Protein-protein interactions among signaling pathways may become new therapeutic targets in liver cancer (Review)

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Abstract. Numerous signaling pathways have been shown to be dysregulated in liver cancer. In addition, some protein-protein interactions are prerequisite for the uncontrolled activation or inhibition of these signaling pathways. For instance, in the PI3K/AKT signaling pathway, protein AKT binds with a number of proteins such as mTOR, FOXO1 and MDM2 to play an oncogenic role in liver cancer. The aim of the present review was to focus on a series of important protein-protein interactions that can serve as potential therapeutic targets in liver cancer among certain important pro-carcinogenic signaling pathways. The strategies of how to investigate and analyze the protein-protein interactions are also included in this review. A survey of these protein interactions may provide alternative therapeutic targets in liver cancer.

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1. Introduction

Liver cancer is the sixth most common cancer and the second most common cause of cancer-associated mortality worldwide (1). Approximately 75% of all primary liver cancer types are hepatocellular carcinoma (HCC) that formed from liver cells. Liver cancer can be formed from other structures in the liver such as bile duct, blood vessels and immune cells. Secondary liver cancer is a result of metastasis of cancer from other body sites into the liver. The major cause of primary liver cancer is viral infection with either hepatitis C virus (HCV) or hepatitis B virus (HBV), which leads to massive inflammation, fibrosis and eventual cirrhosis in the liver. Many genetic and epigenetic alterations have been identified in hepatocytes during HCV and HBV infection (2). Other causes, such as alcohol, aflatoxin, high-grade dysplastic nodules, obesity, diabetes and smoking may increase the risk of liver cancer. Surgical resection is an option for liver cancer treatment (3), whereas liver transplantation can be used in cases of liver cancer where surgical resection can be tolerated and the tumor fits specific criteria, i.e., Milan criteria (4).

Signaling pathways are complex processes of signal transduction involving the mutual activation of a protein cascade transmitting signals from the cell surface to the cytoplasm and the nucleus (5). In recent decades, emerging studies have greatly improved our understanding of liver tumorigenesis through investigation of a series of signaling pathways including PI3K/AKT. Cell signaling receptors, intracellular secondary messengers/molecules and transcription factors are essential components for signaling pathways, and the protein-protein interactions (PPIs) among these components act as connectors that mediate signal transduction from one step to the following within a single signaling pathway, and act as transmitters that play an important role in the crosstalk of several signaling pathways (6). PPIs refer to the intentional physical contacts established between two or more proteins as a result of biochemical events and/or electrostatic forces (7). Proteins rarely act alone at both cellular and systemic levels. A number of essential molecular processes are performed by molecular machines that are constructed from a large number of protein components organized by their PPIs. PPIs have been largely investigated in signal transduction and aberrant PPIs in these signaling pathways are considered the basic events of

liver cancer. Targeting these important PPIs may be useful for the treatment of liver cancer.

In the present review, we focus on PPIs in the PI3K/AKT and other important signaling pathways in liver cancer. The potential antitumor therapies targeting these pivotal PPIs and the strategies of how to investigate and analyze PPIs are also assessed.

2. PI3K/AKT signaling pathway

The PI3K/AKT pathway is an intracellular pathway that is involved in cell cycle, growth, survival, proliferation and migration. Enhanced PI3K/AKT activities have been reported in many human cancer types, including cancers of colon, breast, brain, liver, stomach and lung (8). The PI3K/AKT signaling pathway can be activated by four main types of sensors: the receptor tyrosine kinases (RTKs), cytokines, G protein-coupled receptors and the integrins (9-11). These four types of sensors bind with their cofactors and activate downstream kinases in the PI3K families. PI3K, by transferring a phosphoryl group, converts phosphatidylinositol 4,5-diphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3) (12). PIP3 can interact with AKT which contains pleckstrin homology (PH) domain on the inner surface of the plasma membrane, resulting in conformational changes of these proteins (13). Following binding to PIP3 at the membrane, AKT can then be phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) at threonine 308 or be phosphorylated by the mammalian target of rapamycin complex (mTORC) at serine 473 (14,15). Fully phosphorylated AKT can directly interact with >100 proteins including the mTORC, Bcl-2-associated death promoter (BAD), caspase-9, various forkhead box protein O (FOXO) proteins, glycogen synthase kinase 3 β (GSK3β), mouse double minute 2 homolog (MDM2) and tuberous sclerosis 1 (TSC1) (16).

3. PPIs between PI3K/AKT and other signaling pathways

AKT, the core protein in the PI3K/AKT signaling pathway, can physically interact with proteins in other signaling pathway. Thus, activity of the PI3K/AKT signaling pathway in liver cancer can directly affect the activities of other signaling pathways, such as Hippo/YAP, NF-κB, Wnt/β-catenin, Notch, p53, JAK/STAT and MAPK/ERK signaling pathways.

AKT is shown to physically bind to the proteins, IKKA and IKKB in the NF-κB signaling pathway. AKT phosphorylates IKKA on threonine (Thr) 23, thereby activating the NF-κB signaling pathway and subsequently inducing key immune and inflammatory responses (17,18). In addition, IKKB is a direct target of AKT, and activation of the AKT/IKKB signal is closely correlated with the anti-apoptotic and pro-cell survival function of NF-κB signaling in breast cancer cells (19). Similarly, the activation of AKT can directly induce activation of the NF-κB signaling pathway and eventually suppress apoptosis in liver cancer cells (20,21). By contrast, the inactivation of AKT strongly prevents NF-κB transcription factor p65 from entering the nucleus, the site at which p65 exerts its effects, and subsequently induces apoptosis in the HepG2 liver cancer cell line (22).

AKT is also capable of interacting and phosphorylating MST1/2 kinases, key components of the Hippo/YAP signaling pathway, on Thr 120. Such effects reduce the inhibitory impact of MST1/2 on the activity of YAP, the terminal effector of the Hippo/YAP signaling pathway, and thereby enhance AKT-maintained cell survival signaling (23).

AKT was found to have the ability to activate the Wnt/β-catenin signaling pathway by directly interacting with GSK3β, a natural inhibitor of β-catenin. AKT represses GSK3β by initiating its phosphorylation at serine 9 and vice versa (24). The phosphorylation of β-catenin via GSK3β is then repressed, which facilitates β-catenin translocation from the cytoplasm into the nucleus and Wnt/β-catenin signaling is eventually activated (25,26). Notably, the close relationship between the activation of PI3K/AKT and the upregulation of Wnt/β-catenin activity was also observed in liver cancer (27). Additionally, promoted liver cancer cell growth and proliferation was maintained by the PI3K/AKT and Wnt/β-catenin signaling pathways (28). The interaction between PI3K/AKT and Wnt/β-catenin is also critical for regulation of the cell cycle and epithelial-mesenchymal transition (EMT) during tumor formation, as following the respression of GSK3β by AKT, Wnt/β-catenin signaling down-stream effectors, such as cyclin D1, Snail and Mucin 1, are affected (29-31).

The MAPK/ERK is another important signaling pathway involved in a variety of cell processes including proliferation, differentiation, migration and survival. The MAPK/ERK signaling pathway is frequently activated in liver cancer often due to activating mutations or amplification of several components such as Ras, Raf and MEK (32,33). Notably, emerging evidence suggests that AKT is also capable of activating the MAPK/ERK signaling pathway via interaction with MAPK/ERK signaling components. Firstly, Raf, MAP3K5, MAP3K8 and MAP3K11 are activated by AKT via phosphorylation (34-37). Secondly, MAPK2K4 is phosphorylated by AKT on serine 78 to suppress apoptosis (38). Additionally, MAPK14/p38 is directly bound to and activated by AKT, thus establishing a crosstalk between the MAPK/ERK and PI3K/AKT signaling pathways (39). In addition to the direct binding to the MAPK/ERK components, AKT can physically interact with certain proteins, which can indirectly influence MAPK/ERK signaling activity. For example, AKT is known to interact with MAPK8IP1/JIP1, a regulator of MAPK8/JNK, to control inflammatory responses, cell proliferation and apoptosis (40). Furthermore, the downstream effectors of AKT, such as mTOR and GSK3β bind to the core regulator in the MAPK/ERK signaling pathway. For example, GSK3β physically interacts with and activates MAP3K1 *in vitro* and *in vivo* to regulate cell differentiation and apoptosis (41).

PPIs are also important for the crosstalk between PI3K/AKT and tumor suppressor p53 signaling pathways. AKT can directly interact with and regulate MDM2, one of the most well-characterized oncogenic ubiquitin E3 ligases that negatively regulates p53 transcription activity. Activation of AKT leads to phosphorylation of MDM2 on Ser 166 and 186, whereas inhibition of AKT decreases such phosphorylated levels (42,43). Phosphorylation of MDM2 decreases the protein levels of p53, thereby suppressing apoptosis in liver cancer cells (44). When the cells were pretreated with Wortmannin, a well-known PI3K/AKT inhibitor, to suppress

AKT activation, both the upregulation of phosphorylation of MDM2 and downregulation of p53 were reversed in the HepG2 liver cancer cell line (45).

PPIs between PI3K/AKT and Notch signaling have also been identified (46). Upregulation of AKT is shown to induce the activation of Notch signaling (47), and these two signaling pathways are always activated in liver cancer (48). The simultaneous inhibition of these two pathways has been shown to be an effective option aiming at cancer in clinical treatment (49), suggesting the close relationship between PI3K/AKT and Notch signaling during tumorigenesis.

As for the JNK/STAT signaling pathway, the downstream effector of AKT, mTOR, has been shown to physically interact with STAT1 and STAT3 and regulates the transcription activity of these two transcription factors (50,51).

In general, according to the results of the aforementioned studies, AKT can physically bind to a series of core protein in different signaling pathways and activation of the AKT signaling pathway can directly or indirectly lead to the activation of several other signaling pathways. Thus, we suggest that PI3K/AKT is central to the complex signaling network involved in liver as well as in other organ tumorigenesis.

4. Proteins that physically interact with AKT as its downstream effectors in liver cancer

In liver cancer, deregulated PI3K/AKT signaling pathway often leads to uncontrolled cell growth, metabolism, survival, metastasis and tumor formation. The PPI between AKT and mTOR and the mechanism associated with this interaction has been largely investigated in liver cancer. mTOR exists in two different complexes, mTOR complex 1 (mTORC1) and mTORC2. The mTORC2 complex directly phosphorylates AKT on Ser473 and AKT conversely phosphorylates mTORC1 at two COOH-terminal sites (Thr2446 and Ser2448) (14,53). p70S6 kinase and translational repressor protein 4E-binding protein 1 (4EBP1), the downstream effector of mTOR, are then phosphorylated by mTOR and regulate the translation of several important proliferative and angiogenic factors, such as c-Myc, cyclin D1, hypoxia-inducible factor (HIF) 1α and vascular endothelial growth factor (VEGF) (53-55), which are associated with tumor progression in liver cancer. The deregulated expression of mTOR signaling effectors ia present in 40-50% of HCC and activation of mTOR is correlated with poor prognosis and recurrence in HCC (56,57).

FOXO1 is also regarded as an AKT downstream effector. AKT has been proven to interact with FOXO1, which is a transcription factor involved in the regulation of gluconeogenesis and glycogenolysis via insulin signaling, and FOXO1 is also central to the decision for a preadipocyte to commit to adipogenesis (58). When FOXO1 is phosphorylated by AKT on Thr24, Ser256 and Ser319, it is spatially excluded from the nucleus and is then readily ubiquitinated and degraded (59). The phosphorylation of FOXO1 by AKT also impairs FOXO1-induced hepatic glucose production through a reduction in the transcription of glucose 6-phosphatase (*G6PC*) gene (60).

Additionally, BAD has been shown to physically interact with AKT. BAD protein is a pro-apoptotic member of the Bcl-2 gene family, which is involved in initiating apoptosis. Dephosphorylated BAD forms a heterodimer with Bcl-2 or Bcl-xL, represses them and thus initiates Bax/Bak-triggered apoptosis. When BAD is phosphorylated by AKT, it forms the BAD-14-3-3 protein heterodimer, allowing Bcl-2 to inhibit Bax-induced apoptosis (61). Inactivation of AKT removes its inhibitory effect to BAD, which may also decrease the levels of anti-apoptotic Bcl-2 and Bcl-XL proteins, and eventually lead to mitochondria-induced apoptosis in tumor cells (44). In liver cancer cells, AKT-mediated inhibitory effects on BAD-induced mitochondrial apoptotic signals were also observed (62).

AKT interacts with and activates S-phase kinase-associated protein 2 (Skp2) through phosphorylation of this protein on Ser72 (63,64). Skp2 behaves as an oncogene, and overexpression of this protein is frequently observed in human cancer progression and metastasis (wenxian). In human liver cancer cell lines and a murine liver cancer model, overexpression of AKT also led to the overexpression of Skp2 (65), indicating Skp2 may act as a downstream oncogenic effector of AKT during liver tumorigenesis.

Androgen receptor (AR) is activated by teh binding of androgenic hormones testosterone or dihydrotestosterone in the cytoplasm, and exerting its nuclear receptor function in the nucleus (66). AKT is capable of preventing AR from activation by androgen via the phosphorylation of AR on Ser210 and Ser790 allowing AKT to suppress androgen-induced apoptosis (67). AR has been shown to promote the initiation and development of liver cancer during the early stage of the disease but to suppress liver cancer cell invasion during the later stages of the disease (68). Evidence from Nie *et al* (69) indicates that the activation of AKT directly impacts AR to inhibit apoptosis in HCC cells, suggesting the function of downstream AR responds to the function of upstream AKT.

In addition to the PPIs between AKT and the proteins described above, AKT can also interact with other proteins that are found to play significant roles in liver cancer. These proteins include T-cell leukemia/lymphoma protein 1 (TCL1) (70,71), breast cancer type 1 susceptibility protein (BRCA1) (72), vimentin (73), integrin-linked kinase (ILK) (74,75), and heat shock protein 27 (HSP27) (76). The proteins that AKT can bind to liver cancer are provided in Table I.

5. AKT-involved PPIs revealed in other models

A number of novel AKT-involved PPIs have been identified in other cancer types. For example, Nam *et al* (77) found that Cdc-2-like kinase 2 (CLK2) is phosphorylated by AKT at Ser34 and Thr127 *in vitro* and *in vivo*. This type of phosphorylation significantly increased cell growth whereas it inhibits cell apoptosis in HeLa cells. Snail1, a transcriptional factor essential for triggering EMT, can directly interact and thus enhance AKT-induced open chromatin around the Snail1-binding site within the E-cadherin promoter in different cancer cells (78). Sirtuin-6 (Sirt6), a tumor suppressor that plays negative roles on DNA repair, telomere maintenance, glycolysis and inflammation, is directly inhibited by AKT through phosphorylation and subsequent degradation by MDM2, and this type of PPI between Sirt6 and AKT promotes tumorigenesis in breast cancer (79). Cyclin-dependent kinase inhibitor 1C (CDKN1C), an inhibitor of cyclin-dependent kinases, is pivotal in regulating cell cycle progression. AKT phosphorylates and inhibits

Table I. AKT-involved PPIs in *homo sapiens*.

Interactor		Experimental evidence
BAD	BCL2-associated agonist of cell death; promotes cell death. Successfully competes for the binding to Bcl-X(L), Bcl-2 and Bcl-W, thereby affecting the level of heterodimerization of these proteins with BAX	Affinity Capture-Western (161,162) Biochemical Activity (161,163,164)
BRCA1	Breast cancer 1, early onset; the BRCA1-BARD1 heterodimer coordinates a diverse range of cell pathways such as DNA damage repair, ubiquitination and transcriptional regulation to maintain genomic stability	Affinity Capture-Western (165) Biochemical Activity (165) Reconstituted Complex (165,166)
CHUK	Conserved helix-loop-helix ubiquitous kinase; acts as part of the IKK complex in the conventional pathway of NF-κB activation and phosphorylates inhibitors of NF-κB thereby leading to the dissociation of the inhibitor/NF-κB complex and ultimately the degradation of the inhibitor	Affinity Capture-Western (17,18) Biochemical Activity (17)
CREB1	CAMP responsive element binding protein 1; this protein binds the cAMP response element (CRE), a sequence present in many viral and cellular promoters. CREB stimulates transcription on binding to the CRE	Biochemical Activity (167)
FOXO1	Forkhead box O1; transcription factor	Biochemical Activity (168-170)
FOXO4	Forkhead box O4; transcription factor	Affinity Capture-Western (171) Biochemical Activity (172) Reconstituted Complex (171)
GSK3β	Glycogen synthase kinase 3 β; participates in the Wnt signaling pathway. Involved in the hormonal control of several regulatory proteins including glycogen synthase, MYB and the transcription factor JUN	Biochemical Activity (173) Reconstituted Complex (75,174-176)
IKKB	Inhibitor of κ light polypeptide gene enhancer in B-cells, kinase β; acts as part of the IKK complex in the conventional pathway of NF-κB activation and phosphorylates inhibitors of NF-κB thus leading to the dissociation of the inhibitor/NF-κB complex and ultimately the degradation of the inhibitor	Affinity Capture-Western (19)
CSBP	One of the major pre-mRNA-binding proteins. Binds tenaciously to poly(C) sequences. Likely to play a role in the nuclear metabolism of hnRNAs, particularly for pre-mRNAs that contain cytidine-rich sequences	Affinity Capture-Western (39) Biochemical Activity (39,177) Reconstituted Complex (39)
MDM2	Mdm2 p53 binding protein homolog (mouse); inhibits TP53/p53- and TP73/p73-mediated cell cycle arrest and apoptosis by binding its transcriptional activation domain. Functions as a ubiquitin ligase E3, in the presence of E1 and E2, towards p53 and itself	Affinity Capture-Western (173,178) Biochemical Activity (179,180)
MTOR	Mechanistic target of rapamycin (serine/threonine kinase); kinase subunit of mTORC1 and mTORC2, which regulate cell growth and survival in response to nutrient and hormonal signals	Affinity Capture-Western (181) Biochemical Activity (14,182,183) Reconstituted Complex (52)
NEDD4	Essential E3 ubiquitin-protein ligase which accepts ubiquitin from an E2 ubiquitin-conjugating enzyme in the form of a thioester and then directly transfers the ubiquitin to targeted substrates	Affinity Capture-Western (184) Biochemical Activity (185)
PTEN	Phosphatase and tensin homolog; tumor suppressor. Acts as a dual-specificity protein phosphatase, dephosphorylating tyrosine-, serine- and threonine-phosphorylated proteins	Affinity Capture-Western (186)

Table I. Continued.

Interactor		Experimental evidence
RICTOR	RPTOR-independent companion of MTOR, complex 2; subunit of mTORC2, which regulates cell growth and survival in response to hormonal signals. mTORC2 is activated by growth factors, however, in contrast to mTORC1, seems to be nutrient- insensitive.	Affinity Capture-Western (187) Biochemical Activity (85,188,189) Reconstituted Complex (190)
SIRT1	Sirtuin (silent mating type information regulation 2 homolog) 1 (*S. cerevisiae*); NAD-dependent protein deacetylase, which regulates processes such as apoptosis and muscle differentiation by deacetylating key proteins.	Affinity Capture-Western (191) Biochemical Activity (191)
SKP2	S-phase kinase-associated protein 2 (p45); substrate recognition component of a SCF (SKP1-CUL1-F- box protein) E3 ubiquitin-protein ligase complex that mediates the ubiquitination and subsequent proteasomal degradation of target proteins involved in cell cycle signal progression, transduction and transcription.	Affinity Capture-Western (62) Biochemical Activity (193) Co-localization (64) Reconstituted Complex (64)
MST2	Stress-activated, pro-apoptotic kinase which, following caspase-cleavage, enters the nucleus and induces chromatin condensation followed by internucleosomal DNA fragmentation. Phosphorylates NKX2-1 (by similarity). Phosphorylates and activates LATS1 and LATS2	Affinity Capture-Western (193,194) Biochemical Activity (193,194)
MAPK8IP1	The JNK-interacting protein (JIP) group of scaffold proteins selectively mediates JNK signaling by aggregating specific components of the MAPK cascade to form a functional JNK signaling module.	Affinity Capture-Western (161) Biochemical Activity (40)
MAPK14	Mitogen-activated protein kinase 14; responds to activation by environmental stress, pro- inflammatory cytokines and lipopolysaccharide (LPS) by phosphorylating a number of transcription factors, such as ELK1 and ATF2 and several downstream kinases, such as MAPKAPK2 and MAPKAPK5.	Affinity Capture-Western (39) Biochemical Activity (39,177) Reconstituted Complex (39)
MAPKK4	Mitogen-activated protein kinase kinase 4; dual specificity kinase that activates the JUN kinases MAPK8 (JNK1) and MAPK9 (JNK2) as well as MAPK14 (p38), but not MAPK1 (ERK2) or MAPK3 (ERK1)	Affinity Capture-Western (38) Biochemical Activity (38,161)
MAPKKK5	Mitogen-activated protein kinase kinase kinase 5; component of a protein kinase signaling transduction cascade. Phosphorylates and activates MAP2K4 and MAP2K6, which in turn activate the JNK and p38 MAP kinases, respectively.	Affinity Capture-Western (35) Biochemical Activity (35)
SNAIL1	Snail homolog 1 (*Drosophila*) gene; this protein has many roles during post-implantation development. It is involved in embryonic mesoderm formation and its maintenance and may also be involved in chondrogenesis and in epithelial-mesenchymal inductive interactions.	Affinity Capture-Western (78) Biochemical Activity (78)
SIRT6	Sirtuin (silent mating type information regulation 2 homolog) 6 (*S. cerevisiae*); NAD-dependent protein deacetylase. Has deacetylase activity towards 'Lys-9' and 'Lys-56' of histone H3. Modulates acetylation of histone H3 in telomeric chromatin during the S phase of the cell cycle.	Affinity Capture-Western (79) Biochemical Activity (79)
MAPKKK8	Mitogen-activated protein kinase kinase kinase 8; required for TLR4 activation of the MEK/ERK pathway. Able to activate NF-κB 1 by stimulating proteasome-mediated proteolysis of NF-κB 1/p105. Plays a role in the cell cycle.	Affinity Capture-Western (37) Biochemical Activity (37)
MAPKKK11	Mitogen-activated protein kinase kinase kinase 11; activates the JUN N-terminal pathway. Required for serum-stimulated cell proliferation and for mitogen and cytokine activation of MAPK14 (p38), MAPK3 (ERK) and MAPK8 (JNK1).	Affinity Capture-Western (34) Reconstituted Complex (34)

Table I. Continued.

Interactor		Experimental evidence
AR	Androgen receptor; steroid hormone receptors are ligand-activated transcription factors that regulate eukaryotic gene expression and affect cell proliferation and differentiation in target tissues.	Affinity Capture-Western (67,179) Reconstituted Complex (67)
Notch1	Notch homolog 1, translocation-associated (*Drosophila*); functions as a receptor for membrane-bound ligands Jagged1, Jagged2 and Delta1 to regulate cell-fate determination. Following ligand activation through the released notch intracellular domain (NICD) it forms a transcriptional activator complex with RBP-Jκ and activates genes of the enhancer of split locus.	Biochemical Activity (46)

Affinity Capture-Western: an interaction is inferred when a bait protein is affinity-captured from cell extracts by either polyclonal antibody or epitope tag and the associated interaction partner identified by western blot analysis with a specific polyclonal antibody or second epitope tag. This category is also used if an interacting protein is visualized directly by dye stain or radioactivity. Note that this differs from any co-purification experiment involving affinity capture in that the co-purification experiment involves at least one extra purification step to eliminate potential contaminating proteins. Biochemical Activity: an interaction is inferred from the biochemical effect of one protein on another, for example, GTP-GDP exchange activity or phosphorylation of a substrate by a kinase. The bait protein executes the activity on the substrate hit protein. A modification value is recorded for interactions of this type with the possible values phosphorylation, ubiquitination, sumoylation, dephosphorylation, methylation, prenylation, acetylation, deubiquitination, proteolytic processing, glucosylation, Nedd(Rub1)ylation, deacetylation, no modification, and demethylation. Reconstituted Complex: an interaction is detected between purified proteins *in vitro*. Co-localization: interaction inferred from two proteins that co-localize in the cell by indirect immunofluorescence only when in addition, if one gene is deleted, the other protein becomes mis-localized. Includes co-dependent association of proteins with promoter DNA in chromatin immunoprecipitation experiments. The data are from BioGRID (http://thebiogrid.org).

CDKN1C on Ser 282 or Thr310, and then promotes cell proliferation, transformational activity and tumorigenicity in breast cancer cells (80). Although studies have mainly focused on how AKT regulates activities of other proteins, few have discussed how other proteins regulate AKT. Zeng *et al* (81) reported Jade-1 is a novel tumor suppressor that is bound to the catalytic domain and the C-terminal regulatory tail of AKT. This PPI inhibits AKT kinase activity and reduced Jade-1 expression in clear-cell renal cell carcinoma and is regarded as a poor prognostic factor. Cylindromatosis (CYLD) is a directly deubiquitinating enzyme that triggers deubiquitination of K63-linked ubiquitination and inactivation of AKT. CYLD deficiency releases its inhibition to AKT and thereby promotes cell proliferation, glucose uptake and growth of prostate tumors (82). These AKT-involved PPIs were important in tumor initiation and progression in other cancer types. However, whether and how these PPIs are critical during liver tumorigenesis remains largely unclear. Nevertheless, the role of these PPIs as novel therapeutic targets in clinical treatment remains to be investigated.

6. Therapies targeting PI3K/AKT-involved PPIs

Since the PI3K/AKT signaling pathway is a crucial pathway in liver cancer formation and progression, targeting PI3K/AKT pathway, these PI3K/AKT-involved physical PPIs in particular are novel aspects in the clinical treatment of liver cancer. mTOR inhibitors can abolish the interaction between AKT and mTOR by inhibiting the phosphorylation of AKT on Ser 473 (83-85). As PPI between AKT and mTOR are important in liver cancer, the use of mTOR inhibitors, such as sirolimus, can significantly reduce the recurrence of liver cancer in the post-liver transplantation patient population (86). In a recent meta-analysis including 474 patients, the 1-, 3- and 5-year recurrence-free survival (RFS) and overall survival (OS) was considerably improved for the sirolimus group in comparison with the calcineurin inhibitors (CNIs) group. Lower recurrence, lower recurrence-associated mortality and lower overall mortality were observed in the sirolimus group compared to the CNIs group (87). Other second-generation mTOR inhibitors, such as everolimus, Pp242, OSI027, CC-223 and AZD8055, have similar antitumor efficacy in liver cancer cell lines and xenograft models (88). A phase 1/2 study including 28 patients revealed that everolimus is well tolerated in patients with advanced liver cancer, and 10 mg/day was defined as the phase 2 dose (89). In another cohort of 36 patients, everolimus was observed to repress cancer progression in patients with advanced liver cancer when used at a maximum tolerated dose of 70 mg weekly (88,90).

In addition to the therapy targeting mTOR, which may interrupt the PPI between mTOR and AKT, some drugs have been identified to simultaneous inhibit more than one signaling pathway. For example, hydroxytyrosol is capable of inhibiting cell proliferation and inducing G2/M cell cycle arrest and apoptosis in HCC cells by suppressing the PI3K/AKT and NF-κB signaling pathways (91). OSU-A9, a potent indole-3-carbinol-derived PI3K/AKT/NF-κB signaling pathway inhibitor, can induce apoptosis by inactivating PI3K/AKT/NF-κB signaling and killing HCC cells (92). NS398, a selective cyclooxygenase-2 (Cox2) inhibitor and simvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, were previously simultaneously used and this co-administration significantly reduced the activity of the PI3K/AKT and NF-κB signaling pathways, leading to inhibited liver cancer

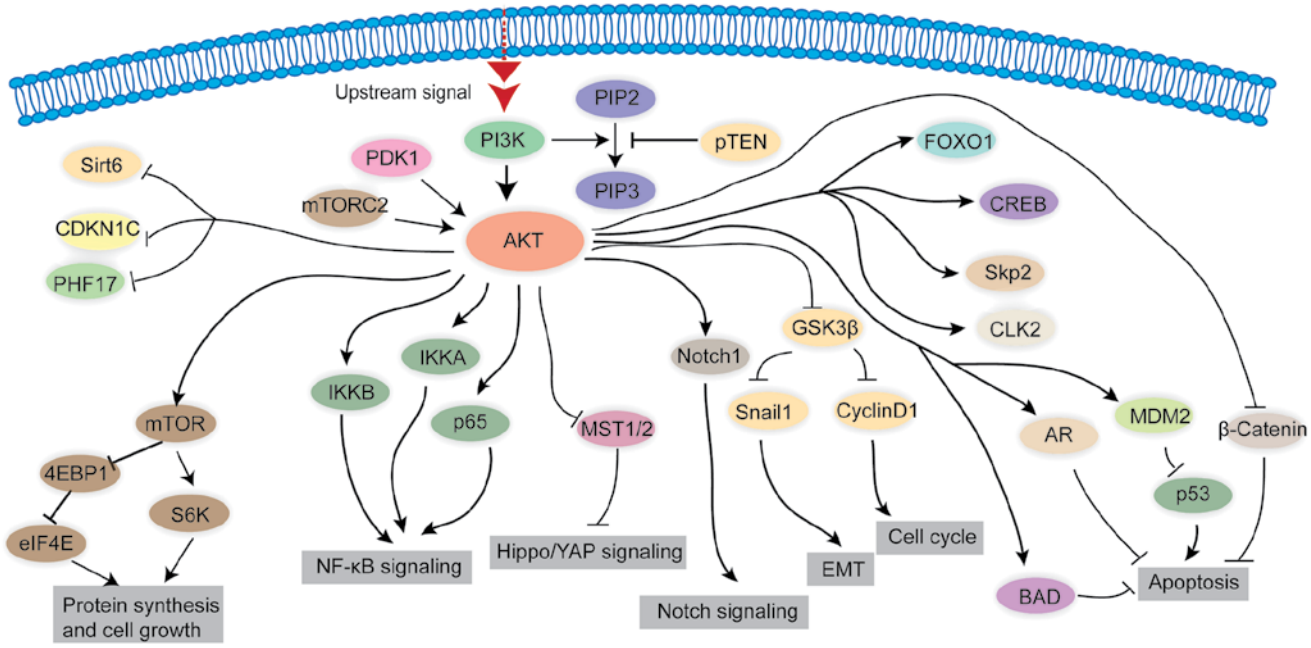

Figure 1. PI3K/AKT-involved PPIs during tumor formation. Image of direct PPIs between AKT and key indicated proteins within certain signaling pathways. The potential therapeutic targets of certain drugs are shown.

cell proliferation and induction of apoptosis (93). Baicalein is another drug that plays a negative role against liver cancer by targeting AKT and β-catenin in clinical treatment (94). An *in vitro* study showed that Baicalein has significant cytotoxicity against liver cancer cells but moderate cytotoxicity against immortalized human hepatocytes, suggesting Baicalein is an ideal drug that is less harmful to normal cells as compared to cancer cells. Baicalein has the ability to induce G0/G1-phase arrest in liver cancer cells by inhibiting PI3K/AKT signaling and promoting degradation of β-catenin, the key factor of Wnt/β-catenin signaling pathway. An *in vivo* study also demonstrated that Baicalein impairs tumor growth in a xenograft mouse model by inhibiting PI3K/AKT and β-catenin. Through a similar mechanism against PI3K/AKT and β-catenin, BCT-100, a new recombinant human arginase, has been revealed to inhibit cell proliferation and enhance caspase-dependent cell apoptosis (95). PI3K/AKT, mTORC1 and MEK signaling can be simultaneously inhibited *in vitro* and *in vivo* by a novel 2-pyrimidyl-5-amidothiazole compound, DC120. Thus, this drug is able to suppress proliferation but also induce apoptosis in liver cancer cells (96).

Other PPIs can also be treated as potential therapeutic targets. Vassilev *et al* (97) developed a class of small molecules, known as Nutlins, that can occupy the p53-binding pocket within the MDM2 protein, thereby blocking the PPI between MDM2 and p53. This effect facilitates the p53 tumor suppressor network to inhibit transformative phenotype of human cancer cells *in vitro* and *in vivo*. Curcumin has also been found to inhibit MDM2 by targeting PI3K/mTOR/ETS2 in several cancer cell lines (98).

The treatment of ANISpm, a novel 3-amino-naphthalimide-spermine conjugate, results in the inactivation of PI3K/AKT signaling followed by dissociation of BAD from BAD-Bcl complexes and the induction of Bcl-mediated apoptosis in liver cancer cells (99). The treatment of 3-nitro-naphthalimide and nitrogen mustard conjugate is another potential therapy that can induce apoptosis in liver cancer cells through the inhibition of PI3K/AKT signaling (100).

Macromolecules (macrodrugs) can be developed that interfere with PPIs by binding with high affinity and specificity to contact surfaces. Although macrodrugs have inherent problems of bio-distribution and delivery to target cells in patients, their efficacies on the inhibition of cancers suggest that efforts to achieve the goal of clinical use should be pursued (101). Targeting physical PPIs especially PI3K/AKT-involved PPIs may become a new aspect in the clinical treatment of liver cancer (Fig. 1).

7. PPIs in other signaling pathways

There are a number of signaling pathways that can play oncogenic roles in liver cancer. For example, the Notch signaling pathway is a highly conserved pathway in most multicellular organisms (102). The Notch system comprises four types of transmembrane Notch receptors (Notch-1, -2, -3 and -4), and two types of ligands, Serrate/Jagged (Jag-1 and -2) and Delta-like (Dll-1, -3 and -4) (103). Extracellular epidermal growth factor (EGF)-like repeats in Notch receptors can interact with the delta serrate LAG-2 (DSL) domain in the ligands. Following ligand binding to the receptors, the extracellular domain of Notch receptors is cleaved by the γ-secretase complex, and the Notch intracellular domain (NICD) is released. NICD then shuttles into the nucleus and interacts with CBF1/*Drosophila* Su (H)/*C. elegans* LAG-1 (CSL)-binding proteins, which are also known as recombination signal binding protein immunoglobulin Jκ (RBP-Jκ). The co-repressors of RBP-Jκ are then replaced and the expression of a set of Notch target genes is activated (104). Notch proteins have been shown to interact with a family of mastermind-like transcriptional coactivators (MAML1, MAML2 and MAML3). MAML1 binds to the

ankyrin repeat domain of the four mammalian Notch receptors, forms a DNA-binding complex with NICD and RBP-Jκ, and amplifies the Notch-induced transcription of HES1 (105). A dominant negative form of MAML can significantly reduce the proliferation of liver cancer cells (106). By contrast, there are also some PPIs that can inhibit Notch signaling. Protein Numb can interact with Notch receptors and antagonize Notch signaling. Mechanically, Numb recruits components of the ubiquitination machinery to the Notch receptor and thereby facilitating ubiquitination of Notch1 at the membrane and promoting the degradation of NICD. Numb acts as a tumor suppressor, and its function of inhibiting tumor cell proliferation occurs largely through the suppression of Notch signaling. In liver cancer cells, the downregulation of Numb is positively associated with activation of Notch signaling-induced cell proliferation and growth (107).

Hedgehog (Hh) is another important pro-tumorigenic signaling pathway that was first identified by the Nobel laureates Nüsslein-Volhard and Wieschaus through mutagenesis screening assays in *Drosophila* (108). In mammals, hedgehog homologues include the Desert hedgehog (DHh), Indian hedgehog (IHh) and Sonic hedgehog (SHh). Hh proteins are synthesized as ~45-kDa precursors, followed by modifications at the amino-terminus with palmitic acids and carboxy-terminus with cholesterol groups (109,110). Hh proteins can bind to the Protein patched homolog (PTCH) receptor, which is a 12-span transmembrane protein (111,112). PTCH is also a negative regulator in Hh signaling because it can inhibit the activity of the 7-pass transmembrane receptor-like protein smoothened (SMO) (113). Binding of Hh proteins to PTCH leads to loss of the inhibitory activity of PTCH on SMO, which initiates an intracellular signaling cascade by releasing GLI proteins, terminal effectors of Hh signaling. These GLI proteins then enter into the cell nucleus to activate the transcription of Hh signaling target genes. The GLI proteins found in mammals include GLI1, GLI2 and GLI3. Numerous genes have been found to be regulated by these three GLI proteins. Overactivation of Hh signaling is responsible for proliferative diseases, including cancer. In 2006, Hh signaling was firstly studied in HCC and investigators identified that SMO and GLI1 proteins are overexpressed in established liver cancer cell lines and liver cancer tissue samples. Furthermore, an increase in the stoichiometric ratio of SMO to PTCH mRNA levels in liver cancer was revealed to correlate with tumor size and be treated as a prognostic marker of liver cancer (114,115). GLI1 expression in HCC tissues was observed to be negatively associated with disease-free and overall survival. Overexpression of GLI1 promotes the proliferation, viability, colony formation, migration and invasion of liver cancer cells, while silencing GLI1 expression in liver cancer cells leads to the opposite output (116). The protein zinc finger of the cerebellum 1 (ZIC1) interacts with GLI1 and repress the activity of GLI1 (117), thus ZIC1 is regarded as a tumor suppressor. In liver cancers, methylation frequencies of ZIC1 promoter are significantly higher than those in the corresponding non-cancerous tissues. Moreover, patients whose ZIC1 promoters are methylated have poorer survival rates than those without such methylation (118).

The Hippo/YAP signaling pathway has become a hot research topic. This pathway was first identified in *Drosophila* and controls organ size through the regulation of cell proliferation and apoptosis. All of the core components of the Hippo/YAP signaling pathway are conserved in mammalians. By phosphorylating the terminal transcriptional regulator of the Yes-associated protein (YAP) signaling pathway, large tumor suppressor kinase (LATS) promotes the PPI between YAP and 14-3-3 proteins, which helps to anchor YAP in the cytoplasm and prevents its transportation into the nucleus. When the upstream Hippo signaling is inactivated, YAP can translocate into the nucleus and bind to several transcription factors including p73, runt-related transcription factor 2 (Runx2) and TEA-domain family member (TEAD) protein families (119). A series of recent studies have demonstrated that the core components of the Hippo/YAP signaling pathway are important for liver tumorigenesis. Approximately 50% of human HCCs show aberrant expression and nuclear localization of YAP (120), with 30% HCCs showing low phosphorylation of YAP on Ser127, a hallmark of the inactivation of YAP (121). PPIs between YAP and other proteins have gradually been identified, including SMADs (122), p73 (123), ErbB4 (124), TEADs (119), RUNX (125), angiomotins (AMOTs) (126-128), zona occludens 1/2 (ZO1/2) (129) and LATS1/2 (130). In liver cancer, these PPIs play pivotal roles in promoting or inhibiting tumor formation. LATS kinases inhibit YAP function by promoting the cytoplasmic retention of YAP by phosphorylating YAP on Ser127 (131). A significant decrease in the expression and activity of LATS kinases is evident in HCC and CCC (132). YAP has been shown to interact with the TEAD family of transcription factors and upregulate genes that promote cell growth and inhibit apoptosis (133,134). The YAP-TEAD complex is important in YAP-overexpressing cancers and disruption of the YAP-TEAD interaction may provide an important approach for the treatment of liver cancer. The treatment of various types of cancer with verteporfin (VP) to disrupt the PPI between YAP and TEAD was suggested to become a new approach in clinical treatment (135). In addition to TEAD, YAP has been found to interact with tight junction proteins angiomotin (AMOT) and zona occludens-2 (ZO-2). AMOT acts as a YAP cofactor, preventing YAP phosphorylation and increasing its activity towards a specific set of genes that facilitate tumorigenesis in liver cancer. However, the functional role of PPI between YAP and ZO-2 in liver cancer remains to be investigated. These direct PPIs in the Notch, hedgehog, and Hippo/YAP pathways that are associated with liver cancer are shown in Fig. 2.

8. How to investigate and analyze PPIs

Methods that detect PPIs can be classified into three categories, i.e., *in vitro*, *in vivo* and *in silico* methods. For *in vitro* methods, a given procedure is carried out in a controlled environment outside a living organism; for *in vivo* methods, a given procedure is carried out inside a living organism; and for *in silico* methods, the procedure is performed in a computer simulation (136). *In vitro* methods for detecting PPIs include affinity chromatography (137), X-ray crystallography (138), co-immunoprecipitation (139), tandem affinity purification (140), protein arrays (141), protein fragment complementation (142) and nuclear magnetic resonance (NMR) spectroscopy (136,143). *In vivo* methods

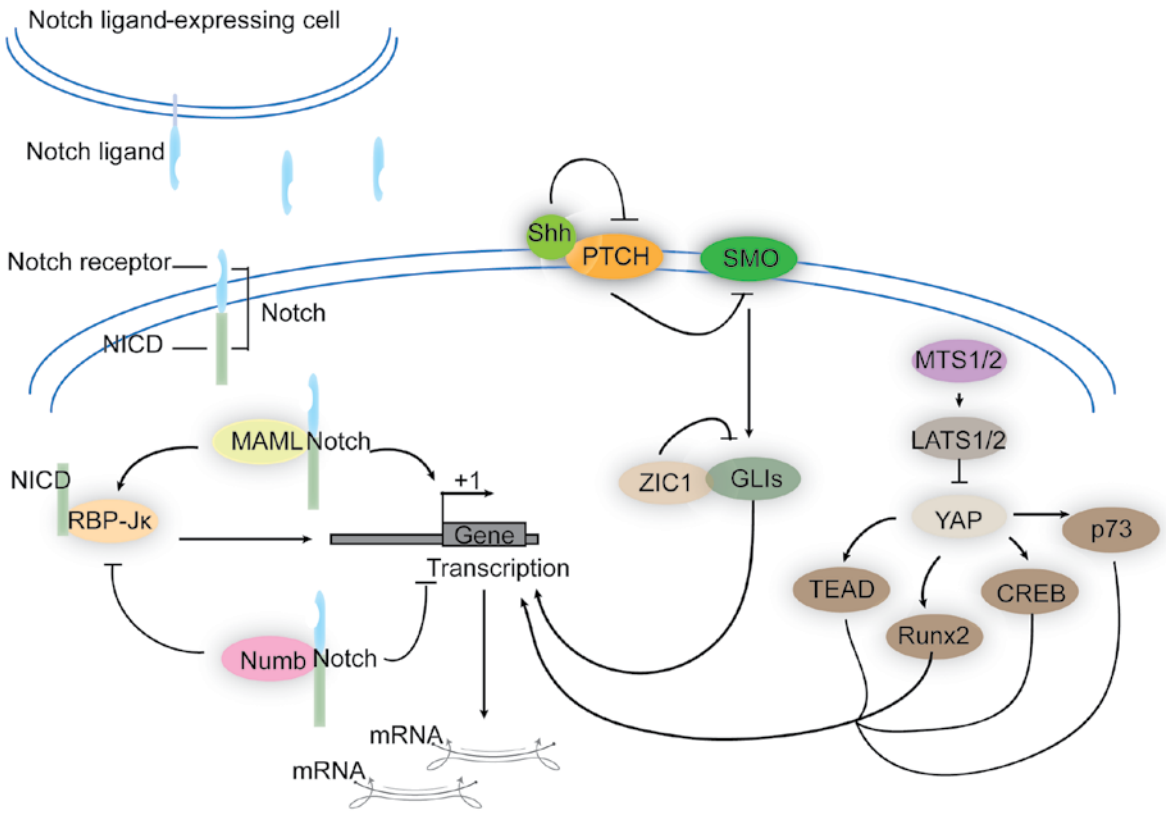

Figure 2. PPIs in Notch, Hedgehog and Hippo/YAP pathway during tumor formation. Direct PPIs in Notch, hedgehog and Hippo/YAP signaling pathway involved in liver cancer are shown.

for detecting PPIs include yeast two-hybrid (Y2H) (144) and synthetic lethality (145). As for *in silico* prediction, there are sequence-based approaches (146), structure-based approaches (147), gene fusion analysis (148), chromosome proximity (149), *in silico* two-hybrid (150), phylogenetic tree (151), mirror tree (152) and gene ontology (153).

Massive identification of PPIs generates numerous interactions, which are collected together in PPI databases that are continuously updated to provide complete interactions. The database of interacting proteins (DIP) is the first PPI database globally (154). The number of public PPI databases has increased rapidly. For example, the Biological General Repository for Interaction Datasets (BioGRID: http://thebiogrid.org) is an open access database that houses genetic and protein interactions curated from the primary biomedical literature for all major model organisms and human. BioGRID contains 749,912 interactions as drawn from 43,149 publications describing studies in >30 model organisms (155). Another example is the Protein Interaction Network Analysis (PINA) platform, which is for protein interaction network construction, filtering, analysis, visualization and management. It integrates PPI data from six public curated databases and constructs a complete, non-redundant protein interaction dataset for six model organisms. PINA also provides a variety of built-in tools to filter and analyze the network to gain insight into the network (156).

Public databases such as HitPredict (http://hintdb.hgc.jp/htp/) (157), IntAct (http://www.ebi.ac.uk/intact/) (158), Agile Protein Interaction DataAnalyzer (APID) (http://bioinfow.dep.usal.es/apid/index.htm) (159) and MINT (http://mint.bio.uniroma2.it/mint/) (160) include the PPIs data and are continuously updated.

9. Conclusion

In summary, signal transduction plays a fundamental role in many biological processes as well as in many diseases. In liver cancer, many signaling pathways including PI3K/AKT, Ras/Mek/ERK, IKK/NF-κB, Wnt/β-catenin, TGF-β, Notch, hedgehog and Hippo/YAP are shown to be dysregulated. The majority of these signaling pathways have PPIs with PI3K/AKT signaling pathways. Identification of PPI is the crucial step involved in identifying the signal transduction pathways. Signals propagation to inside and/or along the interior of cells depends on PPIs between the various signaling molecules. Numerous properties of PPI such as allosteric sites and hotspots, have been incorporated into drug-design strategies. The relevance of PPI as putative therapeutic targets for the development of new treatments is particularly evident in liver cancer. The investigation of PPIs in signaling pathways may provide knowledge on biochemical cascades and disease pathogenesis, and new therapeutic targets in liver cancer.

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