400 μL of phosphate buffered saline. For histological investigation, the biopsies were preserved in 10% formalin. DNA extraction was performed by using innuPREP DNA Mini Kit (analytikjena AG, Germany) and followed the protocol that was given by the manufacturer, as previously described in[30].

Extracted DNA was amplified for the specific *16S* rRNA gene to diagnose and confirm the infection of *H. pylori* using the following primers (primers: F: 5′-GCGCAATCAGCGTCAGGTAATG-3′; and R: 5′-GCTAAGAGAGCAGCCTATGTCC-3′)[31] and the previously described PCR condition[4]. The amplified product for the *16S rRNA* is 522 bp.

Conventional PCR amplification and sequencing of the TNF-A promoter region

To amplify the promotor polymorphisms -308 and -238 of the *TNF-A* gene, primers: F: 5′-GCTTGTCCCTGCTACCCGC-3′ and R: 5′-GTCAGGGGATGTGGCGTCT-3′ were used[32]. The following thermal cycling conditions were used: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 60 s, 58 °C for 30 s and 72 °C for 60 s, with a final extension at 72 °C for 10 min. The amplified PCR product is 691 bp located between -585 bp upstream and 107 bp downstream of the *TNF-A* gene.

Table 1 Demographic characteristics of the study population, *n* (%)

Variable		*Helicobacter pylori* (+ve)	*Helicobacter pylori* (-ve)	Total	*P* value
		n = 61	*n* = 61	*n* = 122	
Mean age in yr ± Standard deviation (range)		44.00 ± 16.99 (15-85)	44.74 ± 18.10 (17-89)	44.37 ± 17.48 (15-89)	0.9184[1]
Gender	Male	36 (50.00)	36 (50.00)	72 (59.02)	1.0000
	Female	25 (50.00)	25 (50.00)	50 (40.98)	
Residence	Urban	24 (44.44)	30 (55.56)	54 (44.26)	0.3622
	Rural	37 (54.41)	31 (45.59)	68 (55.74)	
Hospital	Public	21 (33.87)	31 (49.21)	52 (41.60)	0.0667
	Private	41 (58.57)	29 (41.43)	70 (57.38)	

[1]Mann-Whitney *U*, Gaussian Approximation. +ve: Positive; -ve: Negative.

Table 2 Gastric pathologies of the study population, *n* (%)

Endoscopy results	*n* (%)	*Helicobacter pylori* (+ve)	*Helicobacter pylori* (-ve)	*P* value[1]
Normal gastric findings	9 (7.40)	4 (6.45)	5 (7.94)	0.2686
Esophagitis	4 (3.30)	3 (4.80)	1 (1.59)	
Esophageal varices	6 (4.90)	1 (1.61)	5 (7.94)	
Gastritis	55 (45.10)	28 (45.16)	27 (42.86)	
Duodenitis	6 (4.90)	3 (4.84)	3 (4.76)	
Peptic ulcer	27 (22.13)	17 (27.42)	10 (15.87)	
Cancer	15 (12.30)	5 (8.20)	10 (16.4)	
Total	122 (100)	61 (50.00)	61 (50.00)	

[1]χ^2 test, df: 7.603, 6. +ve: Positive; -ve: Negative.

Fourteen PCR products of *H. pylori*-infected patients, which had the clearest bands, were sent for commercial DNA purification and Sanger dideoxy sequencing by Macrogen Inc., Korea.

Sequence analysis and SNPs detection

Two chromatograms (forward and reverse) for each sequence were visualized, checked for their quality and analyzed with the Finch TV program version 1.4.0[33]. In addition, the Basic Local Alignment Search Tool nucleotide (https://blast.ncbi.nlm.nih.gov/)was used to look for and assess nucleotide sequence similarities[34].

For SNP detection in the *TNF-A* promoter region, multiple alignment sequences were performed for the tested sequences with a reference sequence (*TNF-A*; NG_007462) using Clustal W[35].

Bioinformatics analysis of the TNF-A promoter region in H. pylori-infected patients

In silico prediction of the promoter: In this study, we employed five programs for human promoter prediction and recognition of human PolII promoter region and start of transcription (TSS) which include: Berkeley Drosophila Genome Project (http://www.fruitfly.org/)[36], Promoter 2.0 Prediction Server (http://www.cbs.dtu.dk/)[37], FPROM, TSSG and TSSW (http://softberry.com/) which are based on neutral network and linear discriminant approach[25,38,39].

In silico analysis of the predicted promoter region: Assessment for the presence of promoter-associated features: The computationally predicted promoter regions were additionally assessed for the presence of promoter-associated features using the ENCODE data (https://www.encodeproject.org/)[40-42]. These features include promoter-associated histone marks, DNase I hypersensitivity clusters, broad

chromatin state segmentation, transcription factor ChIP-seq and CpG islands.

Prediction of CpG islands: A CpG island is a DNA segment with high GC and CpG dinucleotide content and often regarded as a marker for the initiation of gene expression. In this study, we applied MethPrimer and GpC finder software (http://www.softberry.com/berry.phtml?topic=cpgfinder&group=programs&subgroup=promoter) to predict CpG islands using default search parameter values for the software: CpG island length >200 bp, CG% > 50%, and Obs/Exp > 0.6[43,44].

Prediction of TFBSs: Prediction of the potentially functional TFBSs is a crucial step in the chain of promoter analytical events. Therefore, we applied five prediction software programs to analyze the predicted promoter region for possible TFBSs: (1) AliBaBa2 (http://www.gene-regulation.com/)[45]; (2) Alggen Promo (http://alggen.lsi.upc.es/cgi-bin/promo_v3/)[46,47]; (3) TF-Bind (http://tfbind.hgc.jp/)[48]; (4) Tfsitescan (http://www.ifti.org)[49]; and (5) Gene Promoter Miner (GPMiner) (http://GPMiner.mbc.nctu.edu.tw/), which is an integrated system. For predicting TFBSs, MATCH tool was utilized to scan TFBSs in an input sequence[50].

Prediction of CEs: CE is the minimal functional unit composed of two closely located DNA binding sites for distinct transcription factors, but its regulatory function is qualitatively different from the regulation effects of either individual DNA binding site. Thus, the MatrixCatch algorithm (http://gnaweb.helmholtz-hzi.de/cgi-bin/MCatch/MatrixCatch.pl) was used to identify the CEs in our region (-146 to +10)[51].

Comparative profiling analysis: ECR Browser (http://ecrbrowser.dcode.org)[52], NCBI Basic Local Alignment Search Tool nucleotide (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Clustal W (https://www.genome.jp/tools-bin/clustalw)[35] were utilized to analyze the possible conservation of the predicted promoter region. The conservation was assessed in 11 species which include: Cow (*Bos taurus*), chimpanzee (*Pan troglodytes*), rhesus monkey (*Macaca mulatta*), dog (*Canis lupus familiaris*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), chicken (*Gallus gallus*), zebrafish (*Danio rerio*), frog (*Xenopus laevis*), opossum (*Monodelphis domestica*), fugu pufferfish (*Takifugu rubripes*) and spotted green pufferfish (*Tetraodon nigrovoridis*). Furthermore, multiple-sequence local alignment and visualization search engine (https://mulan.dcode.org/) was used to evaluate the conservation of the promoter's SNPs and to screen the possible conservation of TFBSs at these SNP locations[53].

Molecular detection of the TNF-A-1030 C/T polymorphism using PCR with confronting two-pair primer method

Two sets of primers were used in PCR with confronting two-pair primer to detect the *TNF-A*-1030 C/T polymorphism as follow: for allele T, the sequences of the primers were F: 5'-AAGGCTCTGAAAGCCAGCTG-3' and R: 5'-CCAGACCCT-GACTTTTCCTTCA-3'; and for allele C, F: 5'-GAAGCAAAGGAGAAGCT-GAGAAGAC-3' and R: 5'-CTTCCATAGCCCTGGACATTCT-3'[54]. The PCR mixture components were the same as in[30]. The amplification conditions were initial denaturation at 95 °C for 10 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 66 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 5 min. The resulting PCR products were visualized by electrophoresis on a 1% agarose gel containing ethidium bromide. The predicted size was confirmed using a 50 bp DNA molecular weight marker. Genotyping was distinguished as follows; 444 bp and 316 bp for TT genotype, 444 bp, 316 bp and 174 bp for TC genotype and 444 bp and 174 bp for CC genotype.

Statistical analysis

Fisher's test or χ^2 test investigated Hardy-Weinberg equilibrium of allele and genotype distributions. Descriptive statistical analysis was applied to calculate percentages, proportions and means ± standard deviation. Regarding the incidence of *H. pylori* infection, differences in frequency distribution by age were examined by the Mann-Whitney test, while differences in the frequency distribution of the categorical demographic and clinical variables of the study population were assessed by Fisher's test or χ^2 test. A multivariate logistic regression model was constructed to estimate OR and their 95%CIs for the association between *TNF-A*-1030, sociodemographic characteristics and *H. pylori* infection. Differences were considered to be statistically significant if $P < 0.05$. The statistical analyses were performed using Stata version11 (StataCorp, College Station, Texas) software.

RESULTS

Nucleotide variations in the 5'-regulatory region (-585 to +107) of the TNF-A gene

In the Sudanese *H. pylori*-infected patients, a total of seven nucleotide variations were detected in the 5′-regulatory region (six substitution mutations and one insertion mutation of C at +70). Among which, five were bimodally mutated heterozygous SNPs, and two are homozygous SNPs, as illustrated in Table 3 and Figure 2. The results of the multiple sequence alignment were visualized in Jalview[55]. The nucleotide sequences of the *TNF-A* 5′-region (-584 to +107) were deposited in the GenBank database under the following accession numbers: from MW251712 to MW251725.

In silico prediction of the TNF-A promoter regions

Five types of promoter prediction programs were applied to predict the promoter regions of the *TNF* 5′-region (-584 to +107), and the results are overviewed in Table 4. Neural Network Promoter Prediction (NNPP version 2.2) predicted one promoter region, located at +1 bp relative to the TNF-α mRNA cap site[26]. In addition, FPROM and TSSG programs predicted two promoter regions -146 bp and +5 bp, respectively. The TSSW program also expected these two regions. In comparison, no promoter region was predicted by Promoter 2.0 Prediction Server.

In silico analysis of predicted TNF-A promoter region

Presence of promoter-associated features: ENCODE data revealed a high level of transcription factor occupancy patterns, promoter-associated histone modifications and DNase I hypersensitivity at the in silico predicted promoter region, from -146 to +10 nt relative to the TNF-α mRNA cap site[26]. Furthermore, CpGFinder and MethPrimer software were employed to predict the presence of CpG islands. However, both software predicted no CpG islands present in the predicted promoter region, and ENCODE data confirmed no presence of the CpG island in the predicted promoter (see Figure 3).

Conservancy of the predicted promoter: As illustrated in Figure 4, the ECR Browser showed mammalian conservation for the (-146 to +10) region in chimpanzee (*Pan troglodytes* - pan-Tro2) (99.4%), rhesus monkey (*Macaca mulatta* - rheMac2) (95.6%), cow (*Bos taurus* - bosTau3) (91.8%), domesticated dog (*Canis lupus familiaris* - canFam^2) (89.3%), rat (*Rattus norvegicus* - rn4) (82.4%), mouse (*Mus musculus* - mm9) (84.3%) and opossum (*Monodelphis domestica* - monDom5) (78.0%). But the region was not conserved in chicken (*Gallus gallus*), frog (*Xenopus laevis*), zebrafish (*Danio rerio*), fugu pufferfish (*Takifugu rubripes*) and spotted green pufferfish (*Tetraodon nigrovoridis*). An overview of the mammalian conservation of the in silico-predicted promoter of *TNF* is shown in Figure 5.

Prediction of TFBSs: In the present study, we used five software programs to predict TFBSs. To ensure accurate analysis, only factors predicted by three out of the five programs or predicted by two programs but verified in the literature were selected. The five prediction programs reported multiple putative TFBSs within the (-146 to +10) region, as illustrated in Table 5. Moreover, screening of this region using the NCBI SNP databases showed four SNPs, as shown in Table 5. The ECR Browser and NCBI Basic Local Alignment Search Tool nucleotide revealed the conservation of these SNPs in chimpanzees, rhesus monkey, cow and dog. Multiple-sequence local alignment and visualization presented multiple TFBSs to be located at rs765073823 and rs537401710. The overview from the multiple-sequence local alignment and visualization software for conserved TFBSs predicted to be conserved (100%) between humans, chimpanzees, rhesus monkeys, cows, and domesticated dogs is summarized in Table 6.

Prediction of CEs: In the present study, CEs were predicted using MatrixCatch, which can find experimentally known regulatory elements (both single sites and pairs) and novel regulatory elements by computational comparison to the known ones in a library of CE models[51]. An overview of predicted CEs by MatrixCatch is presented in Table 7.

Allele and genotype frequencies of TNF-A-1030 and susceptibility to H. pylori infection

Sixty-one participants from each group (*H. pylori*-infected and uninfected) were successfully genotyped for *TNF-A*-1030 polymorphism; a representative gel is shown

Table 3 Nucleotide variations in the 5'-regulatory (-584 to +107) region of tumor necrosis alpha in Sudanese *Helicobacter pylori*-infected patients

SNP	Event	SNP locus[1]	Chromosomeposition	Serialnumber (rs)
C>G (homozygous)	Transversion	-567	NG_007462.1:g.4422 C>G	rs2857711
G>A (heterozygous)	Transition	-375	NG_007462.1:g.4614 G>A	rs1800750
G>A (heterozygous and homozygous)	Transition	-307	NG_007462.1:g.4682 G>A	rs1800629
G>A (heterozygous)	Transition	-237	NG_007462.1:g.4752 G>A	rs361525
T>A (heterozygous)	Transversion	-76	NG_007462.1:g.4913 T>A	rs41297589
A>T (heterozygous)	Transversion	+27	NG_007462.1:g.5016 A>T[2]	-
Insertion of C	-	+70	NG_007462.1:g.5058-5059 InsC	-

[1]Position of the single nucleotide polymorphism is relative to the tumor necrosis factor-alpha mRNA cap site[26].
[2]Novel mutation at the 5′ untranslated region detected in this study. SNP: Single nucleotide polymorphism.

Table 4 Overview of the in silico predicted tumor necrosis factor-alpha promoter regions for the respective prediction programs. The most predicted region is indicated in bold

Prediction program	Predicted promoter regions[1]
BDGP (NNPP version 2.2)[2]	-40 to +10
Promoter 2.0 Prediction Server	no promoter
FPROM	+5
TSSG[3]	-146
TSSW[4]	-140
	+6

[1]All positions are relative to the tumor necrosis factor-alpha mRNA cap site[26].
[2]NNPP: Neural network promoter prediction; Threshold 0.80.
[3]Threshold for LDF- 4.00.
[4]Thresholds for TATA+ promoters - 0.45, for TATA−/enhancers - 3.70.

in Figure 6. Allele and genotype frequencies of *TNF-A*-1030 polymorphism in patients infected with *H. pylori* and uninfected controls are presented in Table 8. Although the TNF-*A*-1030-C allele was more prevalent in patients infected with *H. pylori* compared to uninfected controls, no significant association was found between the allele frequencies of *TNF-A*-1030 and *H. pylori* infection (27.87% *vs* 19.67%, OR = 0.6339, 95%CI: 0.3490-1.151, P = 0.176). However, a significant association was detected between T/C genotype and *H. pylori* infection (39.34% *vs* 19.67%, OR = 2.69, 95%CI: 1.17-6.17, P = 0.020) (Tables 8 and 9). In contrast, no significant association was observed between allele and genotype frequencies of *TNF-A*-1030 and gastric cancer (P = 0.8116 and P = 0.3900, respectively) (Table 10). The allelic and genotype distributions at -1030 of *TNF-A* were in Hardy-Weinberg equilibrium, with non-significant χ^2 values (for uninfected subjects P = 0.1520; and subjects with benign disorders P = 0.2089).

DISCUSSION

Many SNPs have been observed in the vicinity of the *TNF* locus, particularly in the distal *TNF* promoter. Some of these have been implicated in the disease susceptibility, resistance or severity by modifying the transcriptional regulation of *TNF*[13,56-58]. In this study, we functionally analyzed SNPs in the *TNF-A* 5′-region (-584 to +107) of the Sudanese patients infected with *H. pylori* and had divergent clinical outcomes that ranged from simple asymptomatic gastritis to more serious conditions, *i.e.* peptic ulcer disease and gastric cancer. The exploratory sequencing of *TNF-A* 5′-region (-584 to +107) of the Sudanese patients infected with *H. pylori* revealed six previously reported

Table 5 Summary of the in silico predicted transcription factor binding sites for tumor necrosis factor-alpha (-146 to +10) region

TranscriptionFactor	Position[1]		SNP in binding site	Software				
	Start	End		AliBaba2.1	Alggen Promo	Tfsitescan	TF-Binding	GPMiner
Elk-1	-145	-130			X[2]		X	
Sp1	-138	-126		X		X		
AP-2	-129	-118			X		X	
Ets-1	-118	-111				X		
PEA3/ Ets-1	-118	-110				X		
T3R_TRE1	-115	-108			X	X	X	
TNF-alpha-CRE	-112	92				X	X	
C/EBP-beta	-100	-74	rs41297589 [-76][3]			X	X	
NF-kappaB	-99	-86				X	X	
AP-1	-67	-57				X		
NF-kappaB	-55	-46	rs765073823 [-48]rs537401710 [-47]	X				
Sp1	-54	-41	rs765073823 [-48]rs537401710 [-47]	X	X	X		
Sp1	-48	-39	rs765073823 [-48]rs537401710 [-47]	X	X	X		
GATA	-40	-30	rs539666421 [-38]	X	X		X	
AP-2	-38	-27	rs539666421 [-38]	X		X	X	
TBP	-29	-20		X			X	X

[1]Position is relative to the tumor necrosis factor-alpha transcription start site.
[2]X means the transcription factor is predicted by the software that is marked with X.
[3]Observed in this study. SNP: Single nucleotide polymorphism.

Table 6 Overview of conserved transcription factor binding sites predicted by multiple-sequence local alignment and visualization

Transcription Factor	Binding sequence	Position[1]		Conserved SNPs
		Start	End	
V$CETS168_Q6	gCTTCCTC	-115	-108	
V$ETS_Q6	gcTTCCtc	-115	-108	
V$SP1_Q4_01	ttccCCGCCCtcc	-51	-39	rs765073823 [-48]rs537401710 [-47]
V$SP1_Q6_01	tcCCCGCCCt	-50	-41	rs765073823 [-48]rs537401710 [-47]
V$SP1_Q2_01	cCCCGCCCtc	-49	-40	rs765073823 [-48]rs537401710 [-47]

[1]All positions are relative to the tumor necrosis factor-alpha mRNA cap site. Multiple-sequence local alignment and visualization (https://mulan.dcode.org/) to be conserved (100%) between human, chimpanzee, rhesus monkey, cow, and domesticated dog at the (-146 to +10) region. The crucial transcription factor for tumor necrosis factor transcription, nuclear factor, was not included.

SNPs located at -567, -375, -307, -237, -76 and +70 and one novel SNP at +27 relative to the *TNF-A* transcription start site. A C to G homozygous transversion at -567 nt was found in all our studied populations. At the same time, the novel A to T heterozygous transversion at +27 nt was observed in five patients who had normal upper gastro-endoscopy or gastritis. This SNP is located in the 5′ untranslated region. Therefore, it could affect the post-transcriptional regulatory pathways that control mRNA localization, stability and translation efficiency[59]. Further studies are encouraged to investigate this SNP in terms of the frequency of the minor allele (T) in the Sudanese population and its functional significance using computational and experimental

Table 7 Summary of the in silico predicted composite regulatory elements for the -146 to +10 region of tumor necrosis factor-alpha

Composite element	MatrixName for a 1st element	Score, strand	Distance in between	MatrixName for a 2nd element	Score, strand	Position of CE, orientation	orientation	Composite score	*P* value	Sequence
CE00266	V$ETS_Q6	0.991+	12	V$AP1_Q2_01	0.831+	-118	+	0	1.08e-03	GCTTCCTCCagaTGAGCTCATGGG
CE00109	V$AP1_C	0.853-	8	V$NFAT_Q6	0.943-	-66	-	0.007	3.42e-03	TTGAATGATTCTTTCCCCGC
CE00150	V$AP1_C	0.853-	8	V$NFAT_Q6	0.943-	-66	-	-0.237	3.42e-03	TTGAATGATTCTTTCCCCGC
CE00058	V$HMGIY_Q6	0.955-	-5	V$NFKB_Q6_01	0.759-	**-78**	-	-0.09	6.89e-03	GTTTTCCGCTG
CE00058	V$HMGIY_Q6	0.955-	-4	V$NFKB_Q6_01	0.662-	**-78**	-	0.005	9.20e-03	GTTTTCCGCTGG
CE00064	V$HNF3_Q6	0.780+	15	V$NF1_Q6	0.956+	-28	+	-0.254	4.83e-02	ACATATAAAGGCAGtTGTTGGCACACC

CE: Composite regulatory elements.

Table 8 Allele and genotype frequencies of tumor necrosis factor-alpha-1030 polymorphism among *Helicobacter pylori*-infected and uninfected subjects and their contributions to *Helicobacter pylori* infection, *n* (%)

	H. pylori (+ve), *n* = 61	*H. pylori* (-ve), *n* = 61	*P* value	OR (95%CI)
Allele frequency				
TNF-A-1030-T	88 (72.13)	98 (80.33)	0.176	0.63 (0.349-1.151)
TNF-A-1030-C	34 (27.87)	24 (19.67)		
Genotype frequency				
T/T	32 (52.46)	43 (70.49)	-	1 (reference)
T/C	24 (39.34)	12 (19.67)	0.020[a]	2.69 (1.17-6.17)
C/C	5 (8.20)	6 (9.84)	0.862	1.12 (0.31-3.99)

[a]*P* value < 0.05 statistically significant difference. OR: Odds ratio; 95%CI: 95% confidence interval; *H. pylori*: *Helicobacter pylori*; +ve: Positive; -ve: Negative; *TNF-A: Tumor necrosis factor-alpha*.

approaches.

Intriguingly, the heterozygous SNP T>A at -76 was located at the in silico-predicted promoter region. However, in this study, the in silico analysis predicted promoter region was located from -146 to +10 nt relative to the TNF-α mRNA cap site using different algorithms[26]. This finding is in agreement with previous studies conducted on 411 *TNF* promoters from individuals of distinct ethnic backgrounds that concluded that the human *TNF* promoter sequences up to 200 bp relative to the transcription start site involved in enhanceosome formation and the regulation of the gene are completely conserved in humans[21,26,60,61]. Also, the ENCODE data showed a high level of the hallmarks of cis-regulatory regions at the in silico predicted promoter region (-146_+10), which include nuclease hypersensitivity and histone modifications [40-42].

Functional elements in the mammalian gene promoters can be detected by high conservation between species distantly related on the evolutionary scale[62,63]. In this study, the computational comparative analysis revealed mammalian conservation for the -146 to +10 region in chimpanzee (*Pan troglodytes* - pan-Tro2) (99.9%), rhesus monkey (*Macaca mulatta* - rheMac2) (93.7%), cow (*Bos taurus* - bosTau3) (78.2%), domesticated dog (*Canis lupus familiaris* - canFam^2) (77.8%), rat (*Rattus norvegicus* - rn4) (76.3%), mouse (*Mus musculus* - mm9) (78.7%) and opossum (*Monodelphis domestica* - monDom5) (72.2%). In an earlier study conducted by Leung *et al*[64], the region from positions -131 to -63 and -53 and -45 relative to the start site of *TNF* gene transcription showed high sequence conservation among primates, with complete conservation

Table 9 Multivariate analysis of genotype frequencies of tumor necrosis factor-alpha-1030 polymorphism and sociodemographic characteristics in *Helicobacter pylori* infection

Variables		*P* value	OR (95%CI)
Age		0.946	0.99 (0.98-1.02)
Gender	Male		1 (reference)
	Female	0.845	0.93 (0.42-2.02)
Residence	Urban		1 (reference)
	Rural	0.341	1.46 (0.67-3.18)
Hospital	Private		1 (reference)
	Public	0.045[1]	0.45 (0.21-0.98)
Genotype frequency	T/T		1 (reference)
	T/C	0.016[a]	2.87 (1.22-6.78)
	C/C	0.982	1.02 (0.27-3.88)

[1]Weak evidence of significant different.
[a]*P* value < 0.05 statistically significant different. OR: Odds ratio; 95%CI: 95% confidence interval.

Table 10 *Tumor necrosis factor-alpha*-1030 polymorphism and gastric cancer association risk in gastric cancerous patients and subjects with benign gastric disorders, *n* (%)

	Gastric pathologies		OR (95%CI)
	Benign disorders[1], *n* = 108	Cancer, *n* = 14	
Allele frequency			
TNF-A-1030-T	176 (90.28)	21 (75.00)	1.150 (0.4627 - 2.8600)
TNF-A-1030-C	51 (9.72)	7 (25.00)	
P value	0.8116		
Genotype frequency			
T/T	68 (62.96)	7 (50.00)	1.700 (0.5556 - 5.2020)
C carrier	40 (37.04)	7 (50.00)	
P value	0.3900		

[1]Benign disorders, *i.e.* gastritis, duodenitis or ulcer.
OR: Odds ratio; 95%CI: 95% confidence interval; *TNF-A: Tumor necrosis factor-alpha*.

within phylogenetic footprints across seven primate species, including Old World monkeys and New World monkeys[64].

However, the *TNF-A* gene is located within the major histocompatibility complex region of chromosome 6, the highly polymorphic region of the human genome, which includes the human leukocyte antigen (HLA) genes[65]. In fact, all of the common ten detected SNPs (-1030, -862, -856, -574, -375, -307, -243, -237, and +70 nt relative to the start site of transcription) fall outside of the identified *TNF* primate phylogenetic footprints[66-70]. This feature is consistent with the accrual of mutations under neutral evolution[64,72]. Furthermore, several of these SNPs (-1030, -862, -856, -307, -243, -237) have been shown to be in non-random association with extended HLA haplotypes[21,26,71-73], which are HLA regions that exhibit fixity of the genomic interval between HLA-B and HLA-DR, that includes *TNF-A* and complement (complotype) regions[21,74,75].

Intriguingly, we detected one mutation (T>A, -76) located at the identified *TNF* primate phylogenetic footprints[64] in a patient with reinfection with *H. pylori* multiple times. The presence of this SNP in the conserved region that is involved in regulating the *TNF* gene could affect the expression and create a condition of hypoacidity that favors the survival and colonization of *H. pylori*[76,77]. The -76 SNP

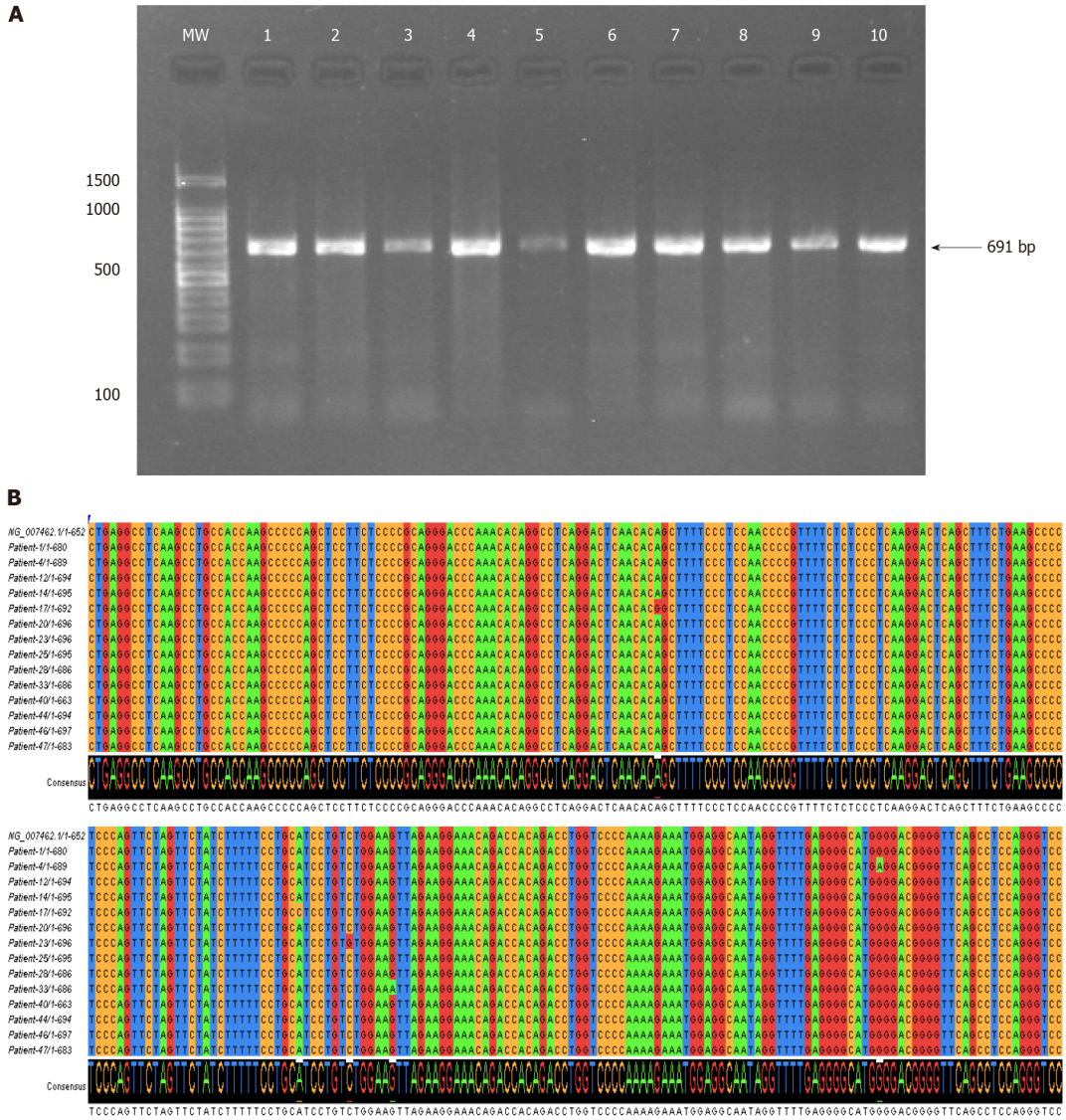

Figure 2 Nucleotide variations of the tumor necrosis factor-alpha 5'-region (-584 to +107) in the Sudanese *Helicobacter pylori*-infected patients. A: PCR amplification results of the tumor necrosis factor-alpha 5'-region (-584 to +107) examined on 1% agarose gel electrophoresis; B: Multiple sequence alignment of the nucleotide sequences of the tumor necrosis factor-alpha 5'-region performed in Clustal W and visualized in Jalview[55]. The levels of identity are visible as histograms at the bottom.

was first discovered with an overall minor allele frequency of 0.031 in the Indian population while studying polymorphisms of *TNF*-enhancer and the gene for FcγRIIa correlated with the severity of *Falciparum malaria* in the ethnically diverse Indian population[78]. It is present at the low-affinity transcription factor Ets-1 binding site in macrophages and the high affinity NFATp binding site in T and B cells[79]. Also, it is located at an in silico-predicted C/EBP-beta (also known as NF-IL6) binding site (in this study, see Table 5), which was experimentally proven in myelomonocytic cells[80-82].

Sequence analysis of the *TNF-A* 5'-region (-584 to +107) of the Sudanese patients revealed relative variability of *TNF-A* SNPs (Figure 2 and Table 3). This finding is in agreement with a study conducted by Baena *et al*[21] that found that the highest relative diversity of *TNF-A* SNPs was revealed among the three African studied populations (Malawian, Southern Nigerian, African-American and African-Caribbean). Also, it is consistent with the "out of Africa" hypothesis based upon human mitochondrial DNA analyses[83]. Despite this high level of variation, here in this study, the sequences involved in enhanceosome formation and gene regulation are highly conserved[64]. Remarkably, we detected only a single SNP (-76) in this region. By contrast, the novel +27 variant and other *TNF-A* SNPs described in this study occur within or immediately adjacent to promoter regions with high diversity in the primate lineage. Unexpectedly, the -243 variant, the first *TNF-A* SNP linked to an African-derived extended HLA haplotype[70], was not detected in our studied population.

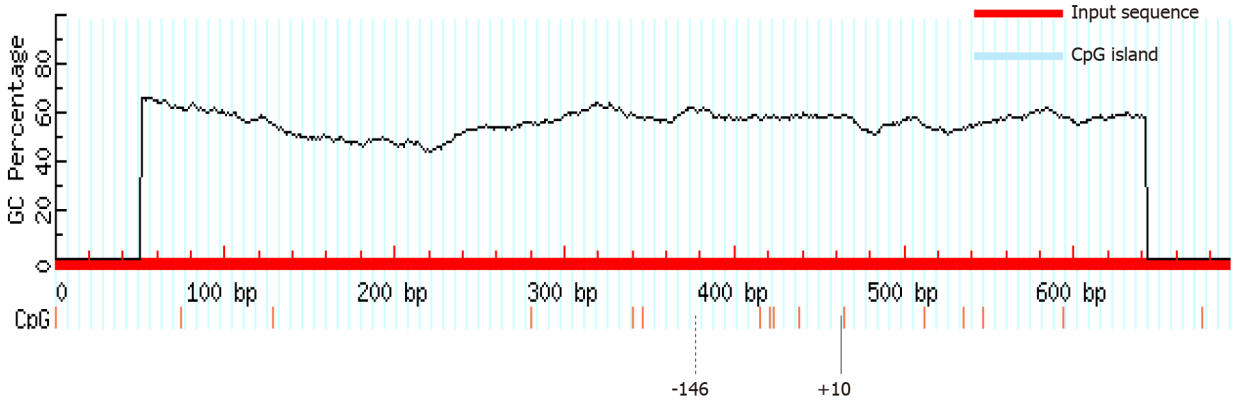

Figure 3 MethPrimer software prediction of no CpG islands in the in silico predicted promoter region.

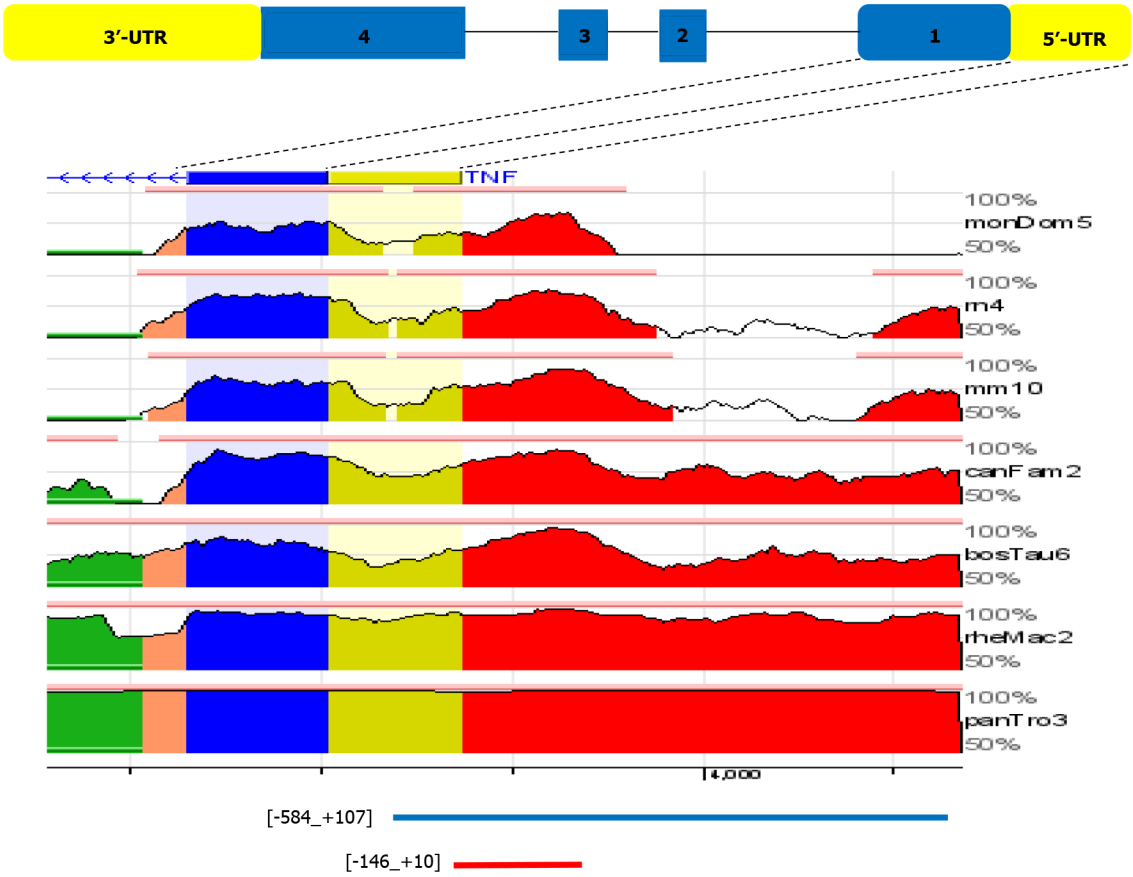

Figure 4 Overview from the ECR Browser shows mammalian conservation of the tumor necrosis factor 5'-upstream region compared to the human sequence in the region (-584 to +107) (hg19 chr6:31542751-31543444). Blue boxes represent the first tumor necrosis factor exon, while yellow indicates the tumor necrosis factor 5' untranslated region. Intragenic positions are highlighted in red or in green when corresponding to transposable elements and simple repeats. UTR: Untranslated region.

Other previously identified SNPs in this region (-584 to +107), which include -567, 375, -307, -237, -76 and +70 nt relative to the start site of transcription, were observed[66-68, 84].

The -1030 (T/C; rs1799964) SNP is frequently observed in African populations (17%-25% Malawian, 7% Nigerian, 29% African-American/Caribbean and 14% Gambia)[21, 22]. In this study, the -1030 SNP was detected in 47 Sudanese (38% of our studied population). Many studies have been published that examine the association between the -1030 SNP and multiple diseases[60,85,86] and diseases related to *H. pylori* such as gastroesophageal reflux disease, gastritis, peptic ulceration and gastric cancer[87-90]. In this study, we found a lack of association between -1030T and susceptibility to *H.*

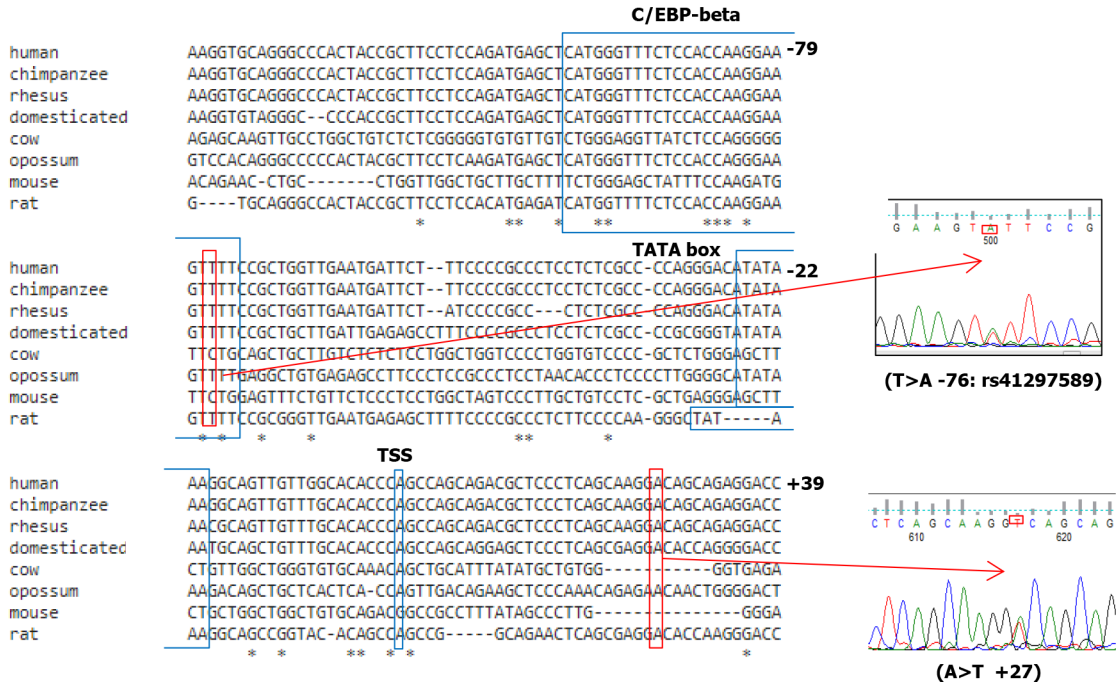

Figure 5 Mammalian conservation of (AT; single nucleotide polymorphism databases dbSNP: rs41297589) at position -76 and the novel mutation (A>T at +27) in the 5' untranslated region among various species. The nucleotides are enumerated at each line on the right side, and the in silico predicted TATA-, C/EBP-beta and transcription start site have marked inboxes. The chromatogram results of the polymorphisms are visualized using Finch TV software. TSS: Transcription start site.

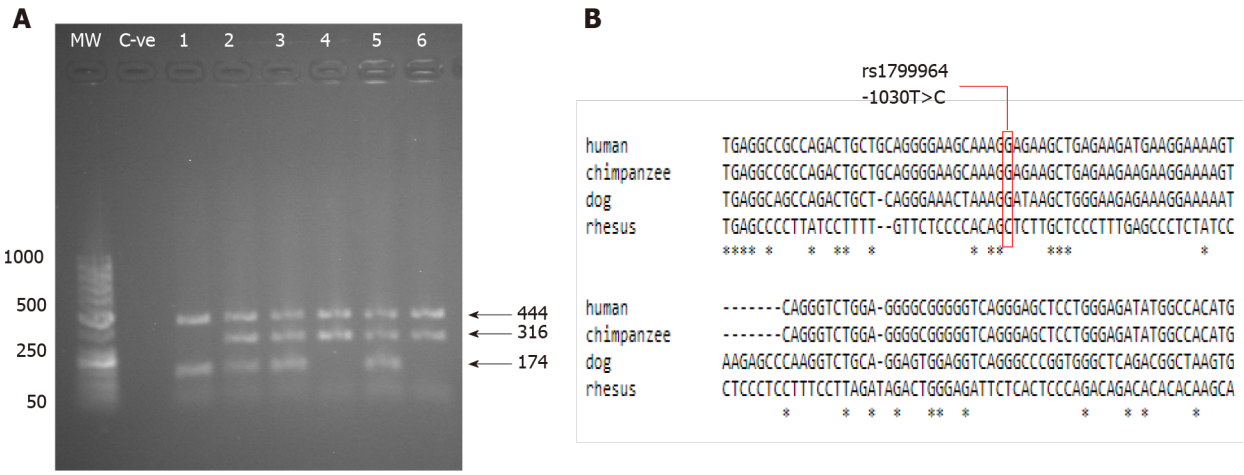

Figure 6 Molecular detection of the tumor necrosis factor-alpha-1030 C/T polymorphism. A: PCR with confronting two-pair primer products analyzed on a 1% agarose gel stained with ethidium bromide. Three genotypes can be seen. Lanes 2, 3 and 5 showed 444 bp, 316 bp and 174 bp, indicating a heterozygous genotype. Lanes 4 and 6 showed 444 bp and 316 bp, which indicated a homozygous T genotype. Lane 1 showed 444 bp and 174 bp, which indicated a homozygous C genotype; B: Mammalian conservation of (TC; dbSNP: rs1799964) position -1030 among different species. MW: Molecular weight marker; C-ve: Negative control.

pylori and gastric cancer in the studied population (P = 0.1756 and P = 0.8116, respectively). This finding is in agreement with previous studies[87,88,91]. In contrast to a study conducted in Japan that observed a significant association between -1030T and gastric cancer[91]. This variation could be attributed mainly to differences in genetic backgrounds of the studied population, the method of genotyping and sample size[76,92].

The study's limitations include the relatively small number of the enrolled subjects and depending on the in silico tools for studying the influence of the detected promoter variants on the *TNF-A* gene expression and *H. pylori* infection (susceptibility and progression). Further large cohort studies are needed to validate the study findings. However, the computational analysis provides a basis for identifying

promoter regions, recognizing regulatory motifs and understanding gene expression patterns[25].

According to the literature, a considerable number of polymorphisms have been discovered in the *TNF-A* promoter. However, a crucial question that remains to be answered is whether these polymorphisms have any functional effect that may significantly impact disease incidence or severity. Identifying which of these many variants are functional is of great significance for discovering new preventive, diagnostic and therapeutic strategies against the incidence and/or progression of multifactorial diseases such as *H. pylori* infection. This study detected seven SNPs in the *TNF-A* 5′-region; only one of them (T>A, -76) was located at the in silico-predicted promoter region (-146 to +10). This SNP could lead to the modification of the transcriptional regulation of *TNF-A* in *H. pylori* infection. However, this conclusion cannot substitute for the experimental proofs (*in vitro* or *in vivo*), but it can provide a direction or insight for such experiments to validate the in silico predictions.

CONCLUSION

In the Sudanese *H. pylori*-infected patients, seven SNPs were observed in the *TNF-A* 5′-region; only one of them (T>A, -76) was located at the in silico-predicted promoter region (-146 to +10). In addition, it was predicted to alter TFBSs and CEs. Furthermore, a novel mutation (A>T, +27) was detected in the 5′ untranslated region, and it could affect the post-transcriptional regulatory pathways. In addition, computational analysis was a valuable method for understanding gene expression patterns and providing guidance for further *in vitro* and *in vivo* experimental validation.

ARTICLE HIGHLIGHTS

Research background

Helicobacter pylori (*H. pylori*) infection represents a major public health challenge in Sudan. However, functional polymorphisms within the tumor necrosis factor-alpha (*TNF-A*) promoter are associated with the incidence and progression of *H. pylori* infection by increasing TNF-α production.

Research motivation

TNF lies within the major histocompatibility complex region of chromosome 6, which is a highly polymorphic region. Therefore, many polymorphisms have been detected in the *TNF-A* promoter and studying which variants could affect *TNF-A* gene expression is relevant. However, the influence of these single nucleotide polymorphisms (SNPs) on TNF-α production is not fully known and still a contradictory topic of debate due to the ethnic differences between populations. Furthermore, to our knowledge, there are no previous studies in Sudan that have addressed the association between *TNF-A* and *H. pylori* infection or *H. pylori*-associated diseases.

Research objectives

To functionally characterize the genetic variations in the *TNF-A* 5′-region (-584 to +107) of Sudanese patients infected with *H. pylori* and predict if these SNPs could alter the regulatory motifs using bioinformatics analyses. Also, to investigate the mammalian conservation of these SNPs using comparative profiling analysis in 11 species.

Research methods

An observational study was conducted in the major hospitals in Khartoum state. Genomic DNA was extracted from 122 gastric biopsies of patients who had been referred for endoscopy. Genotyping of the *TNF-A*-1030 polymorphism was performed using PCR with confronting two-pair primer to investigate its association with *H. pylori* infection in the Sudanese population. Sanger sequencing was applied to detect SNPs in the 5′-region (-584 to +107) of *TNF-A* in *H. pylori*-infected patients; in silico tools were used to predict whether these mutations would alter transcription factor binding sites or composite regulatory elements in this region. In addition, the ECR browser and multiple-sequence local alignment and visualization search engine were

used to study the conservation of the detected SNPs among 11 mammalian species.

Research results

A total of seven SNPs were observed in the *TNF-A* 5′-region of Sudanese patients infected with *H. pylori*. Among them, the SNP (T>A, -76) was located at the in silico-predicted promoter region (-146 to +10), and it was predicted to alter transcription factor binding sites and composite regulatory elements, while the novel mutation (A>T, +27) was detected in the 5′ untranslated region. It could affect the post-transcriptional regulatory pathways. Mammalian conservation was detected for the (-146 to +10) region in chimpanzee (99.4%), rhesus monkey (95.6%), cow (91.8%), domesticated dog (89.3%), mouse (84.3%), rat (82.4%) and opossum (78.0%). Furthermore, genotyping of *TNF-A*-1030 revealed a lack of significant association between -1030T and susceptibility to *H. pylori* and gastric cancer in the studied population (P = 0.1756 and P = 0.8116, respectively).

Research conclusions

Despite the high level of genetic variation in the *TNF-A* 5′-region (-584 to +107) of the Sudanese patients, the sequences involved in enhanceosome formation and gene regulation are highly conserved. Remarkably, only a single SNP (-76) was detected in this region. In addition, computational analysis was a valuable method for studying gene expression patterns and insights for further *in vitro* and *in vivo* experimental proofs.

Research perspectives

Further large cohort studies are needed to assess the association between (T>A, -76) mutation and *H. pylori* infection (susceptibility and progression). Also, further studies are encouraged to investigate the novel mutation (A>T, +27) in terms of the frequency of the minor allele (T) in the Sudanese population and its functional significance using computational and experimental approaches. Identifying which of these detected variants are functional is of great relevance for discovering new preventive, diagnostic and therapeutic strategies against the incidence and/or progression of multifactorial diseases such as *H. pylori* infection.

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ORIGINAL ARTICLE

Prospective Study

Outreach onsite treatment with a simplified pangenotypic direct-acting anti-viral regimen for hepatitis C virus micro-elimination in a prison

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Abstract

BACKGROUND

Prisoners are at risk of hepatitis C virus (HCV) infection, especially among the

people who inject drugs (PWID). We implemented an outreach strategy in combination with universal mass screening and immediate onsite treatment with a simplified pan-genotypic direct-acting antivirals (DAA) regimen, 12 wk of sofosbuvir/velpatasvir, in a PWID-dominant prison in Taiwan.

AIM

To implement an outreach strategy in combination with universal mass screening and immediate onsite treatment with a simplified pan-genotypic DAA regimen in a PWID-dominant prison in Taiwan.

METHODS

HCV-viremic patients were recruited for onsite treatment program for HCV micro-elimination with a pangenotypic DAA regimen, 12 wk of sofosbuvir/velpatasvir, from two cohorts in Penghu Prison, either identified by mass screen or in outpatient clinics, in September 2019. Another group of HCV-viremic patients identified sporadically in outpatient clinics before mass screening were enrolled as a control group. The primary endpoint was sustained virological response (SVR12, defined as undetectable HCV ribonucleic acid (RNA) 12 wk after end-of-treatment).

RESULTS

A total of 212 HCV-viremic subjects were recruited for HCV micro-elimination campaign; 91 patients treated with sofosbuvir/Ledipasvir or glecaprevir/pibrentasvir before mass screening were enrolled as a control. The HCV micro-elimination group had significantly lower proportion of diabetes, hypertension, hyperlipidemia, advanced fibrosis and chronic kidney diseases, but higher levels of HCV RNA. The SVR12 rate was comparable between the HCV micro-elimination and control groups, 95.8% (203/212) *vs* 94.5% (86/91), respectively, in intent-to-treat analysis, and 100% (203/203) *vs* 98.9% (86/87), respectively, in per-protocol analysis. There was no virological failure, treatment discontinuation, and serious adverse event among sofosbuvir/velpatasvir-treated patients in the HCV micro-elimination group.

CONCLUSION

Outreach mass screening followed by immediate onsite treatment with a simplified pangenotypic DAA regimen, sofosbuvir/velpatasvir, provides successful strategies toward HCV micro-elimination among prisoners.

Key Words: Direct-acting antivirals; Sofosbuvir; Velpatasvir; People who inject drugs; Universal screen

Core Tip: We implemented an outreach strategy in combination with universal mass screening and immediate onsite treatment with a simplified pangenotypic direct-acting antivirals egimen, 12 wk of sofosbuvir/velpatasvir, in a people who inject drugs (PWID)-dominant prison. Our study achieved high sustained virological response rate in HCV-infected PWID-dominant prisoners. We provided successful strategies toward HCV micro-elimination among prisoners.

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contributions; all authors participated in universal mass screening, immediate onsite treatment, read and approved the final manuscript.

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INTRODUCTION

Hepatitis C virus (HCV) infection is a progressive and blood-borne infectious disease that can lead to end stage liver diseases, such as hepatic decompensation, liver cirrhosis, and hepatocellular carcinoma[1,2]. Iatrogenic transmission of HCV, such as blood transfusion and surgery, has decreased in developed countries. Whereas people who inject drugs (PWID) has become the major population of HCV transmission, which could consist of approximately 80% of HCV-infected patients[3]. Given that lack of vaccine available, "treatment as prevention" for HCV transmission in PWID is very important for HCV elimination.

Prisoners are at high risk of HCV infection, with prevalence rates ranging from 3.1% to 38%[4,5]. The high prevalence of HCV infection in prisoners is resulted from unsafe lifestyles, psychiatric disorders, and social problems before they are incarcerated. Recently, PWID has been the most important risk factor of HCV infection in prisoners [6]. The anti-HCV prevalence rate could be as high as 91% among PWID prisoners[7]. Screening and eliminating HCV infection in prisoners is therefore an important social health issue.

According to the American Association for the Study of Liver Diseases and European Association for the Study of the Liver (EASL) guidelines, all HCV viremic patients should be treated if life span is expected more than one year[8,9]. HCV therapeutic strategies have been revolutionized significantly because of the availability of direct-acting antivirals (DAA)[10]. Interferon (IFN)-based regimens for HCV infection have serious side effects, long therapeutic duration, and contraindications, leading to the huge gaps in HCV care cascade[11]. The current IFN-free DAA regimens provide shorter treatment duration, very high treatment efficacy and safety profiles, not only for general population[12], but also for special populations[13], such as HCV/human immunodeficiency virus (HIV) coinfected patients, hepatitis B virus (HBV)/HCV coinfected patients and patients with chronic kidney diseases in real-world clinical settings[14,15].

World Health Organization (WHO) set a global goal of HCV elimination by 2030 [16], and Taiwan authority is even ambitious by 2025[17]. To achieve the goal, implementation of the concept of HCV micro-elimination is regarding as an efficient and practical strategy[18]. We have proved that "universal mass screening plus outreach onsite treatment" is the key to achieve HCV micro-elimination among patients under maintenance hemodialysis[19].

Recently, the latest EASL HCV guideline recommended simplified, genotyping/subtyping-free, pangenotypic anti-HCV treatment, either sofosbuvir/ velpatasvir or glecaprevir/pibrentasvir, to increase the accessibility and global cure rates among patients with > 12 years, chronic hepatitis C without cirrhosis or with compensated cirrhosis, with or without HIV co-infection, whatever treatment-naïve or IFN-experienced[8].

Since HCV treatment is not frequently administered to prisoners due to unawareness of HCV infection, difficultly management, easily loss to follow-up, and lack of hepatologist in prison[20], collaboration between hepatologists and prison authorities to carry out strategies for HCV diagnosis and treatment in prisoners in highly demanded. Herein, we implemented an outreach strategy in combination with universal mass screen and onsite treatment with a simplified pan-genotypic DAA regimen, 12 wk of sofosbuvir/velpatasvir, toward HCV micro-elimination in a PWID-dominant prison in Taiwan.

MATERIALS AND METHODS

Patients linked to onsite treatment program for HCV micro-elimination

HCV-viremic patients were recruited from two cohorts in Penghu Prison (Agency of Corrections, Ministry of Justice, Taiwan), a PWID-dominant prison (Figure 1).

HCV-viremic patients identified by a universal mass screening

In September 2019, we conducted a 5 d universal mass screening of viral hepatitis in Penghu Prison. These inclusion criteria were prisoners, who were at least 20 years old, being willing to enter the study for screening of viral hepatitis. The study of mass screening was approved by the Institutional Review Board of Kaohsiung Medical University Hospital (IRB: KMUHIRB-SV(I)-20190033). All participants provided written informed consents. A total of 1137 subjects from 1697 inmates participated the mass screening[21]. Among them, 396 (34.8%) subjects had anti-HCV seropositivity;

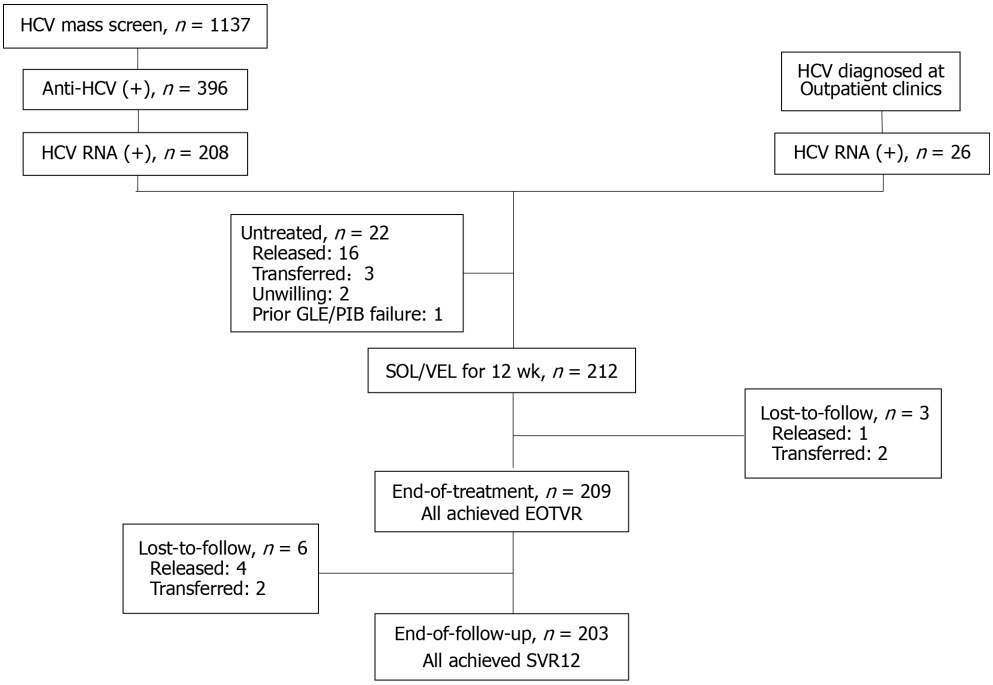

Figure 1 Patient flowchart of hepatitis C virus treatment with a simplified pan-genotypic directly-acting antivirals regimen in Penghu Prison. HCV: Hepatitis C virus; DAA: Directly-acting antivirals; SOL/VEL: Sofosbuvir/velpatasvir; GEL/PIB: Glecaprevir/pibrentasvir; EOTVR: Virological response at end-of-treatment; SVR12: Sustained viral response at post-treatment wk 12.

208 (52.5%) of the 396 subjects were seropositive for HCV ribonucleic acid (RNA) and linked to the onsite HCV treatment program with universal sofosbuvir/velpatasvir regimen.

HCV-viremic patients identified in outpatient clinics during the period of HCV mass screening

Another 26 HCV-viremic subjects identified in outpatient clinics of Penghu Prison between August to December 2019 were also linked to the onsite HCV treatment program with universal sofosbuvir/velpatasvir regimen.

All patients received pretreatment evaluation in December 2019, including medical history, liver and renal function tests, complete blood cell counts, HCV viral loads and genotyping, abdominal sonography and assessment of potential drug–drug interactions. A 12 wk, oral pan-genotypic regimen of sofosbuvir/velpatasvir 400/100 mg fixed-dose combination once daily was initiated in January-February 2020.

Patients identified and treated by DAAs in outpatient clinics before mass screening

A total of 91 HCV-viremic patients identified in outpatient clinics of Penghu Prison and treated with DAA before mass screening from 2017 to 2019 were enrolled as a control. The selection of DAA regimens were based on physician's discretion according to the viral genotype and criteria of reimbursement of National Health Insurance Administration, Taiwan. All patients received pretreatment evaluation, including medical history, liver and renal function tests, complete blood cell counts, HCV viral loads and genotyping, abdominal sonography and assessment of potential drug–drug interactions.

All participants signed informed consent forms. These enrolled inmates of our study were protected according to the guidelines of the Declaration of Helsinki. The current study of DAA therapy was approved by the Institutional Review Board of Tri-Service General Hospital (IRB: TSGHIRB 2-107-05-080).

Assessment, monitoring and endpoints

Anti-HCV antibody was determined by the third generation, commercially available immunoassay (Ax SYM HCV III; Abbott Laboratories, North Chicago, IL). HCV RNA viral loads and genotype were determined by real-time PCR assays [RealTime HCV; Abbott Molecular, Des Plaines IL, United States; detection limit: 12 IU/mL])[22]. Liver cirrhosis was defined by the presence of clinical, radiological, endoscopic or laboratory evidence of cirrhosis and/or portal hypertension or fibrosis-4 index (FIB-4) (> 6.5).

Laboratory data monitoring and assessment of side effects were performed at treatment wk 2, 4, 8 and end-of-treatment (EOT), and 12 wk after EOT.

The primary endpoint was sustained virological response (SVR12, defined as undetectable HCV RNA throughout 12 wk of the post-treatment follow-up period).

Statistical analyses.

The efficacy of all DAA regimens was determined in a intent-to-treat (ITT) population (all enrolled patients with at least one dose of DAA) and a per-protocol (PP) population (subjects receiving at least one dose of DAA and retained in Penghu Prison throughout the DAA treatment and follow-up period). Safety assessments reported adverse event (AE), serious adverse event (SAE) and laboratory abnormalities in the ITT population. Continuous variables are expressed as means ± standard deviation (SD), and categorical variables are expressed as percentages. The differences of continuous variables are estimated by the Student's *t* test. The differences in categorical variables are analyzed using the Chi-square test. The on-treatment and off-treatment virological response rates were analyzed in number and percentages with 95% confidence interval (CI). All data analyses were performed using the SPSS software version 18.0 (SPSS Inc., Chicago, Illinois, United States).

RESULTS

Patient flowchart of HCV micro-elimination campaign

The patient flowchart of HCV mass screen, assessment and treatment was shown in Figure 1. A total of 234 HCV-viremic patients, 208 from mass screening and 26 from outpatient clinics in Penghu Prison were assessed for eligibility of group therapy with sofosbuvir/velpatasvir in December 2019. Twenty-two patients were excluded from anti-HCV therapy due to scheduled to be released from jail (n = 16) or transferred to other jails (n = 3) within 6 mo, unwilling to receive therapy (n = 2) and prior glecaprevir/pibrentasvir treatment failure (n = 1). Finally, 212 patients were recruited for sofosbuvir/velpatasvir therapy initiated in January-February 2020.

Patient characteristics

The baseline characteristics of 303 HCV-viremic patients, including 212 in HCV micro-elimination campaign and 91 sporadic controls from outpatient clinics before micro-elimination campaign were listed in Table 1. They mean age was 48.4 years with male dominant (99.7%). Thirty (9.9%) had HBV coinfection. The mean FIB-4 was 1.3, with 20 (6.6%) had advanced fibrosis (FIB-4 > 3.25). Only one patient (0.3%) had liver cirrhosis. The mean HCV RNA levels was 6.5 Logs IU/mL, dominant with HCV genotype 1 (HCV-GT1, 42.2%), followed by HCV-GT6 (35.3%), HCV-GT3 (11.6%) and HCV-GT2 (10.6%). Three (1%) patients were prior IFN-experienced. The two groups had comparable characteristics in terms of age, gender, HBV co-infection, liver and renal function tests, FIB-4 score, HCV genotype distribution, and prior history of IFN-based therapy. However, the sporadic patients identified in outpatient clinics had significantly higher proportion of comorbidities, including diabetes, hypertension, hyperlipidemia and an estimated glomerular filtration rate (eGFR) < 60 mL/min/1.73 m^2, but significantly lower HCV viral loads. None of patient had decompensated cirrhosis nor liver cancer.

Treatment efficacy

All of 212 patients in HCV micro-elimination campaign received sofosbuvir/velpatasvir treatment; while among 91 sporadic patients with DAA therapy before HCV micro-elimination campaign, 78 (85.7%) received 12 wk of sofosbuvir/Ledipasvir and 13 (14.3%) received 8-12 wk of glecaprevir/pibrentasvir according to the Taiwan HCV guideline[12,13].

In ITT analysis, the overall SVR12 rate was 95.4% (289/303) with comparable SVR12 rates between sporadic HCV control group (94.5%, 86/91) and HCV micro-elimination group (95.8%, 203/212, P = 0.126, Table 2).

During DAA treatment period, all of patients in sporadic HCV control group completed DAA therapy, while 3 patients in HCV micro-elimination group lost-to-follow (2 transferred; 1 released). During the post-treatment follow-up period, 4 patients in sporadic HCV control group lost-to-follow (4 released), while 6 patients in HCV micro-elimination group lost-to-follow (2 transferred; 4 released). In PP analysis, the overall SVR12 rate was 99.7% (289/290) with comparable SVR12 rates between

Table 1 Baseline characteristics of hepatitis C virus-infected patients receiving directly-acting antivirals therapy between sporadic hepatitis C virus therapy in outpatient clinics and campaign of hepatitis C virus micro-elimination in Penghu prison

	Total	Sporadic HCV therapy in outpatient clinics (January 1, 2019 - December 31, 2019)	Campaign of HCV micro-elimination (January 1, 2020 - March 31, 2020)	*P* value
n	303	91	212	-
Age (yr)	48.4 ± 8.2	47.6 ± 8.7	48.7 ± 8.0	0.271
Male	303 (99.7)	90 (98.9)	212 (100.0)	0.126
[1]BMI, kg/m^2	23.9 ± 3.2	23.9 ± 3.3	23.9 ± 3.2	0.986
> 27 kg/m^2	34 (13.8)	11 (13.9)	23 (13.4)	0.960
Diabetes	10 (3.3)	8 (8.8)	2 (0.9)	0.0005[a]
Hypertension	59 (19.5)	25 (27.5)	34 (16.0)	0.021[a]
Hyperlipidemia	8 (2.6)	7 (7.7)	1 (0.5)	0.0003[a]
Cardiovascular disease	2 (0.7)	1 (1.1)	1 (0.5)	0.537
HBsAg (+)	30 (9.9)	9 (9.9)	21 (9.9)	0.997
AST, IU/L	41.3 ± 35.5	45.9 ± 38.9	39.4 ± 33.8	0.168
ALT, IU/L	65.4 ± 77.4	71.6 ± 69.8	62.7 ± 80.4	0.329
Abnormal AST or ALT	159 (52.5)	54 (59.3)	105 (49.5)	0.117
White cell count, × 10^3/iL	6.6 ± 1.9	6.4 ± 2.0	6.7 ± 1.8	0.188
Hemoglobin concentration, g/dL	15.9 ± 1.3	16.0 ± 1.3	15.9 ± 1.3	0.762
Platelet count, × 10^{3}u/L	227.6 ± 67.4	219.4 ± 72.1	231.2 ± 65.1	0.181
Albumin, g/dl	4.5 ± 0.3	4.5 ± 0.4	4.5 ± 0.2	0.233
Total bilirubin, mg/dL	0.8 ± 0.3	0.9 ± 0.4	0.8 ± 0.3	0.003[a]
LC	1 (0.3)	1 (1.1)	0 (0.0)	0.300
FIB-4	1.3 ± 1.0	1.5 ± 1.4	1.2 ± 0.8	0.096
> 3.25	20 (6.6)	10 (11.0)	10 (4.7)	0.044[a]
eGFR, mL/min/1.73 m^2	99.9 ± 17.7	99.1 ± 21.0	100.3 ± 16.4	0.624
< 60	4 (1.3)	3 (3.3)	1 (0.4)	0.048[a]
HCV RNA, $\log_{10}$ IU/mL	6.5 ± 1.1	6.0 ± 1.0	6.7 ± 1.1	< 0.001[a]
HCV genotype, 1/2/1+2/3/6	128 (42.2)/32 (10.6)/1 (0.3)/35 (11.6)/107 (35.3)	38 (41.8)/9 (9.9)/0/11 (12.1)/33 (36.2)	90 (42.5)/23 (10.8)/1 (0.5)/24 (11.3)/74 (34.9)	0.968
DAA regimen				
SOF/VEL	212 (70.0)	0 (0.0)	212 (100.0)	< 0.001[a]
SOF/LDV	78 (25.7)	78 (85.7)	0 (0.0)	
GLE/PIB	13 (4.3)	13 (14.3)	0 (0.0)	
Prior treatment history				
Naïve	300 (99.0)	89 (97.8)	211 (99.5)	0.216
Experienced-IFN	3 (1.0)	2 (2.2)	1 (0.5)	

[1]56 patients did not have body mass index information (12 patients before campaign of hepatitis C virus (HCV) micro-elimination; 44 patients in campaign of HCV micro-elimination).

[a]P < 0.05. DAA: Directly-acting antivirals; HCV: Hepatitis C virus; BMI: Body mass index; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LC: Liver cirrhosis; FIB-4: Fibrosis-4 index; HBsAg: Hepatitis B surface antigen; eGFR: Estimated glomerular filtration rate (mL/min/1.73 m^2); SOF: Sofosbuvir; VEL: Velpatasvir; LDV: Ledipasvir; GLE: Glecaprevir; PIB: Pibrentasvir; IFN: Interferon.

Table 2 Virological responses of hepatitis C virus-infected patients receiving directly-acting antivirals therapy before and during campaign of hepatitis C virus micro-elimination in Penghu prison in Penghu prison

Undetectable HCV RNA, *n*/N (%)	Total	Sporadic HCV therapy in outpatient clinics (January 1, 2019 - December 31, 2019)	Campaign of HCV micro-elimination with simplified pan-genotypic SOF/VEL regimen (January 1, 2020 - March 31, 2020)	*P* value
Intention-to-treat population				
Treatment 4 wk	284/303 (93.7)	85/91 (93.4)	199/212 (93.9)	0.879
End-of-treatment	300/303 (99.0)	91/91 (100.0)	209/212 (98.6)	0.557
End-of 12 wk follow-up	289/303 (95.4)	86/91 (94.5)	203/212 (95.8)	0.126
Per-protocol population				
Treatment 4 wk	284/301 (94.4)	85/90[1] (94.4)	199/211[2] (94.3)	0.964
End-of-treatment	300/300 (100.0)	91/91 (100.0)	209/209[3] (100.0)	-
End-of 12 wk follow-up	289/290 (99.7)	86/87[4] (98.9)e[5]	203/203[6] (100.0)	0.126

[1]One missing data.
[2]One transferred; One missing data.
[3]Two transferred; One released.
[4]Four released.
[5]One relapser.
[6]Four transferred; Five released. HCV: Hepatitis C virus; VEL: Velpatasvir; SOF: Sofosbuvir.

sporadic HCV control group (98.9%, 86/87) and HCV micro-elimination group (100%, 203/203, P = 0.126, Table 2). Only one patient experienced virological failure (54 years old male, treatment-naïve, HCV-GT3 infection with baseline viral loads of 62,883 IU/mL and FIB-4 of 2.37; relapsed from a 12 wk regimen of glecaprevir/pibrentasvir).

Safety profiles

The safety profiles of both groups were shown in Table 3. None of patients had treatment discontinuation other than released or transferred. None experienced serious adverse event. The frequency of adverse events was 4.3% (4/91) and 1.4% (3/212), respectively, among patients in sporadic control group and HCV micro-elimination group. The most reported adverse events were rash in 3 of 13 (23.1%) patients treated with glecaprevir/pibrentasvir and pruritus in 2 of 212 (0.9%) patients treated with sofosbuvir/velpatasvir. None of patients experienced grade 3 or 4 Laboratory abnormality.

DISCUSSION

In the current study, we demonstrated that mass screening combined with onsite group therapy by using a simplified pan-genotypic DAA regimen, 12 wk of sofosbuvir/velpatasvir, provides an "one-size fits all" solution toward the achievement of HCV micro-elimination in prisoners. The SVR rate was 95.6% in ITT population and 100% in PP population after excluding the inmates released or transferred before end-of-follow-up. The high SVR rate was observed in this PWID-dominant population, which HCV genotype distribution was diverse, including genotypes 1a, 1b, 2, 3 and 6.

Recent advance in the development of IFN-free pan-genotypic DAA regimens has remarkably improved the treatment efficacy with an overall SVR rates of > 90%. Therefore, WHO set the global of HCV elimination by 2030, through the achievement of > 90% diagnosis rate and > 80% treatment rate for eligible patients[16]. Nevertheless, there are many barriers in each HCV care cascade toward HCV

Table 3 Safety profiles of hepatitis C virus-infected patients receiving direct-acting antivirals therapy in Penghu prison

n (%)	Total	Sporadic HCV therapy in outpatient clinics (January 1, 2019 - December 31, 2019)	Campaign of HCV micro-elimination with simplified pan-genotypic SOF/VEL regimen (January 1, 2020 - March 31, 2020)
n	303	91	212
Treatment discontinuation other than released or transferred	0 (0.0)	0 (0.0)	0 (0.0)
Serious adverse events	0 (0.0)	0 (0.0)	0 (0.0)
Death	0 (0.0)	0 (0.0)	0 (0.0)
Adverse events	7 (2.3)	4 (4.3)	3 (1.4)
Fatigue	0 (0.0)	0 (0.0)	0 (0.0)
Pruritus	2 (0.7)	0 (0.0)	2 (0.9)
Rash	3 (1.0)	3 (3.2)	0 (0.0)
Nausea	0 (0.0)	0 (0.0)	0 (0.0)
Anorexia	0 (0.0)	0 (0.0)	0 (0.0)
Constipation	0 (0.0)	0 (0.0)	0 (0.0)
Dizziness	0 (0.0)	0 (0.0)	0 (0.0)
Insomnia	0 (0.0)	0 (0.0)	0 (0.0)
Headache	1 (0.3)	0 (0.0)	1 (0.5)
Others	1 (0.3)	1 (1.0)	0 (0.0)
Grade 3 or 4 laboratory abnormalities			
Total blood bilirubin	0 (0.0)	0 (0.0)	0 (0.0)
Alanine aminotransferase	0 (0.0)	0 (0.0)	0 (0.0)

DAA: Directly-acting antivirals; HCV: Hepatitis C virus; VEL: Velpatasvir; SOF: Sofosbuvir.

elimination at the population level[11,23]. To overcome the barriers, combining the concept of micro-elimination and an outreach strategy with immediate onsite treatment would be a more efficient and practical approach to achieve that goal[18,24]. The current study compared the HCV-infected inmates identified sporadically in outpatient clinics of Penghu Prison from 2017 to 2019 before mass screening and the patients identified by mass screening. We found that mass screening identified 208 HCV-viremic patients in a 5 d screening program from 1137 inmates (encountered around two-third of total inmates in Penghu Prison), compared to 91 HCV-viremic inmates treated in outpatient clinics from 2017 to September 2019. Our results demonstrated that mass screening with immediate onsite treatment provide much more efficient and practical solution to overcome the gaps of disease awareness and link-to-care in the HCV care cascades toward HCV micro-elimination in prisoners. In addition, we implemented "HCV reflex testing" in the mass screening program to scale-up and speed-up the diagnosis and link-to-care for treatment uptake of HCV infections[25].

PWID is known as the major risk factor of HCV infection and transmission. Although the anti-HCV prevalence in PWID prisoners decreased from 91% in 2014 to 34.8% in 2019 by the strategy of safe injection in Taiwan[21], almost all (97.6%) of HCV-infected prisoners were PWID. Given the lack of vaccine available and high risk of transmission, the strategy of universal screening and concept of "treatment as prevention" are the keys to HCV elimination in prison as well as PWID.

We observed that the sporadic HCV-infected prisoners identified in outpatient clinics had significantly higher proportion of comorbidities, including diabetes, hypertension, hyperlipidemia and eGFR, than those participating in the HCV micro-elimination campaign. It implicated that a great proportion of identified sporadically in outpatient clinics were due to concomitant morbidities; by contrast, many HCV-infected patients were unaware to their HCV diseases. In our mass screening, only 36.6% (145/396) of HCV-infected prisoners were aware of HCV infection before screening[21]. It indicates that the implementation of an outreach strategy with universal mass screen is necessary for HCV micro-elimination in prison.

Despite of the advances in the management of HCV infections, DAA therapy in incarcerated HCV-infected people remains many obstacles to be resolved, including disease unawareness, lack of updated information about the benefits of new DAA treatment, uncertainty of treatment right[26], poor accessibility due to of onsite treatment facilities or HCV treaters. Another difficulty for HCV treatment in prisoners is the unexpected or scheduled releasing from prison or transferring to other prisons, which frequently leads to the interruption of treatment or lost-to- follow up[20,27]. We are lucky that the Taiwan Health Insurance covered all incarcerated people, including all of the laboratory tests and ultrasound sonography and the cost of DAA regimens. Each prison has a contracted hospital providing point-of-care facility. Before initiating DAA therapy, we excluded the patients with expected release or transfer within 24 wk, and negotiated with the authority to avoid unnecessary transferring to other prisons during the period of HCV treatment and follow-up once the inmates entering the DAA course. Eventually we achieved a high treatment rate of 90.6% (212/234) and a high treatment complete rate of 95.8% (203/212), with a high cure rate at 100% (212/212).

Before the IFN-free DAA available, the lower SVR rate, much longer treatment duration and frequent adverse events of IFN-based treatment discouraged HCV-infected prisoners from receiving treatment[10]. IFN-free DAA regimens revolutionized HCV treatment which has largely extended the indication for various HCV-infected patients. Nevertheless, the application of typical DAA regimens are based on HCV genotype, presence of decompensated cirrhosis, renal function, and prior treatment experience. The two pangenotypic DAA regimens, sofosbuvir/velpatasvir, and glecaprevir/pibrentasvir, have achieved very high SVR rates of > 95%, regardless of HCV GTs, except for treatment-experienced cirrhotic HCV GT3 patients or GT3b patients[8,12,13]. Recently, to improve the access to anti-HCV therapy, reduce the cost of laboratory tests and the relative complexity of genotype-based treatment strategies, simplified treatment without many information needed for treatment decision are recommended to facilitate the care cascade among populations who are historically less engaged in healthcare, such as PWIDs and prisoners[8]. EASL recommends simplified, genotyping/subtyping-free regimens for IFN-free DAA treatment-naïve (except sofosbuvir plus ribavirin), HCV-infected or HCV-HIV coinfected adolescent and adult patients without cirrhosis or with compensated cirrhosis, regardless of HCV genotypes[8]. These recommendations are a universal 12 wk regimen of sofosbuvir/velpatasvir for all patients or glecaprevir/pibrentasvir, 8 wk for non-cirrhotic, 12 wk for compensated cirrhotic, and 16 wk for HCV GT3 patients, respectively. There are only four information needed before treatment, including the presence of HCV viremia, potential drug-drug interactions, and prior treatment experience, and presence of cirrhosis. The advantages of glecaprevir/pibrentasvir is a shorter 8 wk regimen for treatment-naïve HCV patients and IFN-experienced non-cirrhotic patients with compensated liver diseases, which would be benefit for prisoners who are expected to be released or transferred in a short term. However, glecaprevir, a protease inhibitor, is contraindicated for patients with hepatic decompensation and at risk for rare occurrence of serious drug-induced liver injury[28]. Also, glecaprevir/pibrentasvir has higher pill burden, three tablets a d. The advantages of sofosbuvir/velpatasvir include a universal fixed 12 wk regimen, one tablet a d, for all HCV patients with compensated liver diseases, less frequency of potential drug-drug interactions[29], and safety for those with hepatic decompensation. However, a 12 wk regimen with sofosbuvir/velpatasvir needs one more visit and monitoring when compared to an 8 wk regimen with glecaprevir/pibrentasvir. Therefore, we select sofosbuvir/velpatasvir as the antiviral regimen for our outreach onsite treatment. In

our study, all HCV-viremic prisoners fit the criteria of simplified, genotyping/subtyping-free regimens, except one who failed to prior glecaprevir/pibrentasvir therapy and was not enrolled for sofosbuvir/velpatasvir treatment. In our PP analysis, the overall SVR12 rate was comparable between HCV patient group (98.9%, 86/87) and HCV micro-elimination group (100%, 203/203). Our study provided evidence for the concept that simplified, genotyping/subtyping-free regimens can achieve high SVR12 rate in HCV-infected PWID-dominant prisoners.

In our study, none of prisoners had DAA treatment discontinuation due to adverse events. None experienced serious adverse event. These data indicated that the simplified, genotyping/subtyping-free regimen, sofosbuvir/velpatasvir, was safe and well tolerated for HCV-infected PWID-dominant prisoners. Very few adverse events were reported in both groups, whatever using sofosbuvir/Ledipasvir, glecaprevir/pibrentasvir and sofosbuvir/velpatasvir, when compared to the data from clinical trials[30,31]. It might be due to that current population was younger and less patients with advanced fibrosis or chronic kidney diseases.

There were some limitations in our study. First, not all inmates in Penghu Prison participated our mass screening. Strategies and policy to encourage inmates to receive HCV screening is mandatory to achieve the goal of WHO. Second, unexpected prisoners' transferral and release could not be completely avoided, which caused incomplete treatment and follow-up. Successfully linking the released or transferred people to another HCV treaters could help completing HCV treatment and follow-up. Third, there was no reimbursement for the retreatment of prior DAA failed patients in Taiwan at the time of the current study.

CONCLUSION

Well-designed strategies for mass screening and treatment for HCV-infected prisoners can be implemented successfully by the collaboration between physicians and prison authorities. We demonstrated that mass screening followed by immediate onsite treatment with a simplified pangenotypic DAA regimen, sofosbuvir/velpatasvir, provides successful strategies toward HCV micro-elimination among prisoners.

ARTICLE HIGHLIGHTS

Research background

Prisoners are at high risk of hepatitis C virus (HCV) infection. To screen and treat HCV infection in prisoners is an important social health issue. It can be the start for HCV micro-elimination.

Research motivation

HCV treatment is not frequently administered to prisoners due to multiple factors. Therefore, we implemented an outreach strategy in combination with universal mass screen and onsite treatment in a prison.

Research objectives

To implement an outreach strategy. HCV-infected prisoners received a simplified pan-genotypic direct-acting antivirals (DAA) regimen, 12 wk of sofosbuvir/velpatasvir. The primary endpoint was sustained virological response (SVR12, defined as undetectable HCV RNA throughout 12 wk of the post-treatment follow-up period).

Research methods

All participants received blood tests. We used reflex testing. All HCV-infected prisoners received DAA therapy. Laboratory data monitoring and assessment of side effects were performed at treatment wk 2, 4, 8 and end-of-treatment (EOT), and 12 wk after EOT.

Research results

DAA regimen with sofosbuvir/velpatasvir achieved high SVR12 rate. There was no virological failure, treatment discontinuation, and serious adverse event among sofosbuvir/velpatasvir-treated patients in the HCV micro-elimination group.

Research conclusions

Well-designed strategies for mass screening and treatment for HCV-infected prisoners can be implemented successfully by the collaboration between physicians and prison authorities.

Research perspectives

Our study provided evidence for the concept that simplified, genotyping/subtyping-free regimens can achieve high SVR12 rate in HCV-infected prisoners. In the future, it is possible to implement the strategy to all prisoners in our country.

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