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Review

Roles of DLK1 in Liver Development and Oncogenesis

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Abstract: Hepato Cellular Carcinoma (HCC) has an increased mortality rate in the last decade. Protein expression in HCC has similarities to the liver cells during the development process. One of the proteins expressions is Delta-Like 1 homolog (DLK1) that was found during liver development and oncogenesis. This permits the opportunity to study pathophysiology and signaling pathway of DLK1 and to find an early detection and therapeutic target for HCC. This review will explain the signalling mechanism and the roles of DLK1 during liver development, oncogenesis of HCC, as a tumor marker and targeting therapy.

Keywords: Hepatocellular Carcinoma, DLK1, Liver Development, HCC Oncogenesis, Tumor Marker

Introduction

Hepatocellular Carcinoma (HCC) is one of malignancies which has high mortality rate. American Cancer Society has reported an increase in the mortality rate since 2003 until 2012 as much as 2.7% due to this illness (ACS, 2016). Hepatocellular carcinoma and liver cirrhosis are the main cause of chronic hepatitis B and hepatitis C virus that made patients death. Approximately 1.45 million people are infected by these viruses (WHO, 2016). Because of the HCC incidence and high in mortality, it becomes a concern of researcher to make diagnostic and effective therapeutic strategies. It requires a comprehensive knowledge of liver development to understand the preventive measures, causes determination and pathogenesis of liver diseases (Sokol, 2002). One of the proteins target in liver oncogenesis is Delta-Like 1 homolog (DLK1).

Delta-Like 1 homolog (DLK1) is a candidate biomarker of liver stem/progenitor cells (Wang and Sul, 2006; Bujak *et al*., 2015; Kopan and Ilagan, 2009) and plays a significant role in HCC oncogenesis (Baladron *et al*., 2005; Laborda *et al*., 1993; Nueda *et al*., 2007). DLK1$^+$ HCC cells have the same characteristics as cancer stem/progenitor cells. However, molecular mechanism of HCC is still not yet well-established (Falix *et al*., 2012; Xu *et al*., 2012). This review explains the roles of DLK1 in the process of liver development, HCC oncogenesis, as a tumor marker and targeting therapy.

Delta-Like 1 Homolog

Delta-like 1 homolog is a transmembrane protein that has three major regions, i.e., an extracellular region composed of six EGF-like (epidermal growth factor like), a juxtamembrane region with TACE cleavage site (ADAM17) and an intracellular region (Bujak *et al*., 2015; Wang and Sul, 2006). The repeated structure of amino acid sequences of EGF are very similar to the structure of Delta-Like canonical Ligand (DLL) (Baladron *et al*., 2005; Nueda *et al*., 2007; Bujak *et al*., 2015); however, DLK1 does not have N-terminal Delta-Serrate-LAG-2 (DSL)-domain (Yin *et al*., 2006; Kopan and Ilagan, 2009). Consequently, Delta and OSM-11-like proteins (DOS) co-ligand functioned to inhibit NOTCH signalling. DOS is a specialized tandem of EGF-repeats (Baladron *et al*., 2005; Kopan and Ilagan, 2009; Nueda *et al*., 2007; Fig. 1).

DLK1 shows inhibitory activity in NOTCH signalling pathway (Kopan and Ilagan, 2009). It is expressed widely during embryonic development and controls the determination process of cell fate, proliferation and differentiation (Baladron *et al*., 2005; Falix *et al*., 2012; Nueda *et al*., 2007; Fig. 1).

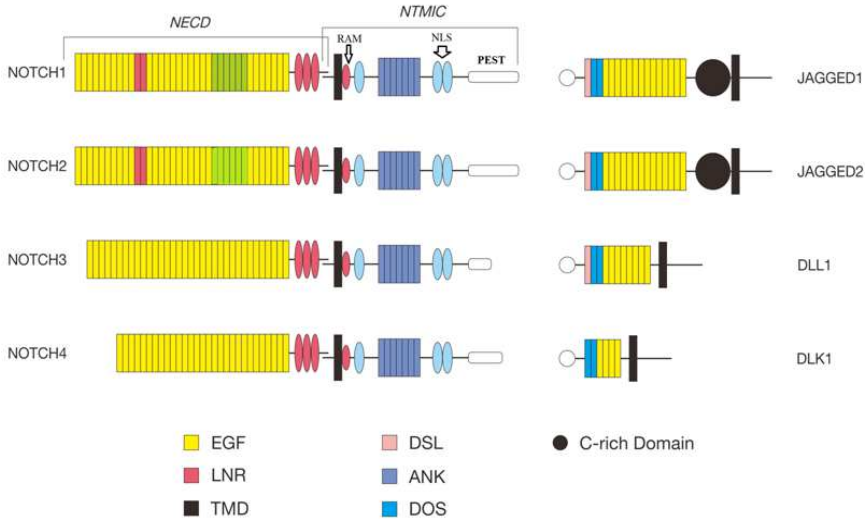

Fig. 1. Schematic diagram of NOTCH1, NOTCH2, NOTCH3 and NOTCH4 receptors and their four ligands (JADDED1, JADDED2, DLL1 and DLK1). NOTCH receptors consist of multiple extracellular EGF-like repeats, which are different in the repeating number in each receptor. NOTCH receptors have two domains, i.e., extracellular (NECD) and transmembrane-intracellular (NTMIC). Ligand binding will release intracellular (NICD) fragment and leads to NOTCH signalling activity. Two NOTCH receptor ligands family are Jagged and DLL ligands. Those canonical ligands contain DSL, DOS and EGF motifs. DLK1 is a non-canonical ligand that lacks of its DSL domain; consequently, it will acts as DOS ligand

DLK1-NOTCH Relationship

In determining the fate of hepatoblast cells and HCC pathogenesis, NOTCH signaling has a significant role (Lu *et al*., 2016). NOTCH signalling is also required for cell commitment, cell spesification and maintenance process of progenitor cells during prenatal development (Chen *et al*., 2011; Dill *et al*., 2013; Lu *et al*., 2016; Loomes *et al*., 2002). NOTCH has four receptor types, i.e., NOTCH1-4 and two families ligands (Jagged and Delta-like) (Falix *et al*., 2012).

NOTCH receptors consist of two domains, i.e. NOTCH Extracellular Domain (NECD) and NOTCH Transmembrane-Intracellular domain (NTMIC) (Kopan and Ilagan, 2009; Falix *et al*., 2012). NOTCH extracellular domain is composed of varian EGF like repeats (29-36 EGF-like repeats), a region that mediates interaction between the receptor and its ligand, especially at EGF-like repeats 11-12 and 24-29 (Kopan and Ilagan, 2009). Epidermal growth factor repeats are followed by negative regulatory region or NRR that contains three cysteine-rich Lin12-Notch Repeats (LNR) and a heterodimerization domain or HD. In the absence of ligand, NRR has a special function to prevent receptor activation (Kopan and Ilagan, 2009; Falix *et al*., 2012; Teodorczyk and Schmidt, 2014).

NOTCH Transmembrane and Intracellular domain (NTMIC) are composed of transmembrane domain or TMD, a RBP-Jκ association module-domain for binding with DNA binding protein CSL (CBF1/RBP-Jκ/Su(H)/Lag-1) (Kopan and Ilagan, 2009), nuclear localization sequences or NLSs, a seven ankyrin reputation-domain that functions to take the coactivator Mastermind/Lag-3 and a Transactivation Domain (TAD) that habors a conserved proline/glutamicacid/serine/threonine-rich (PEST) motifs (Teodorczyk and Schmidt, 2014; Kopan and Ilagan, 2009; Geisler *et al*., 2008). Glutamine-rich repeat (OPA) in Drosophila has a function as transactivation domain (Kopan and Ilagan, 2009).

Two families of ligand have three structural motifs, i.e. an N-terminal DSL motif, a specialized tandem EGF repeats, i.e. DOS-domain and variable EGF-like repeats (Falix *et al*., 2012; D'Souza *et al*., 2010; Teodorczyk and Schmidt, 2014). The difference between Jagged/Serrate ligand and Delta-like ligand are based on the existence of a cysteine-rich domain. Jagged/Serrate ligand has cysteine-rich domain and Delta-like ligand do not have it (Fig. 1). N-terminal DSL and DOS region will bind with NOTCH receptor (Kopan and Ilagan, 2009; Falix *et al*., 2012).

NOTCH signalling pathway begins with the binding of ligand-receptor that induces the receptor. With the aid of proteolytic enzyme, NOTCH receptor releases NICD. In further, NICD enters nucleus to bind with CBF1/RBP-Jκ. It will activate target genes, such as Hes (hairy and enhancer of split homologs; Fig. 2) that has functions in the regulation of proliferation, differentiation and apoptosis of epithelial cells and carcinogenesis (Falix *et al*., 2012; Hansson *et al*., 2004; Lu *et al*., 2016). NOTCH signaling also controls Sox9, HNF1, TGF-β and Homeobox B expressions in the liver, which play a role in determining liver cells commitment (Dill *et al*., 2013; Zong *et al*., 2009; Zong and Stanger, 2011; Figure 2). The absence of RBP-Jκ indicates blocking of NOTCH signalling pathway (Morell *et al*., 2013).

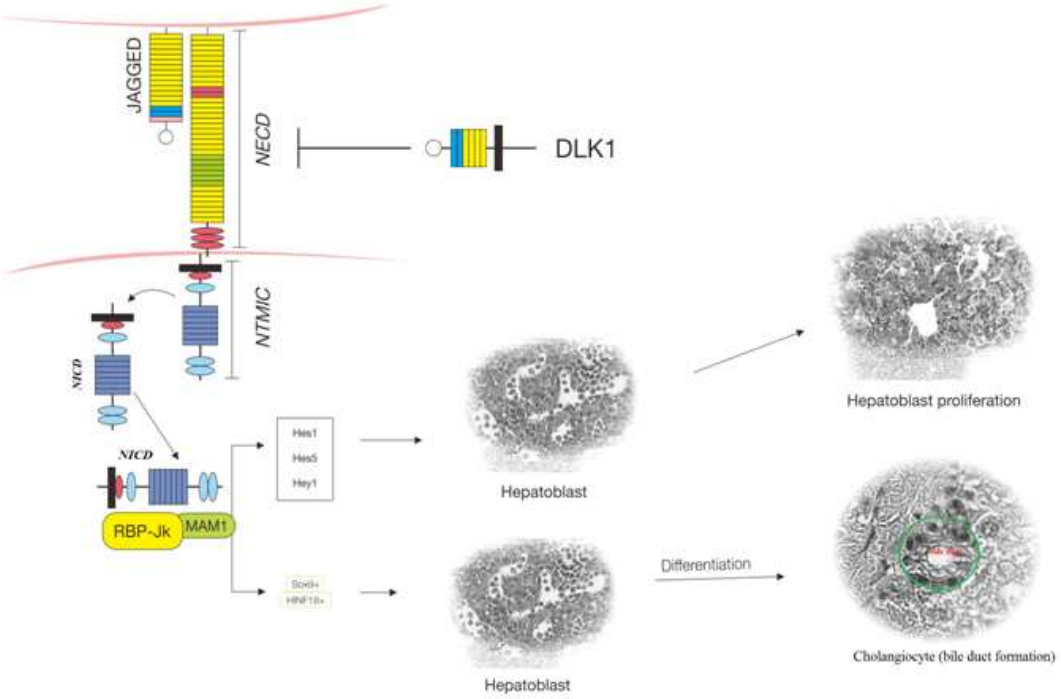

Fig. 2. DLK1 role in the proliferation of liver cells during the development is as the inhibit differentiation of hepatoblast into cholangiocyte. NOTCH receptor-canonical ligand (Jagged) binding will release NICD. After that, NICD enters the nucleus and binds with RBP- Jκ to become a complex protein. This complex protein recruits MAM1 and other co-activators to activate target genes (Hes and Hey) transcription that facilitate hepatoblast differentiation into cholangiocyte. DLK1 shows inhibitory activity at the extracellular domain of NOTCH signalling pathway

In mammals, four NOTCH receptors have their own roles. NOTCH1 has a role in the normal prenatal development; nevertheless, it also involved in the regulation of oncogenesis process in adult (Falix *et al*., 2012). NOTCH2 is responsible for the cholangiocytes differentiation, aggressive behavior and immature morphology of HCC (Falix *et al*., 2012; Hayashi *et al*., 2015). NOTCH3 and NOTCH4 have functions in vascular development (Falix *et al*., 2012). DLK1 role as non-canocical ligand is as inhibitors of NOTCH signaling (Figure 2). It can be seen from the fact that increased DLK1 expression is associated with the decreased of NOTCH and Hes1expressions (Chen *et al*., 2011; Tanimizu and Mitaka, 2014).

DLK1-NOTCH in Liver Development

DLK1 has dynamic expression during liver development. DLK1 expression begins since ED10.5 and increases significantly at ED14.5–ED16.5. DLK1 expression is downregulated concomitantly with the formation of cholangiocytes and the remodelling of ductal plate into intrahepatic ducts. Eventually, DLK1 expression is no longer found after neonate period (Falix *et al*., 2013; Tanimizu *et al*., 2003; Tanaka *et al*., 2009).

DLK1 is a marker for hepatoblast that has high proliferation and bipotentiality. DLK1 is also considered as a marker for immature hepatocytes because $DLK1^{+}$cells express HNF1β, HNF3β, HNF4 and HNF6, but do not express CK19 that is expressed by cholangiocytes (Tanimizu *et al*., 2003; 2004). While DLK1 is down regulated, NOTCH expression is increased and at the same time hepatoblasts differentiates into cholangiocytes (Baladron *et al*., 2005; Gordillo *et al*., 2015; Loomes *et al*., 2002; Yin *et al*., 2006).

NOTCH signalling is responsible for activation of target genes, i.e., Hes, Sox9, HNF1β, that are important for cholangiocytes differentiation (Gordillo *et al*., 2015; Tanimizu and Mitaka, 2014; Shin *et al*., 2015; Jörs *et al*., 2015; Morell *et al*., 2013; Fig. 2). In addition, DLK1 also has a role for progenitor/stem cells' maintenance and differentiation during development (Begum *et al*., 2014). DLK1-Wnt10b-βCatenin pathway contributes in hepatocytes proliferation during liver regeneration. $DLK1^{+}$ hepatocyte cells are induced by hepatic stellate cells through paracrine effects and self-inductive (Zhu *et al*., 2012).

DLK1-NOTCH Role in Hepatocellular Carcinoma Oncogenesis

Some spectrum of tumors, such as breast cancer, small-cell lung carcinoma, leukemia, neuroblastoma,

gliomas, pancreatic cancer, colon cancer, have high DLK1 expression (Begum *et al*., 2014; Bujak *et al*., 2015). In the pathogenesis of liver diseases, NOTCH signaling has a significant role. Increased expression of NOTCH3 and NOTCH4 are found in cancer tissues. Observation of HCC HepG2 cell lines showed a relatively high NOTCH3 expression and a little expression of NOTCH4 (Geisler *et al*., 2008). In the pathogenesis of HCC, there is a mechanism involving DLK1 and NOTCH signaling pathway. Regulation of HCC is through multiple signaling pathways including NOTCH, RAF/MEK/ERK, Wnt/β-catenin, AKT/mTOR, EGFR, HGF/cMET and (Coral *et al*., 2012; Woo *et al*., 2009; Zhao *et al*., 2016; Fig. 3).

DLK1 expression can be induced by hypoxia; therefore, it is possible that oncogenesis process involving DLK1 were affected by hypoxic conditions in the microenvironment (Kim *et al*., 2009). DLK1 is also secreated in hepatic stellate cells selectively and will induce the activation of hepatic stellate cells in vivo and in vitro (Zhu *et al*., 2012). DLK1 will upregulate WNT pathway. Knockdown of DLK1 will lead to the reduction of Wnt10b, Wnt3a, necdin and Shh expression (Zhu *et al*., 2012). In vitro, DLK1 inhibit Mesenchymal Stem Cells (MSC) differentiation (Chen *et al*., 2011). In vivo, over-expression of DLK1will increase the stemness of the tumor cell and tumor growth (Begum *et al*., 2014; Kim *et al*., 2009). DLK^+ HCC showed to form a colony, spheroid colony and higher chemoresistency compared to DLK1 knockdown on HCC cells (Xu *et al*., 2012).

Another oncogenesis process of HCC is by activation of NOTCH signaling pathway that will lead an epithelial tranformation into mesenchymal (Fig. 3). This change will also result in the disappearance of main characteristic of epithel cells and play a role in embryonic development, liver fibrosis and cancer (Zhao *et al*., 2016).

NOTCH2 signalling can lead and accelareated HCC formation that has the same characterictics and expression pattern as hepatoblast (Hayashi *et al*., 2015; Dill *et al*., 2013), but with great migration and invasion capability (Gordillo *et al*., 2015).

NOTCH2 signalling is one of key regulators in HCC. NOTCH2 signalling will increase Hes1 and Sox9 mRNA expression related to HCC formation (Tanimizu *et al*., 2003; Dill *et al*., 2013). Increased proliferation on DEN-N2ICD mice correlated to the increased of NOTCH2 espression. The increased of Hes1 mRNA will eventually form a colony in HCC formation. In addition, NOTCH2 signalling will accelerate the HCC growth (Dill *et al*., 2013).

In the last 5 years, researches have demonstrated the role of hepatic stellate cells and some morphogensin oncogenesis process of HCC (Zhu *et al*., 2012; Fung *et al*., 2016). Activated hepatic stellate cells will express some morphogen such as Wnt, Necdin, DLK1, Shh and NOTCH. Wnt pathway, Necdin and DLK1 have a function in HCC through the regulatory role of $PPAR_\gamma$. $PPAR_\gamma$ plays a role in inhibiting tumor growth and invasion of cells to the surrounding tissues. Another function of PPARγ is to inhibit EMT process which plays a role in HCC (Fung *et al*., 2016; Hsu and Chi 2014; Kimura *et al*., 2012; Shen *et al*., 2012; Zhu *et al*., 2012). Studies that have been conducted gave indirect evidence to the relation of Wnt, Necdin, DLK1 and PPARγ in HCC. Necdin has a role in activation of Wnt pathway by binding with GN boxes on the proximal promoter of Wnt10b resulting in the repression of PPARγ. This proves that there is an opposite role of Necdin and Wnt with PPARy (Ross *et al*., 2000; Zhu *et al*., 2012). In studies of DLK1 knockdown, induction of PPARγ was occured. It was almost identical with Necdin and Wnt mechanism in regulation of PPARγ (Zhu *et al*., 2012; Fung *et al*., 2016). Therefore, the increase of Wnt, Necdin and DLK1 expression will lead to PPARγ suppression resulting in increased expression of HGF, which has a role in EMT that will eventually develop into HCC (Fung *et al*., 2016; Maulik *et al*., 2002; Ding *et al*., 2010; Mizuno *et al*., 2005; Ozaki *et al*., 2003). Additionally, proliferation and invation of HCC will be increased due to HGF stimulation of Matrix metallopeptidase 9 (MMP9) and Matrix metallopeptidase 3 (MMP3) (Wang *et al*., 2007; Ozaki *et al*., 2003; Mizuno *et al*., 2005; Mohammed *et al*., 2005; Lee *et al*., 2010).

DLK1 as Hepatocellular Carcinoma Marker

Delta-like 1 homolog is expressed in malignancy and promotes cancer cell stemness and tumorigenicity so it can be used as therapeutic target and tumor marker for cancer stem/progenitor cells. Li *et al*. (2015) revealed that tumor size of HCC was positively correlated to DLK1 in serum. DLK1 can be a complement to Alpha Feto Protein (AFP) in the diagnosis of HCC (Li *et al*., 2015; Chauhan and Lahiri 2016; Shen *et al*., 2016).

It is possible to use DLK1 as a prognostic factor since there is an evidence that it correlates to survival rate in HCC. HCC patients with $DLK1^+$ cells have shorther survival rate compared to HCC patients without $DLK1^+$ cells (Jin *et al*., 2008; Li *et al*., 2015).

The relationship between expression of DLK1, PPARy and HGF needs to be studied in further, especially as marker of invasion and metastases level of HCC. HCC anti-metastatic proteins, such as E-cadherin, spleen tyrosine kinase or SYK and ECM regulator metallopeptidase inhibitor 3 (TIMP3), act as anti-metastatic in HCC (Hsu and Chi 2014; Shen *et al*., 2012).

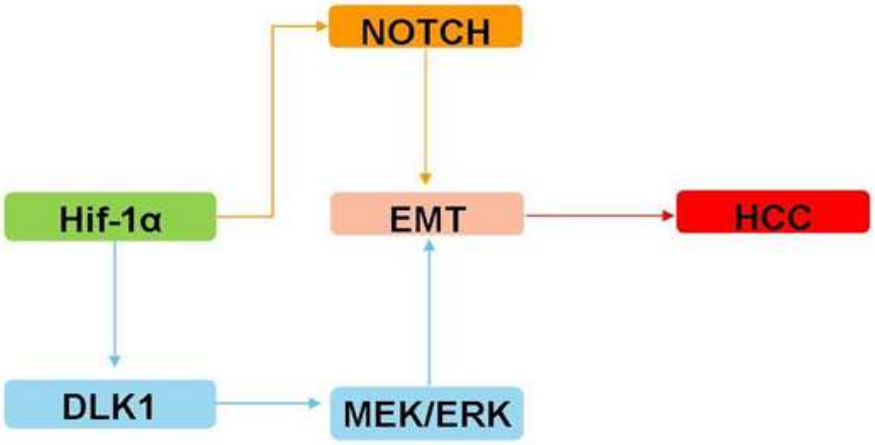

Fig. 3. Schematic diagram of DLK1 and NOTCH role in HCC oncogenesis.HIF-1α and HIF-2α are capable to enhance DLK1 transcription. DLK1 may increase the phosphorylation of MEK/ERK and activates MEK/ERK signaling. MEK/ERK signaling facilitates Epithelial-to-Mesenchymal Transition (EMT) process and Oct4/Nanog-regulated EMT to HCC

Conclusion

HCC and progenitor/stem cell have similar expression pattern. A protein that is found in both condition is DLK1 that can affect cell proliferation and differentiation. Some signaling pathways, such as Wnt and NOTCH pathway, are indirectly affected by DLK1; even though, the signaling pathways are not clearly understood (Fung *et al*., 2016). The role of DLK1 in Wnt pathway are in non-canonical or β-catenin-independent mechanism (Vilchez *et al*., 2016; Fung *et al*., 2016) and also through PPARγ suppression mechanism. In addition, paracrine and self-inductive effect of hepatic stellate cells were able to activate DLK1 to play a role in the process of liver development, regeneration, fibrosis and malignancy (Zhu *et al*., 2012; Fung *et al*., 2016). Further researches are expected to reveal the biological role of DLK1 in the signaling pathway. This knowledge will provide solutions for HCC therapy in the future. Another research opportunity that can be further explored is the relationship between DLK1, Wnt and PPARγ in oncogenesis of HCC.

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Author's Contributions

Each author has an equal contribution in the preparation, development and publication of this manuscript.

Ethics

This review does not have any ethical problems since the subject was published original articles.

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