Challenges in cancer therapy:

molecular targets, signaling pathways and personalization?

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Uitdagingen in de behandeling van kanker:

targets, signaalwegen en personalisatie

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Contents

Abbreviations

BMP	Bone morphogenetic protein
CFU	Colony-forming unit
DMEM	Dulbecco’s modified Eagle’s medium
DMS	N, N-Dimethyl-D-erythro-sphingosine
DMSO	Dimethyl sulfoxide
FACS	Flow cytometric analysis
FBS	Fetal bovine serum
GTP	Guanosine-5'-triphosphate
HCC	Hepatocellular carcinoma
Histone-GFP	Histone 2B-green fluorescent fusion protein
IMP	Inosine 5'-monophosphate
IMPDH	Inosine monophosphate dehydrogenase
mutIMPDH2	Mutated IMPDH2
IP	Intraperitoneal
LRC	Label-retaining cell
LT	Liver transplantation
MMF	Mycophenolate mofetil
MPA	Mycophenolic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa B

PI	Propidium iodide
PPAR	Peroxisome proliferator activated receptor
sh RNA	Short hairpin RNA
SPF	Specific Pathogen Free
SPHK1	Sphingosine kinase-1
rtTA	Reverse Tet transactivator
TGFB	Transforming growth factor beta
TMA	Tissues microarray
mTOR	Mammalian target of rapamycin
TRE	Tetracycline response element
XMP	Xanthosine monophosphate

Chapter 1

General introduction

General concept of Cancer

Cancer cells originate from normal cells in which the DNA within the cell nucleus has become damaged or mutated. The body is made up of trillions of cells that usually grow, divide and die in an organized manner. This process is tightly controlled by the information contained within the DNA machinery of the cells. Cancer occurs when cells in a particular site start to grow and form new, abnormal cells. These abnormal cells can also invade other tissues, a property that normal cells do not possess [1]. Cancer represents a major health problem. Importantly, both prognosis and therapy of cancerous disease depends to a large extent on the organ from which the cancer arises as well as the specific cell type within this organ. Thus although important lessons can be learned from generic cancer cell models (*e.g.* HeLa cells could be considered as such), it is unfortunately also necessary to investigate cancer manifestation within the context of the organ from which it has arisen. In this specific thesis I shall concentrate on cancer of the liver and in particular on primary liver cancer.

Hepatocellular carcinoma & current treatment

Hepatocellular carcinoma (HCC) is a main type of primary liver cancer. It is a major health problem worldwide as more than 700 000 cases are diagnosed worldwide yearly [2]. Treatment options for HCC are not plentiful and generally have limited effect. The only approved drug for the treatment of advanced HCC is sorafenib, a multi-kinase inhibitor that provides an overall survival benefit of only 3 months [3]. About 15% of patients present with early stage disease. For this group of patients liver transplantation (LT) is attractive because resection of the malignant tumor can be achieved along with the replacement of the usually cirrhotic liver. However, at 5 years after LT, 30-50% of patients have developed recurrence of the cancerous disease and thus the development of novel therapeutic strategies against HCC appears mandatory and represents a major challenge in the field. In this thesis, I aim to contribute to this battle and provide novel insights into HCC pathogenesis and devise potential novel avenues of treatment, especially of patients with prior LT.

Molecular pathogenesis of cancer

The molecular pathogenesis of cancer is extremely complex and heterogeneous. In normal cells, genes regulate growth, maturity and death of the cells. Genetic changes can occur at many levels. There could be a gain or loss of entire chromosome or a single point mutation affecting a single DNA nucleotide or even a karyotypically normal cell but in a wrong environment, as can be the case with teratocarcinomas. Typically, changes in many genes are required to transform a normal cell into a cancer cell, highlighting the multitude of innate defense mechanisms present in our genome. Mutations in the genes disrupt regulation. Gene amplification leads to excessive transcription and might cause overproduction of trophic cell receptors. Abnormal signal transduction of key pathways results in uncontrolled cell proliferation, loss of apoptosis or programmed cell death. Tissue invasion and metastasis permit the spread of cancer, and angiogenesis leads to enhanced blood supply of cancer. All of these events are somehow the result of genomic aberrations.

Cancer target therapy

Standard chemotherapy works largely by killing rapidly dividing cells. However, it also results in collateral damage to healthy cells, causing side effects in areas such as the circulatory system, the immune system, the digestive system and others. Because these drugs usually affect processes that are present in all rapidly dividing cells, many normal cells throughout the body are undergoing active growth and cell division is affected as well. Unlike traditional chemotherapy, the current emphasis on so called “target therapy” consists of specific molecules that target the pathways and processes more-or-less specifically used by cancer cells to grow, divide and spread throughout the body. These targeted therapies are designed to be destructive to cancer cells with great precision and thus are associated with fewer toxic side effects, contributing to the better life quality for patients undergoing treatment – if not improved outcome as well.

An example is the tyrosine kinase inhibitor (TKI) sorafenib that has activity against receptor tyrosine kinases (VEGFR-1, 2 and 3, PDGFR-β, c-Kit, Fms-related tyrosine kinase (FLT) 3 and ret proto-oncogene) and cytoplasmic kinase (Raf-1, B-Raf, C-Raf). Sorafenib has anti-angiogenic, anti-proliferative and pro-apoptotic effects. Sixty genes have been reported in the literature to be targeted directly and indirectly by sorafenib. The question is why sorafenib that has effects on multiple receptors, pathways and genes, only has a limited treatment effect, lasting on average only a few months? [4]. The answer to this question remains for now elusive but may lie in the existence of slowly dividing cell pools relatively independent of sorafenib-targeted pathways but capable of re-initiating cancer, but may also be a reflection of interconnectivity of signaling pathways allowing cancer cells to bypass biochemical blocks mounted by sorafenib therapy or for instance the gradual accumulation of sorafenib resistance-conferring mutations. The limited effectivity of this drug does call for further research into HCC molecular pathogenesis.

The ideal target for therapy is a molecule or pathway that is present in cancer cells, and absent in normal cells. This ensures that therapy only attacks cancer cells. Unfortunately, this is not usually the case. It is often difficult to find targets that are present only in cancer cells in part, because cancer cells derive from normal cells. The next optimal target for therapy is a molecule that is present more frequently in cancer cells than in normal cells. In this case, the cancer cells will be killed more often than normal cells. Other possible targets for therapy include molecules that are present on both cancer cells and normal cells, but the patient’s body can replace the normal cells that get destroyed. As a result, the critical issue is to identify molecules in cancer cells that can potentially be exploited therapeutically (Fig. 1).

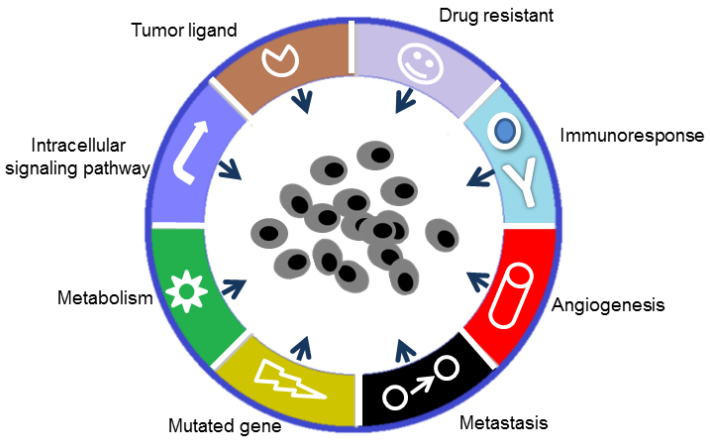

Figure 1. Targets for new drugs should be aimed at reducing the cancer cells capacity to multiply and spread and should have a high barrier to drug resistance[5]

IMPDH as a target in cancer treatment

Life is a physical system that maintains structure and avoids decay by feeding on negative entropy through metabolism converting the organism to low entropy. At the cellular level, normal proliferating cells activate metabolic pathways resulting in cell mass accumulation and DNA synthesis. The by-products of this metabolism itself promotes tumorigenesis [6]. These are general principles. In this thesis I want to explore various details of these processes and exploit the findings for better therapy. An obvious candidate for such an endeavor would be an enzyme associated with nucleotide synthesis, as nucleotide synthesis is *a sine qua non* for any cell proliferation.

The metabolic enzyme, inosine monophosphate dehydrogenase (IMPDH) has been identified as a key enzyme in the biosynthesis of purine nucleotides, which is required for DNA and RNA synthesis. IMPDH has received considerable interest in recent years as an important target enzyme for anticancer drugs. Inhibition of IMPDH causes an overall reduction in cytoplasmic guanine nucleotide pools and as guanosine-5'-triphosphate (GTP) is a cofactor in the conversion of inosine 5'-monophosphate (IMP) to xanthosine monophosphate (XMP), adenylate pools are also diminished. Subsequent interruption of DNA and RNA synthesis results in

cytotoxicity. Human IMPDH is a tetramer consisting of 56KDa monomers. It catalyzes the nicotinamide-adenine dinucleotide (NAD+)-dependent oxidation of IMP to XMP. This is a rate-limiting step in the *de nove* synthesis of guanine nucleotides [7 8]. Human IMPDH has two isoforms, type 1 and type 2. They have 84% sequence identity and show similar kinetic properties. It was found that type 1 IMPDH is prevalent in normal human leukocytes and lymphocytes [9], while type 2 predominated in tumor cells and rapidly proliferating cells [10, 11].

Mycophenolic acid (MPA), a widely used immunosuppressant in organ transplantation, and has shown partial inhibitory activity towards human type 1 and type 2 IMPDH [12, 13]. According to Carret *et al* [14] , the type 2 enzyme (*Ki* = 7 nM) is 4.8-fold more sensitive to MPA than type 1 (*Ki* = 33 nM) (Fig. 2). As IMPDH type 2 appears to be up-regulated in neoplastic cells, the higher inhibitory activity of type 2 may be exploited in designing isoform-selective drugs.

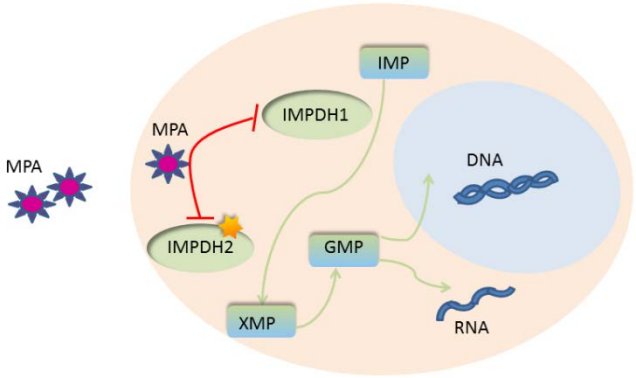

Figure 2. By targeting IMPDH, MPA suppress guanosine nucleotides synthesis, IMPDH isoform 2 is more sensitive than 1 to MPA treatment**.**

SMAD4 as a target in hepatocellular carcinoma therapy

Oncogenes are cancer causing genes. They may be normal genes which are expressed at in appropriately higher levels in patients with cancers or they may be altered or changed into normal genes due to mutation, and then lead to cancerous changes in the tissues.

SMAD proteins are recognized as central mediators of transforming growth factor beta (TGF-β) and/or bone morphogenetic protein (BMP) signaling pathway

(Fig. 3), which regulate a plethora of physiological processes including cell growth and differentiation [14],[15]. Accordingly, deregulation of TGF-β/BMP pathways almost invariably leads to developmental defects and/or diseases, in particular cancer [16]. Already decades ago, one member of the SMAD family, *SMAD4* was originally identified as a candidate tumor-suppressor gene at chromosomal location 18q21.1. SMAD4 binds to receptor-regulated SMADs (r-SMADs) and thus forms heteromeric complexes, which subsequently translocate into the nucleus. Here, the heteromeric complexes bind to promoters and interact with transcriptional activators. Presence of nuclear SMAD4 protein has profound consequences for gene expression. The tumor-suppressive functionality of SMAD4 has now almost achieved dogmatic status and loss of its activity has been implicated in the initiation and progression of a multitude of cancer types [17]. Therefore, this pathway represents an attractive target for new anti-cancer drugs.

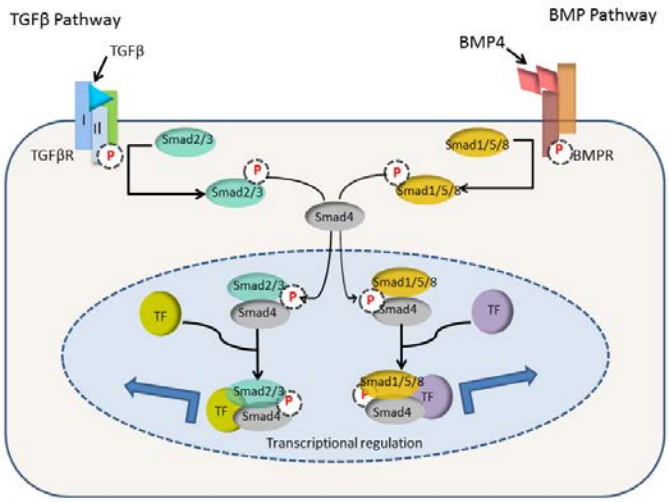

Figure 3. TGF-β/BMP/ SMAD signaling pathway

SPHK1/NF-κB pathway as a target for cancer treatment

The Nuclear factor kappa B (NF-κB) signal pathway in tumor biology is another that has attracted substantial attention, because of its role in inflammation but also as a gene conferring resistance to apoptosis and especially so in the liver. It seems thus highly relevant in the context of this thesis. It has been reported that cells expressing a high level of NF-κB are resistant to chemotherapy and radiotherapy [37]. In tumor cells, NF-κB is active either due to mutations in genes encoding the NF-κB transcription factors themselves or in genes that control NF-κB activity (such as IκB genes). In addition, some tumor cells secrete factors that cause NF-κB to become active. Blocking NF-κB can cause tumor cells to stop proliferating, to die, or to become more sensitive to the action of anti-tumor agents. Thus, NF-κB is the subject of much active research both in academia but also prominently by pharmaceutical companies because of its potential as a target for anti-cancer therapy.

However, caution should be exercised when considering anti-NF-κB activity as a broad therapeutic strategy in cancer therapy. Even though convincing experimental data has identified NF-κB as a critical promoter of cancer development, hence creating a solid rationale for the development of antitumor therapy that suppresses NF-κB activity. Data has also shown that NF-κB activity enhances tumor cell sensitivity to apoptosis and senescence [18-20]. Canonical NF-κB signaling increases transcription of the pro-apoptotic death receptor FAS, whereas alternative NF-κB signaling counteracts transcription of FAS [18]. Therefore, NF-κB can, under certain conditions, promote FAS-mediated apoptosis in cancer cells, and thus drug-induced inhibition of NF-κB may suppress FAS-mediated apoptosis and impair host immune cell-mediated tumor suppression. Hence context-dependent investigation into the role of this signaling system is called for.

Targeting tumor-initiating hepatocellular carcinoma cells (label-retaining cells)

The idea that cancer is constituted of heterogeneous population of cells differing in morphology, marker expression, proliferation capacity and tumorigenicity has been around for over a century [21, 22]. The hypothesis is that this heterogeneity occurs due to the fact that a tumor is hierarchically organized and contains a slow-growing stem cell compartment called the cancer stem cells (CSC) that is the source of fast-growing “normal” tumor cells. It has been hypothesized that CSC are responsible for therapeutic failure, because they are resistant to targeted therapy (Fig. 4). Therefore, new therapies for advanced cancers should effectively target the CSC.

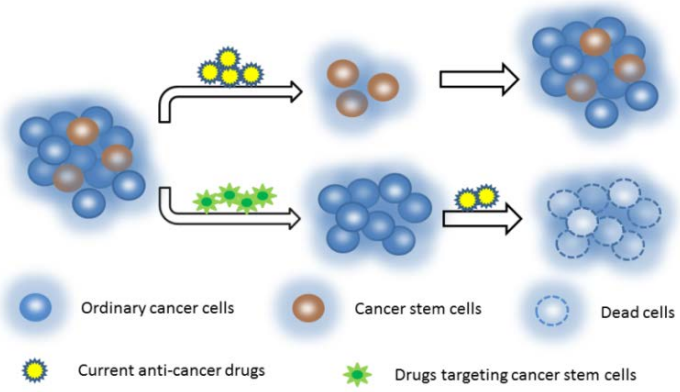

Figure 4. Cancer stem cells are resistant to therapy

Label-retaining cells (LRCs) (Fig. 5)have been demonstrated to possess cancer stem cell characteristics. Evidence showed that LRCs undergo asymmetric cell division, and have the capacity to generate new metastases while having superior resistance to therapy [23, 24]. Many attempts have been made to identify these LRCs. In particular fluorescent-coupled nucleotide analogs are commonly used to identify LRCs in cell culture, allowing their functional study [4]. It is unknown whether LRCs are resistant to targeted therapies at present.

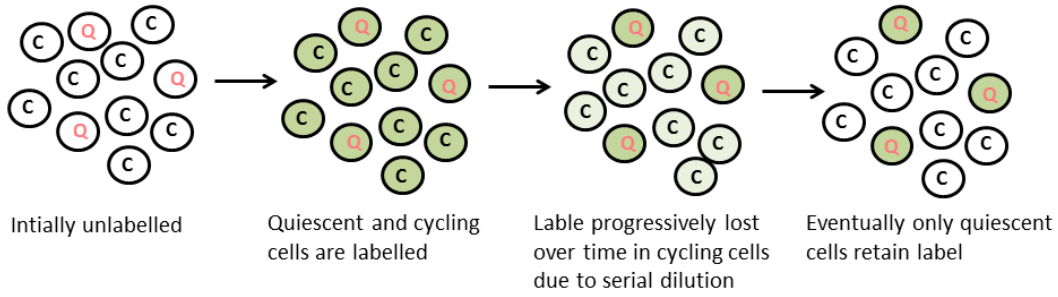

Figure 5. Nucleotide analogs are used to identify label-retaining cancer cells

Personalized therapy

Physicians treating cancer patients have long recognized that some treatments worked well for some patients but not for others. Researchers found that the genetic differences in people and their tumors explained many of these different responses to treatment, but not all. As a result, “personalized” cancer therapy based on genetic make-up and other factors has become a popular approach to improve the choice of proper therapy for a cancer patient. Personalized cancer therapy is a treatment strategy centered on the ability to predict which patients are more likely to respond to specific cancer therapies. This approach is based on the idea that tumor biomarkers are associated with patient prognosis and tumor response to therapy. Tumor biomarkers include DNA, RNA, protein and metabolic profiles that predict therapy response. Identification of these patient-specific tumor characteristics currently requires the sequencing of tumor DNA and is usually done by testing a sample of the tumor obtained through a biopsy or during surgery. Liquid biopsies (*e.g.* a vial of patient blood containing tumor cells or soluble markers) are also gaining popularity. Using this information, doctors can choose optimal treatment strategies that may be more effective and cause fewer side effects than standard treatments. In this thesis I want to contribute to this effort by increasing knowledge on the diversity of mechanisms employed by cancer cells in HCC to grow, metastases and evade therapy.

The future strategy of cancer therapy

Personalized cancer medicine is based on increased knowledge of the cancer mutation repertoire and availability of agents that target altered genes or pathways. With the development of a new “cancer research framework” the genetic basis of a cancer is proposed to determine treatment decision [25].

Mutations are abundant in cancer cells, numbering between thousands and hundreds of thousands per tumor [26]. In addition, specific mutations have been linked to one or more forms of cancer, and mutant gene products have been associated with the biological characteristics of cancer. With the development of DNA sequencing technologies, now quantifying and cataloguing mutations, transcriptomes, and methylomes for many forms of cancer are underway in dozens of countries through coordinated projects of the International Cancer Genome Consortium (ICGC). Already, partial cancer genome data is available for several thousands of tumors with protein-altering mutations affecting more than 7,500 genes (ICGC Data set Version6: http://www.icgc.org). The hypothesis is that if a mutation is predictive of a drug response in one form of cancer, then there may be some likelihood that the same drug could affect tumors from other origins with the same mutation. As a result, rather than approaching each patient’s tumor investigation with an organ-based list of mutation tests, one could systematically perform a global search for all such “actionable” mutations in any type of cancer and test targeted therapeutics in patients with the specific mutations regardless of cancer histology.

Aim of this thesis

In this thesis, I aim to contribute to our understanding of HCC and obtain insight on how to develop novel treatment modalities but also how to better exploit existing approaches towards managing this disease. At present LT remains a powerful approach in managing HCC, not only does it provide a radical removal of a non-metastasized cancer from the patient, it also remove the cirrhotic liver from the cancer originated. The problem is that the cancer often reoccurs: apparently there

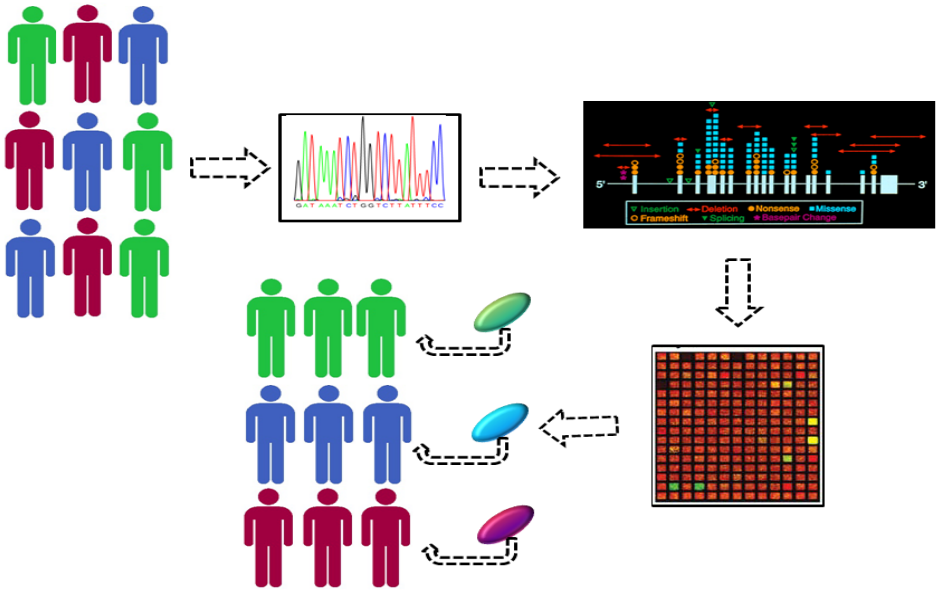

Figure 6. Future strategy for cancer therapy **is** not “organ-based” but “mutation-based”

were undetected metastases hiding in the patient that reignite disease. The relative insensitivity to chemotherapy makes adjuvant protocols unsuitable to deal with this problem. Furthermore, following LT immunosuppressive medication must be given that may hamper immunological combat of the occult HCC. But immunosuppressive medication may sometimes also interfere with HCC growth *per se*. If such an immunosuppressive protocol – one that concomitantly slows cancer growth can be identified, management could be substantially improved. In ***Chapter 2***, I explored this notion and provided evidence for a rationale for personalized immunosuppressive medication for hepatocellular carcinoma patients after liver transplantation. In ***Chapter 3***, the same issue is tackled experimentally and identifies MPA as an immunosuppressant constraining HCC proliferation. In order to further elucidate the potential of MPA in this respect, I investigated its action on different pools of cancer cells. I discovered differential sensitivities of fast- and slow-cycling cancer cells to inosine monophosphate dehydrogenase 2 inhibitions by MPA, suggesting that a pool of cancer cells exist that will evade such treatment and thus MPA can delay disease but not cure it. Nevertheless, its potential is substantial and the notion is further bolstered by the investigations described in ***Chapter 5***, where the tumor suppressing function of IMPDH2 in HCC and also the role of its differential subcellular localization is characterized in detail and linked to outcome of disease.

Results obtained with MPA may however be relevant to liver cancer only. The liver is different and this notion is further highlighted by investigation into the role of the canonical tumor suppressor SMAD4 in HCC. Unexpectedly a tumor promoting role was uncovered for this gene in ***Chapter 6*** and thus HCC differs in important ways from other types of cancers. Highly liver specific is for instance the role of NF-κB signaling in counteracting programmed cell death. Thus therapy directed against this signaling may have promise. It is encouraging that in ***Chapter 7***, I showed that N, N-dimethyl-D-erythro-sphingosine can cause cancer cell death through inhibition of NF-κB signaling in cancer cells, albeit in a generic cancer model, thus obviously further research is needed. The directions such future research should have in the context of the findings described in this thesis are explored in ***Chapter 8***. The main conclusion arising from the discussion involved is that the main challenges in cancer therapy remain targets and signaling pathway identification as well as the personalization of clinical management.

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Chapter 2

Rationale of personalized immunosuppressive medication for hepatocellular carcinoma patients after liver transplantation

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Abstract

Liver transplantation is the only potentially curative treatment for hepatocellular carcinoma (HCC), which is not eligible for surgical resection. However, disease recurrence is the main challenge for the success of this treatment. Immunosuppressants that are universally used after transplantation to prevent graft rejection could potentially have significant impact on HCC recurrence. Nevertheless, current research is exclusively focusing on the mammalian target of rapamycin (mTOR) inhibitors, which are thought to be the only class of immunosuppressive agents that may reduce HCC recurrence. In fact, substantial evidence from the bench to bed has indicated that other classes of immunosuppressants may also exert diverse effects, such as potential anti-tumor effects of inosine monophosphate dehydrogenase (IMPDH) inhibitors. This article aims to provide a comprehensive overview of the potential effects as well as mechanism-of-action of different types of immunosuppressants on HCC recurrence from both experimental and clinical perspectives. To ultimately improve the outcome of HCC patients after transplantation, we propose a concept as well as approaches to develop personalized immunosuppressive medication for either immunosuppression maintenance or during prevention/treatment of HCC recurrence.

Introduction

As the escalating epidemic of viral hepatitis, approximately 500 million people are living with chronic hepatitis B (HBV) or C (HCV) virus infection worldwide. HBV and HCV are the leading causes and accounting for 80% of hepatocellular carcinoma (HCC), a type of very aggressive primary liver tumor [1]. The only potentially curative treatment options are surgical resection or liver transplantation (LT) for patients who are not eligible for resection, although the majority of HCC patients in fact are not eligible for either resection or transplantation. LT is attractive because resection of the malignant tumor can be achieved while also replacing the cirrhotic liver that remains at risk for the development of new lesions. However, outcome after LT clearly depends on recurrence of HCC. Patients with extensive disease had very poor outcome, whereas most patients with small tumors could be cured. Tumor nodule size, histologic grade of differentiation, and both microvascular and macrovascular invasion are currently the common predictors of recurrence after surgical resection and LT [2]. This has led to many controversies around the use of LT in patients with HCC, in particular the selection of patients in the context of worldwide organ shortage.

After LT, immunosuppressants are used to reduce the risk of graft rejection. However, these drugs are generally associated with oncogenic properties [3, 4] and may play important roles in HCC recurrence [5]. The high incidence of malignancy and its aggressive progression are previously thought only to be the consequence of impaired immune-surveillance system due to immunosuppressants. However, more recent evidence pointed out other distinct mechanisms for affecting malignancy that were independent of host immunity [6]. Furthermore, different immunosuppressants likely have differential effects on HCC recurrence [5, 7, 8], although there are still no randomized controlled trials that have confirmed the direct effects of immunosuppressants. Despite a broad implication of different immunosuppressants in HCC recurrence, current attentions have only been extensively paid to the mammalian target of rapamycin (mTOR) inhibitors, including rapamycin and everolimus, which potentially possess anti-tumor property. A prospective randomized, open-labeled trial has just started to accrue on its application in prevention of HCC

recurrence after LT [9]. Based on preclinical research, mTOR inhibitors are expected to be effective in the treatment of tumors with genetic mutations that lead to over-activation of mTOR signaling, such as loss of PTEN, Stk11 (Lkb1), or Tsc2. Since HCC is a rather heterogeneous malignancy with multiple etiologies [10], it is likely impossible that one immunosuppression protocol fits all.

In this article, we aimed to provide an overview of the potential impact and possible mechanism-of-action of different types of commonly used immune-suppressants on HCC recurrence after LT, including corticosteroids, calcineurin inhibitors, mTOR inhibitors and selective anti-proliferative agents. More specifically, we emphasized the rationale and approaches of developing personalized immunosuppressive medication for both maintaining immunosuppression and during prevention or treatment of HCC recurrence.

Glucocorticoids

Glucocorticoids (also called steroids or corticosteroids) have been used since the early years of organ transplantation. Prednisolone and its close analogue dexamethasone are potent suppressors of the immune system, by modulating cellular and inflammatory responses via stimulation or inhibition of gene transcription. Prednisolone was first introduced to the world as an anti-inflammatory agent in the early 1950s and was used in LT in 1968 with some of the first “successful” cases [11]. Nowadays, steroids are often used in combination with newer immunosuppressants [12]. In fact, steroids are widely used for treating lymphoid malignancy [13] as well as adjuvant therapy for solid tumors (mainly for reducing pain), although there is still little clinical evidence supporting the efficacy [14]. In contract, evidence from LT patients suggested that steroids may increase recurrence of HCC [15], which also inspired the notion of steroids withdrawal early after LT in patients with advanced-stage HCC. Subsequently, a randomized clinical trial has reported that steroids withdrawal 3 months after transplantation was not only safe but also significantly decreased tumor recurrence rates and adverse effects, which conceivably led to an increase in long-term survival [16]. However, a recent retrospective study reported no differences regarding HCC recurrence and patients survival between early and late steroids withdrawal [17].

The mechanistic rationale of using steroids treating cancer, especially lymphoid malignancy, is their drastic effects on cell cycle progression. They can impact on cellular processes ranging from proliferation and differentiation to apoptosis. Glucocorticoids exert function by binding to their receptor (GR), a member of the nuclear hormone receptor family, to trigger transcription of target genes [18]. Regarding the mechanism how steroids trigger apoptosis, a serial of studies have demonstrated that steroids can activate cell death through induction of pro-apoptotic members of the Bcl-2 family [19, 20] and suppression of anti-apoptotic members [21, 22]. However, the responsiveness and the mechanism-of-action vary dramatically among different cell types [23]. In particular, the development of resistance has been observed in many types of tumor cells, including acute lymphoblastic leukemia, osteosarcoma and small-cell lung cancer cells [23]. Since hardly experimental research has been done in models of human HCC, it is thus difficult to predict the actual effects on the outcome of HCC recurrence.

Calcineurin inhibitors

Major improvements in the outcomes of LT were attributed to the introduction of a new generation immunosuppressants as calcineurin inhibitors, including cyclosporine A (CsA) and tacrolimus. CsA was discovered in 1970 and approved by FDA in 1983 [24], whereas tacrolimus (FK506) was lately introduced [25]. High dose of CsA has been associated with increased risk of *de novo* malignancy, in particular skin cancer, after kidney [26] or liver transplantation [4]. However, this is still highly controversial regarding the comparison between CsA and tacrolimus. Several studies found no difference in the incidence of *de novo* cancer between CsA-based and tacrolimus-based regimens [27-29], whereas other studies reported a higher risk for CsA-based [30, 31] or tacrolimus-based [32] immunosuppressive protocols.

In the context of HCC patients with LT, a close correlation between the dosage of CsA administered during the first postoperative year and tumor recurrence was observed [33]. When compared with other types of immunosuppressants (steroids or azathioprine), a significant association between CsA medication with the incidence of recurrence has been observed in a retrospective study [5]. In comparison to tacrolimus, one study [34] reported significant higher whereas another study [35] observed lower five-year disease-free survival rate with CsA immunosuppression.

The primary cellular targets of the calcineurin inhibitors are immunophilins to exert immunosuppressive property. CsA binds to cyclophilins and tacrolimus binds to FK binding proteins (FKBPs). Both events result in a profound inhibition of the phosphatase activity of calcineurin. This immunophilin-dependent signal transduction via calcineurin represents a key cascade in the activation of T-cell proliferation by regulating expression of the gene that encodes IL-2 [36]. CsA was reported to be able to increase the invasiveness of both non-transformed [37] and transformed cells and to promote adenocarcinoma cell growth *in vitro* and in mice, via the activation of transforming growth factor-beta[6]. In contrast, CsA did not facilitate tumor progression and it partially inhibited tumor growth in mouse models of squamous cell carcinoma or B-cell lymphoma [38]. Tacrolimus has been reported to promote the growth of HCC cells in vitro [39, 40]. Mechanistically, this was proposed to be linked to the increased CDK4 kinase activity that promoted cell cycle progression [41].

Inosine monophosphate dehydrogenase inhibitors

Mycophenolate mofetil (MMF) is an ester prodrug of mycophenolic acid (MPA), which can be rapidly hydrolyzed in the gastrointestinal tract to MPA. MPA acts as a non-nucleoside non-competitive, reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH) and thereby exerts immunosuppressive capacity by inhibiting lymphocytes proliferation through blocking *de novo* guanine nucleotide synthesis [42]. Currently, MMF/MPA is widely used for prevention of allograft rejection in LT recipients, attributing to the lacking of nephrotoxicity [43].

In fact, the anti-proliferative property of MPA was the initial driving force of developing as an anti-cancer agent [44]. Anti-angiogenesis and anti-proliferation of tumor cells are proposed as the main mechanism-of-action of MPA [45]. Inhibition of cancer cell proliferation and induction of caspase and mitochondrial dependent apoptosis by MPA have been reported in several models of human solid tumors or hematological malignancies [46-49]. MPA was also reported to be able to trigger the necrotic signaling pathway in apoptotic-resistant tumor cells [49]. Many of these cellular functions of MPA are thought to depend on nucleotide pool. When the guanine nucleotide pool is depleted, the cells can no longer make sufficient mRNA and accordingly can’t make those necessary proteins involved in cell proliferation. In addition, the consume of guanine nucleotides can also affect other cellular

machinery, such as protein translation and signal transduction [50]. Several GTP-dependent pathways are of major concern because once these initial pathways are disrupted, they cause a cascade of detrimental cellular effects. In yeast cells, MPA treatment resulted in growth arrest, cell size increase and multinucleated or puffy DNA content, with reduced expression of 27 proteins [51].

Phase I clinical trial has shown a positive correlation between clinical response and depletion of intracellular dGTP level by MMF in advanced multiple myeloma patients [52]. Another clinical study in pancreatic cancer was failed to show any beneficial effects, despite its effects of anti-angiogenesis and anti-proliferation of tumor cells observed in their preclinical models [53]. The discrepancy between preclinical and clinical results could be partially due to the potent immunosuppressive activity of MPA, which probably masks its anti-tumor effects. Thus, transplantation setting is more idea to investigate its potential anti-tumor effects, when compared with other immunosuppressants. A large prospectively conducted observational cohort study observed a tendency toward a lower risk of malignancy in the MMF versus non-MMF treated renal transplanted patients [54]. Although it has been rarely studied in the LT setting, the effects of MMF/MPA on HCC recurrence deserve to be further investigated.

Mammalian target of rapamycin inhibitors

The mTOR inhibitors that are used as immunosuppressants include rapamycin (also known as sirolimus and originally isolated from *Streptomyces hygroscopicus*) and everolimus [the 40-O-(2-hydroxyethyl) derivative of rapamycin]. The molecular mode of action of these rapalogs is well characterized and involves inhibition of the mTOR pathway. These medications are increasing in popularity in the context of transplantation, mainly because of its low nephrotoxicity and potential anti-tumor effect [55]. Rapamycin and everolimus have been extensively evaluated in clinical trials for treating various types of cancer (Table 1). Temsirolimus, another analog of rapamycin and everolimus, has been approved by the Food and Drug Administration for the treatment of advanced renal cell carcinoma after the failure of a first-line treatment with sunitinib or sorafenib. Subsequently, everolimus has also been approved by the Food and Drug Administration to treat tuberous sclerosis complex-associated subependymal giant cell astrocytoma that cannot be surgically

removed [56]. Currently, several ongoing phase III trials are evaluating the efficacy of rapamycin and its analogs for a variety of malignancies, including breast, gastric and hepatic cancer, mantle cell lymphoma and cancers associated with organ transplantation (www.clinicaltrials.gov). Data from phase I and I/II clinical studies suggest that everolimus monotherapy may stabilize advanced HCC progression [57, 58].
everolimus monotherapy may stabilize advanced HCC progression [57, 58].

Table 1. Completed Trails of Rapamycin/Everolimus-Based Regimens for Treating Cancer

Type of mTOR inhibitors	Phase	Condition	Intervention	ClinicalTrials.gov Identifier
Rapamycin-based	1	lung cancer	Rapamycin + Sunitinib	NCT00555256
	1	Advanced Cancers	Rapamycin (liquid/Tablets)	NCT00667485
	1	HIV-related Kaposi's sarcoma	Rapamycin	NCT00450320
	1	Advanced Cancer	Rapamycin	NCT00707135
	1	Neurofibromatosis, angiofibroma, neurofibroma	Rapamycin + Skincerity	NCT01031901
	1	Myeloid leukemias AML Leukemia CML	Rapamycin+Mitoxantrone + Etoposide+Cytarabine	NCT00780104
	2	PTEN hamartomatous tumor syndromes	Rapamycin	NCT00971789
Everolimus-based	1	Head and neck cancer	Everolimus+Docetaxel+ Cisplatin	NCT00935961
	1	Head and neck cancer	Everolimus + Cisplatin	NCT00858663
	1	Breast cancer	Everolimus+Erlotinib	NCT00574366
	1	Advanced solid tumors	Everolimus + Cisplatin	NCT00423865
	2	Malignant pleural mesothelioma	Everolimus	NCT01024946
	2	Small cell lung cancer	Everolimus	NCT00374140
	2	Neuroendocrine carcinoma	Everolimus	NCT00607113
	2	Lymphangioleiomyomatosis	Everolimus	NCT01059318
	2	Renal cell carcinoma, Renal cell adenocarcinoma, Nephroid carcinoma	Bevacizumab+Everolimus	NCT00719264
	2	Hypernephroid carcinoma, Glioma Low-grade Glioma	Everolimus	NCT00782626
	3	Metastatic Renal Cell Carcinoma	Everolimus+ Placebo	NCT00410124

NOTE: The trails are registered at ClinicalTrails.gov

Despite the potential beneficial effects of mTOR inhibitors on HCC recurrence after LT, there are only retrospective and uncontrolled clinical studies that could serve as supportive evidence to some extent. A large prospective and case-controlled data analysis showed a significant better overall survival with sirolimus versus tacrolimus immunosuppression [59]. A subsequent large retrospective registry data analysis also reported a significant improvement of patient survival with sirolimus-based immunosuppression protocols [60]. Some pilot studies [61, 62] also shown that treatment of patients with advanced HCC with sirolimus can induce temporary partial remission (PR) or stable disease (SD). Nevertheless, no significant beneficial effects but only a tendency towards improvement were observed by others [63, 64]. Fortunately, a prospective randomized, open-labeled trial has just started to accrue on the application of mTOR inhibitors in prevention of HCC recurrence after LT [9].

In general, the mTOR pathway is a key regulator of cellular proliferation and angiogenesis implicated in carcinogenesis [65], including in HCC [66]. This pathway consists of two protein complexes that mTOR, raptor and mLST8 proteins constitute to form the mTOR complex 1 (mTORC1) and mTOR, rictor and Sin1 proteins forming mTOR complex 2 (mTORC2) [56]. mTORC1 plays a vital role in cellular growth and associated proliferation by integrating signals from nutrients and energy status. It regulates several processes including ribosome biogenesis, protein synthesis, metabolism and autophagy [67]. mTORC2 plays a role in cytoskeletal organization through protein kinase C and paxillin [68]. Rapalogs can inhibit the mTOR pathway by binding to FK-binding protein-12, which in turn binds to the mTOR protein, and subsequently preventing the assembly of mTORC1 [69]. However, prolonged use of rapalogs can also disrupt mTORC2 [70]. A particular interesting working mechanism of rapalogs is the induction of autophagy formation. It is a process for catabolizing organelles and other cytoplasmic components to balance cellular metabolism and to promote cell survival during stressful conditions. It also plays important but context-dependent and dynamic roles in cancer: either suppresses or promotes tumor progression [71]. This dichotomal effect of autophagy was also reported in various experimental HCC models [72-75]. Furthermore, mTOR is a central element of the phosphatidylinositide 3-kinase (PI3K)/Akt/mTOR signaling. Virtually, HCCs from most of the etiologies can be influenced by this cascade which in turn represents a

druggable pathway [76]. Although the targeting pathways by rapalogs are extensively studied in experimental setting, the exact molecular determinants of drug response however remain largely elusive [77], which are certainly crucial for their clinical implication.

The choice of immunosuppressants during prevention or treatment of HCC recurrence by sorafenib?

Sorafenib, an oral multikinase inhibitor, was approved by FDA to treat renal cancer (in 2005) and advanced HCC (in 2007). Two large randomized control trials demonstrated that it can increase survival of advanced HCC patients with approximately 2-3 months [78] [79]. However, LT patients were excluded in those randomized trails and therefore, only a few retrospective cohort studies have investigated sorafenib in the setting of transplantation [80-84]. In a recent case-control study, sorafenib was reported to be associated with a benefit in survival in recurrent HCC patients, compared to best supportive care [85]. Based on current pre-clinical and clinical data, the concept of integrating sorafenib into an algorithm for the management of post-transplant HCC recurrence is emerging [86].

A unique important issue in the transplantation setting is which immunosuppressive regimen to choose, during sorafenib treatment. To the current knowledge, sorafenib, as a multikinase inhibitor, can suppress cancer cell proliferation by targeting Raf/MEK/ERK signaling and inhibit angiogenesis by targeting vascular endothelial growth factor receptor (VEGFR)-2, VEGFR-3, and platelet-derived growth factor receptor (PDGFR)-β [87]. It is not surprising that combination of sorafenib with rapalogs has been favored in the LT setting [82-86], since rapalogs have the potential of inhibiting cell cellular proliferation and angiogenesis by targeting mTOR pathway [65]. However, the tolerability of sorafenib is rather poor in LT patients [82-85, 88, 89], either with or without mTOR inhibitors, which in turn hampers the proper assessment of its efficacy. A phase I trial has attempted to determine the maximum tolerated dose of everolimus in combination with standard-dose sorafenib in advanced HCC patients. Unfortunately, the inability to achieve a biologically effective everolimus concentration (because of poor tolerability) precluded further study of this combination with a full dose of sorafenib [91]. An intriguing question is the optimal dose of sorafenib for preventing or treating HCC recurrence, a malignant

state that is clearly different from advanced HCC. Future studies for determining the optimal dose of sorafenib, including mTOR and IMPDH inhibitors, are proposed for consideration.

Rationale and necessity of personalized immunosuppression

HCC is a rather complex and heterogeneous malignancy at both cellular and molecular levels [76]. The status of the tumor cells could also evolve with intrinsic and extrinsic changes (eg, their microenvironment and anti-cancer treatment) [91]. The type and the status of the tumor at the time of transplantation can directly influence disease recurrence. Because of this complexity and heterogeneity, personalized management is certainly needed to achieve optimal outcome after LT. Because of the potential impact on HCC recurrence and the distinct models of action of different immunosuppresants, we believe that it is necessary and essential to develop personalized immunosuppression protocol for HCC patients after transplantation (Fig. 1).

A key step towards personalization is the identification of robust biomarkers. As a primary druggable pathway, hyperactivation of mTOR signaling frequently occurs in various cancers and in 15%-50% of liver tumors [62, 92-94]. This is mainly caused by numerous mutations of upstream regulators, including PTEN, Stk11 and Tsc2 [10]. A recent phase I clinical trial has shown substantial anticancer activity of rapamycin in PTEN-deficient glioblastoma patients, supporting the concept of personalization by incorporating molecular tools [95]. Similarly, IMPDH enzymes are the primary targets of MPA, whereas the isoform IMPDH2 exhibit a 4.8 fold higher sensitivity to MPA than IMPDH1 [41]. IMPDH activity can vary between tumors [96] and, therefore, represents a potential molecular marker of the responsiveness to MPA treatment. Thus, the identification of particular molecular determinants of the treatment response will eventually help in the design of an optimal immuno-suppressive protocol for each HCC patients after LT (Fig. 1).

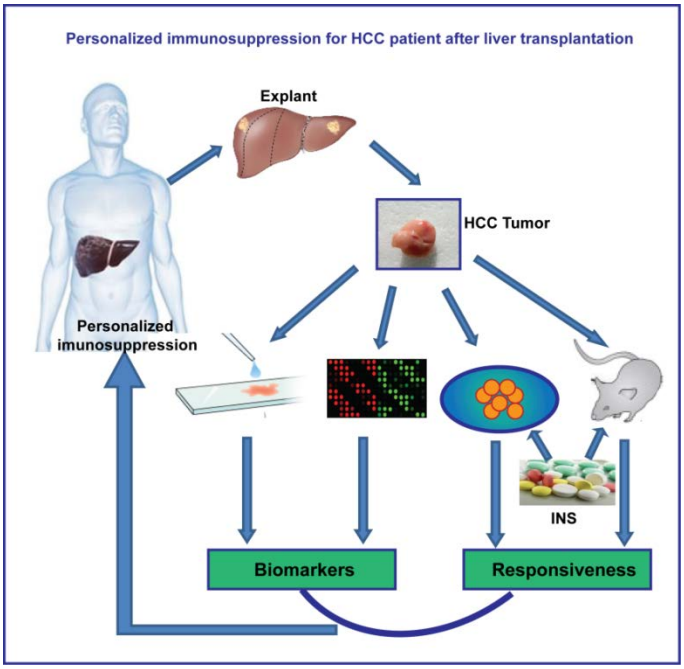

Figure 1. Concept and approaches for achieving personalized immunosuppressive medication for patients with HCC after LT. Conventional approaches (eg, quantitative reverse-transcription polymerase chain reaction and immunohistochemical staining) or high-throughput approaches (eg, transcriptome and proteome profiling) can be used to identify potential biomarkers to predict the responsiveness to a particular immunosuppressant through the use of surgically resected HCC tumors at the time of transplantation. Furthermore, resected tumor tissues can be used to establish ex vivo cell cultures or xenograft tumor models for patients to functionally evaluate the responsiveness.

Besides immunosuppression maintenance, the choice of immunosuppressant during the prevention or treatment of HCC recurrence (particular during sorafenib therapy) should also be justified. Potential drug-drug interactions, the combined anti-tumor activity, and the side effects will determine the choice and also the dosage of a particular immunosuppressant for certain patients undergoing sorafenib treatment.

Advances in molecular and cell biology technologies have enabled us to identify biomarkers with either conventional approaches (eg, quantitative reverse-transcription polymerase chain reaction and immunohistochemical staining) or high-throughput approaches (eg, transcriptome and proteome profiling) through the use of surgically resected HCC tumors at the time of transplantation (Fig. 1). Furthermore, it is even feasible to functionally evaluate the responsiveness by *ex vivo* cell cultures or xenograft tumor models for patient with resected tumors (Fig. 1).

Conclusion

Substantial evidence from the bench to bed has indicated the essential impact of immunosuppressive medication on HCC recurrence after LT. Nevertheless, only mTOR inhibitors are thought to have the potential of reducing recurrence and thus have been exclusively paid attention so far. However, we urge that other classes of immunosuppressive agents may also exert diverse effects, such as the potential anti-tumor effects of IMPDH inhibitors.

Given the complexity and heterogeneity of HCC, we hope that the approach of personalized immunosuppressive medication proposed in this article will help to significantly improve patient outcome during either immunosuppression maintenance or prevention/treatment of disease recurrence. To achieve this ultimate goal, future efforts are required to identify robust biomarkers and to develop sophisticated personalized HCC tumor models.

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Chapter 3

Mycophenolic acid constrains hepatocellular carcinoma

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In preparation

Abstract

Tumor recurrence is a major complication following liver transplantation (LT) as treatment for hepatocellular carcinoma (HCC). Immunosuppression is an important risk factor for HCC recurrence, but conceivably may depend on the type of immunosuppressive medication. This study investigated the effect of mycophenolic acid (MPA) in three experimental HCC cell lines and in liver transplant patients with HCC. The association of treatment with mycophenolate mofetil (MMF), the pro-drug of MPA, with HCC recurrence was retrospectively analyzed in a LT cohort. With clinically achievable concentrations, MPA effectively inhibited HCC cell proliferation and single cell colony-formation unit (CFU). Most importantly, the use of MMF was associated with less HCC recurrence and improved survival after LT. In addition, more potential compounds were found *in vitro* experiment. Thus, we concluded that MPA can specifically counteract HCC growth *in vitro*. In LT patients, the use of MMF is associated with reduced HCC recurrence and improved survival. These results warrant prospective clinical trials into the role of MPA-medicated immunosuppression following LT indicated by liver cancer.

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide [1]. Surgical resection or liver transplantation (LT) are currently the potentially curative treatment options. LT is particularly attractive because of the radical resection of the tumor achieved. Moreover, LT cures the underlying liver disease along with the replacement of the diseased liver that remains at risk for development of new malignant lesions other complications when simple tumor resection is executed. However, tumor recurrence is a common threat for the success of both surgical resection and LT [2]. A unique risk factor strongly associated with recurrence in LT patients is the universal use of immunosuppressants after transplantation, which is to prevent graft rejection [3-5] but concomitantly hampers anti-cancer immunosurveillance.

Importantly, immunosuppression involves inhibition of immune cell proliferation and thus such therapy might have direct effects on the cancerous compartment as well. Besides a general impairment of the immunosurveillance system, different type of immunosuppressant however could thus have effects independent of the host immunity on the malignancy process [5-8]. Current research efforts are now mainly focusing on the mammalian target of rapamycin (mTOR) inhibitors, including rapamycin (sirolimus) and everolimus. They are thought to be the only class of immunosuppressive agents that may reduce HCC recurrence, and this notion is supported by some retrospective and meta-analysis studies [9-13]. However, these studies do not provide firm evidence to establish superiority of mTOR inhibitors on HCC recurrence in comparison to other types of immunosuppression[14]. In a recent prospective study it was shown that sirolimus in LT recipients with HCC does not improve long-term recurrence-free survival beyond 5 years, although a beneficial effect between 3 to 5 years after transplantation in subgroups was suggested [15]. Furthermore, higher rejection rates were reported for monotherapy of sirolimus or everolimus in liver transplanted HCC patients [16, 17]. This latter effect may be a

reflection from the issue that HCC is a heterogeneous malignancy with multiple etiologies [18, 19]. It is unlikely that one immunosuppression protocol fits all cases. Therefore, the impact of other immunosuppressant also deserves to be carefully investigated, in order to define appropriate immunosuppressive regimens for management of HCC recurrence after LT.

Mycophenolic acid (MPA) and its prodrug, mycophenolate mofetil (MMF), are currently widely used for prevention of allograft rejection because of lacking nephrotoxicity [20]. These drugs act through depletion of guanine nucleotide pools by inhibition of inosine monophosphate dehydrogenase (IMPDH), in particular the isoform 2 (IMPDH2) [21] (Fig 1A and B). This results in blockage of *de novo* guanine nucleotide synthesis and inhibition of lymphocyte proliferation [20]. Interestingly, MPA has been reported to be able to inhibit cancer cell proliferation and induce caspase/ mitochondrial-dependent apoptosis in several experimental models of human solid tumors and hematological malignancies[22-25]. A large prospectively conducted observational cohort study observed a tendency toward a lower risk of malignancy in MMF versus non-MMF treated renal transplanted patients [26]. However, this class of immunosuppressant has not been extensively studied in the setting of HCC recurrence after LT. This consideration inspired us to explore the effects and mechanism-of-action of MPA in experimental HCC models and in HCC-related LT patients. The results support a role for MPA in the management of HCC-indicated LT patients.

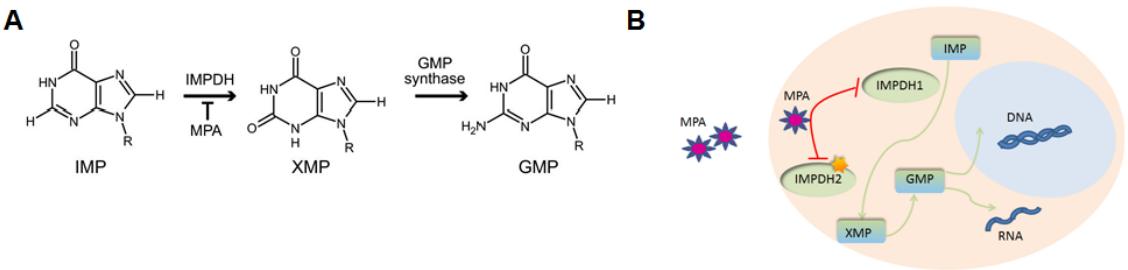


Figure. 1 Schematic overview of the function of IMPDH and MPA. (A) IMPDH is an important enzyme in the pathway of purine *de novo* biosynthesis; (B) By targeting IMPDH, MPA suppress guanosine nucleotides synthesis, IMPDH isoform 2 is more sensitive than isoform 1 to MPA treatment.

Patients, materials and methods

Patient information

A LT database established in our previous study [4] was used for retrospective analysis of the effect of MMF use on HCC recurrence. This cohort included patients transplanted between October 1986 to December 2007 at the Erasmus Medical Centre, Rotterdam, The Netherlands. From this database 44 out of 385 LT patients were identified as HCC-related LT and thus subjected to the analysis described in this study.

Reagents

Stocks of MPA (AMRESCO LLC, USA) were dissolved in dimethyl sulfoxide (DMSO). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich. Antibodies recognizing IMPDH2 (rabbit, monoclonal) and β-actin (mouse, monoclonal) were purchased from Abcam Company. Twenty-three IMPDH inhibitors were kindly provided by Center for Drug Design, University of Minnesota, USA.

Cell culture

HCC cell lines, including HuH6, HuH7 and PLC/PFR/5 were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO Life Technologies), supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone Technologies), 100 units/mL of penicillin and 100 μg/mL of streptomycin. All the cells were incubated at 37℃ in a humidified atmosphere containing 5% CO_2.

MTT assay

Cells were seeded in 96-well plate, at a concentration of 6×10^3 cells/well in 100 μL medium. All of the cells were incubated overnight to attach to the bottom of the wells,

and then treated with serials dilutions of MPA (0, 3, 15, 30 and 60μM). Cell viability was analyzed by adding 5 mg/mL MTT and DMSO. Absorbance was determined using Enzyme mark instrument at the wavelength of 490 nm.

Colony formation assay

Cells were harvested and suspended in medium, then seeded into 6-well plates (500 cells/well). Formed colonies were fixed by 70% ethanol and counterstained with haematoxylin & eosin after two weeks. Colony numbers were counted.

Western blot analysis

For Western blot, the proteins (40 μg) in cell lysates were heated for 5 mins at 95℃, followed by loading onto a 10% sodium dodecyl sulfate polyacrylamide gel and separating by electrophoresis. After 90 mins running in 115 voltages, proteins were electrophoretically transferred onto polyvinylidene difluoride membranes (Invitrogen) for 1.5 hours with an electric current of 250 mA. Subsequently, the membranes were blocked with 5 mL blocking buffer containing 0.05% Tween-20. They were followed by incubation with primary antibodies (1:1000 dilution) overnight at 4℃. Membranes were washed 3 times and then followed by incubation with secondary antibodies (1:10000 dilution) at room temperature for 1.5 hours. Protein bands were analyzed by using Odyssey Imaging system.

Immunofluorescence

Immunofluorescence microscope was performed to investigate the localization of intracellular IMPDH2 protein. HCC cells were cultured on glass cover slides in 6-well plates and treated by MPA at the concentration of 3 μM for 3 days, then cells were fixed with 4% paraformaldehyde and endogenous peroxidase was blocked by 0.6% H_2O_2 at room temperature for 20 mins. The slides were incubated in 5% milk-containing solution followed by overnight incubation with a rabbit monoclonal antibody against IMPDH2 (1:250) at 4℃. As a negative control, the primary antibody

was omitted, positive control were taken from other slides that had been successfully stained before. Following a serious of washed with 5% milk medium containing 0.05% tween20, and then cells were incubated with one drop of secondary antibody at the dilution of 1:1000 (Alexa Fluor 488) for 45 mins under dark environment. After a series of washing, DAPI (1:1000) was used to counterstain nuclear. Cells were imaged on a confocal microscope (Zeiss LSM 510) at 400 × and 920 × magnification respectively. ZenlightEdition software was used to analyze confocal microscope images.

T cell isolation and [3H]-Thymidine assay

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Paque^{TM} (Life technologies, Bleiswijk, Netherland). T cells were isolated by using Pan T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Dynabeads coated with human T-activator CD3/CD28 (Life technologies, Bleiswijk, Netherland) in the concentration of 1×10^4 cells/well were used for T cell expansion and activation. FACS analysis was used to detect the purity of T cells. T cell were finally cultured in round-bottom 96-well plates at the concentration of 1×10^5 cells/well in 200 µL RPMI1640 medium (GIBCO Life Technologies) at 37℃, 5% CO_2, with or without compounds. T cell proliferation was assessed by determination of [3H]-Thymidine (Radiochemical Central, Amersham, Little Chalfont, UK) incorporation, 0.5 µCi/well was added and cultures were harvested 18 hours later.

Statistical analysis

Statistical analysis was performed by using GraphPad InStat software. P-values < 0.05 were considered statistically significant.

Results

Use of MMF is associated with reduced HCC recurrence and improved survival

We investigated the effect of the often-used immunosuppressive post-LT medication MPA on outcome of patients having had LT indicated by HCC. This retrospective analysis performed by a prospectively collected LT cohort [4] showed that 44 out of 385 patients had HCC-related LT. Twelve patients of these HCC patients treated with immunosuppressive regimens containing MMF at any time during the follow up and for any period (Supplementary Table 1), whereas 32 patients were treated with immunosuppressive regimens that did not contain MMF.

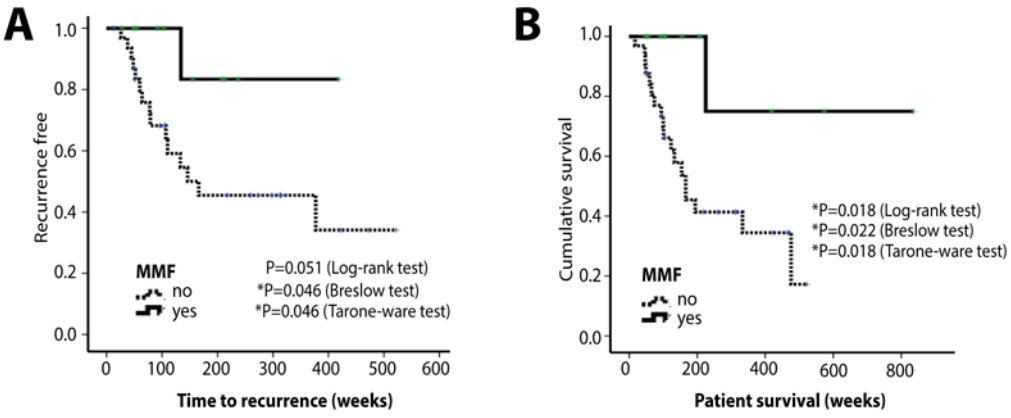
There were no significant differences between these groups regarding to patient characteristics, including age and sex, and regarding known prognostic factors of HCC recurrence after LT [27], including the size of tumor, the number of lesions, tumor differentiation stage, vascular invasion, the level of α- fetoprotein (AFP) before transplantation and time of follow up (Table 1).

However, only one out of twelve patients (8.3%) in the MMF group developed recurrence; whereas fifteen out of thirty-two patients (46.9%) in the control group developed recurrence during follow up. Thus, the use of MMF was significantly associated with lower recurrence rates ($P < 0.05$) and higher survival rates ($P < 0.01$; Table 1). Kaplan Meier analysis confirmed that patients using MMF have significantly temporally delayed HCC recurrence ($P < 0.05$; Fig. 2A) and associated with better survival ($P < 0.05$) (Fig. 2B). Consistently, cox regression analysis revealed that patients using MMF have a lower risk of fast recurrence (progression; HR = 0.169, 95% CI: 0.022-1.284; Fig. 2C) and lower risk of demise (HR = 0.128, 95% CI: 0.017-0.967; Fig. 2D). These results indicate that MMF use is associated with reduced HCC recurrence and improved survival in liver transplant patients.

Table 1. Patient characteristics according to MMF use

No	Characteristics	MMF use		*P*-value[a]
		No(%/Median)	Yes(%/Median)	
1	Age	54.94	56.33	------
2	Sex (% male)	23/32 (71.9%)	10/12(83.3%)	0.446
3	Recurrence*	15/32 (46.9%)	1/12 (8.3%)	0.017*
4	Death**	18/32 (56.2%)	1/12 (8.3%)	0.004**
5	Size of tumor(>= 2 cm)	18/32 (56.2%)	8/12 (66.7%)	0.542
6	Number of lesions(>= 2)	20/31 (64.5%)	8/12 (66.7%)	0.898
7	Differentiation			
	Good	9/31 (29.0%)	3/11 (27.3%)	0.798
	Moderate-Bad	22/31 (71.0%)	8/11 (72.7%)	0.789
8	Vaso - invasion	9/30 (30%)	1/11 (9.1%)	0.176
9	AFP(>25 µg/L) pre-transplantation	11/20 (55%)	4/12 (33%)	0.248

[a] Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Mann Whitney test.



C

MMF use	P-value	HR	95% CI for HR
yes	0.086	0.169	0.022-1.284

D

MMF use	P-value	HR	95% CI for HR
yes	*0.046	0.128	0.017-0.967

Figure 2. MMF use is significantly associated with better clinical outcome in HCC-related LT patients. Kaplan Meier analysis (n = 44) revealed that patients using MMF display significantly longer times to HCC recurrence (P < 0.05) (A) and have a better survival (P < 0.05) (B); Consistently, cox regression analysis showed that patients using MMF have a lower risk of fast recurrence (progression) (C) and lower risk of poor survival (D). HR: Hazard Ratio, *P < 0.05.

MPA inhibited cell proliferation and colony unit formation in HCC cell lines

In order to investigate whether MPA may directly affect cellular physiology of HCC cells, the effects of this drug on cell proliferation and single cell colony unit formation (CFU) were evaluated in different cell line models of this disease. The results showed that MPA inhibits cell proliferation in HuH6, HuH7 and PLC/PRF/5 cell lines at clinically relevant concentrations [28] (Fig. 3C). In apparent agreement, MPA profoundly inhibited the number of colonies formed in the CFU assay. It appears that even at a relatively low concentration (3 μM), MPA already impeded colony formation (Fig. 3D and E). HuH7 cells were more sensitive to MPA treatment compared to the other two HCC cell lines, in this cell models 103 ± 3 colonies/500 cells were formed in untreated cultures but only 22 ± 2 colonies were formed in 15 μM MPA treated groups (mean ± SEM, n = 6, P < 0.01; Fig.3D). We concluded that MPA strongly interferes with HCC expansion *in vitro*.

Exogenous nucleotide supplementation partially counteract the anti-growth effect of MPA

Depletion of intracellular nucleotide pool is the key immunosuppressive mechanism employed by MPA to inhibit lymphocytes proliferation. Supplementation of exogenous guanosine nucleotide indeed partially counteracted the anti-proliferative effects of MPA on HCC cells, this is marked by the effects seen at low doses of MPA (3 μM) in HCC cells, but high doses of MPA out-compete exogenous guanosine nucleotides

(Fig. 4A). This effect was also observed when colony formation by HCC cell line models were studied (Fig. 4B and C).

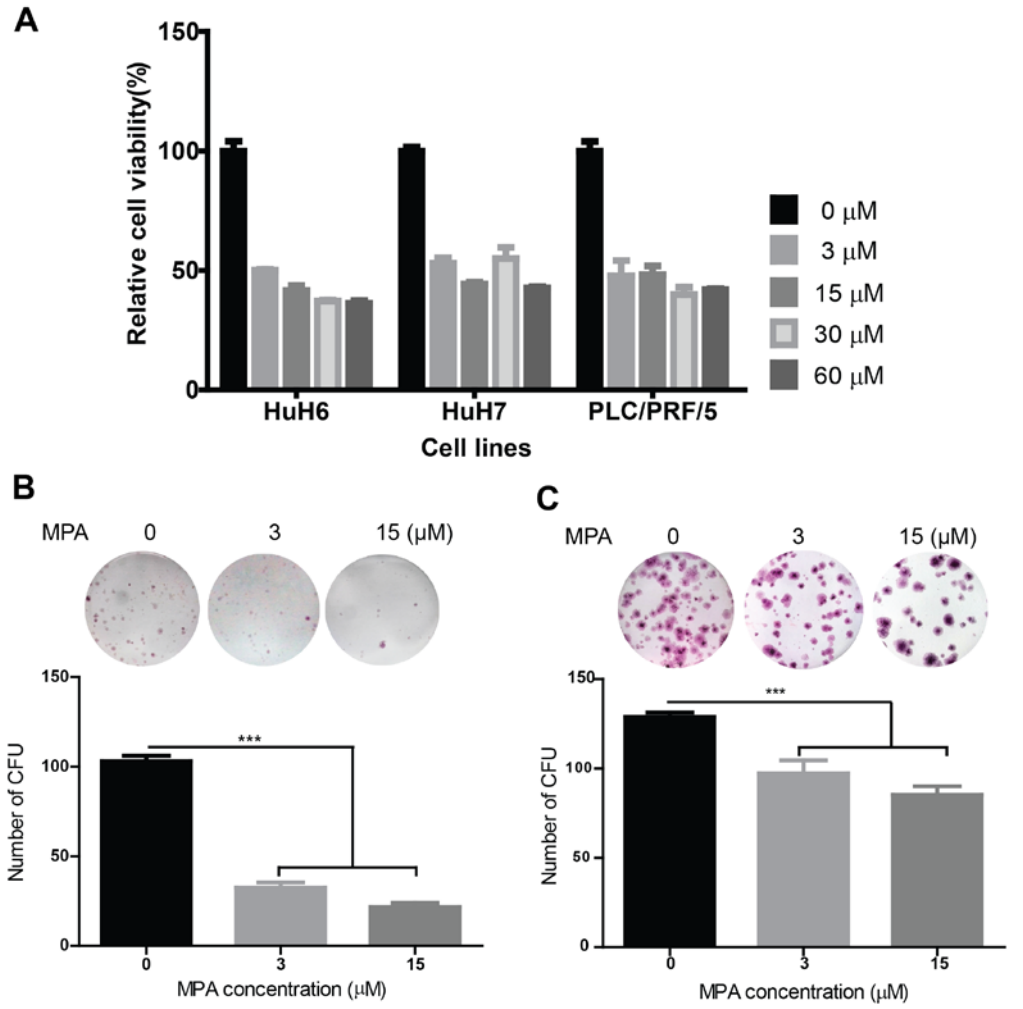


Figure 3. MPA inhibited cell growth in HCC cell lines. (A) With clinically achievable concentrations, MPA potently inhibited cell proliferation, determined by MTT assay (mean ± SD, n = 5); MPA inhibited the ability of colony formation in HuH7 cell line (B) and HuH6 cell line (C). (mean ± SEM, n = 6, respectively, ***P < 0.001).

Other IMPDH inhibitors have potential immunosuppressive and anti-HCC agents

The effects raise the possibility that other IMPDH inhibitors exhibiting superior anti-HCC activity as compared to MPA but with comparable immunosuppressive activity may constitute improved treatment choices following HCC-indicated LT. Thus

we attempted to identify such compounds. Twenty-three IMPDH inhibitors were profiled. Their immunosuppressive capability was evaluated in a T cell proliferation assay. Fourteen of them were more potent than MPA in inhibiting T cell proliferation after 72 h treatment (Fig. 5A). Intriguingly, four of these compounds were identified as more potent inhibitors of HuH6 cell proliferation than MPA using dose-response assays (Fig. 5B). Collectively, three compounds were found possessing both stronger immunosuppressive and anti-tumor activity than MPA, including 1351, 1353 and 1382. Interestingly, the data showed that 6 compounds, 1385, 1393, 1400, 1402, 1406 and 1407 inhibit HCC cell proliferation without affecting T cell growth, which suggests that these six compounds may have potential as new generation of anti-HCC drugs in a non-transplant setting that does not require immunosuppression.

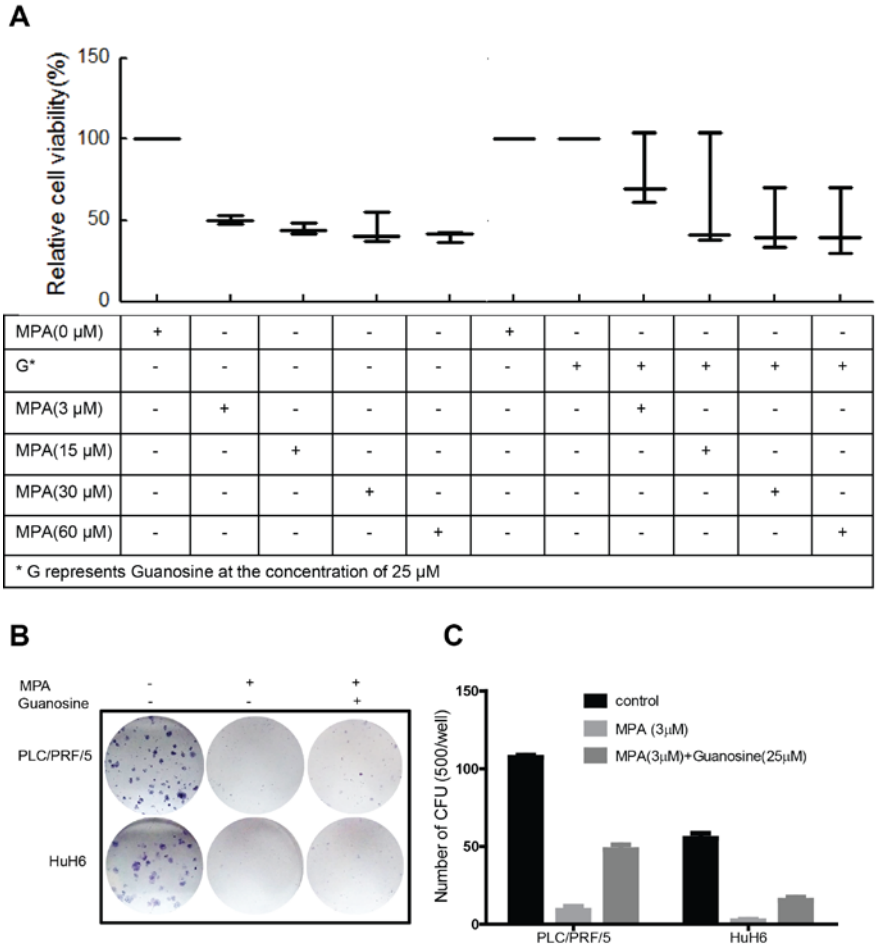


Figure 4. Guanosine supplementation partially counteracts effects of MPA. MTT assay (A) and CFU assay (B) showed that exogenous guanosine could partially counteracted the effect of MPA; (C) Quantification of CFU assay.

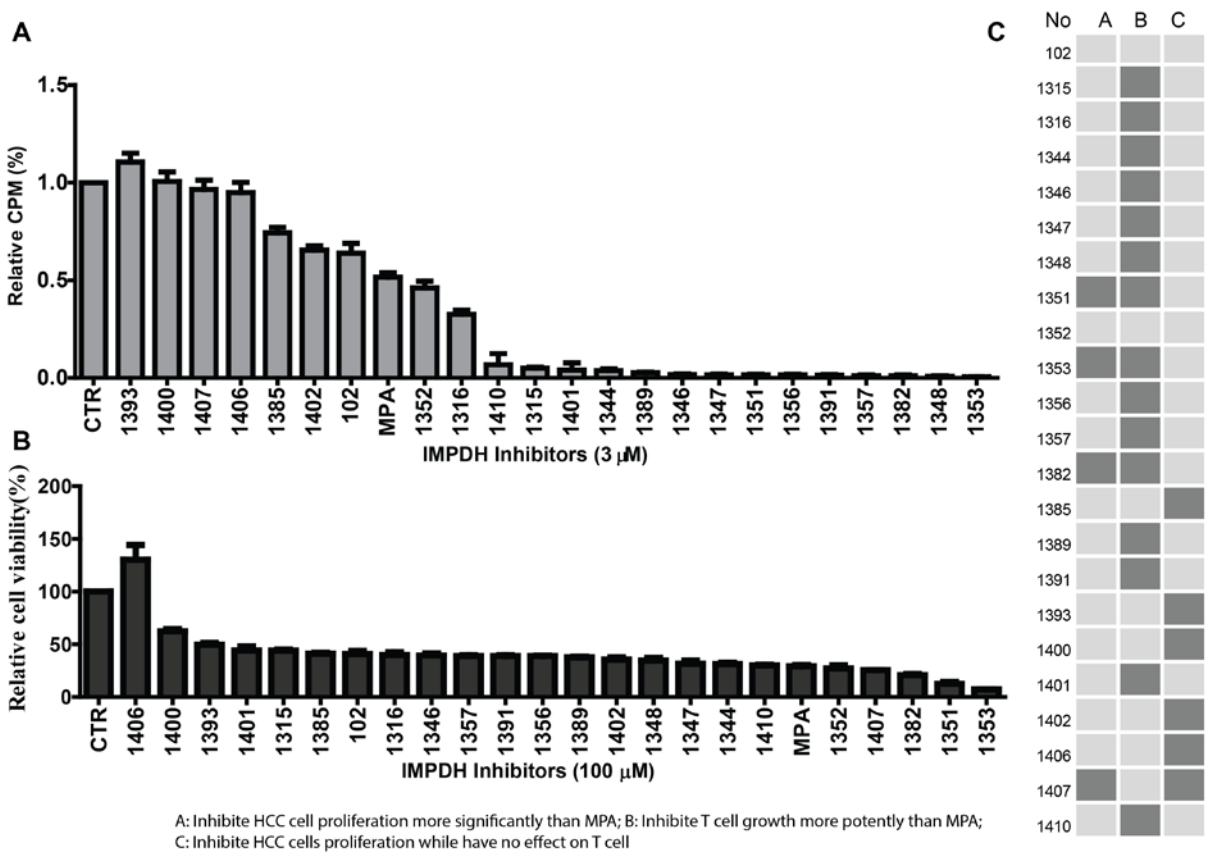


Figure 5. Other IMPDH inhibitors and their immunosuppressive and anti-tumor activity. (A) [3H]-Thymidine assay showed that 14 compounds were more potent than MPA in inhibiting T cell proliferation; (B) MTT assay showed that 4 compounds were more potent than MPA in anti-HCC activity; (C) 3 compounds were verified to be both better immunosuppressants and stronger inhibitors of HCC growth, 6 compounds could inhibit HCC cell proliferation without effects on T cell proliferation.

Discussion

Although it is suspected that immunosuppressive medication following LT facilitates HCC recurrence, the issue of how specific immunosuppressive drugs affect the disease process is only poorly understood [29]. Obviously, a regimen that can perform its immunosuppressive function necessary for preventing graft rejection but that concomitantly exerts anti-tumor effects against HCC should be the preferential clinical choice in this particular setting. In this aspect, mTOR inhibitors attract attention as they are potent renal function-sparing immunosuppressant with anti-proliferative activity on tumor cells [9-13]. However, only approximately 50 % of all HCC exhibit activation of mTOR downstream signaling elements and retrospective or meta-analysis-based studies do not provide firm evidence to conclude that mTOR

inhibitors exert beneficial effects with regard to HCC recurrence after LT [12, 30, 31]. Indeed, both experimental and clinical evidence suggests that tumors bearing different genetic mutations can respond differentially to mTOR inhibitors [32, 33]. Given the heterogeneity of HCC [18], other immunosuppressive regimens also deserve careful attention. In this study, we demonstrated a cancer-constraint effect of MPA in experimental HCC models. We further provided clinical evidence that the use of MMF, a prodrug that metabolized into MPA after administration, is associated with reduced disease recurrence and improved survival in HCC-related liver transplant patients.

A potential anti-malignant effect of MPA in patients has been evaluated in clinical settings other than LT. A phase 1 clinical trial in patients with advanced multiple myeloma showed a positive correlation between clinical responses and depletion of the intracellular deoxyguanosine triphosphate levels by MMF [34]. Our data support that MPA has potent inhibitory effects on HCC growth *in vitro*. Indeed, a tendency toward a lower risk of malignancy in MMF-treated renal transplant patients versus non–MMF-treated renal transplant patients has been reported in a large, prospectively conducted, observational cohort study [26].

Excitingly, after performing a retrospective analysis in our LT cohort, we found a strong association between MMF use and reduced HCC recurrence and improved patients survival. Importantly, there are no significant differences regarding to patient and tumor characteristics [27] between these two groups. It must be said that our observations may also be related to a potential inferior immunosuppressive effect of MMF containing treatment regimens. Still, regardless of the small sample size, the single center setting and retrospective nature of these findings, further clinical evaluation is warranted preferentially in randomized studies to confirm our findings. Moreover, out of 23 other IMPDH-inhibitors studied, three compounds were found to possess both stronger immunosuppressive and anti-tumor activity than MPA and may therefore be considered as promising alternatives for MMF in the LT setting.

In summary, this study demonstrated that clinically relevant concentrations of MPA are capable in constraining HCC cell growth in experimental models. We further provided clinical evidence that MMF is associated with reduced HCC recurrence and improved survival in liver transplant patients. Confirming these experimental findings and retrospective clinical observations by prospective randomized trials could lead to better management of immunosuppressive medication for HCC patients after LT.

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Supplementary Information

Supplementary Table S1. Clinical information on patients using MMF

No	Age (yrs)	LTx date	Recurrence date	Start date MMF	End date MMF	MMF Period (weeks)	AFP before transplantation
1	58	22-May-1992	-	26-Jan-98	21-Feb-08	525	N/D
2	50	18-Jan-1998	-	26-Sep-02	04-Feb-11	436	116
3	55	18-May-2006	-	24-May-06	04-Jul-13	371	15
4	53	28-Jul-2007	-	13-Aug-07	12-Jun-13	304	6
5	60	21-Dec-2005	-	25-Jan-06	07-May-09	171	94
6	69	21-May-2000	-	19-Dec-05	22-Sep-08	144	15
7	63	05-Sep-2007	-	11-Sep-07	15-Jun-10	144	19
8	65	20-Nov-2004	19-Jun-2007	15-Sep-05	17-Mar-08	130	101
9	58	01-Jan-2007	-	29-Jan-07	21-Jan-10	155	9
10	24	09-Feb-2005	-	02-Mar-05	20-Apr-06	59	1
11	65	23-Aug-2007	-	23-Aug-07	21-Sep-07	4	2
12	56	22-Jan-2007	-	26-Jan-07	15-Oct-07	37	200

Note: - no recurrence; N/D not determined.

Chapter 4

Differential sensitivities of fast- and slow-cycling cancer cells to inosine monophosphate dehydrogenase 2 inhibition by mycophenolic acid

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Abstract

As uncontrolled cell proliferation requires nucleotide biosynthesis, inhibiting enzymes that mediate nucleotide biosynthesis constitutes a rational approach to the management of oncological diseases. In practice, however, results of this strategy are mixed and thus elucidation of the mechanisms by which cancer cells evade the effect of nucleotide biosynthesis restriction is urgently needed. Here we explored the notion that intrinsic differences in cancer cell cycle velocity are important in the resistance towards inhibition of inosine monophosphate dehydrogenase (IMPDH) by mycophenolic acid (MPA). In short-term experiments, MPA treatment of fast-growing cancer cells effectively elicited G0/G1 arrest and provoked apoptosis thus inhibiting cell proliferation and colony formation. Forced expression of a mutated IMPDH2, lacking a binding site for MPA but retaining enzymatic activity, resulted in complete resistance of cancer cells to MPA. In nude mice subcutaneously engrafted with HeLa cells, MPA moderately delayed tumor formation by inhibiting cell proliferation and inducing apoptosis. Importantly, we developed a lentiviral vector-based Tet-on label-retaining system that enables to identify, isolate and functionally characterize slow-cycling or so-called label-retaining cells (LRCs) *in vitro* and *in vivo*. We surprisingly found the presence of LRCs in fast-growing tumors. LRCs were superior in colony formation, tumor initiation and resistance to MPA as compared to fast-cycling cells. Thus, the slow-cycling compartment of cancer seems predominantly responsible for resistance to MPA.

Introduction

Uncontrolled cell proliferation resulting from cell cycling deregulation is a hallmark of cancer. Although aggressive cancers are diverse and heterogeneous, they almost universally contain a fast-cycling compartment that can rapidly complete a cell cycle, and these cells are primarily responsible for the increase in tumor mass [1]. This impressive proliferative capacity is, however, dependent on adequate supply of nucleotides. Cellular nucleotide synthesis is biochemically complex, but requires various enzymes that can be clinically targeted, including Inosine monophosphate dehydrogenase (IMPDH), which is a rate-limiting enzyme in *de novo* synthesis of guanine. The enzymatic activity of IMPDH is composed of two separate isoforms, type 1 and 2 [2]. The IMPDH2 isoform is associated with aggressive cancerous disease in experimental cancer [3-6], and related to poor survival in osteosarcoma patients [7]. Mycophenolic acid (MPA) acts as a nonnucleoside, noncompetitive, reversible inhibitor of IMPDH with 5-fold higher potency of inhibiting IMPDH2 than IMPDH1. It has been reported to be able to inhibit cancer cell proliferation and induce apoptosis in several experimental models of human solid tumors and hematological malignancies by depleting guanine nucleotide pools [5, 8-10].

In the last decade, interest into the importance of cell cycle velocity heterogeneity of cancers has increased. Although it was initially thought that cancer cells universally cycle and grow faster than normal cells, recently a slow-cycling (largely quiescent) compartment has been identified in many tumors, which does not divide frequently but with the capacity to generate progeny that can repopulate the fast cycling compartment [11]. Functionally, these slow-cycling cancer cells appear to be associated with capacity to generate new metastases while having superior resistance to therapy [12]. Technically, these slow-cycling cells are identified by their capacity to retain a pulse label as faster cycling cells lose the pulse label at cell division. Thus these cells are indicated with the term label-retaining cells (LRCs) [13].

In this study, we aim to develop a lentiviral vector-based Tet-on label-retaining system that enables to identify slow-cycling cancer cells *in vivo,* which can be subsequently isolated for functional characterization. We exploit this system to investigate the different sensitivity between fast and slow-cycling cancer cells to IMPDH2 inhibition by MPA.

Materials and Methods

Reagents

Stocks of MPA (AMRESCO LLC, USA) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO). Doxycycline, Collagenase IV and DNase were purchased from Sigma-Aldrich Corporation. Antibodies against IMPDH2, p-Histone3 and Cleaved caspase3 were purchased from Abcam Company, Millipore Corporation and Cell Signaling Technology, respectively.

Cell culture

To investigate the effects of MPA on cancer cells, 7 different cancer cell lines derived from various tumor types were cultured. Human hepatoma cell lines HepG2 and HuH7, colon adenocarcinoma cell line Caco2, the epithelioid cervix carcinoma cell line HeLa and ovary adenocarcinoma cell line SKOV-3 were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO Life Technologies). Pancreatic cancer cell lines BxPC3 and PANC-1 were cultured with RPMI-1640. Both of the mediums were supplemented with 10% (v/v) fetal bovine serum (FBS) (Hyclone Technologies), 100 units/mL of penicillin and 100 µg/mL of streptomycin. All the cells were incubated at 37℃ in a humidified atmosphere containing 5% CO_2. All the cell lines were confirmed with mycoplasma free and their STR genotyping were analyzed at the Department of Pathology, Erasmus Medical Center Rotterdam (Supplementary table. 1).

Lentiviral vector-based Tet-on label-retaining system

Lentiviral backbone plasmids pLV.EX3D/EF1A-rtTA(M2)-dsRed-Express2 and pLV.EX2D/TRE-eGFP were used to pack third generation lentiviral vectors (Supplementary Figure S1A). HeLa cells were transduced with both vectors to generate a system (HeLa tet-on) that can express a histone 2B-green fluorescent fusion protein (Histone-GFP) upon induction by doxycycline. GFP expression *in vitro* was analyzed from week 0 to week 3 by flow cytometry analysis (FACS) and confocal microscope (Zeiss LSM 510) (Supplementary Figure S1B and S1C). ZenlightEdition software was used to analyze confocal microscope images. Cells that maintained GFP expression over this period of time were identified as LRCs.

For identification of LRCs *in vivo*, female NOG mice at the age of 8-10 weeks were purchased from Taconic Biosciences (Denmark). Animal experiment was performed with the approval of the institutional animal ethics committee (Dier Experimenten Commissie, Erasmus MC). Mice were breed in Special Pathogen Free (SPF) environment during the whole experimental period. Mice were injected subcutaneously with 5×10^6 HeLa tet-on cells. After engraftment (10-15 days), water containing 1 mg/mL doxycycline and 5% sucrose has been given for 5 days. Mice were sacrificed at different time points after withdrawal of doxycycline. A proportion of harvested tumor samples was fixed with 4% paraformaldehyde and embedded in paraffin for subsequent immunohistochemistry. While the remaining tumor tissue was dissociated with 5 mg/mL collagenase IV and 2 mg/mL DNase at 37℃ for 30min to obtain single cell suspension. Cells were further sorted as singlets for separation into Non-LRCs (GFP^{low}dsRed) and LRCs (GFP^{high}dsRed) by FACS sorter (Supplementary Figure S2). Non-LRCs and LRCs were injected subcutaneously into mice (either 1,000 cells or 10,000 cells *per* injection as appropriate) on four sites in the mice. At the same time, two populations of sorted cells were plated for colony formation unit assay (CFU) (treated with or without MPA).

Colony formation assay

Cells were harvested and suspended in culture medium, yields were quantified through counting and plated in 6-well plates (500 cells/well), and then treated with serial dilutions of MPA (1, 2, 3, 4 and 5 μg/mL). The control group was supplemented with equal volume of PBS. For the cells derived from xenograft tumour, cells were seeded into 12-well collagen coating plates and cultured in medium with or without MPA (10 μg/mL). Formed colonies were fixed by 70% ethanol and counterstained with haematoxylin & eosin after two weeks. Colony numbers were counted and their sizes were measured microscopically through digital image analysis.

MTT assay

Cells were seeded in 96-well plate, at a concentration of 5×10^3 cells/well in 100 μL medium. Cells were incubated overnight to attach to the bottom of the wells, and then treated with serials dilutions of MPA (1, 5, 10, 15, 20, 25 and 30 μg/mL). Cell viability was analyzed by adding 5 mg/mL MTT (Sigma-Aldrich, St Louis, MO) and 150 μL DMSO. Absorbance was determined using Enzyme mark instrument at the wavelength of 490 nm.

Analysis of cell cycle

Cells (5×10^5/well) were plated in six-well plates and allowed to attach overnight, followed by application of MPA at the concentrations of 5, 10, 15, 20 and 25 μg/mL for 48 h. Vehicle control was performed through the addition of an equal volume of PBS. After 48 h, control and treated cells were trypsinized and washed with PBS and then fixed in cold 70% ethanol overnight at 4℃. The cells were washed twice with PBS and incubated with 20 μg/mL RNase at 37℃ for 30 min, and then with 50 μg/mL Propidium Iodide (PI) at 4℃ for 30 min. The samples were analyzed immediately by FACS. Cell cycle was analyzed by ModFit LT 3.0 software.

Analysis of cell apoptosis

Cell apoptosis analysis was performed by staining cells with Annexin V-FITC and PI. Cells (5×10^5/well) were seeded into six-well plates and incubated at 37℃ in 5% CO_2 for overnight, then serials dilutions of MPA (5, 10, 15, 20 and 25 μg/mL) were added; whereas for vehicle control, an equal volume of PBS was used. After 48 h, all of the cells were trypsinized and resuspended in Annexin-binding buffer, and stained with Alexa Fluor 488 AnnexinV and PI at room temperature for 15 min. Detection of apoptosis was performed by FACS.

Xenograft assays in nude mice

The xenograft tumor model was performed using nude mice in accordance with current prescribed guidelines and under a protocol approved by the Institutional Animal Care and Use Committee of Hangzhou Normal University, China. Mice were breed in SPF environment during the whole experimental period. Mice were all female and 4-6 weeks of age at the time of inoculation, and were subcutaneously inoculated with $5X10^6$ of HeLa cells. After 20 hours, mice were divided into 3 groups and were treated with different doses of MPA or PBS (240 mg/kg body weight, n = 10; 60 mg/kg body weight, n = 11 and PBS, n = 10) [2]. MPA was injected via the intraperitoneal (IP) route for 20 consecutive days. Tumor formation was monitored through palpation. At day 30 post-engraftment, mice were sacrificed and tumors were harvested and macroscopically analyzed. Tumor tissues were fixed with 4% paraformaldehyde and embedded in paraffin for evaluation by histology or immunohistochemistry.

Immunohistochemistry

Paraffin embedded tumor tissue slides were deparaffined in xylene, rehydrated in graded alcohols, and rinsed in PBS supplemented with 0.05% Tween 20. Slides were boiled in citrate acid buffer (pH 6.0) for 10 min to retrieve antigen. A 3% H_2O_2 for 20

min at room temperature treatment was used to block endogenous peroxidase activity. The slides were incubated in 5% milk-containing blocking solution followed by overnight incubation with either a rabbit monoclonal antibody against IMPDH2, a rabbit polyclonal antibody against p-Histone H3 or a rabbit polyclonal antibody against cleaved caspase 3, used at a final dilution of 1:500, 1:1000, 1:3000 respectively and then counterstained with hematoxylin according to routine procedures. As a negative control, the primary antibody was omitted; positive controls were taken from other slides that had been successfully stained before. IMPDH2, phosphor-Histone H3 and cleaved caspase 3 staining were scored by two independent expert observers. The numbers of mitotic cells and cleaved caspase 3 positive cells were counted in 10 high-power fields. Median numbers of positive cells in each of the 10 fields were calculated for each sample of the different groups using a semi-quantitative assessment. Three categories were used to evaluate the percentages of apoptotic cells: < 10% mild; 10% - 50% moderate; > 50% high. The intensity of IMPDH2 staining was presented by categories: + weak; ++ moderate; +++ strong.

Statistical analysis

Statistical analysis was performed by using the nonparametric Mann-Whitney test for paired or non-paired data, or the paired T-test using GraphPad InStat software as appropriate. A P value < 0.05 was considered statistically significant.

Results

MPA is very effective for inhibiting cancer cell proliferation in fast-growing cell lines

A first indication as to how MPA effects on cancer cells relate to cell cycle velocity comes from experiments in which we investigated the effects of MPA on cell proliferation and colony-forming potential of different cancer cell lines. To this end, 7

different cancer cell lines derived from various tumor types were compared. Growth curves show substantial variation in the proliferation rate and colony-forming potential of these cell lines; with in general HeLa and Caco2 showing more aggressive behavior as compared to the other cell lines (Figure 1). Challenge with MPA inhibited both cell proliferation and colony unit-forming potential of cancer cell lines, but strikingly fast-growing cell lines are more effected by MPA treatment as compared to slow-growing cell lines (Figure 1), indicating that MPA mainly affects the fast-cycling compartment.

MPA inhibited cell proliferation, arrested cell cycling and induced cell apoptosis in fast-growing cancer cell line

To further understand how MPA acts on fast-growing cancer cells, HeLa cells (the most sensitive cell line to MPA emerged from our panel of cancer cell lines) were treated with clinically relevant MPA concentrations [14] and analyzed in more detail for the effects of MPA on cellular expansion, cell cycle, and programmed cell death. MPA counteracted HeLa cell proliferation and colony-forming potential in a time- and dose-dependent manner (Figure 2A). Indeed, even a relatively low concentration (1 µg/mL) of MPA already substantially impeded colony formation; whereas higher concentrations (2-5 µg/mL) completely inhibited colony formation. The result reports 322 ± 27 colonies/500 cells were formed in untreated, but only 148 ± 27 colonies were formed in 1 µg/mL MPA treated groups (mean ± SEM, $n = 6$, $P < 0.01$) (Figure 2B). Accordingly, the size of CFU was significantly smaller in MPA treated compared to untreated groups (96 ± 5 pixels vs 278 ± 8 pixels, mean ± SEM, $n = 30$, $P < 0.01$) (Figure 2C). Furthermore, MPA dose-dependently provoked the G0/G1-phase arrest (Figure 2D). In addition, MPA dose-dependently triggered both early and late cell apoptosis (Figure 2E). These data suggest that MPA profoundly interferes with the physiology of fast-growing cancer cells and raises questions as to how cancers can escape the effects of MPA.

IMPDH2 is a relevant target for MPA in inhibiting cancer cell growth

The clinical effects of MPA are presumed to be mediated through inhibition of IMPDH enzymatic activity and subsequent inhibition of *de novo* nucleotide biosynthesis. Although two isoforms of IMPDH exist, the type 2 isoform (IMPDH2) exhibits a 5-fold higher sensitivity to inhibition by MPA as compared to the type 1 isoform (IMPDH1) [3].

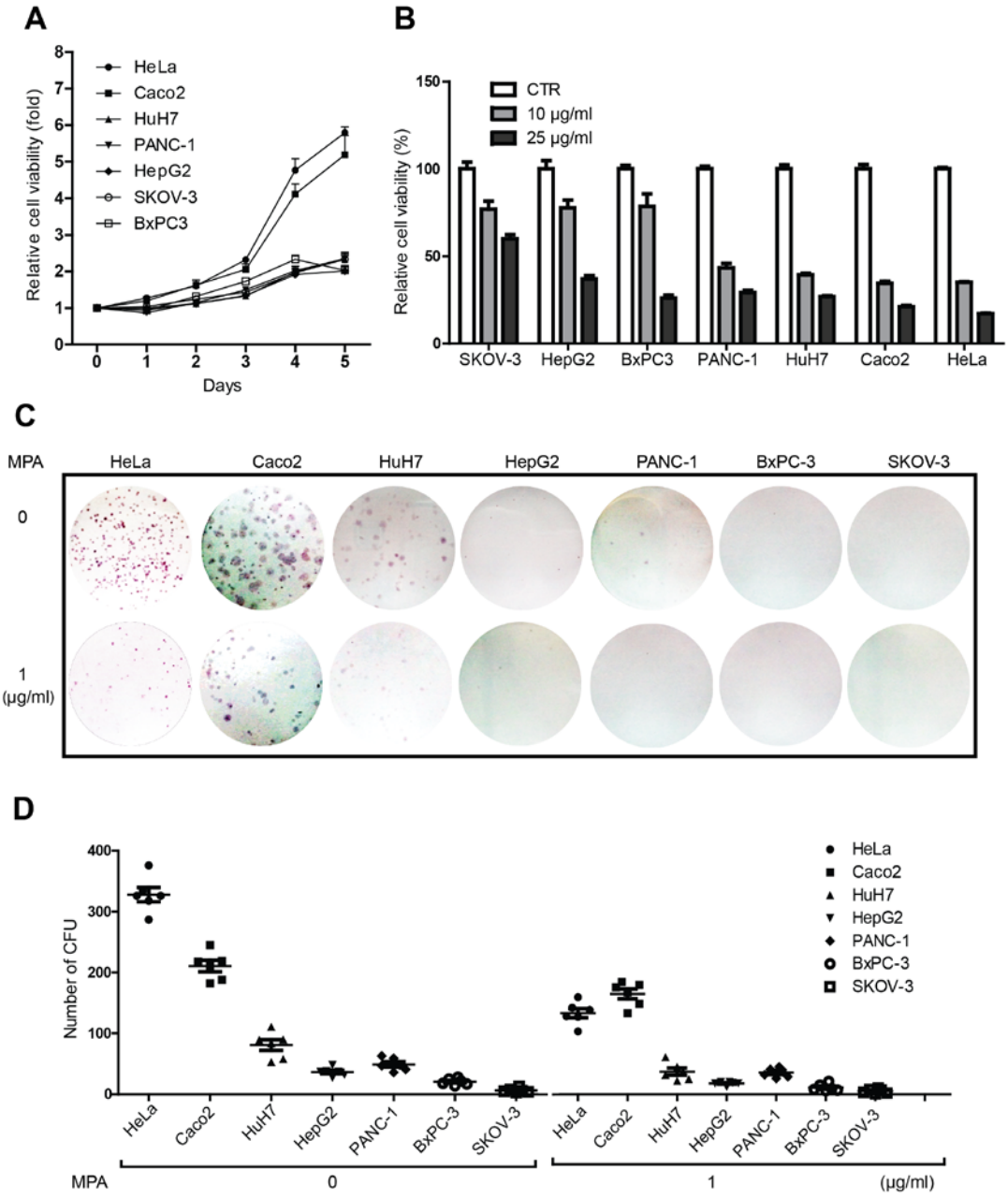


Figure 1. MPA inhibited cell proliferation and colony formation of different cancer cell lines. (A) Growth curve of seven different cancer cell lines show that HeLa cells grow faster than the other cell lines tested (mean ± SD, n = 6). (B) MPA inhibited cell proliferation of all seven cancer cell lines as

determined by MTT, data shown cells were treated by MPA for 72 h (mean ± SD, n = 5). (C) and (D) MPA inhibited single cell colony formation of seven cancer cell lines (mean ± SD, n = 6, ***P < 0.001).

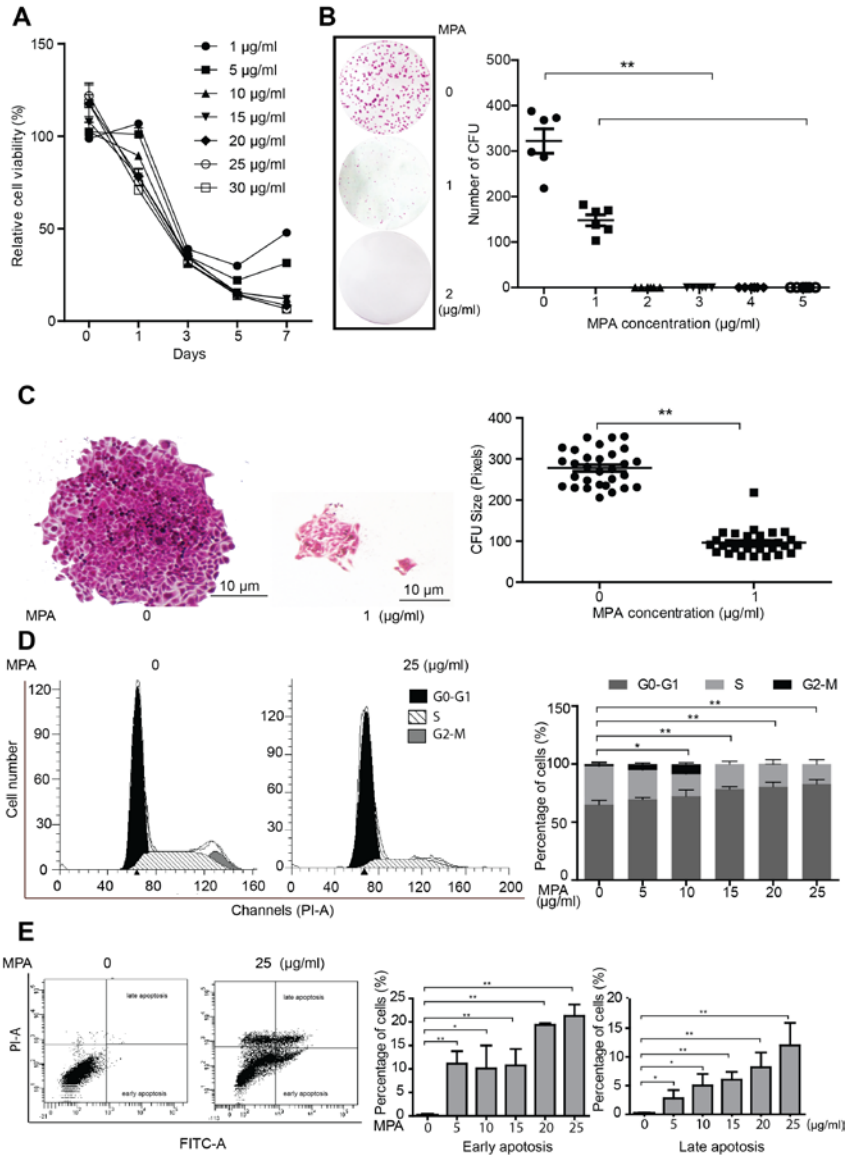


Figure 2. MPA counteracts proliferation of a fast-growing cancer cell line. (A) Clinically-relevant MPA concentrations, potently inhibit proliferation of the HeLa cell line as assessed by MTT activity (mean ± SD, n = 5). (B) Clinically-relevant MPA concentrations impair colony formation of HeLa cells (mean ± SD, n = 6). (C) Clinically-relevant MPA concentrations impair colony growth of HeLa cell as determined by image analysis (mean ± SEM, n = 30, **P < 0.01). (D) MPA treatment causes G0/G1 phase cell cycle arrest. The left panel shows cell cycle phase distribution of a vehicle-treated culture, whereas the middle panel shows cell cycle phase distribution in a MPA-treated culture. The right panel shows a quantification of the MPA effects on the cell cycle of HeLa (mean ± SD, n = 3. *P < 0.05; **P < 0.01). (E) FACS analysis of cellular apoptosis through Annexin-V positivity and PI incorporation. The left panel provides an example of a vehicle-treated HeLa culture, whereas the middle panel provides an example of the effects seen following MPA treatment. The quantification in the right panel shows statically significant stimulation of both early and late apoptosis in the 5-25 µg/mL MPA concentration (mean ± SD, n = 3, *P < 0.05; **P < 0.01).

IMPDH2 is assumed to be the major target of MPA. Moreover, IMPDH2 is often up-regulated in cancer [15], suggesting that IMPDH2 is the relevant target for MPA in the experiments described above. To substantiate this notion, we employed a lentiviral vector expressing an experimentally mutated *IMPDH2* (mut*IMPDH2*) fused to *GFP* [16, 17]. The product of this construct has normal IMP dehydrogenase activity but lacks the binding site for MPA and thus confers MPA-resistance. Transduction of this vector resulted in successful expression of this mutated allele in HeLa cells (Figure 3A). In the CFU assay, forced expression of this mutated *IMPDH2* provoked resistance to MPA treatment (Figure 3B and 3C). Furthermore, the mut*IMPDH2* cells prevented MPA-induced apoptosis (Figure 3D). These results are consistent with a key role of IMPDH2 in mediating the effects of MPA in our experimentation.

MPA delayed tumor initiation, inhibited cancer cell proliferation and induced cell apoptosis *in vivo*

Insight into the effects of MPA on tumor cell *in vivo* was obtained in experiments, in which nude mice were used for subcutaneous engraftment of the HeLa cell line. 20 hours after inoculation, mice were intraperitoneally injected with MPA for 20 consecutive days. In this xenograft model, treatment of MPA (60 mg/kg body weight) significantly ($P < 0.05$) delayed tumor initiation (Figure 4A). In the 240 mg/kg body weight of MPA treated group, one mouse failed to form tumor; while tumor formation also tended to be delayed in the other mice (Figure 4B). Thus MPA counteracts growth of experimental tumors in this model. Immunohistochemical staining of tumors harvested from these mice demonstrated significant down-regulation ($P < 0.05$) of IMPDH2 at the protein level following treatment with MPA (Figure 4C). Concomitantly, MPA inhibited tumor cell proliferation, as shown by a significant reduction ($P < 0.05$) of proliferating cells assessed by the percentage of p-histone H3 positive cells (Figure 4D). Furthermore, MPA treatment provokes substantial apoptosis in the tumor cell compartment, as evidence from the significant increase in the percentage of cleaved

caspase 3 positive cells (Figure 4E). These results show that MPA counteracts tumor growth elicited by a fast-growing cancer cell *In vivo.*

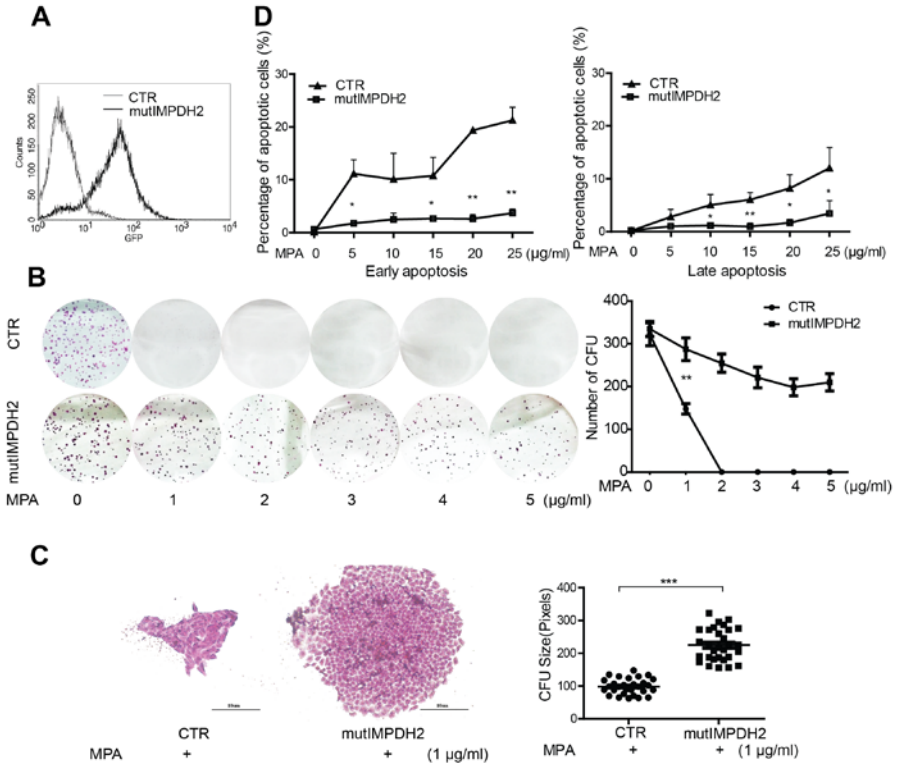
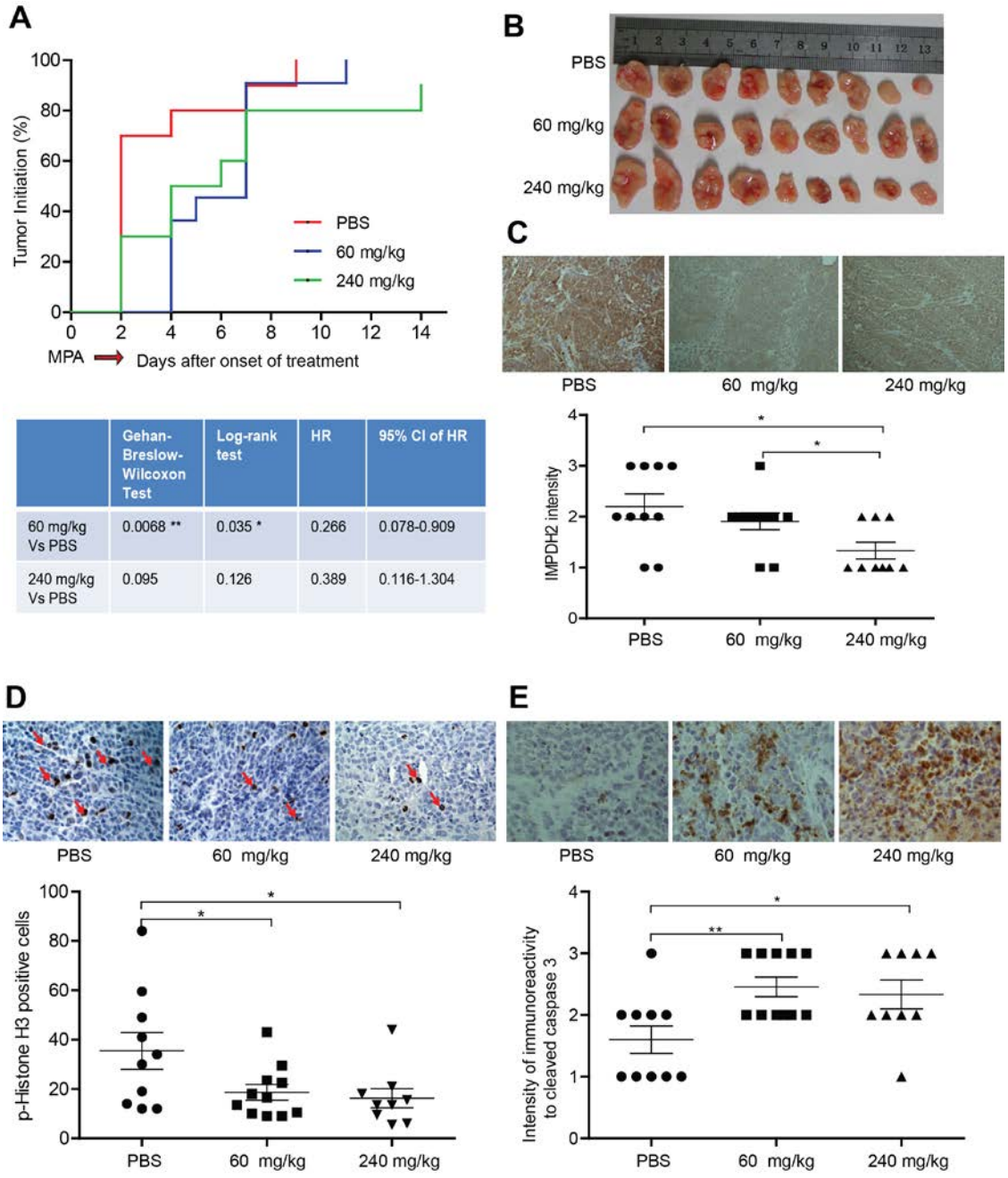


Figure 3. Forced-expression of a mutated IMPDH2, lacking the MPA binding site, results in resistance to MPA. (A) An IMPDH2 variant having normal IMP hydrogenase activity but lacking the MPA binding site was fused to a GFP reporter (mutIMPDH2) and expressed in HeLa cells by a lentiviral vector. FACS analysis showed robust GFP expression in the transduced HeLa cells but not in mock-transduced cells. Transduced cells appear to be resistant to MPA in both colony formation (B) (mean ± SD, n = 6, **P < 0.01) and cell proliferation assays (C) (mean ± SEM, n = 30, ***P<0.001). (D) MPA-mediated induction of both early apoptotic cells as well as late apoptotic cells is significantly reduced in mutIMPDH2 HeLa cells (mean ± SD, n = 3. *P < 0.05; **P < 0.01).



	Gehan-Breslow-Wilcoxon Test	Log-rank test	HR	95% CI of HR
60 mg/kg Vs PBS	0.0068 **	0.035 *	0.266	0.078-0.909
240 mg/kg Vs PBS	0.095	0.126	0.389	0.116-1.304

Figure 4. MPA delayed tumor initiation, inhibited cell proliferation and provokes tumor apoptosis in mice. (A) MPA treatment significantly delays tumor initiation by HeLa cells in nude mice. (B) Following the experiment, animals were sacrificed and tumors were harvested. The photograph illustrates the macroscopic appearance of the tumors in the respective groups. (C) Immunohistochemical staining of harvested tumor tissue sections revealed a significant down-regulation of IMPDH2 protein levels following treatment with MPA. (D) Treatment of MPA significantly reduced the percentage of p-histone H3 positive (proliferating) cells in the tumors. (E) MPA treatment of HeLa-grafted nude mice significantly increased the numbers of anti-cleaved caspase 3 immunoreactive (apoptotic) cells (mean ± SEM, PBS, n = 10; 60 mg/kg, n = 11; 240 mg/kg, n = 9, *P < 0.05; **P < 0.01).

Existence of slow-cycling cancer cells compartment in fast-growing tumors

Substantial evidence indicated that slow-cycling cancer cells can evade therapeutic agents and repropagate the tumor [18]. In order to identify whether there are slow-cycling cancer cells in our experimental system, we established a lentiviral based Tet-on label-retaining system that enables us to isolate slow-cycling cells for further functional characterization. To this end, cells were transduced with two vectors. One vector expresses a reverse Tet trans activator (rtTA), dsRed fluorescent protein and a neomycin-resistance cassette. The other vector expresses a Histone-GFP fusion protein driven by a tetracycline response element (TRE) and puromycin-resistance gene (Supplementary Figure S1A). Stable cell lines can be established by co-transducing with these two vectors and the clones can be selected either via drug resistance or by cell sorting based on fluorescence. This constitutes a genotoxic free and cell proliferation independent approach to identify slow-cycling cells. Upon induction by doxycycline, all the cells are labeled with GFP. After doxycycline withdrawal, dividing cells lose their GFP signal; whereas quiescent or slow-cycling cells retain their GFP expression, which thus serves as a label for LRCs (Figure 5A and Supplementary Figure S1).

HeLa cells engineered with Histone-GFP$^{tet\text{-}on}$ were subcutaneously engrafted in immunodeficient mice (Figure 5B). Once a small tumor was formed, Histone-GFP expression was induced by doxycycline in the drinking water of the animal. Following doxycycline withdrawal, mice were sacrificed at different time points. As shown by both FACS and Immunohistochemical staining, a small population of LRCs was detected in tumors (Figure 5C and 5D). Thus we concluded that even fast-growing tumors harbor LRC compartment.

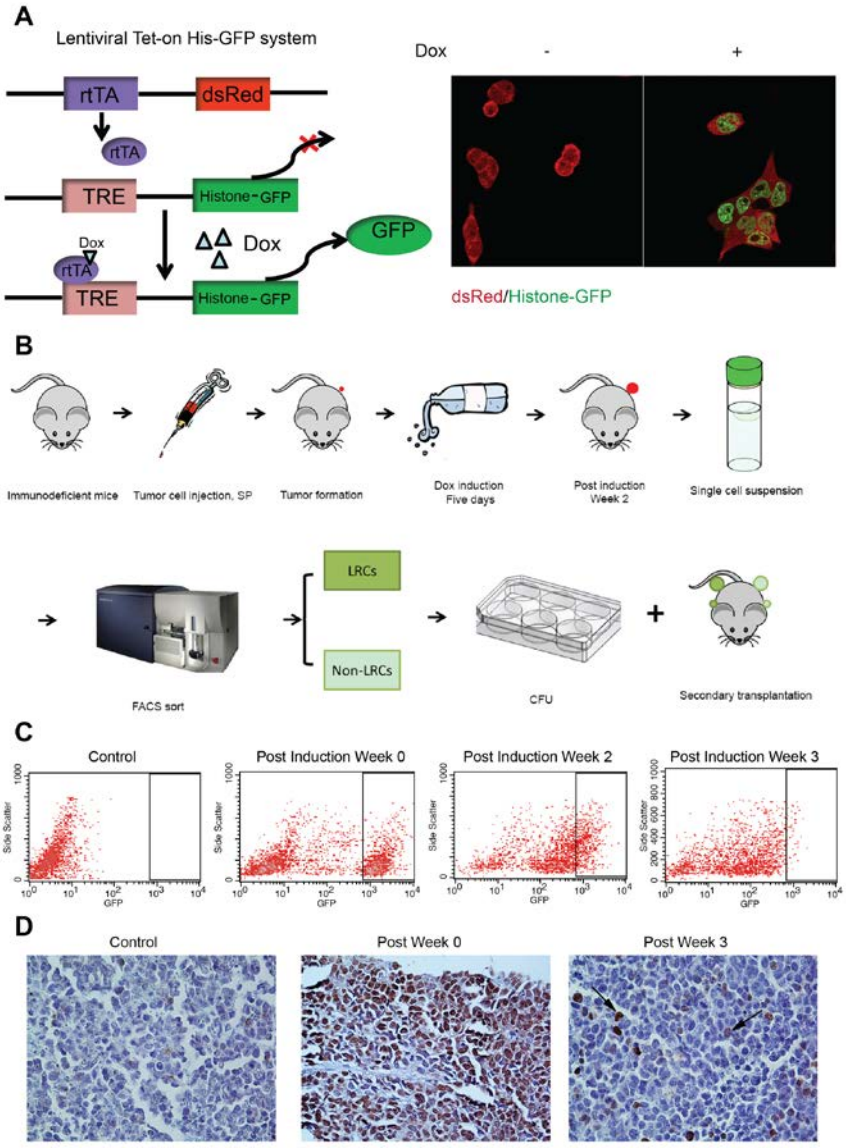


Figure 5. Identification of slow-cycling cancer cells using a lentiviral label-retaining system. (A) Illustration of principle the lentiviral Tet-on Histone-GFP strategy employed. Confocal imaging confirms the specific induction of nuclear Histone-GFP expression in HeLa cells upon doxycycline treatment. (B) Schematic representation of the experimental approach to identify, isolate and characterize the slow-cycling cells, or termed as label-retaining cells (LRCs) *in vivo*. (C) FACS characterization of Histone-GFP expression in transduced HeLa cell during 3 weeks following release from doxycycline induction. (D) Immunohistochemical staining of harvested xenograft tumor tissues confirms the presence of nuclear anti-GFP immunoreactivity in these tissues.

Slow-cycling cells are superior in tumor formation and display more resistance to MPA

To functionally characterize LRCs, LRCs and non-LRCs were isolated from HeLa cell-derived tumors using FACS sorter (Figure 5B and Supplementary Figure S3). Surprisingly, *ex vivo* CFU assay showed that LRCs were significantly more efficient in colony formation (non-LRCs: 2.8 ± 1.3 colonies/100 cells; LRCs: 8.1 ± 2.2, mean ± SEM, n = 19, $P < 0.001$) when compared to fast-cycling cells (Figure 6A). Consistently, LRCs are more efficient in forming tumors, both with regard to size and number in immunodeficient mice upon subcutaneous engraftment (Figure 6B).

Subsequently, we evaluated the relative sensitivity of LRCs and non-LRCs to MPA. Both populations were sorted and colony forming potential was assessed in the presence or absence of MPA. Compared with the control groups, treatment with MPA (10 μg/mL) significantly inhibited the colony formation efficiency of non-LRCs but not of LRCs (where only minor effects were seen; Figure 6C). Thus, slow-cycling cancer cells when compared to fast-cycling cancer cells appear more resistant to MPA and may thus constitute the MPA-resilient reservoir in cancers.

Discussion

IMPDH is a key enzyme in *de novo* guanine nucleotide biosynthesis and is thus a target for oncologic disease. MPA works as a potent IMPDH inhibitor that is used as an immunosuppressive drug [3]. A phase I trial in patients with advanced multiple myeloma showed a positive correlation between clinical responses and depletion of the intracellular deoxyguanosine triphosphate levels by mycophenolate mofetil (MMF), the prodrug of MPA. MMF was administered up to a maximum dose of 5 g/day, which is 2-3 times higher than general use in organ transplantation patients, but was well tolerated in this study [19]. In renal transplant patients, a tendency towards a lower risk of malignancy in MMF-treated patients versus non–MMF-treated has been reported in a large, prospectively conducted, observational cohort study [20]. However, another

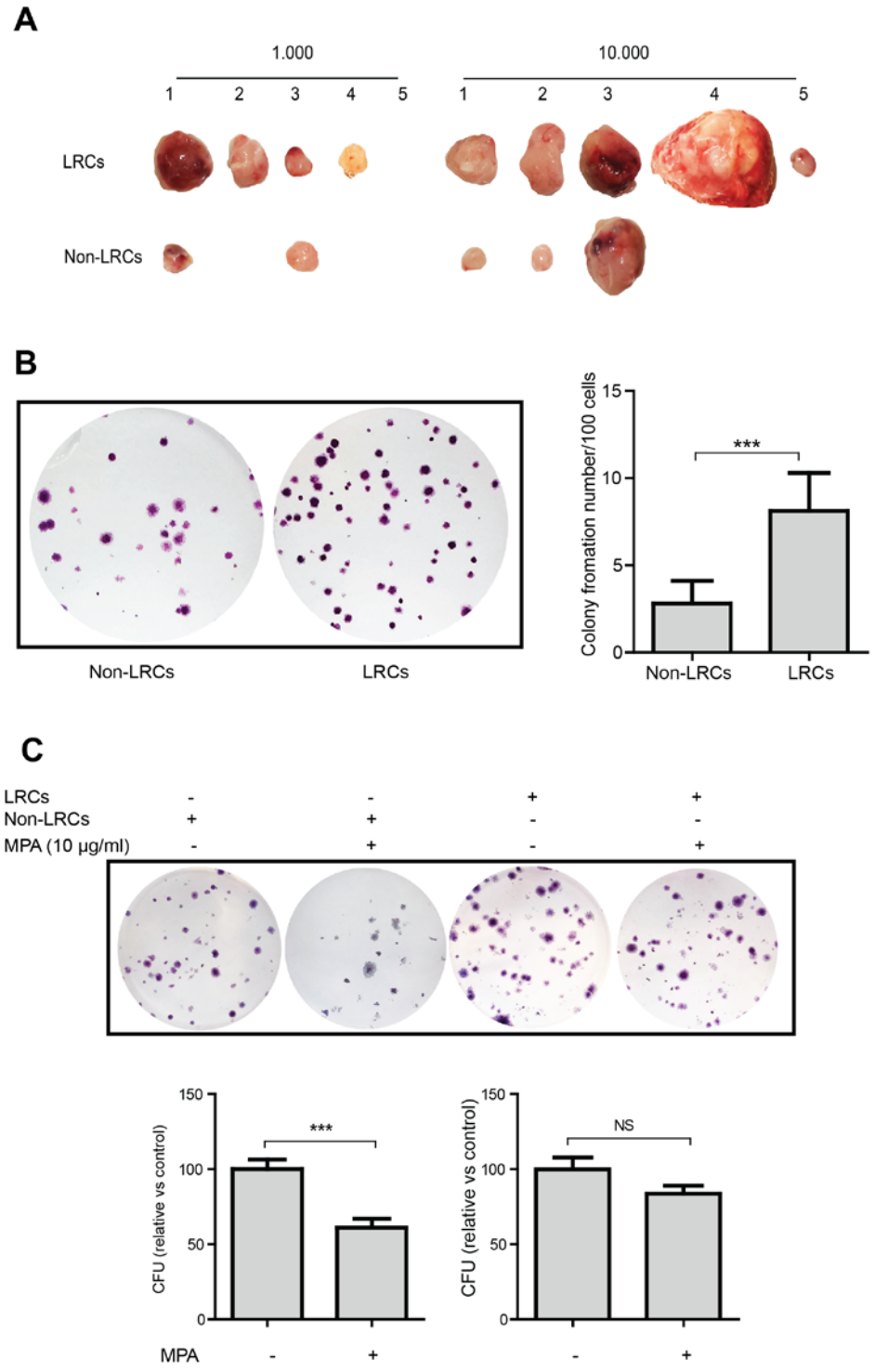


Figure 6. Grafted label-retaining cells are superior in tumor initiation and more resistance to MPA. (A) The macroscopic appearance of the tumors formed following secondary grafting of LRCs and non-LRCs respectively. (B) Colony formation following grafting LRCs and Non-LRCs harvested from xenograft tumor tissues (mean ± SEM, n = 19, ***P<0.001). (C) Colony formation by LRCs and Non-LRCs harvested from xenograft tumor tissues, when treated with MPA at a concentration of 10 µg/mL (mean ± SEM, n = 17, ***P<0.001).

clinical study in pancreatic cancer failed to show any beneficial effects [21]. We found that only fast-cycling, but not slow-cycling, cancer cells are sensitive to the inhibitory effects of MPA. Thus, dissecting the heterogeneity of cancer may help to understand the distinct responsiveness to MPA treatment.

A unique aspect of this study is the use of a label retaining technique that enables to identify and isolate LRCs. Classically, nucleotide analogs, such as 5-iodo-2'-deoxyuridine and Bromodeoxyuridine, are commonly used to identify LRCs. These agents can be used to identify LRCs, but the cells are not able to be isolated for functional study [22]. Thus, fluorescent-coupled nucleotide analogs were developed for identification and isolation of LRCs. However, these agents are not competent for *in vivo* application [23]. Although nucleotide analogs were also able to label cells *in vivo*, subsequent isolation of LRCs from the tissue is often a challenge that hampers further functional investigation [22]. Another major drawback is that most of these labeling methodologies rely on cell division to label cells. Therefore, the real quiescent cells in fact cannot be labeled. Consequently, the introduction of modified nucleotides into cells profoundly alters the status of the cells [24]. Integrating lentiviral-based Tet-on cell labeling system has circumstanced these limitations. Up on induction of the GFP fluorescent protein, all the cells can be labeled without genotoxic effect. It can be used for identification and isolation of slow-cycling cells both *in vitro* and *in vivo*. In this study, we used a Histone-GFP fusion protein that localized in the cell nucleus with prolonged half-life [25]. Thus, this technique bears broad implications for studying cell cycling. Indeed, we found the existence of slow-cycling cancer cell compartment within the fast growing tumors formed by HeLa cells. These cells are superior in tumor initiation and more resistant to MPA. We speculate that the existence of slow-cycling cancer cells in different patient population may affect the ultimate responsiveness of MMF/MPA treatment [19-21].

IMPDH2 is up-regulated in proliferating cells [26], including in various types of cancer cells [27, 28], and exhibits a 5-fold higher (compared to IMPDH1) sensitivity to

MPA. Mechanistically, the effect of MPA appears through inhibition of its canonical target, IMPDH2. Ectopic expression of a mutated *IMPDH2* (mut*IMPDH2*) largely nullifies the anti-proliferative effects of MPA. Since IMPDH is the key enzyme in purine nucleotide synthesis pathway, we studied the effects of modulating purine nucleotide pool on cell growth. Increasing the nucleotide concentration by supplementation of exogenous guanosine did not have major effect on cell growth (Supplementary Figure S4). In addition, we surprisingly found that supplementation of exogenous guanosine counteracts only to a minor extent to the inhibitory effect of MPA (Supplementary Figure S5). Although the anti-proliferative of MPA is mainly dependent on targeting IMPDH2, depletion of nucleotide could only explain part of its mechanism-of-action.

Interestingly, a recent study has demonstrated a double functionality for IMPDH in drosophila. It can also act as a transcription factor in stress conditions that inhibits cell proliferation [29]. Thus we speculate that IMPDH2 might be a ligand-regulated transcription factor and MPA might act as a ligand. Indeed, IMPDH2 is predominantly located in cytoplasm in normal condition of cultured HeLa cells, but efficiently translocated into nucleus upon MPA treatment (Supplementary Figure S6A). Consistently, a mutated *IMPDH2* lacking the binding site of MPA was not able to translate into nucleus even with treatment of MPA (Supplementary Figure S6B). These results appear to support the previous observation in Drosophila cells and our hypothesis. Furthermore it is already known that MPA works as a ligand to activate the activity of peroxisome proliferator activated receptors, such as PPARγ [30], a critical nuclear receptor on adipocyte differentiation. However, gene silencing of PPARα and PPARγ did not affect the sensitivity of HeLa cells to MPA treatment, excluding their potential involvement (Supplementary Figure S7). The scenario that MPA acts as a ligand for ligand-regulated transcription factors to regulate cancer cell growth is certainly interesting and therefore deserves further investigation.

In summary, this study demonstrated that through inhibiting IMPDH2, MPA was capable of constraining the growth of fast-cycling cancer cells. Using a lentiviral Tet-on cell labeling technique, we identified slow-cycling cancer cells within the fast-growing tumors that are superior in tumor initiation but more resistant to MPA. Thus, it is very necessary to develop regimens that can effective target slow-cycling cancer cells. Combining these regimens with agents targeting fast-cycling cancer cells, such as MPA, may be a viable option in cancer therapy.

Conclusion

Slow-cycling cancer cells within fast-growing tumors were identified. These cells, compared to fast-cycling cells, were superior in tumor initiation and resistant to IMPDH2 inhibition by MPA. Thus, simultaneous targeting slow and fast-cycling cells are necessary to eradicate cancer.

Acknowledgements

The authors would like to thank Dr. Lifeng Ni from the Animal Care at Hangzhou Normal University, Hangzhou, China for helping with the animal experiments. We also thank Professor Riccardo Fodde (Department of pathology, Erasmus Medical Center Rotterdam, Netherland) for providing Plasmids pLV.EX3D/EF1A-rtTA (M2)-dsRed-Express2 and pLV.EX2D/TRE-eGFP.

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Supplementary Information

Supplementary Figure 1

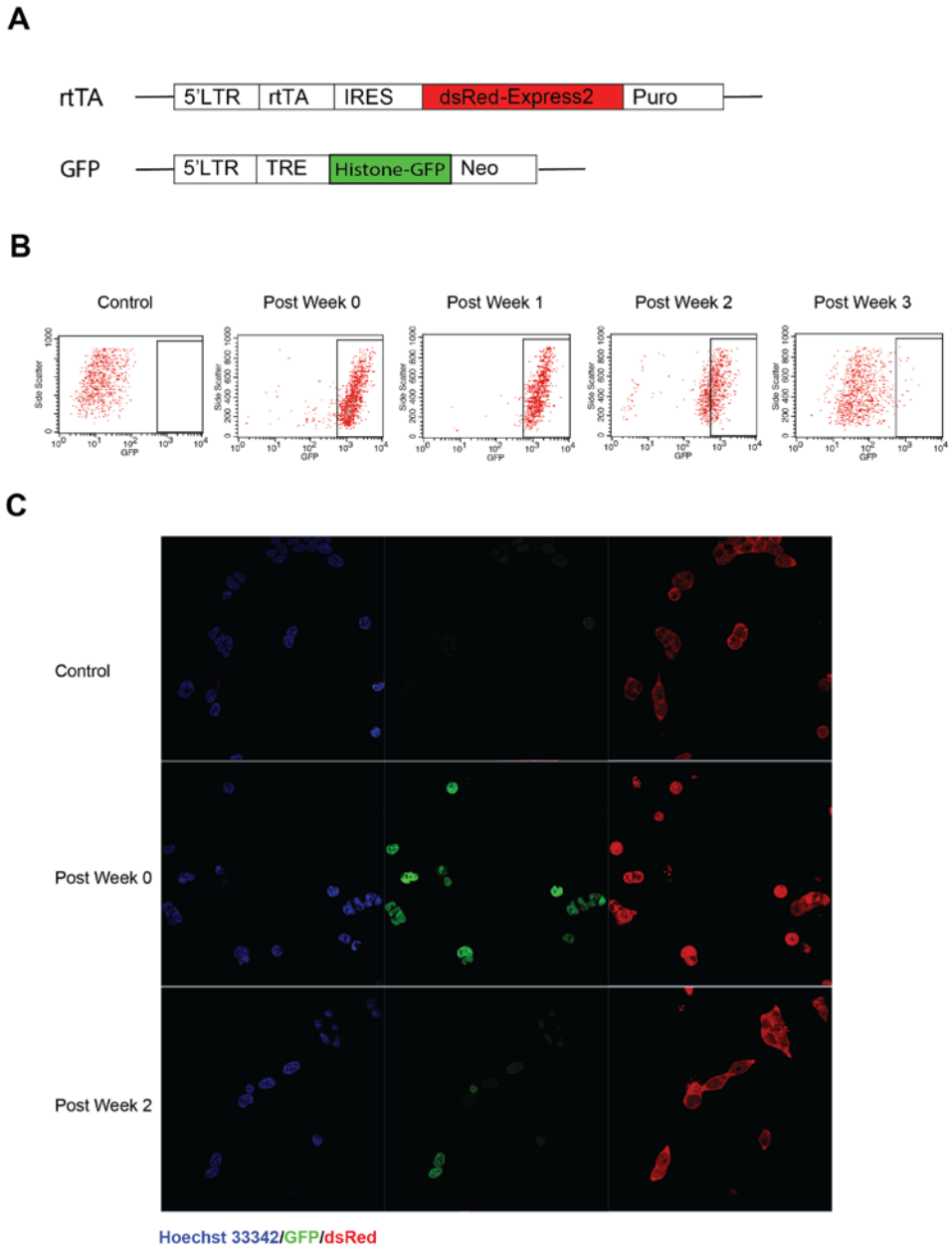

Figure S1. Label retaining Hela$^{tet\text{-}on}$ cell line expressing histone2B-GFP in a Tet-inducible fashion. (A) Plasmids pLV.EX3D/EF1A-rtTA (M2)-dsRed-Express2 and pLV.EX2D/TRE-eGFP were used. (B) and (C) GFP retaining condition *in vitro* could be detected even 3 weeks after doxycycline withdraw.

Supplementary Figure 2

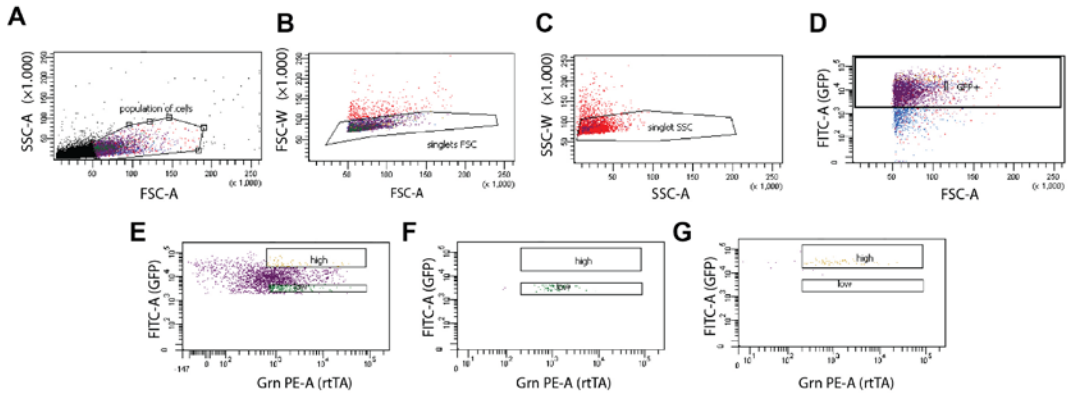

Figure S2. Tumor tissue were dissociated into single cell suspension and sorted for Non-LRCs (GFP^{low}dsRed) and LRCs (GFP^{high}dsRed) by FACS.

Supplementary Figure 3

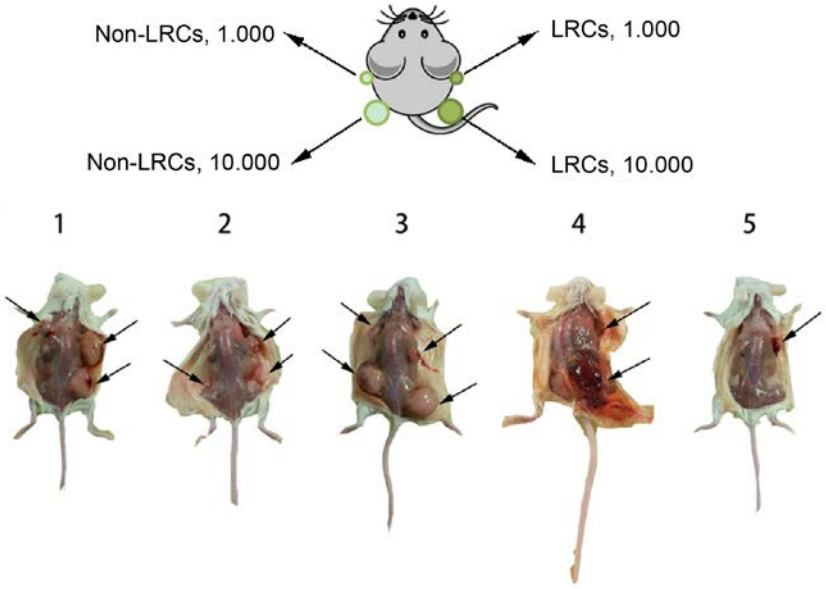

Figure S3. Non-LRCs and LRCs were injected subcutaneously into mice with cell number of 1.000 and 10.000 on four sites. Arrows indicate tumor formation.

Supplementary Figure 4

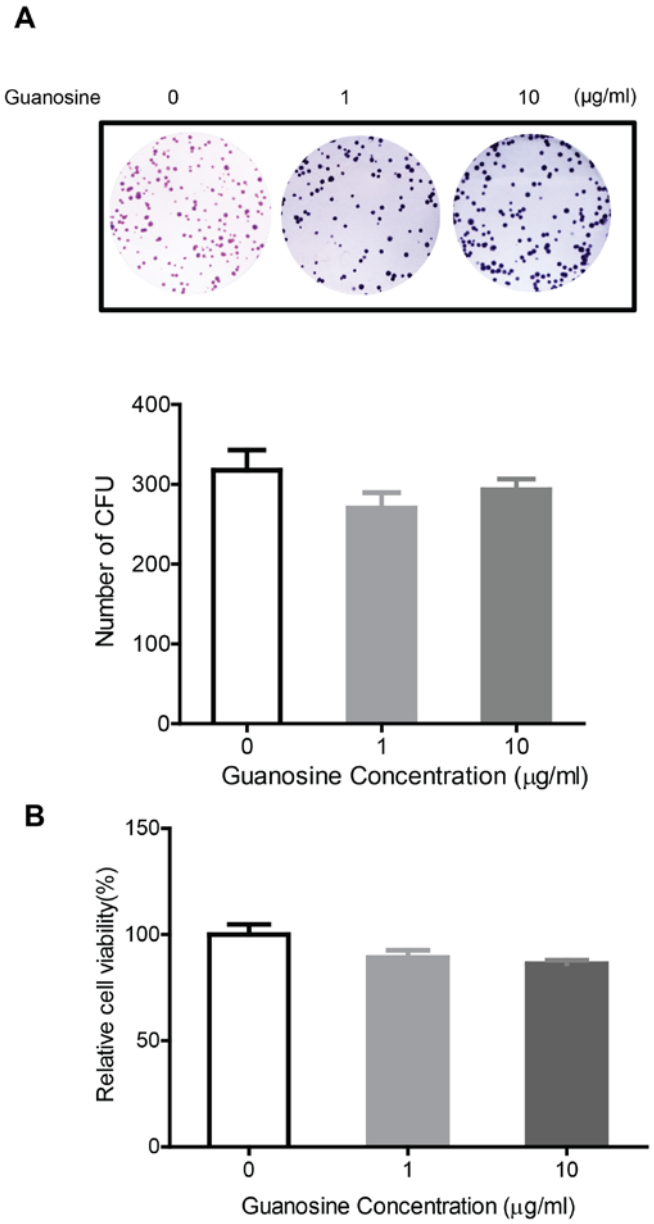

Figure S4. Guanosine alone did not significantly affect cell proliferation. (A) CFU appearance and numbers. (B) MTT assay.

Supplementary Figure 5

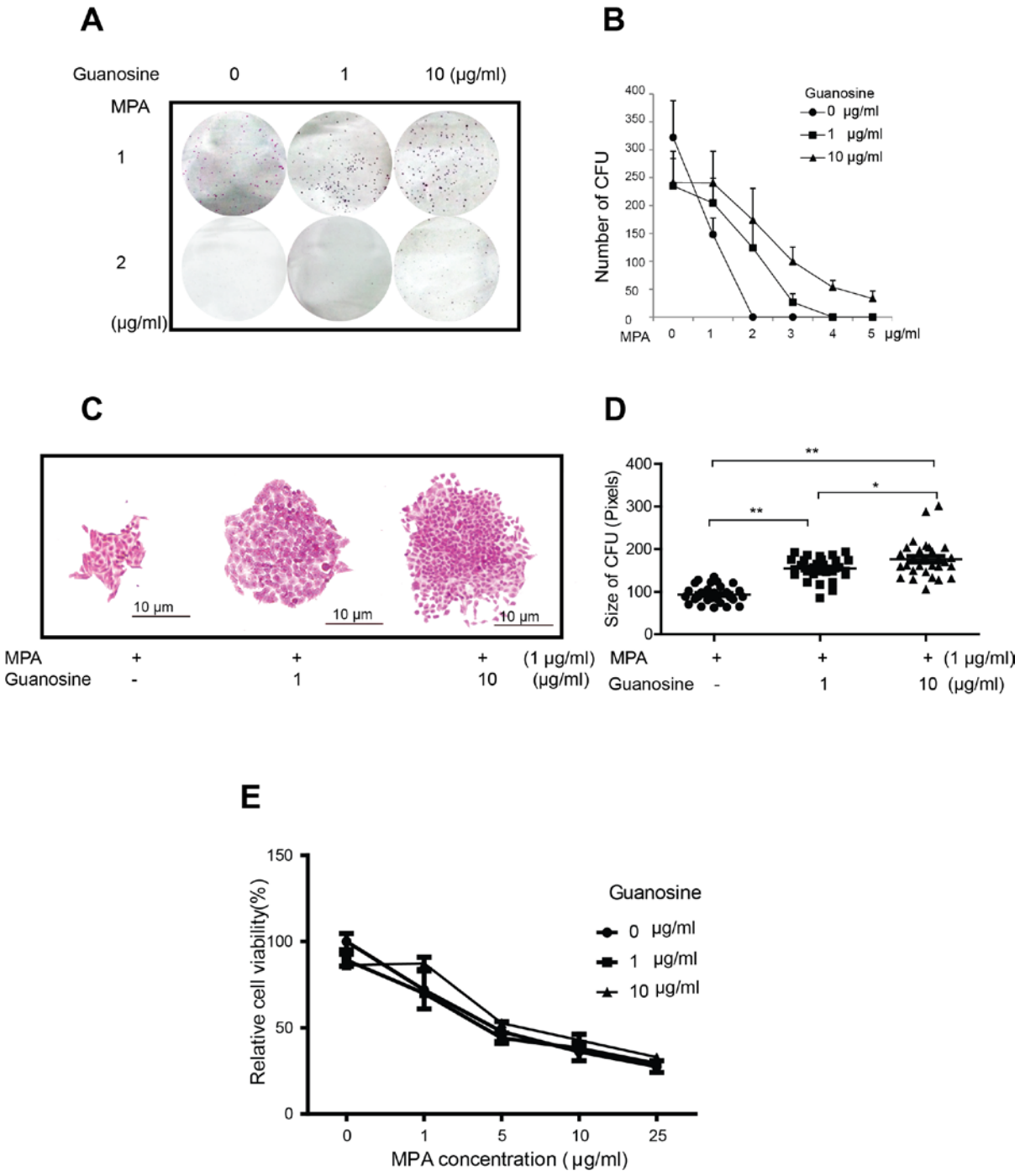

Figure S5. Anti-proliferative effects of MPA were partially restored by supplementation of exogenous guanosine. (A) and (B) The number of CFU, (C) and (D) The size of CFU were partially restored by supplementation of exogenous guanosine during treatment of the HeLa cell line with MPA (mean ± SD or SEM, n = 6 or 30, respectively, *P < 0.05; **P < 0.01).

Supplementary Figure 6

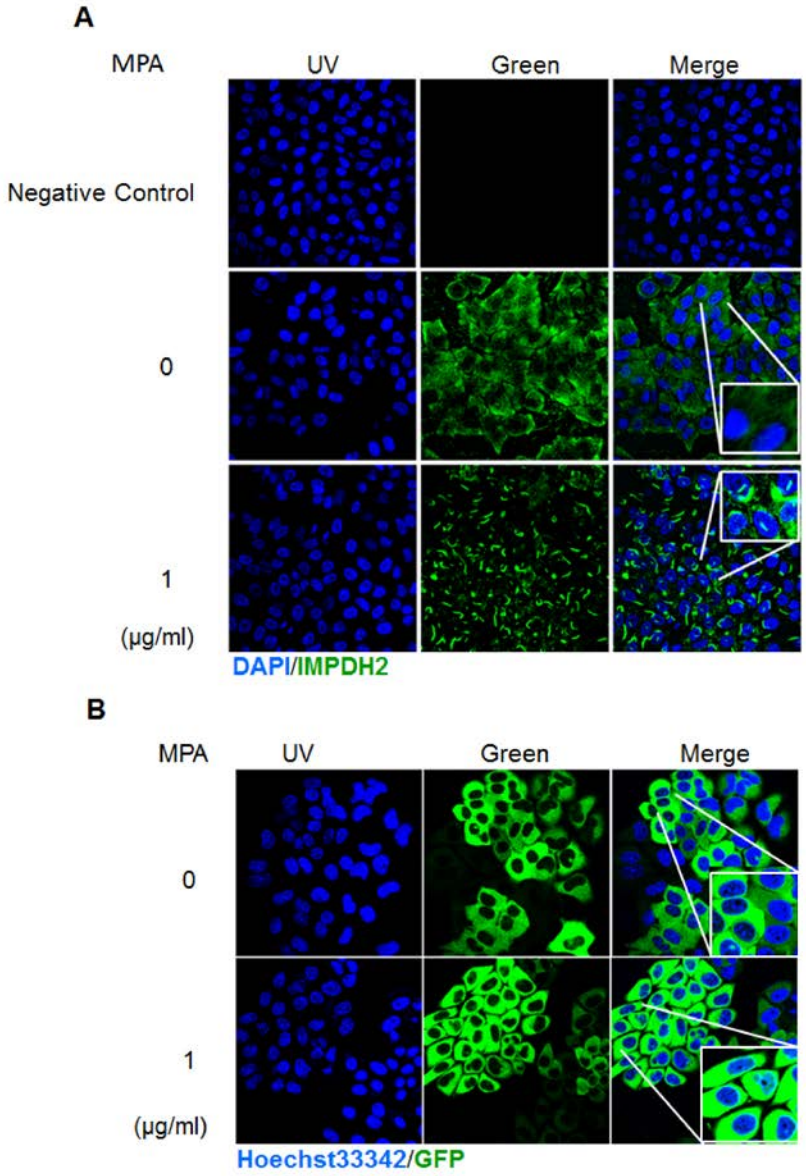

Figure S6. Cellular localization of IMPDH2 protein. (A) IMPDH2 protein predominately located in cytoplasm, but translocate into nucleus upon MPA treatment in HeLa cells. Blue: DAPI nuclear staining. Green: antibody against human IMPDH2. (B) A mutated IMPDH2 (fused with GFP) lacking the binding site of MPA was not able to translate into nucleus even with treatment of MPA in HeLa cells. Blue: DAPI nuclear staining. Green: GFP

Supplementary Figure 7

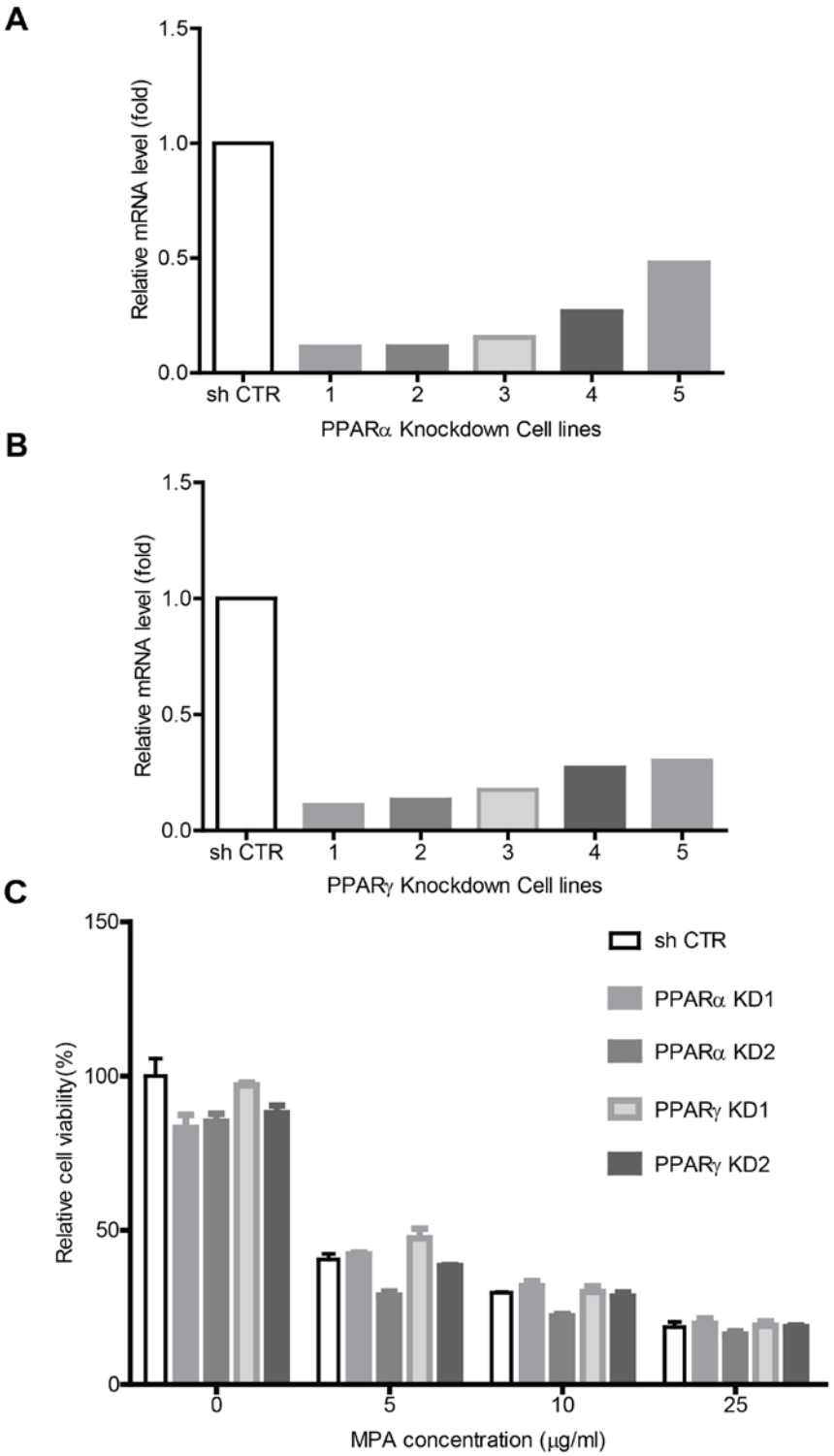

Figure S7. Knockdown of PPARα or PPARγ did not affect the sensitivity of HeLa cells to MPA treatment. (A) and (B) Relative mRNA levels of PPARα or PPARγ in HeLa cells transduced with 5 different lentiviral shRNA clones, GAPDH was used as an internal reference. (C) Clone 1 (KD1) and 2 (KD2) for each gene were selected to perform MTT assay.

Supplementary Table S1

STR genotyping of seven cancer cell lines

The STR match threshold is range from 71% to 94%.

STR	Panc-1	BxPC-3	Caco2	HeLa	HepG2	Huh7	SKOV-3
D3S1358	17	14/16	14/17	18/18	15/16	15/15	14/14
TH01	7/8	9	6	7/7	9/9	7/7	9/9,3
D21S11	28	29	30	27/28	29/31	30/30	30/31,2
D18S51	12	12	12	uitval	13	uitval	16/17/18
Penta_E	7/14	12/14	7	uitval	15/20	11/11	5/13
D5S818	11/13	11	12/13	12/14	11/13	12/12	11/11
D13S317	11	11	11/13/14	12/14	9/13	10/11	8/11
D7S820	8/10	10/13	11/12	8/12	10/10	10/11	13/14
D16S539	11	9/11	12/13	10/10	12/13	10/10	12/12
CSF1PO	10/12	13	11	9/10	10/11	11/11	11/11
Penta_D	14	14	9	8/8	9/13	12/12	12/13
AMEL	X	X	X	XX	XY	XX	XX
vWA	15	14/18	16/18	16/18	17/17	16/18	17/18
D8S1179	14/15	13	12/14	uitval	15/16	14/14	14/15
TPOX	8/11	8	9/11	8/12	8/9	8/11	8/11
FGA	21	20/21	19	21	22/25	22/22	24/25
Match	87%	81%	77%	84%	74%	94%	71%

STR genotyping information comes from the website: http://strdb.cogcell.org/

Chapter 5

A tumor suppressive role of IMPDH isoform 2 in hepatocellular carcinoma

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In preparation

Abstract

Inosine monophosphate dehydrogenases (IMPDH) are key enzymes in the biosynthesis of purine nucleotides with two isoforms, IMPDH1 and IMPDH2, existing in humans. Although high expression of IMPDH2 is reported for various cancers, its regulation and action in hepatocellular carcinoma (HCC) is unknown. Here we examine IMPDH2 expression in a cohort of 152 HCC patients and observe that cytoplasmic IMPDH2 protein levels are significantly lower in HCC as compared to adjacent tissue. Importantly, low tumor IMPDH2 expression associated with poor outcome in these patients. In apparent agreement down-regulation of IMPDH2 by RNAi in an *in vitro* model of HCC interfered with tumor growth in a colony formation assay whereas in an *in vivo* xenograft model of HCC, IMPDH2 knockdown interfered with tumor initiation. Interestingly, nuclear translocation of IMPDH2 was observed in a subset of patient HCC tissues, and was significantly associated with better cumulative survival. Thus, we conclude that IMPDH2 suppresses cell proliferation in hepatocellular carcinoma and assessing both its expression and subcellular localization allow predictions of HCC clinical course.

Introduction

The intimate relationship between cellular metabolism and cancer has been recognized for many decades [1-4]. On one hand, cellular biochemistry is associated with the production of oxygen radicals, which in turn contribute to oncogenic mutations, and metabolism *per se* contributes to the cancerous process [5]. On the other hand, activated oncogenes and inactivated tumor suppressors profoundly alter metabolism, especially resulting in a relatively reduced dependence on oxidative phosphorylation for ATP production; whereas concomitantly the dependence of glycolytic mechanisms and associated increased fermentation of pyruvate to lactic acid becomes larger [3, 4, 6]. This ability to alter metabolic output to fulfill the biosynthetic demands of cell growth and proliferation is considered a defining feature of cancer cells. Liver cells constitute biochemically the most active cells in our body, it is thus to be expected that cancer in this cell type especially would be associated with changes in the expression of enzymes in metabolic processes [7]. Nevertheless, in hepatocellular carcinoma (HCC) the effects on the cancerous process are relatively under-investigated. Nevertheless, in view of it prominence in organismal biochemistry it is to be expected that especially in the liver the occurrence of cancer would be associated with changes in the expression and activity of metabolic enzymes and that characterizing these alteration may provide guidance on the future natural history of HCC and might direct the design of novel rational avenues to combat this disease.

Inosine-5'-monophosphate dehydrogenase (IMPDH) encodes a rate-limiting enzyme which catalyzes the nicotinamide adenine dinucleotide (NAD^+)-dependent oxidation of inosine monophosphate (IMP) to xanthosine monophosphate (XMP) [8, 9]. This is the key step in the pathway of guanosine monophosphate (GMP) biosynthesis, which controls the guanosine nucleotide pool size. Thus it is a key enzyme family for genome replication and transcription. Accordingly, IMPDH is therapeutically highly target for antiviral, anticancer, and immunosuppressive drugs [9, 10]. IMPDH represents two isoforms in mammalian species: type 1 and type 2, which lie on two different chromosomes (chromosome 7 and 3 respectively) and share 84% amino acid similarity and virtually indistinguishable catalytic activity [11]. The two iso-

enzymes differ, however, with respect to sensitivity to pharmacological inhibitors and the regulation of their expression. IMPDH1 is a constitutively expressed gene in most cells, whereas the activity of IMPDH2 is subject to dynamic regulation and in the canonical interpretation especially associated with associated with malignant transformation, poor differentiation, chemo-resistance, and metastasis [12-15]. Liver cells, however, are different from other cell types in that these unusually biochemically active and the alternative epigenetic regulation of enzymes in cellular biochemistry raises questions as to whether also in HCC, IMPDH2 upregulation is associated with the cancerous process. This consideration prompted us to explore the relationship between IMPDH2 action and function in HCC. Remarkably we observed that in contrast with the canonical interpretation of IMPDH2 functionality in cancer, in HCC expression of the IMPDH2 gene is associated with tumor suppression.

Materials and Methods

Reagents

Antibodies recognizing IMPDH1 (rabbit, polyclonal antibody) and IMPDH2 (rabbit, monoclonal antibody) were purchased from Abcam company. Stocks of MPA (AMRESCO LLC, USA) were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO).

Tissue microarray

To construct a tissue micro array (TMA), paraffin-embedded HCC patient tissues (n = 152, between 2004 and 2014) were collected from the Pathology Department of Erasmus Medical Center (Erasmus MC) in Rotterdam and the Academic Medical Center (AMC) in Amsterdam. The use of patient materials was approved by the medical ethical committees of Erasmus MC.

Immunohistochemistry

Paraffin-embedded liver tumor tissue in TMA slides were deparaffinized with xylene and rehydrated in graded alcohols for further immunohistochemistry staining. For antigen retrieval, slides were boiled in citric acid buffer (pH6.0) for 14 min. Peroxidase was blocked by using 3% H_2O_2 for 10 min at room temperature. The slides were incubated overnight with the primary antibody against IMPDH1 (1:150) and IMPHD2 (1:500) at 4℃. Subsequently, peroxidase and hematoxylin were employed to visualize the staining. Negative control was carried out by omitting the primary antibody.

The scoring was based on the cytoplasm staining. The percentage scoring was as follows: grade 0 for 0-5%; grade 1 for 5-30%; grade 2 for 30-70%; grade 3 for > 70% (Fig. 1A and Fig. 5A); the intensity scoring was visually as: grade 1 for weak; grade 2 for moderate; grade 3 for strong (Fig. 1B and Fig. 5B). A final immune-reactivity score (IRS) was obtained for each case by multiplying the percentage and the intensity values. The scorings were done by two investigators and the difference of score was valued by Kappa test.

Lentiviral short hairpin RNA vector

Lentiviral backbone vector pVSVG, pPMD and pREV were obtained from the Erasmus Medical Center for Biomics. A vector expressing short hairpin RNA targeting green fluorescent protein (GFP) (not expressed in HCC cell lines) served as control. Lentiviral vectors of shRNA (Sigma–Aldrich) targeting IMPDH2 were obtained from the Erasmus Center for Biomics and produced in HEK 293T cells as previously described [16].

Cell culture

HCC cell lines (HuH7, HuH6, PLC/PRF/5, SNU398, SNU449, SNU182, HepG2, Hep3B, HepaRG and 97-H) and HEK 293T cell were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and 1% penicillin/streptomycin (Gibco, Bleiswijk). IMPDH2 knockdown cells and control cells were generated by inoculation of lentiviral vectors

and subsequently selected and maintained in DMEM medium with 10% FBS, 1% penicillin/streptomycin and 2 μg/mL puromycin (Sigma-Aldrich). After pilot study, the shRNA vector exerting optimal gene knockdown were selected by q-PCR and western blot assays.

Colony formation assay

Colony formation was performed as described previously [17]. Cells were harvested and resuspended in medium, 500 cells were planted into each well of a 6-well plate. Two weeks later, formed colonies were fixed by 70% ethanol and counterstained with haematoxylin. Colony numbers were counted.

RNA isolation and q-PCR analysis

Total RNA was extracted from HCC cell lines using RNA isolation kit (Sigma). First strand cDNA was synthesis from 1μg of total RNA by using Ready-to-go first strand beads (GE Healthcare). Q-PCR was performed by using GoTaq Real-Time qPCR mix (Promega). Fold changes have been determined by using $2^{\Delta\Delta}$CT and normalized to GAPDH. Finally, the fold changes were obtained by converting the logarithmic scale to an exponential scale (2^ΔΔCT). The primers were used as following table (Table 1).

Western blot analysis

Cells were washed with cold PBS gently, added 250 μL/well cell lysis buffer containing 0.1M dithiothreitol and incubated for 5 min at 95℃. The proteins (40 μg) were separated by 10% SDS-PAGE and then transferred to a PVDF membrane (Millipore, Bedford, MA). Blots were put in blocked buffer at room temperature for 1 h and incubated with primary antibodies against IMPDH1 (1:500 dilution), IMPDH2 (1:1000 dilution) and β-actin protein overnight at 4℃. After washing, the membranes were incubated with secondary antibody (1:5000 dilution) for 1 hour at room temperature. Proteins were analyzed by using the near infrared laser imaging system (LI-COR, Inc.). Quantification was performed by using ImageJ software.

Table 1. The primers used in Q-PCR assay

Genes	Forward primer	Reverse primer
IMPDH1	GCACACTGTGGGCGAT	GAGCCACCACCAGTTCA
IMPDH2	TCTTCAACTGCGGAGAC	CTGTAAGCGCCATTGCT
E2F1	GCATCCAGCTCATTGCCAAG	ACATCGATCGGGCCTTGTTT
E2F2	CAACATCCAGTGGGTAGGCA	GGCAATCACTGTCTGCTCCT
E2F3	GGAGCTAGGAGAAAGCGGTC	TCGAAGAGATCGCTGATGCC
E2F4	TCACAGAGGACGTGCAGAAC	GAGCTCCATGCCTCCTTGTT
E2F5	GAAAGGTGTAGGTGCTGGCT	GAACAGGAAAAACCACGGGC
E2F6	TGGAGCAGTTCCCCAACAAA	CTCTGGGAGCTGGAACATCC
E2F7	ACCAGCCTTCAAGTGGATCG	GGCACTGGCCTTTGAGGTAT
E2F8	CGCCCAGTAGCCCTATCAAG	GGCACTGGCCTTTGAGGTAT
Histone1	GTGCCGAGACCAAGCGACA	TTGGCGTGGCTACCTTCTTG
Histone2A	AGATAGCACACTCAACGCCC	GCTCAGTAGCCTGTCGTTGT
Histone2B	TCACCTCCAGAGCTCCACTT	GCAAGGGTCTTTGGTTTGGC
Histone3A	CGTGTAAAGGGGCCAGGATT	CAGGTCTGCAATGGTGCCTA
Histone3B	GCTGGAATTTTACCGTCGGC	GAAACTGAAAACGCGCTGGT
Histone4	CTGGCATACTTTCGTGCCCT	ACCGTGTGACCCAGTTTTGG
GADPH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA

HCC xenograft tumor in NSG mice

The xenograft tumor model in NSG mice was performed in accordance with current prescribed guidelines and under a protocol approved by the Institutional Animal Care and Use Committee of Erasmus Medical center. Mice were breed in SPF environment during the whole experimental period. Mice were all female and 4-6 weeks of age at the time of inoculation. Six mice were subcutaneously inoculated with 2.5 million control (shCTR) and IMPDH2 knockdown (shIMPDH2) HuH7 cells into the left and right back respectively. Four weeks later, mice were sacrificed and tumors were harvested and weighed.

Statistical analysis

Statistical analysis was performed by using Pearson ×2 test, nonparametric Mann-Whitney test and Kaplan-Meier survival analysis in IBM SPSS Statistical. T-test was also used by using GraphPad Instat software. $P < 0.05$ were considered as statistically significant.

Results

IMPDH2 expression was positively associated with patient's outcome

To assess the expression of IMPDH2 in HCC patients, TMA slides of HCC patients were scored, and the results were compared with adjacent non-transformed tissue. The staining was scored by two independent investigators with a Kappa test of 0.420 (for IMPDH2 in tumor tissue), 0.217 (for IMPDH2 in adjacent normal tissue), which suggest that there was a fair agreement between the two investigators. (Supplementary Table 1 and 2). In surprise, we found that the expression of IMPDH2 in tumor tissues were significantly lower than in adjacent normal tissues ($n = 152$, $P < 0.01$; Fig. 1C and D). Moreover, low IMPDH2 level in surgical resected HCC tumors is significantly associated with poor cumulative patient survival ($n = 152$, $P < 0.05$; Fig. 1E).

Silencing IMPDH2 expression increases colony formation unit potential of HCC and tumor initiatory capacity in HCC xenografts

For reductionist exploration of IMPDH2 expression in the background of HCC, we decided to investigate the effect of forced reduction of IMDH2 expression. To this end, we resorted to an *in vitro* model of HCC as this allows relatively straightforward manipulation of IMPDH2 protein expression. Thus the expression of IMPDH2 protein in ten HCC cell lines (Supplementary Fig. S1A) were investigated to identify cellular models for HCC characterized by relatively high IMPDH2 expression. From this analysis, HuH7, HuH6 and PLC/PRF/5 cell lines were selected to establish stable IMPDH2 knockdown cell lines. Lentiviral RNA interference vectors expressing short hairpin RNA(shIMPDH2) were used in this system. The vector expressing short hairpin RNA targeting GFP worked as control (shCTR). The approach was confirmed by qPCR and western blot, which showed almost absence of IMPDH2 both at mRNA and protein levels in the knockdown cell lines (Supplementary Fig. S1B-E).

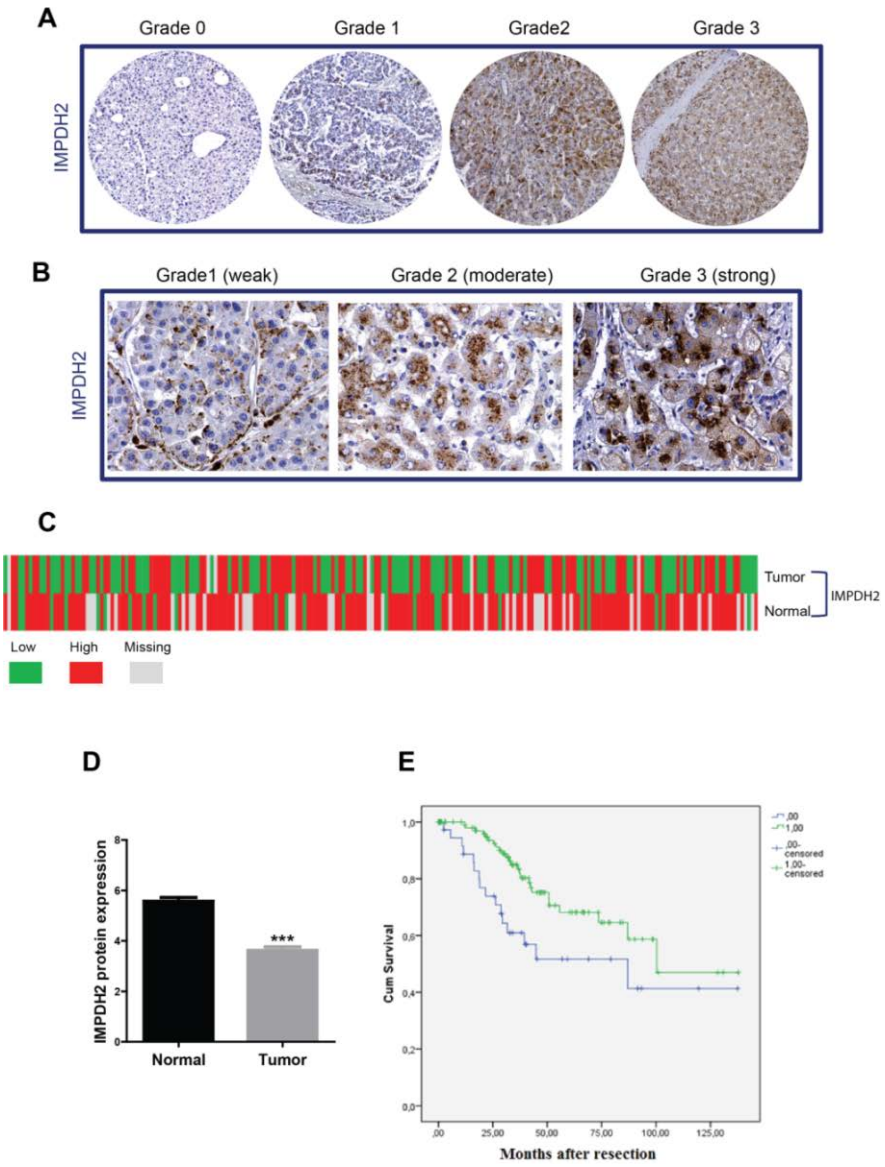

Figure.1 High expression of IMPDH2 significantly associated with better clinical outcome in HCC patients. (A) The levels of IMPDH2 protein positive percentage range from grade 0 (< 5%), grade 1 (5%-30%), grade 2 (30%-70%) to grade 3 (>70%) both in the HCC tumors and their adjacent sites; (B) The levels of IMPDH2 protein intensity range from grade 1 (weak), grade 2 (moderate) and grade 3 (strong); (C) The expressions of IMPDH2 in tumor was inconsistent with adjacent normal tissue in individual patient; (D) Adjacent normal tissue significantly expressed higher IMPDH2 protein than tumor tissue (paired T-test, n = 152, ***P < 0.001); (E) Higher level expression of IMPDH2 patients tend to have better survival (n = 152, P < 0.05). Green = IRS scores 2-9; blue = IRS scores 0-1.99.

To investigate the role of IMDH2 in cancerous process, colony formation unit (CFU) assay was used as it is a robust method to verify whether a single cell is capable of proliferation. We observed that HuH7 cells or shCTR HuH7 cells have some capacity to initiate colony formation, but that knockdown of IMPDH2 substantially increases the capacity (shCTR vs shIMPDH2; 82.5 ± 3.106 vs 115.8 ± 4.438 colonies per 500 cells, mean ± SEM, n = 6, $P < 0.01$; Fig. 2A and B). To assess the *in vivo* importance of this observation we also evaluated the impact of loss of IMPDH2 expression on the propensity to initiate tumors upon xenografting of this HCC cell model in immunodeficient mice. To this either 2.5 million shCTR or 2.5 million shIMPDH2 HuH7 cells were injected into mice subcutaneously on the left or right side on the back of mice, respectively. As shown in Figure 2C, IMPDH2 knockdown HuH7 cells were superior in tumor initiation (6/6 vs 4/6) and tumor growth (Fig. 2D) (weight: 0.2917 ± 0.1635 vs 1.400 ± 0.2266, mean ± SEM, n = 6). We conclude that the association between diminished HCC IMPDH2 expression and poor prognosis observed in our patient cohort correlates to more aggressive HCC cellular characteristics provoked by loss of IMPDH2 expression in experimental HCC.

Nuclear localization of IMPDH2 is significantly associated with better clinical outcome

Because of its action as a metabolic enzyme, it is generally assumed that IMPDH protein should locate in the cytoplasm. However, interestingly, nuclear localization of IMPDH2 protein was observed in our study. We found a subset of patients (n = 24) presenting nuclear IMPDH2 (Fig. 3A), which was significantly associated with better cumulative survival ($p < 0.05$; Fig. 3B). Furthermore, patients presenting nuclear translocation of IMPDH2 were more likely infected by Hepatitis B virus (HBV) (41.7% vs 21.3%; Supplementary Table. 5), and were more likely infiltrated with CD8+ tumor-infiltrating lymphocytes (TIL) (44% vs 22.9%; Supplementary Table. 6).

IMPDH was expected as a nucleic-acid binding protein in previous study [18], To further study the cellular localization of IMPDH2, immunofluorescence and confocal microscopy were used to further investigate the intracellular localization of IMPDH2 protein. Three HCC cell culture models were used for the analysis. The confocal microscope showed that most of the cells involved present cytosol-restricted IMPDH2 protein (Fig. 3C). Next, we wondered whether a change of

metabolic state could affect the localization of IMPDH2. Hydrogen peroxide was used to trigger an oxidative stress in the cells. Almost all HCC cells that had been treated with H_2O_2 for 8 hours, displayed nuclear IMPDH2 localization (Fig. 3D). Thus differences in subcellular IMPDH2 distribution may well relate to differences in cellular redox potential.

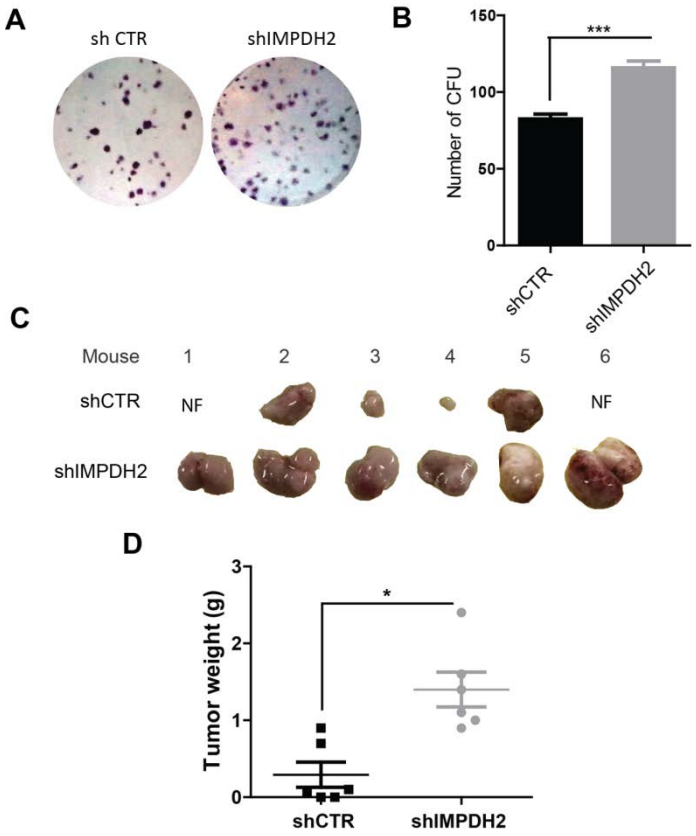

Figure 2. Silencing of IMPDH2 increased colony formation in vitro and enhanced tumor initiation in mice. (A) Appearance of colonies of shCTR and shIMPDH2 cell; (B) Paired t-test showed that shIMPDH2 cells were more capable of colony unit formation; (C) Appearance of tumors from the xenograft model. ShIMPDH2 cells were superior in tumor initiation compared to shCTR cells; (D) Tumors originating from shIMPDH2 treated cells were significantly heavier than the tumors originating from shCTR treated cells.

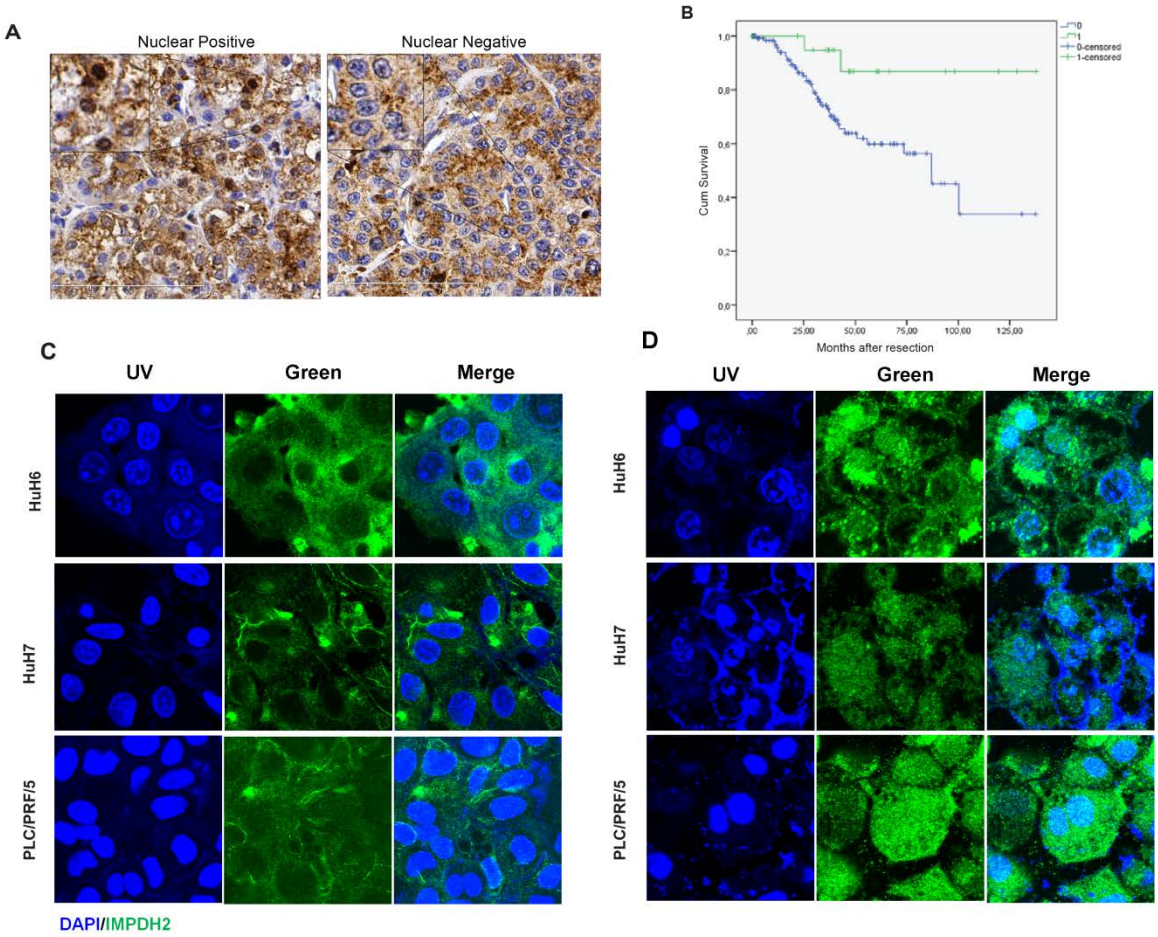

Figure 3. Nuclear localization of IMPDH2 tightly related to better clinical outcome. (A) Cancers are variant as to nuclear aspect of IMPDH2 expression. Most HCC expresses cytosol-restricted IMPDH2 (n = 132), but others also display clear nuclear IMPDH2 (n = 24); (B) The patients exhibiting nuclear IMPDH2 survive longer; (C) Microscopic localization of IMPDH2 in three HCC cell lines; (D) Oxidative stress provokes nuclear localization of IMPDH2.

Silencing IMPDH2 increases the expression of *E2F* and *Histone* genes in HCC cell models

IMPDH functionality in cellular physiology remains subject to an intense research effort but appears also to relate an action of this protein in the control of gene expression [18, 19]. Broad control of gene expression at level of chromatin structure is exerted by Histones. Expression of *Histone* genes is tightly coupled to DNA replication and thus effects on nucleotide synthesis and is limited to the S phase [20]. E2F family members play a major role during the G1/S transition in the cell cycle, through the by upregulation of nucleotide biosynthetic enzymes and thus constitutes a rational partner for IMPDH effects in cellular physiology [21, 22]. Investigation of expression of both classes of genes thus might shed further insight into the mechanisms by which IMDH2 is involved in the HCC process. Hence expression of *E2F* and *Histone* gene cluster were measured at mRNA level. Our q-PCR results showed that both *E2F* and *Histone* genes were up-regulated following IMPDH2 knockdown in the HuH7 cell line (Fig. 4A and C).

Mycophenolic acid (MPA), an uncompetitive inhibitor of IMPDH, can effectively inhibit enzymatic activity of IMPDH. It is reported that IMPDH2 is 4.8-fold more sensitive to MPA than IMPDH1 [23]. Q-PCR were performed to investigate the expression of *Histone* and *E2F* genes under inhibition of IMPDH2 enzymatic activity and compared to vehicle controls. The results show that both *Histone* and *E2F* genes are significantly up-regulated following MPA treatment at concentrations as low as 3 µM (Fig.4B and D). Thus our results reveal an inverse relationship between IMPDH2 enzymatic activity and expression of *Histone* and *E2F* genes.

IMPDH1 and IMPDH2 expression are independently associated with better HCC outcome

The effects seen with IMPDH2 in the present study raise obvious questions as to relative importance for its paralogue IMPDH1 for HCC. Generally speaking, IMPDH1 is expressed at constitutive low levels in most of the tissues [24, 25], but its expression in HCC has not been studied in detail. Intriguingly, recent studies reveal that IMPDH-

1 is likely contributed significantly to the proliferation response of lymphocytes [24, 26, 27]. In order to obtain an insight into the function of IMPDH1 in HCC progression, immunohistochemistry staining was performed by using the antibody to IMPDH1. The staining was scored by two independent investigators with a Kappa test of 0.266 (for IMPDH1 in tumor tissue) and 0.26 (for IMPDH1 in adjacent normal tissue) (Supplementary Table. 3 and 4), which suggest that there was a fair agreement between the two investigators. Unlike IMPDH2, IMPDH1 presented higher expression in tumor as compared to adjacent non-transformed tissues ($n = 152$, $p < 0.001$; Fig. 5C). Mirroring the situation with IMPDH2, higher IMPDH1 levels associated to better clinical outcome in HCC patients (Fig. 5D). To investigate the relationship between IMPDH1 and IMPDH2 in HCC, we also compared the expression of the two isoforms in individual patient. The results reported that the expressions of IMPDH1 and IMPDH2 were independent on each other (Fig. 6A). Moreover, down-regulation of IMPDH2 in HuH7 and PLC/PRF/5 cells had no influence on the expression of IMPDH1 with respect to both mRNA and protein levels (Supplementary Fig. S1 B-E). Finally, by combining IMPDH1 and IMPDH2, we found that the patients expressing both of them had the longest survival, patients expressing either of them were better than the patients expressing none of them (Fig. 6B). *In total*, our results reveal non-canonical tumor suppressive functions of both IMDH1 and IMPDH2 in HCC.

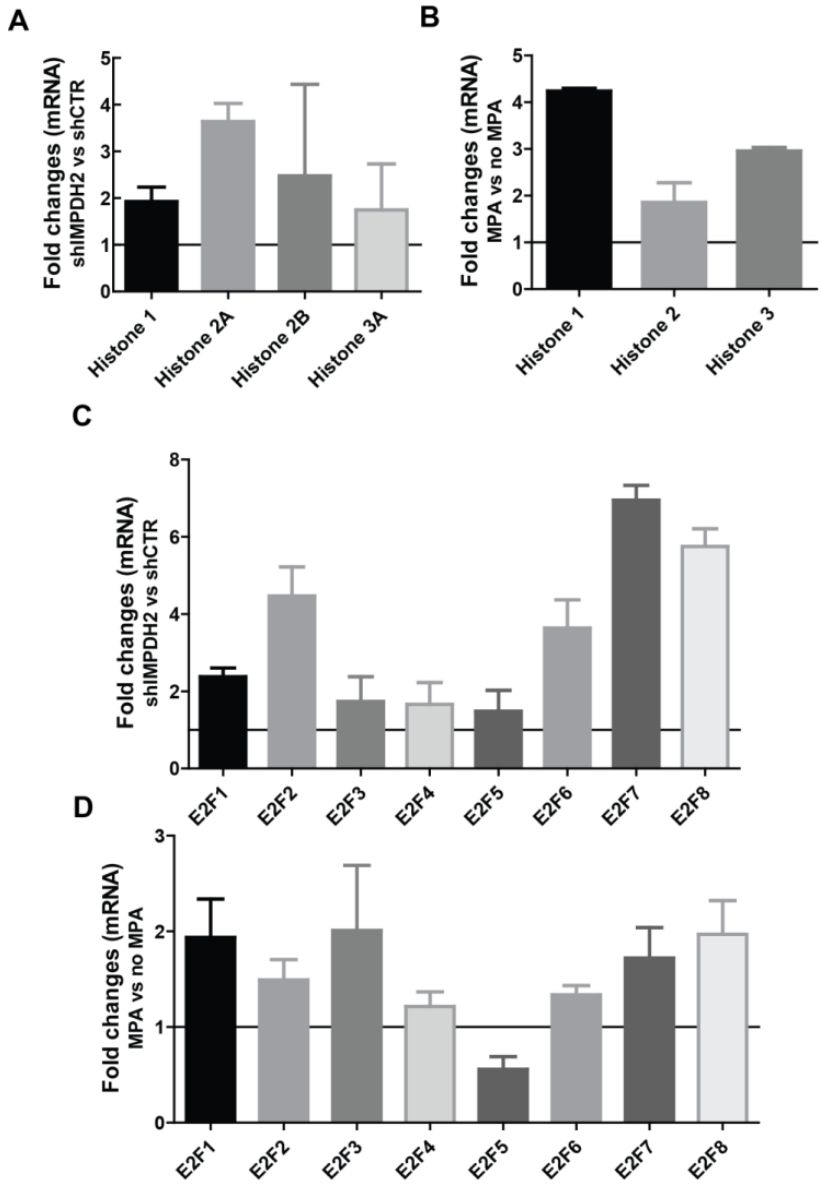

Figure 4. Silencing of IMPDH2 and MPA treatment elevates *E2F* and the *Histone* genes. (A) q-PCR shows that shIMPDH2 elevates ***Histone*** gene expression in the HuH7 cell line;. (B) q-PCR shows that MPA elevates ***Histone*** gene expression in the HuH7 cell line; (C) q-PCR shows that shIMPDH2 elevates *E2F* gene expression in the HuH7 cell line; (D) MPA treatment increases the expression of *E2F* genes at the RNA level.

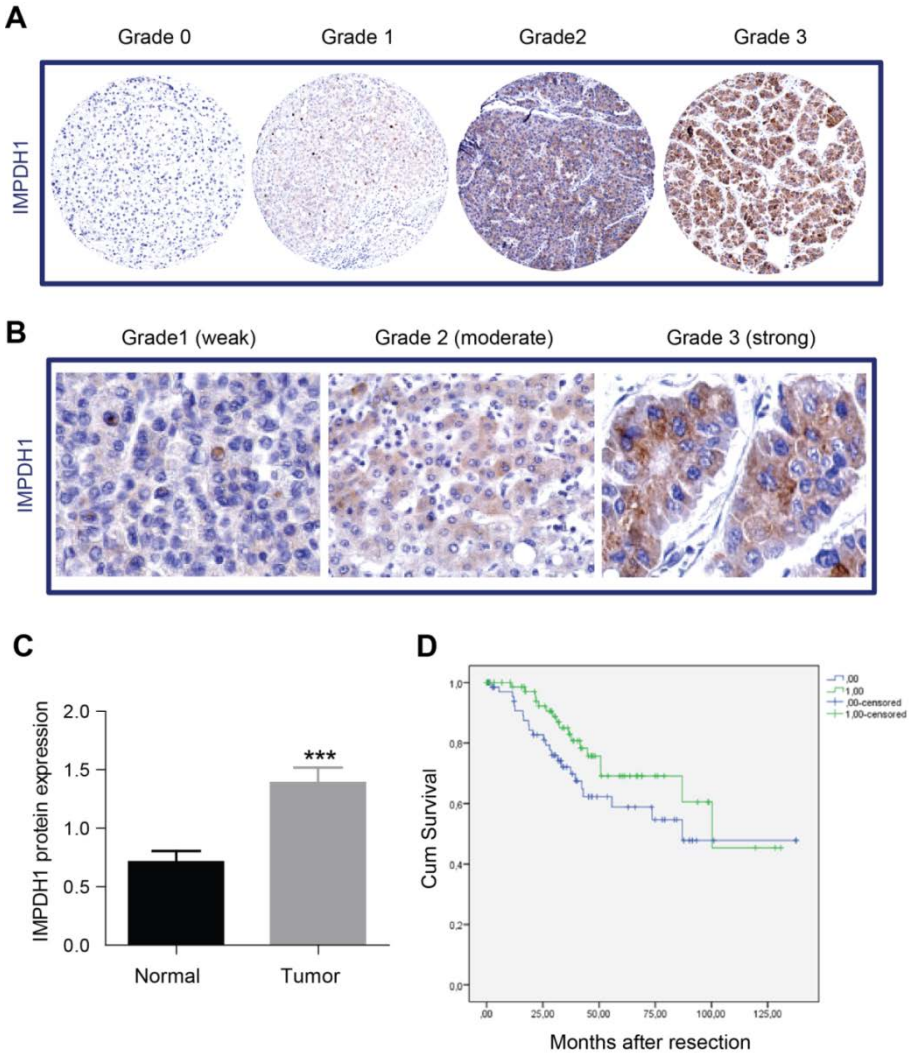

Figure 5. High expression of IMPDH1 significantly associated with better clinical outcome in HCC patients. (A) The levels of IMPDH1 protein positive percentage range from grade 0 (< 5%), grade 1 (5%-30%), grade 2 (30%-70%) to grade 3 (>70%) both in the HCC tumors and their adjacent sites; (B) The levels of IMPDH1 protein intensity range from grade 1(weak), grade 2 (moderate) and grade 3 (strong); (C) The expressions of IMPDH1 in tumor was much higher than in adjacent normal tissue (n = 152, ***p < 0.001); (D) Patients with higher expression of IMPDH1 in tumors tend to have longer survival (n = 152, p<0.05).

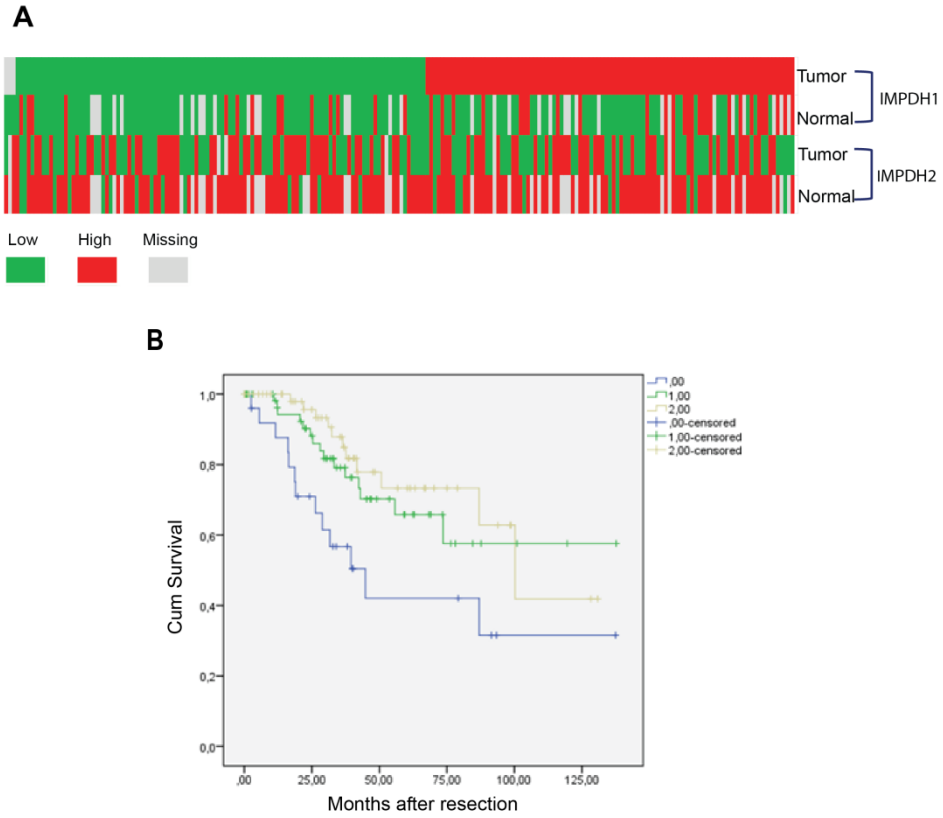

Figure 6. IMPDH1 and IMPDH2 were independently and positively associated with HCC patient's outcome. (A) The expression of IMPDH1 and IMPDH2 does show significant correlation, neither in adjacent normal tissues nor in tumor tissues; (B) Patients expressing both IMPDH1 and IMPDH2 tended to have the best clinical outcome (brown, n = 30), compared to those expressing only one isoform (green, n = 61) and those expressing no IMPDH in tumor or adjacent tissue (blue, n = 61). (***P< 0.005).

Discussion

Ability to support the demands of tumor cellular metabolism features prominently among the defining characteristics of cancer cells [28]. As the rate-limiting enzyme of guanosine nucleotide synthesis, IMPDH enzymes play multifaceted roles in cell growth and differentiation. Conventionally, increased expression of IMPDH2 is an important hallmark of the early phases of tumorigenesis [13]. Remarkably, however in our cohort of HCC patient's expression of IMPDH2 in the tumor, negatively associated to tumor progression. In apparent agreement, IMPDH2 knockdown in HCC cells increased the capacity for colony formation *in vitro* and tumor initiation *in vivo* when compared to control cells. Hence, we concluded that IMPDH2 in HCC has been screwed towards a tumor inhibiting signaling. This is unexpected in view of the elevated expression of IMPDH2 is associated with aggression of cancer [29-31]. A role

for IMPDH enzymatic activity in this respect is further supported by our findings concerning IMPDH1. IMPDH1 is considered a constitutively expressed housekeeping gene subject to little regulation. In HCC, however, we now find that higher expression of IMPDH1 is associated to better outcome for patients. As expression of IMPDH1 and IMPDH2 appears to be correlated, our data strongly support the notion that the overall enzymatic activity of IMPDH family enzymes constrains the cancer process associated with HCC.

Intriguingly, nuclear translocation of IMPDH2 significantly associated with longer survival in HCC patients. The experiments showed that both treating by hydrogen peroxide and MPA (Supplementary Fig S3) could drive IMPDH2 protein moving into nucleus. One possible explain for this is the tumor cells are influenced by microenvironment changes, such as oxidative stress and nucleotide pool depletion, which triggered the translocation of IMPDH2. Clinical analysis confirmed the assume by reporting that nuclear IMPDH2 presenting patients were more likely infected by HBV and infiltrated by TIL. While the mechanism of MPA driving IMPDH2 protein aggregation (Supplementary Fig S3) still need further investigation.

Consistent with previous studies, we observed that IMPDH accumulated in nucleus following DNA replication and suppresses the expression of *Histones* and *E2F* genes [19]. Our qPCR data showed that the expression of *E2F* and *Histone* genes were significantly higher in shIMPDH2 cells than in shCTR cells. These results arouse speculation that by targeting *E2F* and *Histone* genes, nuclear translocation of IMPDH2 maybe plays an important role in the progression of HCC. Obviously, further experimentation is necessary to address this possibility. Mechanistically, the effect of MPA on HCC appears through inhibition of its canonical target, IMPDH. However, our study reported that the content of IMPDH2 increased obviously following MPA treatment both in presence and in absence of exogenous guanosine (Supplementary Fig. S3). In conjunction our results show that MPA and IMPDH2 action in HCC is more complex as previously thought and call for further investigations as their relation to HCC.

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Supplementary information

Supplementary table 1

Symmetric Measures for IMPDH2 in tumor

	Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
Measure of Agreement Kappa	,420	,050	15,373	,000
N of Valid Cases	152			

a. Not assuming the null hypothesis.

b. Using the asymptotic standard error assuming the null hypothesis.

Supplementary table 2

Symmetric Measures for IMPDH2 in normal

	Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
Measure of Agreement Kappa	,217	,053	5,907	,000
N of Valid Cases	109			

a. Not assuming the null hypothesis.

b. Using the asymptotic standard error assuming the null hypothesis.

Supplementary table 3

Symmetric Measures for IMPDH1 in tumor

	Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
Measure of Agreement Kappa	,266	,033	8,938	,000
N of Valid Cases	146			

a. Not assuming the null hypothesis.

b. Using the asymptotic standard error assuming the null hypothesis.

Supplementary table 4

Symmetric Measures for IMPDH1 in normal

	Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
Measure of Agreement Kappa	,260	,047	5,741	,000
N of Valid Cases	113			

a. Not assuming the null hypothesis.

b. Using the asymptotic standard error assuming the null hypothesis.

Supplementary table 5

Nuclear localization of IMPDH2 is associated with HBV infectious

			HBV positive		Total
			0	1	
Nuclear staining	0	Count	118	32	150
		Percentage	78.7%	21.3%	100%
	1	Count	14	10	24
		Percentage	58.3%	41.7%	100%
Total		Count	132	42	174
		Percentage	75.9%	24.1%	100%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	4.671[a]	1	.031		
Continuity Correction[b]	3.672	1	.057		
Likehood Ratio	4.224	1	.040		
Fisher's Exact Test				.040	.033
Linear-by-Linear Association	4.645	1	.031		
N of valid Cases	174				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 5.79.

b. Computed only for a 2×2 tables.

Supplementary table 6

Nuclear localization of IMPDH2 is associated with tumor infiltration lymphocytes infectious

			HBV positive		Total
			0	1	
Nuclear staining	0	Count	128	38	166
		Percentage	77.1%	22.9%	100%
	1	Count	14	11	25
		Percentage	56.0%	44.0%	100%
Total		Count	142	49	191
		Percentage	74.3%	25.7%	100%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	5.076[a]	1	.024		
Continuity Correction[b]	4.029	1	.045		
Likehood Ratio	4.615	1	.032		
Fisher's Exact Test				.046	.026
Linear-by-Linear Association	5.049	1	.025		
N of valid Cases	191				

a. 0 cells (0.0%) have expected count less than 5. The minimum expected count is 6.41.
b. Computed only for a 2×2 tables.

Supplementary Figure 1

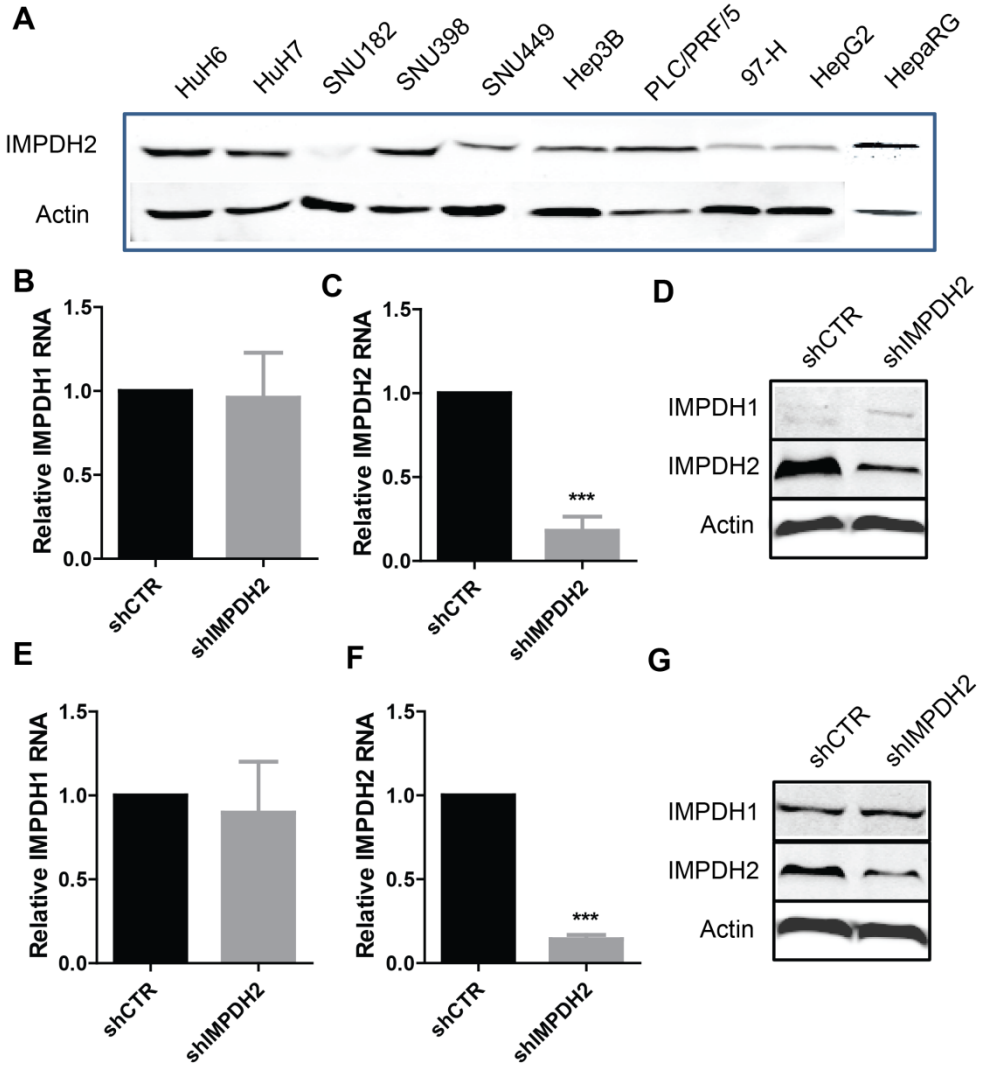

Figure S1. Establishment of IMPDH2 knockdown cells in three HCC cell lines. (A) Measurement of IMPDH2 protein level in 10 HCC cell lines. (B), (C) and (D) Knockdown IMPDH2 did not affect the expression of IMPDH1 both at RNA level and protein level in HuH7 cell; (E), (F) and (G) Knockdown IMPDH2 did not affect the expression of IMPDH1 both at RNA level and protein level in PLC/PRF/5 cell.

Supplementary Figure 2

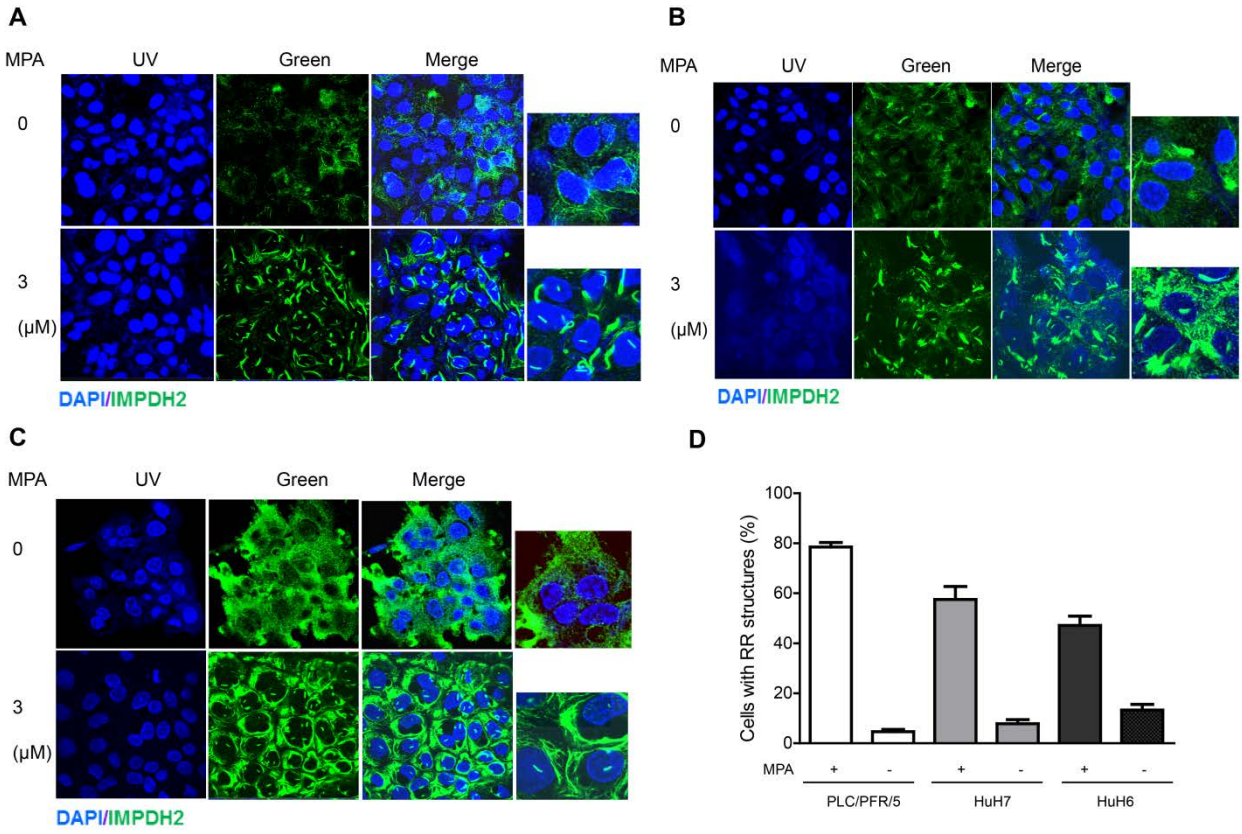

Figure. S2. Effect of MPA on IMPDH2 translocation and aggregation in HCC cell lines. Observed by Immunofluorescent staining, IMPDH2 protein moved into (peri) nucleus after 3 days treatment of MPA at the concentration of 3μM in PLC/PRF/5 (A) HuH7 (B) and HuH6 (C) cell lines respectively; (D) Quantification of IMPDH subcellular distribution. Visualization with confocal microscope was performed at the magnification of ×400 and ×920. Blue: DAPI nuclear staining; Green: antibody against human IMPDH2.

Supplementary Figure 3

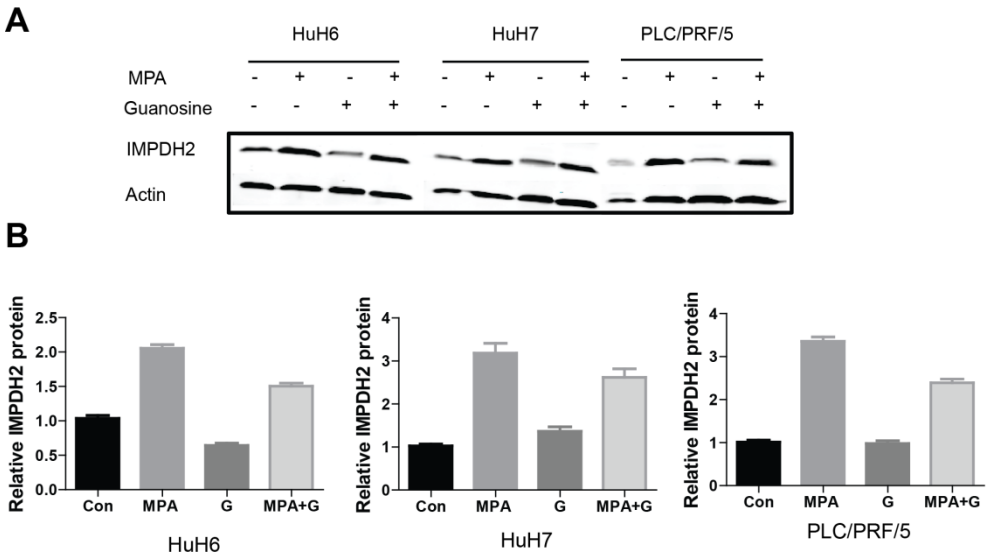

Figure S3 The expression of IMPDH2 protein significantly increased after MPA treatment. (A) Western blot showed that the content of IMPDH2 protein increased after MPA treatment both in present and in absent of supplement of exogenous guanosine; (B) Quantification of western blot.

Chapter 6

SMAD4 exerts a tumor promoting role in hepatocellular carcinoma

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Abstract

Further understanding of the molecular biology and pathogenesis of hepatocellular carcinoma (HCC) is crucial for future therapeutic development. SMAD4, recognized as an important tumor suppressor, is a central mediator of Transforming Growth Factor Beta (TGFβ) and Bone Morphogenetic Protein (BMP) signaling. This study investigated the role of SMAD4 in HCC. Nuclear localization of SMAD4 was observed in a cohort of 140 HCC patients using tissue microarray. HCC cell lines were used for functional assay *in vitro* and in immune-deficient mice. Nuclear SMAD4 levels were significantly increased in patient HCC tumors as compared with adjacent tissues. Knockdown of SMAD4 significantly reduced the efficiency of colony formation and migratory capacity of HCC cells *in vitro* and was incompatible with HCC tumor initiation and growth in mice. Knockdown of SMAD4 partially conferred resistance to the anti-growth effects of BMP ligand in HCC cells. Importantly, simultaneous elevation of SMAD4 and phosphorylated SMAD2/3 is significantly associated with poor patient outcome after surgery. Although high levels of SMAD4 can also mediated an antitumor function by coupling with phosphorylated SMAD1/5/8, this signaling however is absent in majority of our HCC patients. In conclusion, this study revealed a highly non-canonical tumor-promoting function of SMAD4 in HCC. The drastic elevation of nuclear SMAD4 in sub-population of HCC tumors highlights its potential as an outcome predictor for patient stratification and a target for personalized therapeutic development.

Keywords: SMAD4; homeostatic role; tumor promotion; patient outcome

Introduction

SMAD proteins are recognized as central mediators of Transforming Growth Factor Beta (TGFβ) and/or Bone Morphogenetic Protein (BMP) signaling pathways, which regulate a plethora of physiological processes including cell growth and differentiation [1]. Accordingly, deregulation of (TGFβ /BMP pathways almost invariably leads to developmental defects and/or diseases, in particular cancer [2]. These two pathways signal through the family of SMAD proteins to exert their effects. In mammals, there are eight SMADs that are subdivided into 3 distinct classes: receptor-regulated SMADs (R-SMADs) comprising SMAD2 and SMAD3 (transduce (TGFβ signaling) and SMAD1, SMAD5, and SMAD8 (transduce BMP signaling); a common SMAD called SMAD4; and two inhibitory SMADs, namely, SMAD6 and SMAD7 [3]. SMAD proteins are highly conserved within their family and across species, with SMAD4 representing a somewhat divergent subtype which still retains about 40% identity with other family members [4]. SMAD4 binds to R-SMADs and forms heteromeric complexes and facilitating the translocation of these heteromeric complexes into the nucleus. In the nucleus, the heteromeric complex binds to promoters and interacts with transcriptional activators [2, 5] and the presence of nuclear SMAD4 protein has profound consequences for gene expression.

Originally identified as a candidate tumor suppressor gene at 18q21.1 decades ago [6], the tumor suppressive function of SMAD4 has now almost achieved dogmatic status and loss of its activity has been implicated in the initiation and progression of a multitude of cancer types [2, 7-10]. Loss or inactivation of both normal gene copies is associated with carcinoma in several organ systems, including approximately 55% of pancreatic adenocarcinomas [6], 15% to 55% of extrahepatic cholangiocarcinomas [11] and a smaller percentage of gastrointestinal and other carcinomas [12, 13]. Strikingly, loss of SMAD4 expression in hepatocellular carcinoma (HCC) has not been observed, prompting investigations into role and importance of this tumor suppressor in this disease. Hence, we uncovered a non-conventional function of SMAD4 in HCC as a tumor promoter.

Results

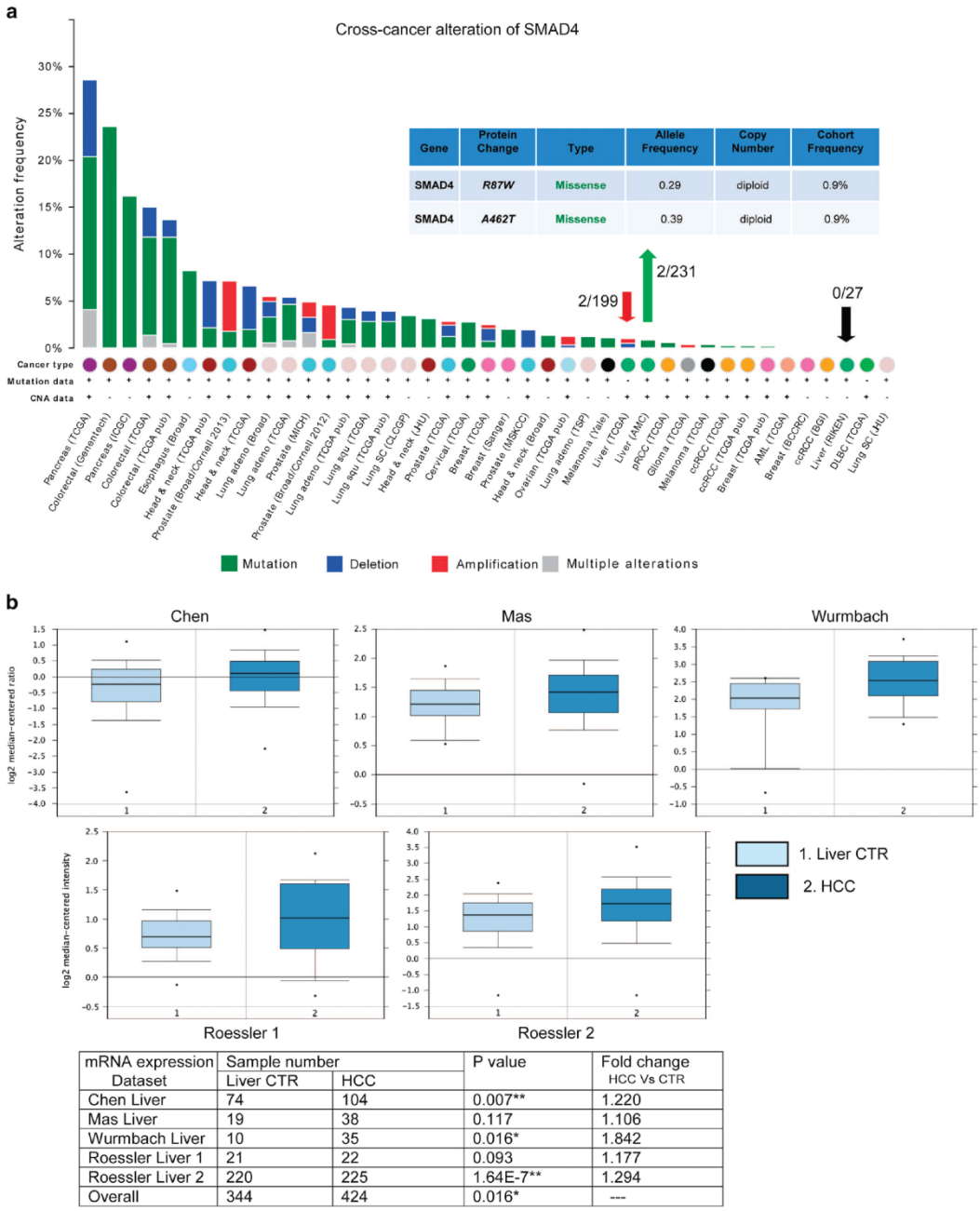
SMAD4 gene mutation is rare in HCC patients but its mRNA expression is significantly upregulated in the tumor tissue

As SMAD4 genomic alterations have been reported for several cancers, we have attempted to analyze its genomic abnormalities in patient HCC tissues. We have searched the database of the cBioPortal for Cancer Genomics. We identified three cohorts of total 457 HCC patients with genomics data of SMAD4 gene (Figure 1a). There are only two patients identified to harbor mutations (R87W; A462T), suggesting that SMAD4 gene mutation in HCC is rather rare, in contrast to pancreatic or colorectal cancers (up to 20–30%) (Figure 1a). In addition, one HCC patient was found to have SMAD4 gene amplification and another one has SMAD4 deletion (Figure 1a).

We further searched the Oncomine microarray database to analyze mRNA expression of SMAD4 in patient HCC. In total, we have identified five cohorts of 424 HCC tissue samples compared with 344 liver tissues. SMAD4 mRNA was upregulated in all the cohorts. By polling all the cohorts, there is a significant increase of SMAD4 mRNA expression in the HCC tumor compared with liver tissue ($P < 0.05$) (Figure 1b). These data indicate that genomic alteration is rare but elevation of mRNA expression is common in patient HCC tumor.

Drastic elevation of nuclear SMAD4 expression in the tumors of sub-population of HCC patients

The paucity of data surrounding the functionality of SMAD4 in HCC prompted us to analyze SMAD4 expression and activation in a panel of resected HCC from 140 individual patients and compare the results with adjacent non-transformed tissue. In these patients, nuclear SMAD4 protein (Figure 2a) was taken as measure of SMAD4 signaling activity, as it is generally assumed that this fraction of the SMAD4 pool represents the transcriptionally active form of the protein. The staining was scored by



mRNA expression Dataset	Sample number		P value	Fold change HCC Vs CTR
	Liver CTR	HCC		
Chen Liver	74	104	0.007**	1.220
Mas Liver	19	38	0.117	1.106
Wurmbach Liver	10	35	0.016*	1.842
Roessler Liver 1	21	22	0.093	1.177
Roessler Liver 2	220	225	1.64E-7**	1.294
Overall	344	424	0.016*	---

Figure 1. *SMAD4* gene mutation is rare but upregulation of its mRNA expression is common in patients. (a) In the database of the cBioPortal for Cancer Genomics (http://www.cbioportal.org/public-portal/), three cohorts of total 457 HCC patients with genomics data of *SMAD4* gene were identified. There are only two HCC patients harboring mutations (R87W; A462T). In addition, one HCC patients has SMAD4 gene amplification and another one has *SMAD4* deletion. Green arrow indicated the cohort identified SMAD4 mutation (2 of 231 HCC patients); red arrow indicated the cohort identified *SMAD4* copy number variation (2 of 199 patients); and dark arrow indicated the cohort without genomic alteration identified (27 patients). (b) The Oncomine microarray database (http://www.oncomine.org) was searched to analyze mRNA expression of SMAD4 in patient HCC. In total, five cohorts of 424 HCC tissue samples compared with 344 liver tissues were identified. SMAD4 mRNA was upregulated in all the cohorts. There is a statistically significant increase of SMAD4 mRNA expression in the HCC tumor compared with liver tissue by polling all the cohorts. *T*-test was used for individual cohort. Meta-analysis of the five cohorts indicated its P-value for the median-ranked analysis. *P<0.05, **P<0.01.

two independent investigators with a Kappa test of 0.773, suggesting that there was an excellent agreement in scoring between the two investigators. The levels of SMAD4 protein positivity range from low (score: 0 –< 2), moderate (score: 2 –< 3) to high (score: 3–4) both in the HCC tumors and their adjacent sites (Figures 2a and b). Interestingly, nuclear SMAD4 levels were considerably higher in human HCC tissue as compared with normal adjacent liver tissue ($n = 140$, $P < 0.01$) (Figures 2a and c), which is consistent with the upregulation of mRNA expression in HCC (Figure 1b). Subsequent subgroup analysis according to the nuclear SMAD4 score in the tumor showed that there was no difference of SMAD4 levels between tumor and adjacent tissue in patients displaying low-to-moderate nuclear SMAD4 scores ($n = 97$, data not shown); whereas a drastic elevation was observed in tumor compared with adjacent tissue in the high SMAD4 expression group, (3.47 ± 0.45 vs 2.27 ± 0.92, mean ± SEM, $n = 43$, $P < 0.001$) (Figure 2c).

Analysis focusing on clinical behavior of the cancer (Supplementary Table S1) revealed that high levels of nuclear SMAD4 were not significantly associated with tumor size ($n = 98$ analyzable patients), number of tumor lesions ($n = 129$ analyzable patients) and vascular invasion ($n = 78$ analyzable patients), but significantly associated with higher levels of alpha-fetoprotein (AFP) pre-resection ($n = 135$ analyzable patients, $P < 0.01$). Serum AFP has been suggested as an independent indicator for HCC prognosis and patients with high AFP levels have been reported to have shorter survival [14]. In addition, SMAD4 is significantly associated with fibrosis ($P < 0.01$) (Supplementary Figure S1). Liver fibrosis is in turn strongly correlated with HCC development [15]. Furthermore, nuclear SMAD4 level was significantly higher in undifferentiated tumor than in well differentiated tumor of HCC (2.53 ± 0.23 vs 1.94 ± 0.11, mean ± SEM, $n = 127$, $P < 0.05$) (Fig. 2d).

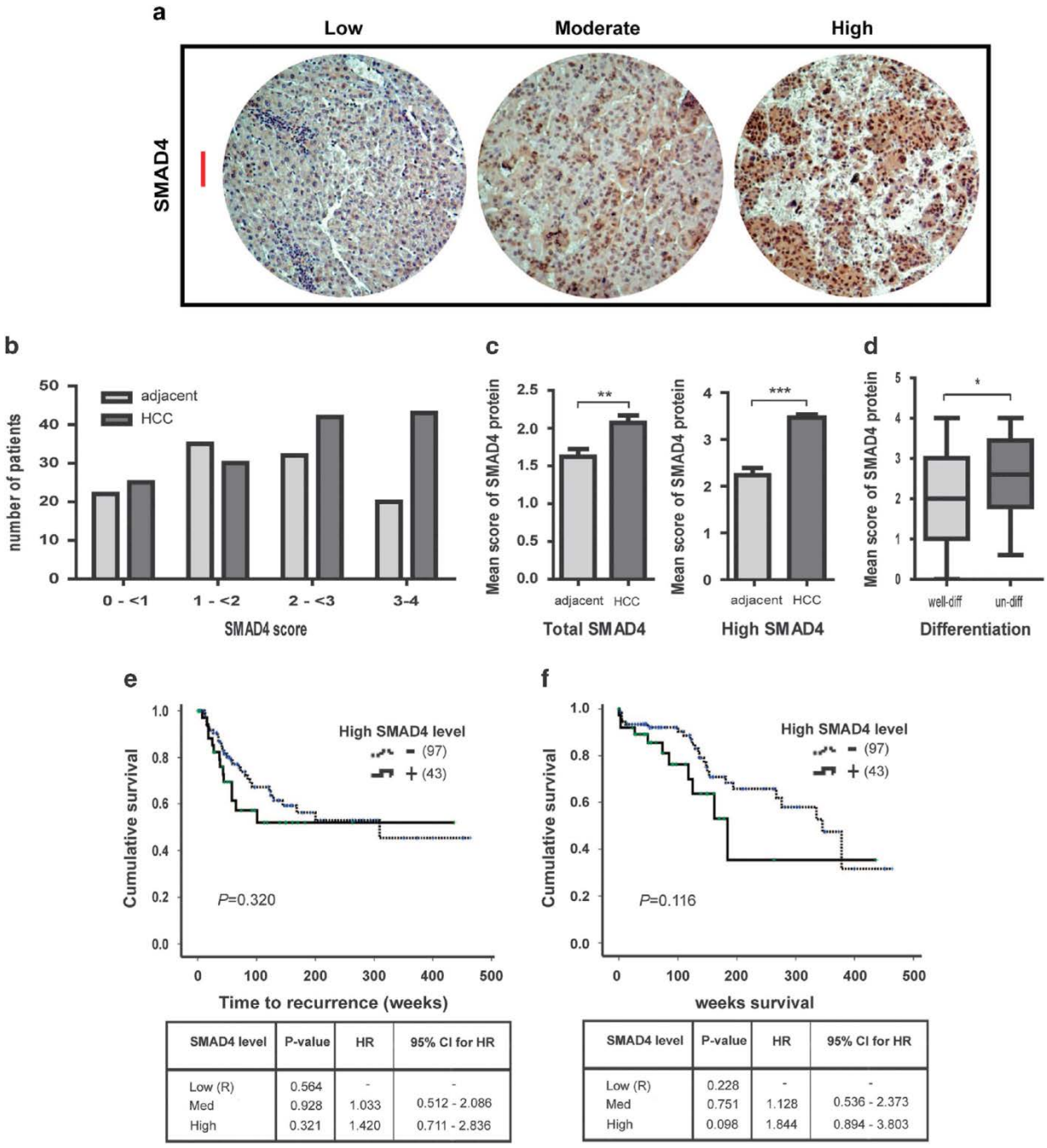
Importantly although, apparently high SMAD4 positivity in surgically resected HCC ($n = 130$ analyzable patients) tend to have higher risk of fast recurrence [Hazard Ratio (HR) = 1.420, 95% confidence interval (CI): 0.711-2.836 in the high-level group] and higher risk of poor survival (HR = 1.844, 95% CI: 0.894-3.803) in the high-level group (Figure 2e and f). Kaplan-Meier analysis ($n = 130$ analyzable patients) also indicated a trend of shorter time to recurrence and lower cumulative

survival in high SMAD4 level patients, although not statistically significant (Figure 2e and f). We interpreted that higher nuclear SMAD4 levels may be associated with more aggressive types of tumors in HCC patients.

Silencing of SMAD4 expression reduced colony formation in human hepatoma cell lines

In order to obtain an insight into the mechanisms possibly mediating the negative relation between SMAD4 signaling and HCC clinical behavior, we used lentiviral RNA interference vectors expressing short hairpin RNA (sh-SMAD4) to stably knockdown SMAD4 expression in human HCC cell lines and subsequently characterized the cellular consequences thereof. Supplementary figure S2 showed the efficacy of gene silencing using this strategy. A vector expressing short hairpin RNA targeting green fluorescent protein served as control (CTR). The success of this approach was confirmed by western blot and probing for SMAD4 protein (Figure 3a), which showed almost absence of the protein in the knockdown cell lines; whereas the control cell lines remain SMAD4 proficient. Using immunofluorescent staining, it has confirmed the efficiency of SMAD4 knockdown in Huh7, Huh6 and PLC cell lines (Figure 3b).

Colony formation assay is a robust tool to evaluate the ability of a single cell to support proliferation. Using this assay, we observed a significant decrease in the numbers of formed colonies in Huh7 cells with SMAD4 knockdown compared with the mock cells (CTR vs sh-SMAD4; 270.8 ± 25.82 vs. 144.8 ± 32.11 colonies per 1000 cells, mean ± SD, $n = 4$, $P < 0.05$) (Figure 3c). Similar results were observed in Huh6 and PLC cells (Figure 3c). Thus, in contrast to most other cell types where SMAD4 expression is associated with reduced cancer cell growth, SMAD4 expression supports proliferation of HCC cells.



SMAD4 level	P-value	HR	95% CI for HR
Low (R)	0.564	-	-
Med	0.928	1.033	0.512 - 2.086
High	0.321	1.420	0.711 - 2.836

SMAD4 level	P-value	HR	95% CI for HR
Low (R)	0.228	-	-
Med	0.751	1.128	0.536 - 2.373
High	0.098	1.844	0.894 - 3.803

Figure 2. Strong elevation of nuclear SMAD4 expression in the tumors of sub-population of HCC patients. (a) The levels of SMAD4 protein positivity range from low (score: 0-<2), moderate (score: 2-<3) to high (score: 3-4) both in the HCC tumors and their adjacent sites. Scale bar, 100 pixels. (b) The distribution of SMAD4 scores among HCC patients. (c) Overall, SMAD4 expression levels were significantly higher in human HCC tissues compared with normal adjacent liver tissues. Error bars represents mean ± SEM from n = 140, paired *T*-test, **P < 0.01. A significant increase was also observed in tumors compared with adjacent tissues in the high-grade patients (n = 43, paired *T*-test, ***P < 0.001). (d) Nuclear SMAD4 level was significantly higher in undifferentiated tumor than in well-differentiated

tumor of HCC. From Cox regression analysis (n = 130 analyzable patients), high SMAD4 level in surgical resected HCC tumor tend to have higher risk of fast recurrence (HR = 1.420) (e) and higher risk of poor survival (HR = 1.844) (f). Kaplan-Meier analysis (n = 130) also indicated a trend of faster disease recurrence (e) and lower cumulative survival (f), although not statistically significant.

Knockdown of SMAD4 attenuated the ability of HCC cell migration

Cell migration is a fundamental function underlying cellular processes including invasion or metastasis of cancer cells. We thus investigated the role of SMAD4 in migration of HCC cells using a ring-barrier system (Figure 4a). Silencing of SMAD4 expression resulted in attenuated migratory capacity toward the cell-free area in Huh7 cells. In Huh7 cells with SMAD4 knockdown, quantification revealed a significant reduction in total migration (CTR vs sh-SMAD4: 174.1 ± 54.3 μm vs 128.7 ± 42.1 μm, mean ± SD, n = 30, $P < 0.01$), effective migration (CTR vs sh-SMAD4: 109.1 ± 33.2 μm vs 55.4 ± 22.4 μm, mean ± SD, n = 30, $P < 0.001$), migration efficiency (CTR vs sh-SMAD4: 63.60 ± 9.60% vs 43.95 ± 16.62% mean ± SD, n = 30, $P < 0.0001$) and migration velocity (CTR vs sh-SMAD4: 7.3 ± 2.3 vs 5.4 ± 1.8 μm/h, mean ± SD, n = 30, $P < 0.001$) (Figure 4b). These results indicate that SMAD4 in HCC cells support migration and in conjunction with the colony formation data support the notion of a non-canonical pro-oncogenic function of SMAD4 in HCC.

Silencing of SMAD4 limited hepatoma initiation and growth in mice

To finally ensure the tumor-promoting effects of SMAD4, we evaluated the impact of SMAD4 loss on tumor initiation and growth in nude mice. One million CTR and SMAD4 knockdown cells were subcutaneously injected into the left or right side of the mice, respectively. As shown in Figure 5, impressively, knockdown of SMAD4 in Huh7 cells resulted in complete abolishment of tumor formation, whereas 7 out of 10 mice in the CTR group formed tumor (weight: 0.59 ± 0.15 g, mean). Collectively, this result is in line with the outcomes of our *in vitro* experimentation and the observation that high SMAD4 expression in human HCC tissue is associated with worse prognoses firmly demonstrates that SMAD4 exerts a tumor promoting role in HCC.

Simultaneous elevation of SMAD4 and phosphorylated SMAD2/3 is significantly associated with poor patient outcome

Upon binding of the cognate ligands to the TGFβ receptor, phosphorylated SMAD2/3 (p-SMAD2/3) binds to SMAD4 to form heteromeric complex, translocate to the nucleus and activate TGFβ signaling [16]. The signaling receptors phosphorylate R-SMAD proteins at the carboxyl-terminal (C-terminal) and the linker region [17].Recent studies uncover a role for agonist-induced phosphorylation of the R-SMAD linker region [18] that may modulate downstream cellular responses to the TGFβ family of ligands. We thus performed immunohistochemistry staining of p-SMAD2/3 both at the C-terminal phosphorylation (p-SMAD2/3C, Ser423/425) and the linker phosphorylation region (p-SMAD2/3L, Thr220/179) in the tissue microarray (TMA) that was used for SMAD4 staining (n = 140). The levels of p-SMAD2/3 protein positivity range from low (score: 0-<2), moderate (score: 2-<3) to high (score: 3-4) both in the HCC tumors and their adjacent sites (Figure 6a and 7a). The patient groups (low, moderate or high) are categorized according to expression levels in the tumors. Although no significant difference overall (n = 140), p-SMAD2/3 levels were significantly lower in HCC tissue as compared with normal adjacent liver tissue in patients with low-to-moderate scores (n = 86, $P < 0.001$, data not shown); whereas it is significantly higher in the tumor of in patients with high scores (n = 54, $P < 0.001$) (Figure 6b). Moreover, overall p-SMAD2/3L expression was significant higher in the HCC tumor than in the adjacent area ($P < 0.001$) (Figure 7b). High p-SMAD2/3L expression in tumor is significantly associated with high recurrence rate (n = 47, $P < 0.05$) and patient death rate (n = 44, $P < 0.05$) (Supplementary Table 2). Cox regression and Kaplan Meier analysis (n = 140) also revealed a tendency of shorter time to recurrence and a trend to less cumulative survival in patients with high levels of p-SMAD2/3-C/L in the tumor (Figure 6c, d and 7c).

As a phosphorylated protein, moderate levels of p-SMAD2/3-C/L would be expected to be already sufficient to trigger the downstream signaling transduction in the presence of SMAD4. A sub-population HCC patients have a simultaneous eleva-

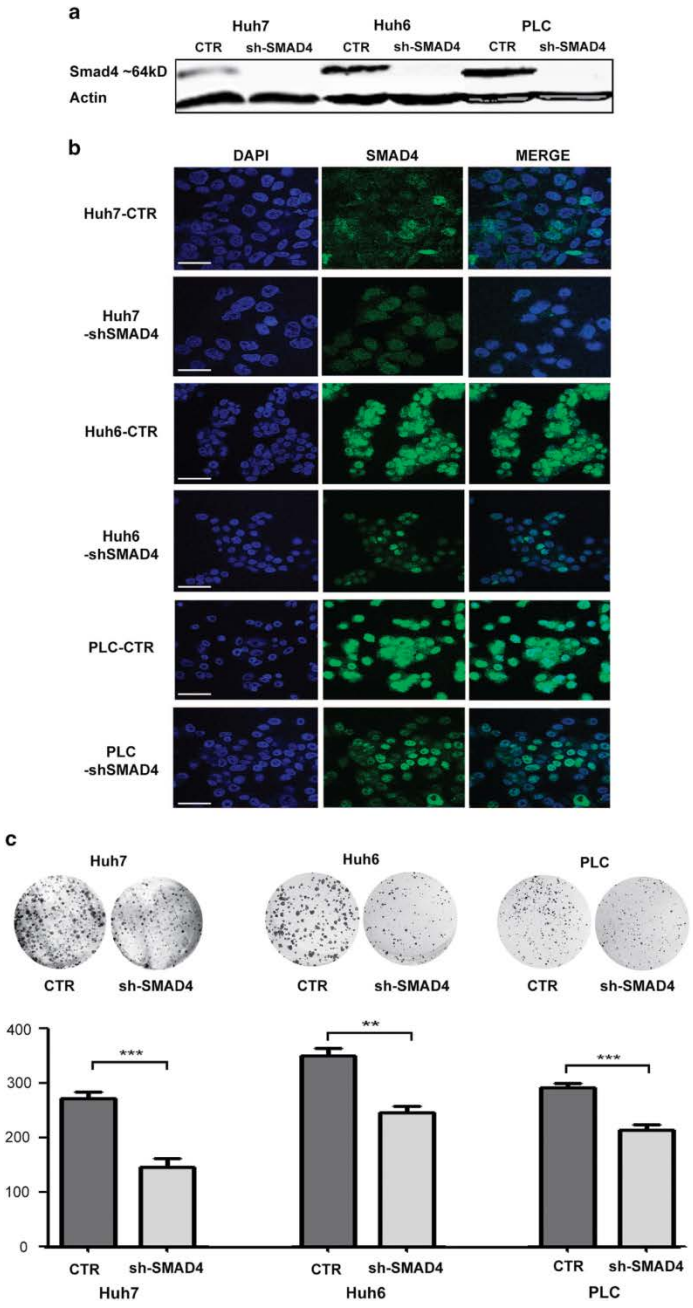


Figure 3. Decreased efficiency of colony formation in SMAD4 knockdown HCC cells. (a) Successful knockdown of SMAD4 in Huh7, Huh6 and PLC cell lines were first confirmed on protein levels by western blot. (b) Immunofluorescent staining confirmed the efficacy of SMAD4 knockdown. Scale bar, 50μm. (c). The control cells (CTR) are significantly more efficient in forming colony than the sh-SMAD4 cells. A significant decrease in the numbers of formed colonies was observed in three HCC cells with SMAD4 knockdown (sh-SMAD4), compared with mock knockdown (CTR). Error bars represent mean ± SD, n = 10, *T*-test, **P < 0.01. ***P < 0.001.

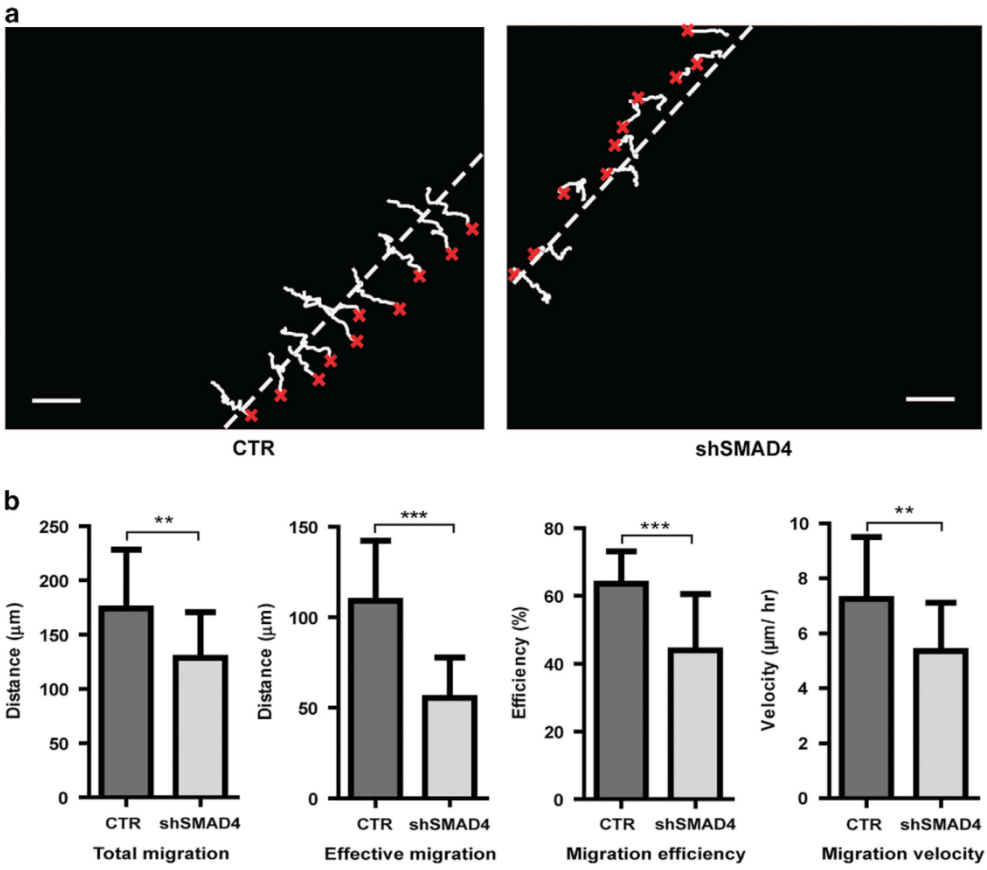


Figure 4. Silencing of SMAD4 inhibited HCC cell migration. (a) Migration assay of Huh7 cells (CTR) and its sh-SMAD4 cells using ring-barrier methods system. (b) Quantification revealed a significant reduces in total migration, effective migration, migration efficiency and migration velocity in 24 h in SMAD4 knockdown cells compared with the control cells. Error bars represent mean ± SD from n = 30, Mann Whitney test, **P < 0.01, ***P < 0.001, NS: not significant. Scale bar, 100 µm.

tion of SMAD4 (n = 22) and p-SMAD2/3C (n = 34), which represents as a hallmark for the activation of the downstream signaling of (TGFβ (Supplementary Figure S3 and S4; Figure 6f and 7c). Cox regression and Kaplan Meier analysis also confirmed that these patients are significantly faster to disease recurrence and worse survival (P < 0.05) (Figure 6f and 7c).

Nuclear p-SMAD3L (Ser213) binds to SMAD-binding element in the promoter with high affinity and specificity [18] and transmits fibrogenic/carcinogenic (fibro-carcinogenic) signaling [19]. Both p-SMAD3C (Ser425) and p-SMAD3L (Ser213) form

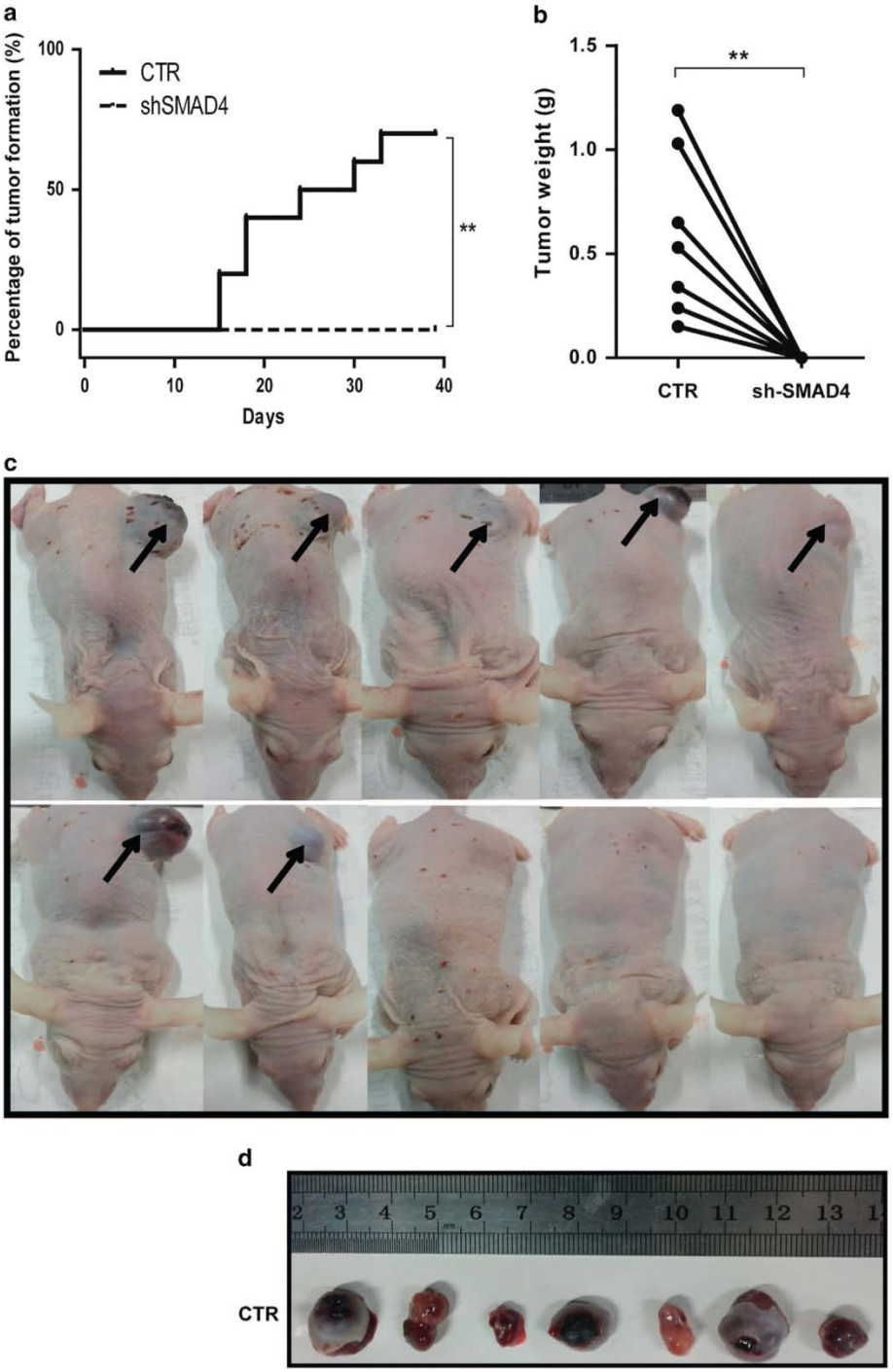
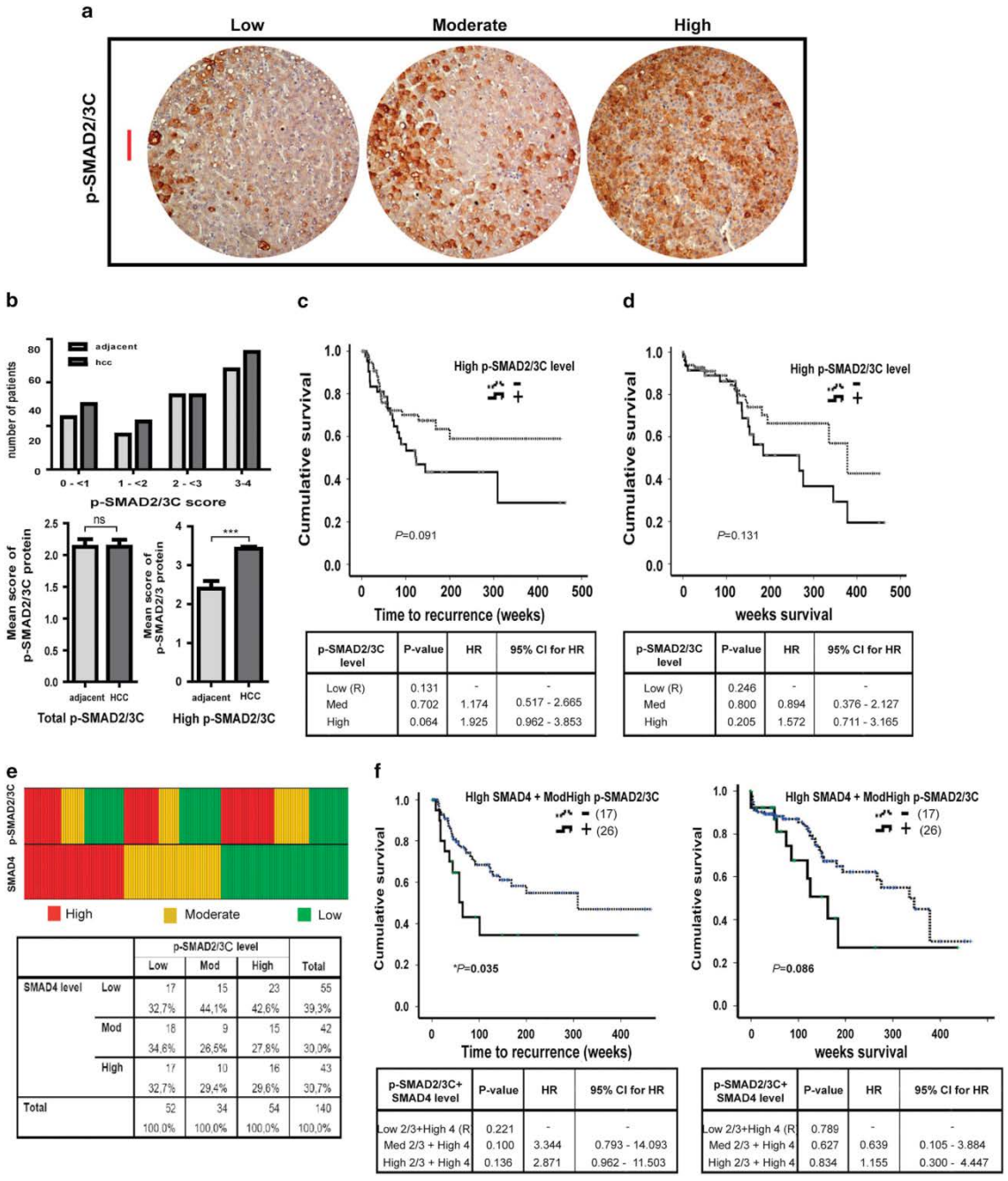


Figure 5. Knockdown of SMAD4 in Huh7 cells failed to initiate tumor in nude mice. (a) Knockdown of SMAD4 in Huh7 significantly abolished the tumor formation, whereas 7 out of 10 mice in the control group formed tumors (paired T- test, **P < 0.01). (b) The weight of formed tumor. (c) The solid arrows indicate Huh7 in control group formed tumors in mice. (d) The appearance of formed tumors in centimeter length.

hetero complexes with SMAD4, and move to nucleus [20]. Chronic inflammation and hepatitis viral additively shift hepatocytes SMAD3 signaling from tumor-suppressive pSMAD3C to fibro- carcinogenic p-SMAD3L [17, 18]. Therefore, we also investigated the expression of this particular form in our cohort. p-SMAD3L levels were significantly higher in HCC tissue as compared with normal adjacent liver tissue. ($P < 0.001$, Figure 7e). Cox regression analysis and Kaplan–Meier analysis ($n = 131$) indicated that patients with high level of p-SMAD3L have worse survival (HR = 1.155, 95% CI: 1.117–5.371 in the high-level group) and these features were also seen in co-expression of SMAD4 and p-SMAD3L (Figure 7f). Taken together (Figures 6 and 7; Supplementary Tables S2–S7), these results indicate that SMAD4 together with p-SMAD2/3, both C-terminal (C) and linker phosphorylation (L), with Ser or Thr residues, exert a tumor-promoting function in HCC patients.

An anti-tumor signaling mediated by phosphorylated SMAD1/5/8 and SMAD4 is inactivated in majority of HCC patients

Upon binding of BMP ligands, phosphorylated SMAD1/5/8 (p-SMAD1/5/8) binds to SMAD4 to form heteromeric complex, translocate to the nucleus and activate BMP signaling. Although the exact role of BMP signaling in cancer is highly context-dependent, a recent study demonstrated that BMP4, a BMP ligand, inhibited the tumorigenic capacity of HCC cells [20]. We further examined the effects of BMP4 on HCC cells. In Huh7 cells, BMP4 significantly reduces colony formation ability of Huh7 cells and knockdown of SMAD4 attenuated the effects of BMP4. The efficiency of colony formation was reduced by BMP4 treatment in CTR cells by 47.02 ± 6.5% but only by 25.3 ± 6.4% in SMAD4 knockdown Huh7 cells (mean ± SD, $n = 4$, $P < 0.01$) (Figure 8a). Consistently, adding BMP inhibitor Noggin appears to increase the efficiency of colony formation in CTR cells (124.5 ± 19.1%, mean ± SD, $n = 4$) but has much less effect (109.6 ± 9.5%, mean ± SD, $n = 4$) in SMAD4 knockdown Huh7 cells (Figure 8b). Thus BMP4 significantly reduced the colony formation ability of hepatoma cells, which was consistent with previous reports in other cancer 19-21, and knockdown of SMAD4 attenuated the effects of BMP4. Western blot analysis showed the effects of BMP4 and Noggin on the protein levels of SMAD4, p-SMAD2/3 and p-SMAD1/5/8. This was in broad agreement with the efficacy of the experimental strategy but also suggested the existence of SMAD4-dependent feed-



p-SMAD2/3C level	P-value	HR	95% CI for HR
Low (R)	0.131	-	-
Med	0.702	1.174	0.517 - 2.665
High	0.064	1.925	0.962 - 3.853

p-SMAD2/3C level	P-value	HR	95% CI for HR
Low (R)	0.246	-	-
Med	0.800	0.894	0.376 - 2.127
High	0.205	1.572	0.711 - 3.165

		p-SMAD2/3C level			
		Low	Mod	High	Total
SMAD4 level	Low	17	15	23	55
		32,7%	44,1%	42,6%	39,3%
	Mod	18	9	15	42
		34,6%	26,5%	27,8%	30,0%
	High	17	10	16	43
		32,7%	29,4%	29,6%	30,7%
Total		52	34	54	140
		100,0%	100,0%	100,0%	100,0%

p-SMAD2/3C+ SMAD4 level	P-value	HR	95% CI for HR
Low 2/3+High 4 (R	0.221	-	-
Med 2/3 + High 4	0.100	3.344	0.793 - 14.093
High 2/3 + High 4	0.136	2.871	0.962 - 11.503

p-SMAD2/3C+ SMAD4 level	P-value	HR	95% CI for HR
Low 2/3+High 4 (R	0.789	-	-
Med 2/3 + High 4	0.627	0.639	0.105 - 3.884
High 2/3 + High 4	0.834	1.155	0.300 - 4.447

Figure 6. Simultaneous elevation of p-SMAD2/3 and SMAD4 is significantly associated with poor clinical outcome in HCC patients. (a) The levels of p-SMAD2/3 protein positivity range from low (score: 0-<2), moderate (score: 2-<3) to high (score: 3-4) both in the HCC tumors and their adjacent sites. Scale bar, 100 pixels. (b) There were more patients with higher p-SMAD2/3 score both in tumor and adjacent sites. No significant overall difference of p-SMAD2/3 expression between HCC tissue and normal adjacent liver tissue. Nevertheless, in the high grade patients group, p-SMAD2/3 expression was significantly higher in HCC tissues compared to adjacent sites (n = 54). Error bars represents mean ± SEM, paired *T*-test, ***P<0.001. From Cox regression and Kaplan-Meier analysis (n = 130), high levels of p-SMAD2/3 tend to have higher risk of fast recurrence (HR = 1.649) (c) and tend to have higher risk of

poor survival (HR = 1.633) (d). (e) Twenty-two out of 140 patients have simultaneously sufficient levels of both p-SMAD2/3 (n = 16 high; n = 6 moderate levels) and SMAD4. (f) These patients have significantly poor clinical outcome as shown by both Cox regression and Kaplan-Meier analysis. *P < 0.05. NS, not significant.

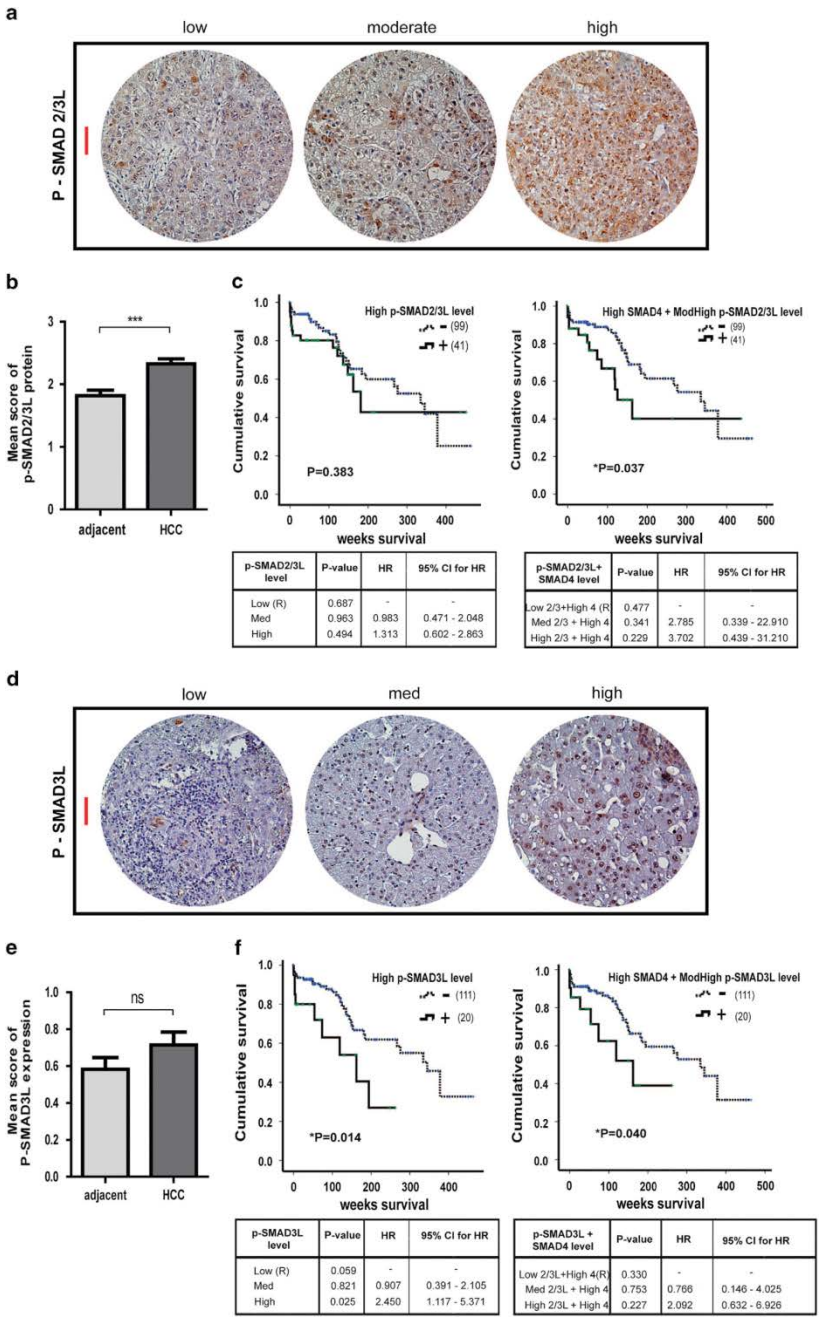


Figure 7. Simultaneous elevation of p-SMAD2/3L and SMAD4 is significantly associated with poor clinical outcome in HCC patients. The levels of p-SMAD2/3L (a) and p-SMAD3L (d) protein range from low (score: 0-<2), moderate (score: 2-<3) to high (score: 3-4) both in the HCC tumors and their adjacent sites. Scale bar, 100 pixels. (b) P-SMAD2/3L expression was significantly higher in HCC tissues compared with adjacent sites. Error bars represents mean ± SEM, paired *T*-test, ***P<0.001. (c) High

levels of p-SMAD2/3L tend to have higher risk of poor survival (HR = 1.313) and 34 out of 140 patients have simultaneously sufficient levels of both p-SAMD2/3L and SMAD4. These patients have significantly poor clinical outcome as shown by both Cox regression and Kaplan-Meier analysis, *P < 0.05. (e) The p-SMAD3L expression had no difference in HCC tissues compared with adjacent sites. Error bars represents mean ± SEM, paired *T*-test, NS, no significant. (f) High levels of p-SMAD3L tend to have higher risk of poor survival (HR = 2.450) and 21 out of 140 patients have simultaneously sufficient levels of both p-SMAD3L and SMAD4. These patients have significantly poor clinical outcome as shown by both Cox regression and Kaplan-Meier analysis, *P < 0.05.

back loops on BMP signaling elements (Figure 8c and d). Our results confirm that activation of BMP signaling, which involves both SMAD4 and p-SMAD1/5/8, exerts anti-HCC effects.

Next, we further explored the role of this pathway in our HCC cohort. Immunohistochemistry staining of p-SMAD1/5/8 was performed in the TMA (n = 140), and was scored and categorized as described for SMAD4 and p-SMAD2/3 (Figure 9a and b). Although p-SMAD1/5/8 is significantly higher in the tumor tissue compared to adjacent liver tissue (Figure 9c), only a small subset of patients have high levels of p-SMAD1/5/8 in the tumor (17 out of 140, see Supplementary Table S8). No significant relation was observed regarding to the size (n = 98) and the number of tumor foci (n = 129) (Supplementary Table S8). Interestingly, a Bonferroni-corrected clinical parameter analysis revealed a negative correlation between tumor p-SMAD1/5/8 level and age (Supplementary Table S8). Patients with high levels of p-SMAD1/5/8 appear to have lower risk of fast recurrence (HR = 0.542, 95% CI: 0.191-1.538) and lower risk to poor survival (HR = 0.596, 95% CI: 0.210-1.697 in the high-level group) (Figure 9d). Kaplan Meier analysis also revealed a trend of longer time to recurrence and higher cumulative survival in these patients (Figure 9d).

As a phosphorylated protein, p-SMAD1/5/8 could sensitively control the downstream signaling transduction. Since the anti-tumor function of this signaling requires both SMAD4 and p-SMAD1/5/8, we further categorized the expression levels of both proteins in the same patients. As shown in figure 9e, there are only eight patients having simultaneously sufficient levels of both SMAD4 and p-SMAD1/5/8 (n = 2 high; n = 6 moderate levels). These results suggest that SMAD4 and p- SMAD1/5/8 mediated anti-tumor signaling is inactivated in majority of our HCC patients.

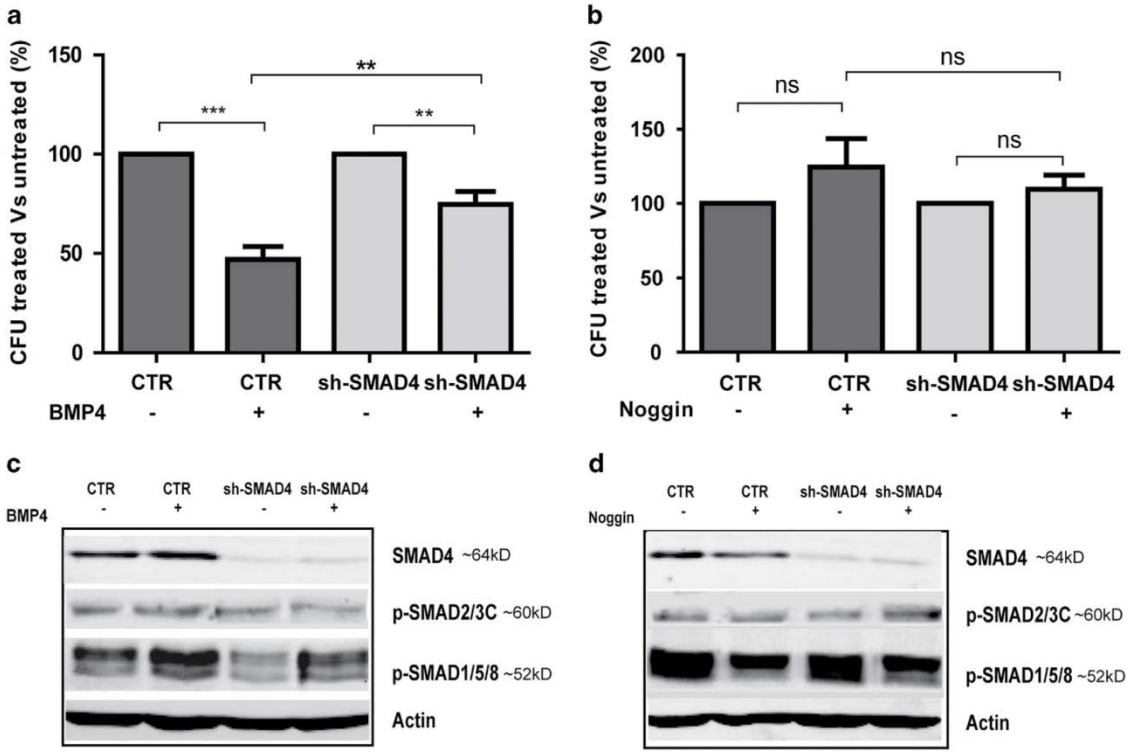
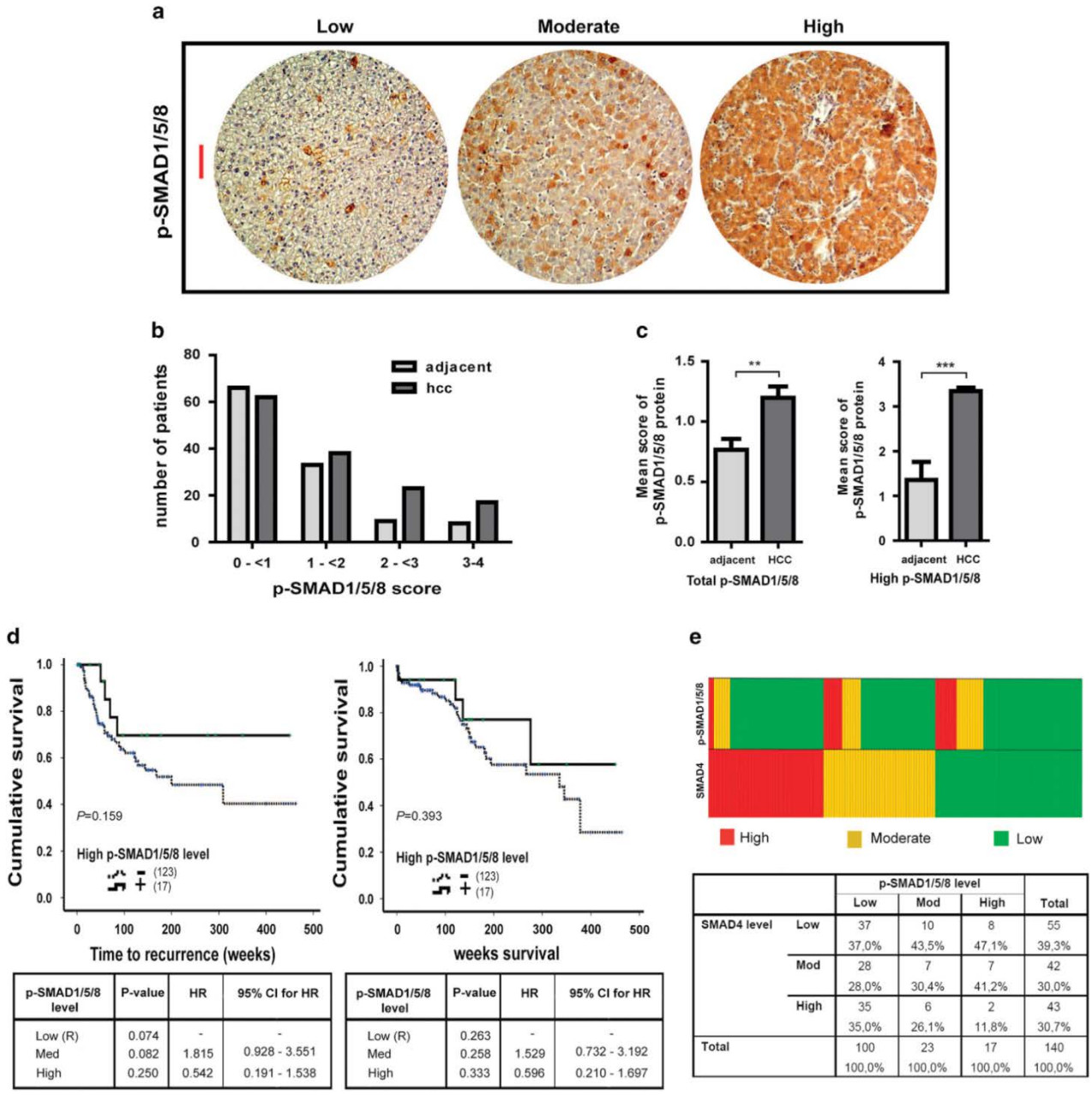


Figure 8. BMP4 significantly reduced the colony formation ability of HCC cells and its ability was attenuated by silencing SMAD4. (a) The efficiency of decreasing colony formation by BMP4 treatment was significantly reduced in Huh7 cells with SMAD4 knockdown. Error bars represent mean ± SD from n = 4, paired *T*-test, **P < 0.01, ***P < 0.001. (b) Although the difference was not statistically significant, adding BMP inhibitor Noggin appeared to increase the efficiency of colony formation in control cells and to a less extend in SMAD4 knockdown Huh7 cells. Error bars represent mean ± SD from n = 4, paired t-test, NS, not significant. (c) Protein levels of SMAD4, phospho-SMAD2/3 and phospho-SMAD1/5/8 after BMP4 treatment and (d) protein levels of SMAD4, phospho-SMAD2/3 and phospho-SMAD1/5/8 after Noggin treatment.



p-SMAD1/5/8 level	P-value	HR	95% CI for HR
Low (R)	0.074	-	-
Med	0.082	1.815	0.928 - 3.551
High	0.250	0.542	0.191 - 1.538

p-SMAD1/5/8 level	P-value	HR	95% CI for HR
Low (R)	0.263	-	-
Med	0.258	1.529	0.732 - 3.192
High	0.333	0.596	0.210 - 1.697

		p-SMAD1/5/8 level			
		Low	Mod	High	Total
SMAD4 level	Low	37	10	8	55
		37,0%	43,5%	47,1%	39,3%
	Mod	28	7	7	42
		28,0%	30,4%	41,2%	30,0%
	High	35	6	2	43
		35,0%	26,1%	11,8%	30,7%
Total		100	23	17	140
		100,0%	100,0%	100,0%	100,0%

Figure 9. The anti-tumor signaling mediated by p-SMAD1/5/8 and SMAD is inactivated in most of the HCC patients. (a) The levels of p-SMAD1/5/8 protein positivity range from low (score: 0-<2), moderate (score: 2-<3) to high (score: 3-4) both in the HCC tumors and their adjacent sites. Scale bar, 100 pixels. (b) Different to SMAD4 or p-SMAD2/3, there were fewer patients with high p-SMAD1/5/8 score both in tumor and adjacent sites. (c) Overall p-SMAD1/5/8 expression was significantly higher in HCC tissue compared with adjacent liver tissue. The p-SMAD1/5/8 levels were also significantly higher in HCC tissue compared with adjacent tissue in the high-grade (n = 17) group. Error bars represents mean ± SEM, paired *T*-test, **P < 0.01, ***P < 0.001. (d) From Cox regression analysis (n = 130), patients with high level of p-SMAD1/5/8 tend to have less risk of fast recurrence (HR = 0.542) and less risk to poor survival (HR = 0.596). Kaplan-Meier analysis (n = 130) showed similar trends. (e) However, there are only eight patients who have simultaneously sufficient levels of both SMAD4 and p-SMAD1/5/8 (n = 2 high; n = 6 moderate levels), suggesting that this signaling is inactivated in most of the HCC patients.

Discussion

In this study, we reported a drastic elevation of nuclear SMAD4 localization in tumors of subset of HCC patients. High expression of SMAD4 was further demonstrated to be functionally important for hepatoma formation and progression. Importantly, simultaneous elevation of SMAD4 and p-SMAD2/3 in sub-population of HCC patients significantly associated with poor outcome after surgery. Although SMAD4 coupled with p-SMAD1/5/8 can also mediate an anti-tumor effect, this signaling, however, is silent in majority of our HCC patients. Thus, we conclude that high nuclear SMAD4 expression has been screwed towards a tumor-promoting signaling in HCC (Supplementary Figure S5). This is unexpected in view of the dogma that SMAD4 is a potent tumor suppressor.

SMAD4 was initially described in pancreatic cancer, named DPC4 (deleted in pancreatic carcinoma, locus 4), and appears critical in pancreatic cancer progression [22, 23]. SMAD4 loss occurs in 40–50% of colon cancers [24], which is associated with metastasis, advanced disease and reduced survival. Similarly, its loss in cholangiocarcinoma [25] or prostate cancer [8] is also related to more progressive disease. The tumor suppressor function of SMAD4 is often closely linked to its capacity to mediate (TGFβ and BMP signals. However, we question whether activation or silencing of (TGFβ /BMP downstream components, including SMADs, is always ligand dependent in cancer? Because in xenografts of human hepatoma cell lines in mice, which are thus unlikely to encounter their (human) ligands, we observed that high expression of SMAD4 is even required for tumor formation and growth. In contrast to our observation, a previous study has reported a lower protein level of SMAD4 in HCC tissue compared with adjacent liver tissue in an Asian cohort [26]. A possible explanation could be that the etiologies of HCC may influence the expression of SMAD4. In Asia, viral hepatitis is the main cause of HCC; whereas only < 30% of patients in our European cohort has viral hepatitis history, although high expression of SMAD4 was also reported in another Asian HCC cohort [27]. In addition, technical differences, including the source of antibody and the protocol of immunohistochemical staining, may also result in discrepancy. In this study, we have used a robust staining protocol for SMAD4 (see Materials and Methods section) that was optimized and established in our previous studies [28, 29].

The essential role of TGFβ/BMP signaling in cancer is certainly well-documented, whereas its exact functions are also context dependent [30]. TGFβ 1 was also well recognized for its dual role in carcinogenesis [31]. It acts as a tumor suppressor in early stages of hepatocarcinogenesis by inducing apoptosis [32] and at a later stage, however, liver tumor cells often become resistant to its pro-apoptotic effect, and produce large amounts of TGFβ themselves [33]. This was in line with our result that the different levels of the phosphorylated R-SMADs are associated with distinct patient outcome. We speculate that dysregulation of key R-SMADs may lead to the opposite effect of the canonical TGFβ and BMP signaling. In our HCC cohort, a sub-population of HCC patients have a simultaneous elevation of SMAD4 and p-SMAD2/3C (n = 22) or p-SMAD2/3L (n = 34), indicating the activation of TGFβ downstream signaling. Both high expression of the C-terminal (C) and linker phosphorylation (L) region of p-SMAD2/3 are associated with worse outcome after surgical resection in our HCC cohort, which is in line with previous report in colorectal cancer [34], confirming a tumor-promoting function of TGFβ signaling in these HCC patients.

Several distinct BMP ligands were reported to act together to promote the migratory and invasive potential of cancer cells [35], including in HCC [36, 37]. In contrast, a recent study demonstrated that BMP4 induced differentiation of HCC cancer stem cells and inhibited their tumorigenic capacity [38]. Our *in vitro* study indicated activating BMP signaling by adding BMP4 ligand in HCC was able to effectively suppress colony formation of HCC cells, which was consistent with previous reports in other cancer [19-21]. However, silencing of *SMAD4* gene attenuated this effect, confirming that these anti-oncogenic actions require basal levels of SMAD4. Despite an antitumor effects of BMP pathway, this signaling, however, is silent in majority of our HCC patients, by losing the key components, either SMAD4 or p-SMAD1/5/8, or both of them. The obvious implication of this observation is that HCC cells should prove exquisitely sensitive to stimulation with BMP ligands mediating such signaling. In conjunction with the recent Food and Drug Administration approval of BMP2 and BMP7 as treatment for certain bone pathologies [39]. However, we have to be cautious that there are also studies reporting pro-oncogenic roles of BMP ligands in particular settings. For instance, BMP7 and BMP9 have been shown to have tumor promoting

functions in some experimental cancer (including HCC) models [36, 40, 41]. Nevertheless, our results call for further study exploiting this Achilles' heel of HCC.

In summary, this study reports a significant elevation of nuclear SMAD4 expression in patient HCC tumors. High nuclear SMAD4 has been screwed towards tumor-promoting effects because of simultaneous elevation of p-SMAD2/3 in subset of patients. SMAD4 can also mediate an antitumor signaling by coupling p-SMAD1/5/8, this complex, however, is absent in majority of patients due to lack of either SMAD4 or p-SMAD1/5/8, or both of them. These results have certainly shed new light on the molecular biology of HCC and more importantly SMAD-based molecules may have potential as outcome predictors for patient stratification.

Material and Methods

Tissue microarray

To make TMA, paraffin-embedded HCC patient tissues (n = 140, between 2004 to 2013) were collected from the pathology department of Erasmus Medical Centre (Erasmus MC) Rotterdam. The use of patient materials was approved by the medical ethical committee of Erasmus MC (Medisch Ethische Toetsings Commissie Erasmus MC) [42, 43].

Bioinformatics analysis of genomics and mRNA assay data sets

To analyze the prevalence of genomic alterations of SMAD4 gene in patient HCC tissues, the database of the cBioPortal for Cancer Genomics was searched (http://www.cbioportal.org/public-portal/). Both copy number variation and gene mutation data were analyzed across cancer types with focusing on HCC.

To analyze mRNA expression of SMAD4 in HCC, the Oncomine microarray database (https://www.oncomine.org) was searched. SMAD4 mRNA expression was analyzed in identified cohorts by comparing expression levels in HCC tumors with liver tissues.

Immunohistochemistry

Paraffin-embedded liver tumor tissue in TMA slides were deparaffinized in xylene, rehydrated in graded alcohols. For antigen retrieval, slides were boiled in Tris/EDTA pH 9.0 for 30 min (for SMAD4 antibody) and 10 min for other antibodies; 3% H2O2 was used to block endogenous peroxidase for 10 min at room temperature. The slides were incubated in 5% milk blocking solution followed by overnight incubation in mouse SMAD4 antibody (1:100 dilution, Santa Cruz Biotechnology, Inc., Huissen, The Netherlands), goat p-SMAD2/3C (Ser423/425) antibody (1:250 dilution, Santa Cruz Biotechnology), mouse p-SMAD2/3L (Thr220/179) (1:250 dilution, Takara Bio, Shiga, Japan), mouse p-SMAD3L (Ser213) (1:250 dilution, Takara Bio), rabbit p-SMAD1/5/8 (1:500 dilution, Cell Signaling, Leiden, The Netherlands) and p-Histone H3 (1:1000 dilution, Merck Millipore, Amsterdam, The Netherlands), and then counterstained with hematoxylin. The SMAD4 scoring was based on the nuclear staining and the p-SMAD2/3 and p-SMAD1/5/8 scoring were based on cytoplasm and/or nuclear staining. The following scores were applied: score 0 for 0-10% positive staining, score 1 for 10-30% positive staining, score 2 for 30-70% positive staining, score 3 for > 70% positive staining, and score 4 for > 70% positive staining + high intensity. The scorings were done by two investigators and the difference of scoring was valued by Kappa test.

Lentiviral shRNA vectors

Lentiviral backbone vectors for SMAD4 knockdown and non-targeting control were obtained from the Erasmus Center for Biomics (the Sigma–Aldrich TRC library, Zwijndrecht, The Netherlands). A vectors expressing short hairpin RNA targeting green fluorescent protein (not expressed in HCC cell lines) served as control (CTR). Lentiviral viral particles were generated as described previously [44].

Cell culture and reagents

Human hepatoma cell lines (Huh7, Huh6 and PLC) were cultured in Dulbecco's modified Eagle's medium (Lonza, Breda, The Netherlands) supplemented with 10% fetal bovine serum (Sigma–Aldrich) and 1% penicillin/streptomycin (Gibco, Bleiswijk, The Netherlands). SMAD4 knockdown cells and control cells were generated by

inoculation of lentiviral vectors and subsequently selected and maintained in Dulbecco's modified Eagle's medium with 10% FBS, 1% p/s and 2 µg/ml puromycin (Sigma-Aldrich). Recombinant human BMP4 protein (100µg/ml, Merck Millipore) and recombinant human Noggin (50µg/ml, R&D System, Oxon, UK) were used to treat cells, respectively.

Colony forming assay

Colony formation was performed in Huh7 cells as described previously [43]. After trypsinizing, 1000 cells were added to each well of a six-well plate and were cultured in Dulbecco's modified Eagle's medium as previously described. The colonies formed are counterstained with hematoxylin and eosin after two weeks.

Western blotting

Sub confluent cells were lysed in Laemmli sample buffer containing 0.1 M dithiothreitol and incubated for 5 min at 96°C. Immunoblotting was performed using fluorescent Odyssey immunoblotting (LI-COR Biosciences, Lincoln, NE, USA). Antibodies used were mouse SMAD4 antibody (1:500 dilution, Santa Cruz Biotechnology, Inc.), goat p-SMAD2/3 antibody (1:500 dilution, Santa Cruz Biotechnology, Inc.) and rabbit p-SMAD1/5/8 (1:500 dilution, Cell Signaling). Quantification was performed using Odyssey LI-COR software.

Ring-barrier migration assay

Ring-barrier based migration assays were performed as previously described [45, 46]. Huh7 and its sh-SMAD4 cells, $3x10^5$ cells were seeded in the ring in Dulbecco's modified Eagle's medium+ 10% fetal bovine serum +1% penicillin/streptomycin. After 24 h, the migration barrier was removed and the cells were washed twice followed by the addition of fresh medium. All cell tracking measurements were conducted using AxioVision 4.9.1 (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA). *P*-values were calculated using the two-tailed Mann-Whitney test. Track diagram images were processed in Adobe Illustrator CS6 (Adobe Systems Inc., San Jose, CA).

HCC xenograft tumor in nude mice

HCC xenograft tumor model in nude mice was established as previously described.[47] Ten mice for each cell line (Huh7), aged 6-8 weeks, were subcutaneously engrafted with 1 million control (CTR) and SMAD4 knockdown cells into the lower left or right flank, respectively. Tumor initiation in the mice was monitored. At the end of experiment, mice were killed and tumors were harvested and weighed. The use of animals was approved by the Animal Care and Ethics Committee at Hangzhou Normal University, Hangzhou, China.

Statistical analysis

Statistical analysis was performed by using Chi Square test, nonparametric Mann–Whitney test, Cox regression analysis and Kaplan Meier survival analysis in IBM SPSS Statistical (IBM Corporation, Armonk, NY, USA). T-test was also used using Graph Pad InStat software (Graph Pad Software Inc., San Diego, USA). P-values < 0.05 were considered as statistically significant.

Acknowledgements

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Supplementary Information

Supplementary Figure 1

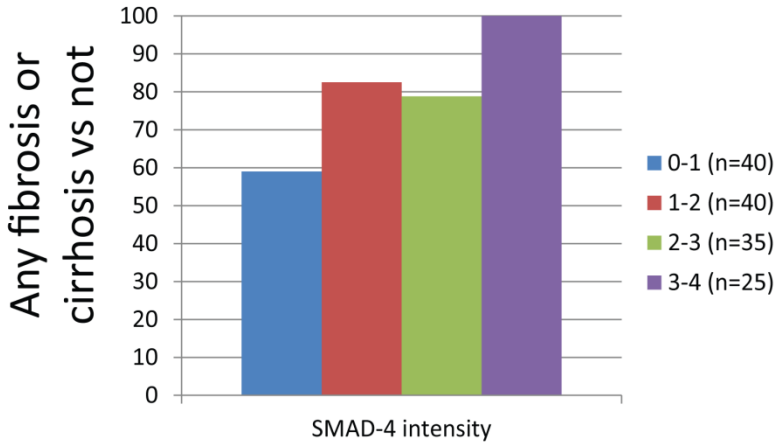


Figure S1. SMAD4 is significantly associated with liver fibrosis. Score 0 for 0-10% positive staining, score 1 for 10-30% positive staining, score 2 for 30-70% positive staining, score 3 for >70% positive staining, and score 4 for >70% positive staining. 0-1 represents any score from 0 to (including) 1; 1-2 represents any score higher than 1 and up to 2; 2-3 represents any score higher than 2 and up to 3; 3-4 represents any score higher than 3 and up to 4. $P < 0.01$; Chi-Square test.

Supplementary Figure 2

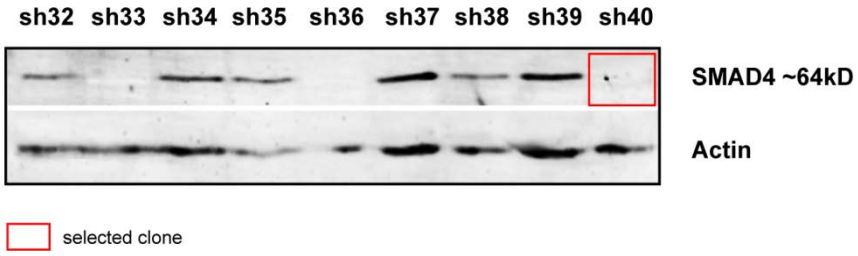
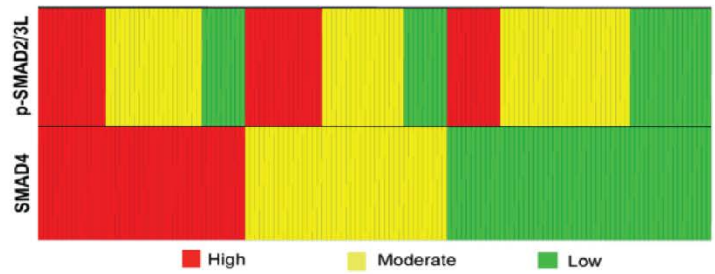


Figure S2. Selection of optimal lentiviral shRNA vectors for targeting SMAD4. Western blotting was used to evaluate the efficacy of SMAD4 knockdown in Huh7 cells. Sh40 was selected for follow-up experimentation.

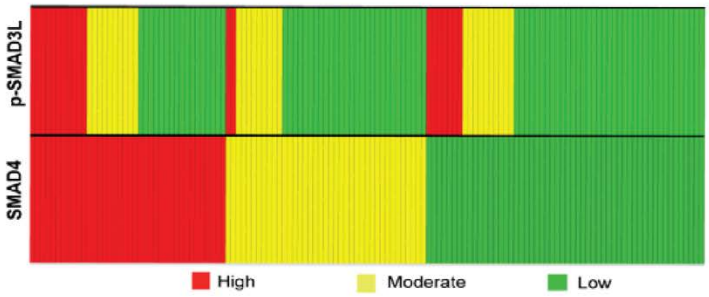
Supplementary Figure 3

A



		P-SMAD2/3L Level			Total
		Low	Moderate	High	
SMAD4 Level	Low	17	27	11	55
		48,6%	42,2%	26,8%	39,3%
	Moderate	9	17	16	42
		25,7%	26,6%	39,0%	30,0%
	High	9	20	14	43
		25,7%	31,2%	34,1%	30,7%
Total		35	64	41	140
		100,0%	100,0%	100,0%	100,0%

B



		P-SMAD3L Level			Total
		Low	Moderate	High	
SMAD4 Level	Low	37	10	7	54
		45,1%	34,5%	35,0%	41,2%
	Moderate	28	9	2	39
		34,1%	31,0%	10,0%	29,8%
	High	17	10	11	38
		20,7%	34,5%	55,0%	29,0%
Total		82	29	20	131
		100,0%	100,0%	100,0%	100,0%

Figure S3. The expression level of SAMD4 together with p-SMAD2/3L (a) and p-SMAD3L (b) in our HCC cohort and its cross tabulation.

Supplementary Figure 4

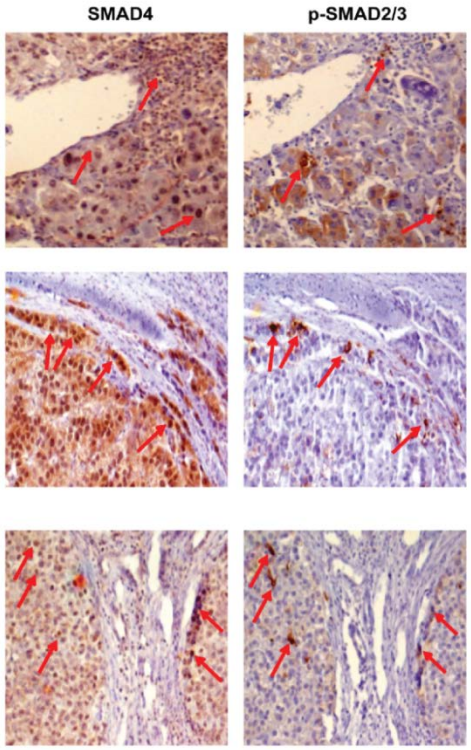


Figure S4. Immunohistochemistry staining of SMAD4 and p-SMAD2/3C in consecutive tissue slices of HCC patient.

Supplementary Figure 5

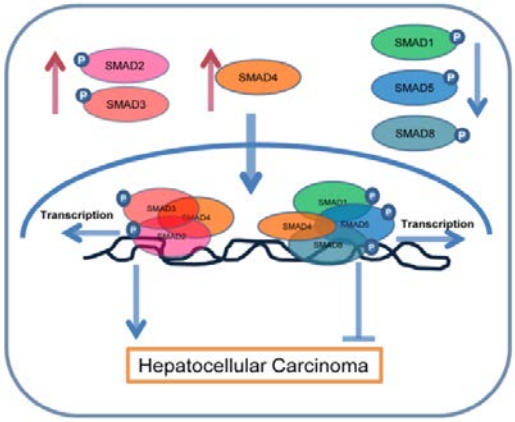


Figure S5. The model of action of SMADs in HCC. SMAD4 and p-SMAD2/3 are elevated in subset of patients that mediate a tumor promoting effect. Although high SMAD4 together with p-SMAD1/5/8 can also exert an anti-tumor effect, this complex, however, is absent in majority patients, due to missing of either SMAD4 or p-SMAD1/5/8, or both of them.

Supplementary Table S1

Patient characteristics according to SMAD4 expression level

No	Characteristics	SMAD4 expression		Total patients	*P*-value[a]
		Low-mod	High		
1	Age	60.70 ± 15.62	59.88 ± 12.61	140/140	0.763
2	Sex (% male)	68/97 (70.1%)	29/43 (67.4%)	97/140	0.753
3	Recurrence	32/97 (33,0%)	15/43 (34.9%)	47/140	0.827
4	Death	30/97 (30,9%)	14/43 (32.6%)	44/140	0.848
5	Size of tumor	6.88 ± 0.63	6.55 ± 0.99	98/140	0.777
6	Number of lesions	1.56 ± 0.11	1.39 ± 0.17	129/140	0.413
7	Vascular invasion	54/85 (63.5%)	24/36 (66.7%)	78/140	0.742
8	AFP before resection*	5.00	16.00	135/140	0.005

* *P* value < 0.01
[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test, median differences were tested using Mann-Whitney test.

Supplementary Table S2

Patient characteristics according to p-SMAD2/3C expression level

No	Characteristics	p-SMAD2/3C expression		Total Patients	*P*-value[a]
		Low-mod	High		
1	Age	58.71 ± 15.76	63.22 ± 12.56	140/140	0.077
2	Sex (% male)	57/86 (66.3%)	40/54 (74.1%)	97/140	0.330
3	Recurrence*	23/86 (26.7%)	24/54 (44.4%)	47/140	0.031
4	Death*	21/86 (24.4%)	23/54 (42.6%)	44/140	0.024
5	Size of tumor	6.68 ± 5.38	6.97 ± 5.13	98/140	0.796
6	Number of lesions	1.44 ± 1.00	1.6 ± 1.20	129/140	0.376
7	Vascular invasion	51/74 (68.9%)	27/47 (57.4%)	78/140	0.199
8	AFP before resection	10.00	7.00	135/140	0.077

* *P* value < 0.05
[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test, median differences were tested using Mann-Whitney test.

Supplementary Table S3

Patient characteristics according to p-SMAD2/3L expression level

No	Characteristics	p-SMAD2/3L expression		Total Patients	*P*-value[a]
		Low-mod	High		
1	Age	61.42 ± 13.12	58.10 ± 17.98	140/140	0.225
2	Sex (% male)	72/99 (72.7%)	25/41 (61.0%)	97/140	0.170
3	Recurrence	34/99(34.3%)	13/41(31.7%)	47/140	0.764
4	Death	30/99 (30.3%)	14/41(34.1%)	44/140	0.656
5	Size of tumor	6.74 ± 4.89	6.90 ± 6.14	98/140	0.893
6	Number of lesions	1.52 ± 1.09	1.41 ± 1.07	129/140	0.520
7	Vascular invasion	56/84 (66.7%)	22/37(59.5%)	78/140	0.445
8	AFP before resection	11.00	7.00	135/140	0.343

[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test, median differences were tested using Mann-Whitney test.

Supplementary Table S4

Patient characteristics according to p-SMAD3L expression level

No	Characteristics	p-SMAD3L expression		Total Patients	*P*-value[a]
		No	Yes		
1	Age	60.77 ± 15.00	60.25 ± 14.46	140/140	0.885
2	Sex (% male)	80/111 (72.1%)	11/20 (55.0%)	97/140	0.127
3	Recurrence	37/111 (33.3%)	8/20 (40.0%)	47/140	0.563
4	Death	32/111 (28.8%)	9/20 (45%)	44/140	0.151
5	Size of tumor	6.92 ± 5.34	6.83 ± 5.27	98/140	0.957
6	Number of lesions	1.55 ± 1.15	1.24 ± 0.56	129/140	0.080
7	Vascular invasion	62/95 (65.3%)	10/17 (58.8%)	78/140	0.610
8	AFP before resection	7.00	16.00	135/140	0.658

[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test, median differences were tested using Mann-Whitney test.

Supplementary Table S5

Patient characteristics according to high SMAD4 expression and moderate-high p-SMAD2/3C expression level

No	Characteristics	High SMAD4 + HighMod p-SMAD2/3C level		Total Patients	*P*-value[a]
		No	Yes		
1	Age	61.04 ± 15.45	57.85 ± 10.82	140/140	0.319
2	Sex (% male)	80/114 (70.2%)	17/26 (65.4%)	97/140	0.633
3	Recurrence	35/114 (30.7%)	12/26 (46.2%)	47/140	0.132
4	Death	34/114 (29.8%)	10/26 (38.5%)	44/140	0.392
5	Size of tumor	6.85 ± 5.23	6.50 ± 5.53	98/140	0.800
6	Number of lesions	1.52 ± 1.08	1.44 ± 1.12	129/140	0.744
7	Vascular invasion	67/100 (67%)	11/21 (52.4%)	78/140	0.203
8	AFP before resection	6.50	14.00	135/140	0.136

* *P* value < 0.05
[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test, median differences were tested using Mann-Whitney test.

Supplementary Table S6

. **Patient characteristics according to high SMAD4 expression and moderate-high p-SMAD2/3L expression level**

No	Characteristics	High SMAD4 + ModHigh p-SMAD2/3L level		Total Patients	*P*-value[a]
		No	Yes		
1	Age	60.92 ± 15.23	58.96 ± 13.18	140/140	0.503
2	Sex (% male)	76/106(71.7%)	21/34 (61.8%)	97/140	0.275
3	Recurrence	36/106 (34.0%)	11/34 (32.4%)	47/140	0.863
4	Death	31/106 (29.2%)	13/34 (38.2%)	44/140	0.392
5	Size of tumor	6.89 ± 5.32	6.48 ± 5.12	98/140	0.738
6	Number of lesions	1.52 ± 1.04	1.47 ± 1.22	129/140	0.831
7	Vascular invasion	58/91 (63.7%)	20/30 (66.7%)	78/140	0.771
8	AFP before resection	5.50	27.00	135/140	0.004

* *P* value < 0.05
[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test, median differences were tested using Mann-Whitney test.

Supplementary Table S7

Patient characteristics according to high SMAD4 expression and moderate-high p-SMAD3C expression level

No	Characteristics	High SMAD4 + ModHigh p-SMAD3C level		Total Patients	*P*-value[a]
		No	Yes		
1	Age	60.54 ± 15.00	59.67 ± 14.06	140/140	0.804
2	Sex (% male)	81/114 (71.7%)	12/21 (57.1%)	97/140	0.206
3	Recurrence	38/114 (33.3%)	7/21 (33.3%)	47/140	1.000
4	Death	35/114 (30.7%)	8/21 (38.1%)	44/140	0.504
5	Size of tumor	6.58 ± 5.12	8.14 ± 6.26	98/140	0.313
6	Number of lesions	1.59 ± 1.16	1.06 ± 0.23	129/140	0.054
7	Vascular invasion	63/98 (64.3%)	12/18 (66.7%)	78/140	0.846
8	AFP before resection	6.50	13.50	135/140	0.154

[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test, median differences were tested using Mann-Whitney test

Supplementary Table 8

Patient characteristics according to p-SMAD1/5/8 expression level

No	Characteristics	p-SMAD1/5/8 expression		Total Patients	*P*-value[a]
		Low-mod	High		
1	Age***	62.24 ± 11.96	47.53 ± 24.29	140/140	0.000
2	Sex (% male)	88/123 (71.5%)	9/17 (52.9%)	97/140	0.119
3	Recurrence	43/123 (35,0%)	4/17 (23.5%)	47/140	0.350
4	Death	40/123 (32.5%)	4/17 (23.5%)	44/140	0.454
5	Size of tumor	6.70 ± 5.14	7.45 ± 6.38	98/140	0.657
6	Number of lesions	1.50 ± 1.07	1.41 ± 1.23	129/140	0.708
7	Vascular invasion	70/105 (66.7%)	78/121 (64.5%)	78/140	0.194
8	AFP before resection	8.00	7.50	135/140	0.664

****P* value < 0.0001

[a]Categorized parameters were compared using Pearson's Chi-Square test, mean differences were tested using Student's *t*-test, median differences were tested using Mann-Whitney test.

Chapter 7

DMS triggers apoptosis via inhibition of SPHK1/NF-κB activation and increase of intracellular Ca^{2+} concentration in human cancer cells

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Abstract

N, N-Dimethyl-D-erythro-sphingosine (DMS) is known to induce cell apoptosis by specifically inhibiting sphingosine kinase-1 (SPHK1) and modulating the activity of cellular ceramide levels. This study aims to investigate the effects and the mechanism-of-action of DMS on human lung cancer cells. We found that DMS does-dependently suppressed cell proliferation and induced cell apoptosis in a human lung cancer cell line, A549. Mechanistically, treatment of DMS suppressed the activation of SPHK1 and NF-κBp65 but increased intracellular $[Ca^{2+}]i$ in A549 cells. In conclusion, this study demonstrated that DMS could trigger apoptosis of human lung cancer cells via modulation of SPHK1, NF-κB and calcium signaling. These molecules may represent as targets for anti-cancer drug design.

Introduction

N, N-Dimethyl-D-erythro-sphingosine (DMS, Fig.1) is biologically derived from sphingosine and has been detected in several tissues [1]. It has been reported that DMS can modulate phosphorylation events by inhibiting protein kinase [2] along with sphingosine kinase [3]. DMS can inhibit the activity of sphingosine kinase-1 (SPHK1), resulting in increase of ceramide level and decreases of sphingosine-1-phosphate （S1P）level within the cellsevents involved in cell differentiation and apoptosis [4-6].

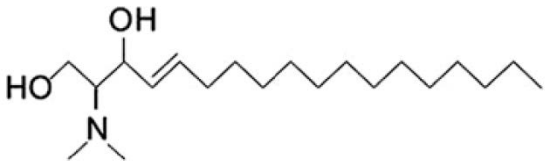

Figure 1. Chemical structure of N, N-dimethyl-D-erythro-sphingosine(DMS)

Nuclear factor kappa B（NF-κB）family is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens. Upon induction, it can transfer to the nucleus and stimulate the expression of various target genes, which play several crucial functions, including resistance to apoptosis and promoting cell survival. Increased SPHK activity can change the sphingolipid signal and NF-κBp65 expression and eventually can lead to drug resistance of breast cancer cells [7]. Inhibition of basal SPHK1 activity has been shown to induce apoptosis in A549 cells through interfering with constitutive NF-κB activity [8].

Calcium is a ubiquitous second messenger that controls a broad range of cellular functions. Previous studies reported that DMS could increase of Ca^{2+} concentration within cells, including T lymphocytes, monocytes, astrocytes, neuronal cells [9-12] and HCT116 human colon cancer cells [13].

Lung cancer is a common devastating malignancy worldwide with limited treatment option available. Experimental research has shown that SPHK1 inhibitor can significantly improve the curative effect of chemotherapy drugs on lung cancer cells as well as other types of cancer cells [14-16]. However, its mechanistic insight has

not been well-studied yet. In this study, the effects of DMS on human lung cancer cells were examined. Further, the mechanistic relationship between DMS and SPHK1 expression, NF-κB pathway and intracellular Ca^{2+} concentration in A549 cells were investigated.

Material and methods

Reagents

DMS was purchased from Enzo Life Science (Enzo, China) and was dissolved in dimethyl sulfoxide (DMSO). The final concentrations of DMSO were 0.1% or less in drugs. Antibodies to SPHK1, NF-κBp65, PARP and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents were purchased from Sigma-Aldrich (St Louis, MO).

Cell culture

The human lung cancer cell line A549 was maintained in RPMI 1640 culture medium (GIBCO Life Technologies) supplemented with streptomycin (100 μg/mL) and penicillin (100 U/mL), glutamine (2 mM) and 10% (v/v) fetal bovine serum. The cells were grown at 37℃ in a humidified atmosphere containing 5% CO_2.

Morphological observation

Cells (5×10^5/well) were plated in six-well plates. After 12 h, cells were exposed to serial dilutions of DMS (1, 2, 4 and 8 μmol/L) for the times indicated. The changes in cell morphology were observed by inverted microscope at the time of 48 h.

Cell proliferation assay

Cells (6×10^3/well) were transferred in five replicates to 96-well plate in 100 μL medium. All the cells were incubated at 37℃ in 5% CO_2 for 12 h to allow the cells to attach to the bottom of the wells. Serials dilutions of DMS (0.5, 1, 2, 4, 8 μmol/L) were added and control group was added with equal volume of PBS. At the culture time of 24, 48 and 72 h, the viability of the cells were analyzed after the addition of MTT and DMSO respectively. Absorbance was determined using Enzyme mark

instrument at a wavelength of 490 nm.

Colony Formation Assay

Cells were washed, trypsinized and resuspended in culture medium, then counted and plated in 60 mm dish (200 cells/dish) in triplicate and cultured in medium with serial dilutions of DMS, grown for 3 weeks, fixed by 10% methanol and stained with 2% crystal violet for 20 mins. The dishes were washed and dried, and the colonies were counted to obtain a cloning efficiency for each DMS concentration.

Analysis of cell apoptosis

Apoptotic cell was measured by staining cell nuclei with the Hoechst33342 dye and apoptotic cells were identified by condensed, fragmented nuclear regions. Cells were stained with 5 μL Hoechst 33342 (1 g/L) at 37℃ dark incubation for 30 mins after treated with DMS at concentrations of, 1 and 2 μmol/L for 24 and 48 h. Cell morphology was observed under fluorescence microscope.

Flow cytometric analysis of apoptosis was performed by staining cells with Annexin V-FITC and propidium iodide (PI). Cells (5×10^5/well) were plated in six-well plates and incubated overnight to attach to the bottom of the wells. Subsequently, serials dilutions of DMS were added and the control group was added with equal volume of PBS. After 48 h, control or treated cells were resuspended in Annexin-binding buffer, stained with fluorescein-conjugated Annexin V and Propidium Iodide (Annexin V-FITC kit, Becton Dickinson) and incubated at room temperature for 15 min. The cells stained only with Annexin V-FITC were used as the positive control to set the apoptotic window, and the cells stained only with PI were used as the positive control to set the necrotic window.

DNA fragmentation analysis

Cells were plated in six-well plate and treated with serial dilutions of DMS. 24 h and 48 h later, cells were collected by scraping and centrifuged at 600×g for 10 min. Cells were then washed twice with PBS and DNA fragmentation was extracted by Genome extraction kit (Generay Biotechnology, China) according to the manufactur-

er's instructions. The DNA samples were subjected to electrophoresis on 2% agarose gel and then visualize under UV light after staining with ethidium bromide.

Caspase-3 activity assay

Cells were plated in six-well plate and treated with serial dilutions of DMS for 24 and 48 h, afterwards, cells were washed twice with cold PBS and lysed in lysis buffer (Beyotime Institute of Biotechnology, China) and placed on ice for 15 mins. Sample of cytosolic protein was formed by centrifugation at 5000 g for 10 mins and protein concentration was determined by Bradford method. Cell extracts (30 μg protein) were incubated in reaction buffer containing the Ac-DEVD-pNA (2 mM) at 37 ℃ for 2 h. Cleavage of the pNA fluorescence was detected by Enzyme mark instrument at an excitation wavelength of 405 nm. Capase 3 activity was presented as units of fluorescence/(mg of protein × h).

Quantitative RT-PCR

Cells were washed with cold PBS, and then harvested in Trizol reagent (Invitrogen, Carlsbad, CA), and total RNA was extracted according to the manufacturer's instructions. The extracted RNA was reverse transcribed to cDNA. Reverse transcription reaction system was 10 μL: 5×reaction buffer 2 μL, M-MuLV Reverse Transcriptase 0.5 μL, primer mix 0.5 μL, RNA 1 μg and add DEPC to final volume of 10 μL. Leave the samples at 37℃ for 15 mins then incubate at 98℃ for 5 mins. Set up the PCR components as follow: 1 μL cDNA product, 12.5 μL Master mix, 10 pmol/μL Forward primer, 10 pmol/μL Reverse primer and add DEPC to final volume of 25 μL. Perform PCR with 30 cycles of denaturation: 5 mins at 95℃, 30 s at 94℃, 40 s at 57℃, and extension 30 s at 72℃. The primers were designed using primer 5 software. SPHK1's Forward: 5'-gtt cca aga cac ctg cct cc-3', SPHK1's Reverse：5'-cac gca acc gct gac cat-3'; GAPDH's Forward: 5'-ggt gtg aac cat gag aag tat gac-3', GAPDH's Reverse：5'-tgg cag tga tgg cat gga ctg tg-3'. The amplified DNA fragment was separated on gel electrophoresis and analyzed by Appied Biosystems 7300 real-time PCR software.

Western Blot Analysis

Cells were washed with cold PBS gently, added 100 uL/per well cell lysis buffer (Beyotime Institute of Biotechnology, China) and placed on the ice for 15 mins. Sample of cytosolic protein was formed by centrifugation at 14000g for 10 mins andprotein concentration was determined by BCA method. The proteins (40 μg) were separated by 12% SDS-PAGE and then transferred to a PVDF membranes (Millipore, Bedford, MA). Blots were blocked with 5% non-fat milk and then probed with primary antibodies (1:1000 dilution) against the SPHK1 and GAPDH protein at 4℃ for overnight. After washing, the membranes were incubated with secondary antibody (1:10000 dilution) at room temperature. Antibodies were diluted in TBS containing 0.05% (v/v) Tween 20 and 5% BSA. Proteins were analyzed using the near infrared laser imaging system.

Measurement of $[Ca^{2+}]i$

Intracellular $[Ca^{2+}]i$ concentration was measured using the fluorescent dye Fluo-4/AM (Dojindo Laboratories, Japan). Cells were treated with serial dilutions of DMS for 24 and 48 h, then resuspended in PBS containing 1% bovine serum and incubated for 30 mins with 5 μM Fluo-4/AM keeping in dark place. After washed with PBS, Fluo-4/AM loaded cells were observed by fluorescent under the inverted fluorescence microscope.

Results

Morphological alteration of A549 cells by treatment of DMS

To explore the effect of DMS on A549 cells, the cells were exposed to serial dilutions of DMS for 24 h. Cell morphology was observed under inverted microscope. DMS dose-dependently changed the morphology of A549 cells. At the concentration of 4 μmol/L, the cells became shrinkage and turned round. Vacuoles were also seen in the cytoplasm (Fig. 2). Induction of apoptosis was further confirmed by Hoechst33342 staining. Under fluorescence microscope, with increasing concentration of DMS, cells showed body shrinking, nuclear fragmentation, nuclear dissolution and apoptosis bodies (Fig. 3).

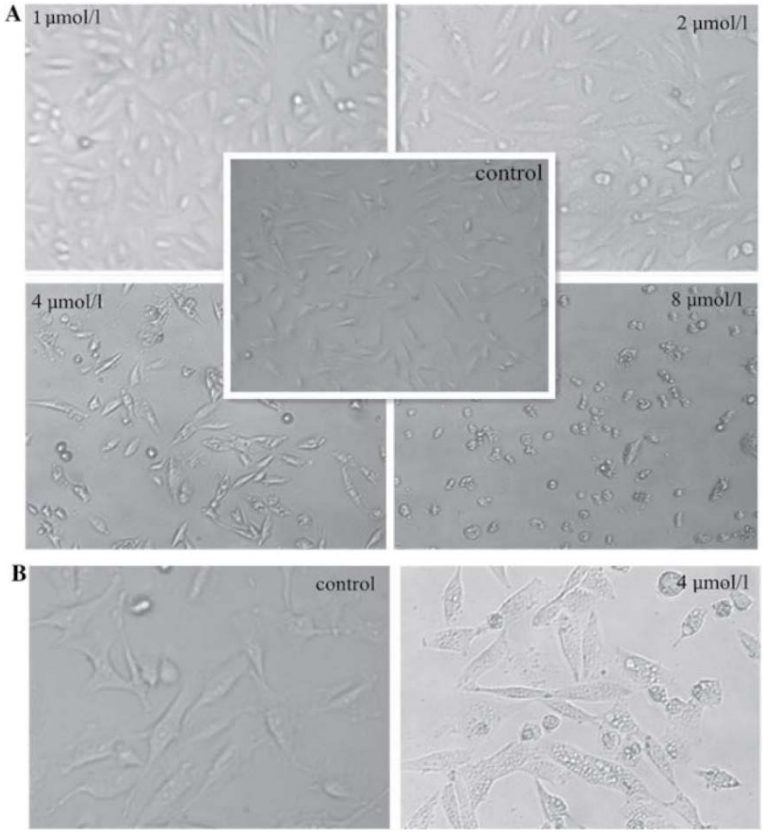

Figure 2. Changes in cell morphology were observed microscopically. (A) The number of viable cells of A549 markedly decreased after treatment with DMS at the concentration of 4 μmol/L for 48 h. Original magnification×100. (B) Cells shrinkage and rounding was observed and vacuoles were also seen in the cytoplasm following treated with DMS at concentrations of 0 and 4 μmol/L for 24h. Original magnification×200.

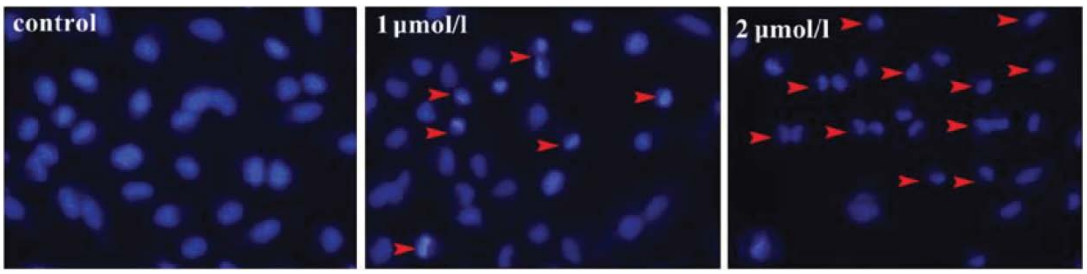

Figure 3. Stained with Hoechst 33342 after treatment with DMS at concentrations of 0, 1 and 2 μmol/L for 24h. Cell showed body shrinkage, nuclear fragmentation, nuclear dissolution and apoptotic bodies. Original magnification×320.

Cytotoxicity of DMS on A549 cells

A549 cells were treated with DMS at serial dilutions for 24, 48, and 72 h. MTT assay were used to measure cytotoxicity of DMS on A549 cells. Treatment of DMS dose- and time-dependently decreased viability of A549 cells (Fig. 4). When DMS concentration reaching 4 μmol/L, cell survival rates were significantly decreased by 37.74% ± 3.1, 36.25% ± 2.82 and 46.5% ± 5.11 (mean ± SD, n = 6, $P < 0.01$) at 24, 48 and 72h, respectively (Fig. 4). The IC50 values for 24, 48 and 72 hours were 4.864 μmol/L, 4.788 μmol/L and 4.456 μmol/L respectively, calculated by the SPSS16.0 software.

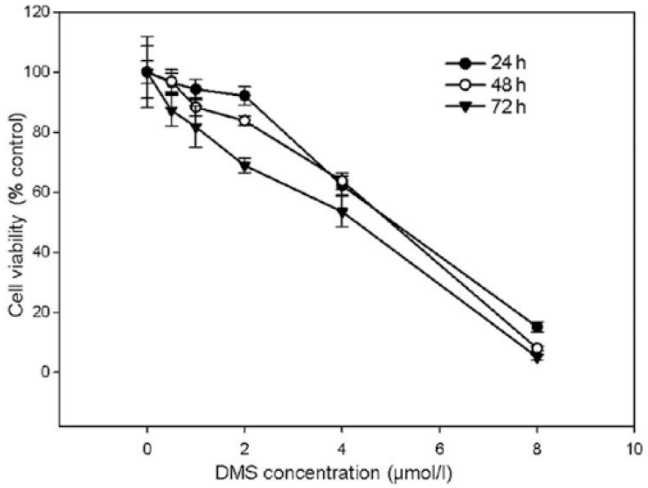

Figure 4. **Cytotoxic activity of DMS on A549 cell**. Cells were treated with DMS at different concentrations for 24, 48 and 72 h. Cell viability was assayed by the MTT test. Shown is a representative result from at least three independent experiments.

Inhibition of cell colony formation by DMS

To determine the effect of DMS on the ability of single cell proliferation, colony formation assay was performed. Colony formation efficiency was calculated with the number of visible colonies divided by the number of plated cells. Treatment with DMS suppressed colony formation in a dose-dependent manner. Once the concentration higher than 2 μmol/L, the growth of A549 was almost completely inhibited, with colony formation rate less than 1% (Fig.5).

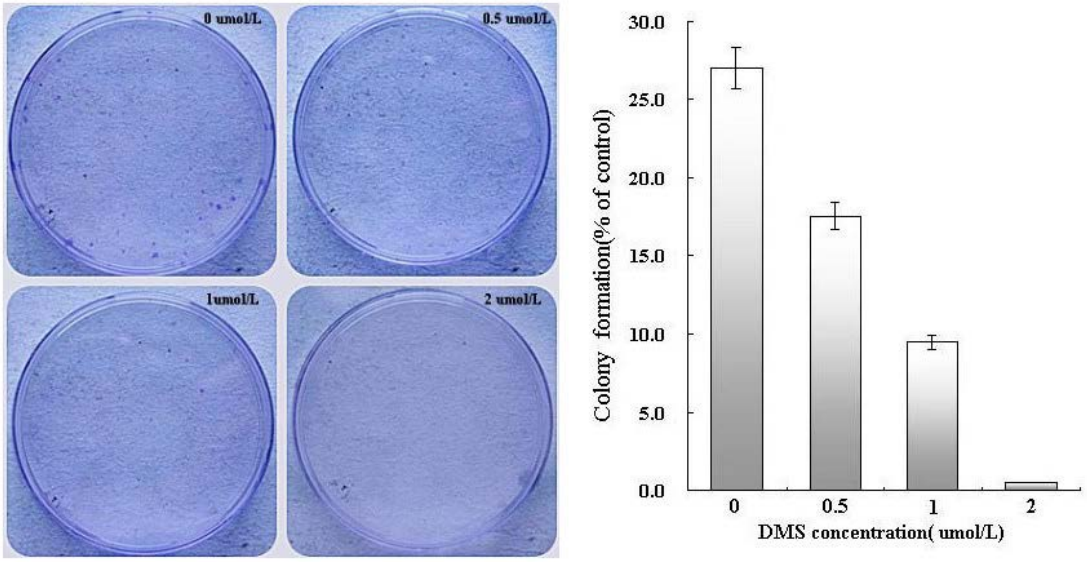

Figure 5. DMS inhibits A549 cell clonogenicity. Data are the means ± SD of 3 independent experiments.

DMS induces cell apoptosis by activating apoptotic signaling pathway

A549 cells were treated with different concentrations of DMS for 24 and 48 h, stained with Annexin V-FITC and PI, and analyzed by flow cytometry (Figure. 6A). FITC single positive region is early apoptosis cells, FITC/PI double positive area represents apoptotic cells and PI single positive region is dead cells. The percentages of apoptotic cells were increased with increased DMS concentrations and prolonged exposure time. However, when treated with 4 μmol/L DMS for 48 h, the percentage of dead cells were reached 40.5% and the number of apoptotic cells was in turn decreased. These results were further confirmed by DNA fragmentation assay. Cells treated with DMS (4 μmol/L for 24 h, 2 μmol/L and 4 μmol/L for 48 h respectively) resulted in a classical laddering pattern, whereas no DNA laddering was visualized in the control (Fig. 6B).

To investigate the signaling involved in DMS stimulation, caspase 3 activation in response to DMS was measured and the expression of poly shear ADP-ribose polymerase (PARP) was analyzed by western blot assay The results showed an increase in caspase 3 activity after treatment with DMS(1 μmol/L). It reached a plateau from 2 to 4 μmol/L. In addition, as substrate of caspase-3, PARP was

sheared and produced PARP shear body, in a time and concentration dependent manner (Fig. 7).

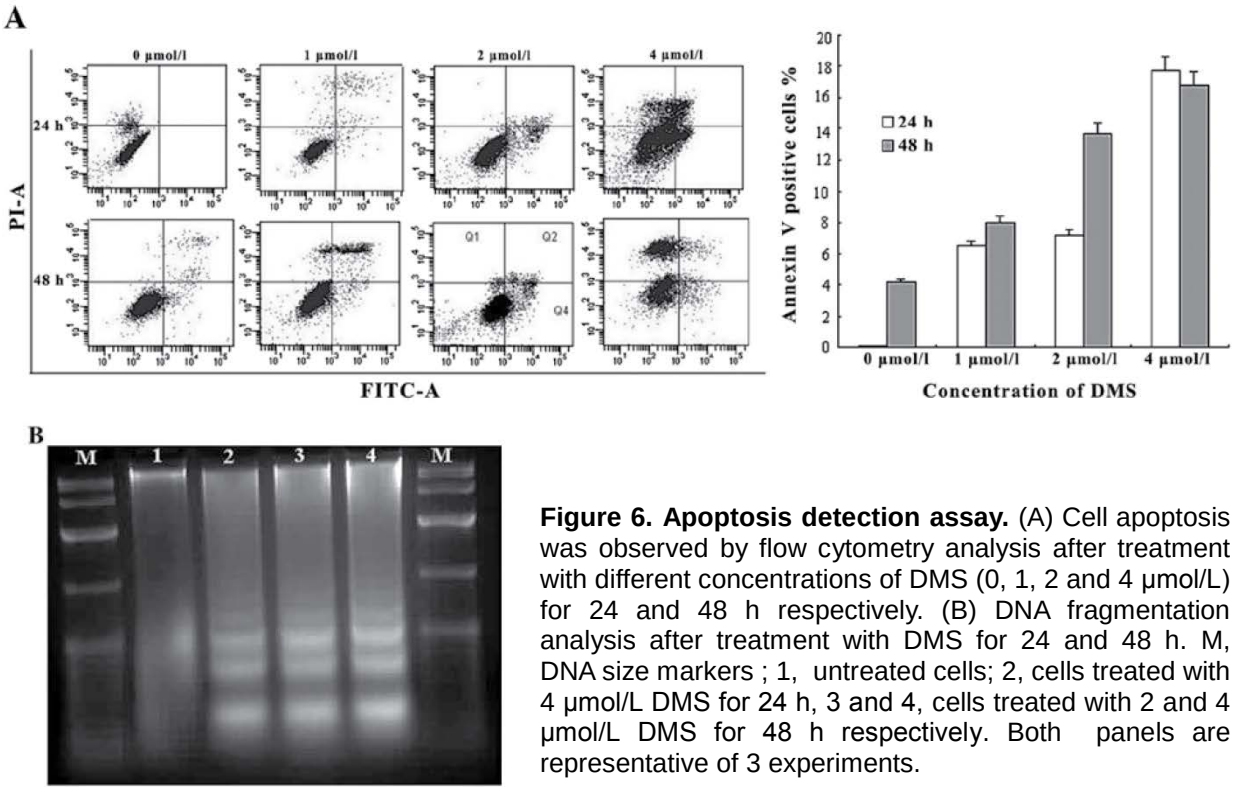

Figure 6. Apoptosis detection assay. (A) Cell apoptosis was observed by flow cytometry analysis after treatment with different concentrations of DMS (0, 1, 2 and 4 μmol/L) for 24 and 48 h respectively. (B) DNA fragmentation analysis after treatment with DMS for 24 and 48 h. M, DNA size markers ; 1, untreated cells; 2, cells treated with 4 μmol/L DMS for 24 h, 3 and 4, cells treated with 2 and 4 μmol/L DMS for 48 h respectively. Both panels are representative of 3 experiments.

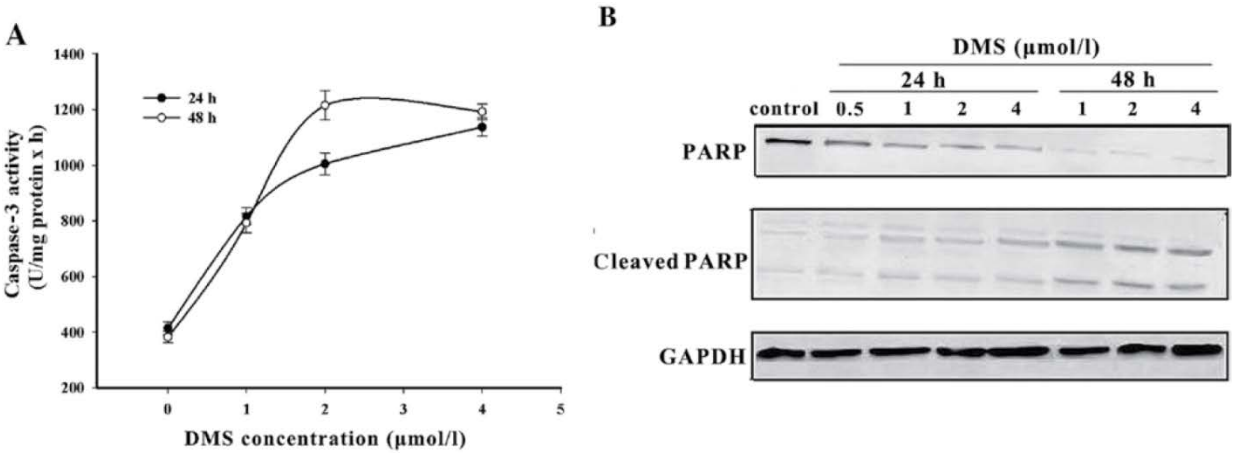

Figure 7. DMS activates the apoptosis signaling pathway. (A) Caspase 3 activity was measured in total lysates obtained from treated or untreated cells at 24 and 48 h after treatment. Data are means ± SD of 3 independent experiments. (B) A549 cells were treated with different concentrations of DMS for 24 h and 48 h. Marked increase of PARP, apoptosis-related protein, was observed using western blot analysis. Data are from 3 independent experiments.

DMS suppresses gene expression of *SPHK1*

To investigate the effect of DMS on *SPHK1* gene expression, we analyzed the mRNA levels of *SPHK1* in A549 cells by RT-PCR. The results showed that mRNA levels of *SPHK1* were dramatically down-regulated after treated with DMS at 2 and 4 μmol/L for 48 h. In addition, Q-PCR assay displayed that mRNA levels of *SPHK1* decreased by 35.28% and 34.64% when cells were treated with 2 and 4 μmol/L DMS respectively for 48 h (Fig. 8), indicating that DMS can inhibit the expression of *SPHK1* at transcriptional level.

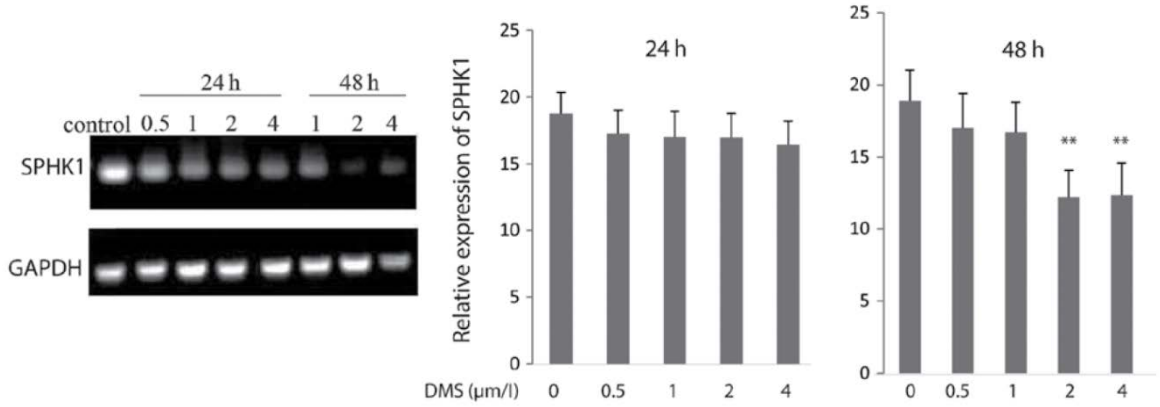

Figure 8. mRNA expression of specifically inhibiting sphingosine kinase 1(SPHK1) in A549 cells. A549 cells were treated with various concentrations of DMS for 24 and 48 h. SPHK1 mRNA expression was determined by RT-PCR and qRT-PCR respectively. Data are the means ± SD of 3 independent experiments.

DMS can inhibit SPHK1 and NF-κB activation

Western blot analysis showed that the expression of both SPHK1 and NF-κBp65 subunit were decreased, with the increase of DMS concentrations and the prolong of treatment time. Conceivably, inhibition of NF-κB activity and SPHK1 expression are probably responsible for the induction of cell apoptosis by DMS (Fig. 9).

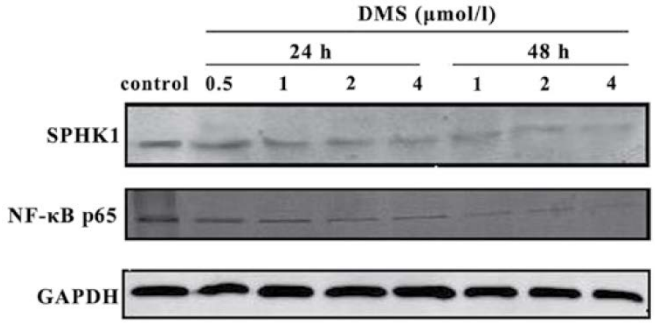

Figure 9. DMS specifically inhibits SPHK1 and the activation of the nuclear factor-κB (NF-κB) signaling pathway. Western Blot assay was used to investigate the protein expressions of SPHK1 and NF-κB subunit p65 in A549 cells, treated with different concentrations of DMS for 24 and 48 h.

DMS increases intracellular Ca2+ concentration

A549 cells were treated with different concentration of DMS for 24 and 48 h respectively, and then incubated with fluo 4-AM for 30min. Since Fluo 4-AM can conjugate with [Ca^{2+}]i and thus generate strong fluorescence in 405 nm after excitation light, therefore, intracellular [Ca^{2+}]i levels can be indirectly visualized by inverted fluorescence microscope. We observed that DMS increased intracellular [Ca^{2+}]i in A549 cells (Fig.10).

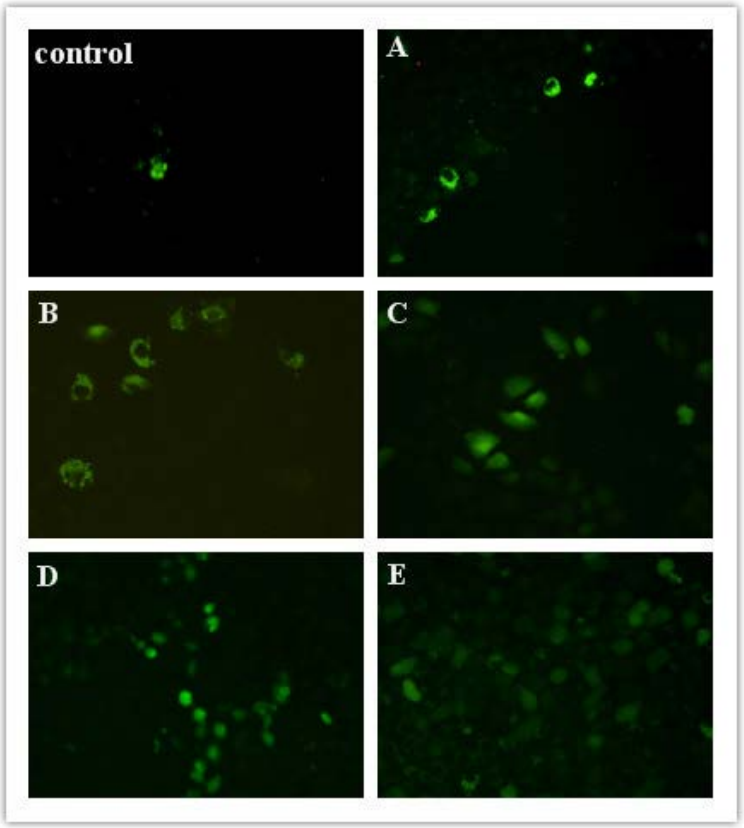

Figure 10. DMS increases intracellular Ca2+ concentration. (Control) , Untreated cells; (A and B), cells treated with 2 and 4 μmol/L DMS for 24 h, respectively; (C-E), cells treated with 1, 2 and 4 μmol/L DMS for 48 h respectively. Original magnification, ×200. Data are representative of 3 independent experiments.

Discussion

Tumor progression depends mainly on the degree of cell proliferation and cell loss; whereas apoptosis is the main source of cell loss. SPHK1 is highly expressed in many type of tumor cells (about 2 ~ 3 times higher) and its ability to prevent apoptosis has been extensively demonstrated (Table. 1) [17]. There is evidence that As an inhibitor of SPHK1, anti-cancer property of DMS has been widely investigated

over expression of SPHK1 contributes to cellular resistance to chemotherapy drugs [7]. in preclinical models. Inhibition of tumor cell growth and migration by DMS, have been reported [18-20], with Ki value of 5 μmol/L [21, 22]. Moreover, the dose of DMS and tumor growth inhibition were positively correlated in animal model of tumor-burdened nude mice.

Table 1. Summary of changes in SPHK1 expression in cancer tissues

Tumor type	SPHK1 expression (Refs.)	Prognostic association (Refs.)	Associated with drug resistance (Refs.)
Breast	Increase	Yes	Yes
Prostate	Increase		Yes
Ovary	Increase		
Glioblastoma		Yes	
Liver	Increase		
Gastrointestinal	Increase		
AML	Increase		Yes
Lung	Increase		Yes
Melanoma		Yes	Yes

The NF-κB signal pathway in tumor biology has attracted substantial attention. It has been reported that cells expressing high level of NF-κB could resist to chemotherapy and radiotherapy [23]. Inhibition of NF-κB activation could sensitize tumor cells to chemotherapy [24, 25] and leads to apoptosis eventually. Consistently, we observed that triggering apoptosis in A549 cells was associated with inhibition of NF-κB activation. In fact, NF-κB is a calcium dependent transcription factor [26]. Disturbance of intracellular calcium triggers the elevation of reactive oxygen species in mitochondria and leads to the translocation of NF-kB into the nucleus [27]. Recent study reported that DMS can increase $[Ca^{2+}]i$ in U937 and HCT116 cells [13]. In this study, we confirmed that DMS can increase intracellular $[Ca^{2+}]i$ level in A549 cells.

Billich's [8] reported that suppression of SPHK1 activation by DMS diminished NF-kB activity due to reduced nuclear translocation of RelA (p65), resulting in spontaneous apoptosis in A549 cells. This is consistent with our experimental results. However, in our study, NF-kB activity does not rise, despite increase of intracellular

$[Ca^{2+}]i$ in A549 cell after treatment of DMS. These results suggest that other mechanism may exist between SPHK1 pathway and intracellular calcium signaling in the term of regulating NF-kB activity. When SPHK1 pathway play a major role, NF-kB activity will probably be diminished. In contrast when intracellular calcium signaling play a dominant role, NF-kB activity will probably be increased. However, the exact role of SPHK1 pathway, calcium channel and NF-κB signal network in regulating the growth of cancer cells remains to be further elucidated.

Acknowledgements

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Chapter 8

Summary and discussion

For the majority of patients with advanced hepatocellular carcinoma (HCC), sorafenib is the only FDA (Food and Drug Administration)-approved drug. However, the overall efficacy of sorafenib is rather limited. For early-stage HCC, surgical resection is a treatment option, whereas for patients not eligible for resection but without metastatic disease, liver transplantation (LT) is possibility. The absence of satisfactory treatment options in advanced disease and the frequent recurrence of HCC following either resection or LT make further research aimed at improved therapy imperative.

The advent of the molecular era in medicine has raised great expectations with respect to the progress in understanding the molecular genesis of cancer and thus is implicitly a promise for translation into great benefits for patients. For some types of oncogenic disease, *e.g.* chronic myeloid leukemia, this notion has partly materialized. However, HCC has proven to be far more complex than we realized, and more genetically heterogeneous, thus defeating even novel systems biology approaches, at least to a certain extent [1]. Hope now focusses on targeted therapy, designed to be destructive towards the cancer cells with greater precision and fewer side effects. The critical issue for HCC is to identify the relevant molecular targets and to determine whether targeting them for treatment is feasible. In the present thesis I strive to make contributions here.

Mycophenolic acid (MPA) has a favorable activity spectrum for immunosuppressive therapy after LT for HCC

LT is an effective treatment for non-metastatic HCC. However, disease recurrence following LT remains a major challenge. Immunosuppressants are widely used after LT to reduce the risk of graft rejection, while use of immunosuppressant following LT is an important risk factor for HCC recurrence. Different type of immunosuppressant evidently acts through highly distinctive mechanisms on recipient immunity and thus could also have widely divergent effects on carcinogenesis *per se*. The present thesis appears to confirm this notion.

I demonstrated that MPAs limit experimental HCC through targeting IMPDH2 in different experimental modalities. Furthermore, I observed that mycophenolate mofetil (MMF, the morpholinoethyl ester prodrug of MPA) therapy is strongly associated with reduced disease recurrence and improved survival in HCC-related liver transplant patients (***Chapter 3***). Interestingly, when investigating the mechanism-of-action of MPA on HCC, I analyzed the intracellular content and subcellular localization of the IMPDH2 protein and surprisingly I found that MPA treatment dramatically enhances the expression of IMPDH2 protein, while concomitantly provoking its translocation from cytoplasm into (peri) nuclear and protein aggregation. Moreover, when I evaluated the anti-HCC effects of 23 IMPDH inhibitors, I found that most of these compounds showed comparable or even more potent anti-HCC activity relative to MPA. As these compounds display inhibitory effects on T cell proliferation as well, it may become worthwhile evaluating their potential clinical use, especially after HCC-indicated orthotropic liver transplantation. However, the same property may hamper their use following simple resection, as T cells are intimately implicated in tumor surveillance and thus the existence of a net protective anti-HCC will have to be analyzed prospectively in a large trial.

Label-retaining cells are responsible for resistance to anti-cancer therapy

Being both highly proliferative cell types with substantial demand for nucleotide synthesis, both T cells and cancer cells might be sensitive to similar therapeutic agents [2, 3]. However, exogenous nucleotide supplementation only partially counteracted the suppressive action of MPA on HCC cells, thus further elucidation of the mechanisms by which cancer cells evade the effect of nucleotide biosynthesis restriction is urgently needed but may require in depth analysis of the mechanisms involved in the synthesis of nucleotides and thus constitute a substantial body of work. Alternatively, cellular subtypes of cancer cells with different metabolic requirements but with intrinsic capacity to reignite the cancer process might exist. Especially label-retaining cells (LRCs), which are slow-cycling cells, have been identified by the capacity to retain a pulse label at cell division, due to frequently but

probably doing so at the time of the pulse labelling. LRCs cells have been reported to be associated with the capacity to generate new metastases while having superior resistance to therapy [4].

It has been reported that MPA can inhibit cancer cells proliferation and induce mitochondrion-dependent apoptosis in several human malignant tumors [5-8]. In ***Chapter 4***, we demonstrate the existence of a slow-cycling cancer cell compartment by the label-retaining technique, within fast growing tumors formed by Hela cells. Our experiments verified that LRCs are superior in tumor initiation, but more resistant to the inhibitory effects of MPA. We speculate that the differences in the existence of slow-cycling cancer cells may affect the ultimate responsiveness of MPA/MMF treatment in individual cancer patients. It may support the complexity of MPA *in vivo*, and highlight that its anti-carcinogenic effects are mainly restricted to fast growing cancer cells, which has important consequences when thinking about the long-term effects of MPA in cancerous disease, more likely to delay disease progression as combating it *per se*.

Pivotal role of inosine monophosphate dehydrogenase 2 (IMPDH2) in the process of HCC progression

Metabolic enzymes have been linked to tumorigenesis for decades [9]. As uncontrolled cell proliferation requires nucleotide biosynthesis, inhibiting metabolic enzymes that mediate nucleotide biosynthesis constitutes a rational approach to oncological disease. Being a rate-limiting enzyme of guanosine nucleotide synthesis, IMPDH plays a multifaceted, almost kaleidoscopic, role in cell growth and differentiation.

In this thesis, we demonstrated that IMPDH2 to be involved in HCC progression (***Chapter 5***). The expression of IMPDH2 in the tumor is positively associated with prognosis of HCC patients. This is unexpected in view of the elevated expression of IMPDH2 is associated with aggression of cancer [10-12]. Intriguingly, in the subpopulation of patients in which nuclear localization of IMPDH2

was detected, it was significantly associated with longer survival (***Chapter 5***). Hence, we concluded that aberrant IMPDH2 expression in HCC may contribute to carcinogenesis, while the relation to subcellular localization warrents further investigation. I also established that IMPDH2 knockdown in HCC cell lines by RNAi significantly increased the expression of *E2F* and *Histone* genes at the mRNA level. Therefore, by targeting *E2F* and *Histone* genes, nuclear IMPDH2 may affect progression of HCC, but further work *e.g*. using heterologous expression of a nucleus–targeted IMPDH2, is obviously required to substantiate this notion.

SMAD4 plays a tumor-promoting role in HCC progression

SMAD4 is originally identified as a candidate tumor suppressor gene, and is recognized as a central mediator of transforming growth factor beta (TGF-β) and/or the bone morphogenetic protein (BMP) signaling pathway. This signaling network allows rational design of novel cancer targeted therapy.

However, in this thesis, a drastic elevation of nuclear SMAD4 levels was observed in HCC patients (***Chapter 6***). High expression of SMAD4 was established to be important for HCC initiation and progression. Importantly, we observed that simultaneous elevation of SMAD4 and p-SMAD2/3 is strongly associated with poor outcome in a subpopulation of HCC patients. Although SMAD4 coupled with p-SMAD1/5/8 canonically mediated antitumor effects, this association is evidently absent in the majority of our HCC patients. Thus, we concluded that SMAD4 expression has been corrupted into a tumor-promoting signal in HCC. This is unexpected in view of the dogma that SMAD4 is a potent tumor suppressor [13-16]. Hepatitis B virus (HBV) encoded oncoprotein pX, which is associated with increased SMAD4 levels and amplifies TGF-β signaling [17], may be one possible reason to explain why SMAD4 in HCC has different behavior compared to other cancers. These results have certainly shed new light on the molecular biology of HCC and have demonstrated that SMADs may have potential as outcome predictors for patient stratification.

Conclusion and future perspective

In this thesis, we first demonstrated that MPA could constrain HCC. Next, we provide evidence for IMPDH2 playing a cardinal role in the progression of HCC. We found that MPA treatment only predominantly affects the function of fast-cycling cancer cells in contrast to the slow-cycling cells. Therefore, MMF treatment may prevent recurrence of HCC after LT mainly by affecting this population of fast-cycling cells, resulting in the improved prognosis that we demonstrate. Whether it can prevent recurrence at all, needs to be evaluated in prospective clinical trials.

Another novel finding is that the nuclear translocation of IMPDH2 in tumor cells is tightly related to better outcomes in HCC patients. It indicates that IMPDH has a previously unappreciated role in tumor cell metabolism and HCC progression. Further analysis of this pathway may identify targets for molecular therapy that may induce such a nuclear translocation.

Discovery of the tumor promoting function of SMAD4 in HCC, as opposed to other cancer types, leads to the question whether the etiology of preexisting liver disease, more specifically, the presence of HBV infection, contributes to aberrant SMAD4 expression and function. This requires further study that may expose targets for intervention in these subsets of patients.

Although I perceive my studies described in this thesis to have contributed to a better understanding of the complex nature of HCC, I fully realize more work is required to eliminate HCC from a top position in the list of lethal cancers. I hope this work can contribute to such efforts.

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Chapter 9

Netherlands samenvatting

Dutch Summary

Publiekssamenvatting in het Nederlands

Het hepatocellulair carcinoom (HCC) is een kwaadaardige wildgroei die ontstaat na genetische beschadiging van levercellen (hepatocyten). Wereldwijd is dit een zeer veel voorkomende kanker, waaraan jaarlijks meer dan een miljoen mensen komen te overlijden. In Nederland is de ziekte relatief zeldzaam, maar wel de meest voorkomende primaire kwaadaardige levertumor. Het Erasmus MC van de Erasmus Universiteit van Rotterdam, waar dit promotieonderzoek is uitgevoerd, heeft relatief van alle Academische Centra in Nederland de meeste HCC patiënten en is dus een voor de hand liggende plaats om deze ziekte te onderzoeken. Een achtergrond en uitvoerige motivatie voor de gestelde doelen van het hier uitgevoerde onderzoek geef ik in Hoofdstuk 1.

De behandeling van HCC is problematisch. Bij niet uitgezaaide ziekte is resectie of levertransplantatie in optie met een matige tot redelijke prognose, maar desalniettemin komt de ziekte toch vaak terug. Momenteel is het nog onduidelijk welke patiënten die resectie of transplantatie ondergaan door de ingreep definitief genezen zullen worden en welke factoren bepalen hoe lang het duurt voordat de ziekte weer terugkomt. Het is van belang om zulke factoren te kennen omdat ze mogelijk gebruikt kunnen worden voor het ontwerpen van therapie met een betere uitkomst voor HCC patiënten. In deze context is met name immuunsuppressie interessant. Na levertransplantatie voor HCC dient immuunsuppressie te worden gegeven, anders zou de nieuwe lever immers worden afgestoten. Er zijn veel verschillende soorten immunosuppressieve geneesmiddelen die alle op andere wijze interacteren met fundamentele aspecten van de cellulaire biochemie. Het zou dus mogelijk kunnen zijn dat sommige soorten immuunsuppressie niet alleen het immuunsysteem afremmen in haar neiging de nieuwe lever af te stoten, maar tegelijkertijd ook de kankercel remmen bij kwaadaardige groei. Middels een literatuuronderzoek (Hoofdstuk 2) maak ik dit inderdaad waarschijnlijk. Aangemoedigd door dit resultaat besloot ik deze hypothese nader uit te werken en in Hoofdstuk 3 laat ik experimenteel zien dat de immuunsuppressieve medicatie mycofenolzuur, groei van HCC remt zowel in weefselkweken van HCC als ook in proefdieren. Sterker nog, ik kon ook laten zien dat HCC patiënten die een levertransplantatie kregen en werden behandeld met mycofenolzuur langer leven en minder vaak in ziekte terugvallen wanneer vergeleken met HCC patiënten die een

levertransplantatie kregen en werden behandeld met andere immunosuppressiva. Mijn onderzoek wijst er in sterke mate op dat het gebruik van mycofenolzuur na levertransplantien bij de behandeling van HCC een goed idee is.

De vraag echter die oprees uit deze resultaten was hoe mycofenolzuur dit effect bewerkstelligde. Mycofenolzuur remt twee enzymen die betrokken zijn bij de nucleotidesynthese, inosine monophosphate dehydrogenase 1 en 2 (IMPDH1 en IMPDH2). Het ligt voor de hand om te veronderstellen dat deze enzymen dan ook wel betrokken zouden zijn bij het antikanker effect van mycofenolzuur. Een eerste aanwijzing dat deze gedachte inderdaad juist was, wordt gegeven in Hoofdstuk 4. Hier laat ik zien dat het specifiek remmen van IMPDH2 de groei van kankercellen remt, wederom zowel in kankerkweekjes alsook in proefdieren. Met name het snelgroeiende compartiment van kankers wordt getroffen door het remmen van IMPDH2, terwijl het langzaam delende compartiment ontzien wordt. Ofschoon deze data impliceren dat de kanker niet volledig opgeruimd kan worden door het remmen van IMPDH2, is het tegengaan de snelle proliferatie per se, vaak een belangrijk doel in het bestrijden van de ziekte. Bovendien kan men zich voorstellen dat door het verminderen van de proliferatieve uitdaging, het immuunsysteem van de patiënt meer tijd krijgt om een efficiënte reactie tegen de kanker op te bouwen. Omdat, zoals gezegd, na levertransplantatie immuunsuppressie sowieso noodzakelijk is bij de behandeling en patiënten dus een relatief zwak immuunsysteem hebben, is dit niet een onbelangrijk punt. Inderdaad laat ik in Hoofdstuk 5 een relatie zien tussen de expressie van IMPDH2 in leverkanker en de prognose van de ziekte, ofschoon ik niet alle mechanistische details kon ophelderen. Toch ondersteunen deze meer mechanistische studies de gedachte dat in ieder geval bepaalde groepen HCC patiënten baat kunnen hebben bij specifieke vormen van immuunsuppressie volgend op levertransplantatie.

In Hoofdstuk 6 richt ik mijn aandacht op de leverkankercel zelf. Hierbij ontdek ik dat leverkankercellen andere eigenschappen hebben in vergelijking tot kankercellen elders uit het lichaam. Waar zogenaamde SMAD signalen normaliter een belangrijke rol spelen bij het voorkomen van kanker, blijken deze zelfde SMAD signalen in de lever juist te leiden tot stimulatie van het oncologisch proces. Met deze kennis kan gepoogd worden therapie te ontwikkelen die juist gericht op het remmen van SMAD signalen en op deze wijze de ziekte te bestrijden. In Hoofdstuk 7

diep ik deze materie nog verder uit en kijk ik met name hoe deze signalen zich verhouden tot de (vermoedelijke) oorzaak van de kanker (virale hepatitis versus andere oorzaken, zoals overmatig alcoholgebruik en de daarop volgende onstekingsreacties). Ik laat zien dat het remmen van biochemische elementen betrokken bij ontstekingsreacties kankercellen tot geprogrammeerde celdood drijft. Samen met de kennis die ik elders in dit proefschrift heb vergaard met betrekking tot de werkingsmechanismen van immuunsuppressie en hun relatie tot het kankerproces kan op deze wijze een meer helder beeld worden geschetst als hoe mogelijke nieuwe therapie gericht op het behandelen en zelfs genezen van HCC er uit zou kunnen zien. Mijn gedachten hierover geef ik in Hoofdstuk 9. Alles te samen hoop ik met dit proefschrift een aanzet te hebben kunnen geven voor zulke nieuwe therapie.

Chapter 10

Appendix

Curriculum vitae

Kan Chen was born on 10th June 1977, in Hanzhong, Shaanxi province, China. She grew up and attended elementary, middle and high school at her hometown, Hanzhong, the northwest part of China.

In 1994, she started her Bachelor study at the Clinical Medical Faculty of Yan'an University, Shaanxi, China. Soon after graduation in 1999, she worked at Central Hospital of Hanzhong, as a clinical pathologist, under supervision of Prof. Lijuan Liu. In 2002, she moved to Xi'an, the capital of Shaanxi province, to start her Master programme on Pathology and Pathophysiology, at the college of Medicine, Xi'an Jiaotong University. Under supervision of Prof. Yili Wang and Prof. Lvsheng Si, she initiated the research on cancer from then on. After graduation in 2005, she subsequently moved to Hangzhou, the east part of China, to start her reseach on tumor stem cell at Zhejiang Sci-Tech University, under supervision of Prof. Xinyuan Liu and Prof. Cheng Qian. At the same time, she worked as a immunology lecturer in college of Life Science, Zhejiang Sci-Tech University.

In 2013, she started her PhD programme at the department of Gastroenterology and Hepatology, Erasmus Medical Center Rotterdam, The Netherlands, to carry out research on liver cancer. Under supervision of Prof. Maikel .P Peppelenbosch, Dr. Dave Sprengers and the sharp-minded, young-talented researcher Dr. Qiuwei Pan, she focused on the role of a metablic enzyme, Inosine monophosphate dehydrogenase (IMPDH) in liver cancer.

PhD Portfolio

Name PhD Student	Kan Chen
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Promotor	Prof. Dr. Maikel P. Peppelenbosch
Co-promotors	Dr. D.Sprengers
	Dr. Q Pan

General Courses

- Biomedical Research Techniques XIII & XIV
- Translational Imaging Workshop by AMIE From mouse to man
- Microscope Image Analysis: From Theory to Practice
- Photoshop and Illustrator CS6 Workshop
- Basic Human Genetics course: Genetics for Dummies
- Basic and Translational Oncology
- Biomedical English Writing Course for MSc and PhD-students
- Molecular Medicine
- Flow cytometry

Oral Presentations at National Conferences

- 2016, the 9th Dutch Experimental Gastroenterology and Hepatology (DEGH)

Poster Presentations at International /National Confercence

- 2016, The 51th Annual Meeting of the European Association for the Study of the Liver (EASL), Barcelona, Spain
- 2016, The 20th Annual Molmed Day, Molecular Medicine Postgraduate School, Rotterdam, The Netherlands
- 2015, The 19th Annual Molmed Day, Molecular Medicine Postgraduate School, Rotterdam, The Netherlands
- 2014, The 49th Annual Meeting of the European Association for the Study of the Liver (EASL), London, United Kingdom
- 2014, The 18th Annual Molmed Day, Molecular Medicine Postgraduate School, Rotterdam, The Netherlands

- 2014, The International Liver Transpaltation Society (ILTS) annual congress

Academic Awarde

- Young Investigator Bursary form the 51th Annual Meeting of the European Association for the Study of the Liver (EASL), Barcelona, Spain

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Publications

1. Chen K, Cao W, Li J, et al. Differential Sensitivities of Fast- and Slow-cycling Cancer Cells to Inosine Monophosphate Dehydrogenase 2 Inhibition by Mycophenolic Acid. Mol Med 2015.
2. Hernanda PY, Chen K, Das AM, et al. SMAD4 exerts a tumor-promoting role in hepatocellular carcinoma. Oncogene 2015;34:5055-68.
3. Chen K, Man K, Metselaar HJ, et al. Rationale of personalized immunosuppressive medication for hepatocellular carcinoma patients after liver transplantation. Liver Transpl 2014;20:261-9.
4. Chen K, Pan Q, Gao Y, et al. DMS triggers apoptosis associated with the inhibition of SPHK1/NF-kappaB activation and increase in intracellular Ca2+ concentration in human cancer cells. Int J Mol Med 2014;33:17-24.
5. Chen K, Yang X, Wu L, et al. Pinellia pedatisecta agglutinin targets drug resistant K562/ADR leukemia cells through binding with sarcolemmal membrane associated protein and enhancing macrophage phagocytosis. PLoS One 2013;8:e74363.
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7. Gao Y, Chen K, Ma JL, et al. Cerium oxide nanoparticles in cancer. Onco Targets Ther 2014;7:835-40.