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The Role Of p62/IMP2 In Hepatocellular Carcinoma Progression

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Dedication

This dissertation is dedicated to my family. All my achievements would not have been reached without their unconditional love and support.

THE ROLE OF p62/IMP2 IN HEPATOCELLULAR CARCINOMA PROGRESSION

by

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Abstract

The family of insulin- like growth factor 2 mRNA binding proteins (IMPs) contains three members: IMP1, p62/IMP2 and IMP3. All these proteins are oncofetal proteins, expressed during embryogenesis and lost in most tissues in adults. However, p62/IMP2 were found overexpressed in various cancers but its function in carcinogenesis remains to be investigated. Our previous studies found that p62/IMP2 was not only overexpressed in hepatocellular carcinoma (HCC) tissues, but also overexpressed in HCC cell lines. To explore the biological roles of p62/IMP2 in HCC progression, p62/IMP2 was knockout in two p62/IMP2 positive HCC cell lines (SNU449, HepG2). Due to the low expression level of p62/IMP2 in SNU449, we overexpressed p62/IMP2 in this cell line. The results from both of wound healing assay and transwell migration assay indicated that overexpressed p62/IMP2 in both cell lines could promote the cell migration significantly ($p<0.05$). On the contrary, the lack of p62/IMP2 expression can reduce the cell migration ability ($p<0.05$). After analyzing the HCC expression data from Gene Expression Omnibus (GEO), the high and low p62/IMP2 expression groups were set up based on the median expression of p62/IMP2 from GSE 14520. CTNNB1 was selected in differential gene expression analysis with a cancer-metastasis related gene profile. Subsequently, western blotting analysis was performed to explore the effect of overexpressed p62/IMP2 on Wnt/β-catenin pathway-related proteins, and we found that overexpressed p62/IMP2 can significantly enhance the expression of Wnt and β-catenin, whereas inhibiting the

expression of Gsk3b and E-cadherin. A Wnt/β-catenin signaling pathway inhibitor XAV939 was used for confirming the results. Nevertheless, although β-catenin expression dramatically reduced in p62/IMP2 knockdown SNU449 cells, their colony formation ability was still enhanced by activating Wnt5-induced non-canonical Wnt signaling. In addition, we validated our results at the phosphorylation level by using Wnt pathway phosphoproteome chips. In summary, our data showed that overexpressed p62/IMP2 can enhance the migration ability of HCC cells via activating Wnt/β-catenin.

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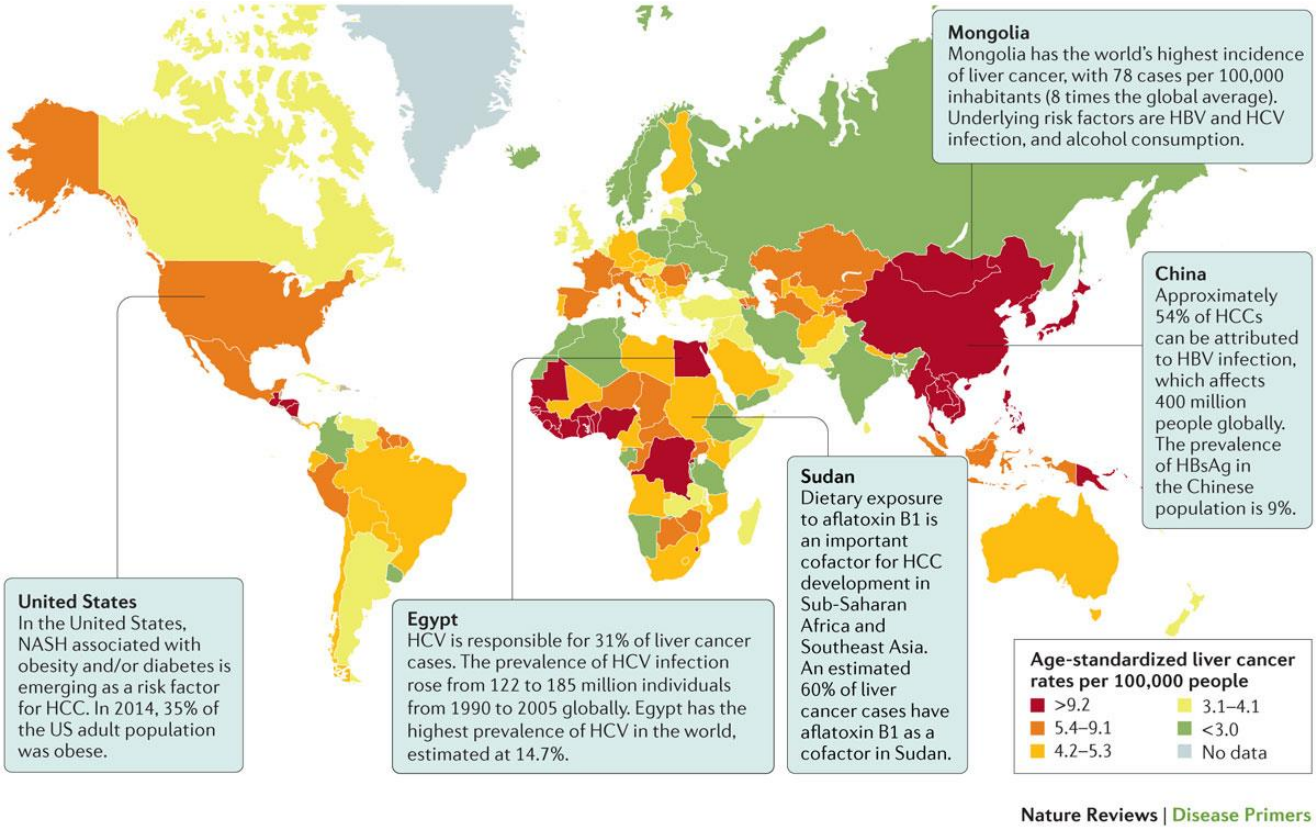
Chapter 1 Background and significance

1.1 Hepatocellular Carcinoma

1.1.1 Epidemiology and etiology

Liver cancer is one of the most frequently occurring malignant tumors in the world, which has a high mortality rate. Liver cancer can be divided into two major categories: primary liver cancer and secondary liver cancer. The cancer which originates and grows in the liver cells is called primary liver cancer. The liver metastasis transferred from cancer originated in other organs of the body is called secondary liver cancer. [1].

Primary liver cancer is the sixth most common cancer (about 850,000 new cases each year) and is the second leading cause of cancer-related deaths (about 800,000 annually) in the world [2,5]. Hepatocellular carcinoma (HCC) is one of the most common primary liver cancers, which accounts for about 90%. In the United States, there were estimated 40,710 new cases of primary liver cancer, of which HCC accounts for three quarters of all cases [3]. The ratio of men to women for HCC in global distribution is 2.4, and the most common age when HCC is diagnosed is usually between 30 and 50 years [4]. HCC occurs mainly in developing countries, including China, Mongolia, Southeast Asia, and sub-Saharan West Africa and Eastern Africa. With exception of Japan, Italy and France, the incidence of HCC in developed countries is low [2,5]. However, it is worth noting that from 1980 to the present, the incidence of liver cancer has increased by 43% in the United States and has increased at a rate of 4% per year [6].



Adapted from Laursen, L. A preventable cancer. *Nature* **516**, S2–S3 (2014), Nature Publishing Group

Figure 1. The global burden of HCC

HCC occurs predominantly in patients with liver cirrhosis [7], so risk factors often include factors that may contribute to chronic liver disease that leads to cirrhosis. The known risk factors include hepatitis B [8] or hepatitis C infection [9], alcohol abuse [10], aflatoxin [11], iron overload (hemochromatosis) [12], nonalcoholic steatohepatitis [13] and type 2 diabetes [14]. The risk factors for HCC are also different in different regions. China is a high-risk country for HCC, and chronic hepatitis B infection is found in 90% of cases [5,8]. However, in Japan, chronic hepatitis C is associated with 90% of cases of HCC. At the same time, another important factor leading to HCC in these areas is aflatoxin, which is widely found in East Asia and Southeast

Asia [11]. In Europe and the United States, hepatitis C and alcohol abuse are the most important factors leading to HCC [9].

The etiology of HCC has not yet been fully elucidated. Many studies indicated that HCC is caused by the accumulation of genomic mutations. In one HCC nodule, the coding region within the genome was accumulated with an average of 40 functional somatic mutations [15, 16]. This can lead to the activation of several cancer-related pathways in HCC. First, the WNT-β-catenin pathway is frequently activated in patients with liver cancer [17, 18], triggering a more aggressive HCC phenotype [104]. The mutations in its associated gene, such as CTNNB1, Axin1, APC, and ZNRF, are also frequently observed in patients with HCC [15]. Second, RB1, CDKN2A, and other tumor suppressor genes are frequently founded mutated in HCC. Inactivation of p53 and alternations in cell cycle also occur frequently in HCC [15, 19]. The third, RAS-RAF-MAPK and PI3K-AKT-mTOR pathways are also frequently activated in HCC [15]. Studies have shown that they are associated with FGF3, FGF4 and FGF19 amplification, and the inactivation of TSC1, TSC2, PTEN, RPS6K3, RSK2. In addition, mutations are also closely related to telomerase overexpression. Two mutations, located at the nucleotide positions 124 and 146 upstream of the ATG, suggest that they can generate new transcription factor binding sites and regulate Telomere reverse transcriptase mRNA expression. In 60% of HCC cases, the TERT promoter contains mutations, whereas telomerase is overexpressed in 90% of HCC cases [20].

1.1.2 Staging, Diagnosis and Treatment

The traditional methods for diagnosing HCC include imaging examination and blood tests. The majority of patients with HCC develop from chronic liver disease and cirrhosis, so related high-risk groups are recommended to use ultrasound for screening. In addition, CT scans and MRI can be used to diagnosis HCC [21,22]. The Liver Imaging Reporting and Data System (LI-RADS) is applicable to HCC for high risk groups. LI-RADS can be used regardless of the presence of nodules in previous ultrasound or other imaging check. If the mass has been treated, it should be classified as LR-treated. Benign and high-possibility benign lesions were classified as LR-1 and LR-2, respectively. If the lesion appears to be a malignant tumor such as cholangiocarcinoma, it should be classified as LR-M (possibly malignant tumor but not specifically HCC). In this case, biopsy is required for confirmed diagnosis. If there is a clear vein thrombus, regardless of whether the primary tumor can be observed, it should be classified as LR-5V. This can be diagnosed as a malignant lesion. Although intravascular tumor emboli are most common in HCC, intrahepatic cholangiocarcinoma may also be accompanied by atumor emboli. Based on specific criteria, lesions can also be classified as LR-3, LR-4, LR-5 [23].

Blood tests are also an essential procedure for the early diagnosis of HCC. At present, alpha-fetoprotein (AFP) is the most widely used serological marker in the diagnosis of HCC, but its specificity is not very high [24]. Therefore, AFP is not an optional biomarker for screening HCC. In recent years, research on autoantibodies to tumor-associated antigens (TAAs) is becoming a challenging area in the early diagnosis of HCC. As early as the 1960s, Robert W.

Baldwin showed that the human immune system can produce autoantibodies in the early stage of cancer development. A widely accepted viewpoint in cancer research field is that mutations, ectopic or recombination can occur early in the development and progression of tumor cells. During tumorigenesis and progression, specific protein products which were excreted by tumor cells, or released, shed by necrosis of cells are recognized by the immune system to produce antibodies against the antigen, which is called tumor-associated autoantibodies [25-27]. Many new TAAs, such as CAPERα/HCC1.4 [28], p62/IMP2 [29] and CIP2A/p90 [30], have been discovered with certain clinical values in the early diagnosis of HCC.

Unlike the TNM staging system, which is used for most tumors, the Barcelona Clinic Liver Cancer (BCLC) staging system is used for HCC [31]. The introduction of this system will help assess the patient's condition, provide accurate treatment options and predict patient outcomes. The clinical staging system for BCLC liver cancer was proposed by Llovet in 1999 and later revised by the American Association for the Study of Liver Diseases （AASLD) in 2005 [24]. Table 1 shows the BCLC staging and the corresponding treatment plan.

Table 1. BCLC staging and preferred treatment plan

Stage	Disease condition description	Preferred treatment plan
Stage 0 (very early stage)	The patient is in good condition and has a single tumor with a diameter of less than 2 cm. His liver function is well maintained.	Surgery Ablation for patients who are not suitable for surgery [32]

Stage A (early stage)	The patient is in good health, with a single to three tumor nodules less than 3 cm in diameter, with good liver function maintenance	Liver transplantation/surgery Ablation for patients who are not suitable for surgery [32]
Stage B (intermediate stage)	Cannot be surgically removed, the patient has multiple nodular asymptomatic tumors, no invasive growth, and good physical condition	Transcatheter arterial chemoembolization (TACE) [33]
Stage C (advanced stage)	Cannot be surgically removed, patients have cancer-related symptoms affecting physical state, symptomatic tumors, portal vein invasion, and extrahepatic spread (lymph node metastases)	Sorafenib [34]
Stage D (end-stage)	Cannot be surgically removed, patients have extensive metastases, and their physical condition is very poor	Palliative care [32]

Just a few years ago, sorafenib was the only drug that proved effective for advanced HCC treatment [34]. In recent years, with the in-depth study of the tumor microenvironment, researchers have discovered that the accumulation of a large number of gene mutations leads to the production of multiple antigens in occurrence and development of HCC, which can be recognized and specifically killed by the immune system [35]. At the same time, HCC cells can evolve, thereby to evade the body's immune surveillance through a variety of mechanisms.

Studies have confirmed that persistent viral infections and tumor neoantigen stimulation result in the formation of immunosuppressive states in the liver and promote development of HCC [36]. Cell populations with immunosuppressive functions such as regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC) are significantly up-regulated. In addition, the continuous exposure of antigens causes the tumor-specific lymphocyte surface to over-express inhibition signaling molecules, such as cytotoxic T-lymphocyte antigen-4 (CTLA-4), programmed cell death 1 (PD-1), lymphocyte activation gene-3 (LAG-3), consequently leaving T cells in a state of disability [37, 38]. Therefore, against the mechanism of immunosuppression in HCC, reverse the disability state of T cells and exert their anti-tumor effect is an important direction of HCC immunotherapy.

PD-1/PD-L1

PD-1 is a member of the CD28 superfamily that binds to ligands PD-L1 or PD-L2 and transmits a co-suppressor signal to T cell receptors [39]. It is mainly expressed on the surface of activated T cells and can also be expressed on B cells, Treg cells, NK cells and MDSC. Its ligands PD-L1 and PD-L2 are mainly expressed in macrophages, monocytes and other inflammatory cells [40].

Activation of T cells requires two signals, the first signal from the specific binding of TCR and antigen, and the second signal from the activation of accessory molecules such as CD80/CD28 [41]. When PD-1 on the surface of T cells binds to ligand, cytoplasmic tyrosine in

immuno-receptor tyrosine switch motifs (ITSM) domain of PD-1will be phosphorylated. Then it can recruit SH2 domain-containing protein-tyrosine phosphatase-2 (SHP-2) to dephosphorylate ZAP-70 and PI3K in the downstream of TCR and CD28, blocking the activation of T cells [42].

PD-1 can be expressed under the action of inflammatory mediators such as IFN-y. IFN-y can induce interferon regulatory factor 9 (IRF-9) binding to PD-1 gene promoter, leading T cells to transcribe PD-1. At the same time, IFN-γ stimulation also up-regulates PD-L1 expression on the cell surface. In the tumor microenvironment, T cells activate and expand into effector T cells by recognizing tumor antigens, specifically killing tumor cells, and secreting a large number of inflammatory factors such as IFN-y. Prolonged antigen stimulation causes activated T cells to overexpress PD-1, leading T cell incapacitation [43]. Most tumor cells escape the attack of immune cells in this way [44]. Therefore, the use of blockers to block the interaction between PD-1 and PD-L1 can restore T cell activity and kill tumor cells.

CTLA-4

CTLA-4 is a protein receptor also known as CD152. CTLA-4 functioning as an immune checkpoint participate in the inhibitory pathways in the immune system, which play an important role in self-tolerance. CTLA-4 is produced by Tregs, sharing the same ligands with CD28. In the process of activating immune cells, antigen-presenting cells (APC) present antigens to T cells and combine with TCRs to generate a first signal for T cell activation, which also need CD28 bind with CD80/CD86 to generate a second signal. Tregs bind to CD80/CD86 molecules on the

T cell surface through CTLA-4, thereby inhibiting the function of effector T cells [45]. CD80/CD86 also expresses on the surface of APC. Regulatory T cells induce APC through binding of CTLA-4 and CD80/CD86 thereby producing IDO, which also can inhibit the function of activated T cells and induces T cell apoptosis [46].

1.1.5 The metastasis and Epithelial-mesenchymal transition in HCC

As much as 90 percent of cancer related deaths can be attributed to metastasis rather than primary tumors [47], and HCC is no exception. According to the BCLC staging system, the patients with early HCC have a good prognosis, 30%-40% of patients can be cured, and the overall survival (OS) is more than 60 months. However, the prognosis of advanced stage patients with portal vein invasion and extrahepatic metastases is poor, whose OS is only 11 months [31]. Despite the remarkable advances in the research, drug development and clinical treatment plan of HCC in recent years, majority of advanced metastatic HCC patients are still facing desperate facts that we do not have an effective curable treatment method, but only limitedly extend their lives.

Epithelial-mesenchymal transition (EMT) refers to a process in which epithelial cells are transformed into a biological process with a mesenchymal phenotype by a de-differentiation procedure [48]. EMT plays an important role in the development of many tissues and organs in embryos [49]. In recent years, many studies have shown that EMT is closely related to fibrosis and cancer metastasis. Its main characteristics include the reduced expression of cell adhesion

molecules (such as E-cadherin), up-regulated expression of markers of mesenchymal cells (such as Vimentin, N-cadherin) and cytoskeletal transformation [50]. Through EMT, epithelial cells lose their cell polarity and epithelial phenotypes such as their attachment ability to the basement membrane, and therefore can acquire high interstitial phenotypes such as high migration and invasion, anti-apoptosis, and ability to degrade extracellular matrix. EMT is an important biological process for the ability of epithelial cell-derived malignant tumor cells to gain migration and invasion [48].

EMT is common in HCC patients. As the most important molecular marker of EMT, E-cadherin is down-regulated in 69% of HCC specimens and is associated with intrahepatic metastasis and invasion [51]. In patients with metastases, E-cadherin expression was significantly lower than the patients without metastasis.

The emergence of EMT requires a series of complex signaling pathway cooperation which are orchestrated by many activated EMT-inducing transcription factors (EMT-TFs). The most important EMT-TFs include the SNAI family (Snail and Slug), the Twist family (Twist 1, Twist 2, E12 and E47, and the ZEB family (ZEB 1 and ZEB 2) [52]. All these EMT-TFs can repress the transcription of E-cadherin by similar mechanisms: to bind with E-boxes which present in the E-cadherin promoter. In HCC patients, Snail and Twist overexpression are associated with larger tumor volume, increased relapse rate, shorter overall survival. *In vitro* experiments have demonstrated that overexpression of Snail or Twist promotes tumor cell invasion and increases

the mesenchymal phenotype of cells [53]. Overexpression of Snail and Twist in Huh7 cells inhibits E-cadherin expression and induces EMT phenotypic transformation [53]. Besides increasing cell invasiveness, EMT also protects HCC cells from extracellular signal-induced apoptosis. For example, EMT can activate epidermal growth factor receptor (EGFR) pathway and help HCC cells escape apoptosis induced by transforming growth factor-β1 (TGF-β1) [54].

Numerous signaling pathways have been shown to be involved in EMT, including the TGF-beta signaling pathway, the Wnt signaling pathway, and the Notch signaling pathway. The TGF-β signaling pathway is a key regulator of EMT and a major inducing factor that plays a crucial role in development, wound healing, fibrosis and tumors [55]. This pathway is initiated by a complex of TGF-β family members with cell surface receptors, which are tetramers composed of two type I and two type II transmembrane protein kinase receptors. This binding first phosphorylates the TβRII receptor and subsequently activates the TβRI receptor. The activated TβRI receptor then recruits and phosphorylates the Smad2 and Smad3 proteins in the cell. The phosphorylated Smad2 and Smad3 can form a trimeric complex with Smad4. As the Smad trimeric complex are translocated into nucleus, a series of its target gene will be regulated by other transcription factors. Smad signaling has been found to regulate EMT transcription factors including Snail1/Snail2 [56], ZEB1/ZEB2 [57], and the expression of Twist [58]. In addition to the classical Smad signal, TGF-β can also induce EMT through other non-Smad

signaling pathways, including Rho-like GTPase [59], PI3K/Akt/Mtor 60], extracellular signal-regulated kinase (ERK) [61], p38 [62], and JNK [63].

Wnt signaling pathway has been extensively studied in tumor progression in past two decades. So far, 19 different Wnt ligands and 11 transmembrane Frizzled receptors (FZD) have been found in humans. The binding of extracellular secreted Wnt ligands to FZD and low-density lipoprotein receptor related protein, (LRP) 5/6L can initiate canonical Wnt signaling pathway (Wnt/β-catenin signaling pathway) and non-canonical signaling pathway (PKC and JNK signaling pathway) [64].

Without Wnt signaling cascade activated, β-catenin is rarely existed in cytoplasm. There is a destruction complex consisting of APC, Axin, GSK3, CK1α and PP2A responsible for the removal of free cytoplasmic β-catenin [65]. However, while Wnt bind with Fz and LPR5/6, the function of degradation of β-catenin by destruction complex will be prevented. The activation of Wnt signaling could trigger the translocation of axin and bind with the cytoplasmic tail of LPR6 which result in the destruction complex loses the ability to bind with β-catenin. In the other way, activated Wnt-Fz-LPR5/6 complex can cooperate transduce the signaling to cytoplasm, leading the inhibition of GSK3β [66]. The consequences of Wnt signaling pathway activation allow β-catenin accumulating in cytoplasm, and finally translocate into nucleus [67].

Once Wnt pathway activated, β-catenin could bind with lymphoid enhancing factor/T-cell factor, LEF/TCF complex, forming β-catenin/LEF/TCF complex in nucleus. Following, the

transcriptional coactivators and histone modifiers, such as Brg1, CBP, Bcl9 and Pygopus will be recruited by the β-catenin/TCF complex and drive the Wnt target genes expression [68]. As a result, the transcription of cell cycle related gene such as cyclin D1 and c-myc will be activated, enhancing the proliferation ability of cells. In addition, a study has found that β-catenin/LEF-1 complex can down-regulate the expression of E-cadherin. Another research demonstrated that this pathway also plays an important role in EMT induced by hypoxia-inducible factor-1α (HIF-1α) [69].

Notch signaling pathway plays a crucial role in many physiological and pathological processes. So far, there are 4 Notch receptors (Notch 1-4) and 5 Notch ligands (Delta-like1,3,4 and Jagged1, 2) were found. Notch ligands and receptors are transmembrane proteins [70].

1.2 Insulin like growth factor 2 mRNA binding protein family

1.2.1 Nomenclature

Due to the family members are identified and assigned different functions in different biological systems, the nomenclature of the IMPs family in the earlier literature was confusing. In the past decade, many synonyms have been used in the literature, including IMP, CRD-BP, VICKZ, ZBP, Vg1RBP/Vera, or KOC [71]. The research scientists in this field finally discovered that all these proteins belonged to the same RNA-binding proteins (RBPs) family, which was so-called insulin-like growth factor 2 mRNA binding proteins (IMPs). Many studies have demonstrated that IMPs could regulate diverse aspects of cell function during development

and cancerIn mammals, three IMP paralogs (IMP1-3), also known as insulin-like growth factor 2 (IGF2) mRNA binding proteins 1, 2 and 3 (IGF2BP1-3) have been identified so far. Some studies also showed that IMPs can regulate the translation of IGF2 mRNA through a high-affinity binding site [72].

1.2.2 Structure of IMPs

IMPs are highly conserved, and they share a similar structure. The molecular weights of human IMP1, 2 and 3 family members are 63, 66 and 64 kDa, respectively. The identity of the overall amino acid sequences approximately is 60-80%. IMP1 and IMP3 are more closely related members in this family, with 73% amino acid sequence identity [71]. The genes for encoding IMP1 and IMP3 contain 15 exons, and the gene for encoding p62/IMP2 protein contains 16 exons [73].

All three members have 6 characteristic RNA binding modules, including two N-terminal RNA recognition motifs (RRMs) and four heteronuclear ribonucleoproteins (hnRNP) K-homology (KH) domains in their C-terminal regions (Fig.2). All members of the IMP family, regardless of the organism they express, show a high affinity for binding RNA. The association with DNA was only reported once for the p62/IMP2 homolog protein Vg1RBP/Vera in *Xenopus* [74]. Due to the lack of highly resolution structural information of the IMPs and their complexes with the target RNA, the current analysis of each domain is based on inferences from biochemical experiments and structural data from RRM and KH domains in other RNA binding

proteins. *In vitro* studies show that KH domains are mainly responsible for RNA binding [75], while the RRM domain is responsible for protein-protein interactions, protein dimerization, and stabilization of IMPs-RNA complexes [76]. However, the KH domains could only recognize short stretch RNAs with weak binding affinities so that a single domain may not be enough to regulate the translation of target mRNAs. Research indicates that human IGF2BP1 KH domains 3 and 4 form an anti-parallel pseudo-dimer conformation, in which KH3 and KH4 recognize their targets through sequence-specific interactions, each in contact with the targeted RNA. Two low-affinity interactions can allow KH3 and KH4 to bind to highly specific RNAs and force their associated transcripts to adopt a specific conformation [77]. It is worth noting that IMP-RNA complexes have a long *in vitro* half-life, suggesting that IMPs play an important role in the formation and maintenance of stable protein-RNA complexes.

1.2.3 Expression of IMPs

In mice, the expression characteristics of the IMP family are biphasic. IMPs were first peaked in oocytes and zygotes, and they have another expression peak in embryos from day 10.5 to day 12.5 [72], then they are continuously reduced till birth. In the second trimester, IMPs could be found in most tissues of embryos. They are expressed most in neurons and epithelial cells, and they also could be detected in the brain, nasal cavity, zygomatic arch, intestine, tail, vertebrae, and skin [78, 79].

After E12.5, the expression of IMPs is differentiated. In E17.5, p62/IMP2 can still be observed in the brain, nasal cavity, lung, liver, intestine and kidney. IMP3 almost disappeared at this time; between E12.5 and E16.5, IMP1 expression was only expressed in stem cells with poorly differentiated levels in the dorsolateral telencephalon (DMT) [80]. IMP1 and p62/IMP2 remained at low levels of expression in some organs until birth, whereas IMP3 was almost undetectable. Taking into account of the striking different expression in fetal and adult stages, IMPs are considered to be oncofetal proteins.

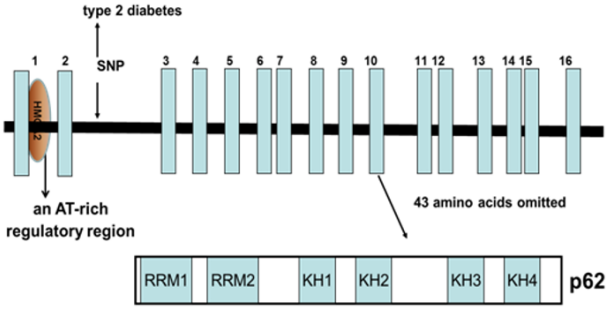
1.2.4 Physiological functions

Studies have shown that IMPs play an important role in embryonic development. Members of the IMP family plays an important role in mRNA processing steps, including localization, stability, translation, and possible nuclear export [71, 72]. IMP1 deficiency cause defects in the development of the intestine in newborn mice, and their size are 40% smaller than normal controls [79]. Also, p62/IMP2 deficiency severely affects the development of neural stem cells in mice [81]. IMPs do not express or express less in adulthood, but they are found overexpressed in many types of cancer.

Although this family of proteins was named as IGF2 mRNA binding proteins, IMPs have a wide range of binding targets. IMP1 was initially described to be involved in the stabilization of c-myc mRNA [82]. IGF2BP2 and IGF2 mRNA both have 3'-UTR and 5'-UTR binding sites, which can upregulate its expression and organize its presentation. p62/IMP2 can also organize

the degradation of c-myc mRNA through a similar mechanism to increase c-myc expression [83]. A comprehensive study showed that 3% of the HEK293 cell transcriptome will be expressed in IMP1 mRNP particles, which implies the number of IMP1 targets may be over 1000 [84]. IMP1 can allow β-actin mRNA to be localized to the leading edge of fibroblasts, thereby increasing the migration ability of cancer cells [85,86]. In addition, the IMP1 gene is amplified in breast cancer and studies have shown that this is associated with the loss of the estrogen receptor [87]. Let-7 is a family of highly conserved miRNAs with a total of 13 members which promote differentiation and inhibit tumor growth. IMPs can protect target genes of let-7, such as LIN28A, LIN28B, and HMGA2 from let-7-dependent silencing, thereby preventing the breakdown of these proteins and thus enhancing the invasiveness of tumors [88-90]. The role of IMP3 in cancer has also been extensively studied. It also influences cancer progression by modulating the stabilization and translation of cytoplasmic fate of mRNAs. IMP3 has been found to have many target genes, such as CD44 [91], H19 [92], HMGA2[93], etc. It is closely related to the invasion of cancer, and its expression is a good indicator of prognosis.

1.2.5 The regulation of p62/IMP2 and its role in cancer



Yang Li, 2015

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Figure 2. p62/IMP2 gene and its structure

The p62/IMP2 gene is located on 3q27.2, which contains 16 exons and 15 introns. The largest isoform of p62/IMP2 is 66KD.When exon10 is not transcribed, another 62KD isoform will be expressed known as p62 (Fig.2). The first intron contains an AT-rich regulatory region. An oncofetal protein, HMGA2, can bind to this AT-rich region to regulate p62/IMP2 expression [94]. In addition, HMGA2 has recently been reported as a driver of cancer metastasis by inducing epithelial-mesenchymal transition (EMT) [95]. Therefore, p62/IMP2 overexpression also has a potential role in promoting cancer metastasis. Besides HMGA2, *Airn*, a long noncoding RNA (lncRNA) could bind p62/IMP2 in cardiomyocytes and upregulate the translation of p62/IMP2. Silencing *Airn* will decrease expression of p62/IMP2 and reduce the binding ability of p62/IMP2 with its target mRNA [97].

p62/IMP2 was originally identified as an autoantigen in HCC [29], which implied that p62/IMP2 may play a role in cancer progression. Furthermore, several cancer-associated p62/IMP2 target mRNAs have been identified, such as CTGF mRNA [96], IGF2 mRNA [72] and c-myc mRNA [59]. Several studies have reported that p62/IMP2 is involved in carcinogenesis. p62/IMP2 might be a driver in cancer proliferation. Phosphorylated by mTOR, p62/IMP2 could stabilize HMGA1 and enhance the expression of IGF2 [101]. By activating the IGF2 / PI3K / Akt pathway, p62/IMP2 can promote the proliferation, migration, invasion and epithelial to mesenchymal transition (EMT) of GBM cells [98]. Analysis of a large number of samples shows

that p62/IMP2 may enhance the proliferation of gallbladder carcinoma by regulating RAC1-induced ROS generation [99]. In esophageal adenocarcinoma, p62/IMP2 expression is abnormally increased and is associated with short survival and metastasis [100].

Recently, more and more evidence showed that p62/IMP2 can also enhance the migration ability of cancer cells and promote cancer metastasis. A cluster of p62/IMP2 targets (LIMS2, TRIM54, and LAMB2) are considered to be involved in cell migration, cell adhesion, and cytoskeleton remodeling. These processes are highly related with cancer metastasis [102]. A recent study from our group found that the overexpressed p62/IMP2 could enhance cell migration and reduce cell adhesion in breast cancer [96]. Other group also reported that p62/IMP2 and IMP3 could cooperate to down-regulate progesterone receptor and promote metastasis in triple-negative breast cancer [103].

Some studies suggest that p62/IMP2 plays an important role in the regulation of EMT in cancer. One of the mechanisms is that p62/IMP2 can be considered as an indicator of Wnt/β-catenin signaling pathway in HCC, thereby inducing genomic instability and an invasive phenotype [104]. In breast cancer, p62/IMP2 could enhance the expression of β-catenin and promote metastasis [96]. Wnt/β-catenin pathway activation triggers the loss of E-cadherin, plays a key role in EMT. In sum, overexpressed p62/IMP2 might promote EMT through the activation of Wnt/β-catenin pathway.

Chapter 2 Hypothesis and Specific Aims

2.1 Hypothesis

Liver cancer is one of the most common types of cancer and the third leading cause of cancer related death in worldwide. There are three major subtypes of liver cancer, including HCC (HCC), cholangiocarcinoma, hepatoblastoma. Among these three types of liver cancer, HCC is the most common type of primary liver cancers in adults. Most HCC cases are caused by liver cirrhosis associated with hepatitis B virus or hepatitis C virus infection and excess alcohol intake. HCC commonly metastasizes to lungs, lymph nodes, adrenal gland and bones, which leads to poor prognosis for patients.

Insulin-like growth factor 2 mRNA binding protein 2 (p62/IMP2/) was originally reported as an autoantigen in HCC which belongs to IMPs family. The IMPs family contains three members: IMP1, p62/IMP2 and IMP3, which share similar protein structure. IMPs are oncofetal proteins, which are expressed during embryogenesis and lost in most tissues in adults. Therefore, p62/IMP2 can be used as a potential biomarker for early diagnosis of HCC. It has been reported that many targets mRNA of p62/IMP2 are highly related with cancer progression, such as c-myc mRNA, CTGF mRNA, p53 mRNA and RAS mRNA. The cluster of p62/IMP2 targets was involved in cell migration, cell polarization and cytoskeleton remodeling, all of which could promote cancer metastasis. Several studies have reported that p62/IMP2 is related to the metastasis of different types of cancer. However, the mechanism is poorly understood. **We hypotheses that overexpression of p62/IMP2 may play an important role in EMT and**

metastasis of HCC. The proposed study will help us to elucidate the roles of p62/IMP2 in HCC and provide the basis for novel biomarker development and further design of anti-cancer drug.

2.2 Specific Aims:

Specific Aim 1: To use bioinformatics and *in vitro* approaches to explore the relevance of p62/IMP2 to the HCC progression.

Specific Aim 2: To investigate the effect of overexpressed p62/IMP2 on HCC progression.

Specific Aim 3: To explore the effect of overexpressed p62/IMP2 on the molecular mechanism of HCC carcinogenesis.

2.3 Significance

In worldwide, primary liver cancer is the sixth most common cancer and is the second leading cause of cancer-related deaths. In China and sub-Saharan Africa, HCC is the most common cancer among men. Once metastasis occurs, the average survival time of patients with HCC will be less than 10 months. Therefore, early diagnosis is very important.

p62/IMP2 was first reported as a tumor-associated antigen in HCC. A series of studies have demonstrated the abnormal overexpression in HCC cell lines and HCC tissues. Since the expression of p62/IMP2 is regulated by HMGA2 and closely related to the activation of the Wnt/β-catenin pathway in HCC, we hypothesize that p62/IMP2 may play an important role in the metastasis of HCC, but the mechanism remains to be investigated.

In the present project, firstly we will use bioinformatics approach to analyze and identify the targets of p62/IMP2, and then conduct *in vitro* studies to explore the role of p62/IMP2 in HCC. This project will also focus on the p62/IMP2

pathways and their target genes in the metastasis of HCC. We propose that the *in vitro* studies will also provide evidence for a better understanding of HCC metastasis at the molecular and cellular levels.

Chapter 3. Materials and Methods

3.1 Data pre-processing

All the original data was downloaded from Gene Expression Omnibus (GEO) for further pre-processing. R software (version 3.4.3) was used to read and analyze data. The bioconductor "affy" package was used to adjust the background and normalize the original microarray data. Probe id was annotated to gene id accordingly. For genes with multiple detecting probes, mean gene expression values were used in the subsequent analyses.

3.2 Differential expression analysis

Gene expression profile from HCC patients or HCC tissues as well as HCC cell lines were included. Due to the nature of different type of sample sources, they were analyzed separately. All samples were separated into two groups, a high expression group and a low expression group by using the median expression value of p62/IMP2 gene. Bioconductor package "limma" was used to compare differentially expressed genes (DEGs) in the high and low p62/IMP2 expression groups. Genes with log2 fold change greater than 1.5 and adjusted p value lower than 0.01 were considered to be significantly different.

3.3 Gene Ontology (GO) and KEGG annotation of DEGs

R Bioconductor package "clusterProfiler" was used to carry out GO and KEGG annotations for the DEGs.

3.4 Cell lines and cell culture

Human HCC cells SNU449 and HepG2 were purchased from ATCC and cultured in RPMI (GIBCO, Life Technologies, Grand Island, NY, USA). Medium was supplemented with 10% FBS and 100 units/ml penicillin plus 100ug/ml streptomycin (Thermo Scientific, Waltham, MA, USA) at 37^{o}C, 5% CO_2. When cells were cultured with Wnt/β-catenin inhibitor, 10 μM of XAV-939 was added into media at 80 percent cell confluence. The cells were collected after 24h of growth.

3.5 Transfection

To overexpress p62/IMP2, SNU449 cells were transfected using Lipofectamine 2000 (Life Technologies, Grand Island, NY, USA) in 6-well plates following the manufacturers' instruction. 800 ng/ml G418 was used to select the transfected cells and to obtain stably transfected clones. The clones were picked with cloning cylinders (Corning, Tewksbury, MA, USA) and expanded in 24-well plates.

To knock-down p62/IMP2, co-transfected IMP2 HDR Plasmid (h) and IMP2 CRISPR/Cas9 KO Plasmid (h) (Santa Cruz Biotechnology, TX, USA) with Lipofectamine 3000 (Life Technologies, Grand Island, NY, USA). Puromycin (1mg/ml) was used to select stable p62/IMP2 knockout variants. 10 clones for each variant were obtained, and they were expanded and tested by western blotting.

3.6 Immunohistochemistry

Immunohistochemistry was performed on a commercially available liver cancer tissue array (BC03116a, US Biomax, Inc.). Briefly, after deparaffinization with xylene and rehydration with ethanol, antigen retrieval was performed by microwave heating methods. 2-3 drops of avidin block solution were applied to the slide to block endogenous biotin activity. After washing, 200µl p62/ IMP2 monoclonal antibody (Cell Signaling Technology, MA, USA) with 1:100 dilution was applied to the cover of every slide and then incubated overnight at 4^{o}C. The slide was then incubated with polyvalent biotinylated linked goat-anti-rabbit secondary antibody for 1 hour at room temperature. 3'-Diaminobenzidine (DAB) was used for detection. Lastly, the slide was counterstained with hematoxylin, dehydrated with ethanol, stabilized with xylene and then processed for imaging.

3.7 Proliferation assay

Cells were seeded at $5x10^3$ cells/well in 96-well plates and grown for 1-5 days. The cell proliferation was determined by a sulforhodamine (SRB) assay. Briefly, cells were fixed with 10% (w/v) trichloroacetic acid (TCA) at 4°C for 1 hour, rinsed 5 times with water, and air dried. The cells were then fixed with 100ul 0.4% (w/v) SRB in acetic acid for 15 minutes. After washing, the unbound dye was removed by washing five times with 1% acetic acid and the plate was air-dried. The binding stain was dissolved by 150 ul 10 Mm Tris base each well. Colorimetric readings were performed in microplate reader at 515nm.

3.8 Transwell migration assay

Cells were detached from culture plate with 0.25% Trypsin-EDTA solution. After centrifugation, the cells were resuspended in serum-free medium, and 100 μl of the cell suspension was added onto the filter of a transwell chamber. The cells were cultured for 16 hours after carefully adding 600 μl of 10% FBS medium in 24-well plate lower chambers. Cells were fixed by 3.7% formaldehyde in PBS and then permeabilized by 100% methanol 20 min at room temperature. Cells were finally stained with 0.1% crystal violet for 15 min and counted under microscope. After each step, cells were washed twice with PBS.

3.9 Plate clonogenic assay

Cells were seeded onto 6-well plates at 500 cells/well and cultured for 2 weeks with 10% FBS. The colonies were washed with PBS, fixed with 100% methanol for 15 minutes and stained with 0.1 % crystal violet for 20 minutes. Colonies with more than 50 cells were counted manually.

3.10 Wound healing assay

Cell were cultured in 6-well plates and cultured in RPMI medium with 2% FBS. Wound scratches were made to the all monolayer cells with pipetting tips. The photographs of the wound scratches were taken every 24 hours. The cell migration levels into the scratch were quantified using Image J software.

3.11 Western blotting analysis

Proteins (20 μg/well) were separated on 10% SDS-PAGE gels and transferred onto nitrocellulose membrane. Blocking in 5% no fat milk TBST buffer for 1 hour in room temperature. Membranes were incubated at 4°C in TBST with primary antibodies overnight, then in TBST with secondary antibody at room temperature for 1 hour. Finally, an enhanced chemilluminescence visualization kit (Thermo Fisher Scientific, MA, USA) was used to visualize the staining bands. Protein expression was detected by western blotting with rabbit monoclonal antibodies: p62/IMP2, β-catenin, Wnt3a, Wnt 5a/b, GSK3β, Snail, N-cadherin, GAPDH (Cell Signaling Technology, MA, USA); and mouse antibodies: E-cadherin (Cell Signaling Technology, MA, USA). Primary antibodies were detected with goat anti-mouse IgG (HPR conjugate) or anti-rabbit IgG (HPR conjugate). The membranes were finally scanned by Invitrogen iBright Imaging System (Thermo Fisher Scientific, MA, USA).

3.12 Immunofluorescence

Cells were seeded onto 8-chamber culture slides and grown for 24 hours fixed with 100% methanol and 100% acetone at -20°C. The cells were subsequently treated with β-catenin monoclonal antibody (Cell Signaling Technology, MA, USA) and incubated with Alexa Flour 488 conjugate secondary antibodies. Mounting medium containing DAPI was then added. Confocal fluorescence images were acquired with a laser scanning microscope (LSM 700; Zeiss, New York, NY, USA).

3.13 Phosphoproteome assay

The Wnt Pathway Phospho Antibody Array (PNT227) was conducted by Full Moon BioSystem Inc, which contains 227 site-specific and phospho-specific antibodies. SNU449 cells, HepG2 cells and their transfected variants were harvested as whole-cell lysates and transferred to Full Moon Biosystem Inc in dry ice. Further, proteins were purified, labeled with biotin and placed on the blocked microarray with coupling solution. 0.1% Cy3-Streptavidin solution was used to detect conjugation-labeled proteins. The phosphorylation degree of a site was represented by the ratio of phosphorylation/unphosphorylation.

Chapter 4. p62/IMP2 is Highly Associated with Hepatocellular Carcinoma

4.1 Overview

The family of insulin- like growth factor 2 mRNA binding proteins (IMPs) contains three members: IMP1, p62/IMP2 and IMP3. All these proteins are oncofetal proteins, expressed during embryogenesis and lost in most tissues in adults. However, IMPs were found to be overexpressed in various cancers. In this project, we will focus on p62/IMP2 and explore its role in HCC progression. In chapter 4, the association between p62/IMP2 and HCC will be investigated.

4.2 Rationale, experimental design and alternative approach

Specific Aim 1: To use bioinformatics and *in vitro* approaches to explore the relevance of p62/IMP2 to the HCC progression.

4.2.1 Rationale

The high frequency of anti-p62/IMP2 autoantibody occurrence in the serum samples from various types of cancer patients implies that p62/IMP2 may play a role in cancer progression. Several cancer-associated p62/IMP2 target mRNAs have been identified. They include CCN2 mRNA (15), IGF2 mRNA (7) and c-Myc mRNA. In previous study, our group has demonstrated that p62/IMP2 is overexpressed in breast cancer. However, we still know very little about the role of p62/IMP2 in the development of HCC.

Many studies have demonstrated that the occurrence of HCC is mainly due to the gene mutations, abnormalities of proteins and the change of cell metabolism. Differentially expressed

genes may be directly or indirectly related to the occurrence and susceptibility of cancer. The significance of detecting these differentially expressed genes lies in the ability to reveal the mechanism of cancer development from the gene level and to better explore the methods for cancer treatment. The formation of tumors is a long-term and complex process that requires the mutations of multiple genes at multiple stages, including changes in tumor suppressor genes and oncogenes, and dysregulation of normal genes. In this specific aim, we plan to find HCC related datasets in Gene Omnibus (GEO) database, and will perform a preliminary analysis of the role of p62/IMP2 in liver cancer and possible regulatory pathways through differential expression analysis.

4.2.2 Experimental Design & Methods:

1）Analysis of p62/IMP2 expression in HCC by *in vitro* methods: to examine the expression of p62/IMP2 in HCC tissues, a multiple HCC tissues microarray was used for performing immunohistochemistry (IHC). The microarray contains 40 tumor tissues with detailed clinical information and 30 non-tumor tissues including tumor adjacent tissues and healthy tissues as control. The microarray was performed by IHC with p62/IMP2 antibody and the results were analyzed between tumor and non-tumor tissues. The expression of p62/IMP2 in normal liver cell lines (L02) and HCC cell lines (HepG2, SNU449, Hep3b, Huh7 and SMMC7721) was detected by western blotting analysis. We subsequently compared the expression differences of p62/IMP2 in normal liver cells, highly differentiated HCC cells and poorly differentiated HCC cells. In addition, we examined the frequency of autoantibody against p62/IMP2 in sera from HCC

patients. A total of 160 HCC sera and 90 normal human sera as controls were obtained from the serum bank of Cancer Autoimmunity and Epidemiology Research Laboratory at the University of Texas, El Paso. Recombinant p62/IMP2 protein was purified and ELISA was used to screen the sera with p62/IMP2 autoantibody.

2) Analysis of p62/IMP2 expression in HCC by bioinformatics approaches: three HCC gene expression datasets (accession numbers GSE 25097, GSE36376, GSE 14520) were downloaded from GEO, the p62/IMP2 mRNA expression data was analyzed in normal tissue group (only in GSE 25097), tumor adjacent tissue group and HCC tissue group. Subsequently, high and low p62/IMP2 expression groups were set up based on the median expression of p62/IMP2 to obtain the DEG profile. Based on the DEG profile, GO analysis, KEGG analysis and FunRich enrichment analysis were performed with R (version 3.4.3) and Funrich (version 3.1.3) software.

4.2.3 Potential problems & alternative approaches:

Due to the high prevalence of autoanbodies against p62/IMP2 was detected in HCC patients, we expect the overexpression of p62/IMP2 is highly related to the progression of HCC. However, the overexpression of proteins is not the only reason to stimulate the immune system to produce autoantibodies. Other factors such as protein structure alternation and chronic inflammation can also result in autoantibodies production. Otherwise, the reliable results of IHC and western blotting analysis are relied on the high sensitivity and specificity of the antibodies. In this project, bioinformatics approaches are performed to provide support for *in vitro* studies.

4.3 Results

4.3.1 *In vitro* studies showed that p62/IMP2 is overexpressed in HCC.

To examine the expression level of p62/IMP2 in HCC tissues, we performed immunohistochemistry (IHC) analysis on a tissue array including 40 HCC tissues and 30 normal liver tissues. The expression of p62/IMP2 was scored by the immune-staining intensity and positive immune-staining cell area. p62/IMP2 protein is overexpressed in human HCC tissues compared with normal human liver tissues (score= 10.30, n=40 vs. score =5.23, n=30, $p<0.05$) (Table 2). The color scores between HCC tissues and normal tissues were significantly different, while the area score results were similar. The representative examples of weak p62/IMP2 staining pattern of adjacent normal tissue and strong p62/IMP2 staining pattern of HCC tissue is shown in Figure 3. In addition, western blotting analysis was performed to examine p62/IMP2 protein expression in the non-tumorigenic liver cell line L02 and three HCC cell lines. p62/IMP2 are overexpressed in all the HCC cell lines; in contrast, L02 cells showed a relatively low expression level of p62/IMP2. Meanwhile, p62/IMP2 was overexpressed in well-differentiated cell lines (HepG2, Hep3b and Huh7) compared to the poorly differentiated cell lines (SNU449) (Fig.5). To detect the anti-p62/IMP2 autoantibody in HCC patients, the p62/IMP2 recombinant protein was purified and used as coating antigen in ELISA for the detection of 160 sera from HCC patients and 89 normal controls (Fig. 4 & Table 3). The average OD value of anti-p62/IMP2 autoantibody in HCC sera is significant higher than that in normal controls. The prevalence of anti-p62/IMP2 autoantibody in HCC sera is 18.1%, which is significantly higher

than that in normal control sera (2.2%), while using Mean+3SD of normal group OD value as the cut-off value.

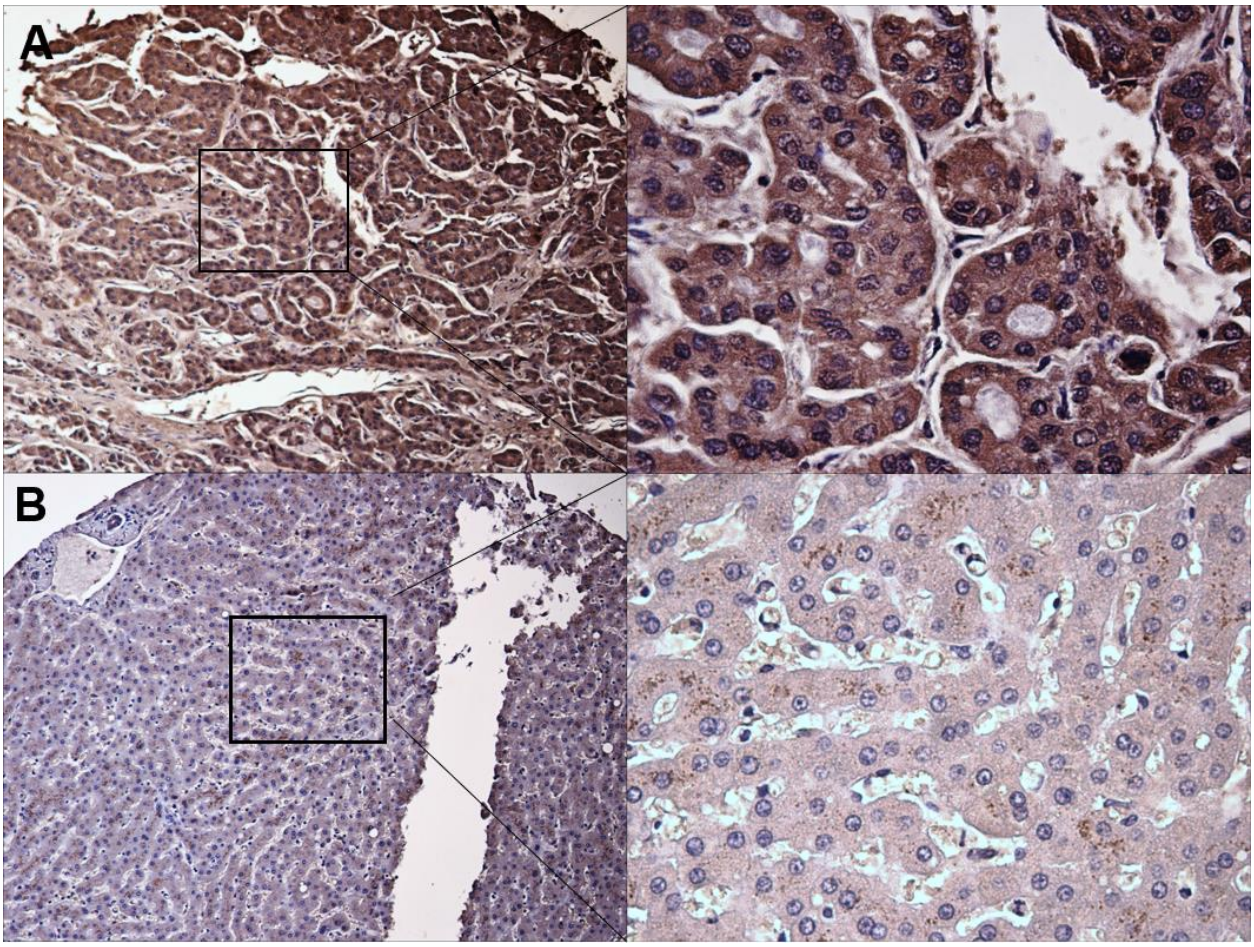


Figure 3. Immunohistochemical staining of p62 in Liver cancer tissue and adjacent normal tissue slides.

A. Positive stain pattern of p62 in representative liver cancer tissue. B. Weak stain pattern of p62 in representative adjacent normal tissue Positive stain pattern of p62 in representative liver cancer tissue. (x100 and x400 magnification).

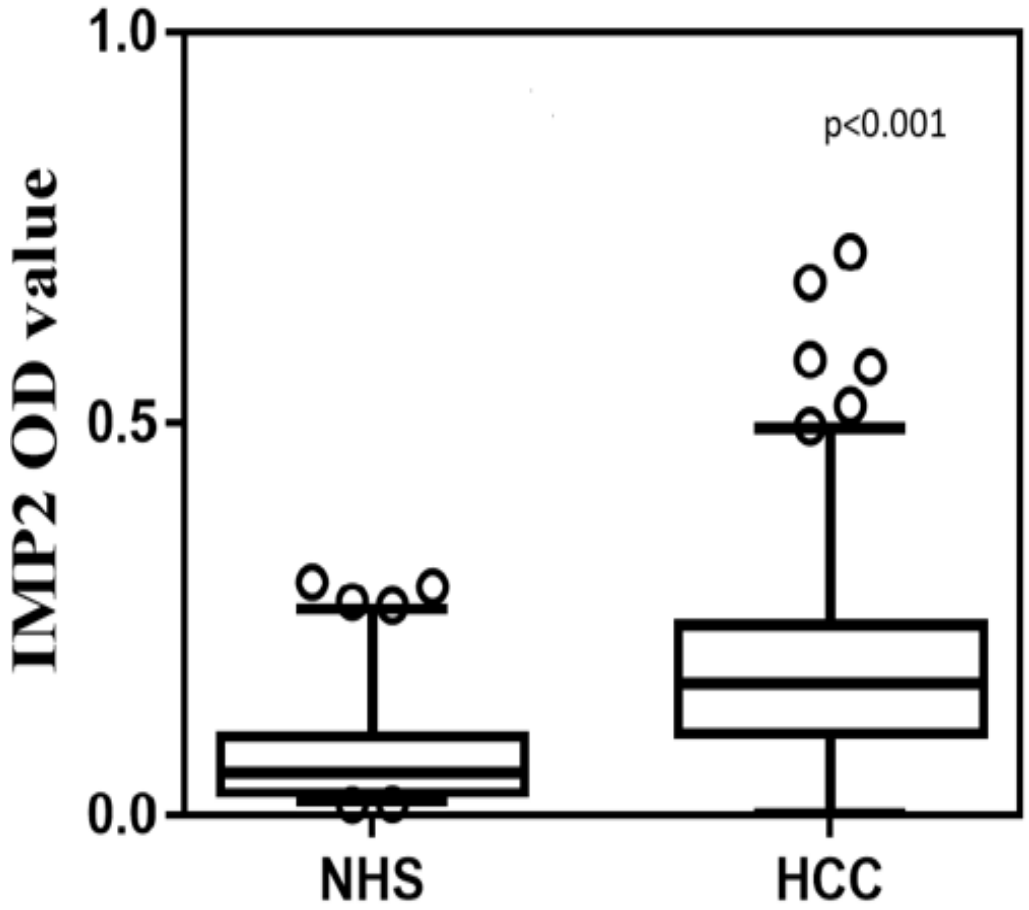


Figure 4. Distribution of autoantibodies against p62/IMP2 in HCC sera and normal human sera.

The distribution of antibody titers is indicated as optical density (OD) obtained from ELISA. HCC: sera from HCC patients. NHS: normal human sera.

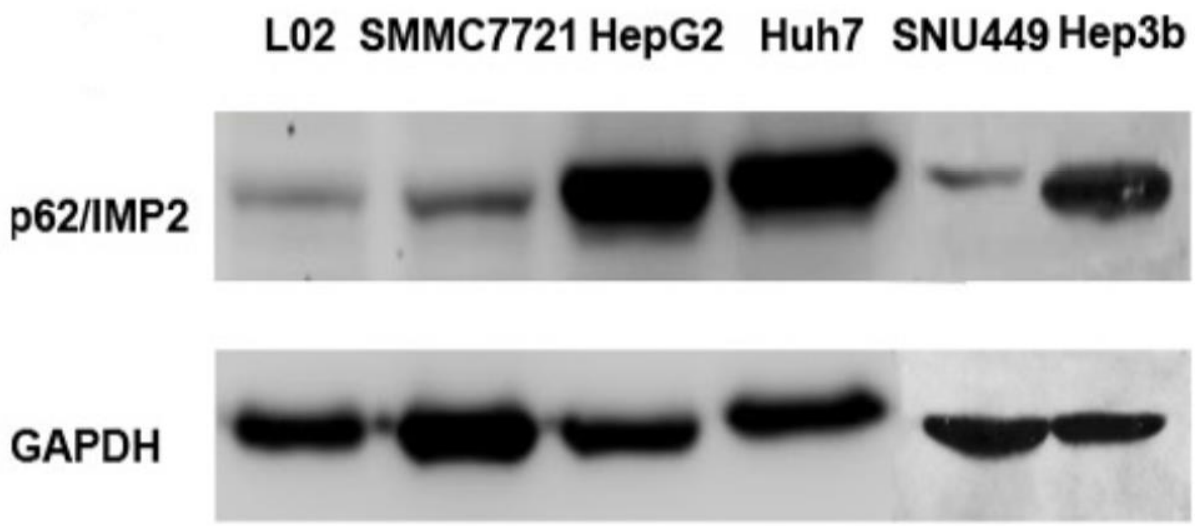


Figure 5. The expression of p62/IMP2 in normal liver cell line L02 and HCC cell lines.

Compare to L02, p62 is slightly overexpressed in SNU449 and SMMC7721 cells and highly overexpressed in HepG2, Huh7 and Hep3b cells.

Table 2. Expression of p62/IMP2 in HCC tissues and adjacent normal tissues.

Type of tissues	IHC score			
	Sample Quantity	Color	Area	Final score
Tumor tissues	40	2.823*	3.68	10.30*
Stage I tumor tissues	4	2.50*	3.75	9.00*
Stage II tumor tissues	13	2.85*	3.54	10.15*
Stage III tumor tissues	23	2.87*	3.74	10.61*
Normal tissues	30	1.5	3.67	5.23

*$p<0.05$, compared with normal group. (one-way ANOVA)

The staining of p62/IMP2 was evaluated by a four-level scoring system for color and area. The final score is the product of color and area score. Here we show the average number of samples.

Table 3. Frequency of autoantibody response to p62/IMP2 in sera from HCC patients (HCC) and normal human sera (NHS)

Group	Positive(%)	Negative (%)	Total
HCC	29/160 (18.1%)*	131/160 (81.9%)	160
NHS	2/89 (2.2%)	87/89 (97.8%)	89
Total	31	218	249

Cutoff value: Mean+3SD of normal group;

HCC group vs Normal group: * $p<0.01$;

HCC: sera from HCC patients; NHS: sera from normal human.

4.3.2 Bioinformatics studies indicated that p62/IMP2 is highly associated with HCC progression.

We investigated p62/IMP2 mRNA expression in a large cohort of HCC patients from the GEO database (accession numbers GSE 25097, GSE36376, GSE 14520). Compared with adjacent tissues of HCC and normal liver tissues from healthy people, p62/IMP2 gene expression in HCC tissues was significantly upregulated in all the three datasets. p62/IMP2 gene expression did not show a distinct difference between normal liver tissues and adjacent tissues in GSE 25097 (Fig. 6). To acquire the DEG profile in HCC, we set up a p62/IMP2 high expression group and a p62/IMP2 low expression group based on the median in GSE 14520 and processed them in R software. Totally 900 DEGs was selected and further used for GO analysis. The cellular component analysis demonstrated that most of the proteins expressed by DEGs are located in the cell membrane and cytoplasm, which might be an indirect evidence that p62/IMP2 is involved in the regulation of cell migration (Fig. 7). In Funrich enrichment biological pathway analysis as well as cellular component analysis, our data supported GO enrichment analysis: 9.9% DEGs were figured out related to EMT ($p<0.01$) and the DEGs are mainly distributed in cell membranes and collagens (Fig.8). Therefore, we select 20 DEGs related with EMT and analyzed their interaction genes and we found that a lot of interaction genes are involved in Wnt signaling pathway (Fig. 9).

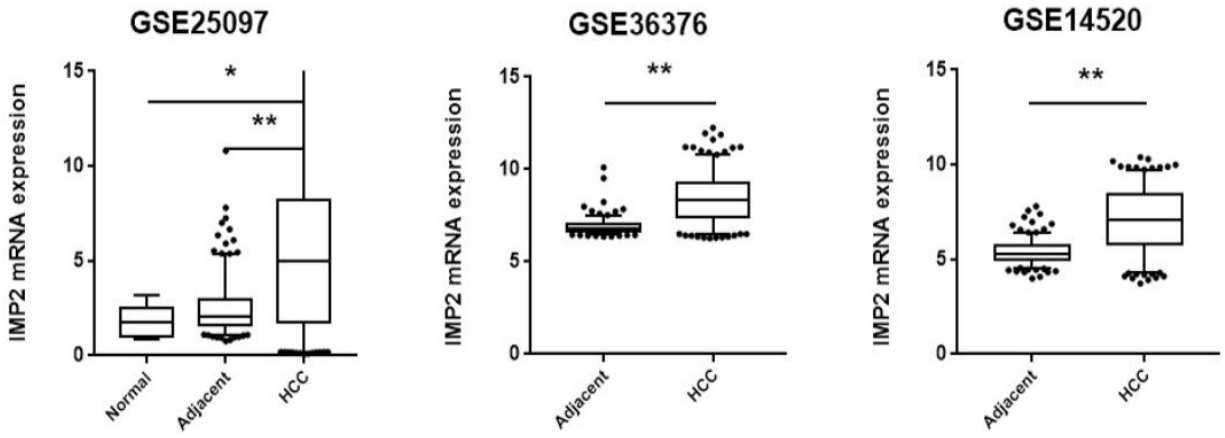


Figure 6. mRNA expression of p62/IMP2 in tumor tissues and their controls in datasets GSE 25097, GSE 36376 and GSE 14520.

Y axis showed the log2 fold change of the p62/IMP2 gene expression. In all the three datasets, p62/IMP2 mRNA expression in HCC tissues is significantly higher than adjacent tumor tissues. *p<0.05, **p<0.01.

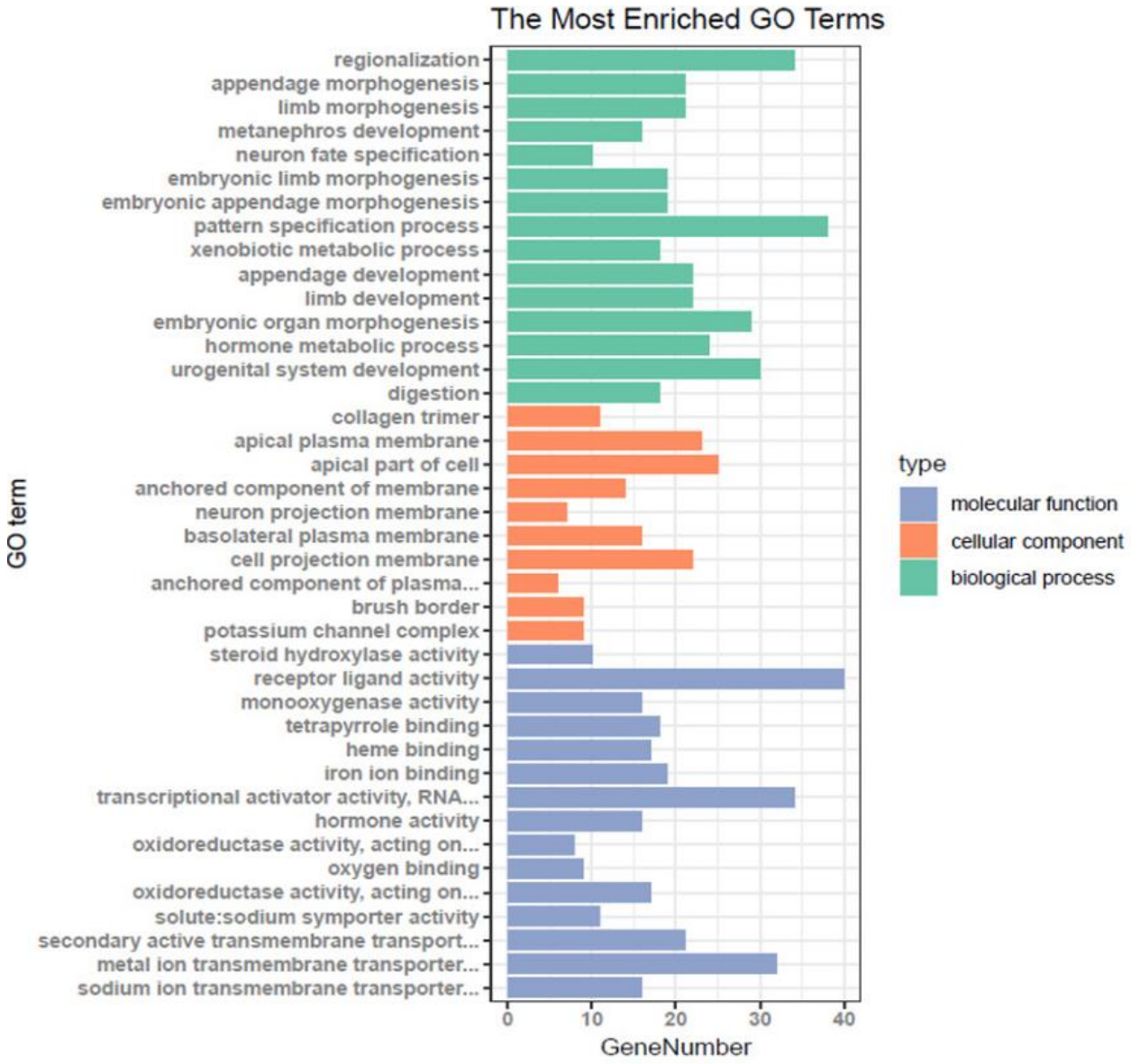


Figure 7. Gene Ontology analysis was performed between p62/IMP2 high expression group and low expression group.

We listed 15 most enriched terms in molecular function and biological process analysis and 10 most enriched terms in cellular components analysis.

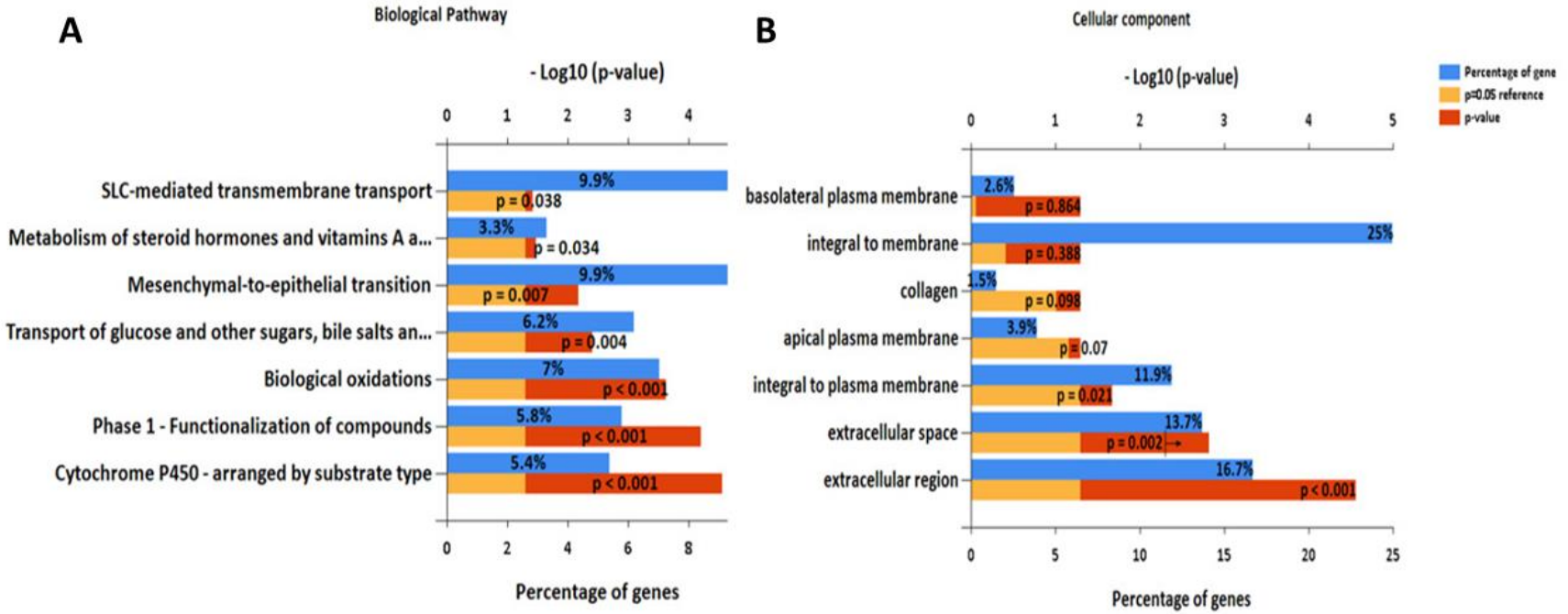


Figure 8. The FunRich enrichment analysis of Biological pathway and Cellular component for 900 DEGs.

The enrichment analyses were performed with FunRich 3.1.3 software.

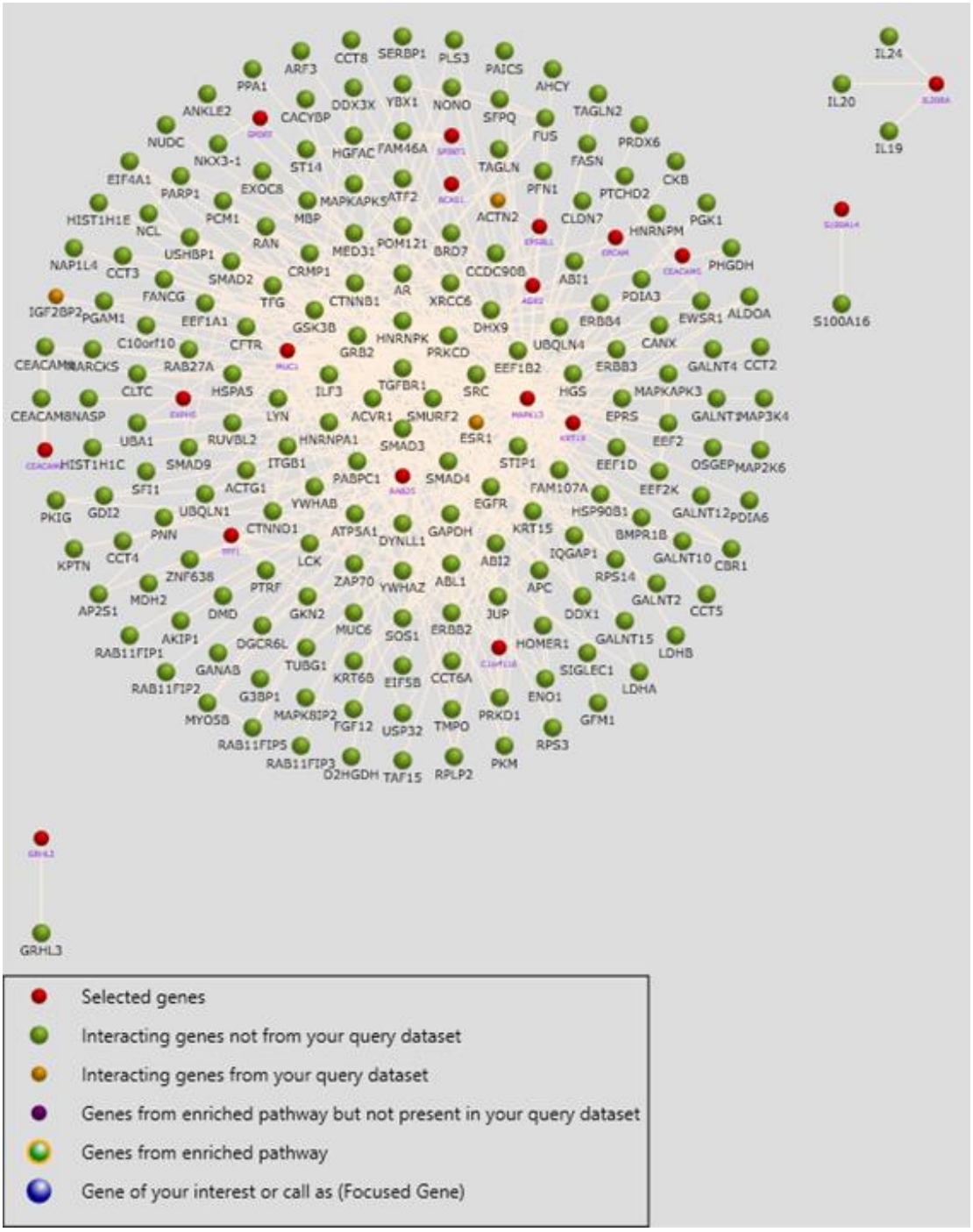


Figure 9. Gene Interaction analysis of metastasis related DEGs.

The selected metastasis related DEGs are shown as red and their directly interacted gene are shown as green.

Chapter 5. The Effect of p62/IMP 2 in HCC Progression

5.1 Overview

In chapter 4, we indicated that overexpression of p62/IMP2 is widely existed in HCC. Hence, we will discuss the role of p62/IMP2 in HCC progression based on it. In the development of cancer, cancer cells will display many new features that are different from ordinary cells, for instance, anti-apoptosis ability, EMT, higher proliferation ability and motility. In chapter 5, we will focus on the functional study of p62/IMP2 and perform a series of *in vitro* studies. Our results in this chapter will reveal the function of p62/IMP2 in HCC and guide us to the next step, the molecular signaling research.

5.2 Rationale, experimental design and alternative approach

Specific aim 2: To investigate the role of p62/IMP2 on HCC progression

5.2.1 Rationale:

In specific aim 1, we explored the expression of p62/IMP2 in HCC and its potential function by using bioinformatics methods. In specific aim 2, we will establish *in vitro* models by knockout p62/IMP2 in HCC cell lines to further study the function of p62/IMP2 in HCC. Although we first discovered autoantibodies to p62/IMP2 in HCC, we know very little about the role of p62/IMP2 in liver cancer. Our group showed that p62/IMP can enhance migration ability and reduce cell adhesion in breast cancer. There are also other studies that clarify that p62/IMP2 plays a role in cancer metastasis. For example, p62/IMP2 has been shown to be closely related to the metastasis of breast and esophageal adenocarcinomas. By activating the IGF2 / PI3K / Akt

pathway, p62/IMP2 can promote GBM cell proliferation, migration, invasion and epithelial-mesenchymal transition (EMT) (98). In addition, several mRNA targets of p62/IMP2 such as CTGF mRNA, IGF2 mRNA and c-myc mRNA have been shown involved in the proliferation of cancer cells. Therefore, in this chapter, we will concentrate on the effect of overexpressed p62/IMP2 on proliferation and migration ability in HCC cell lines.

5.2.2 Experimental Design & Methods:

We have known that p62/IMP2 is overexpressed in most of HCC cell lines in chapter 4. To explore the changes in cell function induced by overexpression of p62/IMP2, our strategy is to overexpress or knockdown p62/IMP2 in HCC cells by transfecting Crisper/Cas 9 plasmids. After establishing stable transfected HCC cell line variants, we will conduct a series of functional studies.

1) The effect of overexpressed p62/IMP2 on proliferation ability. We designed three experiments to examine the cell proliferation ability. The first one is proliferation assay. The sulforhodamine B (Srb) will be used for checking proliferation ability between wild type HCC cells and the transfected variants. Compared to other competitor methods such as MTT assay, it is more reliable and repeatable. We also performed the cell cycle assay by flow cytometry as the second one. It visually reflects the proportion of cells at each stage. When the cells in the G2 and S phases are increased, we believe that the proliferation of the cell line is enhanced. Another test we used is plate clonogenic assay. It not only reflects the proliferative capacity of cells, but also reflects the independent survival ability of cells.

2) The effect of overexpressed p62/IMP2 on cell migration ability. We performed wound healing assay and transwell migration assay to check the cell migration ability. In Wound healing assay, cells were cultured in 6-well plates and the wound scratches were made to the all monolayer cells with pipetting tips. The photographs of the wound scratches were taken every 24 hours so we can compare the cell migration ability between HCC cell variants. Transwell migration assay is another well-established experiment that can examine the cell migration ability. Counted cells were cultured in a chamber in 24-well plates overnight, the number of cells that can pass the membrane shows their migration ability.

5.2.4 Potential problems & alternative approaches:

We expect that a series of *in vitro* experiments, such as the wound healing assay, transwell migration assay, will support our hypothesis that the overexpression of p62/IMP2 to promote the cell migration ability of HCC cells. Our potential problem is that although different cancers exhibit similar properties, the underlying mechanisms of mutations between different cancers are not the same. Therefore, our hypothesis based on past breast cancer research does not necessarily occur in HCC. The molecular mechanism of cancer is very complicated. Although the target of IMP2 is related to the migration ability and proliferation ability of cells, the influence of IMP2 overexpression on cells is unknown. Another potential problem is that different HCC cell lines contain different genetic background. A single cell line can only represent part of the HCC genetics, so we will select two or more representative cell lines for experiments.

5.3 Results

5.3.1 Establishment of stable p62/IMP2 knockdown variants in HepG2, SNU449, and p62/IMP2 overexpression variants in SNU449 cell lines

We discussed the expression of p62/IMP2 in multiple HCC cell lines in chapter 4. Among them, HepG2 is highly differentiated from Caucasians, while SNU449 is from Asians with low degree of differentiation and strong invasion ability. Because they represent diverse genotypes of HCC, we want to explore whether p62/IMP2 plays a similar role in these two cell lines.

To explore the biological roles of p62/IMP2 in HCC progression, p62/IMP2 was knockout in two p62/IMP2 positive HCC cell lines (SNU449, HepG2). Due to the low expression level of p62/IMP2 in SNU449, we performed the transfection experiment to overexpress p62/IMP2 in this cell line (Fig. 10).

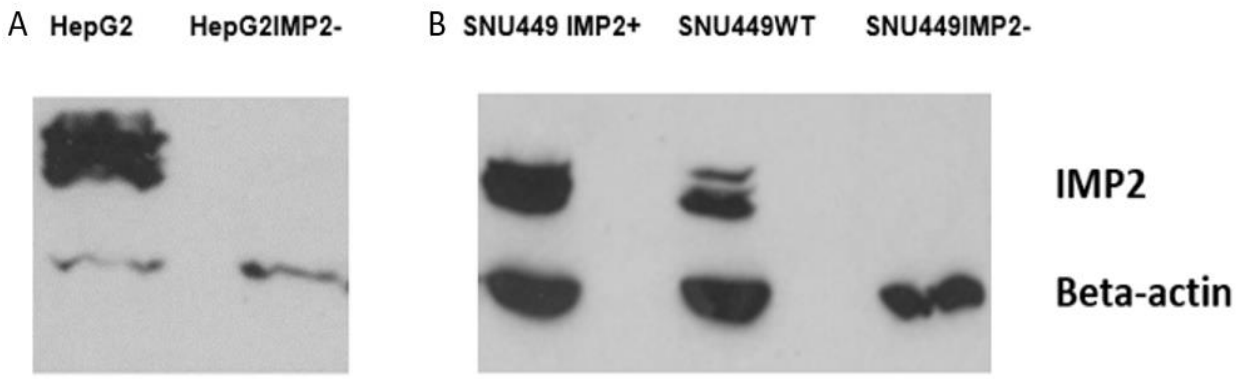


Figure 10. Verification of p62/IMP2 overexpression and knockout cell line with western blotting analysis.

A. Western blotting result of p62/IMP2 knockout in HepG2 cell line. B. Western blotting result of p62/IMP2 overexpression and knockout in SNU449 cell line.

5.3.2 p62/IMP2 does not significantly promote cell proliferation but reduces cell population dependence and enhances colony formation.

All the generated variants were tracked for 5 days in a proliferation assay to examine their relative growth rate. However, regardless of whether p62 was overexpressed or knocked down, we did not observe a consistent impact on the proliferation in either cell line (Fig .11). The cell cycle distribution of each variant was measured by flow cytometry. The results support our proliferation assay results in that the cell cycles profiles were not significantly altered by the transfections. The only exception to this was for the cell ratio in the G2/M phase of IMP2 knockdown cells decreased 7%-9%, compared with their corresponding wild type parental cells (Fig 12). Surprisingly, in a clonogenic assay, p62/IMP2 overexpressing cells showed a higher colony formation rate in both cell lines (Fig. 13). At the same time, the clone formation rate of SNU449 IMP2 knockdown cells had a moderate increase as well. Clone formation rate reflects two important features: cell population dependence, and proliferation ability. Considering that overexpression or knocked out p62/IMP2 did not alter the proliferation ability in both HCC cell lines, our results indicated that the change of p62/IMP2 expression enhanced the ability of growing without population dependence.

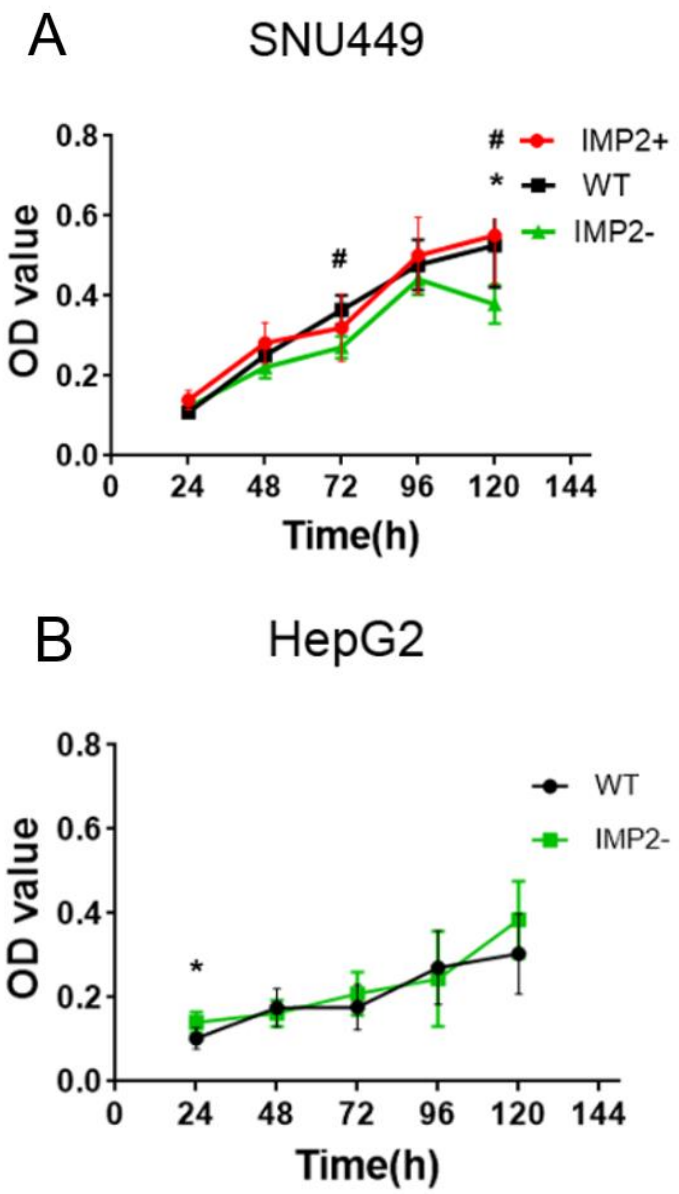


Figure 11. Overexpression of p62/IMP2 does not promote HCC cell proliferation ability.

A. Growth curve of the cell proliferation assay in SNU449. B Growth curve of the cell proliferation assay in HepG2 cells. SNU449 IMP2+ vs SNU449 IMP2-: #$p<0.05$; SNU449(WT) vs SNU449(IMP-): *$p<0.05$ (two-way ANOVA, n=3HepG2(WT) vs HepG2(IMP-): *$p<0.05$ (multiple t-test, n=3).

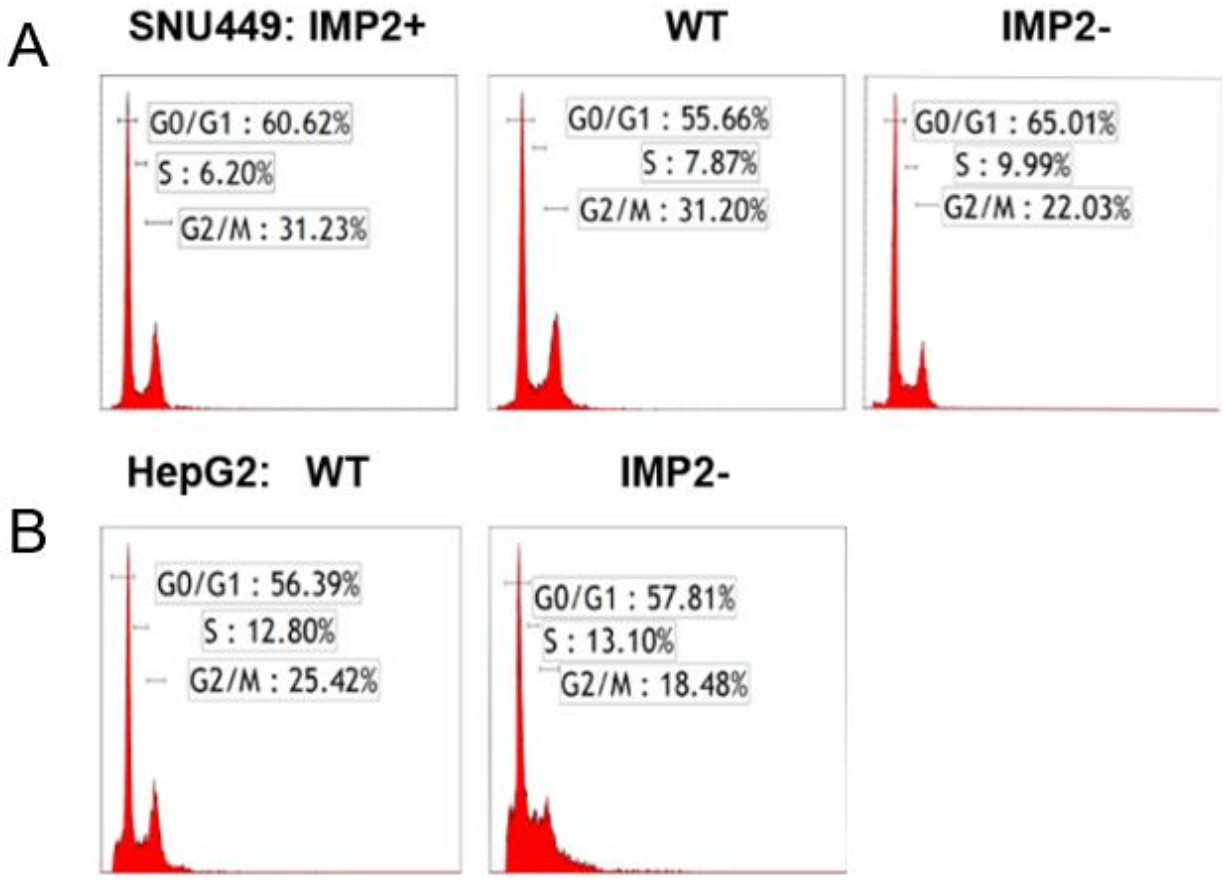


Figure 12. Cell cycle of p62/IMP2 variants tested by flow cytometer.

A. Cell cycle of SNU449 variants. B. Cell cycle of HepG2 variants. The expression of p62/IMP2 does not affect the cell cycle in either cell lines.

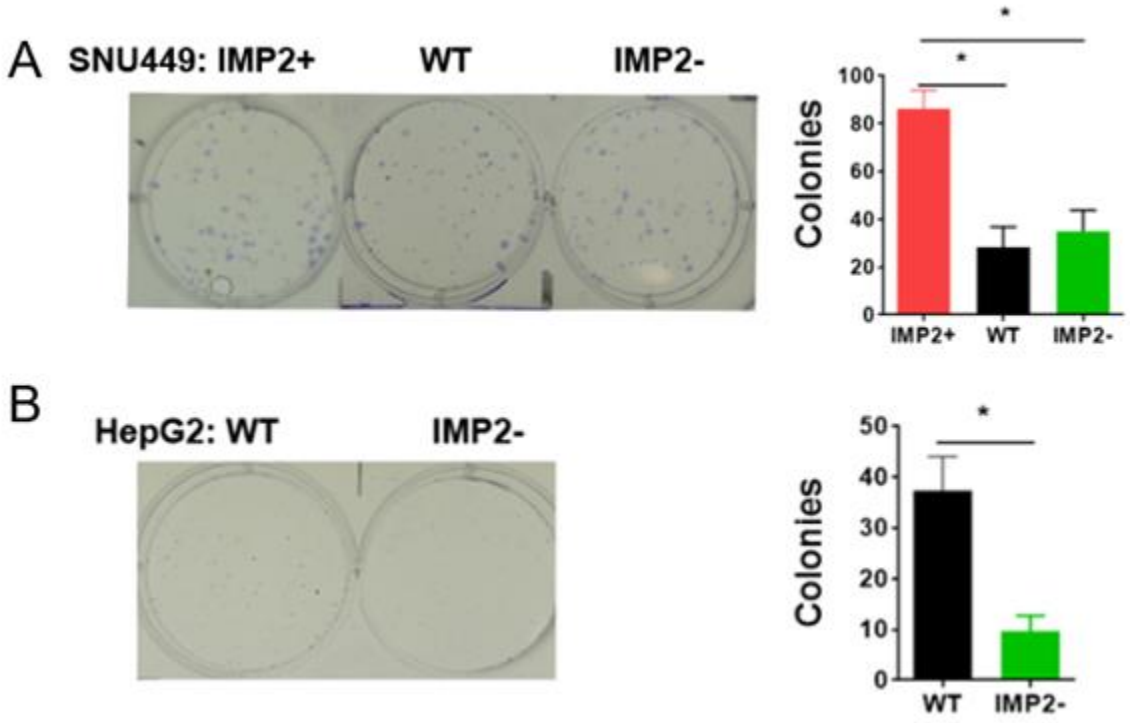


Figure 13. plate clonogenic assay p62/IMP2 variants from two HCC cell lines.

A. Both overexpression and knock down of p62/IMP2 could promote colony formation ability in SNU449 cells. B. Knockdown of p62/IMP2 reduce colony formation ability in HepG2. *$p<0.05$ (one-way ANOVA, n=3).

5.3.3 Overexpression of p62/IMP2 promotes cell migration in HCC

A cellular component of GO classification showed that most proteins expressed by DEGs are expressed in the cell membrane and cytoplasm (Fig. 7). This might be the indirect evidence that p62/IMP2 is involved in the regulation of cell migration. We performed two *in vitro* assays, a wound healing assay and a transwell migration assay, to investigate the cell migration properties. Although the migration rate of hepG2 cells is significantly lower than SNU449 cells, both of assays showed consistent results that overexpressed p62/IMP2 can promote HCC cell migration *in vitro*. Overexpression of p62/IMP2 in HCC cells increased 'wound' closure by 40% to 50% compared to wild-type cells, which also significantly increased the number of transmembrane cells in the transwell migration assay (Fig. 14&15).

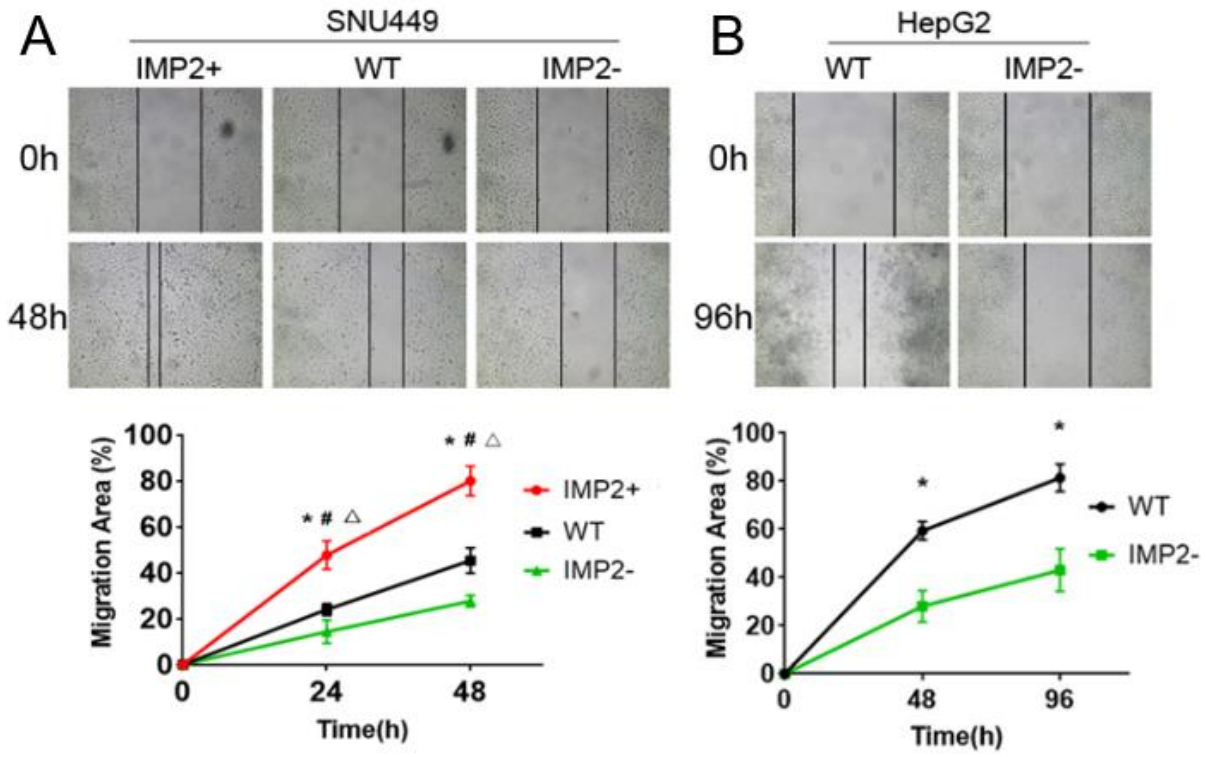


Figure 14. **The wound healing assay showed that overexpression of p62/IMP2 promotes cell migration in HCC.**

A. Overexpression of p62/IMP2 can promote cell migration ability in SNU449. B. Overexpressed p62/IMP2 enhances cell migration ability in HepG2. SNU449 IMP2+ vs SNU449 IMP2-: #p<0.05; SNU449(WT) vs SNU449(IMP-): *p<0.05; SNU449 IMP2+ vs SNU449 WT: △p<0.05 (two-way ANOVA, n=3). *P < 0.05 (t-test, n=3).

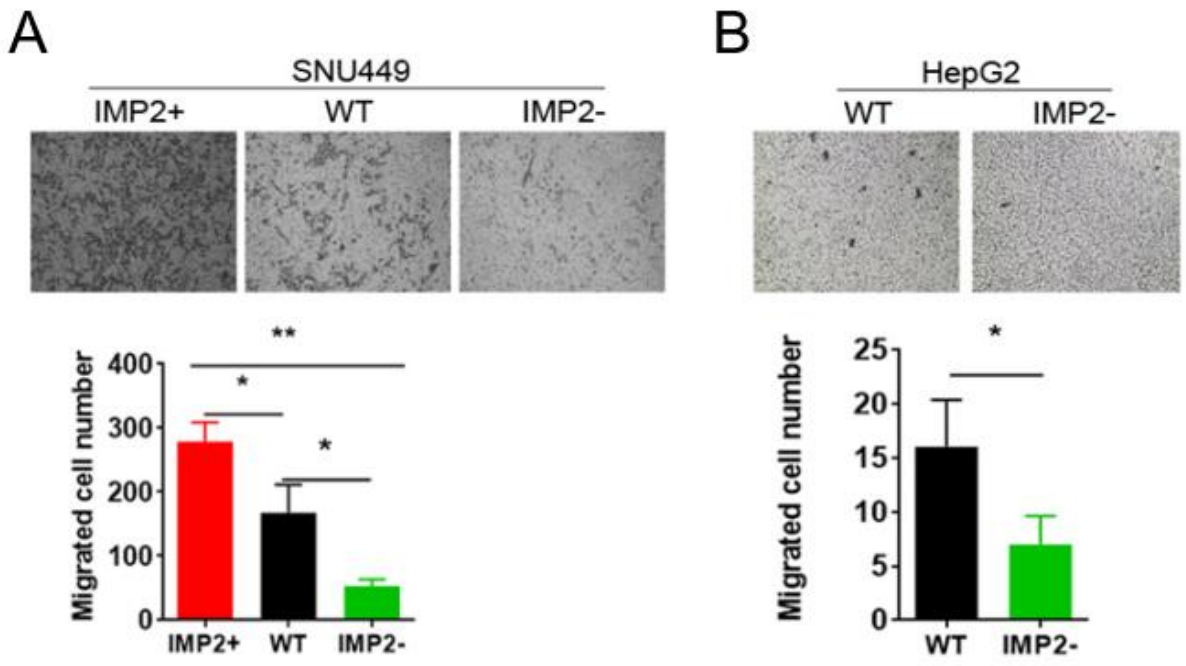


Figure 15. The transwell migration assay in p62/IMP2 variants from two HCC cell lines.

A&B. Overexpressed p62/IMP2 can increase the trans-membrane cell number in both SNU449 and HepG2 cell variants. The image of transwell migration assay for p62/IMP2 variants at random area under 100X microscope. * $p<0.05$, ** $P<0.01$ (One-way ANOVA and t-test, n=3). Values represented mean ± SEM of three independent measurements.

Chapter 6. Identification the Effect of Overexpressed p62/IMP2 on the Molecular Mechanism of HCC Carcinogenesis

6.1 Overview

In chapter 5, we demonstrated that overexpressed p62/IMP2 can enhance the migration ability of HCC cells. However, we still know very little about the molecular mechanism of this phenomenon. Many studies have shown that the classical Wnt signaling pathway plays a very important role in the development of HCC and 40%-70% of the HCC cells have β-catenin aggregation in the nucleus, which is also result from Wnt signaling activation. At the same time, activation of the Wnt pathway is involved in the process of EMT, playing an important role in the metastasis of cancer cells. In this chapter, we will explore the molecular mechanisms of overexpression of p62/IMP2 in cancer metastasis and our understanding of the occurrence of p62/IMP2 autoantibodies will go a step further.

6.2 Rationale, experimental design and alternative approach

Specific aim 3: To explore the effect of overexpressed p62/IMP2 on the molecular mechanism of HCC carcinogenesis.

6.2.1 Rationale

The emergence of HCC is a multi-factor, multi-gene involved, multi-step process. Its 5-year survival rate is not more than 50%, and its treatment is still based on local treatment and systemic therapy [31]. Radiotherapy, Chemotherapy, targeted therapy, and surgery are the mainly treatment strategies, but HCC is not sensitive to radiotherapy and chemotherapy, so the

research on targeted therapy for the pathogenesis of HCC is more urgent. Wnt signaling pathway plays an important role in the occurrence, development, metastasis and prognosis of HCC. In HCC cases, β-catenin gene mutations accounted for 8%-30%, APC and Axin protein gene mutations and loss of function accounted for 1%-3% and 8%-15%, respectively [105]. However, the mutation mechanism of β-catenin gene is quite different from others. Interestingly, studies have shown that the mutation of β-catenin gene appears relatively late in the development and progression of HCC, while the accumulation of β-catenin protein in the nucleus occurs in the early stage of HCC.

6.2.2 Experimental Design & Methods:

The approach we used to identify the targets of p62/IMP2 is DEGs analysis by using a metastasis-related gene profiling. A metastasis gene list was downloaded from QIAGEN's website, which contains 206 key metastasis related genes. The mRNAs of these genes were investigated in high and low p62/IMP2 expression samples as we used in Chapter 4. The DEGs would be the potential targets we will investigate in next step. For potential target proteins and the pathways in which they are located, we will use western blotting analysis and immunofluorescence for further validation. Western blotting analysis and immunofluorescence assay can quantitatively analyze the expression of the protein, and immunofluorescence assay can also show the location of protein expression. After that, we will use the pathway inhibitor to verify the previous experiment. In the regulation of pathways, changes in the phosphorylation level of proteins are also important aspects. Hence, a Wnt Pathway Phospho Antibody Array which contains 227 site-specific and phospho-specific antibodies was used to check the alteration

of phosphorylation degree between each HCC cell variants. Through it we can understand the more comprehensive picture of the changes caused by p62/IMP2 overexpression.

6.2.3 Potential problems & alternative approaches

We expect overexpressed p62/IMP2 can activate the Wnt/ β-catenin pathway, and activation of the Wnt/β-catenin pathway is responsible for the increased migration of HCC cells. The potential problem is that the impact of overexpression of p62/IMP2 is unknown, and there may be a plenty of mRNA targets of p62/IMP2. Therefore, Wnt/β-catenin pathway may not be the only signaling pathway that regulates cell migration. As a way to solve the problem, we will use Wnt pathway inhibitors to verify our results.

6.3 Results

6.3.1 Overexpression of p62/IMP2 can induce EMT through the Wnt/ β-catenin pathway

To better understand how the change of p62/IMP2 can regulate cell migration in HCC cells, we divided the HCC gene expression profile GSE 14520 into 2 groups based on the median of p62/IMP2 expression and performed differential expression gene analysis with a metastasis-related gene list (QIAGEN). One of the top genes we selected was β-catenin (Based on LogFC+ adj. p value) because we considered it as candidate of the targets of p62/IMP2 (Fig.16). It is involved in the regulation of Wnt signaling. Since Wnt/β-catenin pathway plays an essential role in the process of EMT and cancer metastasis, we examined the expression of β-catenin by immunofluorescence (Fig. 18). The results indicated that the expression of β-catenin was significantly enhanced when p62/IMP2 was overexpressed and decreased in

p62/IMP2 knocked out variants (Fig.18). HepG2 and SNU449 cell lines were reported having activated Wnt/β-catenin signaling due to the mutations in their CTNNB1 gene. Due to this, we hypothesized that the translocation of β-catenin into nucleus can be observed in both cell lines and their variants. However, we only observed a stronger nuclear expression pattern of β-catenin in the p62/IMP2 overexpressed cells. When p62/IMP2 is expressed in low quantities, β-catenin exhibited a more cytoplasmic expression pattern. The change of β-catenin expression influenced by p62/IMP2 was confirmed in western blotting analysis. In addition, we tested the expression of several key proteins that are involved in the regulation of Wnt/β-catenin signaling pathway and EMT. In general, the protein expression of Wnt5a/b, Wnt3a, and snail presented a positive correlation with the expression of p62/IMP2, while GSK3β and E-cadherin emerged a negative correlation with the expression of p62/IMP2. However, there are some exceptions in the results of western blotting analysis: For example, Wnt5a/b was lost in the HepG2 cell line. In contrast, it was overexpressed in SNU449 IMP2 knocked down cells (Fig.17).

We further explored whether inhibition of β-catenin expression could attenuate migration ability of HCC cells. The expression of β-catenin in wild type of HepG2 and SNU449 cells is reduced by around 30% to 60%, after being cultured in 10μM Wnt/β-catenin signaling inhibitor XAV939 medium. (Fig.19&20). Consequently, *in vitro* Wound healing assay provided evidence that inhibition of β-catenin can suppress the migration ability in wildtype and p62/IMP2 overexpressed HCC cells, whereas no significant change was observed in p62/IMP2 knockdown HCC cells (Fig. 21).

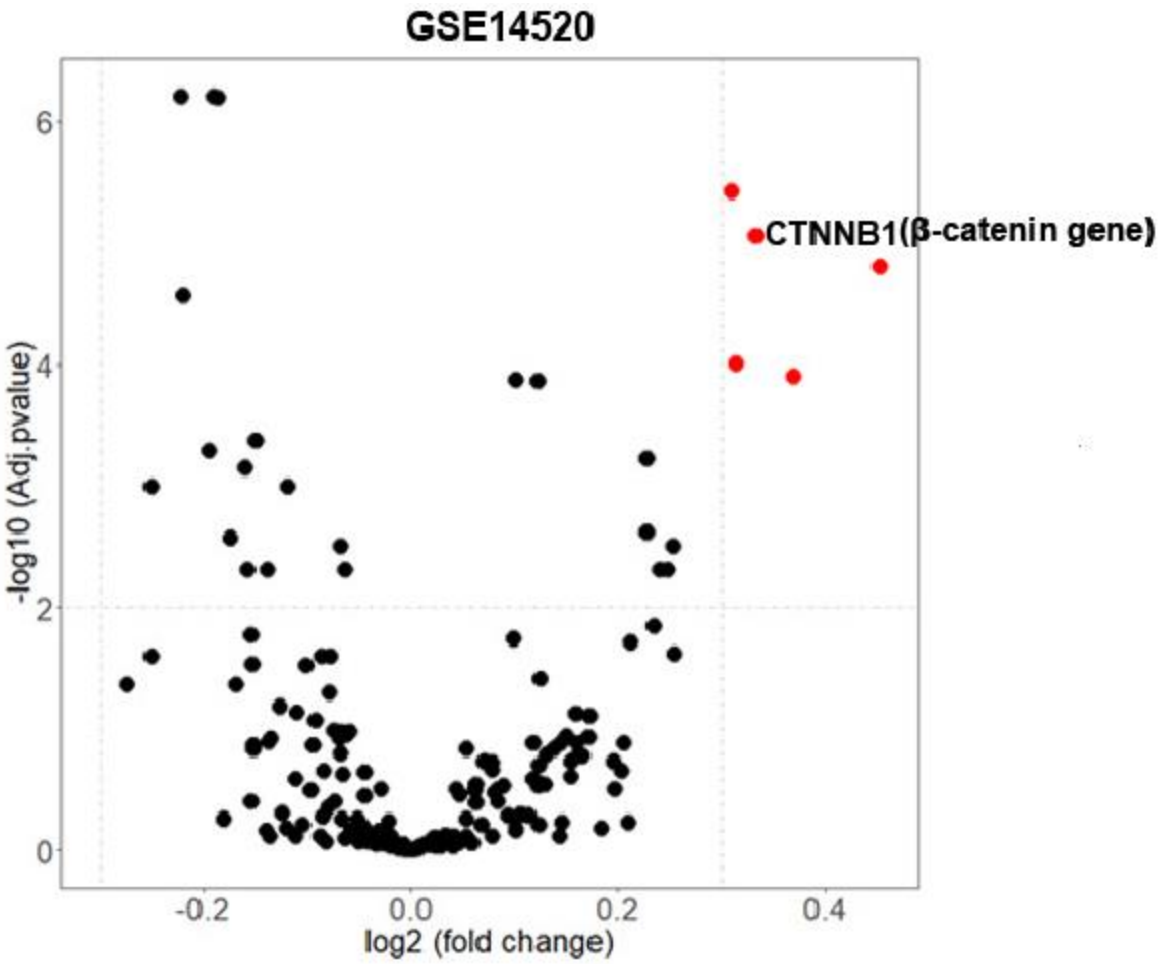


Figure 16. DEGs analysis with a metastasis related gene list.

The -log10 (adj. p value) and LogFC (log 2 conversion of the fold change) between p62/IMP2 high expression group and p62/IMP2 low expression group was shown in a scatter diagram. The gene of β-catenin (CTNNB1) is selected for future research.

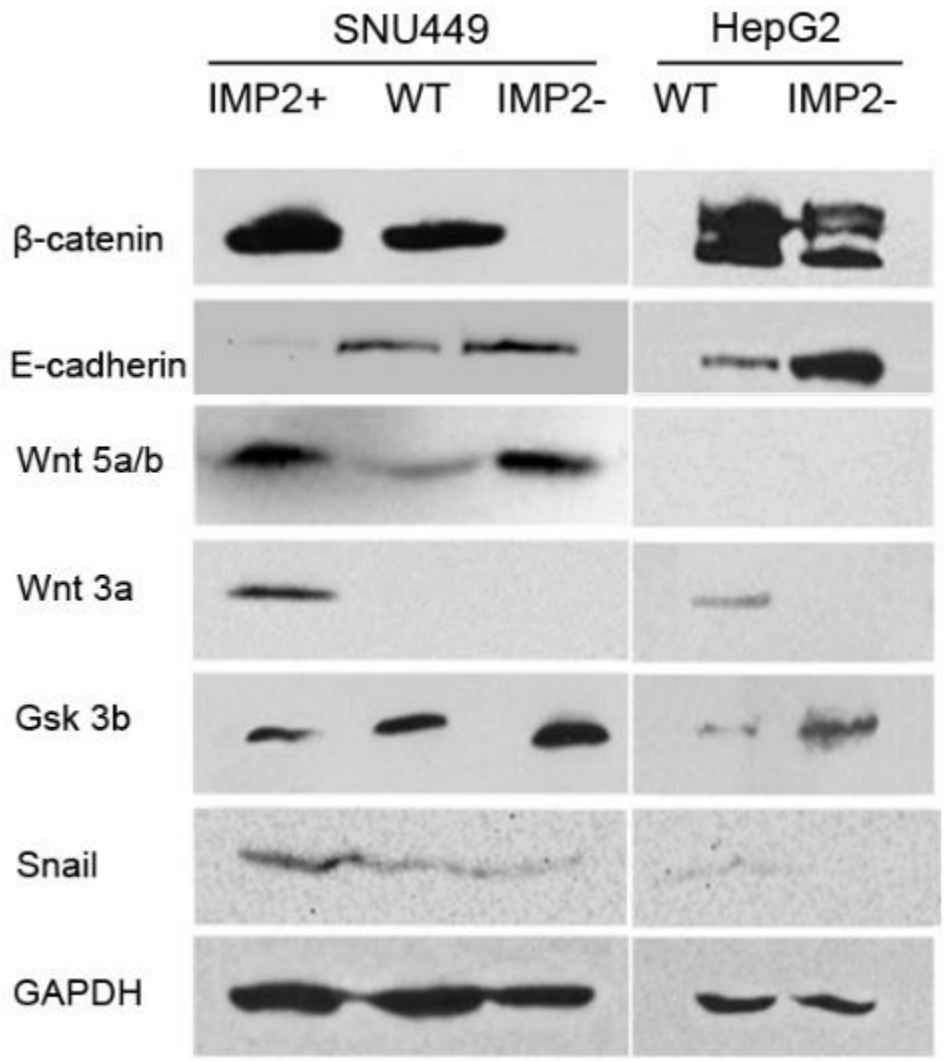


Figure 17. Western blotting analysis of Wnt/ β-catenin signaling and EMT related proteins.

Overexpressed p62/IMP2 enhanced the Wnt signaling and the expression of β-catenin, inhibited the expression of GSK3b and finally triggered the loss of E-cadherin.

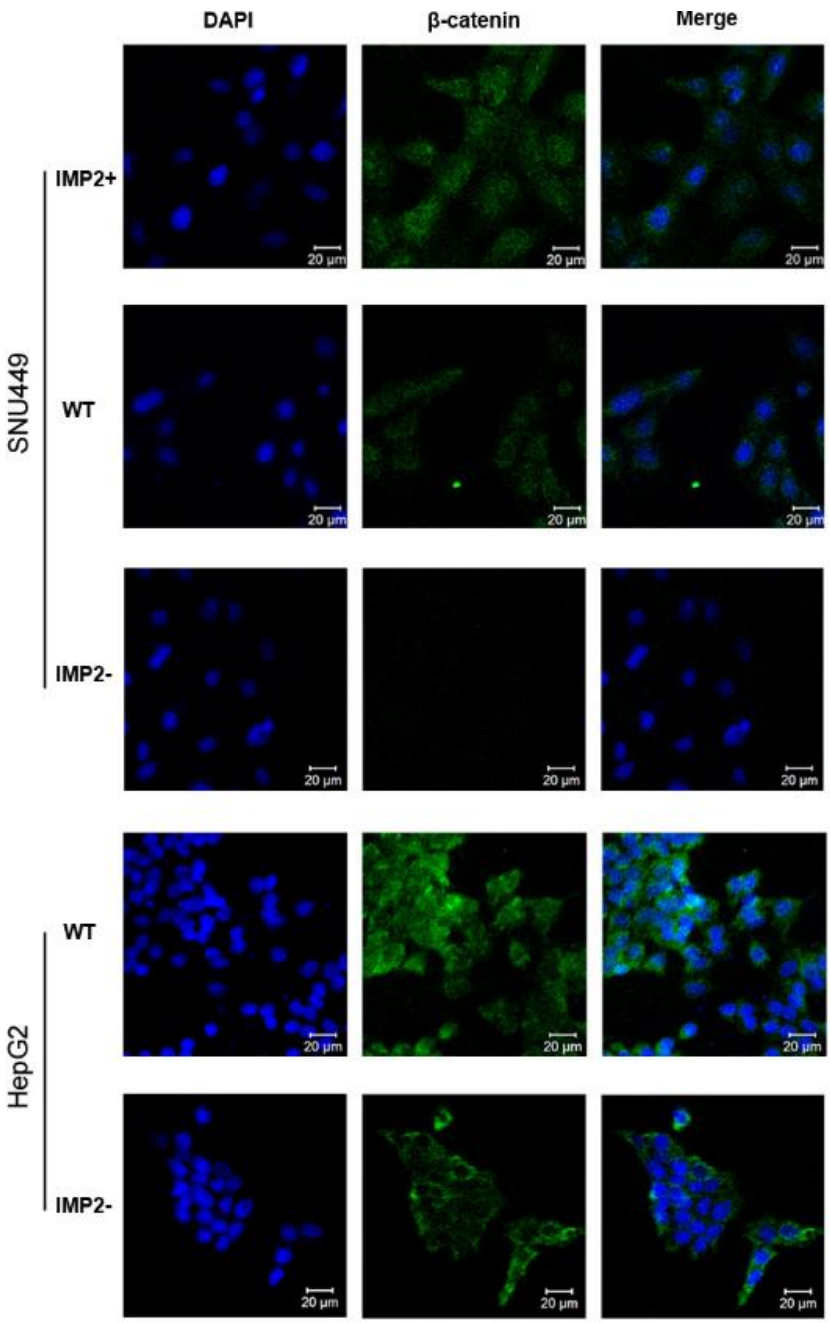


Figure **18**. **Immunofluorescence staining of β-catenin in SNU449 and HepG2 cells.**

Clearly nuclear expression pattern of β-catenin was shown when p62/IMP2 expressed high. Cell nucleuses were stained with DAPI (blue). In contrast, knockdown of p62/IMP2 significantly reduced the expression of β-catenin.

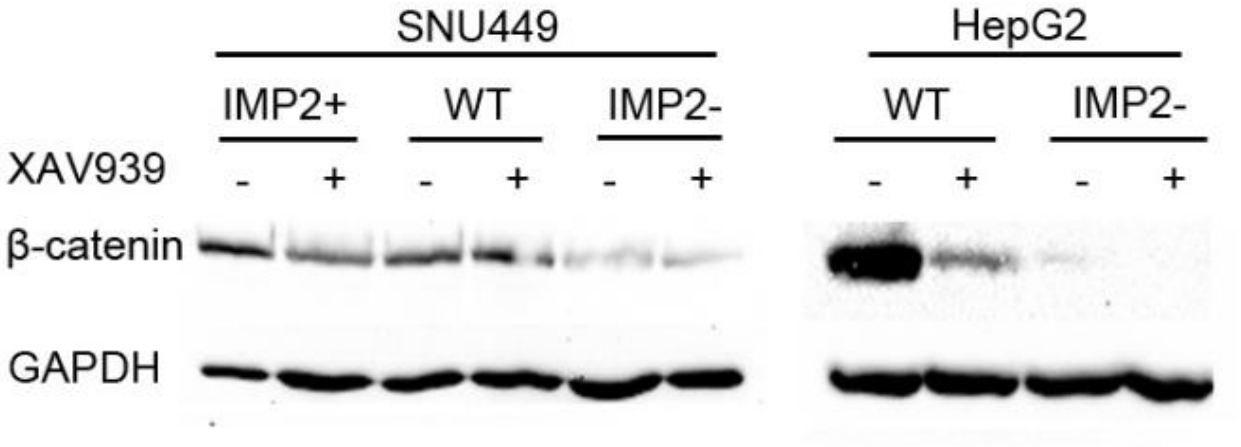


Figure 19. Western blotting analysis showed β-catenin expression was significantly inhibited by XAV939.

In all SNU 449 and HepG2 variants, 10 μM XAV939 can significantly inhibit the expression of β-catenin.

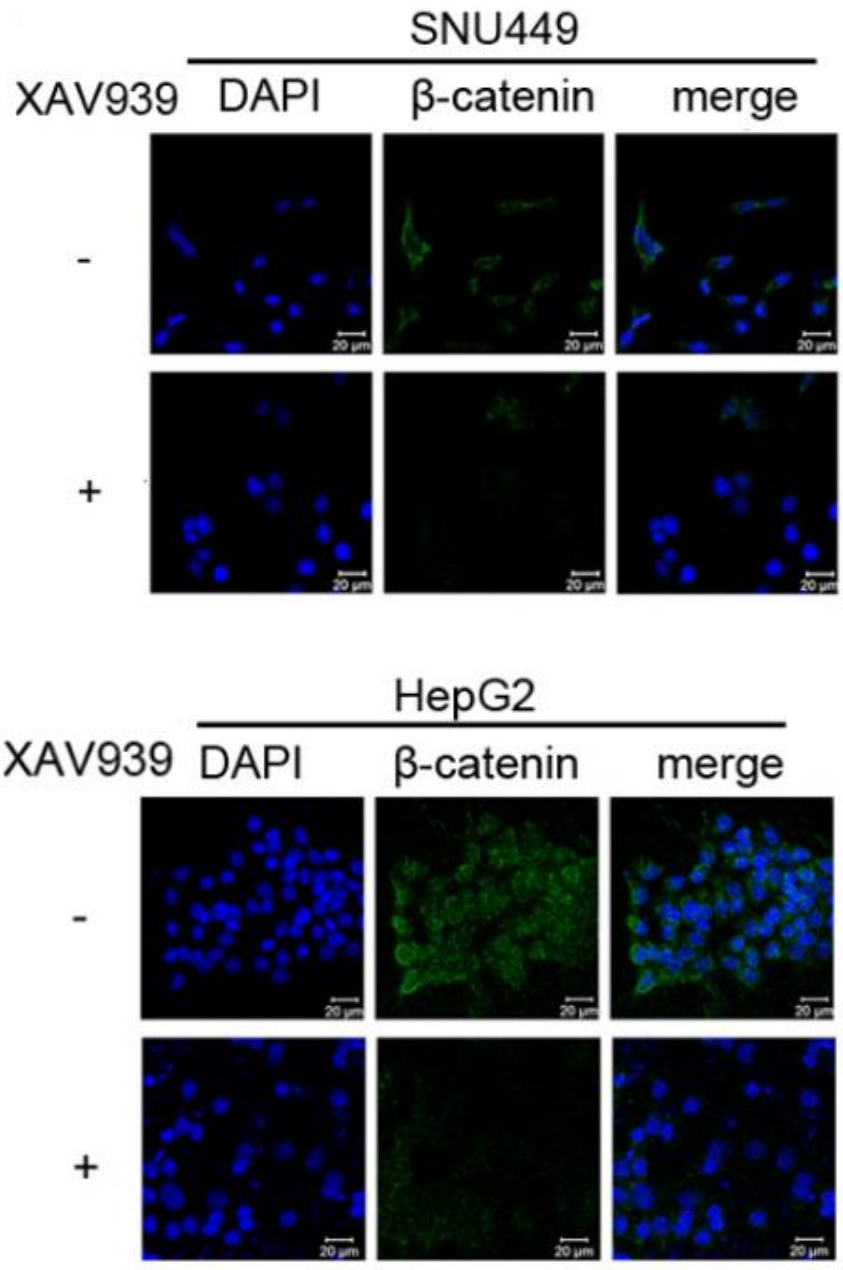
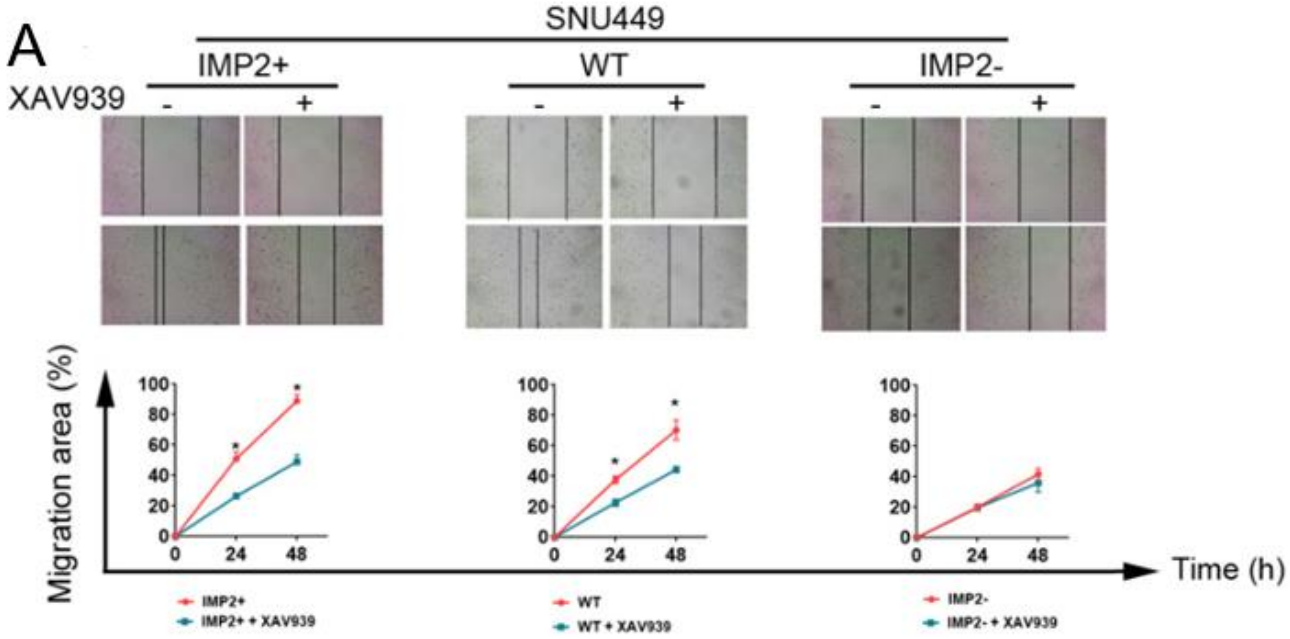


Figure 20. Immunofluorescence showed β-catenin expression was significantly inhibited by XAV939.

In both SNU449 and HepG2 cells, 10 μM XAV939 can significantly inhibit the expression of β-catenin.


A
SNU449
IMP2+
WT
IMP2-
XAV939
-
+
Migration area (%)
Time (h)
IMP2+
IMP2+ + XAV939
WT
WT + XAV939
IMP2-
IMP2- + XAV939

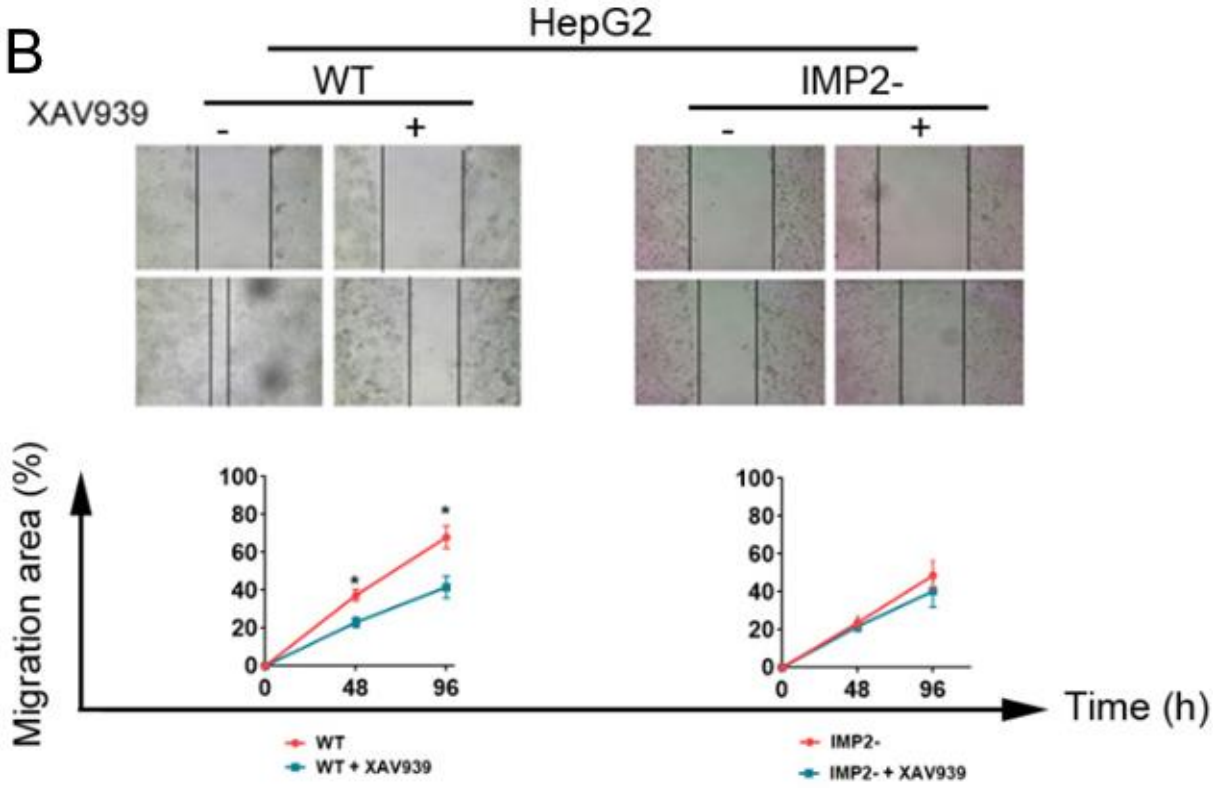

B
HepG2
WT
IMP2-
XAV939
-
+
Migration area (%)
Time (h)
WT
WT + XAV939
IMP2-
IMP2- + XAV939

Figure 21. Inhibition of Wnt/ β-catenin signaling can reduce migration ability in HCC cell lines.

XAV939 repressed migration ability in p62/IMP2 overexpression and wildtype HCC cells. In contrast, XAV 939 did not show inhibition of migration ability in IMP2 knockdown cells. Wound scratch image was taken each 24h, the wound healing area was quantified and each point (48h per time in HepG2 line). *$p<0.05$ (Two-way ANOVA, n=3)

6.3.2 IMP2 can regulate the phosphorylation of Wnt/β-catenin signaling

IMP2 was reported to be involved in the post-translational modification of their targets, including phosphorylation [106]. Therefore, we performed a phosphoproteome analysis by phospho explorer antibody microarrays and then characterized and compared phos/unphos (phosphorylation/unphosphorylation) ratio between transfected and wild type cell lines. There are 93 well-characterized phospho-specific antibodies in the microarray, and we selected 23 Wnt related phosphorylation sites from them. Interestingly, the pattern of phosphorylation is diverse in each group. In general, overexpression of p62/IMP2 enhanced canonical Wnt signaling in both SNU449 and HepG2 cell lines, but the pattern of affected phosphorylation sites was different. In SNU449, the change of p62/IMP2 mainly regulates the cytoplasmic phosphorylation sites associated with β-catenin degradation, such as β-catenin Y654, T41/S45, S37 and APC S2054. Among them, phosphorylation ratio of β-catenin S37 increased by 77 percent in p62/IMP2 knocked down cells, while it decreased 19 percent in p62/IMP2 overexpressed cells, which promoted the degradation of β-catenin induced by GSK3b and supported our western blot results in Fig.17. In contrast, change of p62/IMP2 in HepG2 primarily regulates the Wnt signaling receptor-associated phosphorylation site on the cell membrane (Src Y529, S75 and CKIα Y321) (Fig 24). At the same time, in SNU449 cells, the expression of p62/IMP2 was negatively correlated with the phosphorylation site that activated non-canonical Wnt signaling (Fig. 22&23), whereas we did not find a correlation between the expression level of p62/IMP2 and the phosphorylation level of non-canonical Wnt signaling in HepG2 cells (Fig. 24).

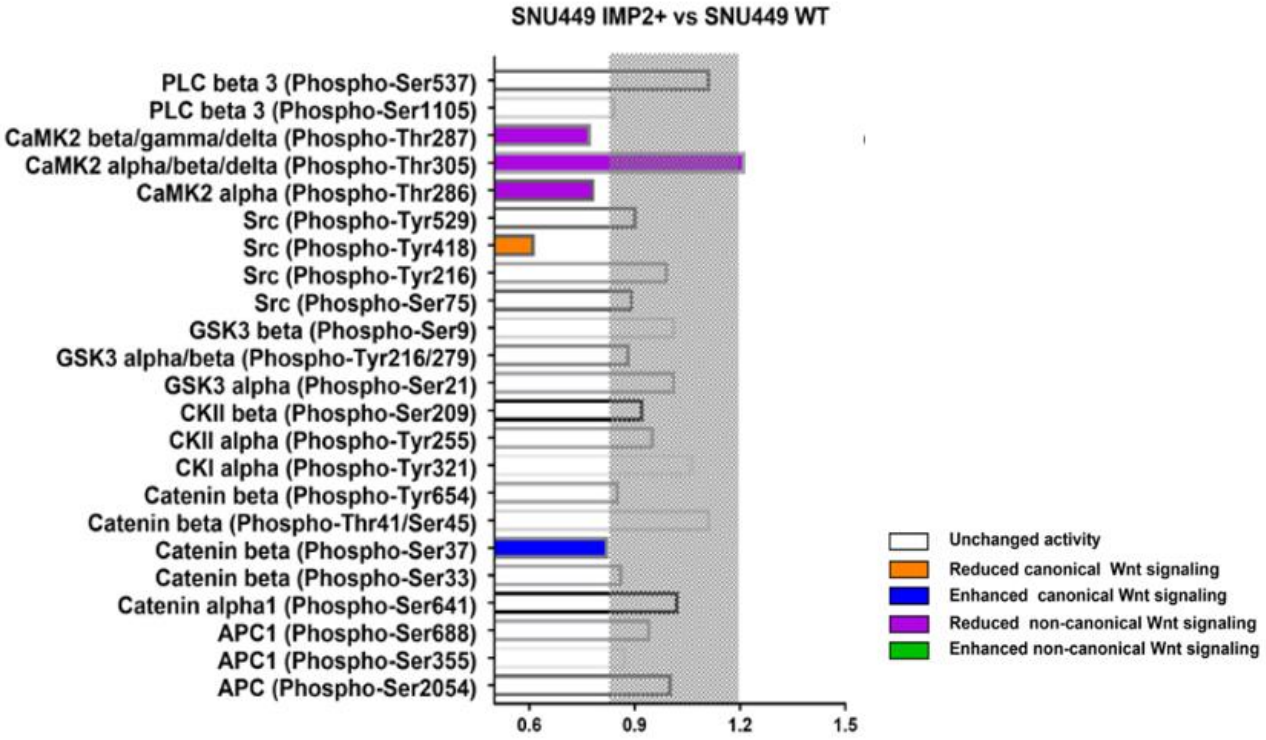


Figure 22. Overexpression of p62/IMP2 enhanced both Canonical Wnt and non-canonical Wnt signaling.

The change of Phosphorylation rate (phosphorylated protein/phosphorylated protein) between SNU449 IMP+ and SNU449 WT cells was tested by a phosphoproteome array which screened the total cell lysates. The gray area was defined as “no change area” (≥83%/≤120%). The induction/reduction of phosphorylation sites was shown with color when their bar is out of the gray area boundaries.

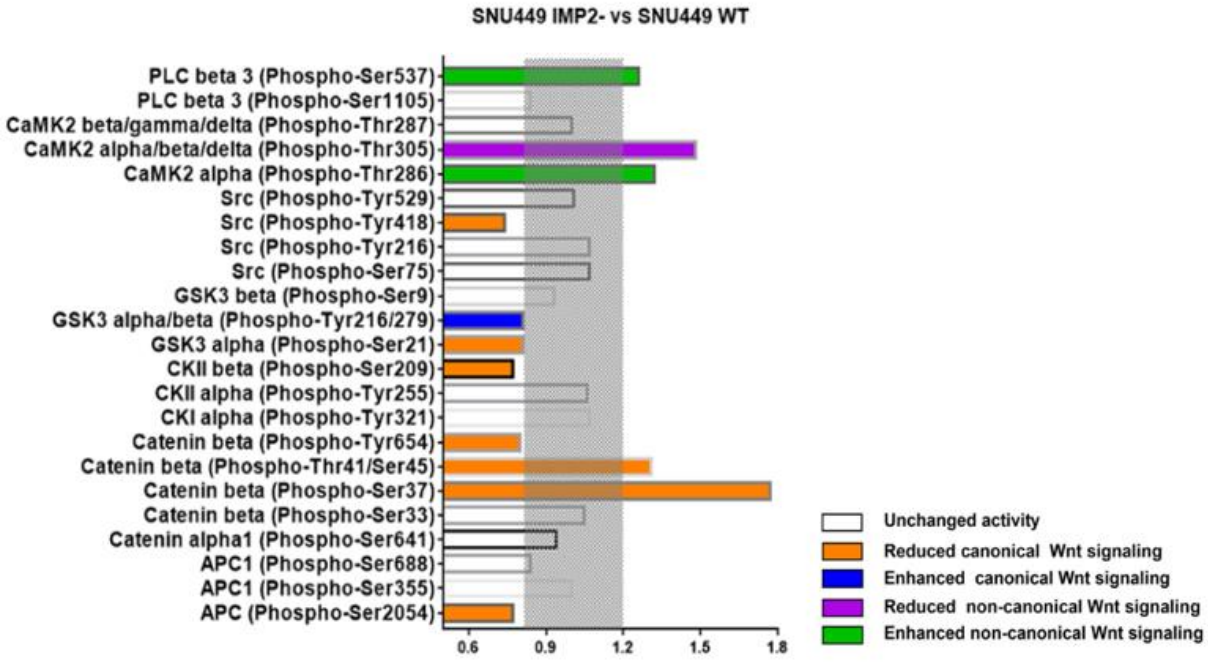


Figure 23. Knockdown of p62/IMP2 reduces the canonical and non-canonical Wnt signaling.

The change of Phosphorylation rate (phosphorylated protein/phosphorylated protein) between SNU449 IMP knockdown and SNU449 WT cells was tested by a phosphoproteome array which screened the total cell lysates. The gray area was defined as “no change area” (≥83%/≤120%). The induction/reduction of phosphorylation sites was shown with color when their bar is out of the gray area boundaries.

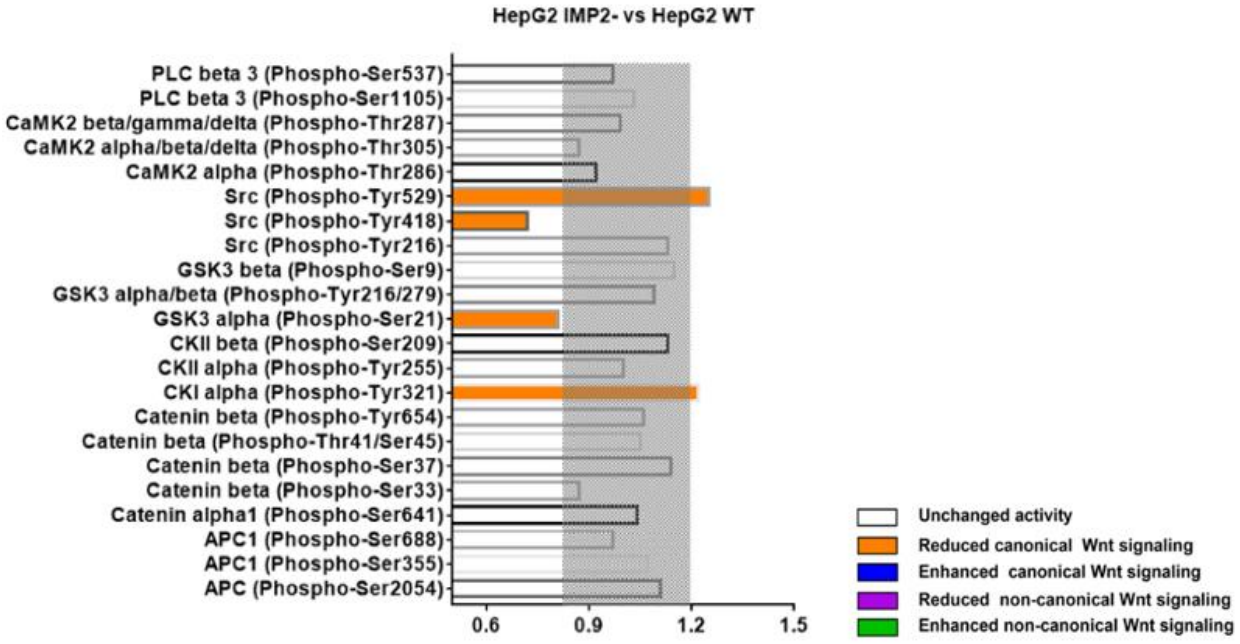


Figure 24. Knockdown p62/IMP2 inhibit canonical Wnt signaling in HepG2 cells.

The change of Phosphorylation rate (phosphorylated protein/phosphorylated protein) between HepG2 IMP2 knockdown and HepG2 WT cells was tested by a phosphoproteome array which screened the total cell lysates. The gray area was defined as “no change area” (≥83%/≤120%). The induction/reduction of phosphorylation sites was shown with color when their bar is out of the gray area boundaries.

Chapter 7. Discussion and Future Direction

7.1 Discussion

In 1999, p62/IMP2 was first identified as a TAA in HCC [29]. Our previous work showed that a high frequency of its autoantibodies can be detected in sera from HCC patients, which suggests that p62/IMP2 can be used as a biomarker of HCC [107]. Nevertheless, the mechanism underlying the production of p62/IMP2 autoantibodies in HCC remains to be explored. Previous studies revealed that most TAAs are non-mutated and but overexpressed in tumor tissues. Autoantibodies against these TAAs are produced when the antigenic peptides presented on human leukocyte antigen (HLA) class I molecules exceeds the TCR threshold which is required for CD4+ T cell activation [108]. In this study, we provided evidence for the hypothesis: p62/IMP2 is aberrantly overexpressed in both HCC tissues and cell lines, and our IHC results showed that the expression of p62/IMP2 reached a high level in the early stage of cancer, which is consistent with a previous study showing that autoantibodies can be detected early in HCC [109]. Considering that the overall survival time is less than 1 year for late stage HCC patients (based on the BCLC staging system), early diagnosis is very important for improving the prognosis of patients. In summary, using autoantibodies against a panel of cancer-associated antigens including p62/IMP2 may be a potential approach for future diagnosis of early HCC [107].

As much as 90 percent of cancer related deaths can be attributed to metastasis [47]. Through the establishment of stable p62/IMP2 overexpressed and knocked down cell lines, we found that

aberrantly expressed p62/IMP2 can regulate the cell migration ability of HCC cells via regulating the expression of β-catenin. β-catenin is known to play a key role in cadherin-mediated cell adhesion system. Without Wnt signaling cascade activation, the β-catenin-α-catenin complex bridge the cadherin to the actin cytoskeleton physically in the cytoplasm [65]. Once Wnt binds with Fz and LPR5/6, the Wnt/β-catenin signaling pathway activates and allows β-catenin to accumulate in the cytoplasm in order to finally translocate into the nucleus. β-catenin in p62/IMP2 overexpressed cells showed a clear nuclear expression pattern. On the contrary, β-catenin is localized in the cytoplasm of HepG2 p62/IMP2 knockdown, SNU449 WT, and SNU449 p62/IMP2 knockdown cells. At the same time, a canonical Wnt ligand, called Wnt3a, was expressed only in p62/IMP2 overexpressed cells, which implies that Wnt/β-catenin signaling is enhanced by the overexpression of p62/IMP2. In the activated Wnt/β-catenin signaling pathway, reduction of GSK3β reduces the phosphorylation of Snail, thereby enhancing its stability [110], inhibiting the expression of E-cadherin, and ultimately, inducing the occurrence of EMT.

Surprisingly, although the expression of β-catenin was intensely repressed, the p62/IMP2 knockdown SNU449 cells also expressed higher colony formation ability than SNU449 wild type cells. Accordingly, we examined the noncanonical Wnt signaling pathway, which is β-catenin independent and can antagonize canonical Wnt signaling in HCC [111]. Wnt receptor Frizzled 2 and its ligand Wnt5a/b were found to be elevated in various cancers that also promote proliferation and cell migration ability [112]. HCC cell lines express Wnt ligands diversely.

Briefly, well-differentiated cell lines such as HepG2 mainly express Wnt3a, whereas poorly differentiated cell lines like SNU449 mainly secrete Wnt5a/b [113]. In the scenario of the sharply down regulation of β-catenin SNU449 knockdown cells, Wnt5a/b may be oppositely upregulated and thus trigger the process of proliferation and EMT. P62/IMP2 is the only IMP family member which was reported existed in adult liver tissues [71]. Our study suggests that the trace of p62/IMP2 expression is important to maintain the normal function in hepatocytes.

The protein activity, stability that involved in Wnt signaling were regulated by phosphorylation status at multiple steps [114]. In this study, we show that the manipulation of the expression of p62/IMP2 triggers a series of changes in the phosphorylation of various Wnt pathway proteins. In the absence of a Wnt stimulus, free β-catenin in cytoplasm will be degraded by a "destruction complex" including APC, GSK3, Axin, and CK1 [115]. In SNU449 p62/IMP2 knockdown cells, CK1α-mediated phosphorylation of ser45 on β-catenin was elevated, which allowed β-catenin to enter the "destruction complex" and specifically interact with Axin [116]. Subsequently, with the dramatic increase of GSK3-mediated phosphorylation of Ser-37 and Thr-41, β-catenin binds with APC, stays in this complex, and is eventually degraded [117]. Simultaneously, the affinity for cadherins is strengthened with the reduction of Tyr 654 phosphorylation on β-catenin [118]. Decreased phosphorylation of APC at ser 2054 can facilitate the APC to be transported to the nucleus [119]. Besides that，the AKT-mediated phosphorylation of GSK3α at ser 21 significantly inhibits the activity of itself [120] (Fig. 23). In summary, all of these changes in phosphorylation strengthens the stability of β-catenin and enhances the Wnt/β-catenin signaling.

In HepG2 cells, the phosphorylation regulated by p62/IMP2 showed a different pattern. Phosphorylation of Tyr 418 of Src was inhibited after knocking down p62/IMP2. Activated proto-oncogene tyrosine kinase Src can enhance the cap-dependent translation, and thus elevate the expression of β-catenin accumulation and its transcriptional activity. Tyr 418 and Tyr 529 are two major phosphorylation sites on Src, Tyr 418 can be auto-phosphorylated, and then displaced from binding pockets to allow substrate access. On the contrary, phosphorylated Tyr 529 can let the tyrosine group interact with SH2 domain and inactive Src [121]. In addition, the reduced ser 21 on GSK3α also downregulated Wnt signaling. We did not observe the phosphorylation change on β-catenin, which may explain why the downregulation of β-catenin is less in p62/IMP2 knockdown cells of HepG2 cells than SNU449 cells (Fig. 24).

Wnt5a can induce CaMKII to weaken the protein stability of a co-repressor of Notch signaling and the silencing mediator of retinoic acid and thyroid hormone receptor (SMRT). Phosphorylated Tyr 287 of CaMKII (Tyr 286 in the α isoform) plays an important role in it: increase more than 1000-fold binding affinity between CaM and CaMKII; preventing auto-inhibition when CaM dissociates from CaMKII due to [Ca2+] i decrease [122]. Moreover, CaMKII can phosphorylate Ser 537 of PLCβ3 and thus enhance its basal activity [123]. Our phosphoproteome analysis data supported that loss of p62/IMP2 augments the Wnt5-mediated non-canonical Wnt signaling, thereby explaining why colony formation ability is improved in SNU449 IMP2 knockdown cells. Our results showed that p62/IMP2 regulates the Wnt/beta-catenin pathway through multiple pathways: regulation of mRNA expression, affecting multiple upstream and downstream genes and changes of phosphorylation levels.

In the differential gene expression analysis, we found that 900 genes were regulated by the change of expression; in addition, their encoded proteins were mainly distributed in the cell membrane and cytoplasm, rather than in the nucleus. Another study showed that 3 percent of the HEK293 cell transcriptome will be expressed in IMP1 messenger ribonucleoprotein (mRNP) particles, which implies that there may be over 1000 targets of IMP1 [124]. Given that IMP family members share a highly conserved structure with 60%-80% identity amino acid sequences [125], there may be many unknown IMP2 targets in these differentially expressed genes, which can help regulate complex pathways, their phosphorylation and improve the migration ability of cancer cells.

In summary, our study revealed that p62/IMP2 is overexpressed in human HCC tissues and cancer cell lines, thereby augmenting cell migration ability through activation of the canonical Wnt signaling pathway. In SNU449 p62/IMP2 knockdown cells, colony formation ability was promoted by noncanonical Wnt pathway. Our differential expression analysis indicates that abnormally high expression of p62/IMP2 in HCC may regulate cells through a variety of pathways, resulting in more malignant HCC phenotypes.

7.2 Future directions

7.2.1 To explore novel mRNA targets of p62/IMP2 and evaluate their value as early diagnosis biomarkers

Recently, more and more novel targets of IMPs have been identified. Due to the highly conserved structure and similar function of IMPs, p62/IMP2 may have a plenty of unknown binding targets. To identify novel targets of p62/IMP2, RNA immunoprecipitation will be used firstly to isolate the mRNA that bind with p62/IMP2 in cytoplasm. We will collect cell lysates from p62/IMP2-positive cells and use the p62/IMP2 antibody to precipitate the entire complex of p62/IMP2-target from cell lysates. The target mRNA is finally isolated and separated by a solution for reverse transcription using a RIP-Assay kit. Subsequently, we plan to use RNA sequencing and qPCR to analyze the isolated RNA and obtain the list of p62/IMP2 targets. After that, we will design a mini-array including p62/IMP2 and its potential targets as well as other characterized TAAs to further enhance the autoantibody detection efficiency in the diagnosis of HCC. ELISA will be used to examine the autoantibodies expression level. The data will be presented as means ± standard deviation (SD). Unpaired two-tailed t test was used to compare different groups. Receiver operating characteristic (ROC) analysis was performed to evaluate the diagnostic value for each TAA, which leading to calculate the area under the curve (AUC). We expect to identify some novel TAAs and enhance the early diagnosis accuracy through our future directions.

7.2.2 To determine the role of p62/IMP in HCC by *in vivo* study

In this project, our data from in vitro studies demonstrated that p62/IMP2 can promote cell migration ability in HCC via activation of Wnt/ β-catenin signaling pathway. To verify our results, the HCC cell variants will be injected into the subcutaneous tissue. Thereafter, the body weight and tumor size of the mice will be recorded daily. After 3-4 weeks, the mice will be dissected, while the primary tumor and metastatic tumor will be taken out for analysis. The metastatic sites of primary liver cancer are mainly concentrated in the lungs and bones, so we will focus on their metastatic tumors for collecting statistical data. We will also analyze the molecular biology differences between metastatic tumors and primary tumors.

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Appendix I

List of abbreviations

APC - Adenomatous polyposis coli

BCLC – Barcelona clinic liver cancer

CDKN2A - Cyclin dependent kinase inhibitor 2A

CIP2A - Cancerous inhibitor of protein phosphatase 2A

DAPI - 4’,6’-diamidino-2-phenylindole

DEG - Differential expression gene

ECL- Enhanced chemiluminescence

ELISA - Enzyme linked immunosorbent assay

EMT - Epithelial mesenchymal transition

ERK - Extracellular signal-regulated kinases

EGFR - Epidermal growth factor receptor

FGF - Fibroblast growth factor

GEO - Gene omnibus

HCC - Hepatocellular carcinoma

HRP - Horseradish peroxidase

IHC- Immunohistochemistry

IMP - Insulin-like growth factor 2 mRNA binding protein

LEF - Lymphoid enhancer binding factor

MAPK - mitogen-activated protein kinase

mTOR - Mammalian target of rapamycin

NHS - Normal human sera

OD - Optical density

PBS - Phosphate-buffered saline

PI3K - Phosphoinositide 3-kinases

PKB - Protein kinase B

TBS – Tris base saline

TCF - Transcription Factor

TERT – Telomerase reverse transcriptase

TSC - Tuberous sclerosis 1

ZEB - Zinc finger E-Box binding homeobox

List of Publications and Manuscripts

1. **Xing M**, Wang X, Li P, Li J, Qin J, Zhang X, Ma Y, Francia G, Zhang JY. Overexpression of p62/IMP2 can promote cell migration in hepatocellular carcinoma (HCC) via activation of the Wnt/ *β-catenin* pathway *(Submitted to Hepatology, under Review)*
2. Dai X, Theobard R, Cheng H, **Xing M**, Zhang J. Fusion genes: A promising tool combating against cancer. *Biochim Biophys Acta Rev Cancer.* 2018;1869(2):149-160.
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9. Wang LD, Bi X, Song X, **Xing M**, et al. A sequence variant in the phospholipase C epsilon C2 domain is associated with esophageal carcinoma and esophagitis. *Mol Carcinog*. 2013;52 Suppl 1(0 1):E80–E86. doi:10.1002/mc.22016

Conference Poster Presentation

1. **Mengtao Xing**, Pei Li, Jianxiang Shi, Liping Dai, Jitian Li, Jianying Zhang. Evaluation of the diagnostic value of serum anti-cyclin B1 autoantibody as a biomarker in lung cancer. [abstract]. In: Proceedings of the 107th Annual Meeting of the American Association for Cancer Research; 2016 Apr 16-20; New Orleans, LA. Philadelphia (PA): AACR; Cancer Res 2016;76(14 Suppl): Abstract nr 2255.
2. **Mengtao Xing**, Pei Li, Jitian Li, Jianxiang shi, Liping Dai, Giulio Francia, Jianying Zhang. Overexpression of P62/IMP2 promotes metastasis in hepatocellular carcinoma via inhibition of E-cadherin expression [abstract]. In: Proceedings of the American Association for Cancer Research Annual Meeting 2017; 2017 Apr 1-5; Washington, DC. Philadelphia (PA): AACR; Cancer Res 2017;77(13 Suppl): Abstract nr 4850. doi:10.1158/1538-7445.AM2017-4850
3. **Mengtao Xing**, Jianxiang Shi, Jitian Li, Beibei Chen, Giulio Francia, Jianying Zhang. P62/IMP2 promotes metastasis in hepatocellular carcinoma (HCC) by regulating EMT in different signaling pathway [abstract]. In: Proceedings of the American Association for Cancer Research Annual Meeting 2018; 2018 Apr 14-18; Chicago, IL. Philadelphia (PA): AACR; Cancer Res 2018;78(13 Suppl): Abstract nr 2027.
4. **Mengtao Xing**, Jianxiang Shi, Pei Li, Jiejie Qin, Xiaojun Zhang, Yangcheng Ma, Xiao Wang, Giulio Francia, Jianying Zhang. Overexpression of p62/IMP2 can promote cell migration in hepatocellular carcinoma (HCC) via activating Wnt/ *β-catenin* pathway

[abstract]. In: Proceedings of the American Association for Cancer Research Annual Meeting 2019; 2019 Mar 29-Apr 3; Atlanta, GA. Philadelphia (PA): AACR; Cancer Res 2019;79(13 Suppl): Abstract nr 4591.

Curriculum Vita

Mengtao Xing earned his B.S. degree in Pharmaceutics from China Pharmaceutical University, Jiangsu, China in 2010. After graduation, he worked in rekoo technology for mobile game development in Beijing and learned experiment skills in cancer research. He got his M.S. degree in biotechnology from Roosevelt University, IL in 2014 and starting his doctoral training in the Department of Biological Sciences at the University of Texas at El Paso (UTEP) in spring, 2015. He has been working at Dr. Zhang's lab and his research project focus on the role of p62/IMP2 in Hepatocellular carcinoma progression.

While pursuing his degree, Mengtao maintained 4.0/4.0 GPA and worked as an associate instructor for the Department of Biological Sciences. In his career as a Ph.D. student, he presented his research in several international and regional meetings, such as AACR 2016-2019 and BBRC 2017. He also received travel awards from the Department of Biological Sciences and College of Science at UTEP.

Mengtao has submitted two first-author manuscripts and several co-authors papers. He was also invited by several prestigious journals in cancer area to act as a reviewer for the manuscripts from other scientists. After graduation, he will be looking for a research position for combating cancer and try to profoundly understand the mechanism of cancer.

This dissertation was typed by Mengtao Xing.