Aus der Medizinischen Universitätsklinik Heidelberg

Pathologisches Institut

Ärztlicher Direktor: Prof. Dr. med. Peter Schirmacher

FUNCTIONAL ANALYSIS OF PROTO-ONCOGENE c-KIT IN THE DEVELOPMENT AND PROGRESSION OF HEPATOCELLULAR CARCINOMA

Inauguraldissertation

zur Erlangung des Doctor scientiarum humanarum (Dr. sc. hum.)

an der

Medizinischen Fakultät Heidelberg

der

Ruprecht-Karls-Universität Heidelberg

vorgelegt von

Qiangnu Zhang

aus

GanSu, VR. China

2018

Dekan: Prof. Dr. med. Andreas Draguhn

Doktorvater: PD Dr. rer. nat. Kai Breuhahn

TABLE OF CONTENTS

ABBREVIATIONS

ACC	Adenoid cystic carcinoma
ATCC	America Type Culture Collection
BCLC	Barcelona clinic liver cancer (BCLC) staging
bp	Base pair
BSA	Bovine serum albumin
c-DNA	complementary DNA
CLIP	Cancer of the Liver Italian Program
DKFZ	Deutsches Krebsforschungszentrum
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FCS	Fetal calf serum
GEO	Gene Expression Omnibus
GIST	Gastrointestinal stromal tumor
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HGF	Hepatocyte growth factor
HIPO	The Heidelberg Center for Personalized Oncology
HSCH	Human stem cell factor
IGF	Insulin-Like growth factor
JCRB	Japanese cancer research resources bank
MEM	Modified Eagle's Medium
NASH	Non-alcoholic steatohepatitis
PAGE	Polyacrylate gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PEI	Polyethylenimine
SCF	Stem cell factor
SDS	Sodium dodecyl sulfonate
SIRT	Selective internal radiation therapy
TACE	Transhepatic arterial chemotherapy and embolization
TBST	Tris buffered saline tween
TCGA	The Cancer Genome Atlas
VEGF	Vascular endothelial growth factor

TABLE-INDEX

FIGURE-INDEX

1. INTRODUCTION

1.1 Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) represents about 75% of all liver cancer cases according to the data of American Cancer Society in 2015 (Moukhadder *et al.*, 2017). It is the most common type of primary liver cancer and the second leading cause of cancer-related death worldwide. The morbidity and mortality of HCC remain high and are showing a trend of global increase (Weinmann *et al.*, 2014). HCC can be induced by multiple risk factors and is influenced by many etiologies, hence HCC has a complex pathogenesis (Petrick *et al.*, 2018). Despite recent improvements in diagnostic methods, surgical techniques, chemoradiotherapy, radiotherapy, and targeted agents the long-term survival rate for advanced HCC remains dismal (Li *et al.*, 2014).

1.1.1 Epidemiology and risk factors of hepatocellular carcinoma

According to the statistical data from the World Health Organization, 0.8 million new cases of HCC were diagnosed for the year 2012 (Bertuccio *et al.*, 2017). The global epidemiological distribution varies by geographic location due to factors of the underlying disease. More than 80% new cases of HCC occurred in developing regions such as sub-Saharan Africa and eastern Asia, with typical incidence rates of more than 20/100,000 individuals (Mittal and El-Serag, 2013). Especially, China comprises more than half of new cases recorded with over 55% of diagnosed patients (Lu *et al.*, 2014). Southern European countries have mid-incidence levels (10.0-20.0/100,000 individuals), whereas, Northern Europe, North America, and Oceania have a relatively low incidence of HCC (Gan *et al.*, 2018). However, the incidence of HCC tends to increase in developed and developing countries. For instance, the incidence rate of HCC has increased from 1.4/100,000 cases/year to 6.2/100,000 cases during 1976–1980 in the USA as reported in 2011 by a USA surveillance program (Abbas *et al.*, 2014). Control of HBV and hepatitis HCV infections,

optimized management of cirrhosis, HCC diagnosis and treatment have contributed to the decrease in HCC-related mortality, e.g., Eastern Asia (Yeo *et al.*, 2010). However, in some developed regions, e.g. USA, due to alcohol consumption, increased obesity, and diabetes HCC mortality increased (Kew, 2000; Pradat *et al.*, 2018).

The most common risk factors for HCC development are chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections. An estimated 80% of all HCC cases are due to chronic infection with hepatitis B (HBV) or hepatitis C (HCV) viruses (El-Serag, 2012; Rongrui *et al.*, 2014). Chronic HBV and HCV infections are mainly responsible for a significant proportion of HCC cases by promoting cirrhosis. Approximately 5% of the world population (350~400 million people) is chronically infected with HBV (Samji *et al.*, 2017). Most of these cases are in developing countries, such as Asia (except Japan) and Africa. Prospective cohort studies showed a 5- to 100-fold increase in the risk of developing HCC among persons chronically infected with HBV (Brechot *et al.*, 2000). Instead of HBV, HCV is regarded as the major risk factor for HCC in the United States and Europe (Beguelin *et al.*, 2017).

Other major risk factors include alcoholic liver disease, non-alcoholic steatohepatitis (NASH), consumption of aflatoxin-contaminated foods, and exposure to chemical carcinogens (Sanyal *et al.*, 2010). Heavy alcohol use, 40 to 60 grams of alcohol daily, significantly increases the risk for HCC and has been reported to have synergistic effects with other risk factors (Younossi *et al.*, 2016). There is more recent recognition that the metabolic syndrome and its components such as diabetes and obesity also increase the risk for HCC. These patients have a twice higher chance of developing HCC compared to those who are not obese and do not have diabetes (Estes *et al.*, 2018; Polesel *et al.*, 2009).

1.1.2 Molecular pathogenesis of hepatocellular carcinoma

HCC is a heterogeneous malignancy resulting from diverse risk factors mentioned above. The pathogenesis of HCC is a multistep process involving the progressive accumulation of complex genetic and epigenetic alternations and cellular events (**Figure 1.1**). Recent evidence suggests that cancer stem cells are also involved in hepatocarcinogenesis but this hypothesis is not yet supported by consistent data (Liu *et al.*, 2011; Yao and Mishra, 2009).

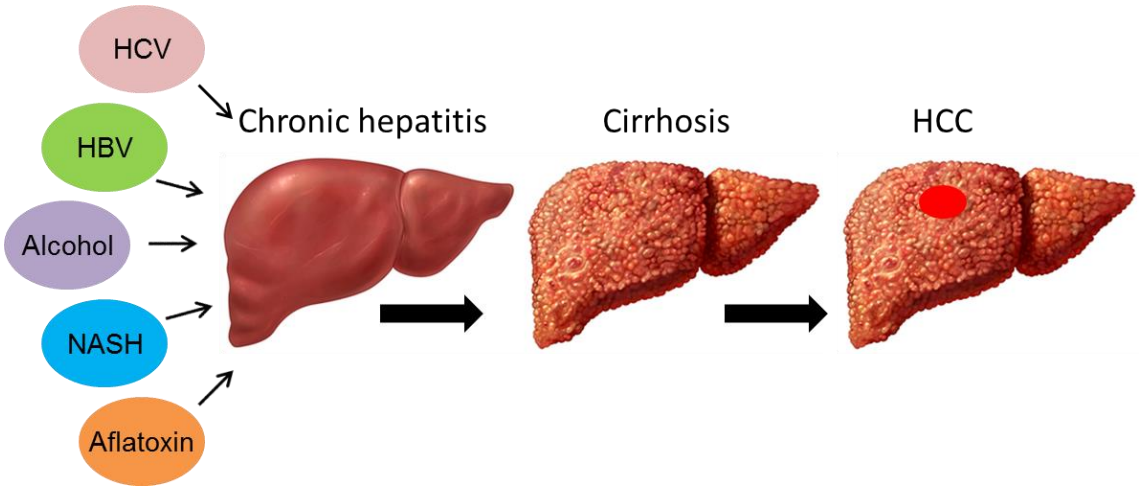


Figure 1.1: Molecular pathogenesis of hepatocellular carcinoma (HCC). Chronic exposure to a hepatitis virus, alcohol abuse or non-alcoholic steatohepatitis leads repetitious hepatocyte damage and triggers a vicious cycle of cell death and regeneration which caused cirrhosis and subsequently genomic instability including gene rearrangements, somatic mutations, copy number alterations, epigenetic changes, and growth factor pathway alterations. Progressive accumulation of multiple genetic events eventually leads HCC and metastases. Adapted from Dhanasekaran and Bandoh 2016 (Dhanasekaran *et al.*, 2016)

1.1.2.1 Genetic and epigenetic changes

With advances of the sequencing technique, more and more genomic landscapes of HCC have been revealed. Major groups of genes altered during the pathogenesis of HCC include DNA damage response, cell cycle control, apoptosis, cell-cell interaction and signal transduction-related genes. Common

genetic changes include gene mutations, copy number variations, and gene rearrangements (Dhanasekaran *et al.*, 2016; Sato and Mori, 2011). TP53 mutations are a classic example for genetic changes in HCC which are most prevalent in HBV- or aflatoxin-related HCC. The frequency of TP53 gene mutation in HCC can reach up to 50% in these etiologies (Puisieux and Ozturk, 1997). TP53 inactivating mutations lead to loss of cell cycle control and affected cells are unable to undergo apoptosis which leads to accumulation of additional mutations leading to disease progression. Thus, TP53 mutation is common in some etiologies and may induce more aggressive development of HCC (Liao *et al.*, 2017). Other common somatic mutations involve the Wnt/β-catenin pathway such as CTNNB1, AXIN1, and AXIN2 (Tang *et al.*, 2004). Interestingly, also mutations in non-coding areas of a gene, such as the promoter of TERT which occurs about 60% of HCC, may lead to altered gene expression, thereby promoting HCC development (Sullivan *et al.*, 2018). Apart from mutations, copy number variations may induce the expression of oncogenes or reduce the expression of tumor suppressors. For instance, a recent study of 125 HCC cases reported that 32% of patients had focal amplifications and 40% of patients had focal deletions (Schulze *et al.*, 2016). Another form of somatic variation contributing to HCC are chromosomal rearrangements that cause gene fusions or gene activation through integration of HBV into the host genome (Herath *et al.*, 2006).

Epigenetic modifications, including DNA methylation, histone modification, and chromatin remodeling are active research topics at the moment (Nakamura *et al.*, 2018). In addition, epigenetic modifications by non-coding RNAs such as microRNA, long non-coding RNAs, and circular RNAs also play a pivotal role in hepatocarcinogenesis through regulating their target genes by diverse mechanisms (Negrini *et al.*, 2011; Peng *et al.*, 2018).

1.1.2.1 Key signaling pathways in hepatocellular carcinoma

Signaling pathways are not only utilized to explain the molecular pathogenesis of HCC but may potentially to be a source of targets for novel therapies. Alterations in various signaling pathways are involved in cell proliferation, angiogenesis, invasion, and metastasis of HCC (Minguez *et al.*, 2009). Several pathways have been studied in depth and observed to be dysregulated in HCC. The most frequently reported pathways are (1) pathways governing growth factors signaling, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF) and hepatocyte growth factor (HGF) pathways (Furuse, 2008; Zender *et al.*, 2010); (2) pathways associated with angiogenesis such as the vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) pathways (Finn and Zhu, 2009; Morse *et al.*, 2018). (3) Pathways correlated to cell differentiation such as the WNT/β-catenin, Hedgehog, and Notch pathways (Pez *et al.*, 2013; Takigawa and Brown, 2008). Among others, receptor tyrosine kinases play a major role leading to signal transduction by phosphorylation cascades. Aberrant activation of receptor tyrosine kinases, including EGFR, HGFR/c-MET, and VEGFR, could be frequently observed and plays a key role in the pathogenesis of HCC (Fan *et al.*, 2017; Huynh *et al.*, 2011). For example, EGFR mutations play a significant role in HCC initiation, hyperactive HGFR/c-MET was found in 40% of HCC patients and is associated with vascular invasion and poor prognosis; over-expression of VEGFR has been shown in HCC and results in poor prognosis by inducing angiogenesis (Giordano and Columbano, 2014).

The main downstream pathways of receptor tyrosine kinases are Ras-mitogen-activated protein kinase (RAS/MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT kinase signaling pathways (Li *et al.*, 2018). In approximately half of all HCC cases, RAS/MAPK and PI3K/AKT kinase signaling pathways are induced by receptor tyrosine kinases. RAS/MAPK

pathway activation could induce downstream pathway activation, such as proto-oncogene cFos and transcription factor AP-1/c-Jun, which may promote transcription of genes that drive HCC cell proliferation (Song, 2009). PI3K/AKT kinase activation, in turn, activates mammalian target of rapamycin (mTOR) pathway which occurs in about 40% of HCC patients (Wang *et al.*, 2018).

1.1.3 Therapy options

Multiple treatment options available for HCC depend on the tumor stage, patient performance status and liver function reserve. Conventional treatment includes curative resection, liver transplantation, radiofrequency ablation (RFA), trans-arterial chemoembolization (TACE), radioembolization and systemic targeting therapy (Kudo, 2018; Kuyvenhoven *et al.*, 2001). However, only resection and liver transplantation are potentially curative. Liver transplantation (LT) is considered the best therapeutic option and potentially curative treatment for HCC, Unfortunately, only patients within the Milan criteria for HCC (one lesion<5 cm or up to 3 lesions with each ≤3 cm) are suitable candidates for LT (Costentin *et al.*, 2018). Patients with single nodules, good liver function, and no underlying cirrhosis, are considered the ideal candidates for surgical resection. In the last decade, satisfactory results have been obtained with RFA, which is recommended as a standard of care for the patients who are not suitable for surgery but with early-stage tumors (e.g., BCLC 0-A). For patients with intermediate-stage HCC (e.g., Child-Pugh class A or B), TACE is currently recommended as a standard of care (Tang, 2001). Excepting of TACE, radioembolization or selective internal radiation therapy (SIRT) has recently emerged as a therapeutic option for these intermediate-stage HCC patients (Harris *et al.*, 2018; Llovet *et al.*, 2018). However, despite surveillance programs for high-risk patients, most patients are diagnosed with advanced stage disease. Systemic targeted therapies, which focus on the critical steps of the carcinogenic pathways and limit widespread systemic toxicity, bring hope to patients with advanced cancer

(Keane *et al.*, 2018). However, compared with other tumor entities, the development of systemic targeted therapies in HCC is lagging behind.

For patients with advanced disease, sorafenib is the only approved therapy. But sorafenib has multiple side effects and the overall survival benefit is only 2-3 months (Mendez-Blanco *et al.*, 2018). Although several novel targeted therapies are currently under evaluation in different clinical trials, current first-line treatment studies have not achieved positive results , e.g., brivanib, linifanib, dovitinib (Fujiwara *et al.*, 2018). Reasons for the lack of effective systemic therapies are the high heterogeneity between HCC patient subgroups and that the molecular mechanisms underlying HCC pathogenesis remain poorly understood, hence identification of novel oncogenic targets in HCC has become a research priority, which is also a motivation for the present thesis work.

1.2 c-KIT and carcinoma

The proto-oncogene c-KIT, also known as stem cell growth factor receptor or tyrosine-protein kinase Kit or CD117, is a receptor tyrosine kinase that is encoded by the KIT gene (located on 4q12). After binding of its ligand, stem cell growth factor (SCF), c-KIT activates various downstream signal transduction pathways (Lennartsson and Ronnstrand, 2012). c-KIT expression is widely distributed in hematopoietic cells and other tissues, e.g., germ cells, mast cells, melanoma cells, and Cajal cells of the gastrointestinal tract(Yoshida *et al.*, 2001). As evident by its diverse distribution, c-KIT plays an important role in several physiological processes such as erythropoiesis, hematopoiesis, lymphopoiesis, pigmentation, and fertility(Klump *et al.*, 2018). In addition, c-KIT is deregulated in several diseases, especially in carcinoma. For example, excessive c-KIT signaling caused by mutation of epigenetic regulation results in leukemia, and tumors of the gastrointestinal tract and germ cells (Stankov *et al.*, 2014). Depending on the mechanism of c-KIT is overactivation, c-KIT may

therapeutically targeted by kinase inhibitors such as imatinib or sorafenib (Abbaspour Babaei *et al.*, 2016). However, little is known about the funtion of c-KIT in HCC and to expand clinical exploitation, the c-KIT signal pathway needs to be studied in-depth.

1.2.1 c-KIT gene and protein structure

The KIT gene is located on chromosome segment 4q11 in humans and is comprised of 21 exons, spanning more than 34 kb of the genome (Heinrich *et al.*, 2002). The promoter of the KIT gene is located 139 bp upstream of the translation initiation codon and contains three functional AP-2 binding sites and the transcription factors microphthalmia-associated transcription factor (MITF), MYB and ITS2 serve as regulators of c-KIT expression by binding to the promoter of KIT(Blume-Jensen *et al.*, 1991).

c-KIT is a transmembrane protein. The extracellular domain is encoded by exons 2~9 of KIT gene and consist of five immunoglobulin-like (Ig-like) domains followed by a single spanning transmembrane region encoded by exon 10 of the KIT gene (Besmer *et al.*, 1986). The intracellular domain of c-KIT, comprises a juxtamembrane region, a kinase domain and a COOH-terminal tail (Ma *et al.*, 1999). The juxtamembrane region is important for c-KIT kinase activity regulation. The kinase domain is divided into two subdomains, tyrosine kinase domain 1 and 2, by an about 80 amino acids long kinase insert region (Qiu *et al.*, 1988). Alternative splicing of c-KIT mRNA results in the occurrence of at least four isoforms of c-KIT in humans. Two of these isoforms are characterized by the presence or absence of the four amino acids Gly-Asn-Asn-Lys (GNNK) just outside the plasma membrane. In addition, two isoforms are defined according to the presence or absence of a single serine residue in the kinase insert region of c-KIT (**Figure 1.2**) (Crosier *et al.*, 1993; Reith *et al.*, 1991).

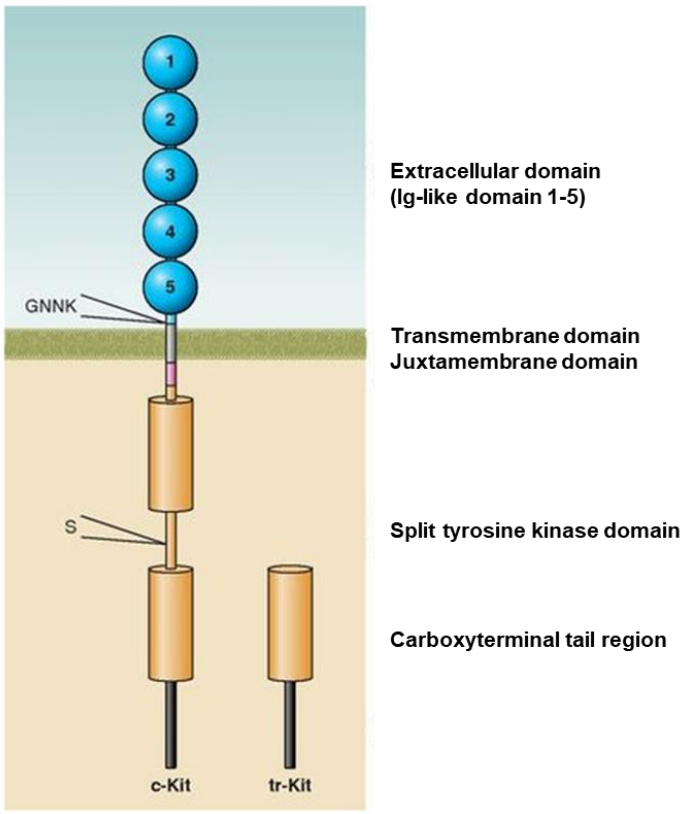


Figure 1.2: Schematic representation of the c-KIT protein structure. Five immunoglobulin-like (Ig-like) domains constitute the extracellular domain of c-KIT. After the transmembrane domain, intracellular domain starts with the juxtamembrane region followed by a kinase domain and ends with a COOH-terminal tail. Adapted from Lennartsson and Rönnstrand 2012 (Lennartsson and Ronnstrand, 2012)

1.2.2 Activation of c-KIT and its downstream pathways

Activation of c-KIT requires dimerization of monomeric receptor molecules which is provoked by ligand binding. The ligand of c-KIT is stem cell factor (SCF, also called Steel factor or Kit ligand) which exists both as a membrane-bound and soluble form (London *et al.*, 1996). The c-KIT activation process is summarized in **Figure 1.3 A**. In brief, the first three Ig-like domains of c-KIT have a complementary shape and are responsible for tight binding to SCF. After binding to each other, two additional Ig-like domains drive dimerization of two c-KIT monomers (Casteran *et al.*, 2003). Conformational changes of the c-KIT extracellular domains results in close proximity of the

transmembrane domains of two c-KIT receptors (Broudy *et al.*, 1998). As a consequence, the intracellular regions of c-KIT facilitate tyrosine kinase activation and consequent transphosphorylation in the juxtamembrane domain at Tyr568 and 570, in the tyrosine kinase domains at Tyr823 and 900, in the kinase insert region at Tyr703, 721, 730, and 747 and in the COOH-terminal tail at Tyr936 (Qiu *et al.*, 1988). The phosphorylation sites in c-KIT and their binding partners are shown in **Figure 1.3 B.**

To meet the biological needs of transient signaling activity, negative feedback regulation of c-KIT's activation is important. This generally includes intracellular degradation of c-KIT, inactivation of the kinase domain by phosphorylation of serine residues 821 and 959, and tyrosine dephosphorylation caused by phosphatases (Blume-Jensen *et al.*, 1995).

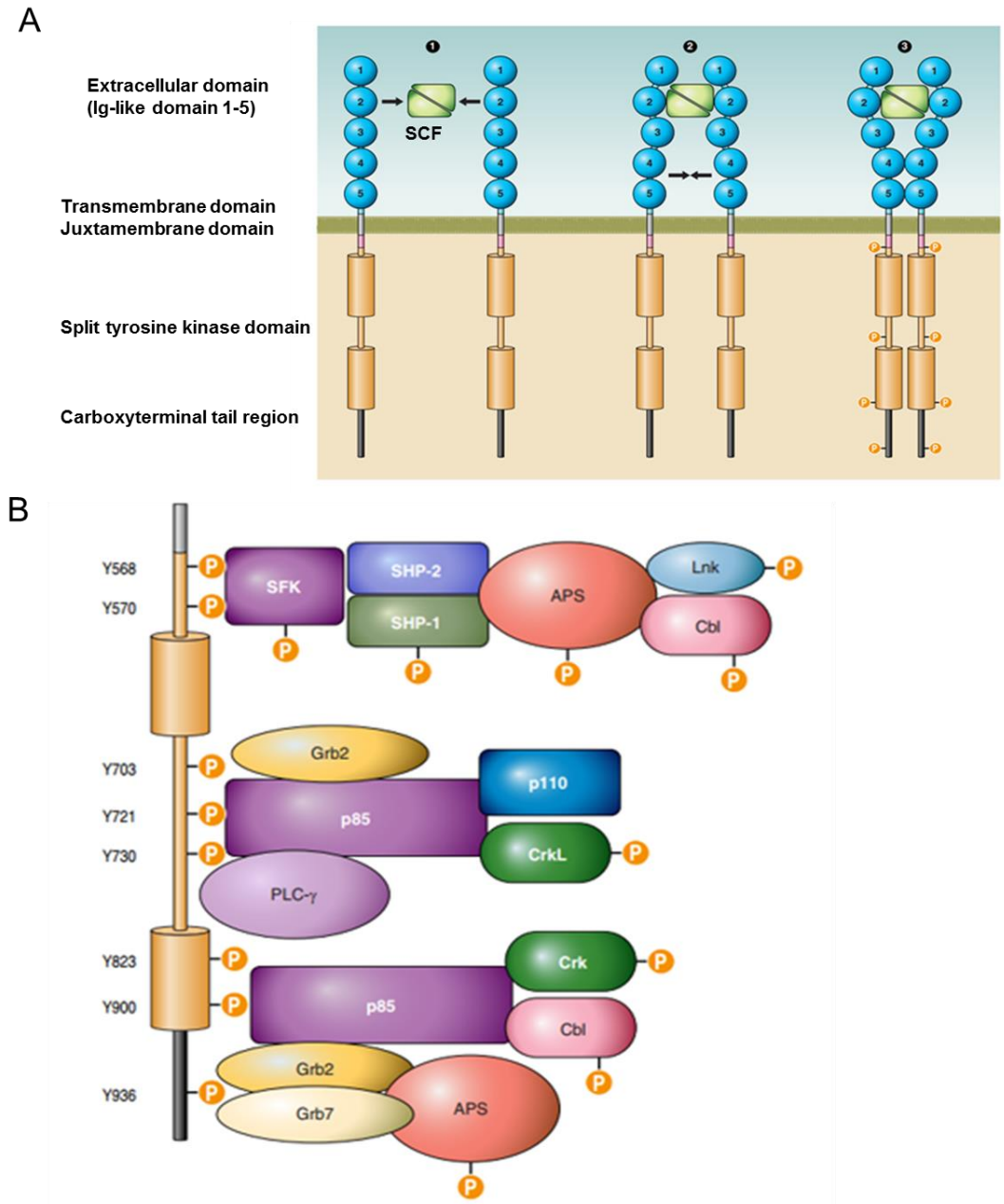


Figure 1.3: Schematic representation of SCF-induced c-KIT activation. (A) SCF binds to the Ig-like domains 1-3 of c-KIT, followed by binding of the Ig-like domains 4 and 5 which leads to dimerization of c-KIT. The dimerization brings the intracellular regions into proximity of each other, allowing for tyrosine kinase activation and transphosphorylation. (B) Depicted are the intracellular region of c-KIT and its phosphorylation sites following SCF stimulation. Adapted from Lennartsson and Rönnstrand 2012 (Lennartsson and Ronnstrand, 2012).

Several downstream signal transduction pathways of c-KIT have been studied in different cell lines. Reported pathways include phosphatidylinositol 3′-kinase/AKT (PI3K/AKT) Signaling, mitogen-activated protein kinase (MAPK) Signaling, JAK/STAT Signaling, Src Family Kinase Signaling and Phospholipases C and D Signaling (Kim *et al.*, 2016;Qiu *et al.*, 1988). They are integrated into a signaling circuit where one pathway may influence others. However, these pathways are cell type dependent and not all of the pathways described here are activated in different cell types. Rather only some of these downstream signaling routs are activated. But c-KIT downstream effectors have not yet been studied in all cell linages and a conclusive overview of the downstream effector pathways c-KIT is still missing (Herraiz *et al.*, 2011). Therefore, the downstream pathways mentioned above are described as separate pathways below and in this thesis special emphasis lays on the two well-established signaling routes, the PI3-kinase/ AKT and MAPK pathways, which were found to be regulated in HCC.

A. c-KIT and PI3K/AKT Signaling

After c-KIT activation, phosphatidylinositol 3′-kinase (PI3K) is activated through direct binding to phosphorylation site Tyr-721 of c-KIT and indirectly through binding to the tyrosine phosphorylated adaptor protein GAB2 (**Figure 1.3 B**). Activated PI3K is recruited to the membrane and induces downstream signals (Wandzioch *et al.*, 2004). Signal transduction molecules downstream of PI3K play a role in cell survival, migration, and angiogenesis. The serine/threonine kinase AKT is a key downstream element of PI3K signaling. AKT was initially discovered as a viral oncogene that promotes cell survival (Whittaker *et al.*, 2010). In response to c-KIT/SCF activation, AKT is activated in a PI3K -dependent manner and then induces phosphorylation of Bad, FOXO, and activation of nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) (Liu *et al.*, 2010). The later regulates the initiation of apoptosis which

could explain how AKT regulates cell survival (An *et al.*, 2017). One of the downstream pathways of AKT is the element serine/threonine kinase mammalian target of rapamycin (mTOR) pathway. The mTOR complex 1 (mTORC1) is located downstream of AKT and c-KIT/SCF has been reported to induce proliferation by activating mTOR through PI3K/AKT in mesenchymal stem cells cells (Lee *et al.*, 2013).

B. c-KIT and MAPK Signaling

MAPK signaling is a downstream effector of multiple cell surface receptors including the activated c-KIT/SCF pathway. MAPK plays pivotal roles in a multitude of biological processes such as cell proliferation and migration, both under normal and pathological conditions including carcinoma (Orouji *et al.*, 2016). In the pathogenesis of carcinoma, three major groups of MAPKs are involved: ERK1 and 2 (ERK1/2), p38, and JNK. Consistently stimulation of c-KIT has been shown to activate ERK1/2, p38, and JNK (Zhang *et al.*, 2016). Among them, ERK1/2 has been studied in more detail: The mechanism of ERK1/2 activation downstream of c-KIT appears to be cell-type specific. For example, in mature mast cells, c-KIT induces ERK1/2 activation by RAS/Raf/Mek1/2 with the assist of additional proteins, e.g., SHC, SOS, and SHP2(McDaniel *et al.*, 2008). However, in primitive hematopoietic cells, it depends on PI3K (Herraiz *et al.*, 2011). On the other hand, the activation of Src family kinase by c-KIT at Tyr570 and Tyr 568 is important for ERK1/2 activation but not in all cell types. Many of the proteins downstream of ERK1/2 are transcription factors or cytoplasmic substrates (Ueda *et al.*, 2002). p38 has also been demonstrated to be activated in response to SCF stimulation. c-KIT/SCF induced p38 activation depends on Src family kinase members Fyn, PTPα and kinase PAK1 (Samayawardhena *et al.*, 2006). Besides, studies suggest that the JNK pathway is activated by c-KIT activity. SFK-dependent phosphorylation of GAB2 induced by c-KIT could recruit SHP2 which is critical

for the SCF-induced JNK activation. Moreover, adaptor protein Lnk serves as a negative regulator for c-KIT-mediated activation of ERK1/2, p38, and JNK (Chen *et al.*, 2013).

1.2.3 The role of c-KIT in carcinogenesis

Tumor progression is strongly associated with the activity of receptor tyrosine kinases and their intracellular signal transduction pathways. Hence as a receptor tyrosine kinase, c-KIT signaling has implicated in the pathogenesis of carcinogenesis and target-based approaches for cancer treatment (Abrams *et al.*, 2018). Progression of various human tumors including gastrointestinal stromal tumors (GISTs), mast cell tumors, melanoma, small cell lung carcinoma, testicular carcinoma, acute myeloid leukemia, and colorectal cancer are strongly associated with the activity of c-KIT and its intracellular signal transduction pathways (Abbaspour Babaei *et al.*, 2016; Choudhary *et al.*, 2016; Lebron *et al.*, 2014). In these tumor entities, different types of deregulation of c-KIT such as gain or loss of function, overexpression, and point mutations may occur (Longley *et al.*, 2001). Most publications emphasize mutations of c-KIT, especially oncogenic mutations. Oncogenic mutations in the c-KIT gene are commonly observed in more than 90% of cases of GISTs and more than 70% of patients with mast cell tumor (Lin *et al.*, 2006). These oncogenic mutations of c-KIT protein can induce downstream phosphorylation cascades in an SCF-independent manner, or excessive activation, quantitatively and also qualitatively, after SCF-binding (Chen *et al.*, 2013). Thus, the downstream pathways which support proliferation, survival, apoptosis, migration, and adhesion are continuously activated leading to tumor development. Some of the mutations may cause resistance to kinase inhibitors hence resulting in poor patient outcome (Papaspyrou *et al.*, 2011). The reported mutations of c-KIT were summarized in **Figure 1.4**.

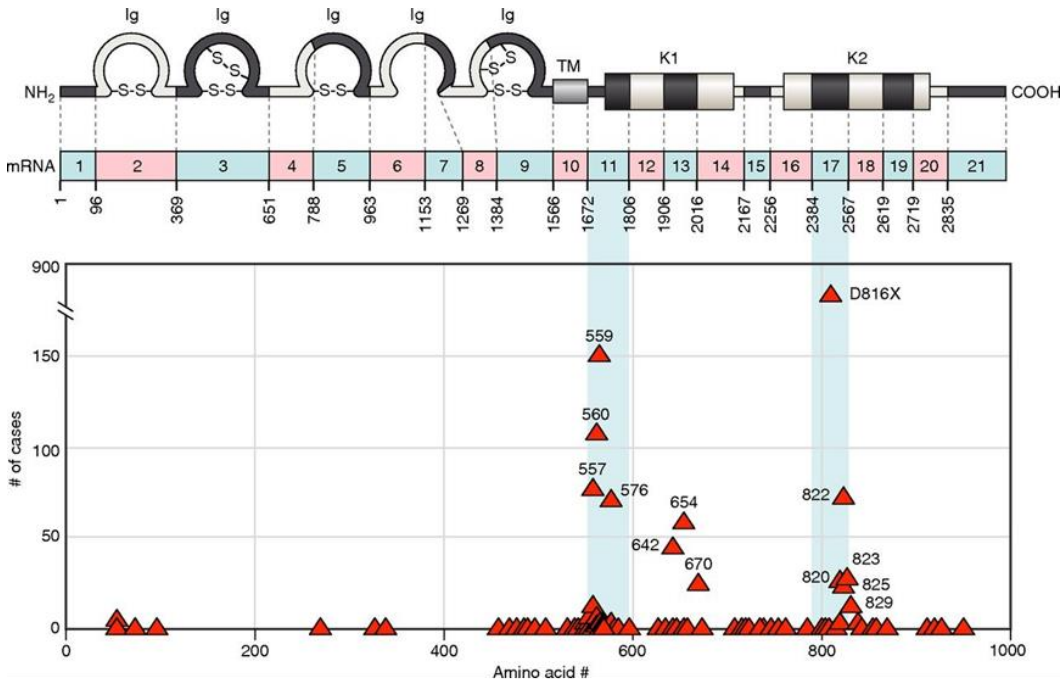


Figure 1.4: The relative abundance and distribution of various reported c-KIT mutations in human malignancies are depicted. Exons 11 and 17 are mutation hotspots. D816X mutations are the most common mutations. Adapted from Lennartsson and Rönnstrand 2012 (Lennartsson and Ronnstrand, 2012)

As can be noted, most common mutations cluster in exon 11 and exon 17. A large proportion of oncogenic c-KIT mutations are in the juxtamembrane region, e.g., V560G or within the kinase domain, e.g., D816X (Maier *et al.*, 2013). However, the exact mechanism behind constitutive activation is not very clear and still needs more investigation. Some of the reported mechanisms are as follows: Mutations within the kinase domains results in a structural change that impedes autoinhibition, e.g., D816V or increase the affinity for ATP and hyper-activation, e.g., D816Y (Agarwal *et al.*, 2015; Beghini *et al.*, 1998). Mutations in the juxtamembrane region could potentially promote dimerization in the absence of SCF, e.g., V560G (Jung *et al.*, 2011; Kitayama *et al.*, 1995).

Interestingly, instead of gain-of-function mutations, in certain tumors such as melanoma, thyroid carcinoma and breast cancer loss-of-function mutations of c-KIT were observed. In fact, in metastatic melanoma gain-of-function could induce apoptosis (Franceschi *et al.*, 2017;Vita *et al.*, 2014).

Besides c-KIT mutation, the role of c-KIT protein level changes also has been demonstrated. In several tumors, c-KIT overexpression could be detected without any mutation. For instance, the concurrent overexpression of c-KIT and SCF occur in neuroblastoma, malignant mesothelioma, colorectal carcinoma, breast carcinoma, and small-cell lung cancer (Topcagic *et al.*, 2018). In these cases, upregulated expression of c-KIT could promote cancer proliferation and induce resistance to chemotherapy (Abbaspour Babaei *et al.*, 2016).

1.2.4 c-KIT-targeted therapies in cancer

The fact that activating mutations or overexpression of c-KIT are found in various types of human cancers has suggests that c-KIT may be a potential target for cancer therapy (Demetri, 2001). Imatinib was the first tyrosine kinase inhibitor to be used in treating chronic myelogenous leukemia. Later studies found that Imatinib can also inhibit c-KIT and benefits patients with GIST (Pandey and Kochar, 2012). However, in GIST imatinib is effective for wild-type c-KIT and some of the exon11 mutants but ineffective in patients with for several other c-KIT mutants, e.g., D816V. Because this mutation causes a continuous active conformation of c-KIT, however, Imatinib can only inhibit c-KIT in its inactive conformation (Wada *et al.*, 2016). To overcome this issue, novel drugs targeting the kinase activity of c-KIT have been developed. For example, dasatinib and nilotinib, as second-generation dual-specificity Src/Abl inhibitors, could target mutant c-KIT D816V in its active conformation (Tobio *et al.*, 2015). Other relevant targeted drugs include sorafenib, sunitinib, and axitinib. More details about c-KIT targeted drugs can be found in Abbaspour

Babaei's reports (Abbaspour Babaei *et al.*, 2016). On the other hand, c-KIT is also expressed in normal tissues and has crucial functions, thus, inhibition of wildtype c-KIT may cause side effects. To target the specific oncogenic mutants of c-KIT, a deeper understanding of the c-KIT signaling machinery is a prerequisite (Grillo *et al.*, 2018).

1.2.5 c-KIT in hepatocellular carcinoma

So far few studies have reported expression changes of c-KIT in HCC patients. However, the functional and mechanistic details of c-KIT in HCC are still unclear. Chung et al. reported that 25.6% cases of HCC tissues expressed c-KIT protein and indicated c-KIT expression might be a good prognostic indicator for HCC (Chung *et al.*, 2005). Mansuroglu et al. found that c-KIT expression was detected in 70% of HCC tissues by immunohistochemistry. They considered the role of c-KIT/SCF pathway in the regulation of the proliferative activity of tumorous hepatic cells. But further confirmation was not performed *in vivo* or *vitro* (Mansuroglu et al., 2009). In contrast, Becker et al., only observed a 2.3% c-KIT-positive rate in HCC patients, they suggested that c-KIT may not have a significant role in HCC, but they also discussed that this low c-KIT-positive rate may result from the quality if antibody (Becker *et al.*, 2007).

In addition, c-KIT has been recognized as marker of hepatic progenitor cells/stem cells, such as Oval cells. Several studies showed that c-KIT is involved in tumorigenic progenitor cells or tumor stem cells in HCC (Wang *et al.*, 2014). The occurrence of c-KIT-positive tumor cells and its potential were reported by some publications but the mechanism of c-KIT function in normal and tumor stem cells has not been elucidated. For instance, Lee et al. found c-KIT-positive tumor cells in tumor tissue of 80% of HCCs. Moreover, they indicated c-KIT-positive tumor cells might be associated with the progression of HBV–associated HCC as progenitor cells (Lee *et al.*, 2005). Belinda et al.,

found c-KIT inhibition by imatinib attenuates progenitor cell proliferation and inhibits HCC formation in mice (Knight *et al.*, 2008).

Andres's group described the interactions between TGF-β and c-KIT signaling. They demonstrated that a positive TGF-β/c-KIT feedback loop drives tumor progression in HCC. In short, aberrant activation of the c-KIT/STAT3 signaling axis causes constitutively activated TGF-β/SMAD2-signaling which results in worse outcome (Rojas *et al.*, 2016). In order to decipher the role of c-KIT in the pathogenesis of HCC and estimate the clinical potential of c-KIT targeted treatment, better understanding the functional role of c-KIT and its underlining mechanisms in HCC need to be obtained in further studies.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell line

Table 2.1: Information of cell lines used

Cell line	Origin	Supplier	Ordering number	Cultivation medium
HUH7	Well-differentiated HCC	JCRB	JCRB0403	DMEM
HEP3B	HCC	ATCC	ATCC HB-8064	MEM
HLE	Undifferentiated HCC	JCRB	JCRB0404	DMEM
SNU-182	HCC, grade III/IV	ATCC	ATCC CRL-2235	RPMI-1640
HLF	Undifferentiated HCC	JCRB	JCRB0405	DMEM
HEK293T	Human embryonic kidney cells expressing SV40 large T antigen	ATCC	ATCC CRL-3216	DMEM

2.1.2 Plasmids

Table 2.2: Information of plasmid used

Plasmid	Supplier	Ordering number
pCMV-c-KIT/WT	Angelika Fraas, Manuel Grez	
pCMV-c-KIT/C674F	Angelika Fraas, Manuel Grez	
pCMV-c-KIT/W262C	Angelika Fraas, Manuel Grez	
pDONR-201	ThermoFisher, Dr. Stefan Pusch (DKFZ)	
pTRIPZ	ThermoFisher, Dr. Stefan Pusch (DKFZ)	
psPAX2	Dr. Didier Trono	Addgene #12260
pMD2.G	Dr. Didier Trono	Addgene #12259

2.1.3 Bacteria

For molecular cloning, competent MACH1mE. coli (High Efficiency) were prepared by AG Roessler, Institute of Pathology, Heidelberg, Germany.

2.1.4 Enzymes

Table 2.3 Information of enzymes used

Enzymes	Supplier	Ordering number
FastDigest Bsp1407I	Thermo Fisher Scientific Inc., Waltham, MA, USA	FD0933
Gateway BP Clonase II Enzyme mix	Thermo Fisher Scientific Inc., Waltham, MA, USA	11789-020
Gateway LR Clonase II Enzyme mix	Thermo Fisher Scientific Inc., Waltham, MA, USA	11791-020
RevertAid H Minus Reverse Transcriptase	Thermo Fisher Scientific Inc., Waltham, MA, USA	EP0452

2.1.5 Antibodies

Table 2.4: Information of antibodies used

Antibodies	Species	Supplier	Ordering number	Dilution
Primary antibodies				
c-KIT antibody (E1)	Mouse	Santa Cruz Biotechnology Inc., Dallas, Texas, USA	sc-17806	1:200
p-c-KIT antibody (Tyr719)	Rabbit	Cell Signaling Technology, Inc., Boston, MA,USA	3391S	1:1000
AKT antibody	Rabbit	Cell Signaling Technology, Inc., Boston, MA,USA	9272S	1:1000
p-AKT antibody(Ser473)	Rabbit	Cell Signaling Technology, Inc., Boston, MA,USA	4060s	1:1000
STAT1 antibody	Rabbit	Cell Signaling Technology, Inc., Boston, MA,USA	9172S	1:1000
p-STAT1 antibody (Tyr701)	Rabbit	Cell Signaling Technology, Inc., Boston, MA,USA	7649S	1:1000
STAT3 antibody (124H6)	Mouse	Cell Signaling Technology, Inc., Boston, MA,USA	9139S	1:1000
p-STAT3 antibody (Tyr705)	Rabbit	Cell Signaling Technology, Inc., Boston, MA,USA	9145S	1:1000
JAK2 antibody (D2E12)	Rabbit	Cell Signaling Technology, Inc., Boston, MA,USA	3230S	1:1000
p-JAK2 antibody (Tyr1007/1008)	Rabbit	Cell Signaling Technology, Inc., Boston, MA,USA	3776S	1:1000
ERK1/2 antibody	Rabbit	Cell Signaling Technology, Inc., Boston,	9102S	1:1000

		MA,USA		
p-ERK1/2 antibody (Y204/Y187)	Mouse	Cell Signaling Technology, Inc., Boston, MA,USA	5726S	1:1000
JNK JNK antibody (FL)	Rabbit	Santa Cruz Biotechnology Inc., Dallas, Texas, USA	sc-571	1:100
p-JNK antibody (Thr183/Tyr185)	Rabbit	Cell Signaling Technology, Inc., Boston, MA,USA	9251S	1:1000
GAPDH	Chicken	Merck Chemicals GmbH, Darmstadt, Germany	AB2302	1:10000
Secondary antibodies				
IRDye 680LT anti-mouse IgG	Donkey	LI-COR Biosciences GmbH, Bad Homburg, Germany	926-68022	1:20 000
IRDye 800CW anti-mouse IgG	Donkey	LI-COR Biosciences GmbH, Bad Homburg, Germany	926-32212	1:20 000
IRDye 800CW anti-rabbit IgG	Donkey	LI-COR Biosciences GmbH, Bad Homburg, Germany	926-32213	1:20 000
IRDye 800CW anti-goat IgG	Donkey	LI-COR Biosciences GmbH, Bad Homburg, Germany	926-32214	1:20 000

2.1.6 Chemicals and kits

Table 2.5: Information of kits used

Chemical and kits	**Supplier**	**Ordering number**
Nucleo Spin® Gel and PCR Clean-up Kit	MACHEREY-NAGEL, Düren, Germany	740609.250
Nucleeo Spin® Plasmid Kit	MACHEREY-NAGEL, Düren, Germany	740588.250
PurreYield™ Plasmid Midprep	Promega GmbH, Mannheim, Germany	A2492
Extractme® Total RNA Kit	DNA-GDAŃSK, Gdańsk, Poland	EM09.1-250
CellTiter-Blue Cell Viability Assay kit	Promega GmbH, Mannheim, Germany	G8080
Lipofectamine RNAiMAX Transfection Reagent	Thermo Fisher Scientific Inc., Waltham, MA, USA	13778150
Lipofectamine 2000 Transfection Reagent	Thermo Fisher Scientific Inc., Waltham, MA, USA	11668019
1 Kb Plus DNA Ladder	Thermo Fisher Scientific Inc., Waltham, MA, USA	10787018
Agarose	Carl Roth GmbH &Co. KG, Karlsruhe, Germany	2267.4

Poly-L-lysine hydrobromide (PLL)	Sigma-Aldrich GmbH, Taufkirchen, Germany	P6282
Albumin Bovine, DNase/RNase-Free (BSA)	SERVA Electrophoresis GmbH, Heidelberg, Germany	11967.09
Kanamycin	Sigma-Aldrich GmbH, Taufkirchen, Germany	60615
Carbenicillin	Carl Roth GmbH, Karlsruhe, Germany	6344.2
Puromycin	Thermo Fisher Scientific Inc., Waltham, MA, USA	A1113803
Ammonium Persulfate (APS)	Bio-Rad Laboratories GmbH, Munich, Germany	1610700
TEMED	Carl Roth GmbH &Co. KG, Karlsruhe, Germany	2367.3
Bradford Reagent	Sigma-Aldrich GmbH, Taufkirchen, Germany	B6916
Milk powder	Carl Roth GmbH &Co. KG, Karlsruhe, Germany	T145.2
PhosSTOP	Roche Diagnostics GmbH, Mannheim, Germany	4906845001
PageRuler Prestained Protein Ladder, 10 to 180 kDa	Thermo Fisher Scientific Inc., Waltham, MA, USA	26616
Tween 20	Carl Roth GmbH &Co. KG, Karlsruhe, Germany	9127.1
dNTP Mix (10 mM each)	Thermo Fisher Scientific Inc., Waltham, MA, USA	R0192
Random Hexamer Primer	Thermo Fisher Scientific Inc., Waltham, MA, USA	SO142
RiboLock RNase Inhibitor	Thermo Fisher Scientific Inc., Waltham, MA, USA	EO0382
Human stem factor	Thermo Fisher Scientific Inc., Waltham, MA, USA	RP-8631
Human hepatocyte growth factor	R&D Systems, Minneapolis, MN,USA	294-HG

2.1.7 Oligonucleotides

Table 2.6: Information of primers used

Primers	Sequences 5'-3'
primers for molecular cloning	
c-KIT-attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTccATGAGAG GCGCCAGAGGGGCCTGG
c-KIT-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTtTCAGACGT CGTCGTGCACCAGCAG
Sequencing primers	
CMV fwd	CGCAAATGGGCGGTAGGCGTG
M13 fwd (-20)	GTAAAACGACGGCCAGT
pDONR201-fwd	TCGCGTTAACGCTAGCATGGATCTC
c-KIT _seq_1207	ATTGCCTTCAACGTGTACG
c-KIT _seq_1945	AACCACATGAACATCGTG
c-KIT _seq_587	TGAGCGAGAAGTTCATCC
qRT-PCR primers	
c-KIT	Forward: GCACAATGGCACGGTTGAAT
	Reverse: GGTGTGGGGATGGATTTGCT
c-KIT (codon optimized)	Forward:GCTGATCGGCTTTGTGATCG
	Reverse:TGCCGTTGATCTCTTCACC
Snail	Forward:CTTCTCTAGGCCCTGGC
	Reverse:GACAGGAGAAGGGCTCTCG
Twist	Forward:GACAAGCTGAGCAAGATTCAGACC
	Reverse:CTGGAGGACCTGGTAGAGGAAG
Vimentin	Forward: GAAAGTGTGGCTGCCAAGAACC
	Reverse: CAGCCTCAGAGAGGTCAGCAA
E-cadherin	Forward: CTTTGACGCCGAGAGCTACA
	Reverse: TCGACCGGTGCAATCTTCAA
N-cadherin	Forward: TGGCAGCTGGACTTGATCGAG
	Reverse: GACATCTGTCACTGTGATGACGG
ZEB1	Forward:GATCCAGCCAAATGGAAATCAG
	Reverse:CTGAGCTAGTATCTTGTCTTCATC
ZEB2	Forward: CTTCTGCGACATAAATACGAACAC
	Reverse:CGCTTTCTTACAAATCTGACAC
PLXDC1	Forward: CATCCAATGCTGCGCTCTTC
	Reverse: GTGGCTGCGAAACTTCATGG
KCNJ2	Forward: CACTTCCACTCCATGTCCCC
	Reverse: GGGAGCCTTGTGGTTCTACC
JAM3	Forward: GACAAGTGACCCCAGGATCG
	Reverse: ACCTCACAGCGATAAAGGGC
GJA1	Forward: TCTGAGTGCCTGAACTTGCC
	Reverse: CCCTCCAGCAGTTGAGTAGG

2.1.8 Medium and solutions

Table 2.7: Information of medium and solutions used

Medium and solutions	Supplier	Ordering number
DMEM (Dulbecco's Modified Eagle Medium	Sigma-Aldrich GmbH, Taufkirchen, Germany	D5796
MEM (Minimum Essential Medium)	Sigma-Aldrich GmbH, Taufkirchen, Germany	M4655
RPMI 1640 (Roswell Park Memorial Institute 1640)	Sigma-Aldrich GmbH, Taufkirchen, Germany	R8758
Opti-MEM® I Reduced Serum Medium	Thermo Fisher Scientific Inc., Waltham, MA, USA	31985047
PBS (Dulbecco's phosphate-buffered saline	Thermo Fisher Scientific Inc., Waltham, MA, USA	14190169
Fetal Bovine Serum, charcoal stripped, USDA-approved region	Thermo Fisher Scientific Inc., Waltham, MA, USA	
Trypsin-EDTA solution	Sigma-Aldrich GmbH, Taufkirchen, Germany	T3924
Penicillin-Streptomycin Solution (10,000 units ea.)	Thermo Fisher Scientific Inc., Waltham, MA, USA	15140130
Dimethyl sulfoxide ≥99.9%	Sigma-Aldrich GmbH, Taufkirchen, Germany	D8418

2.1.9 Buffers and solutions

Table 2.8: Information of buffers used

Buffers and solutions	Composition	Application
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4	Cell harvest
RIPA Buffer	50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0,1% SDS, 1 mM EDTA pH 8.0	Protein isolation
4x Loading Buffer	250 mM Tris HCl pH 6.8, 8% SDS, 40% glycerol, 0.04% bromphenol blue, 100 mM DTT	SDS-PAGE
Running Buffer (pH 8.5 – 8.7)	0.25 M Tris base, 2 M glycine, 1% SDS	SDS-PAGE
Transfer Buffer	25 mM Tris base, 200 mM glycine, 20% methanol	Western Blot
TBST	25 mM Tris base, 140 mM NaCl, 3 mM KCl,	Western Blot

	0.02% Tween 20	
LB medium	10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl	Bacteria cultivation
TAE Buffer	40 mM Tris base, 10 mM EDTA pH 8.0, 6% acetic acid	Agarose gel

2.1.10 Equipment

Table 2.9: Information on equipment used

Equipment	Company
AlphaImager MultiImage Light Cabinet	Alpha Innotech Corp., San Leandro, CA, USA
CO_2 incubator INCOmed	Memmert GmbH + Co. KG, Schwabach, Germany
DNA Engine PTC 200	BioRad Laboratories GmbH, Munich, Germany
Eppendorf 5424R Centrifuge	Eppendorf AG, Hamburg, Germany
Eppendorf pipettes Research plus	Eppendorf AG, Hamburg, Germany
FLUOstar Omega Microplate Reader	BMG LABTECH GmbH, Ortenberg, Germany
Fume hood **Secuflow**	Waldner Laboreinrichtungen GmbH & Co. KG, Wangen, Germany
Hettich Universal 32R centrifuge	Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany
Inverted Fluorescence Microscope Olympus IX81	Olympus Deutschland GmbH, Hamburg, Germany
NanoDrop ND-1000	Thermo Fisher Scientific Inc., Braunschweig, Germany
Neubauer Counting Chamber	Karl Hecht "Assistent" GmbH, Altnau TG, Switzerland
Odyssey Sa Infrared Imaging System	LI-COR Biosciences GmbH, Bad Homburg, Germany
StepOnePlus Real-Time PCR System	Thermo Fisher Scientific Inc., Waltham, MA, USA

2.1.11 Software

Table 2.10: Information on used software

Software	Version	Company
Adobe Photoshop CS5	12.0	Adobe Systems Inc., San José, CA, USA
Fiji/ImageJ	1.51j8	Schindelin J et al., 2012
GraphPad Prism	11.00	GraphPad Software, Inc., La Jolla, CA, USA
Image Studio	3.1.4	LI-COR, Inc., Lincoln, NE, USA
StepOne Software	2.3	Thermo Fisher Scientific Inc., Waltham, MA, USA
R Language	3.4.2	R Development Core Team

2.2 Methods

2.2.1 Cell biological methods

2.2.1.1 Cell culture

HCC cell lines including HUH7, HEP3B, HLE, SNU-182, HLF, and HEK293T cells were used in this study. SNU-182 and HEK293T cells were obtained from American Type Culture Collection (ATCC). HUH7, HEP3B, HLE and HLF cells obtained from the Japanese Cancer Research Resources Bank (JCRB). HUH7, HLE, HLF, and HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, high glucose). HEP3B cells were cultured in Minimum Essential Media (MEM, high glucose). SNU-182 cells were cultured in RPMI 1640 Medium. All medium was supplemented with 10% fetal calf serum (FCS) and 1% Penicillin (100 IU/m)-Streptomycin (100 g/ml) solution. Cells were maintained in an incubator with 37°C, 5 %CO_2, and 95% relative saturation of humidity.

2.2.1.2 Cell seeding and splitting

Cells were split and seeded into new dishes or plates when they achieved ~80% confluence. The medium was discard and cells were washed with PBS. A small volume of trypsin-EDTA (0.25%) was added to the cells. After 5-10 min, an equal volume of medium containing 10% FCS was added to stop the reaction. Then the cell suspension was mixed by pipetting and cells were seeded into new dishes or plates. The medium and trypsin volume and density of cells used in different culture dishes are shown in **Table 2.11**.

Table 2.11: Cell density and medium volumes for various sizes of cell culture dishes and plates

	Surface area (cm^2)	Seeding density	Cells at confluency	Trypsin-EDTA (mL)	Growth medium (mL)
Dishes					
100 mm	60	2×10^6	8×10^6	2	10
60 mm	20	0.8×10^6	3×10^6	1	4
Plates					
6-well	10	0.3×10^6	1.2×10^6	0.5	2.5
12-well	4	0.1×10^6	0.5×10^5	0.3	1
96-well	0.3	0.8×10^4	4×10^4	0.1	0.2

2.2.1.3 Cell cryopreservation and thawing

Surplus cells were frozen as seed stock. Cells were treated with 0.25% trypsin-EDTA when achieved ~80% confluence. A cell suspension was prepared and centrifuged at 110 x *g* for 5 min at room temperature. Then, the supernatant was discarded, and cells were resuspended using freezing medium (Medium: dimethylsulfoxide: FCS=7:2:1). Resuspended cells were transferred into a freezing tube, placed in a freezing container and temporarily stored at -80 °C for 24 h. After 24 h, cells were transferred into liquid nitrogen (-196 °C) for long-term storage. For thawing, frozen cells were rapidly placed in a 37°C water bath. Thawed cells were diluted using pre-warmed growth medium and seeded in a 100 mm dish at a relatively high density. After culturing for 24 h, medium was changed.

2.2.2 Overexpression c-KIT using a lentiviral expression system

To generate inducible expression of wild-type or mutant c-KIT (C674F and W262C) in HUH7 and HEP3B cells pTRIPZ, a lentiviral vector for doxycycline-inducible expression using the Tet-On system was used.

A. Preparation of pTRIPZ vectors

Wild-type c-KIT and mutant c-KIT (C674F and W262C) DNA sequence was amplified from pCMV-c-KIT/WT, pCMV-c-KIT- C674F or pCMV-c-KIT- W262C by PCR with the primers named c-KIT-attB-1 and c-KIT-attB-2, respectively.

PCR products were purified using Nucleo Spin® Gel and PCR Clean-up Kit. The Gateway Cloning System was used to clone the wild-type and mutant c-KIT PCR products into pTRIPZ vectors. By BP reaction, the PCR products flanked by attB recombination sites were inserted into pDONR vectors to create entry vectors. The product of BP reaction was transformed into bacteria. Shortly, the BP reaction and bacteria were incubated on ice for 30 minutes, Heat-shocked at 42°C for 30 seconds, 250 µL of S.O.C. medium was added, and bacteria were incubated at 37°C for 1 hour with shaking. Then 150 µl of the reaction was plated onto kanamycin-selective plates. A single clone was picked, and Nucleeo Spin® Plasmid Kit was used to extract the entry vectors. Obtained vectors were confirmed by agarose gel electrophoresis and sequencing. Primer sequences are shown in **Table 2.6**). Next, in the LR reaction wild-type and mutant c-KIT DNA sequence transferred from the pDONR entry vectors to using LR cloning pTRIPZ vectors. After the LR reaction, bacteria were transformed and selected on carbenicillin plates. pTRIPZ vectors were extracted using a PureYield® Plasmid Midiprep Kit. The obtained pTRIPZ vectors were also confirmed by gel electrophoresis and sequencing. pTRIPZ vectors were named pTRIPZ-c-KIT/WT, pTRIPZ-c-KIT/C674F, and pTRIPZ-c-KIT/W262C, respectively. Meanwhile, to create a control vector for further assay, an empty pTRIPZ without CcdB region was used. The reaction details about BP and LR reaction was shown in **Table 2.12**. More information about Gateway Cloning System can be found on the website of Thermofisher:

(https://www.thermofisher.com/de/en/home/life-science/cloning/gateway-cloning.html).

Table 2.12: Reaction mixes for Gateway BP and LR reaction

Gateway cloning reaction	Component	Final amount
BP reaction	PCR product	40-120 ng
Incubate @25°C overnight	pDONR vector	100 ng
Stop reaction with 1 µl Proteinase-K	BP Clonase II enzyme mix	2 µL
@37°C for 10 min	TE buffer	add up to 10µL
LR reaction	pDONR vector	30 ng
Incubate @25°C overnight	pDest vector	15 ng
Stop reaction with 0.5 µl Proteinase-K	LR Clonase II enzyme mix	1 µL
@37°C for 10 min	TE buffer	add up to 10 µL

B. Production of lentivirus

Lentivirus was produced by the psPAX2 and pMD2.G packaging system. Firstly, the transfection mixture was prepared. Using Polyethyleneimine (PEI) pTRIPZ, psPAX2 and pMD2.G vectors were transfected into HEK293T cells. HEK293T cells were seeded into 100 mm dishes at a density of $5x10^6$ per dish 24 h before transfection and must more than 70% confluence. Transfected cells were routinely cultured overnight (37°C, 5% CO_2). The following day, the medium was removed, and the fresh complete medium was added. Cells were incubated for 48 h (37°C, 5% CO_2). Then medium containing lentivirus was harvested and filtered by a 0.45 µm Millipore filter. According to the different sequence inserted into pTRIPZ vectors, lentivirus was named as Lv-control, Lv-c-KIT/WT, Lv-c-KIT/C674F and Lv-c-KIT/W262C, respectively. The obtained virus may be stored at 4 °C for short periods (hours to days) but was frozen at -20 °C or -80 °C for long-term storage.

C. Cells infection

HUH7 and HEP3B were seeded into 100 mm dishes and cultured until the achieved 70% confluence. Growth medium was removed and 5 mL fresh complete medium +5 mL medium containing lentivirus + polybrene was added (final concentration:8 µg/ml). Cells were incubated overnight, and the medium was removed the following day. After washing with PBS, fresh complete medium was added.

D. Selection and induction

Selection started at 48 h after infection. Medium was removed and replace with 10 ml fresh growth medium containing puromycin (2 µg/mL). Fresh selection medium was added every 48 h. For induction, 2 µg/mL doxycycline was added.

2.2.3 Knock-down using c-KIT siPool

The siPool technology was utilized to knock-down endogenous c-KIT protein levels in HLE, SNU-182 and HLF cells. The siPool tools are composed of 30 selected siRNA that target c-KIT mRNA. Using the reverse transfection protocol c-KIT siPool or control siPool were transfected into cells. For one well in 6-well plate, 210 µL Opti-MEM medium and 40 µL c-KIT or control siPool (0.20 µM) were pipetted into a reaction tube (siPOOL dilution), mixed well by vortexing or pipetting up and down and centrifuged to spin down droplets. In parallel, 246 µL Opti-MEM medium and 4 µL Lipofectamine RNAiMAX Reagent were added to another reaction tube (RNAiMAX dilution). Both reactions were mixed by vortexing or pipetting up and down, combined in a 1:1 ratio and incubated 10 min at room temperature before transfection. To one 6-well, 30 000 cells in 1.5 ml together with 500 µL Transfection mix were added. Then, cells were cultured at 37°C, 5%CO_2. For the mRNA level changes by qPCR, cells were harvested 24h after transfection for RNA extraction. For different analyses (e.g., functional assays or protein analysis), extended incubation periods to 48 h and reduced cell seeding density to 15 000 cells/well.

2.2.4 Phenotypical analyses

2.2.4.1 Cell viability assay

Cell viability assay was performed using the CellTiter-Blue® Cell Viability Assay Kit. Control cells, c-KIT overexpressing HUH7 and HEP3B cells, c-KIT knock-down HLE, SNU-182, and HLF cells were seeded into 12-well plates at

the density of 15 000 cells/well and cultured for 24 h. The cell titer-blue reagent was diluted with relevant medium in a 1:10 ratio. Medium in 12-well plates was discarded, and 500 µL diluted CellTiter-Blue reagent was added in each well. Empty wells with CellTiter-Blue reagent served as blank control. Cells were incubated for 1 h under 37°C, 5%CO_2. After incubation, the CellTiter-Blue reagent was moved into 96-well plate (55 µL/well) from the 12-well plate and the fluorescence was recorded (560_{EX}/590_{EM}) using Omega Fluostar Microplate Reader. Meanwhile, the used 12-well plated was washed using PBS and added into fresh medium. Cells were cultured continually until the next measurement. The first measurement was defined as day 1, and the measurement was repeated after another 24 h, 48h and 72, respectively. In total, 4 time points were collected (day 1, 2, 3 and 4). Human stem cell factor (HSCF) was added on day 2 as mentioned in the results section. For each group, data were normalized by the value obtained from day 1.

2.2.4.2 Cell migration assay

The migration ability of cells was estimated using a wound healing assay. Control cells, c-KIT overexpressing HUH7 and HEP3B cells, or c-KIT knock-down HLE, SNU-182, and HLF cells were seeded into 12-well plates at the density of 12 000 cells/well and cultured until confluence achieved 100%. The growth surface was gently and slowly scratched with a sterile 100 µL pipette tip across the center of the well creating a cross in each well. To remove floating cells, each well was washed by PBS. Then, fresh medium (with 0% FCS) was added. To increase the migration capacity, hepatocyte growth factor (HGF) was added. According to the study design, HSCF was also added or not. Images of gaps in each well were taken at 0 h and 24 h using a light microscope. The area of the gap was measured using Image J software (version 1.51j8).

2.2.5 Nucleic acid analysis

2.2.5.1 RNA extraction and quality control

Total RNA was extracted from treated cells using Extractme® Total RNA Kit following the protocol from manufacturer's instructions. The concentration and quality of total RNA were measured using Nano Drop™ microvolume spectrometer. The RNA samples with high quality were used for cDNA synthesis.

2.2.5.2 Reverse PCR

For cDNA synthesis, reverse PCR was performed using RevertAid H Minus First Strand cDNA Synthesis Kit. The reverse PCR reaction was prepared according to Table, and the PCR program was shown in **Table 2.13 and 2.14**. cDNA samples were stored at -20°C before real-time fluorescence semi-quantification PCR.

Table 2.13: Reaction mixes for reverse PCR (1x reaction)

Master Mix 1	**Volume (µL)**
Random Hexamer Primer	1
RNA 1500 ng	Variable
Nuclease-free water	up to 12.5
Total volume	12.5
Master Mix 2	**Volume (µL)**
5 X RT buffer	1
Ribolock/**RNase** inhibitor	4
dNTP’s	0.5
RT mix RevertAid Minus	1
Total volume	7.5

Table 2.14: Cycling program for reverse PCR

Step 1 for Master Mix 1	Temperature	Time of duration
	65°C	5 min
Step 2 for Master Mix 1+7.5 µL Master Mix 2		
	25°C	10 min
cDNA synthesis	42°C	2 h
Terminate the reaction	70 °C	10 min

2.2.5.3 Real-time fluorescence semi-quantification PCR

cDNA templates obtained from reverse transcription were diluted 1:50 with nuclease-free H_2O. For real-time fluorescence semi-quantification PCR, the prima-QUANT SYBRGreen kit was used. The reaction system was prepared according to **Table 2.15**. All samples were analyzed in triplicates on the StepOnePlus Real-Time PCR System using the PCR program shown in **Table 2.16**. Serine/arginine-rich splicing factor 4 (SRSF4) was used as an internal reference gene. The relative mRNA level of target genes was calculated using the $2^{-\Delta\Delta CT}$ method. All primers were designed using NIH Primer-BLAST online tools and obtained from ThermoFisher Scientific (**Table 2.6**)

Table 2.15: Reaction mixes for qRT-PCR (1x reaction)

	Volume (µL)	Final concentration
primaQUANT CYBR mix	5	1X
Forward Primer (1 µM)	0.8	80 nM
Reverse Primer (1 µM)	0.8	80 nM
Template cDNA (1:50)	2	<180 ng
Nuclease-free water	1.4	-
Total volume	10	-

Table 2.16: Cycling program for qRT-PCR

PCR step	Temperature	Time of duration	Number of cycles
Enzyme activation	95 °C	15 min	1
Denaturation	95 °C	15s	40
Primer annealing/ amplification	60 °C	1 min	
Melting curve			
Denaturation	95 °C	30s	1
Starting temperature	60 °C	30s	1
Melting step	till 95°C in 0.5°C steps		

2.2.6 Protein analysis

2.2.6.1 Protein isolation, quantification and sample preparation

Cell culture dishes or plates were placed on ice and washed with ice-cold PBS. Adherent cells were scraped off the dishes or plates in RIPA buffer supplemented with PhosStop and protease inhibitor using a clean plastic cell scraper and subsequently transferred into a microcentrifuge tube. Cells were lysedfor 30 min on ice. After centrifugation for 15 min at 14 000 x g, 4°C, the supernatant was transferred to a fresh tube and kept on ice. For determination of protein concentration, a Bradford assay was performed. Standard curves were established using known concentration protein standards (0.1, 0.25, 0.5, 1.0, 1.2, and 1.5 µg/µl). To denature, protein samples were diluted with a 4x loading buffer containing sodium dodecyl sulfate (SDS), and boil at 95 °C for 8 min.

2.2.6.2 Western blot analysis

Bis-polyacrylamide gels were prepared using the recipes shown in **Table 2.17**. Denatured protein samples (30-50 µg/well) and prestained molecular weight markers (10 µL/well) were loaded on SDS-PAGE gels. Gel electrophoresis started in a standard migration buffer at 80 V for stacking and then changed to 100 V for resolving. After electrophoresis, gel and membrane are sandwiched between sponge and filter paper. Proteins were transferred to an equilibrated nitrocellulose membrane in standard transfer buffer at 90 V for 2 h by the wet

transfer method. After transfer, the membrane was blocked by 5% defatted milk (diluted by TBST) for 1 h at room temperature. Blocked membrane was incubated with TBST diluted primary antibodies for 2 h at room temperature or 12 h at 4 °C. The antibodies information was shown in Table. GAPDH was used as a reference. Then the membrane was washed by TBST 3 times and each time 10 min. After that membrane was incubated with IRDye secondary antibodies for 2 h at room temperature. Protein bands were detected using the LI-COR Odyssey Imaging System and quantified using Image Studio software.

Table 2.17: Recipes for stacking and resolving gels for SDS-PAGE

	resolving gel (8%)	resolving gel (10%)	Stacking gel (5%)
1.5 M Tris-HCl, pH 8.8	2.5 mL	2.5 mL	-
1 M Tris-HCl, pH 6.8	-	-	380 µL
30% Acrylamide/Bis-acrylamide	2.7 mL	3.3 mL	500 µL
10% SDS	100 µL	100 µL	30 µL
10% APS	100 µL	100 µL	30 µL
TEMED	6 µL	4 µL	3 µL
distilled H_2O	4.6 mL	4 mL	2.1 mL

2.2.7 Public dataset analysis

To analysis the c-KIT expression changes and its potential target genes in HCC patients, four public HCC datasets (GSE14520, 36376, 76242 and 76297, **Table 2.18**) was downloaded from Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo), analyzed and visualized using R software (version3.2.4). Difference expression genes analysis performed by R software with limma package obtained from Bioconductor (https://bioconductor.org). Survival analysis was performed using R software with the survminer package. Heatmaps were drawn using R software with the pheatmap package. GO Enrichment Analysis was performed using the data from Gene Ontology Consortium (http://geneontology.org) and visualized by the Goplot online tools (http://wencke.github.io). The HCC dataset from the Cancer Genome Atlas

(TCGA-LIHC) was accessed and analyzed using Gene Expression Profiling Interactive Analysis online tools (http://gepia.cancer-pku.cn/).

Table 2.18: Information for used GEO public datasets used

Accesion ID	Platform	Sample numbers used	
		Non-Tumor	Tumor
GSE14520	Affymetrix Human Genome U133A 2.0 Array	239	247
GSE36376	Illumina HumanHT-12 V4.0 expression beadchip	193	240
GSE76427	Illumina HumanHT-12 V4.0 expression beadchip	52	115
GSE76297	Affymetrix Human Transcriptome Array 2.0	59	62
GSE22058	Rosetta/Merck Human RSTA Custom Affymetrix 1.0 microarray	97	100
GSE25097	Rosetta/Merck Human RSTA Affymetrix 1.0 microarray, Custom CDF	243	268

2.2.8 Patient samples used for whole exome sequencing in DKFZ-HIPO project

Whole exome sequencing was performed by the DKFZ-HIPO (Heidelberg Center for Personalized Oncology) project 14 using a cohort of 57 hepatocellular carcinoma patients of which fresh frozen tumor and paired non-tumor tissues were available. All tissue samples were provided by the Tissue Bank of the National Center for Tumor Diseases (NCT, Heidelberg, Germany) in accordance with the regulations of the NCT Tissue Bank. Informed consent in writing was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approval of the ethics committee of the Heidelberg University (S-206/2005, S-207/2005 and S-539/2012). Within the presented thesis the finalized results of c-KIT mutations were obtained from Matthias Bieg (DKFZ, Heidelberg)

2.2.9 Statistical analyses

Statistical analyses were performed by R software (version 3.4.2) and GraphPad Prism (version 11.0) built-in statistical analysis function. Normal distribution data was shown as mean±SD. Non-normal data distribution was

presented as median. According to the character of data, for estimating differences between the groups one-way analysis of variance, unpaired t-test, and Mann-Whitney U test were used. Survival analysis was performed by Kaplan–Meier method followed with the log-rank test. A *P* value<0.05 suggested the statistical difference.

3. RESULTS

3.1 Expression of c-KIT in HCC tissue samples and its clinical relevance

To explore the expression alteration of c-KIT in tissue samples from patients with HCC and to identify its correlation with clinical features and prognosis, four publicly available gene microarray datasets derived from tissues of patients with HCC (accession ID: GSE14520, GSE36376, GSE76427 and GSE76297, more details see Table 2.2.6) were retrieved from the Gene Expression Omnibus database. As shown in **Figure 3.1A**, among the four datasets, c-KIT expression was slightly but statistical significant higher in tumor tissue, compared with non-tumor tissue (P<0.05). According to the Barcelona clinic liver cancer (BCLC) staging system and the Cancer of the Liver Italian Program (CLIP) staging system, patients in dataset GSE14520 were divided into two groups including early stage (BCLC stage 0-A or CLIP stage 0-2) and advanced stage (BCLC stage B-C or CLIP stage 3-5). In the tumor tissue samples from advanced stage patients, the c-KIT level was higher compared with early stage patients (P<0.05; **Figure 3.1B**). Moreover, using the median of c-KIT expression as a cut-off, patients in GSE14520 and the TCGA HCC dataset (TCGA-LIHC) were divided into two groups. Kaplan-Meier analysis followed with Log-rank test was performed to determine the difference in survival between patients with high c-KIT level and patients with low c-KIT level. This showed that patients with higher c-KIT level had poor prognosis (**Figure 3.1C**; P<0.05). These observations in HCC patients suggested that c-KIT may play a role in the progress of HCC. Thus, further functional and molecular mechanisms are worth investigating.

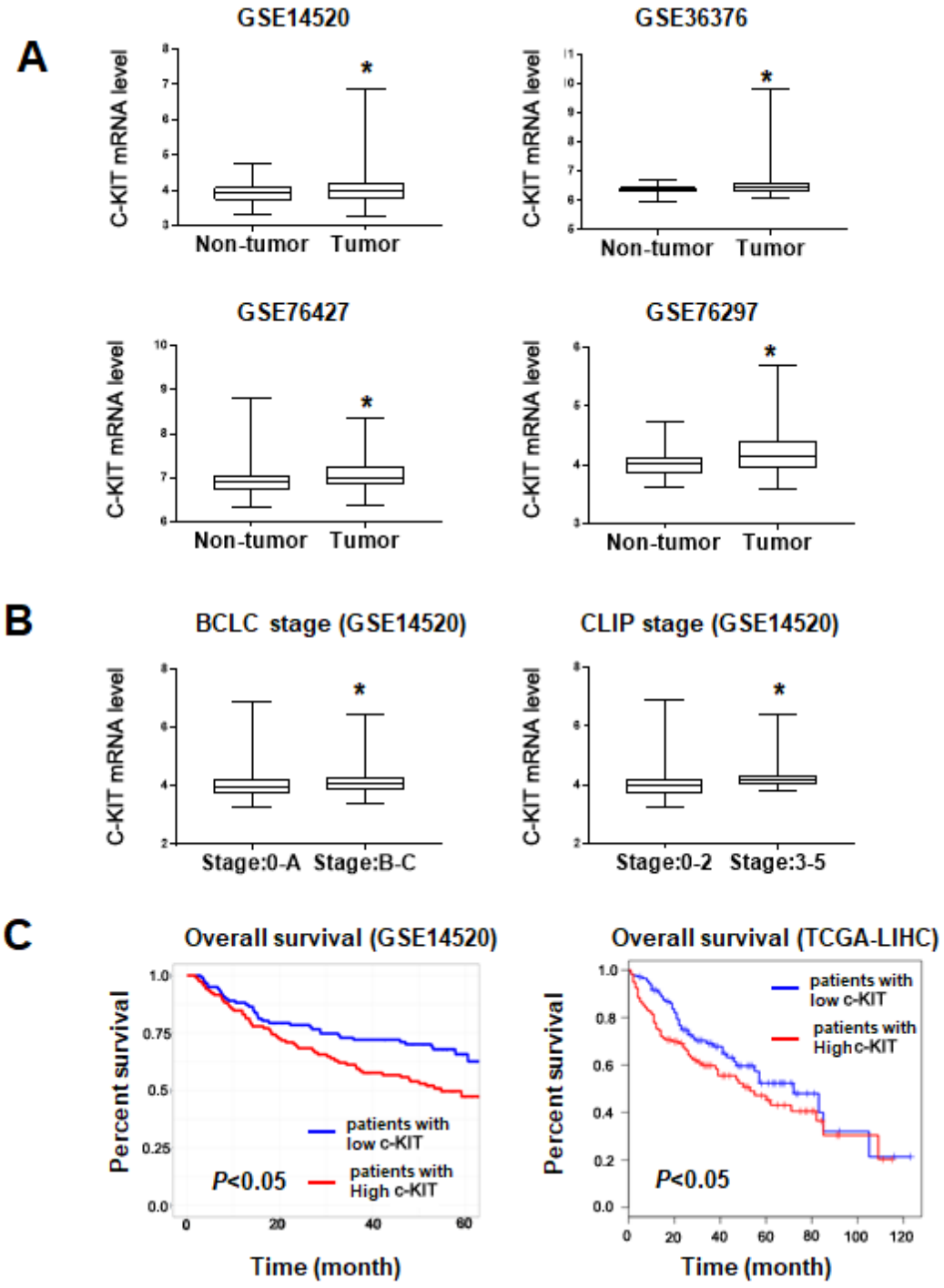


Figure 3.1: c-KIT expression is higher in tumor compared to non-tumor tissues from patients with HCC and is correlated with clinical characteristics. (A) The expression difference of c-KIT between tumor and non-tumor tissues was compared in multiple microarray datasets of patients with HCC retrieved from Gene Expression Omnibus with accession IDs: GSE14520, GSE36376, GSE76427, and GSE76297. All

of four datasets show statistically significant higher c-KIT level in tumor tissues. *P<0.05 compared with non-tumor tissues. (B) In dataset GSE14520, c-KIT was associated with advanced stage. In the BCLC and CLIP stage system, the c-KIT level was higher in tumor tissues from patients of advanced stage. *P<0.05 compared with early-stage patients. (C) In GSE14520 and TCGA-LIHC datasets, patients were divided into two groups using the median of c-KIT level in tumor tissues as a cut-off value.

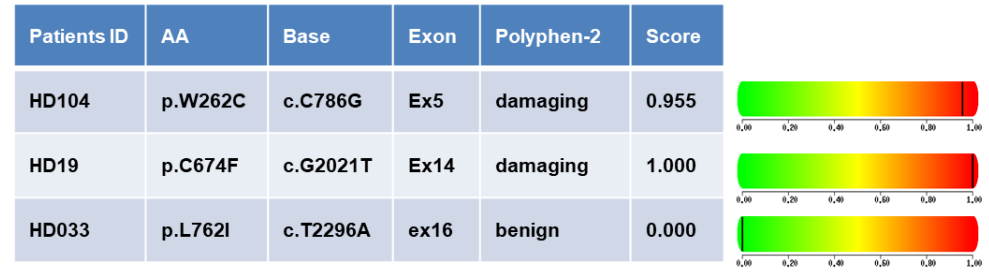
3.2 Mutations of c-KIT in tissue samples from patients with HCC

Oncogenic mutations of c-KIT have been shown to be critically involved in the development of several of carcinomas. But c-KIT mutations have not yet been studied in HCC so far. In project 14 of the Heidelberg Center for Personalized Oncology (HIPO) program, whole exome sequencing of 57 HCC patients was performed and three c-KIT mutations were observed. The possible impact of these three mutations on the structure and function of c-KIT protein was predicted by Polymorphism Phenotyping software (version 2.0; **Figure 3.2A**). The PolyPhen-2 scores of mutation W262C and C674F are 0.955 and 1.000, respectively, which indicated they are most likely damaging mutations. Mutation L762I was predicted as a benign mutation as evident by score 0. Furthermore, all observed mutations were confirmed to be somatic in tumor and non-tumor tissues from patients with HCC using by Sanger sequencing. As shown in **Figure 3.2B**, all three mutations were only present in the respective tumor tissues. The position of W262C, C674F and L766I mutations in the c-KIT protein are shown in **Figure 3.3C.** Mutation C674F is located in exon 14 of the c-KIT gene (G2021T) and is accordingly located in the kinase domain of the c-KIT protein. This suggests that C674F may affect the kinase activity of c-KIT. Mutation W262C within exon 5 on the other hand may have an influence on the Ig-like domain of the c-KIT protein. Based on this evidence, I hypothesized that mutation C674F and W262C might induce altered activation

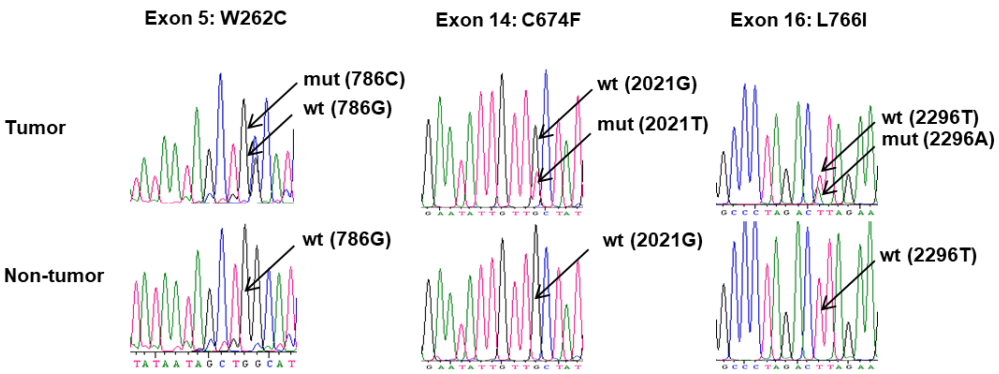
of c-KIT signaling in HCC. The effect of thess two mutations was determined by *in vitro* experiments (see below).

A

Patients ID	AA	Base	Exon	Polyphen-2	Score
HD104	p.W262C	c.C786G	Ex5	damaging	0.955
HD19	p.C674F	c.G2021T	Ex14	damaging	1.000
HD033	p.L762I	c.T2296A	ex16	benign	0.000



B



C

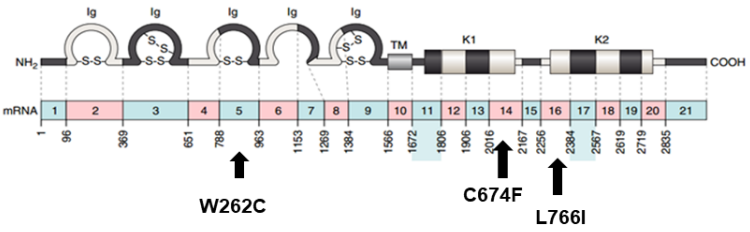


Figure 3.2: Mutations of c-KIT were detected in tissue samples from patients with HCC by whole exome sequencing. (A) c-KIT mutations were detected in 3 of 57 HCC patients. The possible impact of amino acid substitutions in these 3 mutations on the structure and function of c-KIT protein was predicted by Polymorphism Phenotyping software PolyPhen v2.0. W262C and C674F mutations were predicted as damaging amino acid substitutions. (B) All mutations were confirmed by Sanger sequencing and were only observed in tumor tissues of HCC patients. (C) The position of W262C, C674F and L766I mutations in the c-KIT protein domains are depicted.

In the Cancer Genome Atlas Liver Hepatocellular Carcinoma (TCGA-LIHC) data, ten mutations were found in 357 patients with HCC (Table 3.1). The somatic mutation frequency was 2.8%. Among ten mutations, P573Q and D696Y were identified as oncogenic mutations and the other mutations have unknown clinical implications.

Table 3.1 Mutations of c-KIT observed in TCGA-LIHC dataset

Sample ID	Protein Change	Mutation Type	Mutation numbers in Sample
TCGA-ZP-A9D4-01	P141S	Missense_Mutation	61
TCGA-UB-AA0U-01	D696Y	Missense_Mutation	72
TCGA-DD-AAVP-01	S197L	Missense_Mutation	72
TCGA-DD-AAED-01	T847M	Missense_Mutation	59
TCGA-DD-A73D-01	X935_splice	Splice_Site	66
TCGA-DD-A11D-01	P754S	Missense_Mutation	112
TCGA-CC-A8HT-01	D765G	Missense_Mutation	184
TCGA-CC-A7IH-01	S197*	Nonsense_Mutation	685
TCGA-CC-A5UD-01	P573Q	Missense_Mutation	185
TCGA-CC-5264-01	C809*	Nonsense_Mutation	90

3.3 Expression of c-KIT in HCC cell lines

To select appropriate HCC cell lines for functional analyses and further experiments the endogenous expression of c-KIT in HCC cell lines was detected by qRT-PCR and Western blot in nine different HCC cell lines including HEPG2, HLF, HLE, HUH1, HUH6, HUH7, SNU182, SNU387, HEP3B and the immortalized normal hepatocyte cell line HHT4. The relative mRNA level of c-KIT in HCC cells was normalized to the house keeper GAPDH and subsequently to the value of HHT4 cells as reference. In addition, c-KIT protein expression in the same HCC cell lines was measured by Western blot. Botch mRNA and protein expression exhibited large differences among the cell lines tested. HLE, HLF, and SNU-182 showed clear expression at the mRNA and endogenous protein level (**Figure 3.3 A & B**). In the other cell lines, the mRNA level of c-KIT was extremely low and endogenous protein bands were not detectable. Thus, HUH7 and HEP3B cells were selected for c-KIT

overexpression experiments, whereas, HLE, HLF, and SNU-182 were utilized for c-KIT knock-down experiments.

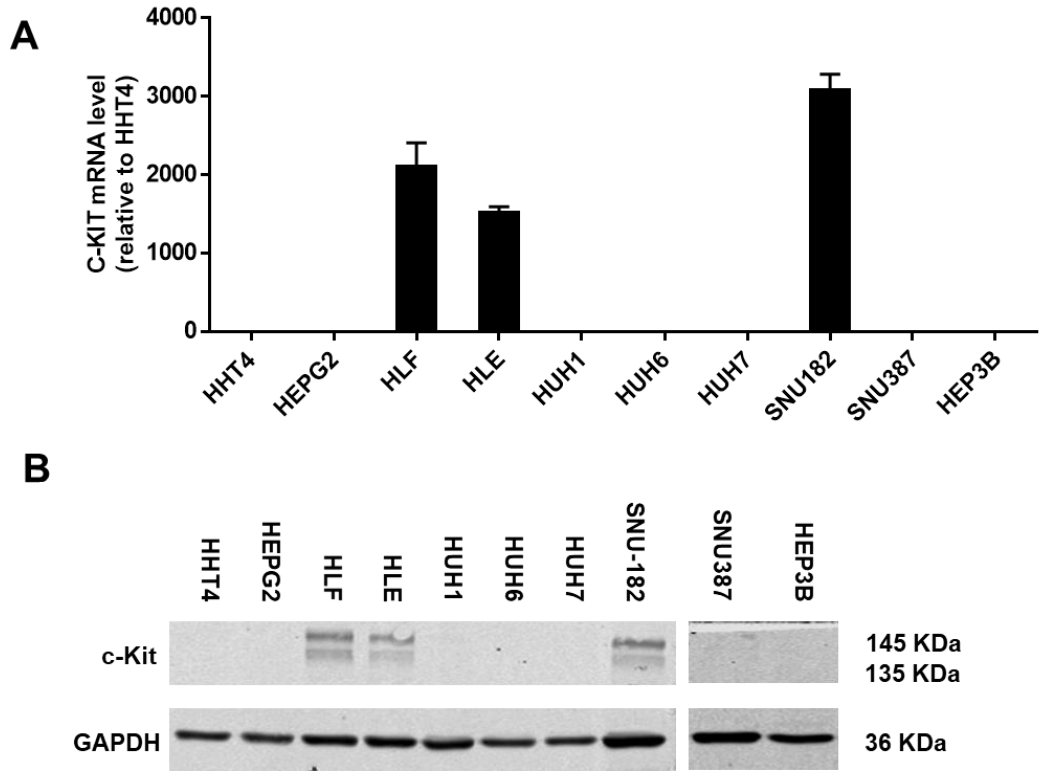


Figure 3.3: Endogenous expression of c-KIT in HCC cell lines. (A) mRNA level of c-KIT in HCC cell lines was measured by qRT-PCR. The CT value was normalized by GAPDH, and then the fold changes relative to HHT4 cells were calculated by the delta delta CT method. (B) c-KIT protein expression in HCC cell lines was detected by Western blot. In accordance with the mRNA results, only in HLF, HLE and SNU-182 cell lines showed detectable c-KIT protein expression.

3.4 Effect overexpression and mutations of c-KIT on relevant downstream pathways in HCC cell lines

As a tyrosine kinase receptor, c-KIT mainly acts by regulating its downstream pathways. Both aberrant expression and mutation of c-KIT may cause pathological downstream signal transduction subsequently promoting the initiation or progression of carcinoma. However, the situation about c-KIT and

its downstream pathway are still unclear in HCC. In this work, three typical c-KIT downstream signaling pathways including AKT, MAPK and JAK/STAT were investigated. Due to undetectable endogenous c-KIT expression, wild-type c-KIT was overexpressed in HUH7 and HEP3B. To identify the feature of c-KIT mutation C674F and W262C, c-KIT containing these two mutations was also expressed in HUH7 and HEP3B cell lines, respectively. On the other hand, the c-KIT expression was knocked down in HLE, SNU-182 and HLF cells using c-KIT-siPooL which is a mixture of 30 selected different siRNAs. To obtain a comparable baseline of phosphorylation level, all cells were starved in serum-free medium for 24 h before Western blotting to detect the phosphorylation level of target pathways. To activate c-KIT signaling, cells were incubated with 100 ng/mL human stem cell factor (HSCF) for 15 min prior to harvesting cells for analysis. The time of duration for stimulation was decided by preliminary experiment. Either shorter or longer time could weaken the detectable phosphorylation of target components.

For overexpression of c-KIT wildtype, c-KIT C674F or c-KIT W262C a lentiviral inducible system was used to ensure homogeneous and consistent expression with minimal selection pressure on the respective cells. The doxycycline (Dox)-inducible lentiviral expression system effectively induced c-KIT protein expression in HUH7 cells (**Figure 3.4**). HSCF treatment for 15 min induced significant phosphorylation of c-KIT at Tyr791. Compared with c-KIT/WT and c-Kit-CF (C674F mutation), cells expressing c-KIT-WC (W262C mutation) showed lower p-c-KIT-Tyr791 (p-c-KIT) level (P<0.05). Furthermore, HSCF induced phosphorylation of AKT at Serine 473 (p-AKT) in c-KIT overexpressing cells. Consistent with the p-c-KIT level, the p-AKT level was also lower in cells expressing c-KIT-WC (P<0.05). For statistical analyses, band intensities of three independent Western blot experiments were quantified and normalized to GAPDH as loading control (**Figure 3.4 B**). Thus,

c-KIT stimulation with HSCF leads to AKT phosphorylation and the patient-derived W262C mutation of c-KIT impairs c-KIT/AKT signaling.

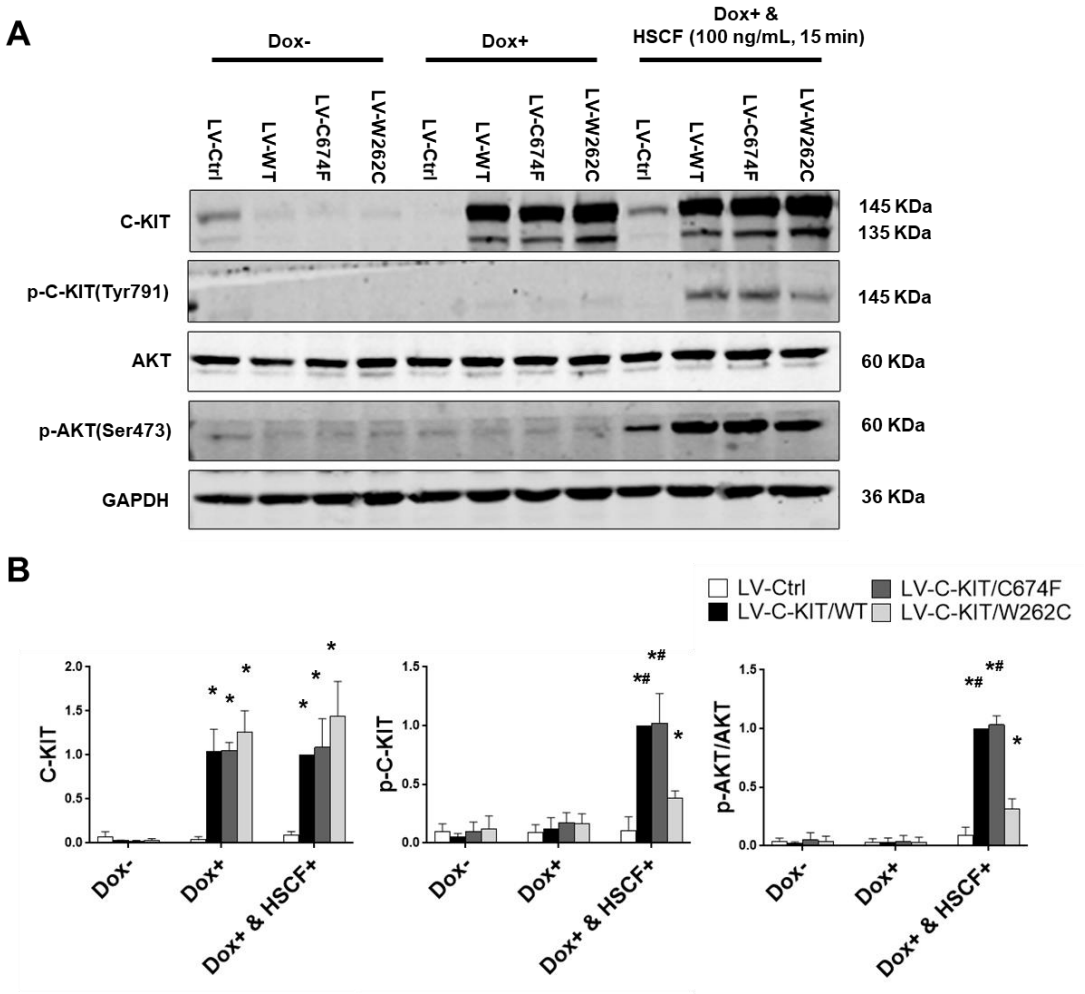


Figure 3.4: c-KIT overexpression induced downstream AKT signal transduction in HUH7 cells. c-KIT wild-type (WT), C674F and W262C mutant protein expression was induced in HUH7 using a pTRIPZ-based doxycycline-inducible lentiviral system. Control lentivirus (LV-Ctrl) cells were prepared using empty pTRIPZ vector. (A) Cells were treated with 100 ng/mL human stem cell factor (HSCF) for 15 min before protein collection. Total c-KIT, phosphorylation level of c-KIT at Tyr791, total AKT and phosphorylation of AKT at Ser473 were measured by Western blot. (B) Data of three independent experiments were quantified and normalized to HSCF-treated LV-c-KIT/WT infected cells. HSCF activated wildtype c-KIT, however, c-KIT/W262C had lower effects on phosphorylation of c-KIT compared to c-KIT/WT and

c-KIT/C674F. Consistently, in c-KIT/W262C infected HUH7 cells less p-AKT levels were observed. *P<0.05 compared to LV-Ctrl infected cells in each of condition. $^{\#}P$<0.05 compared to LV- c-KIT/W262C infected cells in each of condition.

Two routes of MAPK signaling, JNK and ERK1/2, were analyzed in this work because they have been implicated in HCC previously. As shown in **Figure 3.5**, the phosphorylation of JNK at Thr183 and Tyr185 (p-JNK) level was increased by HSCF stimulation. Similarly to AKT, HUH7 cells expressing c-KIT-WC mutant protein showed less pronounced increase of p-JNK levels (P<0.05). But no difference in the phosphorylation of ERK1/2 at Tyr204 and Tyr 187 (p-ERK1/2) level was observed in these conditions. Neither c-KIT up-regulation nor HSCF treatment had any effect on the phosphorylation of ERK1/2. Thus, c-KIT signaling in the HCC cell lines HuH7 did not affect ERK signaling and ERK signaling seemed to be activated by other mechanisms as unstimulated cells already displayed basal p-ERK1/2 levles wich could not be further augmented. Taken together, JNK signaling is activated by c-KIT signaling through stimulation with HSCF but c-KIT mutation W262C led to reduced JNK activation.

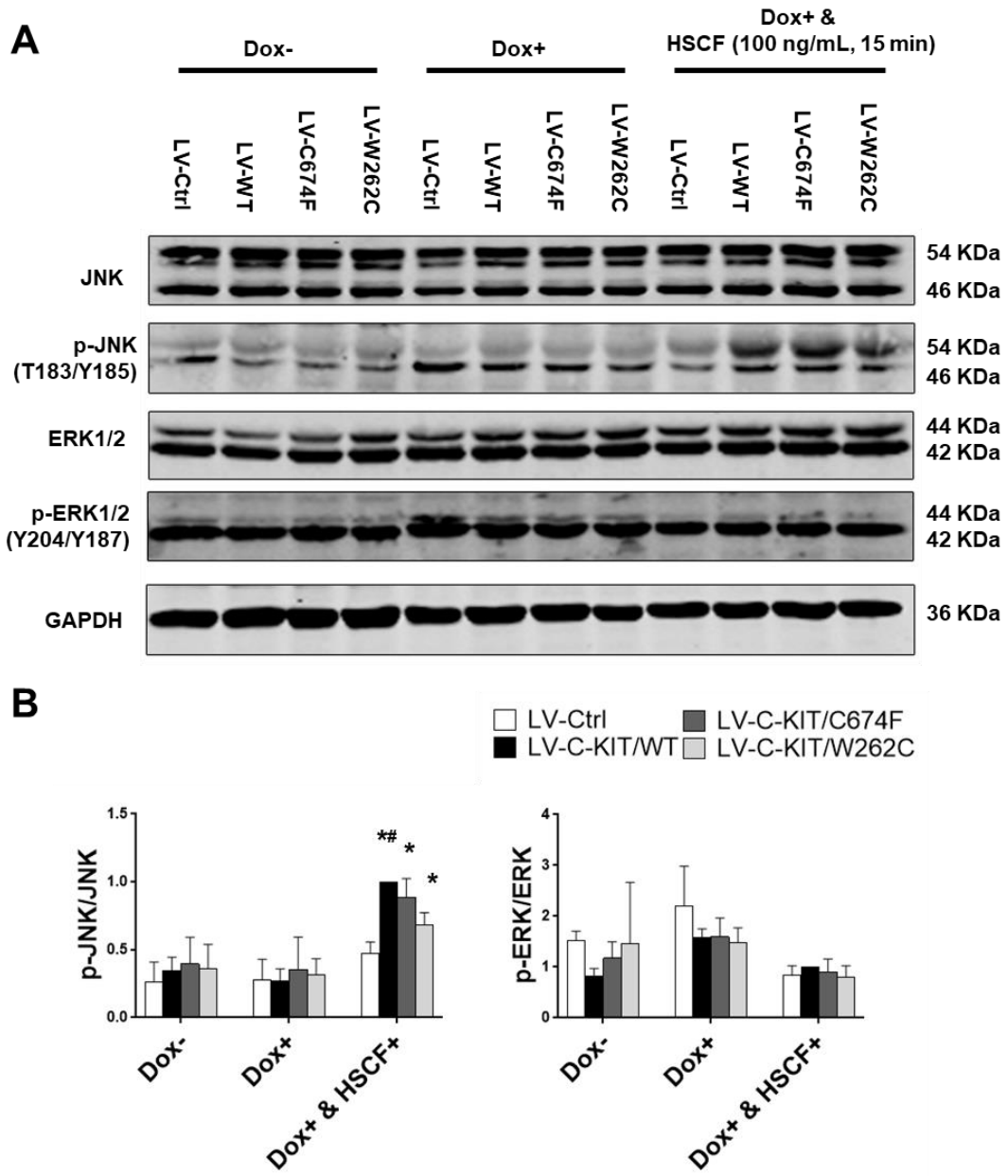


Figure 3.5: c-KIT overexpression influences JNK but not ERK signal transduction in HUH7 cells. c-KIT wild-type (WT), C674F and W262C mutant protein expression was induced in HUH7 using a pTRIPZ-based doxycycline-inducible lentiviral system. Control lentivirus (LV-Ctrl) cells were prepared using empty pTRIPZ vector. (A) Cells were treated with 100 ng/mL human stem cell factor (HSCF) for 15 min before protein collection. Total JNK, phosphorylation level of JNK at Thr183 and Tyr185, total ERK1/2 and phosphorylation of ERK1/2 at Tyr204 and Tyr 187 were measured by Western blot. (B) Data of three independent

experiments were quantified and normalized to HSCF-treated LV-c-KIT/WT infected cells. *P<0.05 compared to LV-Ctrl infected cells in each of condition. $^{\#}P$<0.05 compared to LV- c-KIT/W262C infected cells in each of condition.

Next, the influence of HSCF/c-KIT signaling on the JAK/STAT pathway was studied. The STAT family members STAT1 and STAT3 have been shown previously to be involved in HCC. STAT1 is believed to be tumor suppressive, whereas, STAT3 acted oncogenic in cell culture experiments and animal studies. Thereby, the phosphorylation of STAT proteins is mediated by JAK1, JAK2 or TYK2. Using different antibodies, no phosphorylation of JAK1 or TYK2 could be observed (data not shown). In addition, no alteration in JAK2 total protein or phosphorylation could be detected upon c-KIT wildtype or c-KIT mutant protein expression (**Figure 3.6**). STAT1 signaling was activated by phosphorylation upon treatment with HSCF in c-KIT wildtype overexpressing cells.However, c-KIT-WC showed lower p-STAT1 levels (P<0.05). Interestingly, I found treatment with HSCF only without expression of c-KIT induced activation of STAT3 in HUH7 control cells. The p-STAT3 level was not increased in c-KIT overexpressing cells and the effect of HSCF was lost upon c-KIT expression.

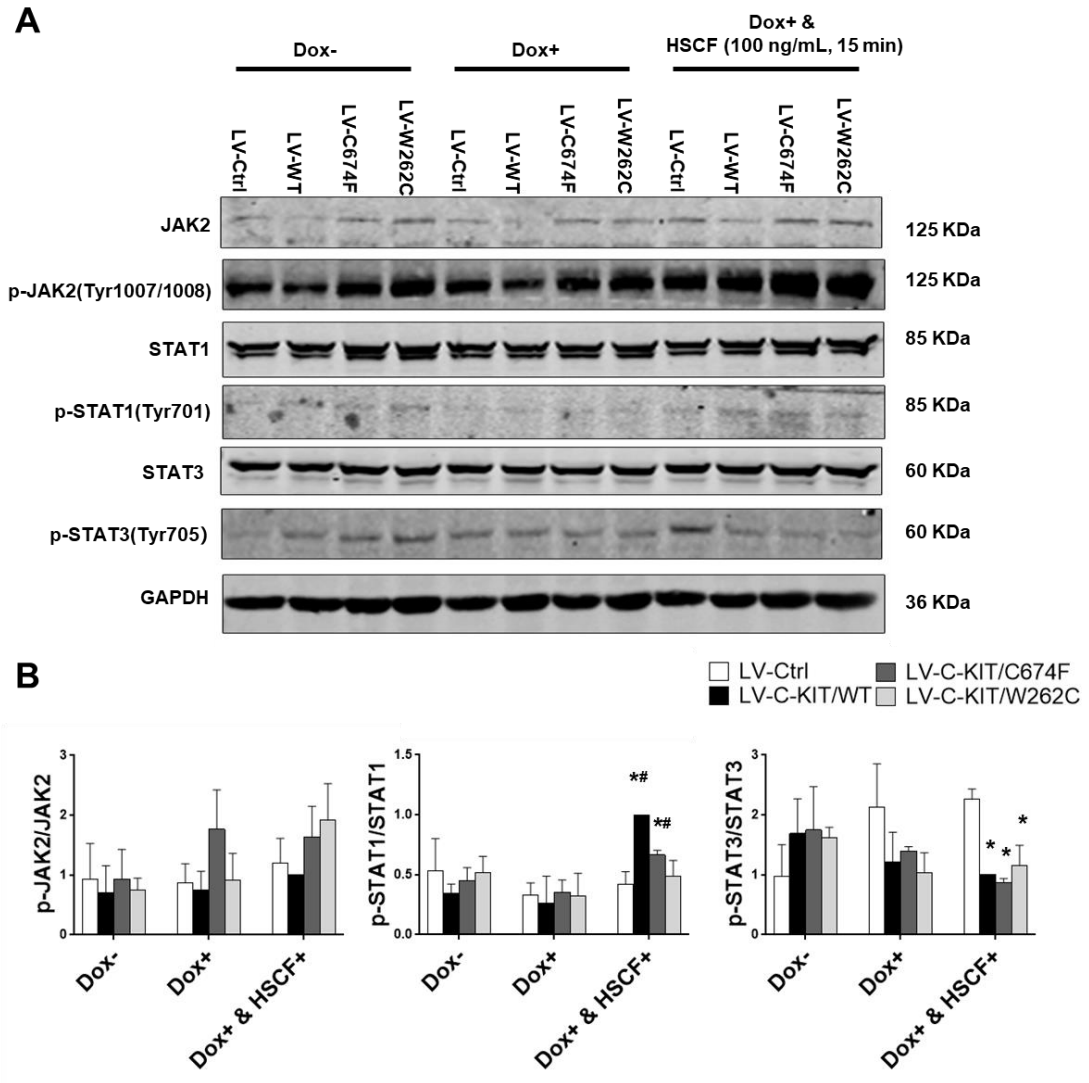


Figure 3.6: c-KIT overexpression influences STAT1 signal transduction in HUH7 cells. c-KIT wild-type (WT), C674F and W262C mutant protein expression was induced in HUH7 using a pTRIPZ-based doxycycline-inducible lentiviral system. Control lentivirus (LV-Ctrl) cells were prepared using empty pTRIPZ vector. (A) Cells were treated with 100 ng/mL human stem cell factor (HSCF) for 15 min before protein collection. Total JAK2, phosphorylation level of JAK2, total STAT1, phosphorylation of STAT1 at Ser701, total STAT3 and phosphorylation of STAT3 at Ser705 were measured by Western blot. (B) Data of three independent experiments were quantified and normalized to HSCF-treated LV-c-KIT/WT infected cells. **P*<0.05 compared to LV-Ctrl infected cells in each of condition. $^{\#}P$<0.05 compared to LV-c-KIT/W262C infected cells in each of condition.

To exclude cell line-specific effects observed in c-KIT signaling in the HUH7 cell line, the same experiments were performed in the HCC cell line HEP3B. The results in HEP3B cells were overall similar with HUH7 but still had some minor differences. As shown in **Figure 3.7**, the Dox-inducible lentiviral expression systems effectively worked in HEP3B cells. After treatment with HSCF, phosphorylated c-KIT followed with activated AKT was observed in c-KIT overexpression cells. Comparable to the results in HUH7, c-KIT with W262C mutation showed less activity, compared with c-KIT/WT and c-KIT-CF (P<0.05).

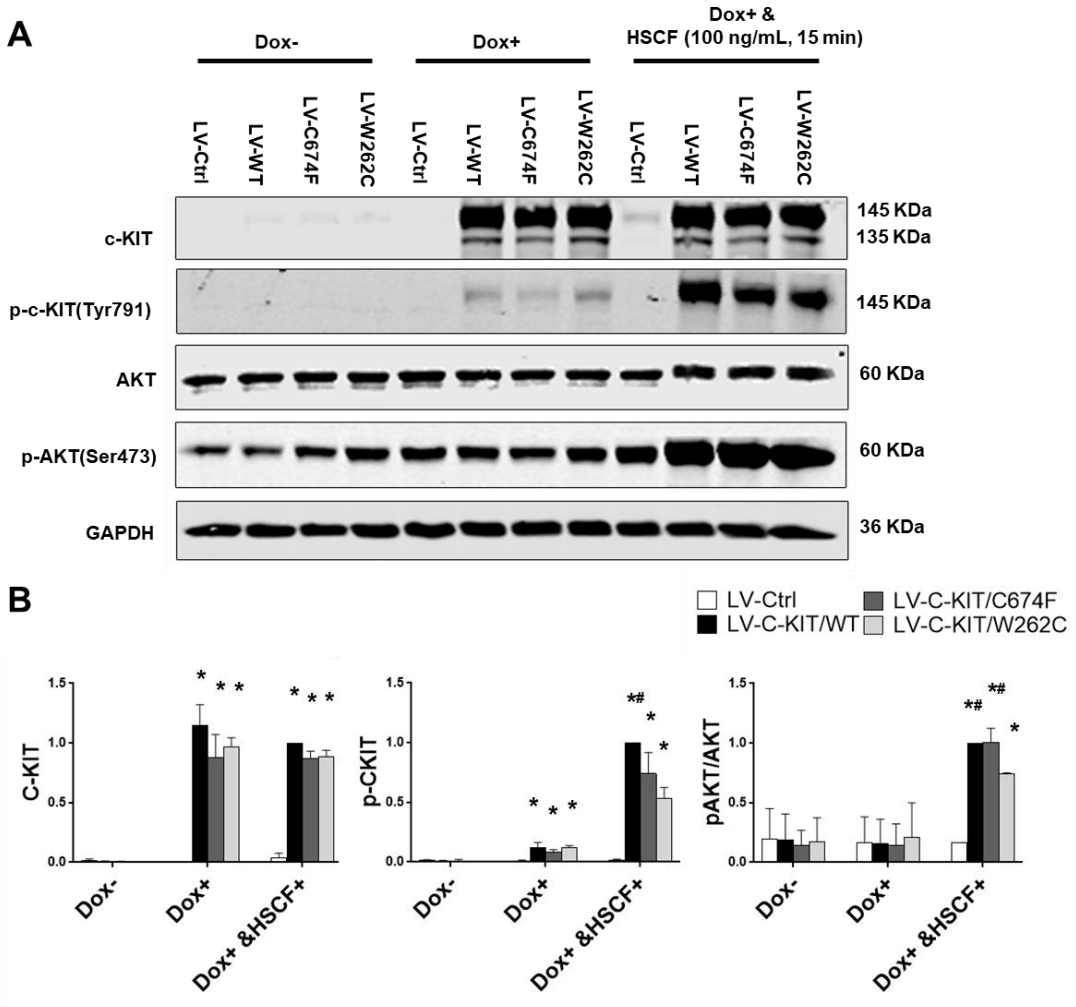


Figure 3.7: c-KIT overexpression induced downstream AKT signal transduction in HEP3B cells. c-KIT wild-type (WT), C674F and W262C mutant protein expression was induced in HEP3B using a pTRIPZ-based doxycycline-inducible lentiviral system.

Control lentivirus (LV-Ctrl) cells were prepared using empty pTRIPZ vector. (A) Cells were treated with 100 ng/mL human stem cell factor (HSCF) for 15 min before protein collection. Total c-KIT, phosphorylation level of c-KIT at Tyr791, total AKT and phosphorylation of AKT at Ser473 were measured by Western blot. (B) Data of three independent experiments were quantified and normalized to HSCF-treated LV-c-KIT/WT infected cells. HSCF activated wildtype c-KIT, however, c-KIT/W262C had lower effects on phosphorylation of c-KIT compared to c-KIT/WT and c-KIT/C674F. Consistently, in c-KIT/W262C infected HEP3B cells less p-AKT levels were observed. *P<0.05 compared to LV-Ctrl infected cells in each of condition. $^{\#}P$<0.05 compared to LV- c-KIT/W262C infected cells in each of condition.

Different from HUH7, both JNK and ERK1/2 were activated by HSCF in c-KIT expressing HEP3B cells. In c-KIT/WT and c-KIT-CF overexpressing HEP3B cells stronger p-JNK and p-ERK1/2 bands were detected after HSCF stimulation, compared with control (**Figure 3.8**, P<0.05). However in c-KIT-WC overexpressing HEP3B cells, the phosphorylation of JNK and ERK1/2 was not induced.

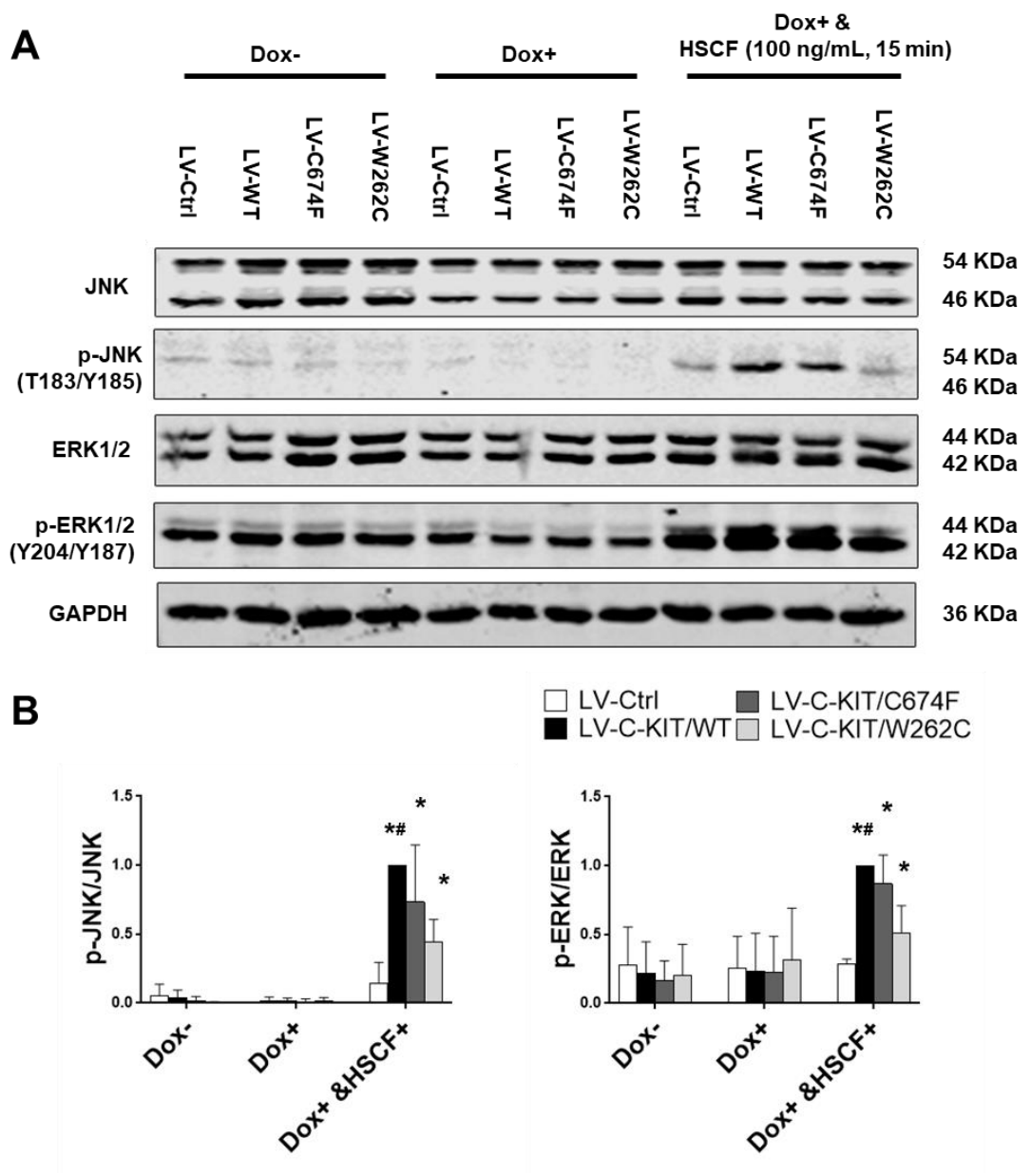


Figure 3.8: c-KIT overexpression influences JNK but not ERK signal transduction in HEP3B cells. c-KIT wild-type (WT), C674F and W262C mutant protein expression was induced in HEP3B using a pTRIPZ-based doxycycline-inducible lentiviral system. Control lentivirus (LV-Ctrl) cells were prepared using empty pTRIPZ vector. (A) Cells were treated with 100 ng/mL human stem cell factor (HSCF) for 15 min before protein collection. Total JNK, phosphorylation level of JNK at Thr183 and Tyr185, total ERK1/2 and phosphorylation of ERK1/2 at Tyr204 and Tyr 187 were measured by Western blot. (B) Data of three independent experiments were quantified and normalized to HSCF-treated LV-c-KIT/WT infected

cells. *P<0.05 compared to LV-Ctrl infected cells in each of condition. $^{\#}P$<0.05 compared to LV- c-KIT/W262C infected cells in each of condition.

Lastly, the JAK/STAT pathway activation was analyzed in c-KIT-inducible HEP3B cells upon HSCF-treatment (**Figure 3.9,** P<0.05). In contrast to HUH7 cells, c-KIT/WT expressing HEP3B cells showed an induction of STAT1 and STAT3 phosphorylation. However, no statistical difference was found in c-KIT-CF and c-KIT-WC compared with control cells. Similarly to HUH7, in HEP3B cells no alteration in JAK2 was detected. Thus, STAT1 and STAT3 signaling was induced in c-KIT/WT expressing cells upon HSCF stimulation and both patient-derived c-KIT mutations abrogated this activation.

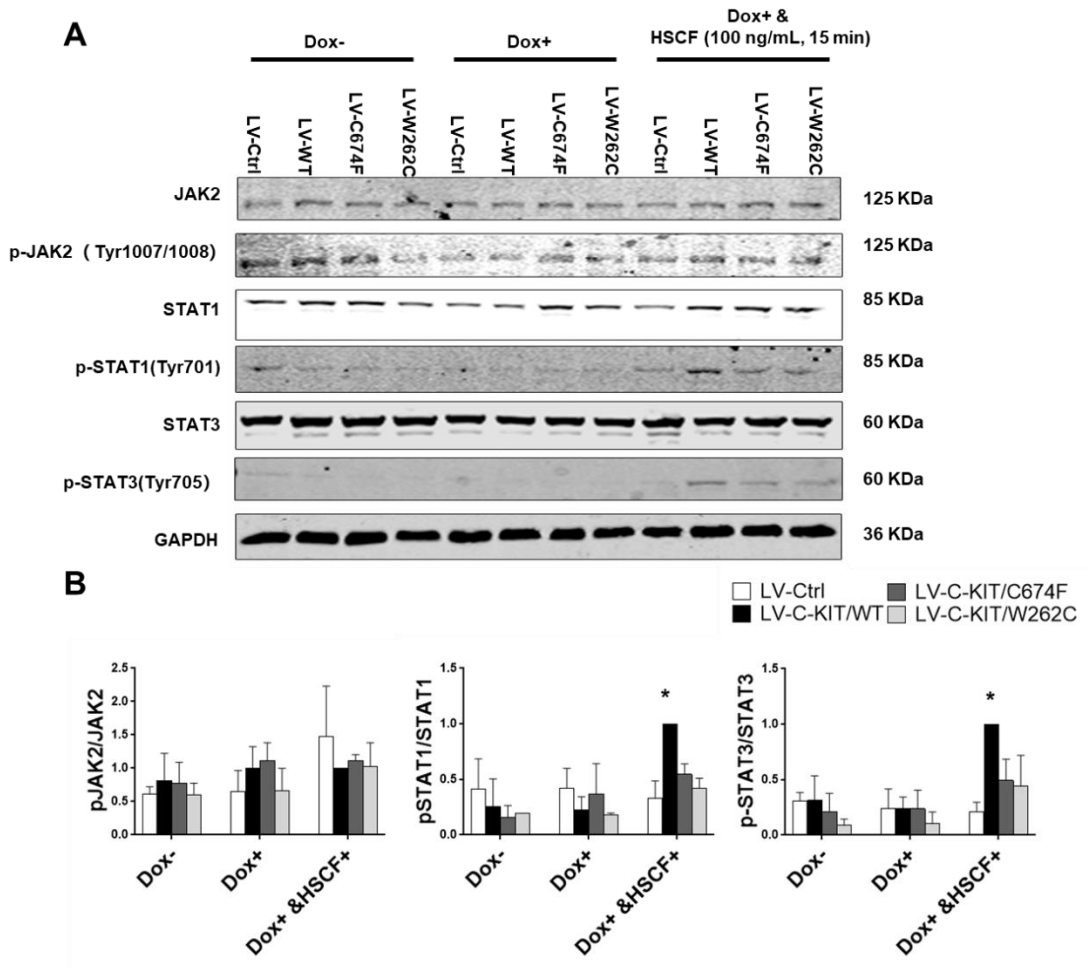


Figure 3.9: c-KIT overexpression influences STAT1 signal transduction in HEP3B cells. c-KIT wild-type (WT), C674F and W262C mutant protein expression was induced in HEP3B using a pTRIPZ-based doxycycline-inducible lentiviral system. Control lentivirus (LV-Ctrl) cells were prepared using empty pTRIPZ vector. (A) Cells were treated with 100 ng/mL human stem cell factor (HSCF) for 15 min before protein collection. Total JAK2, phosphorylation level of JAK2, total STAT1, phosphorylation of STAT1 at Ser701, total STAT3 and phosphorylation of STAT3 at Ser705 were measured by Western blot. (B) Data of three independent experiments were quantified and normalized to HSCF-treated LV-c-KIT/WT infected cells. $*P<0.05$ compared to LV-Ctrl infected cells in each of condition. $^{\#}P<0.05$ compared to LV-c-KIT/W262C infected cells in each of condition.

3.5 Effect of c-KIT knock-down on downstream pathways in HCC cell lines

As the overexpression of c-KIT may impose artifacts due to high level expression in a non-physiological situation, the influence of knock-down of endogenous c-KIT in HCC cell lines was studied. The activity changes of AKT, MAPK and JAK/STAT signaling were measured after c-KIT knock-down in cell lines which express c-KIT and most likely enhibit functional downstream signaling pathways. As shown in **Figure 3.10**, siPool effectively silenced the c-KIT expression and inhibited the activation of c-KIT caused by HSCF in HLE, SNU-182, and HLF cells. Thereby, siPool is a mixture of different siRNAs and it has been demonstrated that unspecific effects of single siRNAs are minimized by using multiple different siRNAs in the siPool. Therefore, it is sufficient to use one siPool sample instead of at least two different conventional siRNAs. As expected, HSCF did not induce phosphorylation of AKT in c-KIT knock-down cells. In the control cells of HLE, SNU-182 and HLF, however, HSCF activated c-KIT through the phosphorylation of c-KIT and subsequently AKT was activated by phosphorylation at Ser473. In all three c-KIT knock-down cell lines, phosphorylation of AKT after HSCF treatment was significantly reduced.

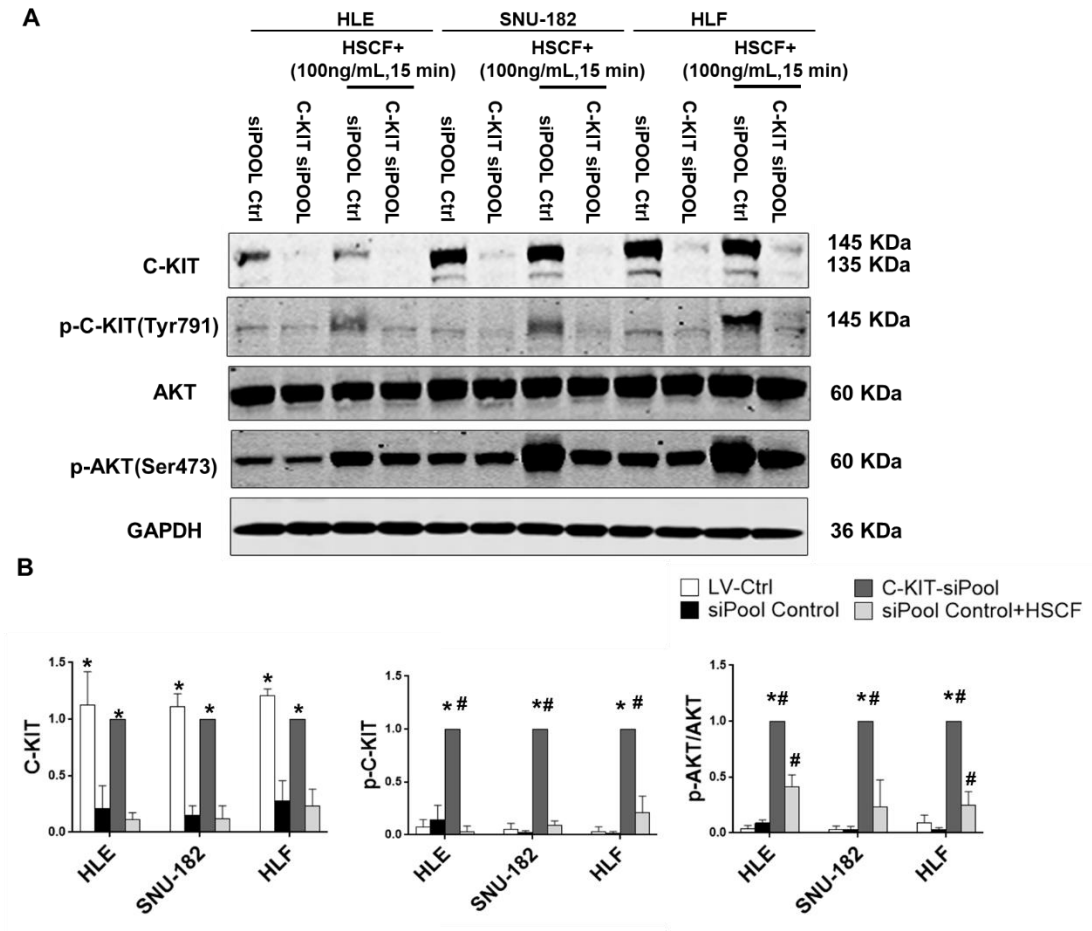


Figure 3.10: c-KIT knock-down inhibited downstream AKT signal transduction in HLE, SNU-182 and HLF cells. To knock-down c-KIT a selected siRNA mixture (sipool) was transfected into cells. (A) Cells were treated with 100 ng/mL human stem cell factor (HSCF) for 15 min before protein collection. Total c-KIT, phosphorylation level of c-KIT at Tyr791, total AKT and phosphorylation of AKT at Ser473 were measured by Western blot. (B) Data of three independent experiments were quantified and normalized to HSCF-treated cells. *P<0.05 compared with c-KIT siPool-treated in each of condition. $^{\#}P$<0.05 compared with cells without HSCF treatment.

In all three cell lines expressing endogenous c-KIT, JNK and ERK1/2 were activated through phosphorylation upon HSCF treatment. The knock-down of c-KIT by c-KIT-siPool treatment antagonized this effect suggesting that c-KIT is necessary for JNK and ERK1/2 signaling (**Figure 3.11**).

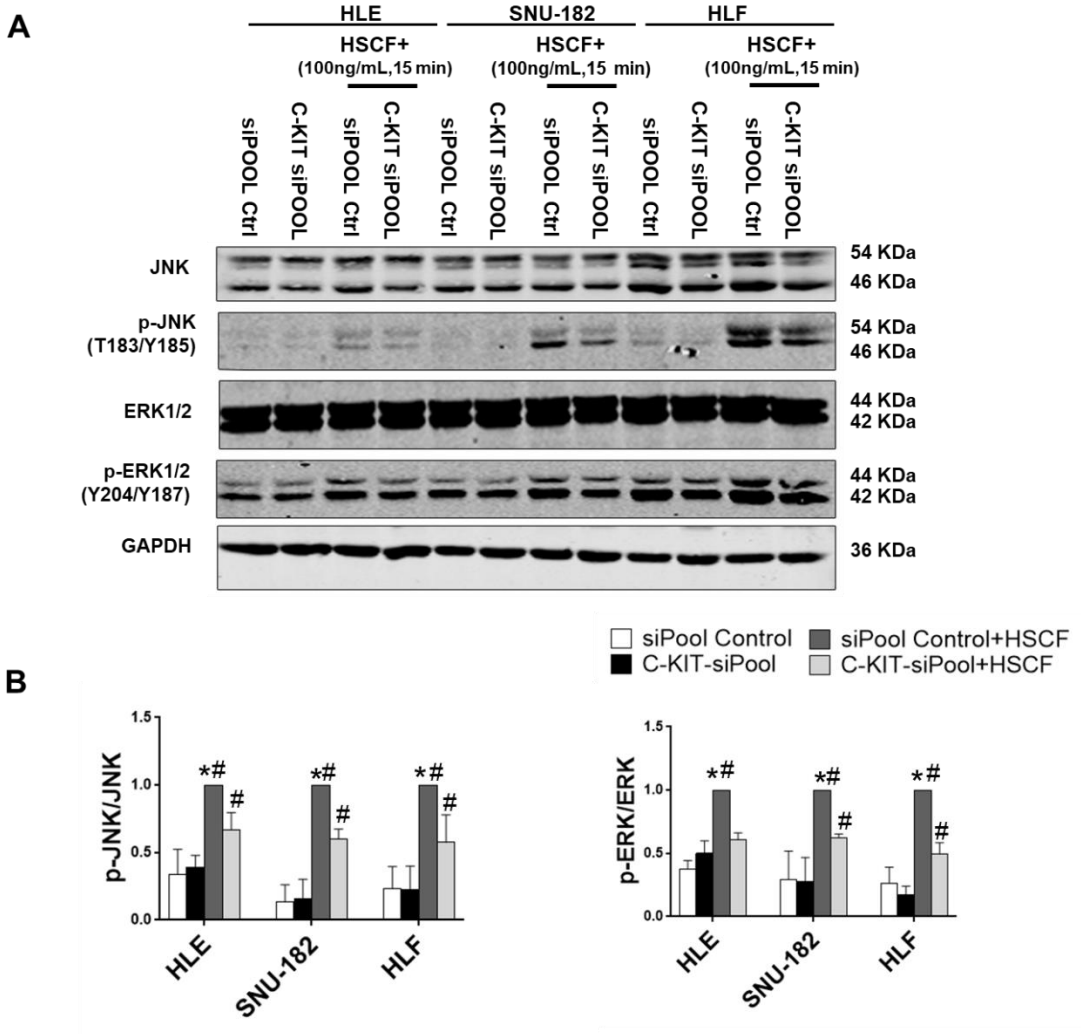


Figure 3.11: c-KIT knock-down inhibited downstream JNK and ERK1/2 signal transduction in HLE, SNU-182 and HLF cells. To knock-down c-KIT a selected siRNA mixture (sipool) was transfected into cells. (A) Cells were treated with 100 ng/mL human stem cell factor (HSCF) for 15 min before protein collection. Total JNK, phosphorylation level of JNK at Thr183 and Tyr185, total ERK1/2 and phosphorylation of ERK1/2 at Tyr204 and Tyr 187 were measured by Western blot. (B) Data of three independent experiments were quantified and normalized to HSCF-treated cells cells. **P*<0.05 compared with c-KIT siPool-treated in each of condition. $^{\#}$*P*<0.05 compared with cells without HSCF treatment.

Only slight inconsistent effects were observed on STAT1 and STAT3 signaling. In HLE and SNU-182 cells, c-KIT knock-down reduced the activation of JAK2

even without the stimulation of HSCF but no effect was detected in HLF cells (**Figure 3.12**). In HLE cells, knock-down of c-KIT reduced HSCF-induced p-STAT1 but decreased the p-STAT3 level in an HSCF-independent manner. No difference on p-STAT1 was observed in treated SNU-182 and HLF cells. Interestingly, HSCF reduced the p-STAT3 level in SNU-182 and HLF even in cells with normal c-KIT expression. Thus, only small cell line dependent effects have been detected after c-KIT knock-down suggesting that STAT1 and STAT3 signaling are not crucial downstream effectors of c-KIT signaling.

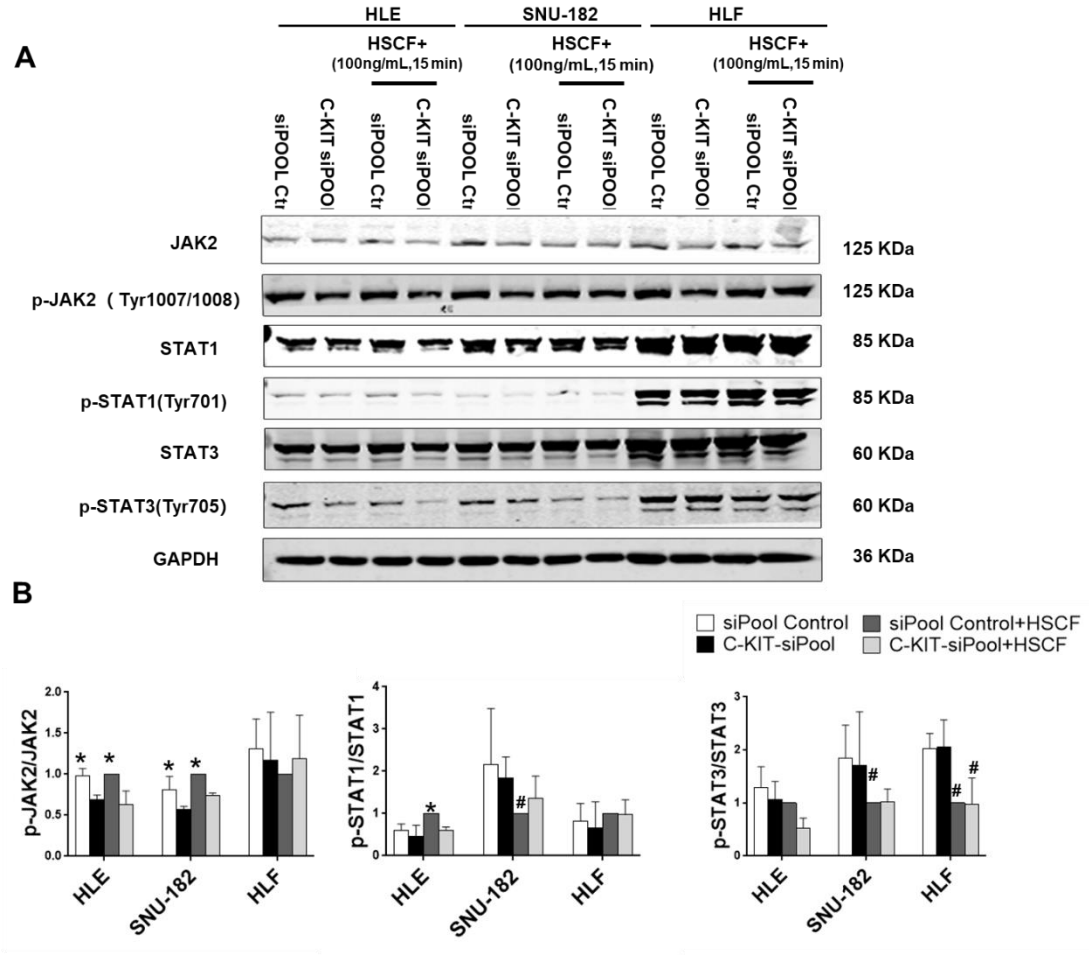


Figure 3.12: c-KIT knock-down had variant effect on JAK/STAT signal transduction in HLE, SNU-182 and HLF cells. To knock-down c-KIT a selected siRNA mixture (sipool) was transfected into cells. (A) Cells were treated with 100 ng/mL human stem cell factor (HSCF) for 15 min before protein collection. Total JAK2,

phosphorylation level of JAK2, total STAT1, phosphorylation of STAT1 at Ser701, total STAT3 and phosphorylation of STAT3 at Ser705 were measured by Western blot. (B) Data of three independent experiments were quantified and normalized to HSCF-treated cells cells. *P<0.05 compared with c-KIT siPool treated in each of condition. $^{\#}P$<0.05 compared with cells without HSCF treatment.

Taken together, the result of all five cell lines, HUH7, HEP3B, HLE, SNU-182, and HLF, suggested that c-KIT could activate AKT, MAPK and partly JAK/STAT signaling in HCC cells but not all the element had been involved equally in the different cell lines. It should be noted that the effect of activated c-KIT on certain specific elements of the downstream pathway was different among different HCC cells. In most settings no significant difference between c-KIT wildtype and c-KIT/C674F mutation were observed. In contrast, the c-KIT/W262C mutation showed a reduced response to HSCF. Hence, this indicated that the W262C mutation maybe a loss of function mutation of c-KIT. Unlike other tumor entities, the two in HCC tested mutations are not hyperactive mutations.

3.6 The effect of c-KIT expression alteration and mutation on the proliferation of HCC cell lines

It was described in several types of carcinomas that continuous activation of downstream signaling induced by abnormal expression or mutation of c-KIT could promote the proliferation of tumor cells. The effect of c-KIT/HSCF signaling on the proliferation of HCC still needs to be determined. I detected the cell viability in c-KIT overexpressing or knock-down HCC cells at four time points. The viability data of each day was normalized by day one of the respective cell line and normalized to control (Ctrl) virus-infected cells. First, HUH7 and HEP3B cells were used for expression of wildtype and mutant c-KIT protein. Second, endogenous c-KIT protein was inhibited by siPool-mediated knock-down in HLE, SNU-182 and HLF cells. The relative cell viability after 2

days showed that neither c-KIT overexpression nor c-KIT knock-down altered cell viability. Overexpression of c-KIT, regardless of wild-type or mutations, could not increase or inhibit cell viability in HUH7 and HEP3B. Likewise, knock-down of c-KIT did not influence the cell viability in HLE, SNU-182 and HLF cells. These results were obtained in cell culture medium supplemented with 10% FCS which contains SCF. To exclude that additional HSCF stimulation is needed, cell culture medium was supplemented with 100 ng/mL human stem cell factor (HSCF). However, HSCF stimulation did not induce any changes in cell viability of all five HCC cell lines (**Figure 3.13**). This suggested that the activation of c-KIT may not have any effect on the cell proliferation in HCC.

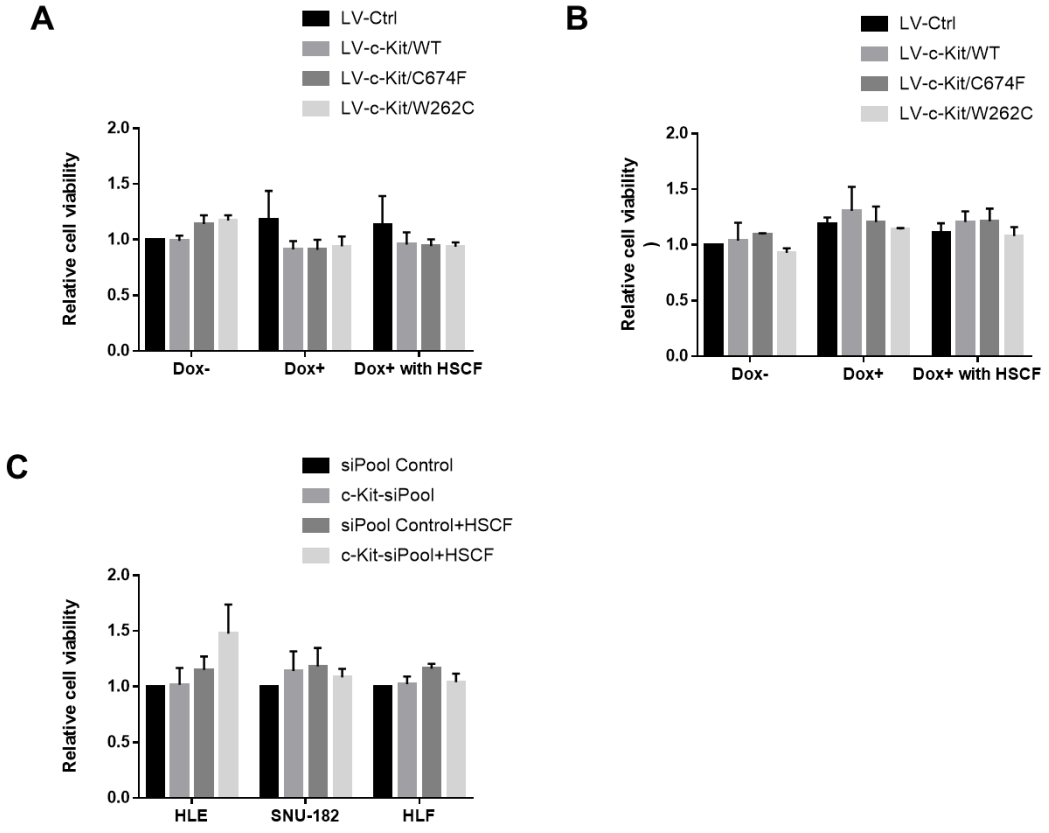


Figure 3.13: Relative cell viability of c-KIT overexpression and knock-down in HCC cells. (A and B) c-KIT wildtype (WT), C674F and W262C mutant protein expression was induced in HUH7 (A) and HEP3B (B) using a pTRIPZ based

doxycycline-inducible lentiviral system. (C) The knock-down of c-KIT in HLE, SNU-182 and HLF cells was conducted using c-KIT siPool tools. Cells were treated with 100 ng/mL human stem cell factor (HSCF) after measurement of day one. Relative cell viability at 48 h (cell viability of day 3 / cell viability of day 1) was calculated and normalized by Ctrl infected cells.

3.7 The effect of c-KIT expression alteration and mutations on the migration of HCC cell lines

Since migration is an important feature of HCC progression and regulation of migration by c-KIT was reported in colorectal carcinoma cells PMID: 28383479, the effect of c-KIT expression alteration and mutation on the migration of HCC cell lines was investigated by wound healing assay. To avoid interference caused by proliferation, c-KIT up- or down-regulated HCC cells were cultured in serum-free medium for 24 h after scratches were performed. To fully activated c-KIT, some of the cell groups received treatment with 100ng/mL HSCF after scratching. As shown in **Figure 3.14**, overexpression of c-KIT promoted migration of HUH7; this effect was stronger in Lv-c-KIT/WT infected cells. Although Lv-c-KIT-CF and Lv-c-KIT-WC infected cells showed perceptible promotion on migration, the difference has no statistical significance. HSCF treatment could not induce faster migration for c-KIT up-regulated HUH7. The results obtained from HEP3B cells were opposite. As shown in **Figure 3.15**, despite HSCF treatment or not, Lv-c-KIT-CF and Lv-c-KIT-WC had hardly any effect on migration of HEP3B cells, overexpression of c-KIT/WT even inhibited the migration.

Under the serum-free condition, there was no difference on migration between normal c-KIT and c-KIT knock-down HLE, SNU-182 and HLF cells without HSCF stimulation. The migration promoting the role of c-KIT emerged when HSCF was added. c-KIT knock-down cells did not respond to HSCF (**Figure 3.16 A**). One possible reason is that c-KIT signaling might not be fully activated

in the serum-free medium due to lack of enough HSCF and limited endogenous c-KIT. To prove this hypothesis, another migration assay performed on c-KIT knock-down cells cultured in complete medium. As expected, c-KIT knock-down cells showed inhibited migration even without the treatment of HSCF (**Figure 3.16 B**). However, HSCF treatment did not extend the difference between siPool-ctrl and c-KIT siPool transfected cells. It indicated that the SCF present in the supplemented FCS was sufficient to fully activated c-KIT. Hence additional HSCF supplementation could not increase migration promoted by activation of c-KIT. Taken together, the results obtained from the wound healing assay indicated that activation of c-KIT may promote migration of HCC cells. The c-KIT mutations C674F and W262C attenuated the role of c-KIT on migration compared with wild-type.

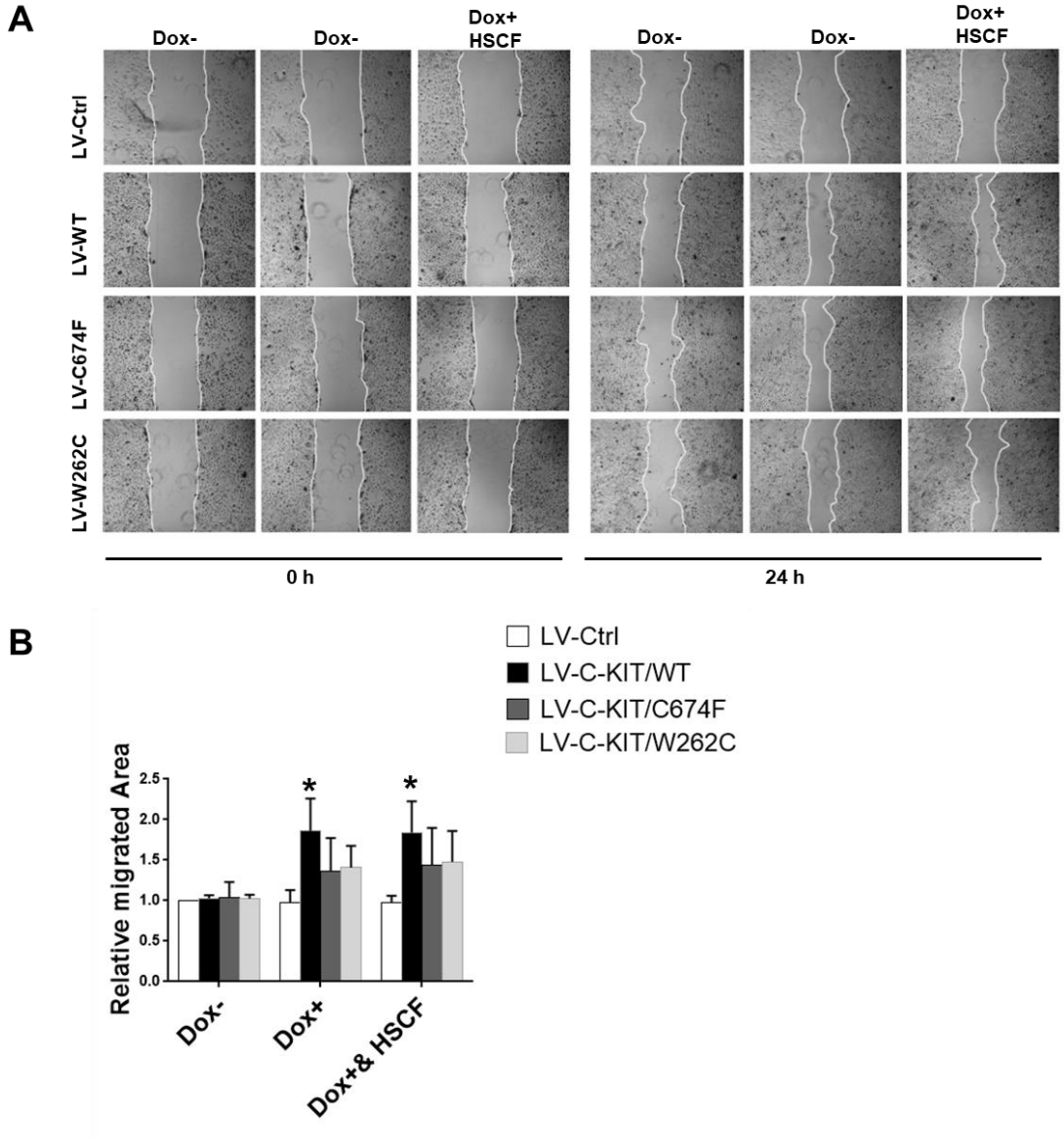


Figure 3.14: Effect of c-KIT overexpression on migration in HUH7. c-KIT wild-type (WT), C674F and W262C mutant proteins were overexpressed in HUH7 cells using a pTRIPZ based doxycycline-inducible lentiviral system. Control lentivirus (Lv-Ctrl) was prepared from an empty pTRIPZ vector. Cells were treated with 100 ng/mL human stem cell factor (HSCF) for 24 h after scratching and cultured in serum-free medium. The migration ability was estimated by wound healing assay. (A) Compared with LV-Ctrl infected cells, infection of LV-c-KIT/WT promoted migration of HUH7 cells in an HSCF independent way. LV-c-KIT/C674F and LV-c-KIT/W262C could also promote migration but without statistic difference. (B) Relative migrated area [1-(gap area of 24 h/ gap area of 0 h)] was calculated. Data were normalized by HSCF-treated and

LV-c-KIT/Ctrl infected cells and presented as mean ± SD of technical duplicates. *P<0.05 compared with LV-Ctrl infected cells in each of condition.

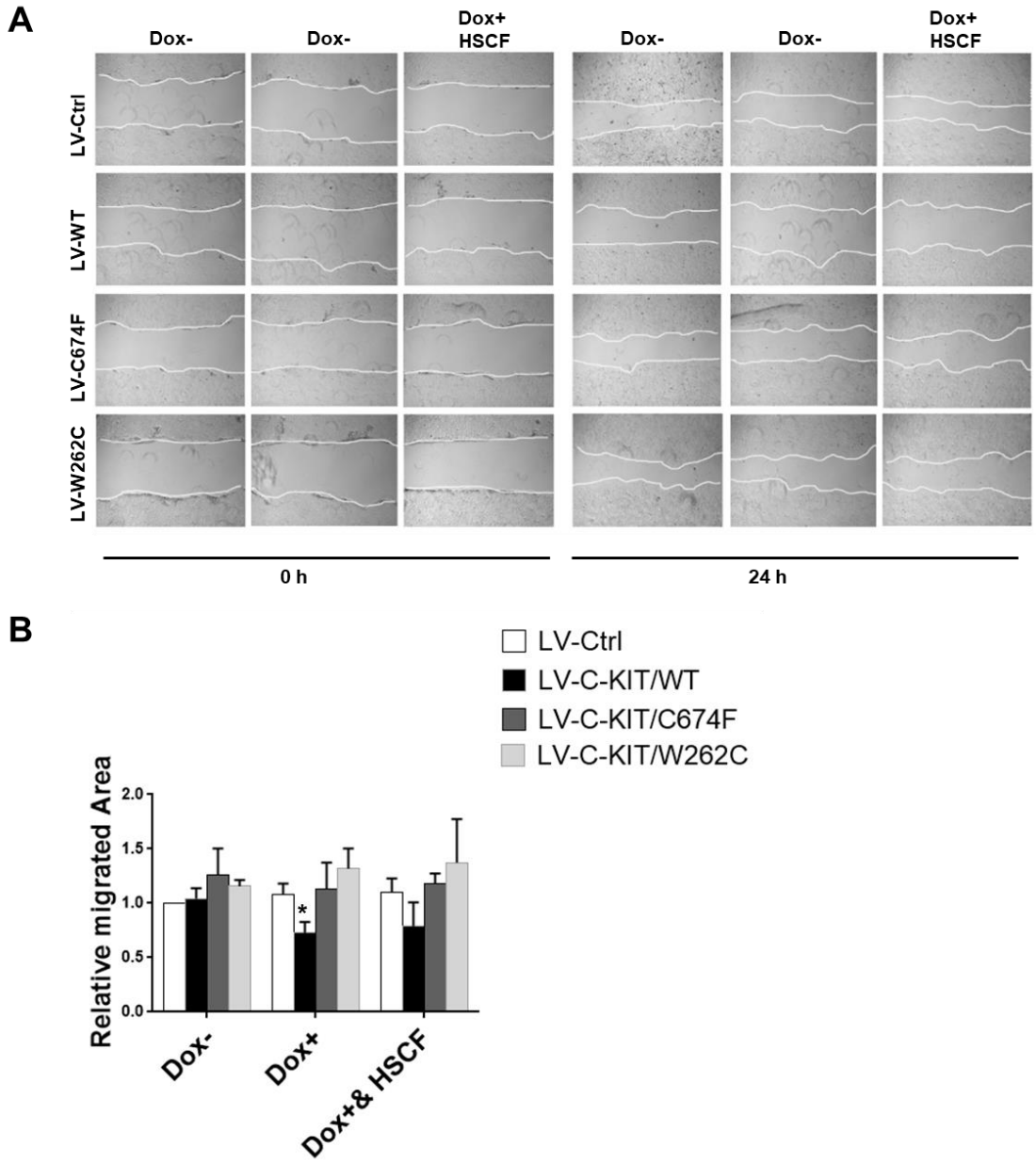
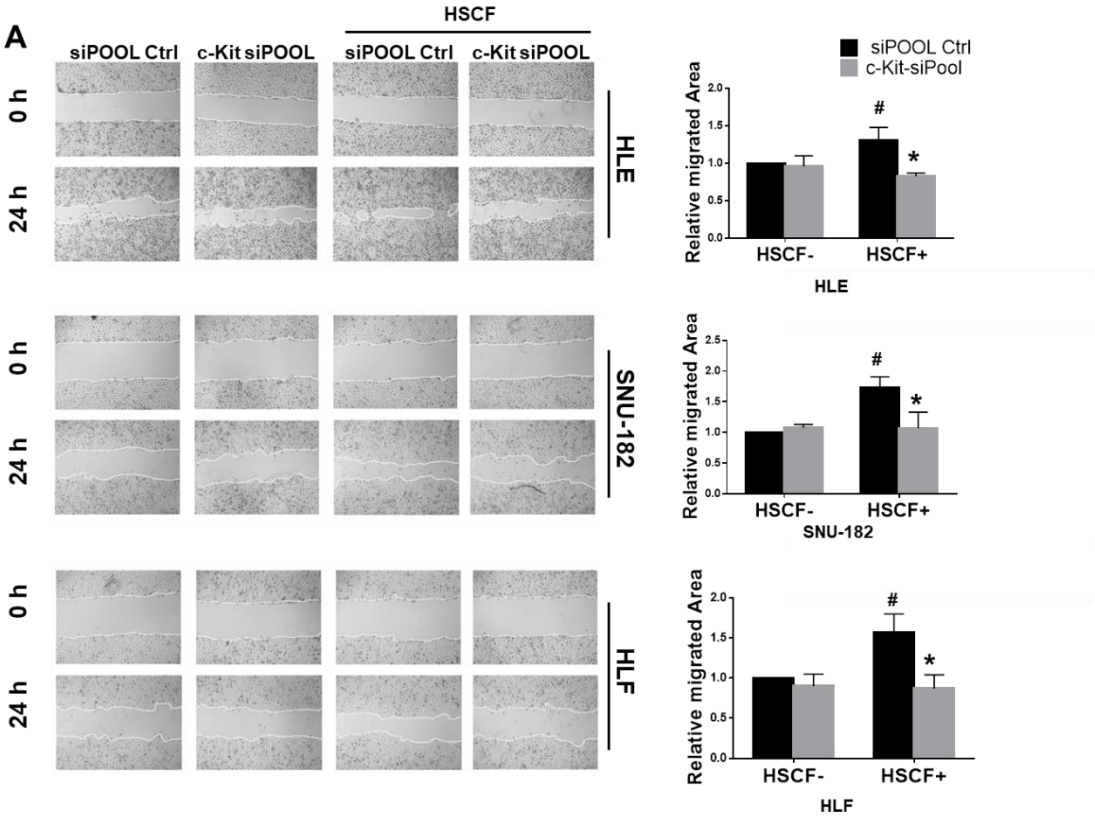


Figure 3.15: Effect of c-KIT overexpression on migration in HEP3B. c-KIT wild-type (WT), C674F and W262C mutant proteins were overexpressed in HEP3B cells using a pTRIPZ based doxycycline-inducible lentiviral system. Control lentivirus (Lv-Ctrl) was prepared from an empty pTRIPZ vector. Cells were treated with 100 ng/mL human stem cell factor (HSCF) for 24 h after scratching and cultured in serum-free medium. The migration ability was estimated by wound healing assay. (A)

Compared with LV-Ctrl infected cells, infection of LV-c-KIT/WT promoted migration of HEP3B cells in an HSCF independent way. LV-c-KIT/C674F and LV-c-KIT/W262C could also promote migration but without statistic difference. (B) Relative migrated area [1-(gap area of 24 h/ gap area of 0 h)] was calculated. Data were normalized by HSCF-treated and LV-c-KIT/Ctrl infected cells and presented as mean ± SD of technical duplicates. *P<0.05 compared with LV-Ctrl infected cells in each of condition.



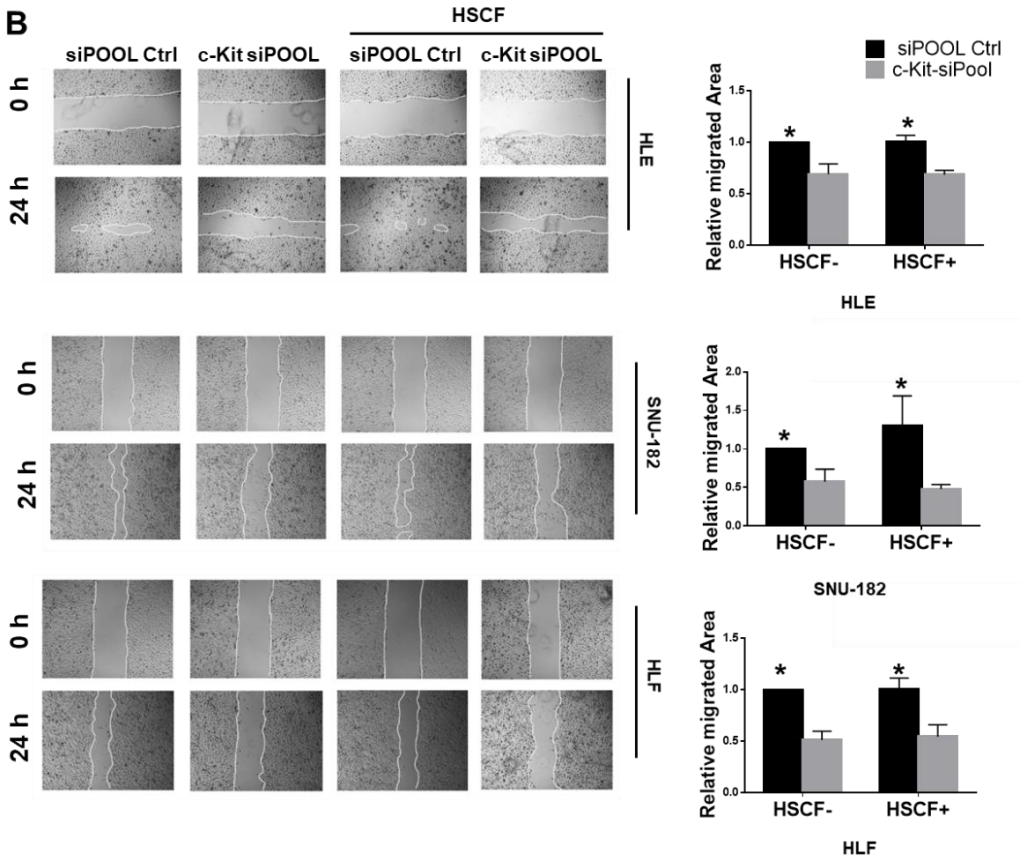


Figure 3.16: Effect of c-KIT knock-down on migration in HLE, SNU-182, and HLF. The migration ability was estimated by wound healing assay. c-KIT siPool (30 selected target siRNAs) was used to knock-down c-KIT expression in HLE, SNU-182, and HLF. Cells were treated with 100 ng/mL human stem cell factor (HSCF) for 24 h after scratching and cultured in serum-free medium (A) or complete medium (B). (A) In serum-free medium, HSCF treatment promoted the migration depended on the c-KIT expression. Relative migrated area [1-(gap area of 24 h/ gap area of 0 h)] was calculated. (B) In complete medium, c-KIT knock-down inhibited cell migration independent of additional stimulation with HSCF. Data were normalized by HSCF-treated and -control transfected cells and presented as mean ± SD of technical duplicates. **P*<0.05 compared with control transfected cells in each of condition. $^{\#}$*P*<0.05 compared with control cells without HSCF treatment.

3.8 c-KIT regulated epithelial-mesenchymal transition in HCC cell lines

Epithelial-mesenchymal transition (EMT) is defined as a process by which cell polarity and cell-cell adhesion of epithelial cells decreases but cells gain migratory and invasive features and acquire a mesenchymal stem cell phenotype. EMT plays an important role in migration and coincides with the initiation of metastasis during cancer progression. As shown above, c-KIT promotes the migration of HCC cell lines. Hence, it was tested whether c-KIT may promote migration by inducing EMT. EMT markers were detected in c-KIT-overexpressing and in c-KIT-knock-down HCC cells by qRT-PCR. First, the effective induction of c-KIT mRNA expression was confirmed in the Dox-inducible lentiviral expression system (Lv-c-KIT/WT) in HUH7 and HEP3B cells. Then, seven EMT markers including Snail1, Twist1, CDH1 (E-cadherin), CDH2 (N-cadherin), Vimentin, ZEB1 and ZEB2 were tested at the mRNA level (**Figure 3.17 A and B**). And it was found that of these 7 EMT genes Twist, N-cadherin, and Vimentin significantly increased in c-KIT overexpressing HUH7 cells (**Figure 3.17 C**; $P<0.05$). However, ZEB1 was the only EMT maker with statistically significant alteration in HEP3B (**Figure 3.17 D**; $P<0.05$). But ZEB1 in contrast to the expected induction of ZEB1, ZEB1 significantly decreased in HEP3B upon c-KIT expression. This finding is in line with cell the migration results of HEP3B. Thus, the decreased ZEB1 matched the c-KIT induced migration changes in HEP3B.

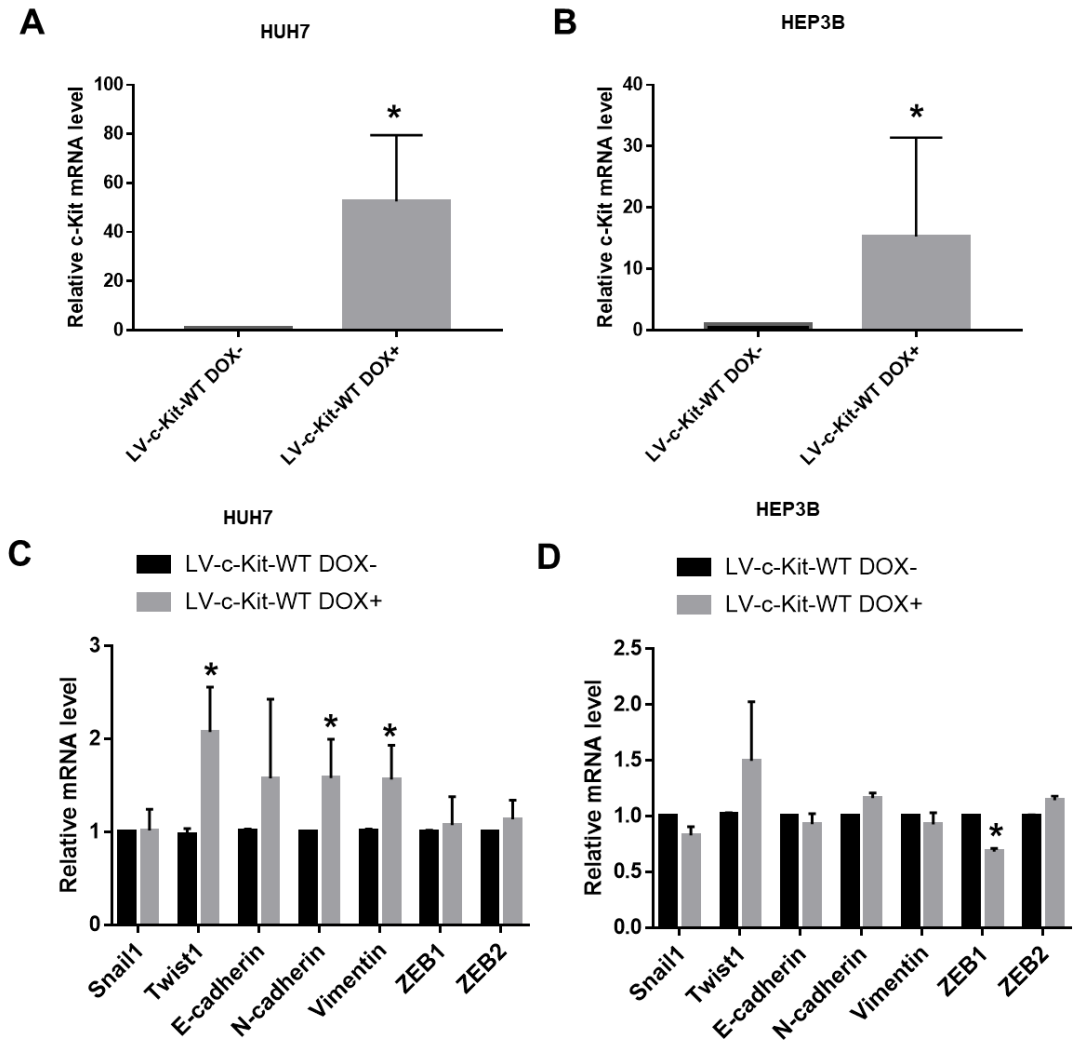


Figure 3.17: Epithelial-mesenchymal transition markers in c-KIT overexpressing HCC cell lines. (A and B) c-KIT mRNA level effectively increased in Lv-c-KIT/WT infected HUH7 and HEP3B cells after doxycycline induction. (C and D) mRNA level of Twist, N-cadherin, and Vimentin significantly increased in c-KIT overexpressed c-KIT overexpressed HUH7 cells. ZEB1 decreased in c-KIT overexpressed HEP3B cells. Data were presented as mean ± SD of technical duplicates. *P<0.05 compared with cells without doxycycline induction.

Next, the expression of these EMT markers was analyzed upon knock-down of endogenous c-KIT protein in HCC cell lines. The results obtained from HLE, SNU-182 and HLE cells were consistent with the results of HUH7. Knock-down of c-KIT significantly inhibited the mRNA levels of Snail1, N-cadherin, Vimentin

and ZEB1 (**Figure 3.18**; *P*<0.05), compared with control cells. The changes of EMT markers indicated that c-KIT induce EMT in HCC cells.

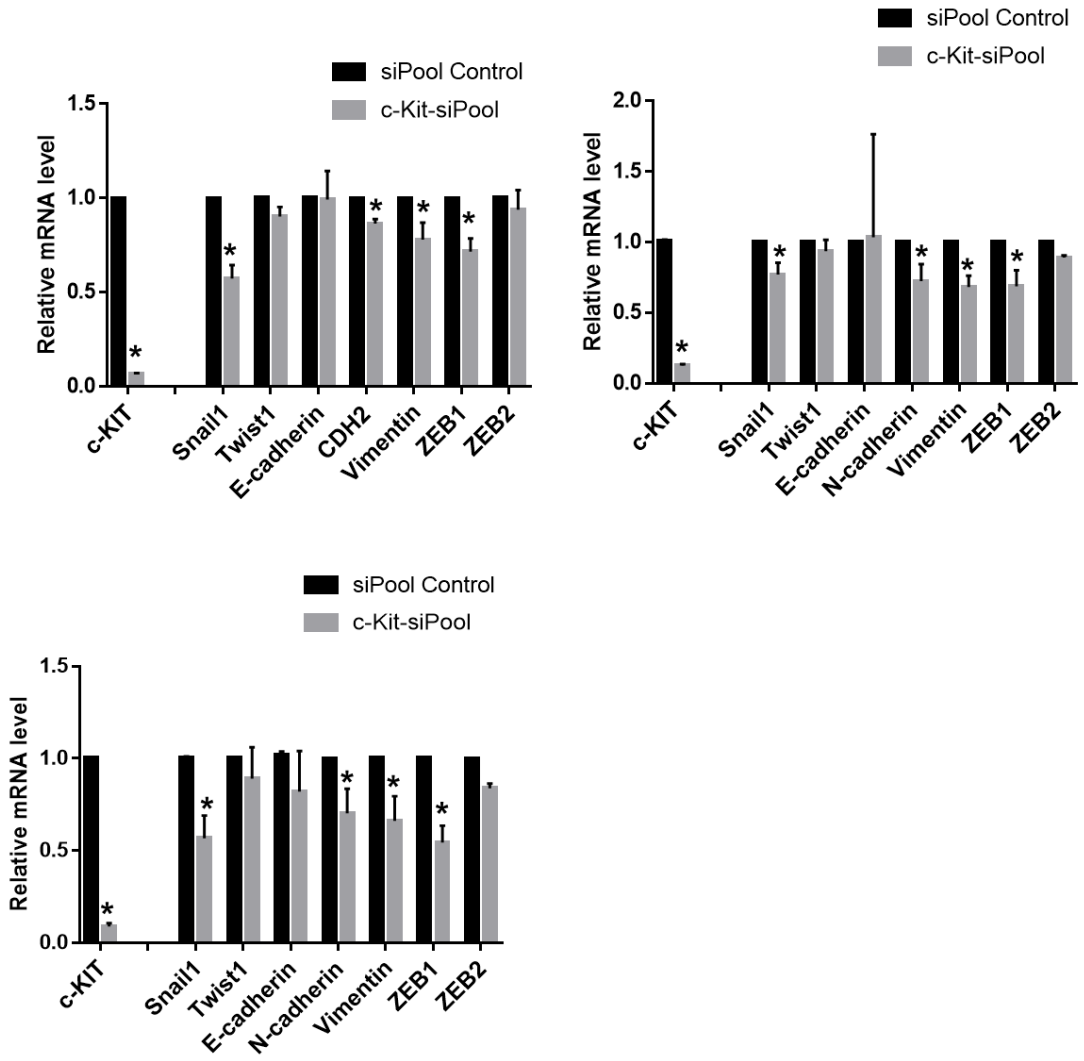


Figure 3.18: Epithelial-mesenchymal transition markers changes in c-KIT knock-down HCC cell lines. (A-B)The mRNA of c-KIT was significantly inhibited by c-KIT-siPool in HLE, SNU-182 and HLF cells. After Knock-down of c-KIT, mRNA levels of Snail1, N-cadherin, Vimentin and ZEB1 significantly declined. Data were presented as mean ± SD of technical duplicates. **P*<0.05 compared with cells transfected siPool control.

3.9 Selection and validation for candidate gene regulated by c-KIT

To select potential target genes of c-KIT four public datasets of HCC patients were analyzed using a series of bioinformatic methods and validated by qRT-PCR. Three datasets (accession ID: GSE14520, GSE 22058 and GSE25097) were retrieved from Gene Expression Omnibus (GEO). More details about these three dataset can be found in **Table 4.6**. Another HCC dataset was downloaded from Cancer Genome Atlas (TCGA-LIHC). Firstly, the c-KIT level in tumor tissues of HCC patients was estimated. Using the median expression of c-KIT as a cut-off value, patients were divided into two groups including patients with high c-KIT expression and patients with low c-KIT expression in each dataset. The differential gene expression profiles between two groups were calculated and genes with fold change>1.2 (P<0.05) were selected. As shown in **Figure 3.19 A**, eight genes were selected through intersecting results from each dataset. In all of 3 GEO datasets, these eight genes are significantly altered in tumor tissues of HCC patients with high c-KIT level (**Figure 3.19 B**).

To further filter the 8 genes mentioned above the clinical roles of these genes on the prognosis of patients was determined. Overall survival was available in datasets GSE14520 and TCGA-LIHC. Thus, patients were grouped according to the median expression level of each of the 8 genes, respectively. Kaplan–Meier curves were drawn followed and statistical analysis was performed using the log-rank test. It appeared that higher GJA1, PLXDC1, CXCR7, and KCJN2 expression were associated with poor prognosis of HCC patients (**Figure 3.20 and 3.21**).

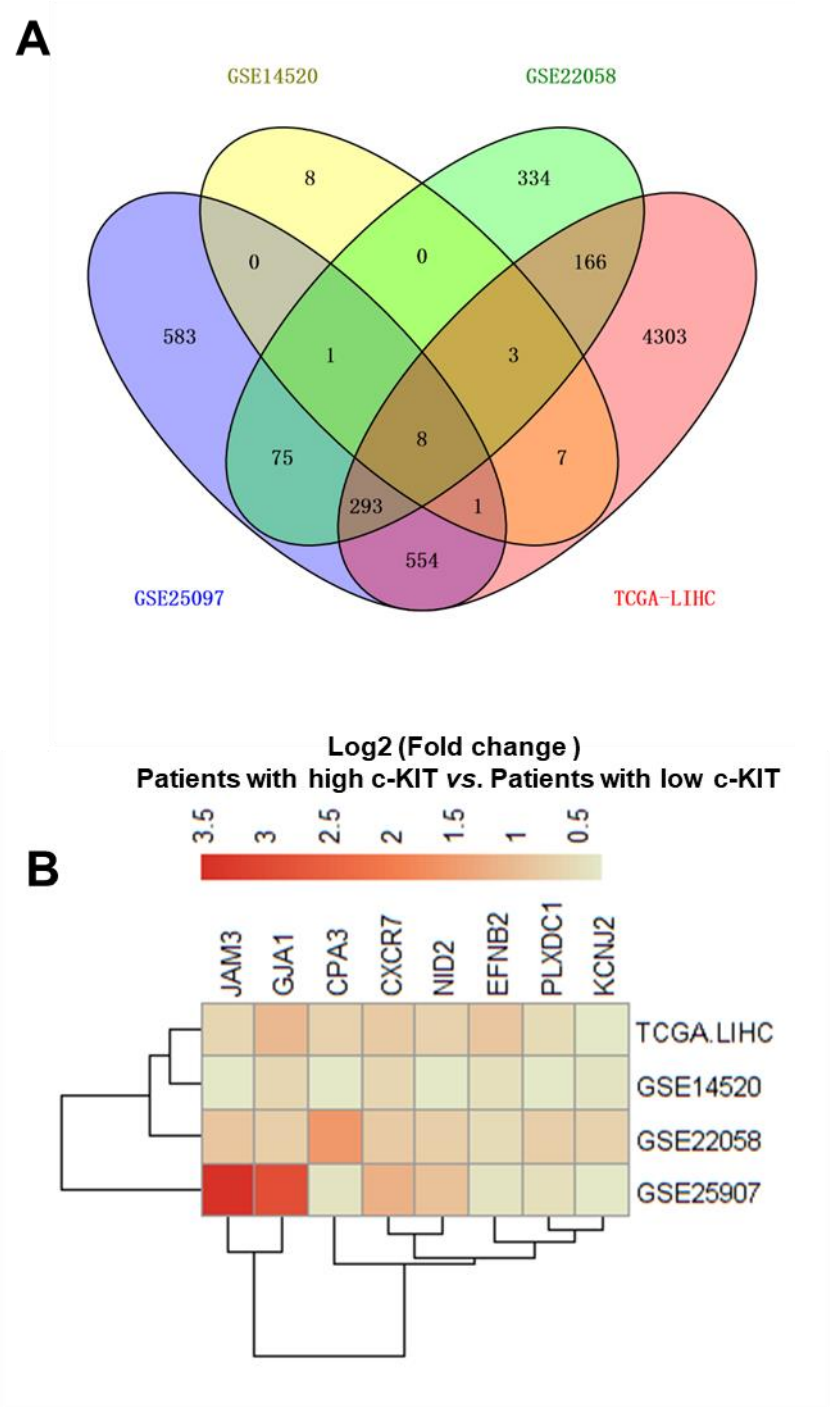


Figure 3.19: Eight potential target genes of c-KIT were selected based on four public HCC patients' datasets. (A) Patients in each dataset were divided into two groups according to the median expression level of c-KIT in tumor tissues. Differential gene expression of patients with high or low c-KIT expression were selected using fold change>1.2, *P*<0.05 as a criterion. Eight genes fulfilled these criteria and were selected after integration of results of the four datasets. (B) The log2 fold change of

the 8 selected genes between high and low expression groups in the three GEO datasets and TCGA-LIHC dataset. All 8 genes were significantly higher in tumor tissues of patients with high c-KIT level. DEG: different expression gene.

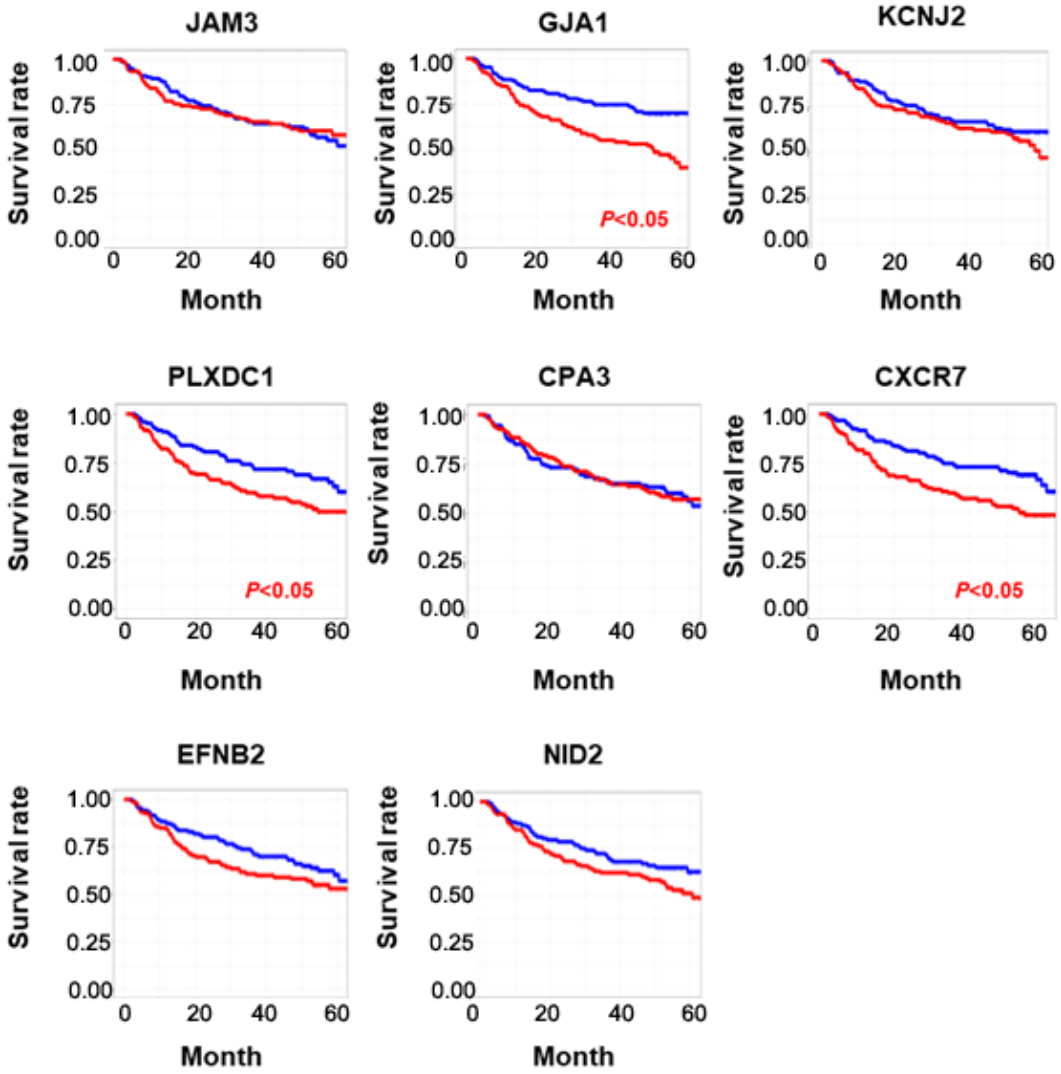


Figure 3.20 the correlation of 8 candidate genes with the prognosis of HCC patients. Patients with HCC from dataset GSE14520 were grouped into two groups using the median of each candidate gene as the cut-off value, respectively.

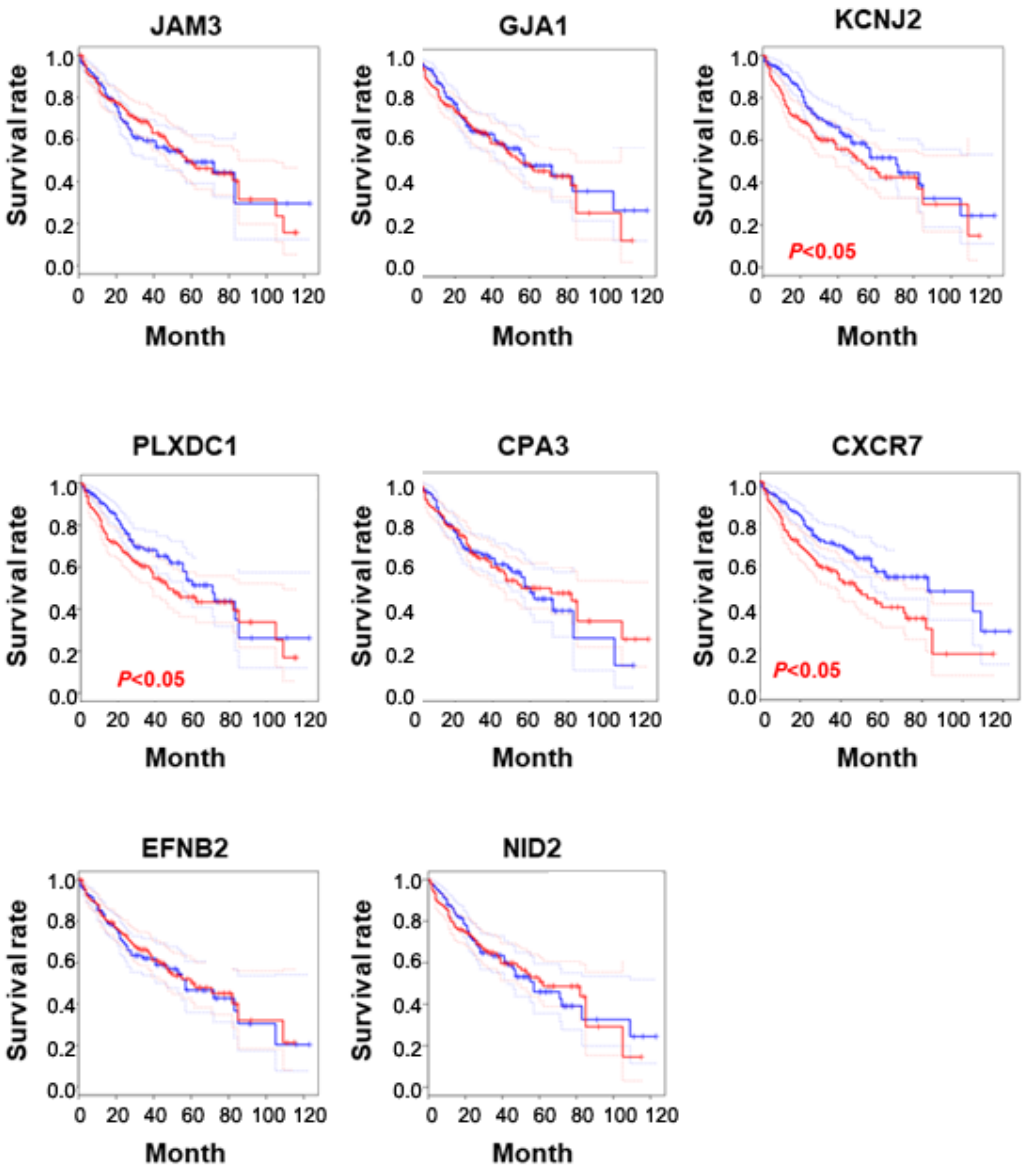


Figure 3.21: the correlation of 8 candidate genes with the prognosis of HCC patients. Patients with HCC from TCGA-LIHC dataset were grouped into two groups using the median of each candidate gene as the cut-off value, respectively. The survival data from TCGA-LIHC dataset was provided by GEPIA online tools.

In addition, to assess the role of the 8 candidate genes on the patient prognosis, univariate and multivariate Cox hazard analysis was performed. Univariate Cox hazard analysis showed that 6 of 8 candidate genes may be regarded as a risk factor for poor prognosis in HCC patients (**Table 3.2**). Furthermore, multivariate Cox hazard analysis was performed for these 6

candidate genes as input (hazard ratio >1.0 and *P*<0.05). After adjustment for confounders, PLXD1 and KCNJ2 were identified as independent risk factors for poor prognosis of HCC patients (**Table 3.3**).

Table 3.2: Univariate Cox hazard analysis for 8 candidate genes

	Coefficients	Hazard ratio	95% CI	*P* value
JAM3	0.15	1.17	0.85-1.59	0.329
GJA1	0.27	1.31	1.09-1.59	0.004
CXCR7	0.16	1.18	1.01-1.38	0.03
NID2	0.43	1.54	1.12-2.10	0.006
PLXDC1	0.26	1.30	1.01-1.67	0.04
EFNB2	0.29	1.35	1.03-1.76	0.02
CPA3	0.001	1.01	0.69-1.46	0.96
KCNJ2	0.334	1.40	1.04-1.87	0.02

Table 3.3: Multivariate Cox hazard analysis for 8 candidate genes

	Coefficients	Hazard ratio	95%CI	*P* value
GJA1	-0.09	0.90	0.53-1.53	0.72
CXCR7	0.01	1.01	0.64-1.59	0.96
NID2	0.37	1.45	0.88-2.38	0.14
PLXDC1	0.53	1.71	1.07-2.72	0.02
EFNB2	-0.04	0.95	0.57-1.59	0.86
KCNJ2	0.48	1.62	1.03-2.59	0.03

Based on the multivariate Cox hazard results, a model was built combining the expression analysis of both genes, PLXDC1 and KCNJ2 which were significant in the multivariate Cox hazard analysis. This resulted in the multivariate-based *risk score y =0.598 PLXDC1+0.520 KCNJ2.* In datasets GSE14520 and TCGA-LIHC, the risk score of patients was calculated by this model. Patients with high-risk score had a lower 5-year survival rate in both independent cohorts confirming that PLXD1 and KCNJ2 may be used as risk factors in HCC (**Figure 3.22**; *P*<0.05). This evidence suggested that GJA1,

PLXDC1, KCNJ2, CXCR7, NID2, and EFNB2, may be potential c-KIT targeted genes, may be involve disease progression and my result in poor prognosis of HCC patients.

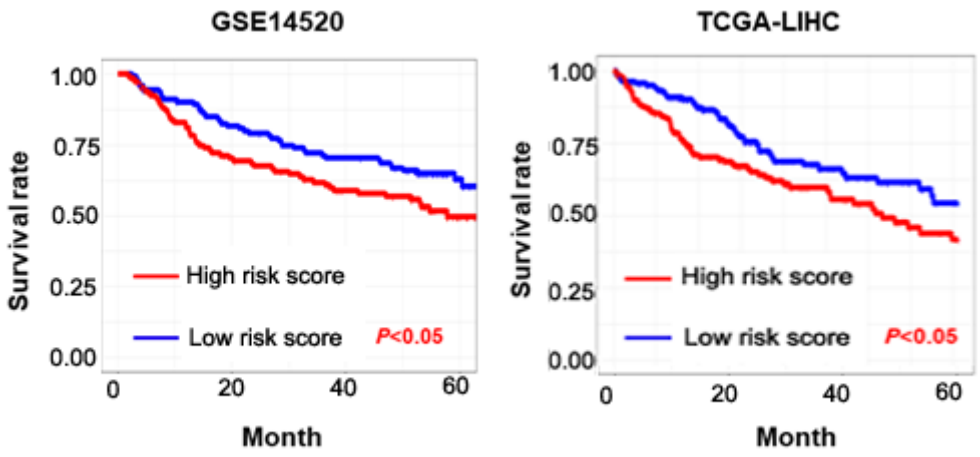


Figure 3.22: Model obtained from Cox hazard analysis may predict patient prognosis. the risk score y =0.598 PLXDC1+0.520 KCNJ2 was built based on Cox hazard analysis which reflects the risk for poor prognosis of HCC patients. In dataset GSE14520 and TCGA-LIHC, the risk score of patients was calculated by this model Kaplan-Meir curves of patients with high risk or low risk score are depicted. P value was estimated by the log-rank test.

Furthermore, the expression of the 8 selected c-KIT-related candidate genes was analyzed in an RNA sequencing dataset of 8 different HCC cell lines (unpublished data of AG Rössler, **Figure 3.23**). According to the endogenous c-KIT expression, HCC cell lines were grouped based on their endogenous c-KIT expression as negative and positive cell lines. The difference in the expression of the 8 candidate genes between c-KIT+ and c-KIT– HCC cell lines revealed that PLXDC1, KCNJ2, JAM3, and GJA1, were higher in endogenous c-KIT-positive HCC cell lines which is in concordance with the results of the patient expression profiles.

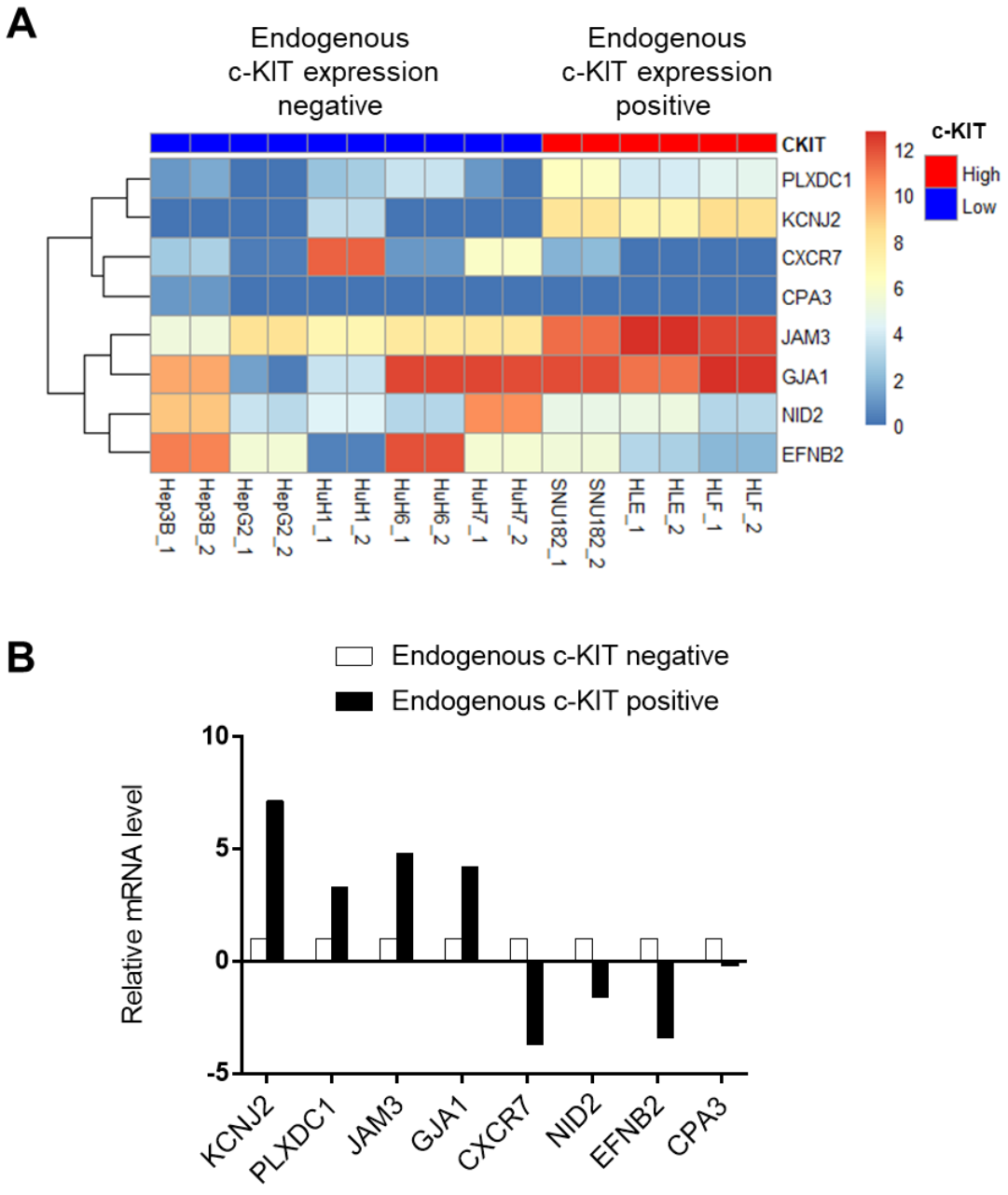
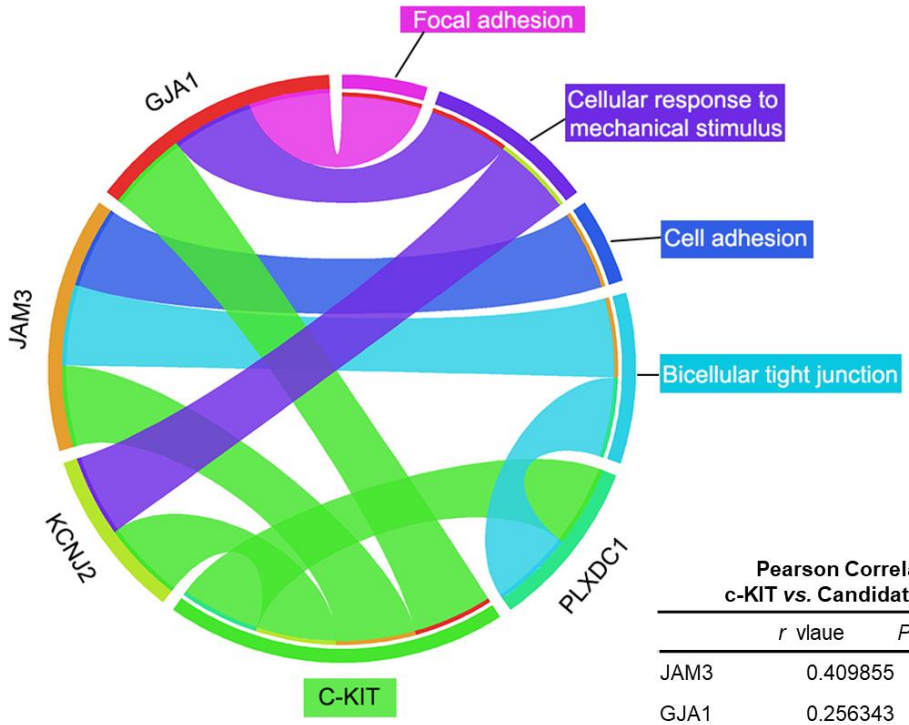


Figure 3.23: Expression difference of 8 candidate genes between endogenous c-KIT-positive and negative HCC cells. (A) Based on the RNA sequencing data, eight HCC cell lines were grouped c-KIT-negative and positive cell lines. The expression profiles of 8 potential c-KIT related genes were shown as a heat map. (B) The relative fold changes of 8 candidate genes between c-KIT-positive and negative HCC cells were calculated.

Furthermore, the correlation coefficient between c-KIT and PLXDC1, KCNJ2, JAM3, or GJA1 was calculated by Pearson Correlation analysis using the data from GSE14520 and matched Gene Ontological (GO) biological process terms were inquired from the database of Gene Ontology Consortium (http://www.geneontology.org/). The GO biological process terms of PLXDC1, KCNJ2, JAM3, and GJA1 included cell adhesion, bicellular tight junction and response to a mechanical stimulus which could be associated with cell migration. Statistically significant correlation of c-KIT with PLXDC1, KCNJ2, JAM3, and GJA1 were observed **(Figure 3.24)**.



Pearson Correlation
c-KIT *vs.* Candidate genes

	r vlaue	*P* value
JAM3	0.409855	2.00583E-11
GJA1	0.256343	4.56935E-05
PLXDC1	0.323884	1.93581E-07
KCNJ2	0.278663	8.76112E-06

Figure 3.24: Gene Ontological (GO) biological process terms of four potential c-KIT targeted genes and the correlation with c-KIT expression levels in HCC tissues. The GO biological process terms of PLXDC1, KCNJ2, JAM3, and GJA1 included focal adhesion, cell adhesion, bicellular tight junction and response to

mechanical stimulus. Statistically significant correlation of c-KIT with PLXDC1, KCNJ2, JAM3, and GJA1 are shown in the table of the bottom right corner.

Combining the evidence obtained from the patients datasets and the HCC cell lines, PLXDC1, KCNJ2, JAM3, and GJA1 were selected forvalidation *in vitro*. Thus, c-KIT expression of HLE, SNU-182 and HLF cells was knockeddown using siPool. the mRNA level changes of PLXDC1 KCNJ2, JAM3, and GJA1, were measured by qRT-PCRJAM3 and GJA1 decreased after c-KIT knock-down in both three cell lines that resulted as expected (**Figure 3.25**). However, the PLXDC1 mRNA level increased in c-KIT silenced HLE and HLF cells. The KCNJ2 mRNA was not changed in c-KIT knock-down HLF cells.

The data mentioned above indicated that c-KIT might regulate some of the genes such as PLXDC1, KCNJ2, JAM3 and GJA1 which were correlated with the prognosis of patients with HCC. c-KIT may also promote migration through these genes. However, further investigation needs to prove this hypothesis.

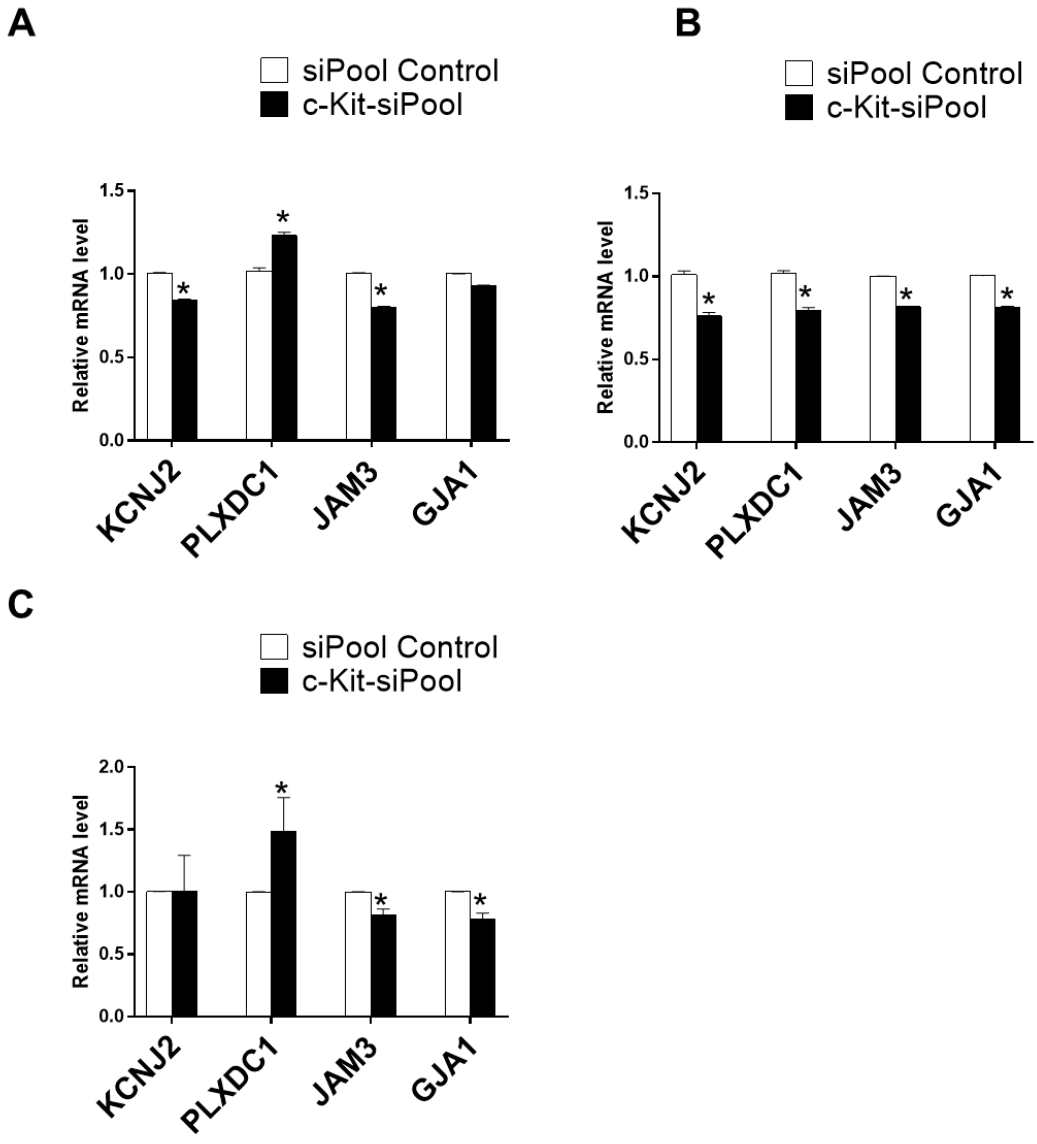


Figure 3.25: PLXDC1, KCNJ2, JAM3 and GJA1 expression changes after c-KIT knock-down HCC cell lines. c-KIT expression of HLE (A), SNU-182 (B) and HLF (C) cells was knocked-down by siPool. the mRNA level changes of PLXDC1 KCNJ2, JAM3, and GJA1, were measured by qRT-PCR. Data were normalized by HSCF-treated and siPool control transfected cells and presented as mean ± SD of technical duplicates. *P<0.05 compared with siPool control transfected cells in each of condition.

4. DISCUSSION

HCC is a leading cause of cancer-related deaths and characterized by a growing incidence worldwide (Cadier *et al.*, 2017). Despite the implementation and improvement of screening for high-risk individuals, most HCC patients are diagnosed in an advanced stage which means that current curative treatment options (e.g., liver transplantation, liver resection, and ablative procedures) cannot be applied Hence, the median overall survival for advanced cases remains poor (Raza and Sood, 2014).The advent and application of molecularly targeted therapy have created an encouraging trend in the management of some advanced cancer patients. For example, a benefit for the survival of advanced non-small lung cancer patients provided by diversified targeted drugs has been well demonstrated (Lategahn *et al.*, 2018). However, the development of HCC targeted drugs has failed so far. Currently, Sorafenib, a multi-targeted kinase inhibitor, is the only drug proven to improve the outcome of patients with advanced HCC providing an overall survival benefit of 2-3 months (Ardelt *et al.*, 2018). The last few years have witnessed the failure of several first and second line phase III clinical trials of novel molecularly targeted therapies. To dissolve this misery, expanded molecular and functional knowledge of the potential target of HCC therapy in drug development is urgently needed (Fujiwara *et al.*, 2018).

The receptor tyrosine kinase c-KIT has been reported to function as an oncogene and to play a crucial role in the development of some cancer entities. Evidence shows that aberrantly activated c-KIT promotes cell proliferation and survival in several cancers (Lamarca *et al.*, 2013). Promising results of using c-Kit as a therapeutic target for treatment of cancers have been observed in GIST, AML, melanoma and colorectal cancer (Shah and van den Brink, 2015; Stankov *et al.*, 2014). However the limitations in clinical application of using c-KIT as target cannot be ignored. For example, in GIST c-KIT inhibitors are

effective only when treating c-KIT wildtype or specific mutations (e.g., D816V.) (Noujaim *et al.*, 2016). A number of c-KIT mutations (e.g., D816V and D816Y) may result in resistance to imatinib but are sensitive to other tyrosine kinase inhibitors (Growney *et al.*, 2005). Hence exploring more mutations of c-KIT and identifying their matched inhibitors are imperative as well as further studies in functions of wild-type c-KIT. Upon activation through binding of stem cell factor (SCF), c-KIT exerts its function by intracellular signaling transduction. The typical downstream signalings of c-KIT include the MAPK pathway, PI3K/AKT, and Jak-STAT signaling which are pathways affected in hepatocarcinogenesis. Thus, c-KIT may be involved in HCC and therefore, it may also be a key therapeutic target for HCC (Salgado *et al.*, 2014). However, the role of c-KIT in HCC has been poorly studied, and results about c-KIT in HCC were inconsistent in previous studies. From the perspective of personalized medicine and precision medicine, c-KIT can be considered as an ideal target as it is a receptor with kinase activity. Therefore, I investigated the functional role and related mechanisms of c-KIT and its mutations in HCC. In addition, the correlation of c-KIT with patients' prognosis was considered to test if c-KIT may also be used as a molecular marker.

4.1 Expression of c-KIT in tissue samples from patients with HCC and its clinical relevance

To make sure that the knowledge of c-KIT could be applied to clinical practices, firstly, I estimated the expression feature of c-KIT in tissue samples from a patient with HCC and analyzed the correlation of c-KIT with clinical characters such as stage and prognosis. For this analysis, gene expression dataset of HCC patients with a relatively large sample size were downloaded from the GEO (GSE14520, GSE76427, GSE36376 and GSE76297) and TCGA-LIHC databases. This included a total of 1148 HCC samples from different geographical regions (China, South Korea, Singapore and Thailand) and with different etiologies (HBV- related or not).

In all four datasets, c-KIT was statistically higher in tumor tissues compared to non-tumor tissues. For dataset GSE14520, patients BCLC and CLIP staging were available. Patients with advanced stage had higher c-KIT expression (P<0.05). This indicates that c-KIT may be involved with the progression of HCC. The elevated expression of c-KIT in tissues and the increased expression in patients with higher stage supported the potential oncogenic role of c-KIT in HCC. The mean difference in c-KIT expression between tumor and non-tumor tissues was relatively small which may be the result of the high heterogeneity among HCC patients. Some patients had high induction of c-KIT, which may indicate that they represent a specific subgroup of HCC patients. Using the median value of c-KIT in tumor tissues as cut-off I grouped patients with HCC into patients with high or low c-KIT expression level. Because the clinical information was not available for two datasets, I only analyzed the correlation of c-KIT with prognosis in the dataset GSE 14520 (Roessler et al.) and the TCGA-LIHC dataset. I found that patients with higher c-KIT expression had poorer prognosis. When using the third quartile as a cut-off, the difference on survival rate because more significant suggesting that one quarter of patients fall in a subgroup of patients with most relevant c-KIT expression (data not shown). Until now characterization of c-KIT expression and its clinical relevance in HCC were only reported in few studies. Yan et al. showed that 48.1% of cases expressed c-KIT in a cohort consisting of 206 HCC cases. High c-KIT expression was considered an independent unfavorable prognostic factor for patients with HCC (Yan *et al.*, 2018). However, other studies did not confirm this relatively high number of c-KIT positive HCC cases. Becker et al. reported that the overall c-KIT-positive rate in HCCs was only 2.3%. Hence, they suggested that c-KIT is not significantly overexpressed in HCC. But this study did not use non-tumor tissue as a control (Becker *et al.*, 2007). Chung et al. reported that 26% of HCC tissues are c-KIT-postive And they found that in contrast to Yan et al. high c-KIT correlated with better prognosis (Chung *et al.*, 2005). Thus, available studies on c-KIT expression in HCC tumor tissues and

on the clinical relevance of c-KIT expression on patient prognosis are inconsistent.

Potential underlying problems are the use of different antibodies, the lack of reference tissue to analyze the relative expression and the overall high heterogeneity within and between the HCC cohorts. As these results were obtained by immunohistochemical staining, the discrepant results may be caused by a wide variety of antibody quality, staining or scoring protocols. Another potential reason includes the cellular heterogeneity of HCC or the distinct stages of cell differentiation. These limitations did not apply to our analysis using mRNA expression profiles of HCC tumor and non-tumor tissues which supported the hypothesis that c-KIT is involved in the HCC progression and be function as an oncogene. Hence, functional assays were performed to analyze the role of c-KIT on cell migration and proliferation.

4.2 Mutations of c-KIT in patients with HCC

Deregulation of c-KIT expression in tumorigenesis may be caused by aberrant epigenetic regulation or mutations (Abbaspour Babaei *et al.*, 2016). Some mutations of c-KIT (such as V560G and L576P) lead to disruption of auto-inhibitory mechanisms, inducing dimerization and continuous activation of kinase domains which subsequently induce excessive activation of downstream pathways. Hence these mutations are regarded as activating or gain-of-function mutations (Ashman and Griffith, 2013). The most striking involvement of c-KIT mutation is observed in GIST. More than 80% of GIST cases display activating c-KIT mutations resulting in constitutive activation. Constitutive activation of c-KIT signaling caused by activating mutations of c-KIT are also observed in AML (Beghini *et al.*, 2004). In these cases c-KIT mutations play a main role in the initiation of tumorigenesis inhibiting the c-KIT kinase activity may be a target for cancer therapy. In contrast, in some cases of melanoma and breast cancer inactivating or loss-of-function mutations of c-KIT

were observed (Eigentler *et al.*, 2013; Tsutsui *et al.*, 2006). In these cases, c-KIT inhibition most likely would not have any benefit and may even have adverse effects. To meet the need for personalized medicine and precision medicine, the molecular function and mechanisms underlying specific c-KIT mutations need to be clarified.

For HCC, mutations of c-KIT have not yet been studied. Recent large scale genome sequencing projects enabled us to search for c-KIT mutations in large HCC cohorts. A relative low frequency of c-KIT mutations was observerd.

For instance, in the TCGA-LIHC dataset, the frequency of c-KIT mutation was 2.8% (10/357). In the HCC data set of the Asian Medical Center (AMC), only 0.83% of HCC patients had c-KIT mutations. In Catalogue of Somatic Mutations in Cancer database (COSMIC, https://cancer.sanger.ac.uk/cosmic) 1057 cases were included which combined the TCGA-LIHC dataset with other genome-wide screens. But only thirteen cases with c-KIT mutations were detected in these 1057 HCC patients. However, no publication has so far identified the role of specific c-KIT mutations in HCC patients. In DKFZ-HIPO (Heidelberg Center for Personalized Oncology) project 14, I analyzed 57 HCC patients by whole exome sequencing. Among the 57 HCC patients, three different c-KIT mutations, W262C, C674F, and L766I, were found in one patient each. All three mutations were validated by Sanger sequencing and confirmed to be somatic Thus, all three mutations are tumor specific and missense mutations. The c-KIT mutations C674F and L766I lie in exon 14 and exon 15, respectively. Exon 14 and exon 15 encode the kinase domain of c-KIT. These two mutations may influence the phosphorylation of c-KIT. The mutation W262C is in exon 5 which encodes the third Ig-like domain of c-KIT. Hence this mutation may impact the binding of SCF. To select the most probably functional mutations, I predicted the possible impact of these mutations on the structure and function of c-KIT using PolyPhen 2.0. The W262C and C674F

mutations yielded a high PolyPhen score which suggested an impact on protein structure and function. As the L766I mutation had a score 0 which implicated that the protein structure and function are not affected by this mutation, it was not included in further analyses. I tried to test whether these mutations are activating mutations or inactivating mutations. Moreover, their function on proliferation and migration of HCC cell lines was determined.

4.3 The endogenous expression of c-KIT IN different HCC cell lines

I selected appropriate HCC cell lines for further analysis according to the endogenous expression of c-KIT. The endogenous c-KIT expression among nine HCC cell lines was detected by Western blot and qRT-PCR. Results showed that the endogenous expression of c-KIT was completely absent or present. An unknown feedback mechanism might play a role to ensure the optimal c-KIT level that can facilitate the growth of specific cells. In addition, the different endogenous expression of c-KIT may be caused due to the heterogeneity of HCC. Thus, HCC cells coming from different origins may have different endogenous c-KIT levels. This could also explain why in previous studies inconsistent rates of c-KIT-positive HCCs were reported. In c-KIT-negative cell lines, the c-KIT-related downstream signaling pathways may also be defective as they are not required for the tumor cell. Hence in these cell lines only overexpression of c-KIT may not cause any expected phenotypic changes. In a clinical perspective, absent expression of c-KIT may be a barrier for the clinical application when using c-KIT inhibitors.

4.4 The effect of c-KIT expression alteration and mutations on relevant downstream pathways in HCC cell lines

As a tyrosine kinase receptor, the main function of c-KIT is mediating downstream intracellular signaling transduction by inducing phosphorylation. In the present work, the effect of c-KIT expression alteration and mutations on relevant downstream pathways in HCC cell lines was investigated by

measuring the phosphorylation level of relevant components. Frequently reported and HCC-related downstream signaling pathways of c-KIT are PI3K/AKT, MAPK, and JAK/STAT pathways (**Figure 4.1**). Few reports showed the downstream regulating pattern of c-KIT in HCC. Moreover, it is necessary to examine the effect of mutations on downstream signaling. Hence these three pathways were investigated in this thesis work.

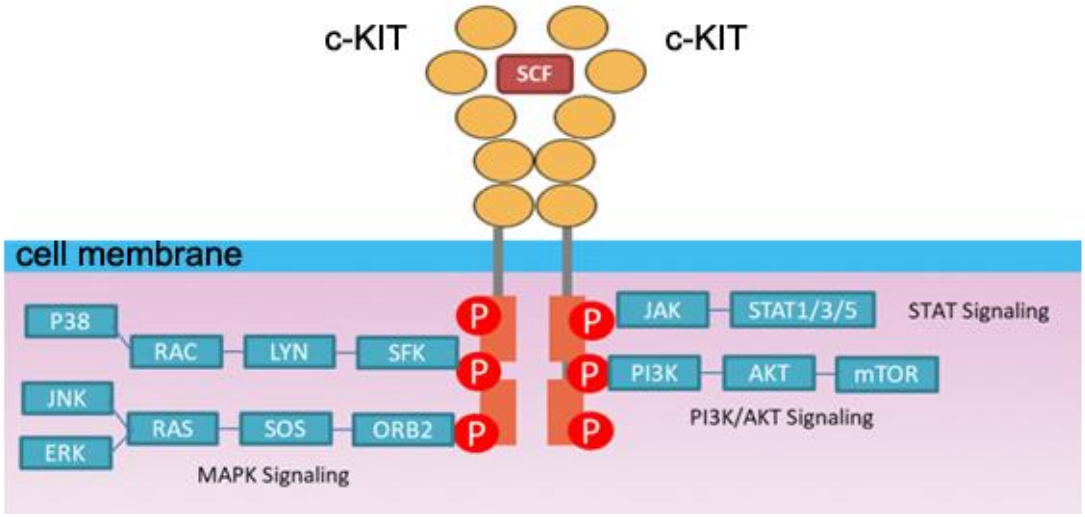


Figure 4.1: Schematic model of downstream pathways regulated by c-KIT. The binding of SCF to the c-KIT receptor results in activation of the MAPK signaling pathway, the PI3K/AKT pathway and the JAK2/STAT pathway.

Firstly, I examined the activation changes of c-KIT after HSCF treatment in HCC cell lines. c-KIT/W262C overexpressing HUH7, and HEP3B cells showed significantly lower p-c-KIT level, compared with cells with c-KIT/WT and c-KIT/C674F. this indicated that the W262C mutation inhibits HSCF-induced activation of c-KIT. The W262C mutation is located in exon 5 of c-KIT cDNA which encodes the third Ig-like domain. Therefore, I hypothesized that W262C might interfere with the HSCF binding. c-KIT/C674F showed no difference in the activation of c-KIT. As expected, p-c-KIT was reduced in c-KIT siPool-treated HCC cells due to the effective knock-down of total c-KIT protein expression.

Next, I analyzed the phosphorylation level of AKT to reflect the changes on PI3K/AKT signaling. PI3K/AKT signaling, which is subsequently activated by phosphorylation of c-KIT, is the most reported pathway involving c-KIT (Zhang *et al.*, 2011). Activation of PI3K/AKT mediated an apoptosis inhibitory and proliferation promoting effect of c-KIT/SCF signaling in GIST (Chen *et al.*, 2017) and AML (Yu *et al.*, 2016). As expected, increasing p-AKT was observed in c-KIT overexpressing HCC cells but not found in c-KIT knock-down cells. However, the p-AKT levels increased significantly less in c-KIT/W262C expressing cells compared to c-KIT/WT and c-KIT/C674F. This evidence indicated that the PI3K/AKT signaling is efficiently regulated by c-KIT in HCC and may be impaired by c-KIT/W262C mutation.

The MAPK family consists of highly conserved serine/threonine protein kinases involved in proliferation, motility, and cell survival in HCC. Todd et al. showed that oncogenic c-KIT mutants contribute to the progression of some melanomas by activating MAPK signaling (Todd *et al.*, 2014). Here, I examined the phosphorylation level of ERK1/2 and JNK in HCC cell lines and found that ERK1/2 increased and JNK signaling is activated by c-KIT signaling through stimulation with HSCF. However, c-KIT mutation W262C led to reduced JNK activation. Thus, the observed low c-KIT phosphorylation levels of c-KIT/W262C did not efficiently activate downstream MAPK signaling.

Finally, I investigated the activation changes of JAK/STAT signaling. The JAK/STAT pathway is a pleiotropic cascade used to transduce signals, involved in the tumor formation and proliferation in HCC (Wen *et al.*, 2018). Calvisi et al. demonstrated the importance of the constitutive activation of the JAK/STAT signaling in the development of HCC and found that negative regulators of JAK/STAT signaling showed growth-suppression activity (Calvisi *et al.*, 2006). Here, the phosphorylation level of JAK2, STAT1, and STAT3 were detected. Previous studies showed that c-KIT could elicit the activation of

STAT1 and STAT3 through activating JAK2 (Deberry *et al.*, 1997; Linnekin *et al.*, 1996). However, I observed varying effects. In HUH7 cells, only wild-type c-KIT overexpressing cells showed increased p-STAT1 level. The p-STAT3 level was decreased in c-KIT/WT, c-KIT/C674F and c-KIT/W262C overexpressing cells which is inconsistent with previous studies in other tumor entities. In HEP3B the phosphorylation level of STAT1 and STAT3 were like in previous studies. The W262C mutation also showed less effects as well as results obtained from PI3K/AKT and MAPK signaling. No significant difference in p-JAK2 was found in each condition. As Chaix et al. showed, JAK3 rather than JAK2 induced the activation of STAT1 and STAT3 in neoplastic mast cells with oncogenic KIT mutants (Chaix *et al.*, 2011). Hence, I assume that STAT1 and STAT3 were regulated by other members of JAK family excepting JAK2 in HUH7 and HEP3B. So far, I did not detect expression changes of JAK2 and JAK3 expression after c-KIT overexpression or knock-down. The lack of good Western blot antibodies against additional JAK family members prevented further analyses. It is a limitation of this work. On the other hand, I found the p-JAK2 level was decreased in c-KIT knock-down HLE and SNU-182 cells. However, I obtained inconclusive results for p-STAT1 and p-STAT3 changes upon knock-down of endogenous c-KIT. The p-STAT1 and p-STAT3 level decreased after stimulation with HSCF in a c-KIT-independent way. The knock-down of c-KIT in all three HCC cell lines tested did not alter the p-STAT1 and p-STAT3 levels. Taken together, it appears that p-STAT1 and p-STAT3 level is not a significant downstream pathway of c-KIT signaling in HCC cells.

In summary, c-KIT activated PI3K/AKT and MAPK pathways in HCC cells. However, c-KIT's role in JAK/STAT signaling still remains unclear. Most importantly, I confirmed that C674F is not an activating mutation and W262C mutation appears to be an inactivating mutation of c-KIT. Tsutsui et al. showed loss of c-KIT expression is associated with an advanced stage and poor

prognosis in breast cancer Tsutsui *et al.*, 2006). Thus, c-KIT inactivation may be clinically relevant.

4.5 The effect of c-KIT expression alteration and mutations on the proliferation and migration of HCC cell lines

Oncogenic c-KIT activation caused by overexpression or mutation could promote proliferation and migration in cancers through regulating various downstream pathways. Yasuda et al. showed proliferation and invasion were significantly enhanced in c-KIT-positive pancreatic cancer cells (Yasuda *et al.*, 2006). In the other study, they also found c-KIT may enhance the proliferation and invasion in colorectal cancer cells (Yasuda *et al.*, 2007). In c-KIT-positive small cell lung cancer cells, SCF increased cellular proliferation. Inhibition of PI3K/AKT signaling could alleviate this effect (Kijima *et al.*, 2002) In HCC, Rojas et al. showed that activating of c-KIT/JAK1/STAT3 axis induces migration of HCC combining with TGF-β related signaling(Rojas *et al.*, 2016). To our knowledge, except the study by Roja et al. until now there is no other direct evidence showing whether c-KIT controls proliferation or migration in HCC cell lines.

In this thesis work, I investigated the effect of c-KIT overexpression and knock-down. Surprisingly, in HUH7 and HEP3B cells overexpression of c-KIT could not promote proliferation even with the HSCF stimulation. Certainly, no significant difference between wild-type and mutated c-KIT was found. On the other hand, knock-down of c-KIT in HLE, SNU-182, and HLE did not inhibit the proliferation compared with control cells. Moreover, HSCF did not induce more proliferation in control cells which had enough endogenous c-KIT. I adjusted seeding density, the time point for measurement and time of duration for HSCF treatment. One possible reason is that the proliferation of these HCC cell lines is relatively fast. It may be hard to promote the proliferation further. In this way, the effect of c-KIT on the proliferation of these cells may be slight and hard to

detect. In preliminary experiments, I examined the cell cycle changes in Huh7 and HLF cells, respectively but no significant difference was found in this analysis (data not shown). Thus, c-KIT had no effect on the proliferation of HCC cells.

To avoid interference from proliferation, HCC cells were cultured in the serum-free medium after scratching in the migration assay. In HUH7 cells, overexpression of wild-type c-KIT significantly promoted cell migration. The two mutant forms of c-KIT also promoted slightly faster migration but without statistical difference. The migration-promoting effect of c-KIT overexpression could be observed without HSCF treatment and HSCF treatment did not enhance this effect. This might be explained by autocrine SCF which could induce enough c-KIT activation in c-KIT overexpressing HCC cells. Another mechanism might be the enhanced dimerization of c-KIT due to ectopic overexpression of the receptor. Interestingly, in HEP3B I observed an opposite result. Wild-type c-KIT inhibited migration and mutant c-KIT had no effect on migration.

Thus, the analysis of c-KIT knock-down might be a more physiological model of c-KIT function in HCC cells. HSCF stimulation promoted migration in HLE, SNU-182, and HLF which was not observed in c-KIT knock-down cells. Without HSCF stimulation no difference was found between c-KIT knock-down cells and control cells. In addition, in control knock-down cells HSCF had a positive effect on cell migration. Thus, the three c-KIT-positive cell lines, HLE, SNU-182 and HLF, cell migration was reduced by lack of HSCF or by c-KIT knock-down. This result may suggest that in HLE, SNU-182, and HLF autocrine HSCF levels and the endogenous c-KIT might be too low in the serum-free medium to promote c-KIT activation. Hence in another migration assay, cells were cultured in medium with 10% FCS to prove our assumption. This confirmed that even without HSCF-treatment a significant difference was

observed between c-KIT knock-down and control cells with c-KIT knock-down cells showing slower migration. In summary, functional assays showed that SCF/c-KIT signaling may promote cell migration.

4.6 c-KIT regulated EMT in HCC cell lines

To explain the molecular mechanism how migration was regulated by c-KIT activation in HCC cell lines, I analyzed changes of EMT markers. EMT is defined as trans-differentiation of stationary epithelial cells into motile mesenchymal cells. During EMT a series of changes such as cell junction loss, cytoskeleton reorganization and gene expression reprogramming occur which increase the migration of individual cells and enables the development of an invasive phenotype (Meng *et al.*, 2018). Numerous studies have reported that some genes influence the migration of HCC cells *in vitro* through regulating EMT (Ibrahim *et al.*, 2017; Zhou *et al.*, 2018). It has been confirmed that EMT plays a crucial role in the early steps of metastasis in HCC and affects the clinical outcome of patients (Giannelli *et al.*, 2016). Tang et al. found a significant association between the positive expression of c-KIT and EMT in salivary adenoid cystic carcinoma (ACC). They indicated that c-KIT participates in the invasion and metastasis of salivary ACC by promoting EMT (Tang *et al.*, 2010). A panel of cell-surface proteins, cytoskeletal proteins, and transcription factors, involved in the occurrence of EMT, are regarded as EMT markers. Their function and more details could be seen in Zeisberg's review (Zeisberg and Neilson, 2009). Here, I examined the mRNA level of seven EMT markers including Snail1, Twist1, CDH1 (E-cadherin), CDH2 (N-cadherin), Vimentin, ZEB1, and ZEB2. In HUH7 c-KIT overexpression increased the mRNA level of Twist and Vimentin. In HEP3B ZEB1 decreased and no other EMT markers changed. This could be used to explain why c-KIT overexpressing HEP3B cells showed opposite effects of HUH7. In c-KIT knock-down HLE, SNU-182 and HLF cells, decreased mRNA levels of Snail1, Vimentin, N-cadherin and ZEB1 were found. Collectively, I suggested that

c-KIT could control the migration of HCC cells through regulating EMT. However, to make this evidence stronger the protein level of the EMT markers needs to be examined in the future.

4.7 Selection and validation for candidate gene regulated by c-KIT

I also sought to identify novel genes which may be regulated by c-KIT. To achieve this goal, I analyzed a series of datasets from HCC patients. In three large datasets from GEO and the TCGA-LIHC dataset, differentially expressed genes (DEGs) between patients with high c-KIT level and patients with low c-KIT level were extracted. Finally, PLXDC1, KCNJ2, JAM3, and GJA1 were selected as candidate c-KIT-regulated genes, as these four genes were higher in patients with high c-KIT level. A limitation of the tissue analysis is that HCC tumor tissues contain a mixture of different cell types. It cannot be pinpointed which cells express the measured genes. Also it cannot be ruled out that the here identified genes are upstream of c-KIT or are co-regulated by unknown mechanisms. The advantage of such gene expression studies is the high sample number and that the candidate genes obtained from patient tissues might have high clinical relevance and may be closer to the potential of clinical application. Furthermore, I found that these genes associated with prognosis of patients with HCC. This underpinned that the identified genes may be relevant in HCC. Bioinformatic query of cellular functions suggested that these genes may be involved with cancer-associated biological process (e.g. cell adhesion). However, the function of these genes needs to be confirmed in further experiments in HCC. It is not clear if these genes are up or downstream of c-KIT. In this work, only mRNA level of KCNJ2, JAM3, and GJA1 inhibition was observed in c-KIT silenced HCC cells. Unfortunately, in c-KIT overexpressing HCC cells, the regulatory effect of c-KIT on these genes could not be confirmed. One possible reason is that c-KIT could only regulate these genes through specific downstream pathway but c-KIT-negative cells may lack these downstream pathways. Although c-KIT was overexpressed in these cells,

due to the lack of specific downstream pathways, the regulatory function could not be performed. Another reason might be that I only tested the expression changes of these genes after c-KIT overexpression at a single time point. Longer time points might be necessary for indirect effects. Thus, the analysis of four large gene expression data sets including a total of 970 patients (GSE22058,GSE25097,GSE14520 and TCGA-LIHC), resulted in the identification of the c-KIT co-regulated genes PLXDC1, KCNJ2, JAM3, and GJA1 of which KCNJ2, JAM3, and GJA1 could be validated *in vitro* by c-KIT knock-down.

4.8 Outlook

So far, the data generated in this work partly revealed the role of c-KIT and encouraged further study. I found c-KIT could promote migration of HCC cells *in vitro* the possible mechanism may be involved with EMT. Apart from that several genes which may be regulated by c-KIT were selected and verified *in virto*. Black boxes of molecular mechanism obstruct us to acquire the overall perspective about the role of c-KIT in HCC. To light up the black boxes, further experiments should be designed.

I have already confirmed that PI3K/AKT and MAPK pathways have been activated by c-KIT/SCF in HCC cell lines. Meanwhile, I found that c-KIT promoted migration of HCC cell lines. However, present data did not show a link between the PI3K/AKT or MAPK pathways between c-KIT's migration regulatory effect. I plan to use specific chemical inhibitors to suppress the activation of PI3K/AKT or MAPK pathways and then observe whether the migration regulatory effect of c-KIT still retains. Excepting PI3K/AKT and MAPK pathways, in future more potential downstream pathways regulated by c-KIT should be revealed. To achieve this goal, a human phospho-kinase antibody array will be used to explore more proteins that can be phosphorylated by c-KIT. I have already found c-KIT regulated EMT in HCC

cell lines that could be used to partly explain how c-KIT promotes cell migration. Based on the patient's data I selected and validated several candidate genes that may be regulated by c-KIT. Next, I will investigate whether these genes are involved in HCC cell migration through functional rescue experiments. RNA microarray profiling will be performed using c-KIT up- or down-regulated HCC cells to discover more genes targeted by c-KIT. Noncoding RNA such as microRNA or LncRNAs will be also considered in microarray assay. Finally, until now all presented data in this thesis are restricted to *in vitro* settings. To address this limitation the role of c-KIT will be examined in an orthotopic mouse model using hydrodynamic tail vein injection.

In conclusion, high c-KIT levels correlated with the stage of HCC and indicated poor patients outcome. *In vitro*, c-KIT activated PI3K/AKT or MAPK pathways in HCC cell lines. The patient-derived c-KIT mutations W262C, and C674F mutations were found to inactivate c-KIT’s function limiting the clinical application in HCC. Wildtype c-KIT increased cell migration through regulating EMT. c-KIT which suggests that overexpression of wildtype c-KIT might be clinically relevant. Thus, c-KIT might be a therapeutic option for specific patients with c-KIT-high HCC tumor tissue.

5. SUMMARY

Hepatocellular carcinoma (HCC) is the most common primary hepatic malignancy and second leading cause of cancer-related deaths worldwide. Despite recent advances in diagnosis and molecular understanding, limited success was obtained in improving the survival of HCC patients. Molecular targeted therapies provide an opportunity to expand life expectancy of patients. However, up to now only sorafenib, a multikinase inhibitor, showed survival benefits in some patients. Thus, there is an urgent demand to discover novel molecular targets in HCC to improve therapeutic outcome. As a receptor tyrosine kinase, c-KIT participates in intracellular signaling. Aberrant expression or mutation of c-KIT plays an oncogenic role and inhibition of c-KIT has shown promising results in some cancer. However, the role of c-KIT in HCC is still unclear and the potential of utilizing c-KIT as a therapeutic target in HCC needs to be assessed. Thus, the main aim of this thesis was to analyze the functional role of c-KIT and illuminate its relevant mechanism in the progression of HCC.

Using public datasets of patients with HCC, the clinical relevance of c-KIT was analyzed. In vitro, activation of downstream pathways which are regulated by c-KIT was investigated. Moreover, functional analysis was performed using c-KIT overexpression and knock-down in HCC cell lines. Meanwhile, two patient derived mutations of c-KIT (W262C and C674F) were considered in the process of analysis. Based on RNA microarray data from patients and RNA sequencing data from HCC cells, candidate genes that are regulated by c-KIT were selected and validated using bioinformatics methods and in vitro experiments. The results showed that high c-KIT indicated poor outcome of patients. c-KIT activation regulated PI3K/AKT and MAPK pathways in HCC cells. No effect of c-KIT on proliferation was observed, but the migration of HCC cell lines was promoted by c-KIT possibly by induction of epithelial-mesenchymal transition. The c-KIT mutation W262C led to loss of

functional c-KIT signaling. The analysis of large HCC gene expression data sets revealed the genes PLXDC1, KCNJ2, JAM3, and GJA1 which may be regulated by c-KIT. Interestingly, PLXDC1, KCNJ2 and GJA1 expression correlated with prognosis of HCC patients and their function is very likely involved in cell migration. Taken together, the data presented here support that c-KIT plays an oncogenic role in HCC and targeting c-KIT may benefit patient outcome.

Zusammenfassung

Das hepatozelluläre Karzinom ist die häufigste primäre Tumorerkrankung der Leber und die zweithäufigste Tumor-bedingte Todesursache weltweit. Trotz Fortschritten in der Diagnose und dem molekularen Verständnis von HCC, hat sich das allgemeine Überleben von HCC Patienten nur leicht verbessert. Molekulare zielgerichtete Therapien bieten die Möglichkeit die Lebenserwartung von Patienten zu erhöhen. Bisher konnte jedoch nur für den Multi-Kinase-Inhibitor Sorafenib ein verbessertes Überleben in manchen HCC Patienten gezeigt werden. Aus diesem Grund besteht ein großer Bedarf neue molekulare Zielstrukturen im HCC zu identifizieren, um den Therapieerfolg zu verbessern. Die Rezeptortyrosinkinase c-KIT partizipiert in der intrazellulären Signaltransduktion. Die veränderte Expression oder Mutation von c-KIT spielt eine onkogene Rolle und die Inhibition von c-KIT zeigte in manchen Tumorentitäten vielversprechende Ergebnisse. Die Rolle von c-KIT im HCC ist jedoch noch unklar und die potentielle Verwendung von c-KIT als therapeutisches Ziel im HCC muss noch untersucht werden. Deshalb war das Ziel der vorliegenden Arbeit die Funktion von c-KIT und die relevanten Mechanismen der Progression des HCC zu untersuchen.

Unter Verwendung von öffentlichen Datensätzen von HCC Patienten wurde die klinische Relevanz von c-KIT untersucht. Die Aktivierung von nachfolgenden Signaltransduktionswegen von c-KIT wurde in vitro untersucht. Zusätzlich wurden funktionale Analysen mittels Überexpression und Inhibierung der c-KIT Expression in HCC Zelllinien durchgeführt. In HCC Patienten konnten vereinzelt c-KIT Mutationen gefunden werden. Zwei c-KIT Mutationen, W262C und C674F, wurden im Rahmen dieser Arbeit untersucht. Basierend auf RNA Microarraydaten und RNA Sequenzierungsdaten von HCC Patienten, wurden Gene, die potentiell durch c-KIT reguliert werden, bioinformatisch identifiziert und mittels in vitro Experimenten validiert. Eine hohe c-KIT Expression korrelierte mit schlechtem Überleben und die

Aktivierung von c-KIT induzierte die PI3K/AKT und MAPK Signaltransduktionswege. Es konnte kein Effekt auf die Zellproliferation von HCC Zellen beobachtet werden, aber die Zellmigration wurde durch c-KIT erhöht, was möglicher Weise durch die Induktion von Epithelial-Mesenchymaler-Transition erklärt werden kann. Die c-KIT Mutation W262C führte zu einem Funktionsverlust der c-KIT Signaltransduktion. Anhand großer Genexpressionsdatensets wurden PLXDC1, KCNJ2, JAM3 und GJA1 als mit c-KIT koregulierte Gene identifiziert, und diese wurden nach c-KIT knock-down vermindert exprimiert. Des Weiteren korrelierte die Expression von PLXDC1, KCNJ2 und GJA1 mit vermindertem Überleben und diese Gene spielen wahrscheinliche eine Rolle in der Zellmigration. Zusammenfassend zeigen diese Ergebnisse, dass c-KIT eine onkogene Rolle im HCC spielt und dass die Hemmung von c-KIT in diesen Patienten möglicher Weise das Überleben dieser Patienten verbessern könnte.

6. REFERENCES

Abbas, A., Medvedev, S., Shores, N., Bazzano, L., Dehal, A., Hutchings, J. and Balart, L. (2014). **Epidemiology of metastatic hepatocellular carcinoma, a nationwide perspective**. Dig Dis Sci *59*, 2813-2820, doi: 10.1007/s10620-014-3229-9.

Abbaspour Babaei, M., Kamalidehghan, B., Saleem, M., Huri, H. Z. and Ahmadipour, F. (2016). **Receptor tyrosine kinase (c-Kit) inhibitors: a potential therapeutic target in cancer cells**. Drug Des Devel Ther *10*, 2443-2459, doi: 10.2147/dddt.s89114.

Abrams, T., Connor, A., Fanton, C., Cohen, S. B., Huber, T., Miller, K., Hong, E. E., Niu, X., Kline, J., Ison-Dugenny, M., Harris, S., Walker, D., Krauser, K., Galimi, F., Wang, Z., Ghoddusi, M., Mansfield, K., Lee-Hoeflich, S. T., Holash, J., Pryer, N., Kluwe, W., Ettenberg, S. A., Sellers, W. R., Lees, E., Kwon, P., Abraham, J. A. and Schleyer, S. C. (2018). **Preclinical Antitumor Activity of a Novel Anti-c-KIT Antibody-Drug Conjugate against Mutant and Wild-type c-KIT-Positive Solid Tumors**. Clin Cancer Res *24*, 4297-4308, doi: 10.1158/1078-0432.ccr-17-3795.

Agarwal, S., Kazi, J. U., Mohlin, S., Pahlman, S. and Ronnstrand, L. (2015). **The activation loop tyrosine 823 is essential for the transforming capacity of the c-Kit oncogenic mutant D816V**. Oncogene *34*, 4581-4590, doi: 10.1038/onc.2014.383.

An, X., Long, C., Deng, X., Tang, A., Xie, J., Chen, L. and Wang, Z. (2017). **Higenamine inhibits apoptosis and maintains survival of gastric smooth muscle cells in diabetic gastroparesis rat model via activating the beta2-AR/PI3K/AKT pathway**. Biomed Pharmacother *95*, 1710-1717, doi: 10.1016/j.biopha.2017.08.112.

Ardelt, M. A., Frohlich, T., Martini, E., Muller, M., Kanitz, V., Atzberger, C., Cantonati, P., Messner, M., Posselt, L., Lehr, T., Wojtyniak, J. G., Ulrich, M., Arnold, G. J., Konig, L., Parazzoli, D., Zahler, S., Rothenfusser, S., Mayr, D., Gerbes, A., Scita, G., Vollmar, A. M. and Pachmayr, J. (2018). **Inhibition of Cyclin-dependent Kinase 5 - a Novel Strategy to Improve Sorafenib Response in HCC Therapy**. Hepatology, doi: 10.1002/hep.30190.

Ashman, L. K. and Griffith, R. (2013). **Therapeutic targeting of c-KIT in cancer**. Expert Opin Investig Drugs *22*, 103-115, doi: 10.1517/13543784.2013.740010.

Becker, G., Schmitt-Graeff, A., Ertelt, V., Blum, H. E. and Allgaier, H. P. (2007). **CD117 (c-kit) expression in human hepatocellular carcinoma**. Clin Oncol (R Coll Radiol) *19*, 204-208, doi: 10.1016/j.clon.2006.12.009.

Beghini, A., Cairoli, R., Morra, E. and Larizza, L. (1998). **In vivo differentiation of mast cells from acute myeloid leukemia blasts carrying a novel activating**

ligand-independent C-kit mutation. Blood Cells Mol Dis *24*, 262-270, doi: 10.1006/bcmd.1998.0191.

Beghini, A., Ripamonti, C. B., Cairoli, R., Cazzaniga, G., Colapietro, P., Elice, F., Nadali, G., Grillo, G., Haas, O. A., Biondi, A., Morra, E. and Larizza, L. (2004). **KIT activating mutations: incidence in adult and pediatric acute myeloid leukemia, and identification of an internal tandem duplication**. Haematologica *89*, 920-925.

Beguelin, C., Moradpour, D., Sahli, R., Suter-Riniker, F., Luthi, A., Cavassini, M., Gunthard, H. F., Battegay, M., Bernasconi, E., Schmid, P., Calmy, A., Braun, D. L., Furrer, H., Rauch, A. and Wandeler, G. (2017). **Hepatitis delta-associated mortality in HIV/HBV-coinfected patients**. J Hepatol *66*, 297-303, doi: 10.1016/j.jhep.2016.10.007.

Bertuccio, P., Turati, F., Carioli, G., Rodriguez, T., La Vecchia, C., Malvezzi, M. and Negri, E. (2017). **Global trends and predictions in hepatocellular carcinoma mortality**. J Hepatol *67*, 302-309, doi: 10.1016/j.jhep.2017.03.011.

Besmer, P., Murphy, J. E., George, P. C., Qiu, F. H., Bergold, P. J., Lederman, L., Snyder, H. W., Jr., Brodeur, D., Zuckerman, E. E. and Hardy, W. D. (1986). **A new acute transforming feline retrovirus and relationship of its oncogene v-kit with the protein kinase gene family**. Nature *320*, 415-421, doi: 10.1038/320415a0.

Blume-Jensen, P., Claesson-Welsh, L., Siegbahn, A., Zsebo, K. M., Westermark, B. and Heldin, C. H. (1991). **Activation of the human c-kit product by ligand-induced dimerization mediates circular actin reorganization and chemotaxis**. EMBO J *10*, 4121-4128.

Blume-Jensen, P., Wernstedt, C., Heldin, C. H. and Ronnstrand, L. (1995). **Identification of the major phosphorylation sites for protein kinase C in kit/stem cell factor receptor *in* vitro and in intact cells**. J Biol Chem *270*, 14192-14200.

Brechot, C., Gozuacik, D., Murakami, Y. and Paterlini-Brechot, P. (2000). **Molecular bases for the development of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC)**. Semin Cancer Biol *10*, 211-231, doi: 10.1006/scbi.2000.0321.

Broudy, V. C., Lin, N. L., Buhring, H. J., Komatsu, N. and Kavanagh, T. J. (1998). **Analysis of c-kit receptor dimerization by fluorescence resonance energy transfer**. Blood *91*, 898-906.

Cadier, B., Bulsei, J., Nahon, P., Seror, O., Laurent, A., Rosa, I., Layese, R., Costentin, C., Cagnot, C., Durand-Zaleski, I. and Chevreul, K. (2017). **Early detection and curative treatment of hepatocellular carcinoma: A cost-effectiveness analysis in France and in the United States**. Hepatology *65*, 1237-1248, doi: 10.1002/hep.28961.

Calvisi, D. F., Ladu, S., Gorden, A., Farina, M., Conner, E. A., Lee, J. S., Factor, V. M. and Thorgeirsson, S. S. (2006). **Ubiquitous activation of Ras and Jak/Stat pathways in human HCC**. Gastroenterology *130*, 1117-1128, doi: 10.1053/j.gastro.2006.01.006.

Casteran, N., De Sepulveda, P., Beslu, N., Aoubala, M., Letard, S., Lecocq, E., Rottapel, R. and Dubreuil, P. (2003). **Signal transduction by several KIT juxtamembrane domain mutations**. Oncogene *22*, 4710-4722, doi: 10.1038/sj.onc.1206587.

Chaix, A., Lopez, S., Voisset, E., Gros, L., Dubreuil, P. and De Sepulveda, P. (2011). **Mechanisms of STAT protein activation by oncogenic KIT mutants in neoplastic mast cells**. J Biol Chem *286*, 5956-5966, doi: 10.1074/jbc.M110.182642.

Chen, L., Faire, M., Kissner, M. D. and Laird, D. J. (2013). **Primordial germ cells and gastrointestinal stromal tumors respond distinctly to a cKit overactivating allele**. Hum Mol Genet *22*, 313-327, doi: 10.1093/hmg/dds430.

Chen, W., Kuang, Y., Qiu, H. B., Cao, Z., Tu, Y., Sheng, Q., Eilers, G., He, Q., Li, H. L., Zhu, M., Wang, Y., Zhang, R., Wu, Y., Meng, F., Fletcher, J. A. and Ou, W. B. (2017). **Dual Targeting of Insulin Receptor and KIT in Imatinib-Resistant Gastrointestinal Stromal Tumors**. Cancer Res *77*, 5107-5117, doi: 10.1158/0008-5472.can-17-0917.

Choudhary, S., Pardo, A., Rosinke, R., Batra, J. K., Barth, S. and Verma, R. S. (2016). **Targeting c-kit receptor in neuroblastomas and colorectal cancers using stem cell factor (SCF)-based recombinant bacterial toxins**. Appl Microbiol Biotechnol *100*, 263-277, doi: 10.1007/s00253-015-6978-2.

Chung, C. Y., Yeh, K. T., Hsu, N. C., Chang, J. H., Lin, J. T., Horng, H. C. and Chang, C. S. (2005). **Expression of c-kit protooncogene in human hepatocellular carcinoma**. Cancer Lett *217*, 231-236, doi: 10.1016/j.canlet.2004.06.045.

Costentin, C. E., Bababekov, Y. J., Zhu, A. X. and Yeh, H. (2018). **Is it time to reconsider the Milan Criteria for selecting patients with hepatocellular carcinoma for deceased-donor liver transplantation?** Hepatology, doi: 10.1002/hep.30278.

Crosier, P. S., Ricciardi, S. T., Hall, L. R., Vitas, M. R., Clark, S. C. and Crosier, K. E. (1993). **Expression of isoforms of the human receptor tyrosine kinase c-kit in leukemic cell lines and acute myeloid leukemia**. Blood *82*, 1151-1158.

Deberry, C., Mou, S. and Linnekin, D. (1997). **Stat1 associates with c-kit and is activated in response to stem cell factor**. Biochem J *327 (Pt 1)*, 73-80.

Demetri, G. D. (2001). **Targeting c-kit mutations in solid tumors: scientific rationale and novel therapeutic options**. Semin Oncol *28*, 19-26.

Dhanasekaran, R., Bandoh, S. and Roberts, L. R. (2016). **Molecular pathogenesis of hepatocellular carcinoma and impact of therapeutic advances**. F1000Res *5*, doi: 10.12688/f1000research.6946.1.

Eigentler, T. K., Meier, F. and Garbe, C. (2013). **Protein kinase inhibitors in melanoma**. Expert Opin Pharmacother *14*, 2195-2201, doi: 10.1517/14656566.2013.827172.

El-Serag, H. B. (2012). **Epidemiology of viral hepatitis and hepatocellular carcinoma**. Gastroenterology *142*, 1264-1273 e1261, doi: 10.1053/j.gastro.2011.12.061.

Estes, C., Razavi, H., Loomba, R., Younossi, Z. and Sanyal, A. J. (2018). **Modeling the epidemic of nonalcoholic fatty liver disease demonstrates an exponential increase in burden of disease**. Hepatology *67*, 123-133, doi: 10.1002/hep.29466.

Fan, Y., Arechederra, M., Richelme, S., Daian, F., Novello, C., Calderaro, J., Di Tommaso, L., Morcrette, G., Rebouissou, S., Donadon, M., Morenghi, E., Zucman-Rossi, J., Roncalli, M., Dono, R. and Maina, F. (2017). **A phosphokinome-based screen uncovers new drug synergies for cancer driven by liver-specific gain of nononcogenic receptor tyrosine kinases**. Hepatology *66*, 1644-1661, doi: 10.1002/hep.29304.

Finn, R. S. and Zhu, A. X. (2009). **Targeting angiogenesis in hepatocellular carcinoma: focus on VEGF and bevacizumab**. Expert Rev Anticancer Ther *9*, 503-509, doi: 10.1586/era.09.6.

Franceschi, S., Lessi, F., Panebianco, F., Tantillo, E., La Ferla, M., Menicagli, M., Aretini, P., Apollo, A., Naccarato, A. G., Marchetti, I. and Mazzanti, C. M. (2017). **Loss of c-KIT expression in thyroid cancer cells**. PLoS One *12*, e0173913, doi: 10.1371/journal.pone.0173913.

Fujiwara, N., Friedman, S. L., Goossens, N. and Hoshida, Y. (2018). **Risk factors and prevention of hepatocellular carcinoma in the era of precision medicine**. J Hepatol *68*, 526-549, doi: 10.1016/j.jhep.2017.09.016.

Furuse, J. (2008). **Growth factors as therapeutic targets in HCC**. Crit Rev Oncol Hematol *67*, 8-15, doi: 10.1016/j.critrevonc.2008.02.009.

Gan, L., Liu, Z. and Sun, C. (2018). **Obesity linking to hepatocellular carcinoma: A global view**. Biochim Biophys Acta Rev Cancer *1869*, 97-102, doi: 10.1016/j.bbcan.2017.12.006.

Giannelli, G., Koudelkova, P., Dituri, F. and Mikulits, W. (2016). **Role of epithelial to mesenchymal transition in hepatocellular carcinoma**. J Hepatol *65*, 798-808, doi: 10.1016/j.jhep.2016.05.007.

Giordano, S. and Columbano, A. (2014). **Met as a therapeutic target in HCC: facts and hopes**. J Hepatol *60*, 442-452, doi: 10.1016/j.jhep.2013.09.009.

Grillo, F., Florio, T., Ferrau, F., Kara, E., Fanciulli, G., Faggiano, A. and Colao, A. (2018). **Emerging multitarget tyrosine kinase inhibitors in the treatment of neuroendocrine neoplasms**. Endocr Relat Cancer *25*, R453-R466, doi: 10.1530/erc-17-0531.

Growney, J. D., Clark, J. J., Adelsperger, J., Stone, R., Fabbro, D., Griffin, J. D. and Gilliland, D. G. (2005). **Activation mutations of human c-KIT resistant to imatinib mesylate are sensitive to the tyrosine kinase inhibitor PKC412**. Blood *106*, 721-724, doi: 10.1182/blood-2004-12-4617.

Harris, W. P., Wong, K. M., Saha, S., Dika, I. E. and Abou-Alfa, G. K. (2018). **Biomarker-Driven and Molecular Targeted Therapies for Hepatobiliary Cancers**. Semin Oncol, doi: 10.1053/j.seminoncol.2018.03.002.

Heinrich, M. C., Blanke, C. D., Druker, B. J. and Corless, C. L. (2002). **Inhibition of KIT tyrosine kinase activity: a novel molecular approach to the treatment of KIT-positive malignancies**. J Clin Oncol *20*, 1692-1703, doi: 10.1200/jco.2002.20.6.1692.

Herath, N. I., Leggett, B. A. and MacDonald, G. A. (2006). **Review of genetic and epigenetic alterations in hepatocarcinogenesis**. J Gastroenterol Hepatol *21*, 15-21, doi: 10.1111/j.1440-1746.2005.04043.x.

Herraiz, C., Journe, F., Abdel-Malek, Z., Ghanem, G., Jimenez-Cervantes, C. and Garcia-Borron, J. C. (2011). **Signaling from the human melanocortin 1 receptor to ERK1 and ERK2 mitogen-activated protein kinases involves transactivation of cKIT**. Mol Endocrinol *25*, 138-156, doi: 10.1210/me.2010-0217.

Huynh, H., Ong, R. W., Li, P. Y., Lee, S. S., Yang, S., Chong, L. W., Luu, D. A., Jong, C. T. and Lam, I. W. (2011). **Targeting receptor tyrosine kinase pathways in hepatocellular carcinoma**. Anticancer Agents Med Chem *11*, 560-575.

Ibrahim, A. A., Schmithals, C., Kowarz, E., Koberle, V., Kakoschky, B., Pleli, T., Kollmar, O., Nitsch, S., Waidmann, O., Finkelmeier, F., Zeuzem, S., Korf, H. W., Schmid, T., Weigert, A., Kronenberger, B., Marschalek, R. and Piiper, A. (2017). **Hypoxia Causes Downregulation of Dicer in Hepatocellular Carcinoma, Which Is Required for Upregulation of Hypoxia-Inducible Factor 1alpha and Epithelial-Mesenchymal Transition**. Clin Cancer Res *23*, 3896-3905, doi: 10.1158/1078-0432.ccr-16-1762.

Jung, C. L., Kim, H. J., Kim, D. H., Huh, H., Song, M. J. and Kim, S. H. (2011). **CKIT mutation**

in therapy-related acute myeloid leukemia with MLLT3/MLL chimeric transcript from t(9;11)(p22;q23). Ann Clin Lab Sci *41*, 193-196.

Keane, F. K., Hong, T. S. and Zhu, A. X. (2018). **Evolving Systemic Therapy in Hepatocellular Carcinoma: Current Management and Opportunities for Integration With Radiotherapy**. Semin Radiat Oncol *28*, 332-341, doi: 10.1016/j.semradonc.2018.06.006.

Kew, M. C. (2000). **Hepatocellular cancer. A century of progress**. Clin Liver Dis *4*, 257-268.

Kijima, T., Maulik, G., Ma, P. C., Tibaldi, E. V., Turner, R. E., Rollins, B., Sattler, M., Johnson, B. E. and Salgia, R. (2002). **Regulation of cellular proliferation, cytoskeletal function, and signal transduction through CXCR4 and c-Kit in small cell lung cancer cells**. Cancer Res *62*, 6304-6311.

Kim, S. R., Im, J. E., Jeong, J. H., Kim, J. Y., Kim, J. T., Woo, S. J., Sung, J. H., Park, S. G. and Suh, W. (2016). **The cKit Inhibitor, Masitinib, Prevents Diabetes-Induced Retinal Vascular Leakage**. Invest Ophthalmol Vis Sci *57*, 1201-1206, doi: 10.1167/iovs.15-18065.

Kitayama, H., Kanakura, Y., Furitsu, T., Tsujimura, T., Oritani, K., Ikeda, H., Sugahara, H., Mitsui, H., Kanayama, Y., Kitamura, Y. and et al. (1995). **Constitutively activating mutations of c-kit receptor tyrosine kinase confer factor-independent growth and tumorigenicity of factor-dependent hematopoietic cell lines**. Blood *85*, 790-798.

Klump, J., Phillipp, U., Follo, M., Eremin, A., Lehmann, H., Nestel, S., von Bubnoff, N. and Nazarenko, I. (2018). **Extracellular vesicles or free circulating DNA: where to search for BRAF and cKIT mutations?** Nanomedicine *14*, 875-882, doi: 10.1016/j.nano.2017.12.009.

Knight, B., Tirnitz-Parker, J. E. and Olynyk, J. K. (2008). **C-kit inhibition by imatinib mesylate attenuates progenitor cell expansion and inhibits liver tumor formation in mice**. Gastroenterology *135*, 969-979, 979 e961, doi: 10.1053/j.gastro.2008.05.077.

Kudo, M. (2018). **Systemic Therapy for Hepatocellular Carcinoma: Latest Advances**. Cancers (Basel) *10*, doi: 10.3390/cancers10110412.

Kuyvenhoven, J., Lamers, C. B. and van Hoek, B. (2001). **Practical management of hepatocellular carcinoma**. Scand J Gastroenterol Suppl, 82-87.

Lamarca, A., Moreno, V. and Feliu, J. (2013). **Thymoma and thymic carcinoma in the target therapies era**. Cancer Treat Rev *39*, 413-420, doi: 10.1016/j.ctrv.2012.11.005.

Lategahn, J., Keul, M. and Rauh, D. (2018). **Lessons To Be Learned: The Molecular Basis of Kinase-Targeted Therapies and Drug Resistance in Non-Small Cell Lung Cancer**. Angew Chem Int Ed Engl *57*, 2307-2313, doi: 10.1002/anie.201710398.

Lebron, M. B., Brennan, L., Damoci, C. B., Prewett, M. C., O'Mahony, M., Duignan, I. J., Credille, K. M., DeLigio, J. T., Starodubtseva, M., Amatulli, M., Zhang, Y., Schwartz, K. D., Burtrum, D., Balderes, P., Persaud, K., Surguladze, D., Loizos, N., Paz, K. and Kotanides, H. (2014). **A human monoclonal antibody targeting the stem cell factor receptor (c-Kit) blocks tumor cell signaling and inhibits tumor growth**. Cancer Biol Ther *15*, 1208-1218, doi: 10.4161/cbt.29523.

Lee, E. S., Han, E. M., Kim, Y. S., Shin, B. K., Kim, C. H., Kim, H. K., Won, N. H., Yeom, B. W., Kim, I. and Leong, A. S. (2005). **Occurrence of c-kit+ tumor cells in hepatitis B virus-associated hepatocellular carcinoma**. Am J Clin Pathol *124*, 31-36, doi: 10.1309/lettwn3luf516.

Lee, Y., Jung, J., Cho, K. J., Lee, S. K., Park, J. W., Oh, I. H. and Kim, G. J. (2013). **Increased SCF/c-kit by hypoxia promotes autophagy of human placental chorionic plate-derived mesenchymal stem cells via regulating the phosphorylation of mTOR**. J Cell Biochem *114*, 79-88, doi: 10.1002/jcb.24303.

Lennartsson, J. and Ronnstrand, L. (2012). **Stem cell factor receptor/c-Kit: from basic science to clinical implications**. Physiol Rev *92*, 1619-1649, doi: 10.1152/physrev.00046.2011.

Li, J., Wang, L., Cong, N., Shi, C., Bu, W., Song, J. and Chen, H. (2014). **Efficacy of Sorafenib for Advanced Hepatocellular Carcinoma and Prognostic Factors**. Hepatogastroenterology *61*, 954-957.

Li, X., Xu, A., Li, H., Zhang, B., Cao, B. and Huang, J. (2018). **Novel role of apatinib as a multi-target RTK inhibitor in the direct suppression of hepatocellular carcinoma cells**. Biochim Biophys Acta Mol Basis Dis *1864*, 1693-1701, doi: 10.1016/j.bbadis.2018.02.014.

Liao, P., Zeng, S. X., Zhou, X., Chen, T., Zhou, F., Cao, B., Jung, J. H., Del Sal, G., Luo, S. and Lu, H. (2017). **Mutant p53 Gains Its Function via c-Myc Activation upon CDK4 Phosphorylation at Serine 249 and Consequent PIN1 Binding**. Mol Cell *68*, 1134-1146 e1136, doi: 10.1016/j.molcel.2017.11.006.

Lin, S. C., Liu, C. L., Wang, T. I., Chang, W. S., Tzen, C. Y. and Huang, M. J. (2006). **Clinical implications of C-kit gene mutation in patients with large gastrointestinal stromal tumors**. J Gastroenterol Hepatol *21*, 1604-1608, doi: 10.1111/j.1440-1746.2006.04322.x.

Linnekin, D., Weiler, S. R., Mou, S., DeBerry, C. S., Keller, J. R., Ruscetti, F. W., Ferris, D. K. and Longo, D. L. (1996). **JAK2 is constitutively associated with c-Kit and is phosphorylated in response to stem cell factor**. Acta Haematol *95*, 224-228, doi: 10.1159/000203882.

Liu, L. L., Fu, D., Ma, Y. and Shen, X. Z. (2011). **The power and the promise of liver cancer stem cell markers**. Stem Cells Dev *20*, 2023-2030, doi: 10.1089/scd.2011.0012.

Liu, S., Wu, L. C., Pang, J., Santhanam, R., Schwind, S., Wu, Y. Z., Hickey, C. J., Yu, J., Becker, H., Maharry, K., Radmacher, M. D., Li, C., Whitman, S. P., Mishra, A., Stauffer, N., Eiring, A. M., Briesewitz, R., Baiocchi, R. A., Chan, K. K., Paschka, P., Caligiuri, M. A., Byrd, J. C., Croce, C. M., Bloomfield, C. D., Perrotti, D., Garzon, R. and Marcucci, G. (2010). **Sp1/NFkappaB/HDAC/miR-29b regulatory network in KIT-driven myeloid leukemia**. Cancer Cell *17*, 333-347, doi: 10.1016/j.ccr.2010.03.008.

Llovet, J. M., Montal, R., Sia, D. and Finn, R. S. (2018). **Molecular therapies and precision medicine for hepatocellular carcinoma**. Nat Rev Clin Oncol *15*, 599-616, doi: 10.1038/s41571-018-0073-4.

London, C. A., Kisseberth, W. C., Galli, S. J., Geissler, E. N. and Helfand, S. C. (1996). **Expression of stem cell factor receptor (c-kit) by the malignant mast cells from spontaneous canine mast cell tumours**. J Comp Pathol *115*, 399-414.

Longley, B. J., Reguera, M. J. and Ma, Y. (2001). **Classes of c-KIT activating mutations: proposed mechanisms of action and implications for disease classification and therapy**. Leuk Res *25*, 571-576.

Lu, T. F., Hua, X. W., Cui, X. L. and Xia, Q. (2014). **Liver transplantation for hepatocellular carcinoma: recent advances in China**. J Dig Dis *15*, 51-53.

Ma, Y., Longley, B. J., Wang, X., Blount, J. L., Langley, K. and Caughey, G. H. (1999). **Clustering of activating mutations in c-KIT's juxtamembrane coding region in canine mast cell neoplasms**. J Invest Dermatol *112*, 165-170, doi: 10.1046/j.1523-1747.1999.00488.x.

Maier, J., Lange, T., Kerle, I., Specht, K., Bruegel, M., Wickenhauser, C., Jost, P., Niederwieser, D., Peschel, C., Duyster, J. and von Bubnoff, N. (2013). **Detection of mutant free circulating tumor DNA in the plasma of patients with gastrointestinal stromal tumor harboring activating mutations of CKIT or PDGFRA**. Clin Cancer Res *19*, 4854-4867, doi: 10.1158/1078-0432.ccr-13-0765.

Mansuroglu, T., Baumhoer, D., Dudas, J., Haller, F., Cameron, S., Lorf, T., Fuzesi, L. and Ramadori, G. (2009). **Expression of stem cell factor receptor c-kit in human**

nontumoral and tumoral hepatic cells. Eur J Gastroenterol Hepatol *21*, 1206-1211, doi: 10.1097/MEG.0b013e328317f4ef.

McDaniel, A. S., Allen, J. D., Park, S. J., Jaffer, Z. M., Michels, E. G., Burgin, S. J., Chen, S., Bessler, W. K., Hofmann, C., Ingram, D. A., Chernoff, J. and Clapp, D. W. (2008). **Pak1 regulates multiple c-Kit mediated Ras-MAPK gain-in-function phenotypes in Nf1+/- mast cells**. Blood *112*, 4646-4654, doi: 10.1182/blood-2008-04-155085.

Mendez-Blanco, C., Fondevila, F., Garcia-Palomo, A., Gonzalez-Gallego, J. and Mauriz, J. L. (2018). **Sorafenib resistance in hepatocarcinoma: role of hypoxia-inducible factors**. Exp Mol Med *50*, 134, doi: 10.1038/s12276-018-0159-1.

Meng, J., Chen, S., Han, J. X., Qian, B., Wang, X. R., Zhong, W. L., Qin, Y., Zhang, H., Gao, W. F., Lei, Y. Y., Yang, W., Yang, L., Zhang, C., Liu, H. J., Liu, Y. R., Zhou, H. G., Sun, T. and Yang, C. (2018). **Twist1 Regulates Vimentin through Cul2 Circular RNA to Promote EMT in Hepatocellular Carcinoma**. Cancer Res *78*, 4150-4162, doi: 10.1158/0008-5472.can-17-3009.

Minguez, B., Tovar, V., Chiang, D., Villanueva, A. and Llovet, J. M. (2009). **Pathogenesis of hepatocellular carcinoma and molecular therapies**. Curr Opin Gastroenterol *25*, 186-194, doi: 10.1097/MOG.0b013e32832962a1.

Mittal, S. and El-Serag, H. B. (2013). **Epidemiology of hepatocellular carcinoma: consider the population**. J Clin Gastroenterol *47 Suppl*, S2-6, doi: 10.1097/MCG.0b013e3182872f29.

Morse, M. A., Sun, W., Kim, R., He, A. R., Abada, P. B., Mynderse, M. and Finn, R. S. (2018). **The Role of Angiogenesis in Hepatocellular Carcinoma**. Clin Cancer Res, doi: 10.1158/1078-0432.ccr-18-1254.

Moukhadder, H. M., Halawi, R., Cappellini, M. D. and Taher, A. T. (2017). **Hepatocellular carcinoma as an emerging morbidity in the thalassemia syndromes: A comprehensive review**. Cancer *123*, 751-758, doi: 10.1002/cncr.30462.

Nakamura, M., Chiba, T., Kanayama, K., Kanzaki, H., Saito, T., Kusakabe, Y. and Kato, N. (2018). **Epigenetic dysregulation in hepatocellular carcinoma: an up-to-date review**. Hepatol Res, doi: 10.1111/hepr.13250.

Negrini, M., Gramantieri, L., Sabbioni, S. and Croce, C. M. (2011). **microRNA involvement in hepatocellular carcinoma**. Anticancer Agents Med Chem *11*, 500-521.

Noujaim, J., Gonzalez, D., Thway, K., Jones, R. L. and Judson, I. (2016). **p.(L576P) -KIT mutation in GIST: Favorable prognosis and sensitive to imatinib?** Cancer Biol Ther *17*, 543-545, doi: 10.1080/15384047.2016.1156263.

Orouji, E., Orouji, A., Gaiser, T., Larribere, L., Gebhardt, C. and Utikal, J. (2016). **MAP kinase pathway gene copy alterations in NRAS/BRAF wild-type advanced melanoma**. Int J Cancer *138*, 2257-2262, doi: 10.1002/ijc.29970.

Pandey, R. and Kochar, R. (2012). **Management of gastrointestinal stromal tumors: looking beyond the knife. An update on the role of adjuvant and neoadjuvant imatinib therapy**. J Gastrointest Cancer *43*, 547-552, doi: 10.1007/s12029-012-9423-0.

Papaspyrou, G., Garbe, C., Schadendorf, D., Werner, J. A., Hauschild, A. and Egberts, F. (2011). **Mucosal melanomas of the head and neck: new aspects of the clinical outcome, molecular pathology, and treatment with c-kit inhibitors**. Melanoma Res *21*, 475-482, doi: 10.1097/CMR.0b013e32834b58cf.

Peng, L., Yuan, X. Q., Zhang, C. Y., Peng, J. Y., Zhang, Y. Q., Pan, X. and Li, G. C. (2018). **The emergence of long non-coding RNAs in hepatocellular carcinoma: an update**. J Cancer *9*, 2549-2558, doi: 10.7150/jca.24560.

Petrick, J. L., Campbell, P. T., Koshiol, J., Thistle, J. E., Andreotti, G., Beane-Freeman, L. E., Buring, J. E., Chan, A. T., Chong, D. Q., Doody, M. M., Gapstur, S. M., Gaziano, J. M., Giovannucci, E., Graubard, B. I., Lee, I. M., Liao, L. M., Linet, M. S., Palmer, J. R., Poynter, J. N., Purdue, M. P., Robien, K., Rosenberg, L., Schairer, C., Sesso, H. D., Sinha, R., Stampfer, M. J., Stefanick, M., Wactawski-Wende, J., Zhang, X., Zeleniuch-Jacquotte, A., Freedman, N. D. and McGlynn, K. A. (2018). **Tobacco, alcohol use and risk of hepatocellular carcinoma and intrahepatic cholangiocarcinoma: The Liver Cancer Pooling Project**. Br J Cancer *118*, 1005-1012, doi: 10.1038/s41416-018-0007-z.

Pez, F., Lopez, A., Kim, M., Wands, J. R., Caron de Fromentel, C. and Merle, P. (2013). **Wnt signaling and hepatocarcinogenesis: molecular targets for the development of innovative anticancer drugs**. J Hepatol *59*, 1107-1117, doi: 10.1016/j.jhep.2013.07.001.

Polesel, J., Zucchetto, A., Montella, M., Dal Maso, L., Crispo, A., La Vecchia, C., Serraino, D., Franceschi, S. and Talamini, R. (2009). **The impact of obesity and diabetes mellitus on the risk of hepatocellular carcinoma**. Ann Oncol *20*, 353-357, doi: 10.1093/annonc/mdn565.

Pradat, P., Virlogeux, V. and Trepo, E. (2018). **Epidemiology and Elimination of HCV-Related Liver Disease**. Viruses *10*, doi: 10.3390/v10100545.

Puisieux, A. and Ozturk, M. (1997). **TP53 and hepatocellular carcinoma**. Pathol Biol (Paris) *45*, 864-870.

Qiu, F. H., Ray, P., Brown, K., Barker, P. E., Jhanwar, S., Ruddle, F. H. and Besmer, P. (1988). **Primary structure of c-kit: relationship with the CSF-1/PDGF receptor kinase family--oncogenic activation of v-kit involves deletion of extracellular domain and C terminus**. EMBO J *7*, 1003-1011.

Raza, A. and Sood, G. K. (2014). **Hepatocellular carcinoma review: current treatment, and evidence-based medicine**. World J Gastroenterol *20*, 4115-4127, doi: 10.3748/wjg.v20.i15.4115.

Reith, A. D., Ellis, C., Lyman, S. D., Anderson, D. M., Williams, D. E., Bernstein, A. and Pawson, T. (1991). **Signal transduction by normal isoforms and W mutant variants of the Kit receptor tyrosine kinase**. EMBO J *10*, 2451-2459.

Rojas, A., Zhang, P., Wang, Y., Foo, W. C., Munoz, N. M., Xiao, L., Wang, J., Gores, G. J., Hung, M. C. and Blechacz, B. (2016). **A Positive TGF-beta/c-KIT Feedback Loop Drives Tumor Progression in Advanced Primary Liver Cancer**. Neoplasia *18*, 371-386, doi: 10.1016/j.neo.2016.04.002.

Rongrui, L., Na, H., Zongfang, L., Fanpu, J. and Shiwen, J. (2014). **Epigenetic mechanism involved in the HBV/HCV-related hepatocellular carcinoma tumorigenesis**. Curr Pharm Des *20*, 1715-1725.

Salgado, E., Maneiro, J. R., Carmona, L. and Gomez-Reino, J. J. (2014). **Safety profile of protein kinase inhibitors in rheumatoid arthritis: systematic review and meta-analysis**. Ann Rheum Dis *73*, 871-882, doi: 10.1136/annrheumdis-2012-203116.

Samayawardhena, L. A., Hu, J., Stein, P. L. and Craig, A. W. (2006). **Fyn kinase acts upstream of Shp2 and p38 mitogen-activated protein kinase to promote chemotaxis of mast cells towards stem cell factor**. Cell Signal *18*, 1447-1454, doi: 10.1016/j.cellsig.2005.11.005.

Samji, H., Yu, A., Kuo, M., Alavi, M., Woods, R., Alvarez, M., Dore, G. J., Tyndall, M., Krajden, M. and Janjua, N. Z. (2017). **Late hepatitis B and C diagnosis in relation to disease decompensation and hepatocellular carcinoma development**. J Hepatol *67*, 909-917, doi: 10.1016/j.jhep.2017.06.025.

Sanyal, A., Poklepovic, A., Moyneur, E. and Barghout, V. (2010). **Population-based risk factors and resource utilization for HCC: US perspective**. Curr Med Res Opin *26*, 2183-2191, doi: 10.1185/03007995.2010.506375.

Sato, K. and Mori, M. (2011). **Evolving molecular mechanism-based strategies for control of hepatocellular carcinoma**. Curr Med Chem *18*, 4375-4388.

Schulze, K., Nault, J. C. and Villanueva, A. (2016). **Genetic profiling of hepatocellular carcinoma using next-generation sequencing**. J Hepatol *65*, 1031-1042, doi: 10.1016/j.jhep.2016.05.035.

Shah, Y. M. and van den Brink, G. R. (2015). **c-Kit as a Novel Potential Therapeutic Target in Colorectal Cancer**. Gastroenterology *149*, 534-537, doi: 10.1053/j.gastro.2015.07.027.

Song, I. H. (2009). **Molecular targeting for treatment of advanced hepatocellular carcinoma**. Korean J Hepatol *15*, 299-308, doi: 10.3350/kjhep.2009.15.3.299.

Stankov, K., Popovic, S. and Mikov, M. (2014). **C-KIT signaling in cancer treatment**. Curr Pharm Des *20*, 2849-2880.

Sullivan, K. M., Kenerson, H. L., Pillarisetty, V. G., Riehle, K. J. and Yeung, R. S. (2018). **Precision oncology in liver cancer**. Ann Transl Med *6*, 285, doi: 10.21037/atm.2018.06.14.

Takigawa, Y. and Brown, A. M. (2008). **Wnt signaling in liver cancer**. Curr Drug Targets *9*, 1013-1024.

Tang, Y., Liang, X., Zheng, M., Zhu, Z., Zhu, G., Yang, J. and Chen, Y. (2010). **Expression of c-kit and Slug correlates with invasion and metastasis of salivary adenoid cystic carcinoma**. Oral Oncol *46*, 311-316, doi: 10.1016/j.oraloncology.2010.02.001.

Tang, Z. Y. (2001). **Hepatocellular carcinoma--cause, treatment and metastasis**. World J Gastroenterol *7*, 445-454.

Tang, Z. Y., Ye, S. L., Liu, Y. K., Qin, L. X., Sun, H. C., Ye, Q. H., Wang, L., Zhou, J., Qiu, S. J., Li, Y., Ji, X. N., Liu, H., Xia, J. L., Wu, Z. Q., Fan, J., Ma, Z. C., Zhou, X. D., Lin, Z. Y. and Liu, K. D. (2004). **A decade's studies on metastasis of hepatocellular carcinoma**. J Cancer Res Clin Oncol *130*, 187-196, doi: 10.1007/s00432-003-0511-1.

Tobio, A., Alfonso, A. and Botana, L. M. (2015). **C-kit mutations determine dasatinib mechanism of action in HMC-1 neoplastic mast cells: dasatinib differently regulates PKCdelta translocation in HMC-1(560) and HMC-1(560,816) cell lines**. Immunopharmacol Immunotoxicol *37*, 380-387, doi: 10.3109/08923973.2015.1064132.

Todd, J. R., Scurr, L. L., Becker, T. M., Kefford, R. F. and Rizos, H. (2014). **The MAPK pathway functions as a redundant survival signal that reinforces the PI3K cascade in c-Kit mutant melanoma**. Oncogene *33*, 236-245, doi: 10.1038/onc.2012.562.

Topcagic, J., Feldman, R., Ghazalpour, A., Swensen, J., Gatalica, Z. and Vranic, S. (2018). **Comprehensive molecular profiling of advanced/metastatic olfactory neuroblastomas**. PLoS One *13*, e0191244, doi: 10.1371/journal.pone.0191244.

Tsutsui, S., Yasuda, K., Suzuki, K., Takeuchi, H., Nishizaki, T., Higashi, H. and Era, S. (2006). **A loss of c-kit expression is associated with an advanced stage and poor prognosis in breast cancer**. Br J Cancer *94*, 1874-1878, doi: 10.1038/sj.bjc.6603183.

Ueda, S., Mizuki, M., Ikeda, H., Tsujimura, T., Matsumura, I., Nakano, K., Daino, H., Honda Zi, Z., Sonoyama, J., Shibayama, H., Sugahara, H., Machii, T. and Kanakura, Y. (2002). **Critical roles of c-Kit tyrosine residues 567 and 719 in stem cell factor-induced chemotaxis: contribution of src family kinase and PI3-kinase on calcium mobilization and cell migration**. Blood *99*, 3342-3349.

Vita, M., Tisserand, J. C., Chauvot de Beauchene, I., Panel, N., Tchertanov, L., Agopian, J., Mescam-Mancini, L., Fouet, B., Fournier, B., Dubreuil, P., Bertucci, F. and De Sepulveda, P. (2014). **Characterization of S628N: a novel KIT mutation found in a metastatic melanoma**. JAMA Dermatol *150*, 1345-1349, doi: 10.1001/jamadermatol.2014.1437.

Wada, N., Kurokawa, Y., Takahashi, T., Hamakawa, T., Hirota, S., Naka, T., Miyazaki, Y., Makino, T., Yamasaki, M., Nakajima, K., Takiguchi, S., Mori, M. and Doki, Y. (2016). **Detecting Secondary C-KIT Mutations in the Peripheral Blood of Patients with Imatinib-Resistant Gastrointestinal Stromal Tumor**. Oncology *90*, 112-117, doi: 10.1159/000442948.

Wandzioch, E., Edling, C. E., Palmer, R. H., Carlsson, L. and Hallberg, B. (2004). **Activation of the MAP kinase pathway by c-Kit is PI-3 kinase dependent in hematopoietic progenitor/stem cell lines**. Blood *104*, 51-57, doi: 10.1182/blood-2003-07-2554.

Wang, S., Zhu, M., Wang, Q., Hou, Y., Li, L., Weng, H., Zhao, Y., Chen, D., Ding, H., Guo, J. and Li, M. (2018). **Alpha-fetoprotein inhibits autophagy to promote malignant behaviour in hepatocellular carcinoma cells by activating PI3K/AKT/mTOR signalling**. Cell Death Dis *9*, 1027, doi: 10.1038/s41419-018-1036-5.

Wang, X., Ren, H., Zhao, T., Chen, J., Sun, W., Sun, Y., Ma, W., Wang, J., Gao, C., Gao, S., Lang, M., Jia, L. and Hao, J. (2014). **Stem cell factor is a novel independent prognostic biomarker for hepatocellular carcinoma after curative resection**. Carcinogenesis *35*, 2283-2290, doi: 10.1093/carcin/bgu162.

Weinmann, A., Koch, S., Niederle, I. M., Schulze-Bergkamen, H., Konig, J., Hoppe-Lotichius, M., Hansen, T., Pitton, M. B., Duber, C., Otto, G., Schuchmann, M., Galle, P. R. and

Worns, M. A. (2014). **Trends in epidemiology, treatment, and survival of hepatocellular carcinoma patients between 1998 and 2009: an analysis of 1066 cases of a German HCC Registry**. J Clin Gastroenterol *48*, 279-289, doi: 10.1097/MCG.0b013e3182a8a793.

Wen, L. Z., Ding, K., Wang, Z. R., Ding, C. H., Lei, S. J., Liu, J. P., Yin, C., Hu, P. F., Ding, J., Chen, W. S., Zhang, X. and Xie, W. F. (2018). **SHP-1 Acts as a Tumor Suppressor in Hepatocarcinogenesis and HCC Progression**. Cancer Res *78*, 4680-4691, doi: 10.1158/0008-5472.can-17-3896.

Whittaker, S., Marais, R. and Zhu, A. X. (2010). **The role of signaling pathways in the development and treatment of hepatocellular carcinoma**. Oncogene *29*, 4989-5005, doi: 10.1038/onc.2010.236.

Yan, W., Zhu, Z., Pan, F., Huang, A. and Dai, G. H. (2018). **Overexpression of c-kit(CD117), relevant with microvessel density, is an independent survival prognostic factor for patients with HBV-related hepatocellular carcinoma**. Onco Targets Ther *11*, 1285-1292, doi: 10.2147/ott.s157545.

Yao, Z. and Mishra, L. (2009). **Cancer stem cells and hepatocellular carcinoma**. Cancer Biol Ther *8*, 1691-1698.

Yasuda, A., Sawai, H., Takahashi, H., Ochi, N., Matsuo, Y., Funahashi, H., Sato, M., Okada, Y., Takeyama, H. and Manabe, T. (2006). **The stem cell factor/c-kit receptor pathway enhances proliferation and invasion of pancreatic cancer cells**. Mol Cancer *5*, 46, doi: 10.1186/1476-4598-5-46.

Yasuda, A., Sawai, H., Takahashi, H., Ochi, N., Matsuo, Y., Funahashi, H., Sato, M., Okada, Y., Takeyama, H. and Manabe, T. (2007). **Stem cell factor/c-kit receptor signaling enhances the proliferation and invasion of colorectal cancer cells through the PI3K/Akt pathway**. Dig Dis Sci *52*, 2292-2300, doi: 10.1007/s10620-007-9759-7.

Yeo, W., Chen, P. J., Furuse, J., Han, K. H., Hsu, C., Lim, H. Y., Moon, H., Qin, S., Yeoh, E. M. and Ye, S. L. (2010). **Eastern Asian expert panel opinion: designing clinical trials of molecular targeted therapy for hepatocellular carcinoma**. BMC Cancer *10*, 620, doi: 10.1186/1471-2407-10-620.

Yoshida, H., Kunisada, T., Grimm, T., Nishimura, E. K., Nishioka, E. and Nishikawa, S. I. (2001). **Review: melanocyte migration and survival controlled by SCF/c-kit expression**. J Investig Dermatol Symp Proc *6*, 1-5, doi: 10.1046/j.0022-202x.2001.00006.x.

Younossi, Z. M., Koenig, A. B., Abdelatif, D., Fazel, Y., Henry, L. and Wymer, M. (2016). **Global epidemiology of nonalcoholic fatty liver disease-Meta-analytic assessment of**

prevalence, incidence, and outcomes. Hepatology *64*, 73-84, doi: 10.1002/hep.28431.

Yu, G., Yin, C., Jiang, L., Zheng, Z., Wang, Z., Wang, C., Zhou, H., Jiang, X., Liu, Q. and Meng, F. (2016). **Amyloid precursor protein cooperates with c-KIT mutation/overexpression to regulate cell apoptosis in AML1-ETO-positive leukemia via the PI3K/AKT signaling pathway**. Oncol Rep *36*, 1626-1632, doi: 10.3892/or.2016.4963.

Zeisberg, M. and Neilson, E. G. (2009). **Biomarkers for epithelial-mesenchymal transitions**. J Clin Invest *119*, 1429-1437, doi: 10.1172/jci36183.

Zender, L., Villanueva, A., Tovar, V., Sia, D., Chiang, D. Y. and Llovet, J. M. (2010). **Cancer gene discovery in hepatocellular carcinoma**. J Hepatol *52*, 921-929, doi: 10.1016/j.jhep.2009.12.034.

Zhang, M., Ma, Q., Hu, H., Zhang, D., Li, J., Ma, G., Bhat, K. and Wu, E. (2011). **Stem cell factor/c-kit signaling enhances invasion of pancreatic cancer cells via HIF-1alpha under normoxic condition**. Cancer Lett *303*, 108-117, doi: 10.1016/j.canlet.2011.01.017.

Zhang, Y., Liu, Y., Duan, J., Yan, H., Zhang, J., Zhang, H., Fan, Q., Luo, F., Yan, G., Qiao, K. and Liu, J. (2016). **Hippocalcin-like 1 suppresses hepatocellular carcinoma progression by promoting p21(Waf/Cip1) stabilization by activating the ERK1/2-MAPK pathway**. Hepatology *63*, 880-897, doi: 10.1002/hep.28395.

Zhou, W., Gong, L., Wu, Q., Xing, C., Wei, B., Chen, T., Zhou, Y., Yin, S., Jiang, B., Xie, H., Zhou, L. and Zheng, S. (2018). **PHF8 upregulation contributes to autophagic degradation of E-cadherin, epithelial-mesenchymal transition and metastasis in hepatocellular carcinoma**. J Exp Clin Cancer Res *37*, 215, doi: 10.1186/s13046-018-0890-4.

7. CURRICULUM VITAE

Name: Qiangnu zhang

Gender: Male

Data of birth: 22-05-1987

Place of birth: Gansu, V.R.China

Citizenship: Chinese

Marital status: Single

EDUCATION

09/2005-06/2010: (Bachelor’s Degree), Medical College of Lanzhou University.

09/2010-06/2013: (Master’s Degree), Medical College of Lanzhou University.

09/2015-11/2018: Institute of Pathology, Heidelberg University Hospital.

8. ACKNOWLEDGMENTS

First, I would like to extend my sincere gratitude to Prof. Dr. Peter Schirmacher, PD. Dr. Kai Breuhahn and Dr. Stephanie Rössler for giving me such a valuable opportunity to study and finish this thesis work in the Pathological Institute of the University Clinic Heidelberg with an excellent environment.

Dr. Stephanie Rössler, as my supervisor and group leader, gives me full support, great instructions, encouragements, and trust during the research work for my doctorate degree. What is more, she also gives me a lot of assistance in my life in Heidelberg. Without her support and help, I cannot finish this thesis work.

I am grateful to those who have offered me support in AG Rössler. They are Carolin Plöger, Sarah Luiken, Angelika Fraas, Felicia Truckenmueller, Raisatun Sugiyanto and Jun Hu. Special acknowledgment is given to Sarah Luiken and Angelika Fraas. Sarah Luiken gives me a lot of academic advice that benefit my project. Angelika Fraas always gives me help from my first day in the lab and provide me with technical training.

I also want to thank all the members of AG Breuhanh who have helped me in my project. Special thanks should give Michaela Bissinger, she provides me with technical training and survival skills in the lab.

My thanks also go to other researchers who give me advice during every seminar. My Chinese colleagues like Shan Wan, Teng Wei, Jun Hu, and Jing Xu give me much help and encouragement in my life and study. My gratitude would be expressed to them.

I would like to extend my deep gratefulness to my friends in Heidelberg who give me support like family. They are Kunsheng Li, Yuechen Wang, Luyu

Huang, Chengxi Xing, Xiaozhong Zhang, Yanyan Liu, Zihan Liu and Danchi Liu (not in any order). I apologize to those who are not mentioned here but helped me.

Finally, my thanks would go to my beloved family for their loving considerations and great confidence in me all through these years. A special thanks to my cousin Dr. Liao Juan who supported me a lot.

9. EIDESSTATTLICHE VERSICHERUNGE

1. Bei der eingereichten Dissertation zum Thema "**FUNCTIONAL ANALYSIS OF PROTO-ONCOGENE c-KIT IN THE DEVELOPMENT AND PROGRESSION OF HEPATOCELLULAR CARCINOMA**" handelt es sich um meine eigenständig erbrachte Leistung.
2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.
3. Die Arbeit oder Teile davon habe ich bislang nicht an einer Hochschule des In- oder Auslands als Bestandteil einer Prüfungs- oder Qualifikationsleistung vorgelegt.
4. Die Richtigkeit der vorstehenden Erklärungen bestätige ich.
5. Die Bedeutung der eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung sind mir bekannt. Ich versichere an Eides statt, dass ich nach bestem Wissen die reine Wahrheit erklärt und nichts verschwiegen habe.

Ort und Datum (city, date) Unterschrift (signature)