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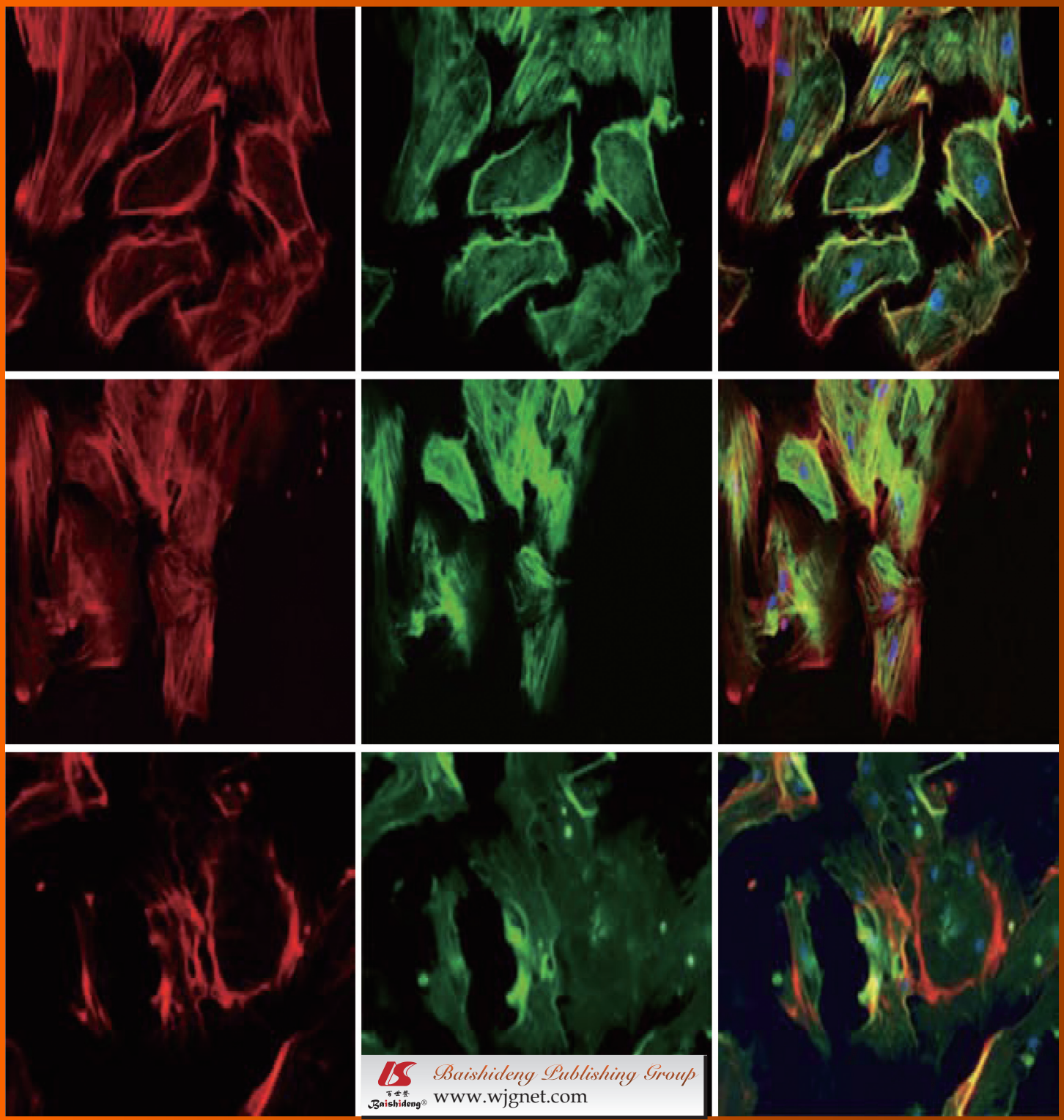

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EDITORIAL

A survey on herbal management of hepatocellular carcinoma

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Abstract

In this review we outline the different mechanisms mediating hepatocarcinogenesis. We also discuss possible targets of bioactive herbal agents at different stages of hepatocarcinogenesis and highlight their role at each individual stage. We gathered information on the most common herbal prescriptions and extracts thought to be useful in prevention or sensitization for chemotherapy in management of hepatocellular carcinoma (HCC). The value of this topic may seem questionable compared to the promise offered for HCC management by chemotherapy and radiation. However, we would recommend the use of herbal preparations not as alternatives to common chemo /and or radiotherapy, but rather for prevention among at-risk individuals, given that drug/herb interactions are still in need of extensive clarification. The bioactive constituents of various herbs seem to be promising targets for isolation, cancer activity screening and clinical evaluation. Finally, herbal preparations may offer a cost effective protective alternative to individuals known to have a high risk for HCC and possibly other cancers, through maintaining cell integrity, reversing oxidative stress and modulating different molecular pathways in preventing carcinogenesis.

Key words: Active ingredients; Chemoprevention; Chemosensitization; Hepatocellular carcinoma; Herbs; Molecular targets

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third deadliest and fifth most common malignancy worldwide[1-3]. It is a highly malignant tumor having high morbidity and motality. HCC has a poor prognosis due to its rapid infiltrating power which leads to complicating liver cirrhosis[4]. The rate of HCC is increasing worldwide between 3% and 9% annually[5,6]. The incidence ranges from less than 10 cases per 100 000 in North America and Western Europe to 50-150 cases per 100 000 in parts of Africa and Asia[7]. Hepatocarcinogenesis is associated with a background of chronic and persistent infection of hepatitis B virus (HBV) and hepatitis C virus (HCV)[8]. These infections along with alcohol and aflatoxin B1 exposure are widely recognized etiological agents in HCC[9].

In Egypt, epidemiology of HCC is characterized by marked demographic and geographic variations[10,11]. Over

the last decade, a remarkable increase, from 4.0% to 7.2%, was observed in the proportion of chronic liver disease (CLD) patients with HCC. The predominant age group (40-59 years) showed a slight increase compared with older groups (> 60 years). A significant increase, from 82.5% to 87.6%, was observed in the proportion of HCC among males. The calculated risk of HCC development is nearly three times higher in men than in women[12]. A unique invisible risk factor for development of HCC in Egypt could be Schistosomal infection and its injection therapy. Schistosomiasis induces immune suppression, which could result in increased persistence of viremia following acute infection of both hepatitis B and C[13].

HCCs are phenotypically (morphology and microscopy) and genetically heterogenous tumors, possibly reflecting the heterogeneity of etiological factors implicated in HCC development, the complexity of hepatocyte functions and the late stage at which HCCs usually become clinically symptomatic and detectable[14,15]. Hepatocarcinogenesis is a multi-factor, multi-step and complex process[8]. It involves three distinguishable but closely connected stages: initiation (normal cell → transformed or initiated cell), promotion (initiated cell → preneoplastic cell), and progression (preneoplastic cell → neoplastic cell)[16]. Malignant transformation of hepatocytes may occur, regardless of the etiological agent, through a pathway of increased liver cell turnover, induced by chronic liver injury and regeneration in a context of inflammation, immune response, and oxidative DNA damage[17-19].

MOLECULAR TARGETS FOR HERBAL COMPOUNDS DURING HCC PROGRESSION

Since ancient times, natural products, herbs and spices have been used as remedies for various diseases, incluing cancer (Table 1). The term chemoprevention was coined in the late 1970s and referred to a pharmacological intervention aimed to arrest or reverse the process of carcinogenesis[20]. Previous attempts were made to identify agents or combinations which could exhibit any of the following characteristics: (1) prevention of tumor initiation; (2) delay or arrest of the development of tumors; (3) extention of cancer latency periods; (4) reduction in cancer metastasis and mortality; and (5) prevention of recurrence of secondary tumors[21]. Recently, the focus has been directed towards molecular targeting of herbal compounds to identify the mechanism(s) of action of these newly discovered bioactive compounds. Moreover, it has been recognized that single agents may not always be sufficient to provide chemopreventive efficacy and therefore the new concept of combination chemoprevention by multiple agents or by the consumption of "whole foods" has become an increasingly attractive area of study[22]. Steps in the development of cancer at cellular level are described below.

Initiation

Initiation involves gene mutation, carcinogen metabolism and aberrant DNA repair. In this initial stage, environmental carcinogens (e.g. dietary, tobacco, pollution) induce one or more simple mutations, including transitions or small deletions in genes which control the process of carcinogenesis. Activated carcinogens exert their effects by forming covalent adducts with individual molecules of DNA or RNA, causing deletions of genetic material or mistranslation of the DNA sequence which may produce mutations in critical genes, such as tumor suppressors and oncogenes[23]. Reactive oxygen species (ROS) are generated normally as part of the normal oxidative metabolism or may be end-products of the breakdown of xenobiotic compounds (Figure 1). Oxidative stress can result in extensive DNA damage. Antioxidant herbs which scavenge activated oxygen species are able to stimulate DNA repair pathways to prevent or overcome oxidative DNA damage. Vitamin C, genistein and compounds originating from cruciferous vegetables are among the most well-studied for their scavenger properties[24]. In addition, chronic inflammation may predispose individuals to certain cancers. Most precancerous and cancerous tissues show signs of inflammation involving the movement of innate immune cells into the tissue, the presence of specific inflammatory signaling molecules (i.e. cytokines and chemokines), changes in tissue structure (remodeling) and the formation of new blood vessels (angiogenesis). Further studies have found that cancer-associated inflammation actually promotes tumor growth and progression[25]. Several pro-inflammatory gene products (i.e. TNF-α , IL-6) have a critical role in regulation of apoptosis, proliferation, angiogenesis, invasion and metastasis. Their expression is mainly regulated by the transcription factor NF-κB, which is constitutively active in most tumors and is induced by carcinogens and chemotherapeutic agents. TNF-alpha can initiate signaling pathways which lead to the activation of NF-κB, the initiation of MAPK cascades, and cell death[26]. These observations imply that anti-inflammatory agents that suppress NF-κB or NF-κB-regulated products should have a potential in both the prevention and treatment of cancer[27].

Recently, diallyl sulphide (DAS) obtained from garlic and vitamin C were reported to decrease the levels of circulatory TNF-α and IL-6 in DENA-induced hepatocarcinogenesis[28]. Previous reports showed that vitamin C can inactivate nuclear factor kappa B in endothelial cells during the inflammation process, independently of its atioxidant activity. Therefore, the anti-inflammatory activity of ascorbic acid (AA) may be mediated by multifactorial mechanisms, which are not necessarily associated with its intrinsic antioxidant activity[29]. DAS also was found to promote an anti inflammatory environment by cytokine modulation, leading to an overall inhibition of NF-kB activity in the surrounding tissue[30]. In addition, DAS may enhance antioxidants and suppresses inflammatory cytokines through the activation of Nrf2 transcription factor[31].

Table 1 Summary of the effects of some herbs and other natural compounds on hepatocellular carcinoma

Compound	Ref.	Composition	Effect
Herbs with cancer chemotherapeutic effect			
Geiji-Bokryung-Hwan	[78,79]	It is composed of five different herbs of Cinnamomi Ramulus, Poria Cocos Hoelen (Pachymae Fungus), Moutan Cortex Radicis, Paeoniae Radix, and Persicae Semen. The active constituents are antioxidative phenolic compounds, trans-cinnamic acid, taxifolin, protocatechuic acid, trans-o-hydroxy cinnamic acid, protocatechuic aldehyde, benzoic acid, trans-o-methoxy cinnamic acid, cis-o-methoxy cinnamic acid, 4-hydroxybenzoic acid, coumarin, daucosterol, Paeoniflorin, albiflorin and benzoylalbiflorin, paeonol and paeoniflorin.	The inhibitory effects of Geiji-Bokryung-Hwan (GBH) on the growth of cancer cell lines (HepG2 and Hep3B) and cancer chemopreventive activity were investigated. Tumor inhibition was found to be mediated via the inhibition of COX-1 activity.
Ganfujian granules	[80]	Ganfujian granules are an oral preparation consisting of dietary and medicinal Chinese herbs including Chinese yam (Rhizoma Dioscoreae), hawthorn fruit (Fructus Crataegi) and Chinese date (FructusZiziphi Jujubae). The active constituents are flavonoids including oligomeric procyanidins (OPCs), vitexin, vitexin 4'-O-rhamnoside, quercetin, and hyperoside	The herb was found to reduce and delay the incidence of diethylnitrosamine-induced hepatocarcinoma by exerting direct or indirect effects on the cell cycle and inhibiting uncontrolled proliferation of rat hepatocytes.
Maharishi amrit kalash	[81]	Maharishi Amrit Kalash (MAK) is composed of a mixture of two herbal mixtures, MAK-4 and MAK-5. The active constituents are multiple antioxidants including alpha-tocopherol, beta-carotene, ascorbate, bioflavonoid, catechin, polyphenols, riboflavin and tannic acid.	MAK was found to inhibit liver carcinogenesis when given as supplement to diet. The authors of this study suggested that the mechanism of this inhibition involved the prevention of excessive oxidative damage.
Scutellaria baicalensis and Bupleurum scorzonerifolfium willd	[43]	Chinese medicinal herbs. The active constituents are antioxidant flavonoids, baicalein, wogonin, neo-baicalein, and skullcapflavone.	The these herbs were found to enhance the chemopreventive effect of selenium on N-nitosobis (2-oxopropyl) amine-induced liver cancers in Syrian hamsters.
Huqi san (Qi-protecting powder)	[28,82]	Huqi san is composed of eight medicinal herbs including (Ramulus Visci, Radix Astragali seu Hedysari, Radix Curcumae, Radix Salviae Miltiorrhizae). The active constituents are polysaccharides, flavonoids, alkaloids and tanshinones.	The inhibitory effect of Huqi san on rat prehepatocarcinoma, which was induced via diethylinitrosamine (DEN), was investigated. It was found to inhibit the over-expression of c-jun, c-fos, and c-myc oncogenes, which were shown to play an important role in the pathogenesis of hepatocellular carcinoma. Huqi san was also reported to inhibit DEN induced oxyradical formation in cultured hepatocytes, leading to suppression of oxidative DNA damage.
Milk thistle	[83,84]	Milk thistle, commonly known as silymarin, is extracted from Silybum marianum. The active constituents are flavonoids from which silibinin and silymarin are the biologically most active compound.	It has been shown that a topical application of silymarin on mice results in complete inhibition of an epidermal carcinogen and prevents the formation of pyrimidine dimers, which are considered to be potential skin cancer agents.
Herbs with cancer chemotherapeutic effect			
Songyou Yin	[85]	This herbal extract is composed of a mixture of 5 Chinese medicinal herbs (Salvia miltiorrhiza, Astragalus membranaceus, lycium borbarum crataegus pinnatifida and trionyx sinensis). The active constituents are diterpenoid tanshinones, flavonoids and saponins.	"Songyou Yin" attenuates tumor proliferation and prolongs survival of nude mice bearing hepatocellular tumors without distinct toxicity. These findings suggest that "Songyou Yin" has some potential in the treatment of hepatocellular carcinoma.
Millettia reticulata benth	[86]	Millettia reticulata Benth is one of the oldest tonic herbs in traditional Chinese medicine. The active constituents are flavonoid derivatives: (-)-epicatechin, naringenin, 5,7,3',5'tetrahydroxyflavanone, formononetin, isoliquiritigenin, and genistein.	It was demonstrated that Millettia reticulata Benth flavonoid derivatives have a positive inhibitory effect on the viability of human cancer cells (including HepG2, SK-Hep-1, Huh7, PLC5, COLO 205, HT-29, and SW 872 cells). This Chinese herb also induces apoptosis in hepatocellular carcinoma cells via both Fas- and mitochondria-mediated pathways.
Bushen huayu jiedu recipe	[87]	"bushen huayu jiedu recipe" (BSHYJDR) is a mixture of several herbs including Chinese Cassia Bark, Psoralea, Zedoary, Rhubarb. The active constituents are alkaloids, flavonoid, arsenic trioxide, cinnamic acid, rhubarb and rhubarb substance.	BSHYJDR was found to inhibit transplanted hepatocarcinoma in mice. This effect is improved in combination with chemotherapy (cisplatin (DDP)).

Star 99	[88]	Chinese herbal compound	Human hepatocellular carcinoma was transplanted in nude mice and treated with Star 99 (intratumoral injection 10 days following to cancer transplantation). The herbal compound was shown to inhibit and destruct liver cancer cells, in particular the membrane, cytoplasm and nucleus of the caner hepatocyte.
Daesungki-Tang	[89]	This is a preparation consisting of four herbs: Rhei radix et rhizoma (the roots of Rheum coreanum Nakai), Aurantiii frutus immaturus (immature fruits of Poncirus trifolita Rafin), Magnoliae cortex (the stem bark of Magnolia officinalis Rehd. Et Wils), and Mirabilite (Matrii sulfas). The active constituents are magnolol, honokiol, physcion, chrysophanol, emodin, rhein, and aloe-emodin, naringenin glucuronide and hesperetin glucuronide.	This herb is widely used in the treatment of cancer metastasis. DST extracts were shown to inhib the invasion of the human hepatocellular carcinoma cell line, Hep 3B. On this basis, DST may be a promising antitumor agent.
Lycium barbarum and rehmannia glutinosa	[90]	Lycium barbarum (LBE) and Rehmannia glutinosa (RGE) are traditionally used as chinese medicines and herbal foods in China. The active constituents are beta-carotene, vitamin C, vitamins B1 and B2, beta-sitosterol, linoleic acid, immunologically active polysaccharides, sesquiterpenoids (cyperone, solavetivone), tetraterpenoids (zeaxanthin, physalin), and betaine .	Hot water-extracted Lycium barbarum (LBE) and Rehmannia glutinosa (RGE) were found to inhibit cell proliferation and induce p53 mediated apoptosis in hepatocellular carcinoma and inhibit oxidative DNA cleavage induced by various DNA damage chemicals. It also has immunological functions which lead to suppression of malignant cell growth.
Semen coicis	[91]	Semen Coicis is a traditional chinese herbal medicine which yields the extract Kang-Lai-Te (KLT). The active constituents are protein, fat, carbohydrate, vitamin B1, amino acids (leucine, lysine, arginine, tyrosine), Coix factors, Coix esters, triterpenoids.	KLT was found to inhibit HepG2 cell growth via a mechanism involving induction of apoptosis through activation of the Fas/FasL pathway.
Paeoniae radix	[58]	This crude drug from the root of Paeonia lactiflora Pallas is used in many traditional prescriptions in China and Japan. The active constituents are Paeoniflorin, albiflorin and benzoylalbiflorin.	Paeoniae Radix was found to inhibit the growth of hepatoma cell lines HepG2 and Hep3B via induction of apoptosis in a p53 independent pathway.
Qingrejiedu, huoxuehuayu, and fuzhengguben	[92]	Qingrejiedu, Huoxuehuayu, and Fuzhengguben (QHF) medicinal herbs. The active constituents are chlorogenic acid, geniposide, baicalin, forsythin, indirubin. ligustrazine chuanxiong, saponins, and isoflavonoids.	The QHF mixture was found to be more efficient in combating cancer than its separate ingredients. It was also reported to relieve symptoms that appear in patients with hepatocellular carcinoma and to decrease tumor growth by increasing the antitumor effect of cisplatin (DDP).
Delisheng	[93]	Delisheng is a n atural medicinal compound composed of ginseng, milk vetch root, secretion bufonis and cantharidium.	The activity of Delishng on the human hepatocellular carcinoma cell line HepG2 was investigated using the MTT assay, and compared to that of the chemotherapeutic drugs 5-fluorouracil and adriamycin. Delisheng was proved to have a positive anti-tumor activity, comparable to that of the chemotherapeutic drugs used.
Astragalus membranaceus	[94]	This herb, also known as aka huang chi, is one of the fundamental herbs used in traditional Chinese medicine. The active constituents are polysaccharides, saponins, flavonoids, amino acids.	The herb was found to improve the function of T lymphocytes in cancer patients compared with untreated cells.
Morarah and khaltita	[95]	Medicinal herbs. The active constituents is Kahalalide F.	Morarah and Khaltita were found to induce cell death in a heptoma (Huh-7) cell line, suggesting that these herbs could have a promising anti-cancer effect.

Possible molecular targets of herbal agents in different stages of hepatocarcinogenesis.

Promotion

This stage is characterized by dysregulation of signaling pathways which normally control cell proliferation and apoptosis (Figure 1). Apoptotic signaling within the cell is transduced mainly via two molecular pathways: the death receptor pathway (also called the extrinsic pathway) and the mitochondrial pathway (also called the intrinsic pathway)[32]. Both pathways activate a variety of proteases, mainly caspases (cysteinyl aspartate-specific proteases), and endonucleases, which finally degrade cellular components. Caspases are constitutively expressed as inactive proenzy-mes, generally require proteolytic processing for their acti-vation, and are capable of self-activation as well as activa-ting each other in a cascade-like process[33]. The extrinsic and the intrinsic pathways are not mutually exclusive and hepatocytes require mitochondrial involvement to amplify the apoptotic signal initiated by death receptors. The intrinsic pathway is triggered by various extra- or intracellular signals that induce mitochondrial dysfunction, resulting in altered membrane permeability and release into the cytosol of mitochondrial proteins, including pro-apoptogenic factors such as cytochrome c[34]. The Bcl-2

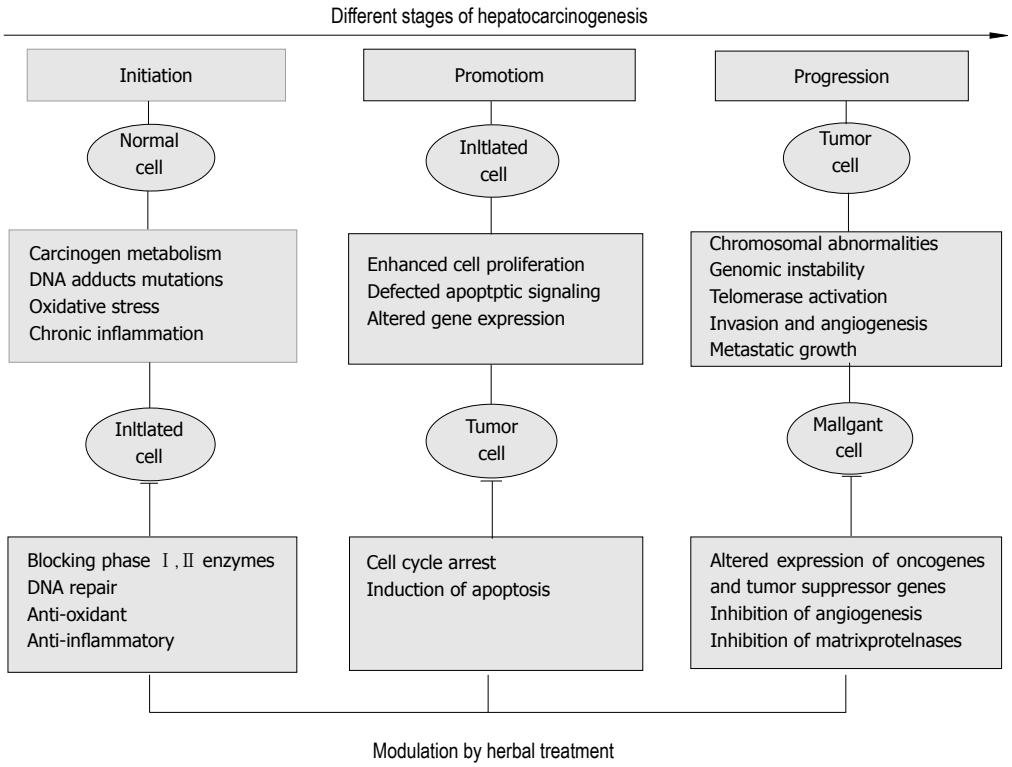

Figure 1 Molecular targets for herbal compounds during hepatocellular carcinoma progression. It is believed that hepatocarcinogenesis involve three main stages: initiation, promotion and progression. Herbal treatment can target multiple biochemical pathways and molecular events involved in different stages of cancer progression and thus offers both chemopreventive protection for healthy or high risk patients and chemotherapeutic potential for cancer patients receiving chemotherapy[23-58].

family is the best characterized protein family involved in the regulation of apoptotic cell death. The anti-apoptotic members of this family, such as Bcl-2, prevent apoptosis either by sequestering proforms of death-driving cysteine proteases called caspases (a complex called the apoptosome) or by preventing the release of mitochondrial apoptogenic factors such as cytochrome c and apoptosis-inducing factor into the cytoplasm. After entering the cytoplasm, cytochrome c and apoptosis inducing factor directly activate caspases that cleave a set of cellular proteins to cause apoptotic changes[35,36]. In contrast, pro-apoptotic members of this family, such as Bax, trigger the release of caspases from death antagonists via heterodimerization and also by inducing the release of mitochondrial apoptogenic factors into the cytoplasm via acting on mitochondrial permeability transition pores, thereby leading to caspase activation. Thus, the Bcl-2 family of proteins is crucial in critical life-death decisions within the common pathway of apoptosis[37].

Many of the molecular events altered in HCC progression are compromise the balance between survival and apoptotic signals in preneoplastic hepatocytes. Some physiological proapoptotic molecules (e.g. Bax) are down-regulated or inactivated in HCC, but the balance between death and survival is mainly disrupted by over activation of anti apoptotic signals (e.g. Bcl-2). Cancer cells show stronger requirements for these intracellular pathways to survive[38] and many cancer cells resist apoptosis through the upregulation of Bcl-2 gene[39,40]. This resistance allows damaged and mutated cells to survive, and ultimately proliferate. It also prolongs the lifespan of cells and makes them more likely to develop mutations. Cells also become resistant to the cytotoxic action of various agents, such as chemotherapy[38,41-43]. Thus, induction of apoptosis in tumor cells as well as the inhibition of increased cell proliferation are vital therapeutic goals for herbal treatment of malignancies. Many herbal agents appear to target signaling intermediates in apoptosis-inducing pathways. Thus, targeting apoptosis pathways in premalignant cells, where these pathways are still relatively intact, may be an effective mechanism for chemoprevention[40].

Previous studies have shown that treatment with DAS significantly modulates DNA levels in DENA-initiated hepatocarcinogenesis, suggesting interference with mitotic pathways and enhancement of apoptosis of cancer cells[44,45]. This effect may be related to the ability of DAS to induce direct perturbation of mitochondria, resulting in apoptotic damage to the cancer cells[46,47]. Other studies have reported that ascorbate induces cell cycle arrest and apoptosis in various tumor cells such as lymphoma, leukemia[48], melanoma[49], brain tumor[50], prostate cancer[51] and stomach cancer cells[52]. It is possible that AA exerts this effect by inhibiting either gene expression and/or activity of mutant p53, vascular endothelial growth factor (VEGF), phosphotyrosine kinase, and protein kinase C or by enhancing gene expression and/or activity of p53 wild-type, transforming growth factor beta (TGFb), mitogenactivated protein (MAP) kinase, caspase, cyclin A and D and their kinases[53,54]. These anti-promotional agents can also target specific signaling pathways for hormone receptors, cell cycle check-point markers, transcription factors, mitogen-activated protein kinases, rate-limiting enzymes (e.g. cyclooxygenases), cell junctions and tumor suppressor genes (e.g. p53). Promotion, unlike initiation, is reversible and so identifying agents which

can stop or reverse the process of promotion is of a great importance[55].

This stage is characterized by invasion, angiogenesis, metastatic growth, and genetic alterations within the karyotype of the cells due to accumulation of mutated genes, resulting in chromosomal abnormalities (see Figure 1). Angiogenesis, the development of new blood vessels from endothelial cells, is a crucial process which allows the malignant cells to get the nutrients and oxygen, which are essential for cancer progression[56]. Tumors that outgrow their oxygen supply cannot form masses greater than 1-2 mm in diameter without developing central necrosis. Neoplasms are genetically plastic and often adapt by switching on genes that increase their ability to invade and metastasize. Tumours do not grow progressively unless they induce a blood supply from the surrounding stroma. The tumour angiogenic switch seems to be activated when the balance shifts from angiogenic inhibitors to angiogenic stimulators[57]. During angiogenesis, endothelial cells are stimulated by various growth factors, including vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). Thus, blocking the growth of new blood vessels, and thereby reducing nutrients and oxygen supply to tumour cells seems to be a successful strategy to prevent cancer metastasis[58].

The process of cancer metastasis consists of a series of interrelated sequential steps, each of which is rate-limiting and may be a target for therapy. The outcome of the process depends on both the intrinsic properties of the tumour cells and the responses of the host. These steps are summarized as follows: (1) Transformation of normal cells into tumour cells; (2) Extensive vascularization (angiogenesis) involving production and secretion of pro-angiogenic factors by tumour cells and host cells to establish a capillary network from the surrounding host tissue; (3) Local invasion to the host stroma via thin-walled venules, fragmented arterioles, and lymphatic channels which offer little resistance to penetration and entry of tumour cells into the circulation; (4) Detachment and embolization, in which most circulating tumour cells are rapidly destroyed, but those that survive arrest in the capillary beds of distant organs by adhering either to capillary endothelial cells or to the exposed subendothelial basement membrane; (5) Extravasation into a new host organ or tissue; and (6) Proliferation within the new host organ or tissue with the micrometastasis developing a vascular network and evading destruction by host defenses. The cells can then continue to invade blood vessels, enter the circulation, and produce additional metastases[59-61].

Recently, there has been significant interest in developing agents which can delay cancer cell progression to metastasis. Many anti-angiogenic herbs, such as curcumin[62], grape seed extract[63,64], and green tea, have been identified[65,66]. These phytochemicals interact at multiple levels to suppress the inflammatory, hyperproliferative and transformative processes that promote angiogenesis. They inhibit aminopeptidase-N (CD13), a member of the matrix metalloproteinase family that is implicated in the angiogenic switch process. They can also interfere with the expression of VEGF by suppressing a series of angiogenic pathways including production of transforming growth factor beta (TGF-B), amplification of cyclooxygenase-2 (COX-2) and epidermal growth factor receptor (EGFR), and amplification of nuclear factor kappa-B (NF-κB) signaling. They may also interfere with endothelial cell function by inhibiting the engagement of specific integrins. Other anti-angiogenic herbs include Chinese wormwood, Chinese skullcap, resveratrol and Chinese magnolia tree, ginkgo biloba, quercetin, ginger, panax ginseng[67,68].

Most anti-cancer herbs can exert both chemopreventive and chemotherapeutic actions. Taking into consideration the sequence of events in carcinogenesis (i.e. initiation, promotion and progression), the boundary between the two actions of herbal agents during progression of cancer is unclear. In other words, the same herbal agent can both act as a chemopreventive agent for healthy or high risk patients, and can be used as a therapeutic agent or chemotherapy adjuvant to increase efficacy, decrease side effects of conventional cytotoxic drugs, and prevent tumour metastasis and recurrence in cancer patients. This dual action of herbal medicines combined with their ability to target multiple biochemical and physiologic pathways involved in tumour development and to minimize normal-tissue toxicity emphasize their importance as an attractive alternative means of controlling malignancy[19].

HERB-DRUG INTERACTIONS

Although herbal medicine has become a popular complementary and alternative strategy for cancer, doubts concerning interference with the action of conventional chemotherapeutic drugs have been raised recently. Considering the narrow therapeutic borders of oncolytic drugs, the usef of herbs could increase the risk of clinically relevant herb-anticancer drug interactions. In addition, the lack of sufficient information about possible mechanisms for such interactions makes it very difficult to accurately evaluate their possible adverse effects[69]. We have tried to highlight the negative side of random use of herbal treatments without medical supervision and the extent to which they can affect the safety and efficacy of chemotherapy in cancer patients.

Herb-drug interactions can occur at different levels (pharmaceutical, pharmacodynamic or pharmacokinetic), but pharmacokinetic interactions are the most likely to occur and can result in changes in absorption, distribution, metabolism, or excretion of chemotherapeutic drugs[70]. Drug-metabolizing systems are among the main targets for such interactions. Phase I enzymes, mainly cytochrome P450, detoxify a variety of endogenous and exogenous chemicals and activate many carcinogens[71]. Phase Ⅱ enzyme systems, which include glutathione S-transferase (GST), 3-quinone reductase, sulfotransferases, and UDP-glucuronosyl-transferase, catalyze the reduction or conjugation of phase I metabolites to various watersoluble

molecules and accelerate the rate of metabolite excretion[72,73]. Herbs can either inhibit or induce these systems, thus modulating the action of oncolytic drugs. Inhibition occurs when a herbal agent reduces the normal activity level of a certain metabolic enzyme or drug transporter involved in the disposition of the chemotherapeutic agent via a competitive or noncompetitive mechanism, thereby leading to higher plasma levels of the cytotoxic drug[74,75]. On the other hand, induction is a much slower process, in which herbs increase the mRNA and protein levels of the relevant metabolizing enzyme or drug transporter, resulting in lower plasma levels of chemotherapeutic agent. In either case, significant clinical interactions can occur which may cause greater toxicity or therapeutic failure[70,76,77].

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ORIGINAL ARTICLE

Nonmuscle myosin Ⅱ regulates migration but not contraction in rat hepatic stellate cells

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Abstract

AIM: To identify and characterize the function of nonmu-scle myosin Ⅱ (NMM Ⅱ) isoforms in primary rat hepatic stellate cells (HSCs).

METHODS: Primary HSCs were isolated from male Spra-gue-Dawley rats by pronase/collagenase digestion. Total RNA and protein were harvested from quiescent and culture-activated HSCs. NMM Ⅱ isoform (Ⅱ-A, Ⅱ-B and Ⅱ-C) gene and protein expression were measured by RealTime polymerase chain reaction and Western blot analyses respectively. NMM Ⅱ protein localization was visualized *in vitro* using immunocytochemical analysis. For *in vivo* assessment, liver tissue was harvested from bile duct-ligated (BDL) rats and NMM Ⅱ isoform expression determined by immunohistochemistry. Using a selective myosin Ⅱ inhibitor and siRNA-mediated knockdown of each isoform, NMM Ⅱ functionality in primary rat HSCs was determined by contraction and migration assays.

RESULTS: NMM Ⅱ-A and Ⅱ-B mRNA expression was increased in culture-activated HSCs (Day 14) with significant increases seen in all pair-wise comparisons (Ⅱ-A: 12.67 ± 0.99 (quiescent) *vs* 17.36 ± 0.78 (Day 14), $P < 0.05$; Ⅱ-B: 4.94 ± 0.62 (quiescent) *vs* 13.90 ± 0.85 (Day 14), $P < 0.001$). Protein expression exhibited similar expression patterns (Ⅱ-A: 1.87 ± 2.50 (quiescent) *vs* 58.64 ± 8.76 (Day 14), $P < 0.05$; Ⅱ-B: 1.17 ± 1.93 (quiescent) *vs* 103.71 ± 21.73 (Day 14), $P < 0.05$). No significant differences were observed in NMM Ⅱ-C mRNA and protein expression between quiescent and activated HSCs. In culture-activated HSCs, NMM Ⅱ-A and Ⅱ-B merged with F-actin at the cellular periphery and throughout cytoplasm respectively. *In vitro* studies showed increased expression of NMM Ⅱ-B in HSCs activated by BDL compared to sham-operated animals. There were no apparent increases of NMM Ⅱ-A and Ⅱ-C protein expression in HSCs during hepatic BDL injury. To determine the contribution of NMM Ⅱ-A and Ⅱ-B to migration and contraction, NMM Ⅱ-A and Ⅱ-B expression were downregulated with siRNA. NMM Ⅱ-A and/or Ⅱ-B siRNA inhibited HSC migration by approximately 25% compared to scramble siRNA-treated cells. Conversely, siRNA-mediated NMM Ⅱ-A and Ⅱ-B inhibition had no significant effect on HSC contraction; however, contraction was inhibited with the myosin Ⅱ inhibitor, blebbistatin (38.7% ± 1.9%).

CONCLUSION: Increased expression of NMM Ⅱ-A and Ⅱ-B regulates HSC migration, while other myosin Ⅱ classes likely modulate contraction, contributing to development and severity of liver fibrosis.

Key words: Hepatic stellate cells; Nonmuscle myosin Ⅱ;

Migration; Contraction; Blebbistatin; Hepatic injury

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INTRODUCTION

The progressive pathology of hepatic fibrosis is characterized by continual deposition and accumulation of type I collagen heavily mediated by the hepatic stellate cell (HSC). HSCs are located in the perisinusodial space of Disse between the hepatocytes and endothelial cells and comprise approximately 15% of the normal liver[1]. These lipid rich, vitamin A storing cells produce collagen and other extracellular matrix (ECM) components for maintenance of basement membrane and regulate hepatic microcirculation by modulating sinusoidal diameter[2-4]. In diseased liver, such as steatohepatitis, fibrosis, cirrhosis or hepatocellular carcinoma, damaging stimuli trigger transdifferentiation of quiescent HSCs to an activated, wound-healing myofibroblast-like cell[5]. Activated HSCs proliferate vigorously, lose retinyl ester stores, increase expression of cytoskeletal proteins such as α smooth muscle actin and secrete numerous ECM proteins including type I collagen leading to disruption of normal liver architecture impeding liver microcirculation[5].

In addition to altering the ECM, HSC hypercontractility contributes to increased resistance of sinusoids manifesting in portal hypertension, characterized by both increased portal blood flow and intrahepatic vascular tone[6,7]. Autoregulation of microcirculation is delicately balanced by vasomodulators, such as endothelin-1 (ET-1), a potent vasoconstrictor synthesized by endothelial cells and nitric oxide, a strong vasodilator[8,9], and activated HSCs have been shown to contract in response to ET-1[10,11]. Prior to matrix and microvasculature remodeling, chemotactic factors released during injury stimulate HSC migration to damaged areas. Platelet-derived growth factor, one of the most potent chemotactic molecules, also regulates factors controlling focal adhesion formation, including myosin regulatory light chain phosphorylation[12].

Myosin proteins act as molecular motors and contribute to cellular contraction, cytokinesis and migration. Myosins bind actin filaments and generate force, using energy from ATP hydrolysis. Specifically, class Ⅱ myosins are associated with generation of contractile forces[13]. In nonmuscle cells, three isoforms of nonmuscle myosin Ⅱ (NMM Ⅱ-A, Ⅱ-B and Ⅱ-C) encoded by different genes have been identified and are expressed in multiple tissues[14-16]. Distinct enzymatic properties of each isoform confer specific functions and are important in modulating kinetic properties of the cell[17].

HSC contraction and migration are necessary for the wound-healing process and influence both development and severity of hepatic fibrosis. Recent studies examined the expression and functionality of NMM Ⅱ proteins in mouse HSCs[18,19]. Inhibition of myosin Ⅱ ATPase by blebbistatin, a cell-permeable pharmacological agent, altered HSC morphology and reduced characteristic HSC contraction; however, NMM Ⅱ isoform specificity of blebbistatin is not well understood[20]. While studies have shown the pharmacological inhibitor blocks skeletal muscle and NMM Ⅱ activity with minimal effects on smooth muscle myosin Ⅱ, others have shown that blebbistatin is specific to smooth muscle myosin Ⅱ[20-23]. Lack of specificity associated with blebbistatin requires further investigation into the distinct roles of NMM Ⅱ isoforms in the HSC. Furthermore, expression of NMM Ⅱ isoforms in HSCs *in vivo* has not been investigated. In the present study we examined expression and functionality of NMM Ⅱ isoforms in rat HSCs.

MATERIALS AND METHODS

Materials

Reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise indicated. ET-1 was purchased from American Peptide (Sunnyvale, CA). TRIzol, Lipofecatmine and Superscript Ⅱ kit were purchased from Invitrogen Corporation (Baltimore, MD). Blebbistatin was purchased from Calbiochem (San Diego, CA). Type Ⅰ collagen was purchased from BD Biosciences (Franklin Lakes, NJ). Pronase and SYBR Green were purchased from Roche Molecular Biochemicals (Chicago, IL). Oligonucleotide primers were designed using Primer3 (v0.4.0) and synthesized by Integrated DNA Technologies (Coralville, IA). Monoclonal antibodies specific against NMM Ⅱ-A and Ⅱ-B isoforms, GAPDH, and anti-rabbit (or goat)-HRP secondary antibodies were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). NMM Ⅱ-C antibody was a gift, kindly provided by Dr. Robert Adelstein. Monoclonal antibodies specific against α smooth muscle actin (αSMA) were purchased from Dako (Glostrup, Germany). Chamber slides were purchased from Lab-Tek (Rochester, NY). Secondary fluorescent antibodies (AlexaFluro 488 anti-rabbit and 594 anti-mouse), rhodamine phalloidin and DAPI were purchased from Molecular Probes (Eugene, OR). ECL reagent was purchased from Amersham Biosciences (Piscataway, NJ). siRNAs for NMM Ⅱ isoforms (Ⅱ-A and Ⅱ-B) were purchased from Ambion (Austin, TX). Optiprep was purchased from Axis-Shield (Oslo, Norway).

Animals

Male Sprague-Dawley rats [250 g (bile duct-ligation (BDL) model); 500-650 g (primary cultures)] purchased from Charles River Laboratories (Raleigh, NC) were used in these studies. All experiments were approved by The University of North Carolina at Charlotte Institutional Animal Care and Use Committee and performed in accordance with NIH guidelines.

HSC Isolation and culture

Primary HSCs were isolated from animals following in situ liver perfusion-pronase/type I collagenase digestion[24]. The liver was perfused with calcium free-buffered saline, pronase (0.035% b.w.) and collagenase (1 mg/mL) for 10 min each. Digested liver suspension was centrifuged twice at 50 × r/min for 2 min. Nonparenchymal cells were recovered from the supernatant by centrifugation at 700 × r/min for 3 min. Density gradients were prepared in Optiprep 40% (*v*/*v*) solution. The gradient was centrifuged at 700 × r/min for 17 min at 25°C. HSCs were recovered from the interface between the medium and density layer, washed and centrifuged at 700 × r/min for 5 min. Typical cell purity following isolation was ≥ 95% as determined by autofluorescence of stored retinoid esters in HSCs. Cell viability was determined by Trypan blue exclusion staining. Cells were either used immediately (quiescent) or cultured on plastic using DMEM supplemented with 10% fetal bovine serum, L-glutamine (2 mmol/L) and antibiotics (activated) as described previously[24]. Growth medium was changed on a daily basis for the first week in culture. Culturing HSCs on plastic is routinely used to mimic the *in vivo* activation process[25].

Surgical procedures

Animals were randomized into two groups; sham and BDL and allowed to recover for two weeks.

Sham: Surgical anesthesia was induced by isoflurane inha-lation and a midline laparotomy performed and closed in two layers.

BDL: Surgical anesthesia was induced by isoflurane inhalation and a midline laparotomy performed. The hepatic bile duct was exposed, double ligated, transected and the abdominal incision closed in two layers.

Post-operative care: In all experimental groups, animals received saline [0.9% (*w*/*v*)] resuscitation and buprenorphine (0.03 mg/kg, *s.q.*) immediately after each procedure. Buprenorphine was administered for the duration of the experiment as determined by the in-house veterinarian. The time points for data analysis were chosen as mild fibrosis ensued to identify specific changes in NMM Ⅱ isoform expression in the liver.

Tissue collection: Two weeks after BDL, animals were sacrificed by exsanguination and the liver resected. Tissue samples (100-200 mg) were fixed in formalin solution overnight and paraffin-embedded.

Table 1 Intron-spanning primers for the amplification of NMM Ⅱ isoforms

Gene	Sense	Anti-sense	Product length (bp)
rMyh9	5′ aga aaa ccg cat cac cat tc	5′ tgt tct tca tca gcc act cg	189
rMyh10	5′ ggc act gga gga act ctc tg	5′ ctt ctt cca gca ggg ttg ag	287
rMyh14	5′ gct gct caa gga cca tta cc	5′ gta cca gct tgc cag aga gg	275

Gene name: rMyh9: Rat nonmuscle myosin Ⅱ-A; rMyh10: Rat nonmuscle myosin Ⅱ-B, rMyh14: Rat nonmuscle myosin Ⅱ-C.

mRNA analysis

Total RNA from quiescent and culture-activated HSCs was isolated using TRIzol, DNase treated, reverse transcribed using Superscript Ⅱ following manufacturer's recommendations. RealTime PCR was run at 94°C 15 s; 58°C 25 s; 72°C 20 s, read 5 s using primers specific against NMM Ⅱ-A, Ⅱ-B and Ⅱ-C (Table 1). Reaction mixture consisted of 1 μL each of cDNA, forward and reverse primers (5 nmol), 2 μL DEPC water, and 5 μL of SYBR Green Master Mix. cDNA concentration was used as a reference to normalize samples since the expression of housekeeping genes was modulated through days in culture[24]. Data were reported as cross-point, the point at which the detectable level of SYBR green fluorescence was detected above the background. All experiments were performed a minimum of three times.

Protein analysis

Western blot: Protein expression of NMM Ⅱ isoforms during transdifferentiation of quiescent (freshly isolated), Day 1 (early activation) and Day 14 (late activation) HSCs was determined by an actin-selection assay[26]. Briefly, HSCs were homogenized in lysis buffer (50 mmol/L Tris-HCl, 0.1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 2% (*w*/*v*) SDS, aprotinin and protease inhibitor cocktail). Sample lysates were equalized for protein concentration using the Bradford method and incubated with F-actin (10 mmol/L) for 30 min at 4°C. The protein complex was centrifuged at 320 000 × r/min for 30 min and the pellet suspended in Laemmli buffer. Immunoblot analysis was performed using 8% SDS-PAGE. Primary antibodies were diluted 1:500 (NMM Ⅱ-A, Ⅱ-B, or Ⅱ-C) and incubated at 4°C overnight. Secondary antibody (anti-rabbit-HRP) was used at a dilution of 1:1000 and incubated for 1 h at room temperature. Rat-1 cell line was used as a positive control for NMM Ⅱ isoform detection. Protein expression of NMM Ⅱ-A and Ⅱ-B siRNA inhibition was determined by standard Western blot analysis[27]. Briefly, HSCs were homogenized in lysis buffer, equalized for protein concentration using the Bradford method and immunoblot analysis performed using 8% SDS-PAGE.

Primary antibodies were diluted 1:500 (NMM Ⅱ-A or Ⅱ-B) or 1:1000 (GAPDH) and incubated at 4°C overnight. Secondary antibody (anti-rabbit-HRP or anti-goat-HRP) was used at a dilution of 1:1000-1:5000 and incubated for 1 h at room temperature. Signal intensity was analyzed using a digital camera and densitometric analysis program (Quantity One, Bio-Rad Laboratories, Inc).

Dual fluorescent immunostaining: The expression of NMM Ⅱ isoforms was evaluated by: (1) immunocytochemistry (ICC) of culture-activated HSCs (Day 14) incubated on chamber slides and (2) immunohistochemistry (IHC) of paraffin-embedded liver sections from normal and injured liver. For immunocytochemistry, slides were washed with PBS, blocked with 5% (v/v) normal goat serum, incubated overnight at 4°C using rabbit polyclonal NMM Ⅱ-A, Ⅱ-B and Ⅱ-C antibody (1:100). Samples were washed with PBS, incubated with secondary antibody (AlexaFluro 488: 1:500) for 1 h, followed by rhodamine phalloidin in-cubation for 15 min and finally with 4, 6-diamidino-2-phenylindole, dihydrochloride (DAPI) for 5 min. For immunohistochemistry, sections were de-paraffinized and hydrated in graded ethanol. Cross-linked proteins were exposed using heat-induced epitope retrieval and proteolytic enzyme digestion. Slides were washed, blocked [0.2% (v/v) NGS] and incubated overnight at 4°C using rabbit polyclonal NMM Ⅱ-A, Ⅱ-B and Ⅱ-C antibody (1:500). In addition, mouse αSMA was used to detect activated HSCs. Slides were washed, incubated with fluorescent secondary antibodies (AlexaFluro 488 and AlexaFluro 594: 1:1000) and developed with DAPI. To demonstrate specificity immunoreactions, negative controls (normal serum from the same species replaced the primary antibody) were included for all immunoreactions. Rat-1 cell line (ICC) and lung tissue (IHC) served as positive controls for NMM Ⅱ isoform detection (data not shown). For microscopic images, cells were visualized using the Olympus IX71 microscope (Olympus America, Inc). Images of NMM Ⅱ isoforms, αSMA, F-actin and DAPI were taken separately at identical exposures and color channels merged using IMAGE-PRO software (Media Cybernetics Inc).

siRNA-mediated inhibition

Freshly isolated HSCs were seeded at 2×10^6 cells on p60 tissue culture dishes and transfected on Day 3 of culture-activation with siRNAs for NMM Ⅱ isoforms (Ⅱ-A and Ⅱ-B) using Lipofectamine reagent. In a 5 mL polystyrene tube, NMM Ⅱ isoform or scramble siRNAs (final concentration was 100 nmol/L) was incubated with 600 μL OptiMEM and vortexed. In a separate 5 mL tube, 20 μL Lipofectamine reagent was incubated with 600 μL OptiMEM and vortexed. Solutions were combined, vortexed and incubated for 30 min at room temperature. Cells were washed and incubated with 1.5 mL OptiMEM. Cells were subsequently incubated with siRNA mixtures for 8 h. At the end of the transfection period, fresh media (2.5 mL) was added to wells and incubated for 48 h prior to analysis of mRNA and protein expression, contraction and migration analyses.

Preparation of collagen lattices

Contraction of HSCs was performed in a 24-well tissue culture dish coated with collagen as described with minor modifications[8,9]. Hydrated collagen lattices were prepared using an 8:1:1 of type I collagen: 0.2 mol/L HEPES: 10 × DMEM for final collagen concentration of 3.65 mg/mL. The mixture (300 μL) was aliquoted onto each well of a 24-well plate and allowed to congeal overnight at 37°C. Cells were serum-starved 24 h prior to seeding onto the congealed collagen lattice (3×10^5 cells/well).

Contraction assay

Collagen lattices were prepared and culture-activated HSCs (Day 4) trypsinized and seeded onto the congealed collagen lattice and allowed to recover overnight. Collagen lattices were dislodged from wells with a 10 μL pipette tip. Cells were treated with ET-1(1 nmol/L) to induce contraction. Images were captured with UVP BioSpectrum AC Imagining System at indicated time points and PTI ImageMaster software was used to measure changes in collagen diameter immediately following ET-1 treatment and 24 h later. The differences in collagen diameters were reported as percentage change in collagen lattice circumference, which is reflective of ET-1-induced contraction. Assays were repeated using transfected HSCs to assess effects of siRNA knockdown on ET-1-mediated contraction.

Migration assay

A sterile pipette tip was dragged through the cell sheet, creating a cleared zone 48 h after transfection. Images were immediately taken of the scrape in four locations per dish using the Olympus IX71 microscope. Twenty-four hours later, images were taken in the exact same locations. To assess the number of migrating cells, the PTI ImageMaster software was used to measure changes in the distance traveled into the 'damaged area' (cleared zone).

Blebbistatin treatment

Collagen-seeded HSCs (Day 5) were pre-treated with increasing doses (0-25 μmol/L; 5 μmol/L increments) of the active enantiomer (-)-blebbistatin (Blebbistatin/Bleb) or inactive enantiomer (+)-blebbistatin (Vehicle) for 30 min. The collagen lattices were dislodged from the wells and incubated with ET-1 (1 nmol/L) to induce contraction. To assess HSC contraction, the PTI ImageMaster software was used to measure changes in the collagen diameter over a 24 h incubation period.

Statistical analysis

Data are presented as mean ± SE. One-way ANOVA followed by Student-Newman-Keuls post hoc test was used to assess differences between groups at different stages of activation using Sigma Stat software. Results were considered significant for $P < 0.05$.

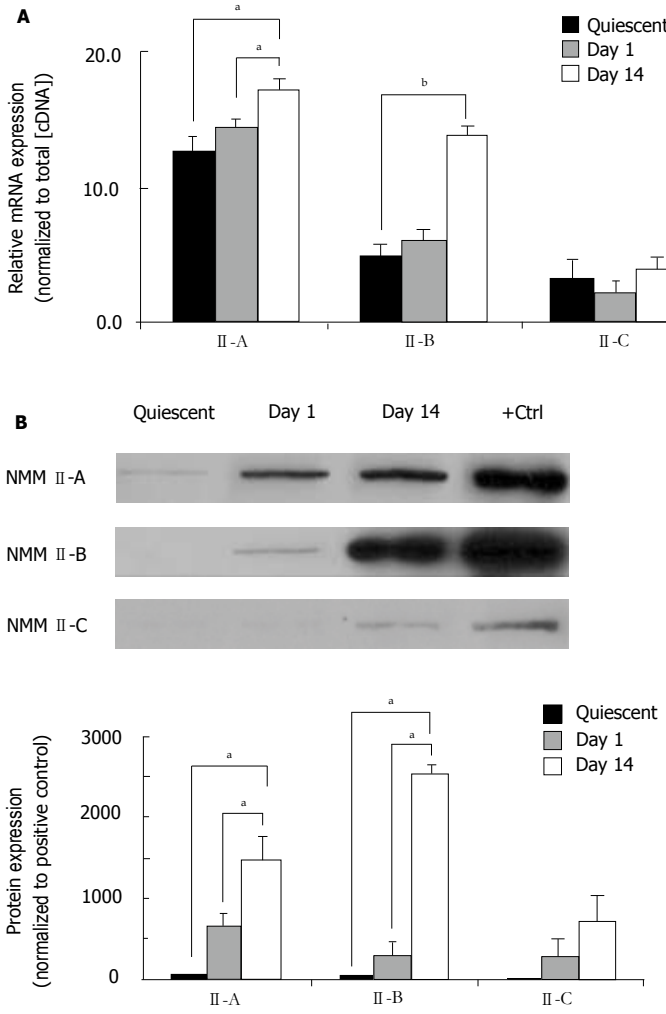


Figure 1 Relative mRNA and protein expression of nonmuscle myosin Ⅱ-A, Ⅱ-B and Ⅱ-C in hepatic stellate cells. A: mRNA expression of all isoforms was assessed in quiescent and culture-activated hepatic stellate cells (HSCs) (Day 1 and Day 14) by RealTime PCR. mRNA expression of all isoforms was normalized to total cDNA concentration. ($^aP < 0.05$; $^bP < 0.001$ as compared to quiescent). B: Protein expression of all three NMM Ⅱ isoforms was determined in quiescent and culture-activated HSCs (Day 1 and Day 14) by an actin-selection assay and subsequent Western blot analysis. Sample lysates were equalized for protein concentration using the bradford method and incubated with F-actin (10 mmol/L). (+ Ctrl; Rat-1 fibroblast cell line). Top panel: representative Western blots. Bottom panel: Western blot quantification using band intensity. ($^aP < 0.05$ as compared to quiescent).

RESULTS

NMM Ⅱ isoform expression

Throughout the HSC transdifferentiation process numerous qualitative and quantitative changes are associated with functional modifications that serve to accommodate normal or injured conditions. Our laboratory has recently demonstrated that HSC transdifferentiation from the quiescent to activated state results in significant morphological and gene expression changes[24]. Furthermore, significant alterations in the classic housekeeping genes are also present in culture activation. In the present study, we normalized gene expression in quiescent (freshly isolated), Day 1 (early activation) and Day 14 (late activation) HSCs to total cDNA concentration. RealTime PCR was performed to quantify mRNA expression of NMM Ⅱ-A and Ⅱ-B isoforms (Figure 1A). Our results demonstrate that expression was increased during transdifferentiation with significant increases seen in all pair-wise comparisons. Interestingly, mRNA expression of NMM Ⅱ-B increased 2.8-fold over culture-activation, whereas Ⅱ-A expression only increased 1.4-fold. NMM Ⅱ-C mRNA expression was not significantly altered following culture-activation. To quantify NMM Ⅱ protein we utilized an actin-selection assay, which takes advantage of the ability of actin to bind myosin in the absence of ATP[26]. Detectable levels of NMM Ⅱ-B and Ⅱ-C isoform protein expression were insignificant in quiescent HSCs (Figure 1B). Protein concentrations were doubled to verify lack of protein expression in quiescent HSCs since these results differed from mRNA expression; however, the intensities still remained negligible. Significant increases in protein expression were seen in Day 1 and Day 14 HSCs for both NMM Ⅱ-A and Ⅱ-B. NMM Ⅱ-C protein expression was also measured; however, significant levels of expression were undetected.

Cellular localization of NMM Ⅱ isoforms

Peak mRNA and protein expression levels were measured

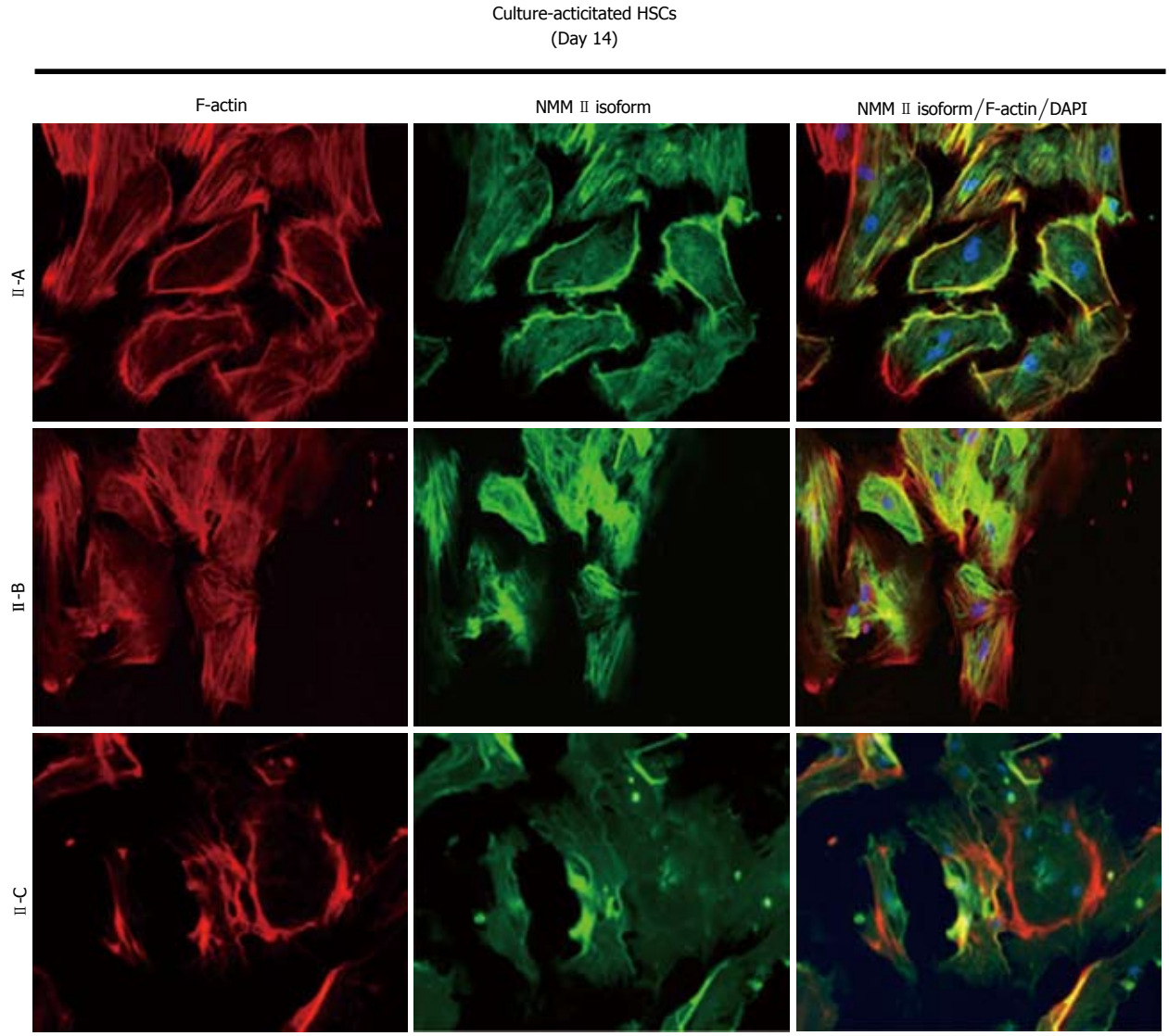


Figure 2 Immunocytochemical analysis of nonmuscle myosin Ⅱ-A, Ⅱ-B and Ⅱ-C in hepatic stellate cells. Expression of all three isoforms was detected in culture-activated cells (Day 14). Specific Ⅱ immunoreactivity of representative fields is shown in green; F-actin stress fibers (phalloidin) are shown in red and cell nuclei are stained blue (DAPI). Images of Ⅱ isoforms, F-actin, and DAPI were taken separately at identical exposures and color channels were merged using IMAGE-PRO software (350 ×).

in fully activated HSCs that are present during wound-healing; therefore, Day 14 cells were utilized for immunocytochemistry (Figure 2). Culture-activated HSCs exhibited characteristic stress fiber formation as detected by F-actin (rhodamine phalloidin) staining. All three NMM Ⅱ isoforms were detected in HSCs, which corresponded with mRNA and protein expression. Merged images revealed a stronger focus (yellow fluorescence) of NMM Ⅱ-A and Ⅱ-B with F-actin compared to Ⅱ-C. Additionally, NMM Ⅱ-A localization with F-actin showed a stronger intensity at the cell periphery, while Ⅱ-B was predominantly located throughout the cytoplasm.

NMM Ⅱ isoform expression in normal vs fibrotic liver

Increased isoform expression was seen in activated HSCs, the main effector cells in hepatic fibrosis. To confirm this observation *in vivo*, a BDL model of liver injury was utilized. BDL-induced fibrosis typically generates lesions surrounding bile duct epithelium and stimulates cholangiocyte proliferation, resulting in hepatic inflammation and injury[28]. HSCs respond to BDL-induced injury and transdifferentiate into activated myofibroblast-like cells, characterized by αSMA expression. NMM Ⅱ-A and Ⅱ-B protein expression was minimally detected in normal liver tissue, while BDL liver tissue showed up-regulation of all three NMM Ⅱ isoforms (green fluorescence), correlating with *in vitro* data. αSMA (red fluorescence) expression was observed in BDL-injured tissue (Figure 3) and, interestingly, NMM Ⅱ-B was the only isoform found to merge (yellow fluorescence) with activated HSCs in BDL liver tissue.

Inhibition of NMM Ⅱ isoforms

siRNA-mediated inhibition was utilized to perform spe-

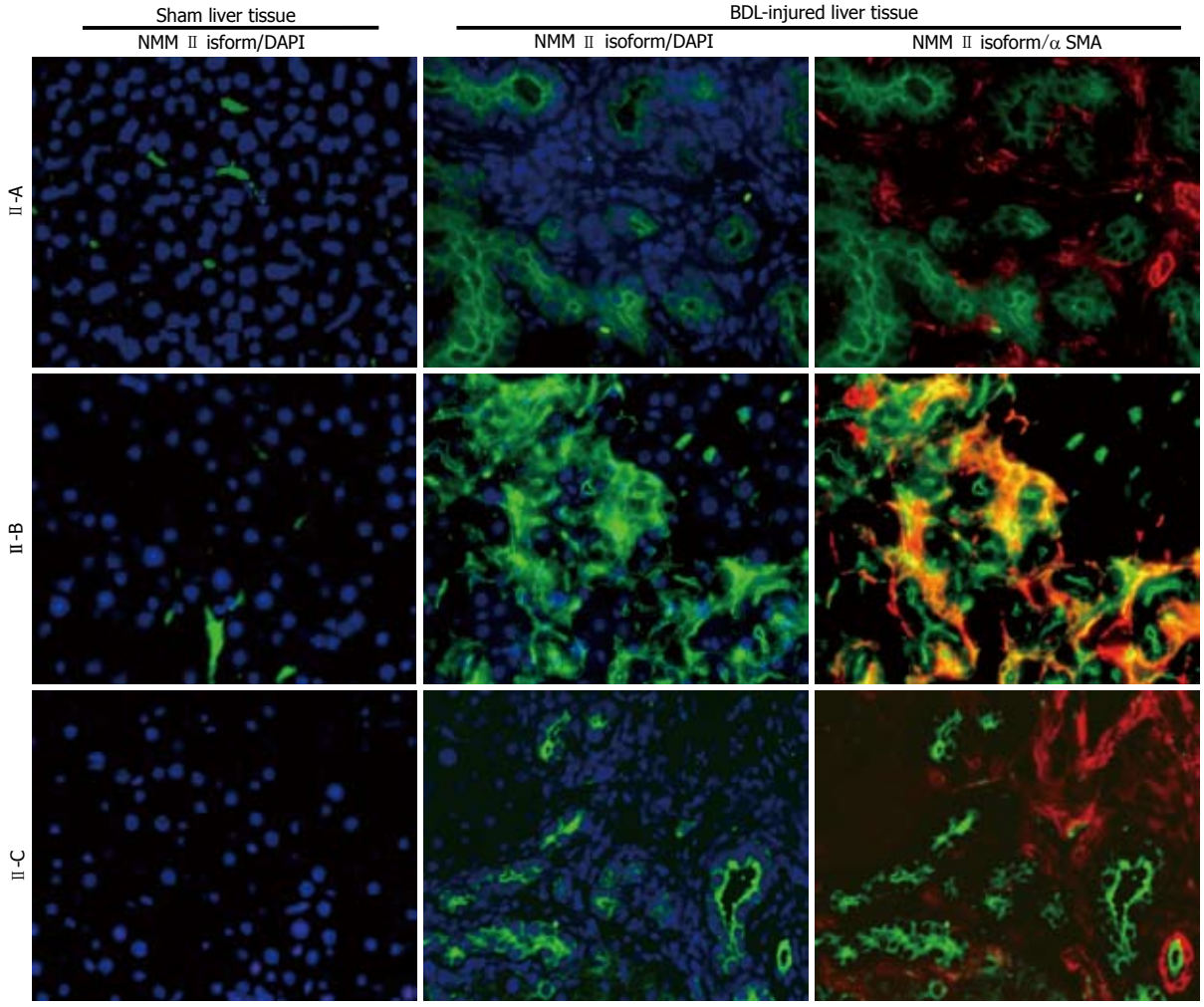


Figure 3 Immunohistochemical analysis of nonmuscle myosin Ⅱ-A, Ⅱ-B and Ⅱ-C in normal and injured liver sections. Specific nonmuscle myosin (NMM) Ⅱ immunoreactivity of representative fields is shown in green; αactin smooth muscle, as a marker of hepatic stellate cell activation is shown in red and cell nuclei are stained blue (DAPI). Images of NMM Ⅱ isoforms, F-actin, and DAPI were taken separately at identical exposures and color channels were merged using IMAGE-PRO software (200 ×).

cific knockdowns of NMM Ⅱ-A and Ⅱ-B in primary activated HSCs given that Ⅱ-C mRNA and protein expression was not significantly altered during transdifferentiation (Figure 3). Cells were transfected with the appropriate siRNA (100 nmol/L). RealTime PCR confirmed successful inhibition of NMM Ⅱ-A (60% reduction compared to scramble) (Figure 4A) and Ⅱ-B (56% reduction compared to scramble) (Figure 4B). Similarly, transfections with NMM Ⅱ-A or Ⅱ-B resulted in reduced protein expression, 52% and 49% respectively (Figure 4C and 4D). Additionally, siRNA inhibition specificity was shown as indicated by no reduction in NMM Ⅱ-A expression when transfected with Ⅱ-B siRNA alone (Figure 4A). Parallel experiments also demonstrated specific knockdown of NMM Ⅱ-B (Figure 4B).

Effect of NMM Ⅱ isoform inhibition on HSC migration and contraction

To determine functional contributions of NMM Ⅱ isoforms in the HSC, culture-activated cells (Day 5) were treated with scramble, Ⅱ-A, Ⅱ-B or Ⅱ-A and Ⅱ-B siRNAs. Using a plate scrape model of injury-induced migration, location-specific images were taken prior to and 24 h following damage and gap distance was marked and measured. As compared to the scramble siRNA, all isoform permutations displayed impaired migratory properties (Figure 5). Quantitative analysis of the change in gap distance revealed significant decreases with both siRNA treatments indicating importance of these molecular motors in HSC migration (bottom panel).

To further investigate additional known functions of NMM Ⅱ-A and Ⅱ-B, HSCs were transfected with specific siRNAs and subsequently treated with ET-1 for 24 h to induce contraction on collagen lattices. The effect of isoform inhibition was determined by changes in gel circumference after 24 h as compared to scramble control. Results indicated that siRNA-mediated knockdown did not result in inhibition of ET-1-induced contraction (Figure 6). Several adjustments to the contraction assay were made to validate results. siRNA concentration and incubation period were changed, in addition to altering matrix stiffness; however, these modifications also demonstrated

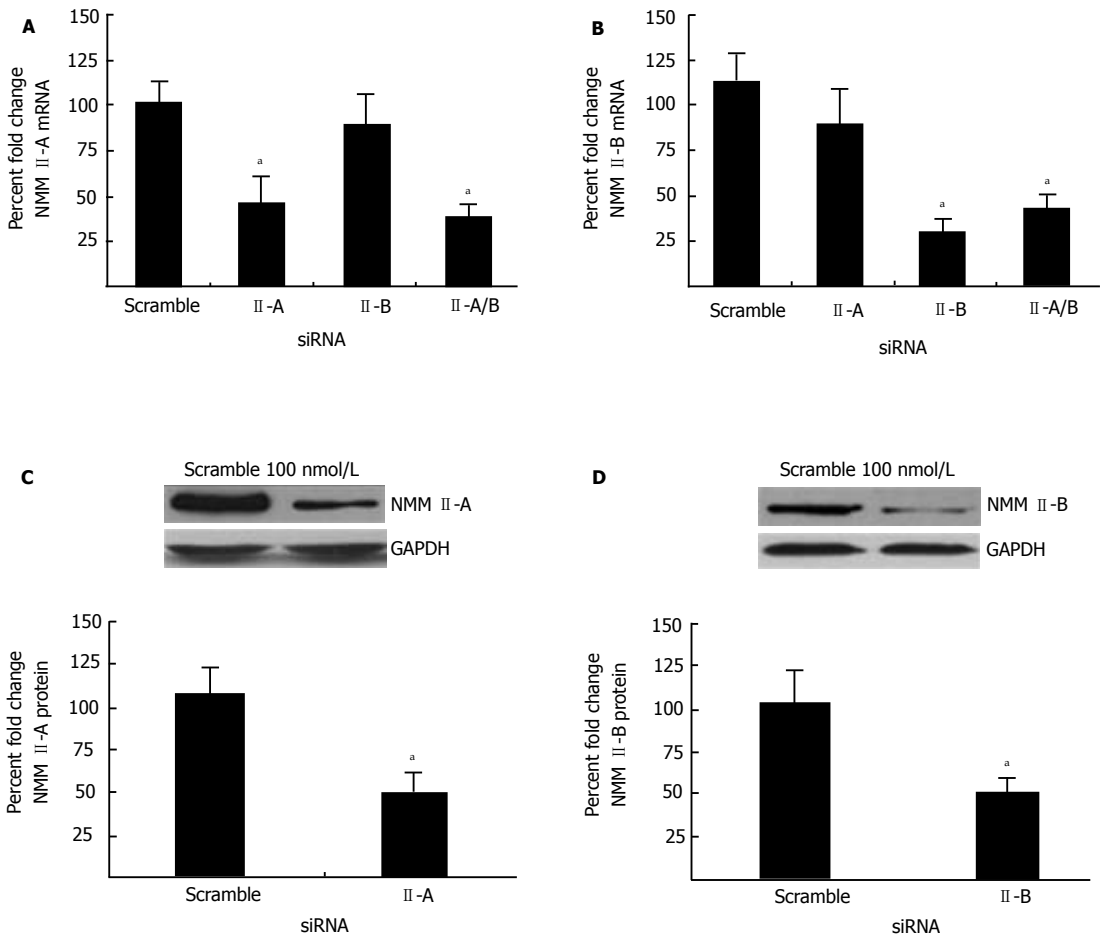


Figure 4 Nonmuscle myosin Ⅱ isoform inhibition. A and B: Culture-activated hepatic stellate cells (Day 3) were incubated with nonmuscle myosin Ⅱ-A or Ⅱ-B, Ⅱ-A & Ⅱ-B, or scramble siRNA (100 nmol/L) for 48 h. RealTime PCR determined isoform-specific inhibition of each isoform as normalized to total cDNA concentration and compared to scramble control. C and D: In parallel experiments cell lysates were isolated and protein inhibition determined by western blot analysis. Protein expression was normalized to GAPDH and compared to scramble control ($^aP < 0.05$ as compared to scramble).

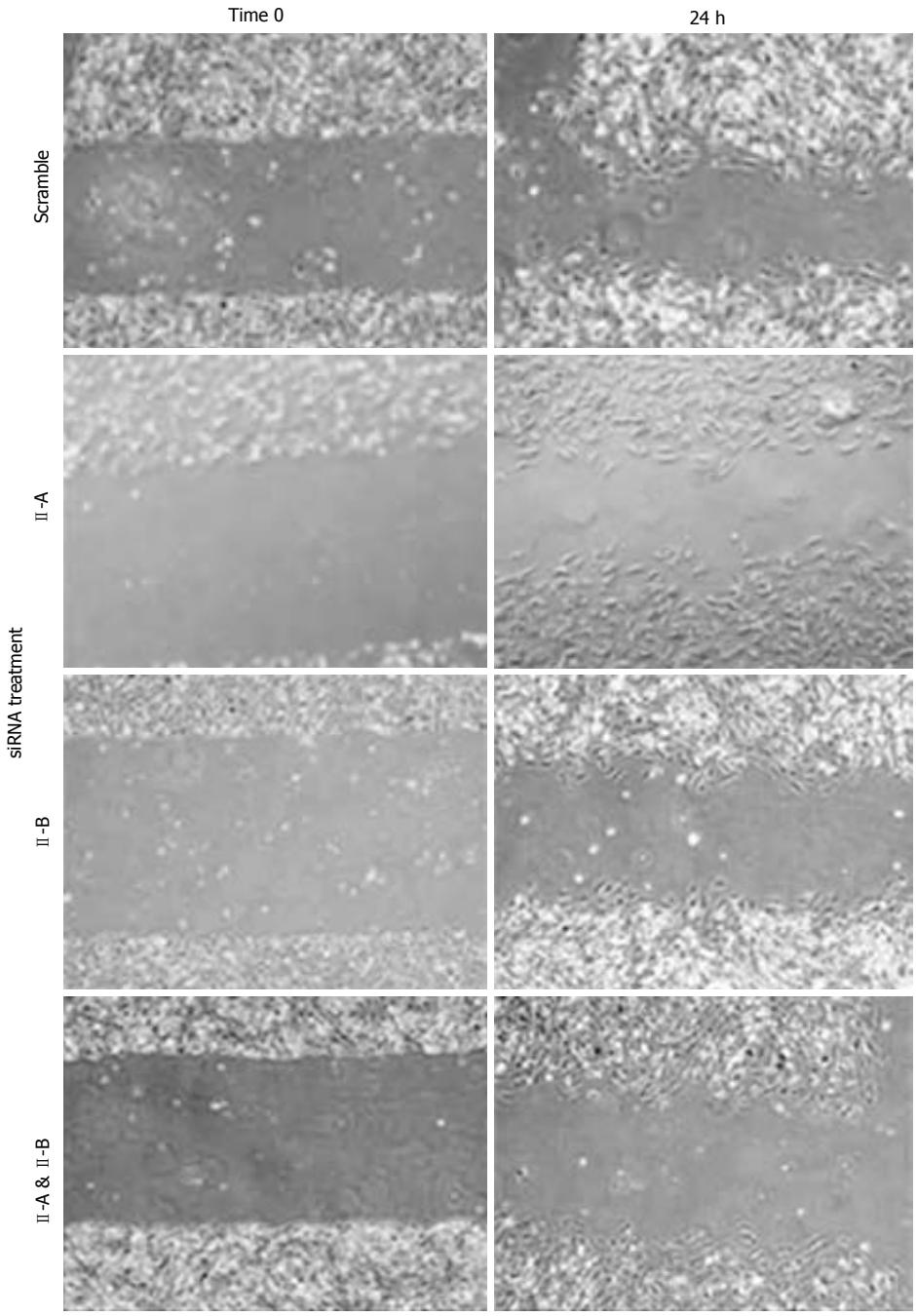
that specific inhibition of NMM Ⅱ isoforms does not alter rat HSC contraction capabilities.

Chemical inhibition of NMM Ⅱ isoforms

Studies conducted utilizing culture-activated HSCs (Day 10) demonstrated myosin Ⅱ chemical inhibition significantly attenuated ET-1-induced HSC contraction[18]. However, our studies demonstrated that gene isoform inhibition by siRNA revealed no effect on contractile properties/function. Therefore, to determine possible contributions of other myosin Ⅱ family members, we used the chemical inhibitor, blebbistatin, in our studies. Day 5 HSCs are most responsive to ET-1-induced contraction[29]; therefore, Day 5 HSCs were pre-treated with increasing doses of blebbistatin (0-25 μmol/L) prior to ET-1 (1 nmol/L) treatment (Figure 7A). Twenty-four hours following chemical treatment, collagen lattice was imaged (top panel) and differences in collagen lattice diameter reported as percentage change in gel circumference (bottom panel). Consistent with previous findings, HSCs exerted a contractile force, which resulted in a 22 ± 3% decrease in collagen lattice circumference (white bar). ET-1 treatment induced hypercontraction and as expected, blebbistatin pretreatment abolished the aforementioned effect in a dose dependent manner (black bars), while vehicle pretreatment permitted ET-1-induced contraction (grey bars). Quantitative analysis revealed that 5 μmol/L blebbistatin treatment significantly reduced HSC contraction, as did higher doses of the pharmacological inhibitor. Consistent with previous findings, micrograph images of HSCs seeded onto collagen lattice exhibited a contractile star-like shape (Figure 7B). In response to ET-1 stimulus, myosin Ⅱ activation resulted in HSC elongation along the cellular axis as previous described[18,30], while chemical inhibition of myosin Ⅱ activation restored original cellular shape.

DISCUSSION

Chronic injury and unresolved fibrosis perpetuates HSC activation and further promotes the deleterious clinical effects of portal hypertension, which is associated with both increased portal blood flow and augmented intrahepatic vascular resistance[6,7]. In characterizing the expression profile of specific NMM Ⅱ isoforms during differ-



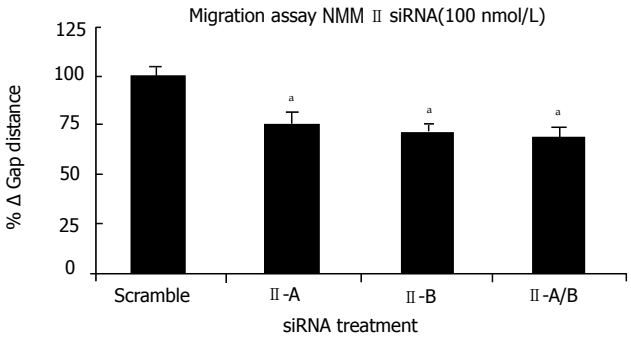


Figure 5 Effect of nonmuscle myosin Ⅱ inhibition on hepatic stellate cell migration. Culture-activated hepatic stellate cells (Day 3) were transiently transfected with siRNA targeted to all nonmuscle myosin (NMM) Ⅱ permutations (NMM Ⅱ-A, Ⅱ-B, Ⅱ-A & Ⅱ-B) or scramble siRNA and incubated for 48 h. A plate scrape model of migration was used to simulate liver injury. After 24 h, migration was calculated as change in wound (gap) diameter over time. Top panel: representative micrographs. Bottom panel: Migration assay quantification. ($^{a}P < 0.05$ as compared to scramble).

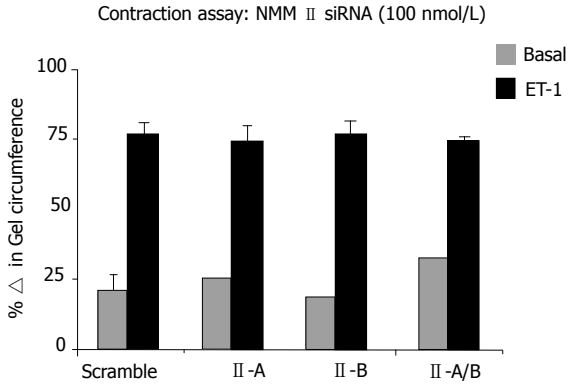


Figure 6 Effect of nonmuscle myosin Ⅱ inhibition on hepatic stellate cell contraction. Culture-activated hepatic stellate cells (Day 3) were transiently transfected with siRNA targeted to nonmuscle myosin(NMM) Ⅱ-A, Ⅱ-B, Ⅱ-A and Ⅱ-B or scramble siRNA (100 nmol/L and allowed to incubate for 24 h. Cells (1×10^5) were seeded onto collagen lattices overnight, serum-starved for 24 h and subsequently treated with ET-1 (1 nmol/L). Twenty-four hours following chemical treatment, hepatic stellate cell contraction was quantified using PTI ImageMaster software and reported as percentage change in gel circumference.

ent stages of HSC transdifferentiation, we were able to evaluate individual components that may be responsible for the fibrogenic response of activated HSC migration. In agreement with our findings, recent studies in mouse HSCs indicated that an increase in NMM Ⅱ isoform expression regulates cellular motility[18,31]; however, in contrast to these studies, we report that NMM Ⅱ isoforms in rat HSCs increases cellular migration.

Isoform-specific localization of NMM Ⅱ along actin stress fibers has previously been ascribed to cellular functions in multiple cell types[32]. Specifically, in bovine aortic endothelial cells, NMM Ⅱ-A merges with actin filaments at the leading edge of cells, while NMM Ⅱ-B merges within the cytoplasm, which facilitates endothelial cell expansion under diseases states[33]. In contrast to these findings, studies in mouse HSCs demonstrated that NMM Ⅱ-A is distributed along αSMA-containing stress fibers following culture-activation, while NMM Ⅱ-B is located at the leading edge of lamellipodia[18]. In our studies, upregulation of NMM Ⅱ-A and Ⅱ-B expression was associated with F-actin stress fibers in the cellular periphery and throughout the cytoplasm of rat HSCs respectively. These results are consistent with previous studies in fibroblast cells suggesting that NMM Ⅱ-A activation facilitates rearrangement of actin bundles into cellular protrusions and NMM Ⅱ-B incorporates into cytoplasmic stress-fibers[34].

While the expression of the NMM Ⅱ isoforms is relatively ubiquitous in most nonmuscle cells it has yet to be determined whether these proteins play redundant, overlapping or distinct roles in performing various mechanical functions in rat HSCs. Therefore, we per-formed migration and contraction studies using siRNA-mediated knockdown of NMM Ⅱ-A and Ⅱ-B, which were upregulated during culture-activation of HSCs *in vitro*. While Liu *et al* reports that siRNA-mediated NMM Ⅱ-A inhibition increased cellular migration in mouse HSCs[18,31], our studies demonstrated that NMM Ⅱ-A and Ⅱ-B knockdown significantly reduced the migratory capacity of rat HSCs. While previously studies have suggested that isoform specific rearrangement of actin stress fibers may be responsible, in part, for differences in migration rates among different cell types[32], further analysis is needed to validate these conflicting species-specific findings.

Based on the kinetic properties of NMM Ⅱ-B, it has been proposed that this isoform may be involved in maintaining tonic force needed for particular cellular functions such as hypercontraction[14]. Furthermore, it has been demonstrated that the loss of NMM Ⅱ-B decreases 3D collagen gel contraction[35]. While Liu *et al* reported that NMM Ⅱ-A is the essential isoform necessary for contraction in mouse HSCs, our results indicated that NMM Ⅱ-A and/or Ⅱ-B knockdown in rat HSCs does not significantly alter basal or ET-1-induced contraction. In order to confirm these results, we optimized our contraction assay by increasing siRNA concentration and incubation times, altering collagen lattice concentration and cell number; however, changing these parameters had no effect on HSC contraction (data not shown). In the studies performed by Meshel *et al*, NMM Ⅱ-B$^{-/-}$ fibroblast demonstrated significant differences in cell movement and contraction depending on experimental substrate[26,35]; therefore, it is possible that technical differences in experimental design may explain our conflicting results. Future studies will more closely examine the contribution of NMM Ⅱ isoforms using a Cre-lox recombination system to completely inhibit these isoforms and assess HSC hypercontraction.

While cellular localization suggests possible mechanisms by which NMM Ⅱ may function in the diseased state, further investigation *in vivo* was explored using a bile-duct ligation (BDL) model of hepatic injury. Obstruction of the common bile duct initiates rapid proliferation of biliary cholangiocytes and inflammation[36]. Following epithelial expansion, sustained blockage of bile flow causes continual activation of HSCs in the periductal region, which promotes biliary fibrosis. In our studies, NMM Ⅱ-B expression was evident in activated HSCs during BDL-induced hepatic fibrosis, while NMM Ⅱ-A and Ⅱ-C were only associated with biliary cholangiocytes. These results may suggest that NMM Ⅱ-A and Ⅱ-C may not be detectable in HSC in the *in vivo* setting. Previous studies have identified an important role for NMM Ⅱ in cellular adhesion and collagen remodeling during wound repair[26,35]. While NMM Ⅱ-B may be the important isoform contributing to the development and progression of hepatic fibrosis, NMM Ⅱ-A and Ⅱ-C may be contributing to the initiation of the inflammatory response by stabilizing integrins and other cellular adhesion molecules. Together, the *in vitro* and *in vivo* data suggest that each NMM Ⅱ isoform may be responsible for specific molecular functions during liver injury.

Although siRNA data confirmed that successful knockdown of one isoform does not influence expression of another NMM Ⅱ isoform (Figure 4A and 4B), it is plau-

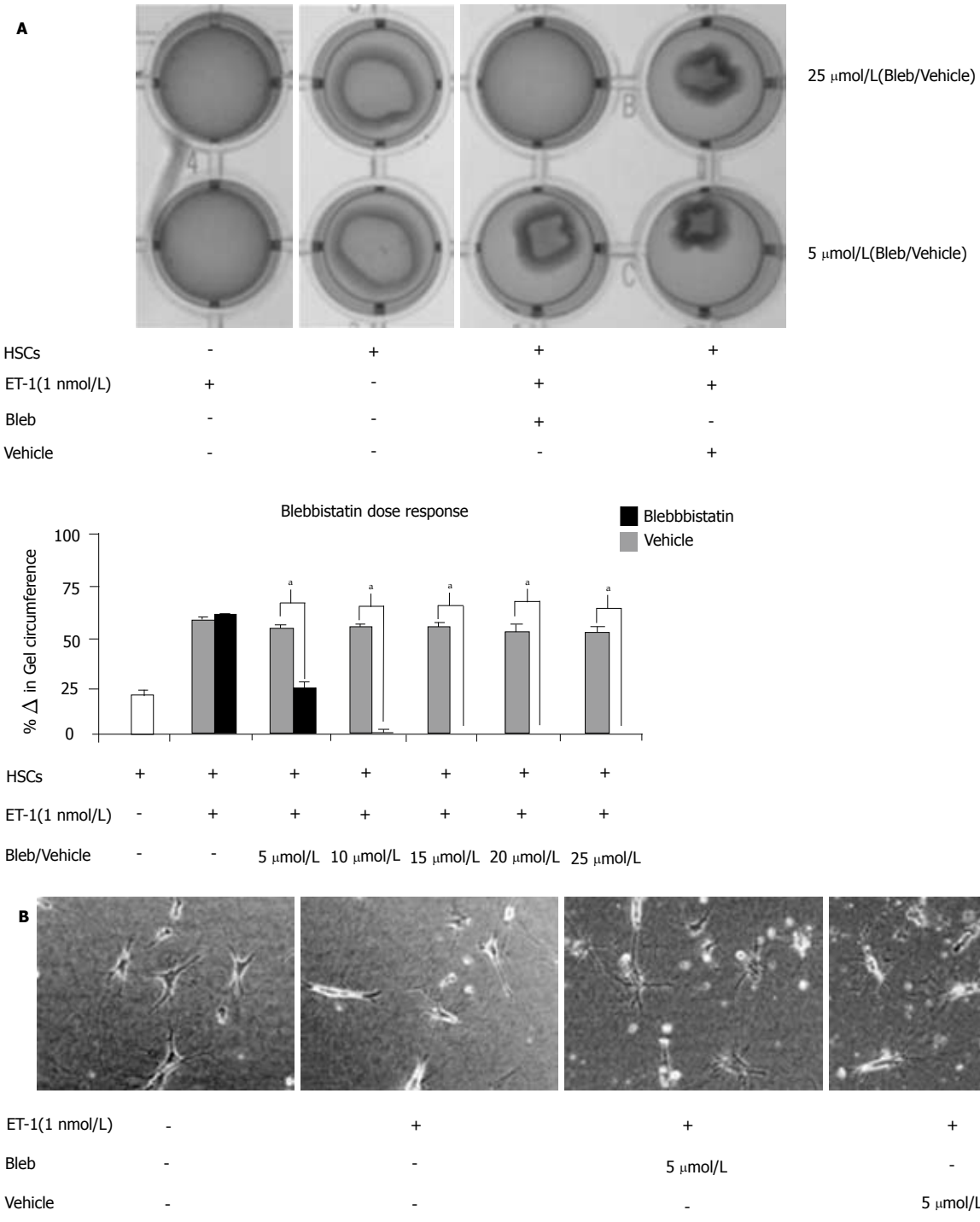


Figure 7 Blebbistatin-inhibited endothelin-1-induced hepatic stellate cell contraction. Culture-activated hepatic stellate cells(HSCs) (Day 4) were serum-starved 24 h prior to seeding onto collagen lattices (3×10^5 cells/well). Cells were pretreated with inactive (Vehicle) or active (Blebbistatin) myosin Ⅱ inhibitor in increasing doses (0-25 μmol/L; 5) and subsequently treated with blebbistatin-inhibited endothelin-1 (ET-1) (1 nmol/L) after 30 min. ($^aP < 0.05$ as compared to scramble). A: Representative light micrographs of collagen lattices: with or without seeded HSCs; with or without chemical treatments (top panel). Twenty-four hours following chemical treatment, hepatic stellate cell contraction was quantified using PTI ImageMaster software and reported as percentage change in gel circumference (bottom panel). B: Representative light micrographs of collagen-seeded HSCs with or without chemical treatments.

sible that these findings resulted from a species-specific replacement to compensate for the deficiency of one isoform by producing a functionally equivalent signaling mechanism to activate migration and contraction using other myosin Ⅱ classes. Since HSCs are nonmuscle cells, the focus of the current study was to evaluate NMM Ⅱ isoforms; however, HSCs express a large number of early and late smooth muscle cell markers including α smooth muscle (SM) actin, SM22a, desmin, SM myosin heavy chain, h1-calponin, h-caldesmon and myocardin, which also may contribute to HSC migration and hypercontraction during hepatic injury[37]. Alternatively,

HSC migration and hypercontraction may be controlled by independent signaling mechanisms that are mutually exclusive, partly explaining the observed species-specific differences. While upregulation of the Rho signaling pathway in activated HSCs confers an increase in contractile potential[38-40], Rac phosphorylation of the MHC induces filament instability, promotes disassembly of actomyosin complexes and decreases migration[41-44]. In the current studies, siRNA knockdown of NMM Ⅱ isoforms does not eliminate the possibility that actin stabilization and/or other myosin Ⅱ sub-classes may be contributing to the hypercontractile phenotype of activated HSCs. Consistent with previous reports, micrograph images from our studies suggest that ET-1-induced contraction is associated with HSC cytoskeletal remodeling and cellular hypercontraction (Figure 7B). In order to facilitate contraction, NMM Ⅱ may mediate cellular stretching and elongation, while other myosin Ⅱ classes may be responsible for coordinated force generation. Therefore, we used the chemical inhibitor blebbistatin, which has previously been shown to block protrusion-mediated lamella formation and cellular contraction[18,45]. By using this selective inhibitor, in combination with our siRNA data, we demonstrate that in rat HSCs myosin Ⅱ is the protein responsible for ET-1 induced cytoskeletal remodeling and hypercontraction; however, we concede that this controversial chemical inhibitor is not specific to NMM Ⅱ. Differences between each experimental approach suggest that other myosin Ⅱ sub-classes may also contribute to the contractile phenotype of rat HSCs.

In addition to sinusoidal constriction being associated with portal hypertension, hepatic microcirculatory failure contributes to end-organ failure in septic patients[46]. Prolonged oxygen deprivation of abdominal organs results in ischemia, tissue damage and necrosis culminating in increased mucosal permeability[47]. Endotoxin and microbial debris can subsequently penetrate the gut wall and permeate into the portal and hepatic artery where sinusoidal endothelial cells (SECs) and Kupffer cells (KCs) establish the first line of inflammatory defense[48]. KCs release a host of inflammatory cytokines, while activated SECs lose their normal anticoagulant state, promote leukocyte infiltration and increase secretion of ET-1. Neighboring HSCs are responsive to these inflammatory and vasoconstrictor signals, which promotes sustained HSC activation and contractility. Compelling evidence has demonstrated the efficacy of targeting SEC/leukocyte interactions, which improved sinusoidal congestion and portal hypertension[49]. Effective experimental treatments have also targeted KC activation, which results in preservation of hepatic function and improved survival after sepsis[50]. In addition to current treatment modalities, manipulation of migrating, hypercontractile HSCs may also improve hepatic microcirculation and patient survival during sepsis. While modulating HSC contraction may improve the microcirculation, controlling migration may prove to be beneficial in ameliorating the severity of fibrosis by decreasing the rate of collagen formation[35]. Portal hypertension remains the main cause of morbidity and mortality in patients with cirrhosis[51]. Although progress has been made in understanding the pathophysiology of portal hypertension, current pharmacological therapies have been limited to non-selective beta-blockers[52] and statins[53]; however,these treatments result in vasomodulation of the *splanchnic circulation*[54]. Given that increased hepatic microvascular resistance to portal circulation is the leading factor in cirrhotic portal hypertension, a direct molecular therapy may be more effective. Modulating intrahepatic vascular tone may provide additive benefit in patients suffering with unresolved fibrosis and cirrhosis, thus targeting all complications associated with portal hypertension. Therefore, delineating the role of NMM Ⅱ isoforms in HSC-associated portal hypertension could lead to new therapeutic targets.

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COMMENTS

Background

Hepatic fibrosis results from normal wound-healing processes going awry and is the main cause of increased intrahepatic vascular resistance during liver injury. When the injury is chronic, type Ⅰ collagen deposition by hepatic stellate cells (HSCs) exceeds collagen resolution as a result of imbalance between fibrogenesis and fibrolysis. Altered extracellularmatrix (ECM) architecture and mechanical distortion culminates in increased blood pressure in the portal venous system, as blood must be diverted away from the liver. In addition to occlusion and compression of the microvasculature by excess collagen deposition, HSC hypercontractility contributes to increased resistance of the sinusoids leading to the clinical manifestation of portal hypertension. HSCs regulate intrahepatic vascular resistance and blood flow at the sinusoidal level through upregulation and activation of motor proteins. Specifically, HSC cytoskeletal remodeling, migration and hypercontraction has been previously associated with nonmuscle myosin (NMM) Ⅱ upregulation and activation. Function of NMM Ⅱ isoforms (Ⅱ-A, Ⅱ-B and Ⅱ-C) have been previously characterized in migrating fibroblasts and mouse HSCs, suggesting an essential role in perpetuation of chronic liver injury.

Research frontiers

Through its effects on cytoskeletal remodeling, targeting NMM Ⅱ may provide a novel mechanism to modulate multiple interrelated pathways such as cellular migration, adhesion and ECM remodeling.

Innovations and breakthroughs

Recently, Liu *et al*[18] reported that siRNA-mediated NMM Ⅱ-A inhibition increased cellular migration in mouse HSCs; however, our results suggest both NMM Ⅱ-A and Ⅱ-B mediate rat HSC migration. Consistent with findings by Vicente-Manzanares *et al*[34], our results demonstrate that NMM Ⅱ inhibition decreases cellular components associated with migration such as cytoskeletal remodeling and elongation. In addition, our studies are the first to report the expression profile of NMM Ⅱ isoforms in a fibrotic injury model *in vivo*. Finally, studies have shown the pharmacological inhibitor, blebbistatin blocks skeletal muscle and NMM Ⅱ activity with minimal effects on smooth muscle myosin Ⅱ, while others have shown that blebbistatin is specific to smooth muscle myosin Ⅱ. Conversely, we demonstrate that in rat HSCs this controversial chemical inhibitor is not specific to NMM Ⅱ.

Applications

Although progress has been made in understanding the pathophysiology of fibrosis and portal hypertension, current pharmacological therapies have been limited to treatments, which result in vasomodulation of the *splanchnic circulation*. Given that increased hepatic microvascular resistance to portal circulation is the leading factor in cirrhotic portal hypertension, a direct molecular therapy may be more effective. Modulating intrahepatic vascular tone may provide additive benefit in patients suffering with unresolved fibrosis and cirrhosis, thus targeting all complications associated with portal hypertension. Therefore, delineating the role of NMM Ⅱ isoforms in HSC-associated portal hypertension could lead to new therapeutic targets.

Terminology

Quiescent HSCs: Under physiological conditions, the inactivated cell projects extensive cytoplasmic processes through the space of Disse and reach between hepatocytes and endothelial cells wrapping around neighboring sinusoids similar to tissue pericytes suggesting a functional role in maintenance of vascular tone similar to smooth muscle cells. Quiescent HSCs also play a vital role in normal matrix maintenance and remodeling, similar to a fibroblast. Activated HSCs: HSCs proliferate, lose retinol droplets, increase expression of alpha smooth muscle actin and secrete excess type Ⅰ collagen for matrix repair. Increased micro-projections from the myofibroblast allow for increased chemotactic signaling, which induces cellular migration to the site of injury. Because of the anatomical location and increased contractile apparatus expression, it has been suggested that HSCs are capable of disrupting liver blood flow by hypercontracting, impeding microcirculation and contributing to portal hypertension. Culture-activated HSCs: Transdifferentiation of quiescent HSCs into the activated state *in vitro* is routinely accomplished by culturing cells on plastic tissue culture dishes, which mimics the *in vivo* environment of hepatic fibrosis. Blebbistatin: A small pharmacological inhibitor with high binding affinity for myosin Ⅱ, which blocks the motor protein in an actin-detached state. Actinomyosin complex: Produced when bipolar myosin filaments interact with polymerized actin filaments to exert tension or produce movement. Lamella: A network of actin fibers which forms the outer edge of cellular protrusions.

Peer review

It's an interesting study and excellent.

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BRIEF ARTICLE

An extended treatment protocol with pegylated interferon and ribavirin for hepatitis C recurrence after liver transplantation

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Abstract

AIM: To evaluate the efficacy and tolerability of an extended treatment protocol and to determine the predictors of sustained virological response (SVR) after liver transplantation (LT).

METHODS: Between August 2005 and November 2008, patients with recurrent hepatitis C virus (HCV) after LT were selected for treatment if liver biopsy showed at least grade 2 inflammation and/or stage 2 fibrosis. All patients were to receive pegylated interferon (PEG)/regimens combining ribavirin (RBV) for an additional 48 wk after HCV undetectability.

RESULTS: Extended protocol treatment was initiated in thirty patients. Overall, 73% had end of treatment response and 60% had SVR. Nineteen patients completed treatment per protocol, of them, sixteen (84%) had end of treatment response, and fourteen (74%) achieved SVR. Both early virological response and 24-week virological response were individually associated with SVR but this association was not significant on multivariate analysis. Eleven patients (37%) discontinued therapy due to adverse effects. Cytopenias were the most common and most severe adverse effect, and required frquent growth factor use, dose adjustments and treatment cessations. The risk of rejection was not increased.

CONCLUSION: Recurrent HCV after LT can be safely treated with extended virological response-guided therpy using PEG/RBV, but requires close monitoring for treatment-related adverse effects, particularly cytopenias.

Key words: Hepatitis C virus; Liver transplantation; Extended treatment protocol

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INTRODUCTION

Hepatitis C virus (HCV)-related end-stage liver disease is the leading indication of liver transplantation (LT) in the United States and Europe[1]. HCV recurrence after LT is almost universal and occurs early, with histological recurrence observed in up to 70% of patients during the first year after LT[2]. Cirrhosis develops in up to 30% of transplant recipients after 5 years with persistant HCV viremia[2], and may be associated with graft failure[3] and the need for re-transplantation. This leads to lower patient survival rates compared to non-HCV transplant recipients[4]. Eradicating HCV using antiviral therapy improves patient and graft survival[5-7].

Regimens combining ribavirin (RBV) with pegylated interferon (PEG) report rates of sustained virological response (SVR), ranging from 28% to 45% with up to 48 wk of treatment[8-13]. Currently, there are no established guidelines to determine the timing and type of HCV treatment after LT. In general, the approach to the treatment of HCV after liver transplantation is similar to the pre-transplant protocol of 48 wk of treatment with viral kinetic evaluation at 12 and 24 wk[8,10-12].

Patients with recurrent HCV after LT are likely to be slow virological responders due to immunosuppression and, therefore, SVR after 48 wk of antiviral therapy is ex-pected to be lower than in immune-competent HCV patients. A few centers have reported improved SVR rates after extending treatment to 72 wk or longer in partial early virological responders (Partial EVR)[14-16]. Partial EVR is currently defined as achieving a 2-log drop in the HCV RNA pre-treatment levels at 12 wk, but not achieving HCV RNA undetectability until after 24 wk of treat-ment. Based on these observations, in August 2005 we designed a viral kinetics-driven treatment protocol that extended PEG/RBV combination therapy in order to maintain viral undetectability for an additional 48 wk of therapy. Our aim was to evaluate the safety and efficacy of this approach in patients who had significant HCV recurrence after LT, as determined by protocol liver biopsies.

MATERIALS AND METHODS

This is an IRB approved study.

Patient selection

Consecutive patients with HCV post-LT who were treated between August 2005 and November 2008 were screened for their eligibility for this study. Six patients were unable to start treatment due to relocation (3), non-compliance (2), or death from early recurrent cirrhosis and sepsis (1). Treatment was initiated in 30 patients. Eligibility criteria were LT for HCV-related end-stage liver disease, the presence of HCV RNA in serum by polymerase chain reaction (PCR), and histologically-proven chronic hepatitis in the graft with at least grade 2 inflammation and/or stage 2 fibrosis on METAVIR scoring of protocol liver biopsies. Additionally, antiviral therapy was initiated if features of aggressive disease (portal fibrosis or moderate-severe necroinflammation) were present on clinically indicated liver biopsies that occurred outside protocol times within the first year. Patients were ineligible for this study if they had unresolved acute or chronic rejection, severe cardiovascular disease, a history of autoimmune disease, coexistent hepatitis B, unresolved biliary complications, active alcohol use, decompensated cirrhosis, renal transplantation, untreated major depression, uncontrolled diabetes, clinically significant retinopathy or thyroid dysfunction, hemoglobin < 10 g/dL, absolute neutrophil count < 1000/mm^3, platelet < 60 000/mm^3, creatinine clearance < 50 mL/min, or patient refusal. Erythropoietin was used preemptively to increase hemoglobin to more than 10 g/dL prior to treatment in otherwise suitable treatment candidates. Data collected and analyzed included the following: patient demographics, viral genotype, interval between LT and initiation of treatment, body mass index, histological features (grade and stage), immunosuppressive therapy used, HCV viral load at baseline and kinetics during therapy, adverse events, dose adjustments and discontinuation, and the need for hematopoietic growth factors. The previous treatment records for these patients were unavailable.

Histology

A liver biopsy was performed, as per protocol, in all patients at 6 mo after LT, and then annually, to evaluate for hepatitis recurrence and to exclude histologic evidence of graft rejection and other viral infections. Samples were evaluated for inflammation (grade) and fibrosis (stage) us-ing the METAVIR scoring system. Histological assessment was carried out by our pathologists utilizing standard pro-cessing techniques and criteria. Patients who had histolo-gical evidence of cirrhosis underwent abdominal ultrasound and upper endoscopy to screen for hepatocellular carcinoma and varices, respectively.

Immunosuppression

Initial immunosuppression in all patients included a calcineurin inhibitor (tacrolimus or cyclosporine) and corticosteroids. Mycophenolate mofetil was used either as part of the initial triple immunosuppressive regimen or added later as maintenance immunosuppression. If possible, pre-dnisone was withdrawn over the first nine months after LT. No adjustments in immunosuppression took place after antiviral treatment had been initiated. Our practice of minimizing overall immunosuppression during the first year after LT was followed in all patients. Antiviral treatment was discontinued once rejection had been determined on histology, which was treated by increasing the calcineurin-inhibitor dose, with or without steroid use.

Virologic assays

Serum HCV RNA was measured by a quantitative assay (COBAS AmpliPrep or TaqMan, Roche Diagnostics, sensitivity limit 600 IU/mL) at baseline. A qualitative assay (Roche, sensitivity limit 50 IU/mL) was used at week 4, 12, at end of treatment, and every 12 wk after the end

of treatment. Rapid virological response (RVR) was defined as viral undetectability at week 4 of treatment. EVR was defined as 2-log drop in viral count at week 12 of treatment. End of treatment (EOT) response was defined as viral undetectability at end of treatment. SVR was defined as a negative qualitative HCV-RNA assay 24 wk after the end of therapy. High viral load was defined as HCV RNA of more than 800 000.

Treatment regimen

All patients were treated with Pegylated Interferon alpha 2a (Pegasys, Hoffman-La Roche, Inc. Nutley, NJ) and RBV. The initial dose of 180 mcg/week was used in patients who had transplants more than two years earlier and had an absolute neutrophil count (ANC) of > 1500/mm^3. Otherwise, an escalating dose regimen starting at 90 mcg/week, increasing as tolerated to a full dose of 180 mcg/week over 8 wk, was used. RBV was started at a dose of 10 mg/kg per day in patients who had transplants more than 2 years earlier and was increased as tolerated to 13-15 mg/kg per day over 4 to 6 wk. If fewer than 2 years had elapsed after transplantation, the starting RBV dose was 8 mg/kg per day, which was slowly increased to 10 mg/kg per day over 4 to 6 wk, then to 13 to 15 mg/kg per day as tolerated and continued at the highest tolerable dose for the duration of therapy.

Regardless of genotype, all patients were treated for a minimum of 48 wk, even if they had undetectable viremia at week 4. Treatment was discontinued if virus was detectable at week 48.

Erythropoietin (40 000 units subcutaneously once a week) was used if hemoglobin dropped below 10 g/dL. Where there was no improvement, the RBV dose was decreased. RBV was discontinued if hemoglobin fell below 8 g/dL. Patients who had hemoglobin < 8 g/dL or became symptomatic received blood transfusions. During the period of dose adjustment, hemoglobin was monitored weekly. Once the hemoglobin had been stabilized or increased by at least 1 g/dL with erythropoietin, RBV dosage was increased gradually as tolerated weekly, aiming for baseline hemoglobin of 10 g/dL and RBV dose of 13-15 mg/kg per day. Patients with ANC < 750/mm^3 were treated with weekly granulocyte colony stimulating factor (G-CSF, Filgrastim 480 mcg subcutaneously) initially, and if there was no improvement, PEG dose was reduced or held. Dose escalation was attempted once ANC increased to 750/mm^3. PEG dose was also reduced if platelet count was < 30 000/L and discontinued if platelet count was < 25 000/L. Antiviral therapy was also reduced or suspended for antidepressantrefractory depression or disabling fatigue.

Statistical analysis

Continuous variables were presented as median (range) or number (percentages) and analyzed with the Wilcoxon rank test. Categorical variables were expressed as percentages and compared with the Fisher's exact test. *P* values less than 0.05 were considered statistically significant.

Table 1 Baseline characteristics (*n* = 30)

Characteristics	Value
Gender (M:F), *n*	23:07
Age (years), median (range)	56 (38-70)
Genotype 1, *n* (%)	23 (77)
Overweight, *n* (%)	22 (73)
Diabetes Mellitus, *n* (%)	18 (60)
Months from Liver Transplant, median (range)	40.5 (2-132)
High HCV RNA, *n* (%)	17 (57)
Cirrhosis, *n* (%)	3 (10)

M: Male; F: Female. Overweight: Body mass index > 25 kg/m^2; High HCV RNA > 800 000 IU/mL.

RESULTS

Patient characteristics

Baseline characteristics of the 30 patients are summarized in Table 1. The median age at inclusion was 56 years (38-70), 23 patients were male. Twenty-two patients (73%) were overweight (body mass index > 25 kg/m^2) and seven (23%) were obese (body mass index > 30 kg/m^2). The median time to treatment from LT was 40.5 mo (2-132). The HCV genotype was 1 in 23 patients (77%), 2 in 4 (13%), and 3 in 3 patients (10%). Three patients had histologic evidence of cirrhosis. There were no patients with evidence of fibrosing cholestatic hepatitis.

Efficacy

Overall twenty-two patients (73%) had EOT response and 18 patients (60%) had SVR. Nineteen patients completed treatment per protocol. Of these, 15 (79%) were aviremic at the end of therapy and 14 (74%) achieved SVR. Eleven patients were unable to complete treatment per protocol and discontinued prematurely at an average of 23 wk due to adverse effects (8 patients) or viral breakthrough (2 patients), and one patient stopped treatment on his own. Of these eleven patients, six (54%) achieved EOT response, and 4 (36%) achieved SVR. The difference between SVR rates among the patients who completed the treatment protocol and those who did not was not statistically significant ($P = 0.052$).

Virologic response

Viral kinetics and virologic response has been summarized in table 3. Six patients (21%) had undetectable virus at week 4 (RVR), and all of them achieved SVR. Among the 21 patients with EVR, 16 (76%) achieved SVR ($P = 0.03$), whereas only two of nine patients (22%) without EVR achieved SVR. Fifteen of eighteen patients (83%) with aviremia at week 24 achieved SVR ($P = 0.008$), whereas the other 3 patients relapsed. Five patients had detectable viremia at week 24, only one (20%) of them achieved SVR. Both early virological response (EVR) and 24-week virological response were individually associated with SVR but this association was not significant on multivariate analysis.

Table 2 Comparison between SVR and Non-SVR groups

Variable	SVR	Non-SVR	*P*-value
Number of patients	18	12	
Age, median (range)	55(38-67)	59 (48-70)	
M: F, *n*	13 : 5	9 : 3	
Overweight, *n*	14	8	0.396
Diabetes Mellitus, *n*	12	6	0.296
Months from LT, median (range)	60 (4-116)	26 (2-132)	
High HCV RNA, *n*	10	7	0.590
CMV antibody positive, *n*	13	8	0.528
Genotype 1: non 1, *n*	13 : 5	10 : 2	0.403
Pre-Treatment biopsy, *n*			
Stage 0-1	3	5	
Stage 2-3	12	7	
Stage 4	3	0	
Grade 0-1	1	2	
Grade 2-3	16	10	
Grade 4	1	0	
Total weeks of treatment, median (range)	56 (13-84)	44.5 (2-60)	
Erythropoietin use, *n* (%)	17 (94)	6 (50)	0.009
G-CSF use, *n* (%)	10 (56)	2 (17)	0.038

M: Male; F: Female; LT: Liver Transplantation; G-CSF: Granulocyte-colony stimulating factors; SVR: sunstianed virological response. Overweight: Body mass index > 25 kg/m^2; High HCV RNA: > 800 000 IU/mL.

Baseline characteristics were compared between the SVR and non-SVR groups (Table 2). The probability of achieving SVR was not related to baseline serum HCV RNA level, genotype, histologic grade or stage, interval between LT and initiation of therapy, BMI, presence of diabetes, duration of steroid use, presence of CMV antibody and total duration of antiviral therapy. SVR rate was 57% in patients with genotype 1 and 71% in genotypes 2 or 3. Sixteen of the 19 patients who completed treatment per protocol were treated for 48 weeks after achieving aviremia. Fourteen (88%) patients in that group achieved SVR. The interval between initiation of therapy and viral eradication ranged between 4-36 wk.

Tolerability and adverse events

Eleven (37%) patients failed to complete therapy, mostly due to treatment-related adverse events. Two (7%) patients developed moderate acute cellular rejection, one at week 2 and another at week 13. Treatment was discontinued and corticosteroids were used to treat both patients. Four (13%) patients discontinued therapy for anemia, one developed pancreatitis, another developed pneumonia requiring hospitalization, two had virological relapse, and one discontinued treatment on his own. Growth factors and transfusions were frequently used. Twenty-three patients (77%) required therapy with erythropoietin for anemia, twelve (40%) required G-CSF, and ten (33%) required blood transfusions. Dose reductions were also instituted frequently. PEG and RBV doses were reduced in four (13%) and twelve (40%) patients, respectively. One patient developed biopsy-proven de novo autoimmune hepatitis 12 mo after completing a 72-week course of therapy and achieving an SVR[17-20].

There was no incidence of chronic rejection.

DISCUSSION

Hepatitis C recurrence remains a major cause of graft loss after liver transplantation. Studies using the same treatment protocol as in the non-transplant population have reported a lower overall sustained virological response among patients who have undergone transplants. Treatment-related adverse effects in transplant recipients are also more severe and dose-limiting. Specifically, cytopenias are more pro-nounced, due to concurrent bone marrow toxicity from immunosuppression. Our treatment protocol was designed to overcome these obstacles by timing the start of treatment to the severity of HCV recurrence, as determined by protocol liver biopsies. To keep dosing as high as possible, we also used growth factors prophylactically with at risk pa-tients, and to maximize the likelihood of response, we ex-tended treatment to maintain 48 wk of viral undetectability.

Our rationale for prolonged treatment was based on the immune-competent experience, where extending treatment beyond 48 wk has led to improved SVR rates in slow responders[21,22] who were likely to be over represented after liver transplant. Compared to a recent single center, observational study treating recurrent HCV after LT for 48 wk after viral undetectability that reported 26% SVR[15], we observed an overall SVR rate of 60%. The reasons for this difference could be attributed to a lower percentage of patients with advanced disease, and a longer interval between transplant and antiviral treatment in our study, as well as possible differences in immunosuppression.

A more recent study by Schmidt *et al*[12], showed that virological response at week 24 has a high predictive value for SVR in patients with recurrent HCV after LT. Similarly, we found that EVR and the 24-week virological response are associated with SVR with a positive predictive value of 76% and 83%, respectively. Only one patient with persistent viremia at week 24 was able to achieve SVR, which suggests that the 24-week stopping rule in the non-transplant population may be applicable to transplanted patients. On the other hand, lack of EVR has a negative predictive value of 98% in the immune-competent population, and has become a treatment stopping point[23-25]. This was not observed in our transplanted cohort where two (22%) of the nine patients who had not achieved EVR, actually went on to achieve SVR. This confers a negative predictive value of 78%. Although the improved SVR rate in the per-protocol group was not statistically significant, our findings suggest that a viral response-guided therapy using this protocol may be considered in a select group of

Table 3 Viral kinetics and outcomes

Serial No.	VL Baseline	VL Week 4	VL Week 12	VL Week 24	EOT	Total Duration of Rx(wk)	Treatment per Protoco	Virological Outcome
1	700 000	< 50	< 50	< 50	Y	46	N	SVR
2	700 000	292 000	45700	22700	Y	84	Y	SVR
3	700 000	< 50	< 50	< 50	Y	52	Y	SVR
4	282 000	62300	< 50	< 50	Y	56	Y	SVR
5	6 870 000	12700	< 50	< 50	Y	60	Y	SVR
6	1 500 000	2750	< 50	< 50	Y	60	Y	Relapsed
7	771 000	18400	< 50	NA	Y	21	N	Relapsed
8	700 000	< 50	< 50	< 50	Y	56	Y	SVR
9	11 000 000	1 980 000	< 50	< 50	Y	60	Y	SVR
10	100 000	15000	< 50	< 50	Y	45	N	Relapsed
11	700 000	700 000	< 50	2420	N	44	N	Breakthrough
12	700 000	33400	< 50	< 50	Y	60	Y	SVR
13	753 000	683 000	309 000	117 000	N	48	Y	NR
14	2 459 000	62100	< 50	< 50	Y	56	Y	SVR
15	6 140 000	311 000	NA	NA	N	7	N	NR
16	2 226 210	3186	< 50	< 50	Y	56	Y	SVR
17	700 000	243 000	1430	< 50	Y	72	Y	SVR
18	962 000	NA	NA	NA	N	2	N	NR
19	101 000	33800	< 50	< 50	Y	42	N	SVR
20	50 000 000	817 000	50 000 000	NA	N	13	N	NR
21	3 200 000	445 000	44500	< 50	Y	72	Y	Relapsed
22	9 340 000	< 50	< 50	NA	Y	13	N	SVR
23	3 550 000	56	< 50	NA	Y	13	N	SVR
24	1 280 000	398 000	20800	670	N	48	Y	NR
25	305 777	36463	1059	NA	N	15	N	Breakthrough
26	3 020 000	< 50	< 50	< 50	Y	52	Y	SVR
27	1 609 966	78800	175	< 50	Y	72	Y	SVR
28	1 360 000	< 50	< 50	< 50	Y	56	Y	SVR
29	11 300 000	1 040 000	1 060 000	1 790 000	N	48	Y	NR
30	15 100 000	295 330	240 000	< 120	Y	72	Y	SVR

VL: Viral load; EOT: End of treatment response; Rx: Treatment; NA: Not applicable; SVR: sunstianed virological response; NR: Non responder; Y: Yes; N: No.

slow responders.

This extended treatment protocol is complex, highly individualized and demanding for both patients and health care providers. The cost in terms of personnel time, laboratory testing and medication use is high, and may be prohibitive for general use. We observed similar treatment-related adverse events leading to dose reduction or treat-ment cessation in 20%-66% of transplanted patients[15,16,24]. Despite the aggressive and pre-emptive use of growth factors and blood transfusions, we observed similar results in our group, where treatment was prematurely discontinued due to severe side effects, principally cytopenias in 37% of patients. Our acute rejection rate of 7% (2 patients) was within the previously reported range of 5%-20%[9,11,14,15].

Our study has several limitations inherent to a retrospective case series. First, we didn't have a comparison group, due to lack of complete virological data on other patients having had transplants who had received HCV treatment prior to the initiation of our current treatment protocol. Second, we cannot explain why 2 patients achieved an SVR after treatment for only a relatively short period. Third, post-treatment liver biopsy data was available in only 8 patients, so we could not examine the changes in liver histology to determine the beneficial effects of prolonged antiviral therapy, i.e., histological improvement or stability, beyond achievement of SVR. Our small number of patients with cirrhosis did not allow us to examine whether achievement of SVR is associated with prevention of hepatic decompensation. Finally, although our results showed a trend towards a positive association between extended treatment protocol and SVR, statistical significance could not be achieved, possibly due to the small sample size.

In conclusion, our single-center observational pilot study suggests that extended treatment protocols may be utilized for HCV recurrence after LT. A response-guided treatment approach that aims to achieve SVR in patients who have viral undetectability by week 24 of treatment is feasible. However, this approach requires intense monitoring, frequent growth factor use and comes at a high cost, both in personnel and medical expense. Further studies comparing extended treatment protocols to standard 48 wk therapy can be helpful to determine the adequate duration of treatment for recurrent HCV after LT before extended treatment can be recommended unequivocally. It also remains to be seen whether the imminent addition of the direct acting antivirals to our armamentarium of treatment for HCV will obviate the need for extended antiviral therapy after LT, as more

patients could potentially achieve desirable viral kinetics earlier in the treatment.

COMMENTS

Background

Hepatitis C virus (HCV)-related end-stage liver disease is the leading indication of liver transplantation (LT) in the United States and Europe. HCV recurrence after LT is almost universal and occurs early. Cirrhosis develops in up to 30% of transplant recipients after 5 years with persistant HCV viremia, and may be associated with graft failure and need for re-transplantation. Patients with recurrent HCV after LT are likely to be slow virological responders due to immunosuppression and, therefore, SVR after 48 wk of antiviral therapy is expected to be lower than immune-competent HCV patients.

Research frontiers

It has been reported that a few centers have improved SVR rates after extending treatment to 72 wk or longer in partial early virological responders (Partial EVR). Partial EVR is currently defined as achieving a 2-log drop in the HCV RNA pre-treatment levels at 12 wk but not achieving HCV RNA undetectability until 24 wk of treatment.

Innovations and breakthroughs

Our single-center observational pilot study suggests that extended treatment protocols may be utilized for HCV recurrence after LT. A response-guided treatment approach that aims to achieve SVR in patients who have viral undetectability by week 24 of treatment is feasible.

Applications

This treatment protocol can be applied to patients who have HCV recurrence after LT to achieve SVR and decrease the incidence of graft loss.

Terminology

"Viral breakthrough" is when the patient goes from undetectable to detectable viral loads while undergoing treatment. "Virological relapse" is when a patient has an undetectable virus at the end of treatment, but also has a detectable viral load after the treatment stops.

Peer review

The authors describe their experience using an extended treatment protocol for recurrent hepatitis C after liver transplantation. Since hepatitis C invariably recurs after transplant and the treatment options are limited, this study is significant and adds important data to the literature.

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Events Calendar 2011

January 14-15, 2011
AGA Clinical Congress of Gastroenterology and Hepatology: Best Practices in 2011
Miami, FL 33101, United States

January 20-22, 2011
Gastrointestinal Cancers Symposium 2011
San Francisco, CA 94143, United States

January 27-28, 2011
Falk Workshop, Liver and Immunology, Medical University, Franz-Josef-Strauss-Allee 11
Regensburg 93053, Germany

January 28-29, 2011
9. Gastro Forum München
Munich, Germany

February 13-27, 2011
Gastroenterology: New Zealand CME Cruise Conference
Sydney, NSW, Australia

February 17-20, 2011
APASL 2011-The 21st Conference of the Asian Pacific Association for the Study of the Liver
Bangkok, Thailand

February 22, 2011-March 04, 2011
Canadian Digestive Diseases Week 2011
Vancouver, BC, Canada

February 24-26, 2011
Inflammatory Bowel Diseases 2011-6th Congress of the European Crohn's and Colitis Organisation
Dublin, Ireland

March 3-5, 2011
42nd Annual Topics in Internal Medicine
Gainesville, FL 32614, United States

March 7-11, 2011
Infectious Diseases: Adult Issues in the Outpatient and Inpatient Settings
Sarasota, FL 34234, United States

March 14-17, 2011
British Society of Gastroenterology Annual Meeting 2011
Birmingham, England, United Kingdom

March 17-20, 2011
Mayo Clinic Gastroenterology & Hepatology 2011
Jacksonville, FL 34234, United States

March 18, 2011
UC Davis Health Informatics: Change Management and Health Informatics, The Keys to Health Reform
Sacramento, CA 94143, United States

March 25-27, 2011
MedicReS IC 2011
Good Medical Research, Istanbul, Turkey

March 26-27, 2011
26th Annual New Treatments in Chronic Liver Disease
San Diego, CA 94143, United States

April 25-27, 2011
The Second International Conference of the Saudi Society of Pediatric Gastroenterology, Hepatology & Nutrition
Riyadh, Saudi Arabia

May 7-10, 2011
Digestive Disease Week
Chicago, IL 60446, United States

May 19-22, 2011
1st World Congress on Controversies in the Management of Viral Hepatitis (C-Hep), Palau de Congressos de Catalunya, Av. Diagonal, 661-671
Barcelona 08028, Spain

May 21-24, 2011
22nd European Society of Gastrointestinal and Abdominal Radiology Annual Meeting and Postgraduate Course
Venise, Italy

May 25-28, 2011
4th Congress of the Gastroenterology Association of Bosnia and Herzegovina with international participation, Hotel Holiday Inn,
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011
The International Digestive Disease Forum 2011
Hong Kong, China

June 13-16, 2011
Surgery and Disillusion XXIV
SPIGC, II ESYS
Napoli, Italy

June 22-25, 2011
ESMO Conference: 13th World Congress on Gastrointestinal Cancer
Barcelona, Spain

October 19-29, 2011
Cardiology & Gastroenterology Tahiti 10 night CME Cruise
Papeete, French Polynesia

October 22-26, 2011
19th United European Gastroenterology Week
Stockholm, Sweden

October 28-November 2, 2011
ACG Annual Scientific Meeting & Postgraduate Course
Washington, DC 20001, United States

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INSTRUCTIONS TO AUTHORS

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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