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Review

JNK1, a potential therapeutic target for hepatocellular carcinoma ☆

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ABSTRACT

Hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide. Despite tremendous efforts to diagnose and institute new treatment regimens, the prognosis is still extremely poor. Therefore, knowledge of the molecular mechanisms governing the initiation, maintenance and progression of HCC is urgently needed. Recently, several groups have attributed an important role for c-Jun N-terminal kinase 1 (JNK1) in the pathogenesis of human HCC and its close association with the expression of HCC signature genes. In this review the various associations between JNK1 and HCC are discussed with the hope that targeting this pivotal kinase may lead to novel therapeutic approaches for this fatal disease.

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1. Introduction

One of the most famous quotations from the eighteenth century German philosopher G.W.F. Hegel is "whatever is real is rational and whatever is rational is real". This is perhaps also true with respect to identification of the c-Jun N-terminal kinase (JNK) in the liver. In 1990, Kyriakis and Avruch [1] isolated a 54-kDa polypeptide with Ser/Thr protein kinase activity from the liver of rats that were given cycloheximide. It was widely known that liver is a master organ for the regulation, synthesis, storage, and metabolism of sugar, fat, protein, minerals, vitamins, hormones, drugs, toxins, and numerous other molecules. The significance of JNK in the function of the liver was unclear at that time. Other studies since then demonstrated that this kinase along with several family members and their alternatively spliced isoforms are ubiquitously expressed in a wide range of tissues or cell types. Association between JNK activation and cancer development was first suspected based on its exclusive role in the phosphorylation of c-Jun, an oncogenic protein essential for oncogene Ras-induced malignant transformation [2,3]. The evidence linking JNK to human cancer was provided by observations of higher JNK activation in a variety of cancer cell lines relative to normal cells [4]. JNK activation, *in situ*, has also been demonstrated in several human cancer samples, including colon cancer [5], pancreatic cancer [6], breast cancer [7], osteosarcoma [8], leukaemia [9], lung cancer [10], prostate cancer [11], head and neck squamous cell carcinoma [12], glioblastoma [13], glioma [14], and gastric cancer [15]. Definitive evidence showing JNK activation in hepatocellular carcinoma (HCC) was not revealed until most recently [16–18], suggesting an unappreciable role of JNK in the liver. In this review, we will discuss the roles of JNK activation in HCC pathogenesis and the possibility of molecular targeting on JNK1 as a potential new therapeutic strategy for HCC.

2. JNK family kinases

2.1. Jnk genes

The JNK family kinases are mitogen-activated protein kinases encoded by three gene loci in human genome: jnk1 located on chromosome 10q11.2, jnk2 located on chromosome 5q35 and jnk3 located on chromosome 4q21.3. JNK1 and JNK2 are ubiquitously expressed, whereas the expression of JNK3 is limited predominantly to the brain, heart, and testis [19]. In-gel protein kinase assay or immunoblotting using antibody against JNK have demonstrated two classes of JNK with apparent molecular weights of 46 and 54 kDa. It was generally viewed that the 46 kDa molecule represents JNK1 and the 54 kDa molecule represents JNK2, respectively. However, it is now recognized that due to alternative splicing of the pre-mRNAs of JNKs, the 46 and 54 kDa variants represent two isoforms of both JNK1 and JNK2. By screening adult human brain cDNA libraries, Gupta et al. [20] showed that at least 4 isoforms of JNK1, 4 isoforms of JNK2 and 2 isoforms of JNK3 were generated from alternative splicing of the corresponding pre-mRNAs. Both JNK1 and JNK2 are represented by two isoforms, α and β, depending on the mutually exclusive use of exon 6α or exon 6β (Fig. 1). An additional alternative splicing of the JNK1 and JNK2 pre-mRNAs can occur at the site between the last intron and exon. Both α1 and β1 transcripts of the JNK1 and JNK2 use the conserved splicing site at this position, yielding a 384 and 382 amino acid polypeptides with a molecular weight about 46 kDa. Alternative splicing at this site by using the cryptic splicing site within the last exon results in deletion of the 5 nucleotides at the 5′-terminus of the last exon and changes of the reading frame, causing an extension of the COOH-terminal region, to generate α2 and β2 isoforms of the JNK1 and JNK2 with a molecular weight of 54 kDa (Fig. 1A and D). The transcript of JNK1 α2/β2 yields a polypeptide of 427 amino acids and the transcript of JNK2 α2/β2 yields a polypeptide of 424 amino acids.

2.2. Predominant JNK isoforms

The expression levels of the JNK isoforms appear to be unequal. In several JNK1 or JNK2 gene knockout studies, the predominant forms of the JNK1 are the α1 or β1 isoforms and the major forms of JNK2 are the α2 or β2 isoforms [21,22] (Fig. 1G). The unequal expression of the different isoforms for a given JNK kinase is possibly determined by some unique characteristics of exons and the splicing sites. In the case of the α and β isoforms of the JNK1, the α exon (exon 6α) may be much favorable for constitutive splicing because of the presence of 2 exonic splicing enhancer elements that were not found in the β exon (exon 6β) (Fig. 1C). At the terminal splicing site of the JNK1 α or β pre-mRNA, the constitutive splicing site has a score of 7.7, close to the average score 7.9 for the majority of splicing sites. In contrast, the cryptic splicing site has a score of 4.2, which is considerably below the average score for most of the splicing sites as determined by using the Splice Site Score Calculation (Fig. 1A). This might account for the predominance of JNK1 α1 being expressed in the majority of tissues or cells (Fig. 1B). The presence of the U2-U12-type hybrid intron 6 that prefers the usage of the α exon, the exon located down stream of the 6β exon in JNK2 genome [23], and the higher score of the cryptic splicing site (Fig. 1D and E) might explain the predominance of JNK2 α2 isoform. Thus, these features of the JNK1 and JNK2 pre-mRNAs may explain why the 46 kDa JNK1 isoform and the 54 kDa JNK2 isoform are dominantly expressed in most cells or tissues. Therefore, the previous assumption that JNK1 and JNK2 correspond to the 46 and 54 kDa isoforms, respectively, is still applicable for most tissues or cells (Fig. 1G).

2.3. Functional differences between JNK1 and JNK2

In addition to some mitogenic signals, JNKs can be activated by a variety of stress signals and proinflammatory stimuli through the upstream MAP3K–MAP2K kinase cascades. Activated JNKs phosphorylate their cognate substrates including c-Jun, JunD and ATF2, the key subunits of the AP-1 transcription factor. Furthermore, JNKs are also capable of phosphorylating a number of other nuclear and non-nuclear proteins important for cell apoptosis, metabolism, and cell motility, such as FoxO4, PPARγ1, c-Myc, p53, NFATc2, STAT1, STAT3, Bcl-2, Smac, Bid, IRS-1, Itch, 14-3-3, etc. [24,25].

A number of studies suggested that the JNK family kinases are functionally redundant in animal development and cell growth regulation. For example, both JNK1 and JNK2 are required for regional specific apoptosis of the brain in early development, neural tube morphogenesis and T cell proliferation [26–28]. However, emerging evidence suggests that the JNK family kinases may play different roles in many instances. Several biochemical experiments have revealed that JNK1, rather than JNK2, is the primary kinase in stress-induced phosphorylation of serines 63 and 73 of the c-Jun protein, which aids cell proliferation. On the other hand, JNK2, which has high affinity for c-Jun in unstimulated cells, mainly serves as a negative regulator for the stability of the c-Jun and consequently, inhibits cell growth [22,29]. A much remarkable distinction between JNK1 and JNK2 is their role in the serine 307 phosphorylation of the insulin receptor substrate-1 (IRS-1), an event associated with insulin resistance and obesity [30]. In studies producing high fat-induced obesity in mice with genetic deficiency in either jnk1 or jnk2 gene, it was found that JNK1 is the key contributor for the serine phosphorylation of the IRS-1. Several other studies also demonstrated importance of JNK1 over JNK2 in lung fibrosis [31], gastrotic cancer [15], histone acetylation [32], Bcl2 phosphorylation [33], repression of the Wnt/β-catenin signaling [34], and myoblast differentiation [35].

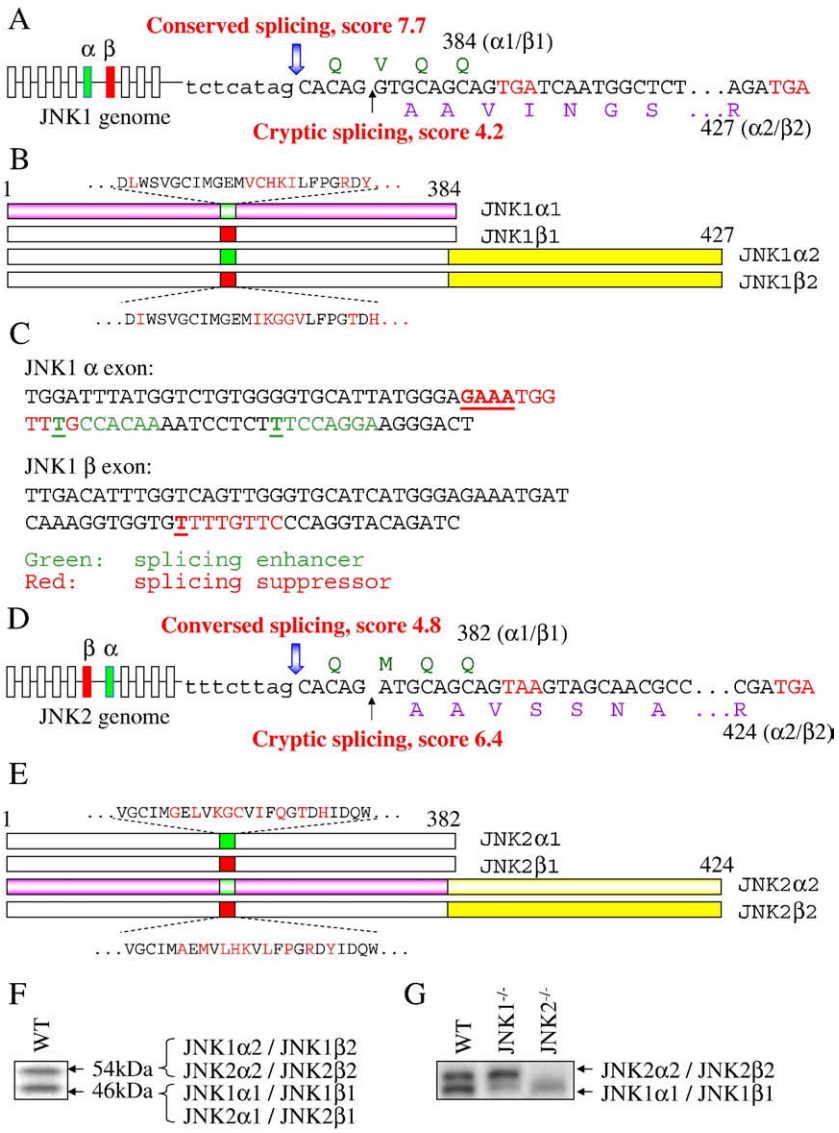

Fig. 1. Genomic structure and alternative splicing of JNK1 and JNK2. A. Schematic structure of JNK1 genome and the alternative splicing site of JNK1 pre-mRNA between the last intron and exon. The exon 6α and 6β are marked as green and red box, respectively. The lower case characters indicate the partial DNA sequence in the last intron; the capitalized characters represent the partial DNA sequence in the last exon. The block blue arrow and the line arrow indicate conserved and cryptic alternative splicing sites with the calculated splicing site scores. The ending amino acid sequence of JNK1 α1/β1 is in green color; the ending amino acid sequence of JNK1 α2/β2 is in purple color. The stop code "TGA" is marked with red color. B. General domain structure of JNK1 isoforms. The amino acid sequences derived from exon 6α and 6β are shown on the top of JNK1α1 and the bottom of JNK1β2. The conserved amino acids derived from these two alternative exons are in black and the non-conserved amino acids are in red. The yellow boxes indicate the extended amino acid sequences of the JNK1 α2 and JNK1 β2 derived from the use of the cryptic alternative splicing site. The starting and ending amino acids are marked with numbers. The JNK1α1 is pink-colored, representing the dominant isoform of JNK1 expressed in tissues or cells. C. DNA sequences of the alternative exon 6α and 6β of JNK1. The elements of exonic splicing enhancers and exonic splicing suppressors are marked by green and red colors, respectively. Only the exon 6α contains two splicing enhancers. D. Schematic structure of the JNK2 genome and the alternative splicing site of JNK2 pre-mRNA between the last intron and exon as marked for JNK1 in A. In contrast to the JNK1 genome, the alternative exon 6β is located at the 5′-upstream of exon 6α. E. General domain structure of JNK2 isoforms. The dominant JNK2 isoform expressed in tissues or cells is pink-colored. F. JNK expression pattern as seen in most Western blotting experiments using anti-JNK antibody. G. The predominant isoforms of JNK1 and JNK2 were detected in Western blotting using cell lysates from mouse embryonic fibroblast cells derived from wild-type, JNK1$^{-/-}$ and JNK2$^{-/-}$ mice, respectively.

The major JNK1 and JNK2 isoforms, JNK1α1 and JNK2α2 proteins, are roughly equally expressed in most type of the cells or tissues. The key domains for the ATP-binding pocket, substrate docking, catalytic loop, and activation loop are highly conserved in JNK1 and JNK2. In view of this it is not yet understood how JNK1 can exert a more dominant effect. One explanation may be the role of the prolonged extension of the carboxy-terminus of the JNK2α2 relative to JNK1α1. This extension may weaken its interaction with upstream kinases, scaffold proteins and substrates through either altering the docking groove or blocking the accessibility of the catalytic or activation loop. An alternative explanation for such unequal activity between JNK1 and JNK2, which might be more sound mechanistically, is the subtle difference in the activation loop that is critical for JNK1 or JNK2 activation by upstream kinases including MEKK1 and MKK4. In JNK2, but not JNK1 molecule, there is a rigid β-strand conformation in that region, which causes a significant reduction of phosphorylation by MEKK1 and MKK4 signals [36].

3. JNK1 in HCC

3.1. JNK activation in human HCC

Aberrant activation of protein kinases has been implicated in playing a causative role for the initiation and progression of the human HCC. These kinases include PI3K-AKT [37], aurora kinase, and receptor tyrosine kinases of VEGF, EGF, PDGF, Heregulin, and IGF-I

[38]. Because of their association with clinicopathological features of HCC, including fast growth of the tumor, stagnated neovascularization and tumor multiplicity, several clinical trails of phase II and phase III had been established by targeting these kinases during the past few years [39].

There were only few scatted reports suggesting potential involvement of JNK in HCC during the past decade. The potentials of JNK activation in the pathogenesis of human HCC was demonstrated only recently [16–18]. In earlier studies using chemical carcinogen-induced mouse HCC model, the involvement of JNK in HCC initiation or progression was largely based on indirect evidence leaving considerable uncertainty. A number of reports suggested potent activation of JNK by the gene products of HBV and HCV, the most common etiological factors of HCC, in a variety of hepatic or non-hepatic cell lines [40,41]. The earliest evidence implying the likelihood of JNK contribution to human HCC is provided by Guo et al. [42] who examined JNK activation among 40 human HCC specimens. By immunohistochemical staining, they showed that positive nuclear staining for the phosphorylated-JNK occurred in 70% of the HCC samples but not in noncancerous liver tissues, indicating higher JNK activation in HCC relative to the noncancerous liver tissues.

A correlation between p21-activated protein kinase 1 (Pak1) and JNK activation was noted in some human HCC samples [43]. It was assumed that the activated JNK is the central player in mediating Pak1-induced HCC cell migration through direct phosphorylation of paxillin, a multi-domain scaffold protein important for focal adhesion and cell motility [44]. Although the percentage of HCC samples exhibiting JNK activation was not shown in the original study, overexpression of Pak1 was observed in about 75% of HCC samples examined. If the Pak1 expression is truly and strongly associated with JNK activation as shown in several selected clinical samples of HCC, it could be estimated that about 70% of the HCC samples would have high JNK activation, which is close to the rate of JNK phoshorylation as reported earlier [42].

Conclusive studies showing importance of JNK activation in the initiation and progression of human HCC were made most recently by two independent groups who simultaneously reported that JNK1, but not JNK2, is over-activated in more than 50% of the human HCC samples [16–18]. Elevated JNK1 kinase activity was observed in 56% of HCC tissue slides relative to the case-matched noncancerous liver tissues in a tissue array study [17]. This observation was supported by immunobloting studies using HCC tissue lysates, which showed that JNK1, rather than JNK2, was highly activated in about 55% of human HCC samples [16]. Both studies also tried to link JNK1 activation to the pathogenesis of HCC. It was believed that JNK1-dependent expression of c-Myc, a transcriptional repressor for the tumor suppressor p21, was responsible for the diminished checkpoint function in the tumorigenic hepatocytes. Evidence supporting this notion is from the observed reduction in HCC size, number and inducibility by chemical carcinogen in JNK1$^{-/-}$, but not in JNK2$^{-/-}$ mice, which correlates with a substantial increase of p21 tumor suppressor in the liver of the JNK1$^{-/-}$ mice [17].

Aberrant activation of JNK1 might define the signature genes crucial for the pathogenesis and prognosis of the human HCC. Gene profiling studies implicated that genes controlling the cell cycle, proliferation, and metabolism were overexpressed, whereas genes for differentiation, morphogenesis, antioxidant response, and p450 family were substantially underexpressed in the HCC tissues with higher JNK1 activation [16]. In addition, a new link between JNK1 activation and alterations in the epigenetic landscape in HCC was proposed based on a positive association of genes encoding histone methyl transferases with the JNK1 activation status in HCC tissues. These histone methyl transferases include EZH2 and SUV39H2 for tri-methylation of lysine 9 and 27 on histone H3 (H3K9me3 and H3K27me3); MLL3, SMYD3 and SMYD5 for tri-methylation of lysine 4 of histone H3 (H3K4me3), and some other potential methyl transferases [16]. Indeed, two histone H3 epigenetic markers, H3K4me3 and H3K9me3, were appreciably elevated in the high JNK1 HCC tissues relative to the case-matched noncancerous liver tissues in immunoblotting experiment. It is unclear whether there is a cause and consequence relationship between JNK1 and histone methyl transferases. In the case of EZH2, our recent investigation implied that JNK1 and JNK2 exhibited opposite regulation on the expression of EZH2 in mouse embryo fibroblasts (MEFs). The expression of EZH2 was JNK1 dependent, which could be antagonized by JNK2 in response to stress signals.

3.2. JNK1 in HCC animal models

In addition to the evidence showing JNK1 activation in human HCC specimens, JNK1 activation had also been demonstrated as a key factor for the chemical carcinogen-induced HCC in mice with Ikkβ gene deficiency specifically in the hepatocytes. An overall 4-fold increase of HCC incidence as well as an elevated total JNK activity was observed in these hepatocyte-specific Ikkβ deficient mice in response to diethylnitrosamine (DEN) treatment [45]. A spontaneous JNK activation and HCC development were noted in the mice with genetic disruption of the Ikkγ, a gene encoding the regulatory subunit of the IKK complex, in the parenchymal cells [46]. Intercross of the hepatocyte-specific Ikkβ deficient mice with the Jnk1$^{-/-}$ mice provided evidence indicating that JNK1, rather than JNK2, is the key mediator for the DEN-induced HCC. Despite a normal expression of the JNK2 protein, the incidence of DEN-induced HCC was significantly decreased in the offspring of the intercrossed mice in which the expression of JNK1 was genetically abolished [47]. As observed in human HCC with high JNK1 activation [16], the tumorigenic property of JNK1 in mice is largely attributed to its promoting effects on the expression of several important cell cycle regulatory proteins including PCNA, cyclin D, CDK1, CDK2, and VEGF [47].

Taken together, all of above evidence suggests that JNK1 is perhaps the most important kinase that is over-activated in HCC. Sustained activation of JNK1 is responsible for the aberrant elevation of several genes critical for hepatocyte proliferation and transformation in both human and experimental animals. In addition to the well-known proteins for cell cycle or growth regulation, JNK1 may also be capable of affecting the epigenetic landscape of the genome to make the hepatocytes more susceptible to carcinogens and DNA damaging agents. The hepatocytes with a strong expression of the histone methytransferases, especially the EZH2 due to high JNK1 activation or activity, may be able to bypass the cellular senescence or apoptotic program, leading to malignant transformation.

4. JNK1 and HCC signature genes

4.1. Current status in HCC signature gene studies

Contribution of JNK to both cell apoptosis and carcinogenic transformation has been well-documented. It remains unclear whether the role of JNK1 played in HCC development is through its pro-apoptotic function or the tumor-promoting property, or both. A number of studies clearly demonstrated that activation of JNK1 or JNK2 is pivotal for the expression of genes important in cell cycle transition, proliferation, metabolism, and metastasis [22]. It is very likely, therefore, that the tumor-promoting, rather than the pro-apoptotic activity of the JNK1 is critical for the pathogenesis of HCC.

A major goal in current HCC research efforts is to define signature genes governing the initiation, maintenance and progression of the HCC [48]. These signature genes should also be able to classify tumor stages and predict tumor recurrence, metastasis and patient survival probability. More significantly, identification of the signature genes will aid in designing the targeted therapies for HCC. Many challenges remain in HCC signature gene discovery. Consistency and reliability

are lacking in reported signature genes so far. Many such reports in the literature have reached dead ends that were viewed as "one-paper wonders" by the HCC research community [49]. The variations in HCC signature genes might be due to the complexity of etiological and pathological features of the HCC. Subtle or dramatic difference in signature genes may occur depending on the nature of infection (HBV or HCV), exposure to different environmental factors, hepatic inflammation, cirrhosis, necrosis, fibrosis, vascular invasion, and tumor cell origination. The complexity is further magnified by the application of different platforms and algorithms used for gene profiling in various studies. All of these factors have hampered the refinement of the HCC signature genes.

4.2. HCC signature genes and JNK1

During the past several years, considerable effort had been made in determining the HCC signature genes. The information emphasizing the link of a unique signaling pathway to the pathogenesis and gene signature of the HCC was very limited. The possible association of JNK1 activation with the biochemical or oncological features of HCC was not revealed until most recently [16].

Considering the fact that JNK1 is an important determinant for HCC development and progression, it is highly perceivable that a set of JNK1-associated HCC signature genes can be defined. By comparing high JNK1 HCC (H-JNK1 HCC) to the low JNK1 HCC (L-JNK1 HCC) in total of 31 clinical samples, Chang et al. demonstrated a substantial increase of tumor size in H-JNK1 HCC relative to the L-JNK1 HCC [16], implying importance of JNK1 in HCC pathogenesis. By analyzing gene profiling data between these two subgroups based on the JNK1 activation status in the HCC, a distinct expression spectrum of the genes was noted between H-JNK1 HCC and L-JNK1 HCC. The genes encoding PEG10, CD24, H19, and KRT19 were expressed at much higher levels in the H-JNK1 HCC relative to the L-JNK1 HCC, whereas the genes reflecting the normal liver function, such as CYP family, NAT2, ADHs, and antioxidant responses were underexpressed. In agreement with earlier reports [22], functional comparison revealed that the genes involved in cell cycle, DNA replication, amino acid metabolism and small molecule biochemistry were overexpressed in the H-JNK1 HCC tissue. In contrast, the genes involved in cell-to-cell interaction, cell signaling, cell morphology, and free radical scavenging were underexpressed in the H-JNK1 HCC tissue.

4.3. JNK1 and HCC prognosis

Considerable differences in the prognosis of HCC patients have long been recognized by clinicians and researchers. A number of previous studies had proposed unique signature genes for the HCC with extremely poor prognosis [50–54]. Because of the increased tumor size and loss of encapsulation seen in the majority of H-JNK1 HCC [16], it is very likely that H-JNK1 HCC has features similar to those HCCs with poor prognosis. Indeed, there is an overlap between the overexpressed genes in H-JNK1 HCC and the poorly prognostic HCC that exhibited impaired patient survival and earlier recurrence of the tumor after surgical resection. These overlapping genes include S100P [55], S100A8 [52], S100A9 [55], CCL20 [37,56], PEG10 [57], CD24 [37,57,58], NTS [51], SPP1 [54], PLAC8 [59], H19 [60,61], SPINK1 [62], GALNT7 [37], HMGA2 [57], C1orf106 [55], AMIGO2 [37,53], AFP [37,52], UBE2C [37,63], SOX4 [37,57,64], IER3 [51], TMED3 [55], IGFBP3 [65], S100A4 [66], and KRT19 [52]. Furthermore, the most down-regulated genes in the H-JNK1 HCC, such as HPD, CYP3A4, SERPINC1, AQP9, GLYAT, OTC, PCK1, CYP2E1, CYP2C9, CYP8B1, FMO3, BAAT, SLC27A5, SLC22A1, CYP2D6 [51], GYS2 [37], F9 [55,67], NAT2 [37,68,69], ADH1B [58], LECT2 [37,70,71], AFM [37], ADH4 [37,50], SLC10A1 [72], and HSD11B1 [37], have also been reported to be substantially down-regulated in the HCC with poor prognosis.

One of the most important factors for the poor prognosis of HCC is its intra- and extra-hepatic metastasis. The lung, lymph nodes and bone are the common metastatic sites found in HCC patients [73]. Although direct evidence indicating JNK1 involvement in HCC metastasis is not available yet, several lines of information clearly suggest contribution of JNK kinase to HCC metastasis. First, JNK1 has long been viewed as a key transcriptional regulator for the expression of matrix metalloproteinases (MMPs), especially MMP2 and MMP9, two important collagenases for tumor cell invasion and metastasis [74]. Silencing JNK1 expression in cancer cells by siRNA substantially reduced the levels of MMP2, MMP9 and tumor metastasis [75]. The expression and activity of both MMP2 and MMP9 are significantly elevated in the metastatic HCC [76]. Secondly, Pak1, a JNK dependent kinase important for cytoskeletal reorganization and cell migration, is overexpressed in human HCC with more aggressive metastatic characteristics [43]. Lastly, JNK1 has been recently viewed as an important signaling component of the osteopontin (SPP1)-induced cancer cell metastasis [77], whereas osteopontin had been previously demonstrated as a molecular biomarker of the metastatic HCC [54].

4.4. JNK1 and liver progenitor cells in HCC

It has been debated for decades whether HCC arises from mature hepatocytes, the liver stem cells or bipotential progenitor cells (oval cells in rodents) that can differentiate into hepatocytes and cholangiocytes [78]. Markers for hepatocytes, such as AFP and albumin, and cholangiocytes, mainly KRT7 and KRT19, are expressed in the progenitor cells. A generally accepted assumption is that if HCC is originated from the progenitor cells, both hepatocytic markers and cholangiocytic markers should be concurrently expressed. Furthermore, the progenitor cell originating HCC should have biochemical, pathological and genetic characteristics of the hepatoblastoma (HB), an infant or child malignancy of the liver precursor cells. As expected, a profound concordance in gene profiles among the H-JNK1 HCC, HB and progenitor cells was noted by comparing the signature genes for both H-JNK1 HCC and HB. The most significant genes in this regard include AFP [57], TACSTD1 (EpCAM) [79], KRT19 [52,57], KRT7 [52], PROM1 (CD133) [79,80], THY1 (CD90) [80] and VIM [52]. An additional feature of the HB and hepatic progenitor cells is the enhanced expression of the imprinted genes, including H19, IGF2, DLK1, PEG3, PEG10, MEG3, SGCE, and NDN [57]. Indeed, all of these mentioned imprinted genes are up-regulated in the H-JNK1 HCC as well [81].

The possibility of progenitor cell origination of the H-JNK1 HCC is also supported by signature genes for $EpCAM^+/AFP^+$ HCC [55]. Based on the expression levels of EpCAM and AFP, stem cell-like HCC ($EpCAM^+/AFP^+$) and hepatocyte-like HCC ($EpCAM^-/AFP^-$), two categories of HCC were characterized. [82]. A number of genes are commonly overexpressed between H-JNK1 HCC and the stem cell-like HCC, such as S100P, S100A9, CCL20, PEG10, CD24, NTS, H19, UBE2C, TMED3, and KRT19. Interestingly, both H-JNK1 HCC and stem cell-like HCC showed poorer prognosis in a Kaplan–Meier survival analysis [81].

"Gene signature" appears to be a fashionable but loosely used terminology. It should be noted that many so-called cancer gene signature studies, in fact, are simply gene profiling because they identified hundreds or even thousands of the differentially expressed genes. A true gene signature should represent a few well-characterized genes that are able to achieve accurate distinction of cancer types and stages, prediction of outcomes or stratification of the patients for proper therapeutic regimen. Furthermore, these signature genes need to be validated by using different cohorts and algorithms. In this context, highlighting the commonly expressed genes among the high JNK1 HCC and the HCC characterized with poor prognosis and progenitor cells may serve as an example for defining the informative HCC signature genes. The genes for S100A8, PEG10, CD24, SOX4,

KRT19, FMO3, GLYAT, and HPD, thus, warrant further testing in HCC classification, diagnosis and the possibility of targeted therapy.

5. How is JNK1 activation sustained in HCC?

5.1. General activators of JNK1 in HCC

The status of JNK1 activation appears to be able to distinguish aggressive HCC from the non-aggressive HCC that can be further stratified by gene expression pattern or unique signature genes [16,17,81]. The question is how JNK1 is sustainedly activated in HCC? Potential activators of JNK1 in the liver include viral proteins derived from HBV or HCV, reactive oxygen species (ROS) and environmental toxicants such as aflatoxin B1 and arsenic (Fig. 2). All of these activators may activate JNK1 through stimulation of the upstream kinases, perturbation of the protein ubiquitination cascade and inactivation of the dual-specific JNK1 phosphatases. The activation of the upstream kinases of the JNK1 by stress signals including ROS and arsenic has been elucidated in much detail [83,84], whereas how the activation or activity of JNK1 is regulated in the pathogenesis of HCC associated with viral infection and aflatoxin B1 exposure is largely speculative.

5.2. Mechanistic insight into preferential JNK1 vs JNK2 activation in HCC

The molecular basis for the preferential JNK1 activation in HCC might be the unique structural features of the JNK1 that are not present in the JNK2 molecule. Despite a general similarity in protein crystallographic structure [85], the differences in some amino acid residues might determine the preference of JNK1 in some cellular responses. Direct evidence supporting this notion was provided by data revealing a more efficient phosphorylation of JNK1 than JNK2 by the MEKK1-MKK4 kinase cascade [36]. It was assumed that Gly177 and Ser179 in the activation loop encompassing the phosphate-acceptor sites at Thr183 and Tyr185 of the JNK1 are responsible for such high efficiency in phosphorylation. These two residues were replaced by Cys177 and Asn179 in JNK2 molecule, resulting in the formation of an additional β-sheet structure that is refractory in transmitting upstream kinase signals. In contrast, Gly177 and Ser179 at this region along with other residues are very likely to form a less ordered conformation that favors interaction and phosphorylation of the JNK1 by upstream kinases. This molecular feature may explain why JNK1 is the dominating kinase in the activation of the $CD8^+$ T cells [86], obesity [30], steatohepatitis [87], lung fibrosis [31], histone acetylation [88], Bcl2 phosphorylation and autophagy [33], gastric cancer [15], and UV- or TNFα-induced stress responses [21], whereas JNK2 activity is negligible for most JNK signaling outputs [89].

5.3. Viral activators for JNK1

Key kinases critical for extracellular signal-induced JNK activation include members of the PKC family, MEKK1, MEKK2, MKK4, MKK7, ASK1, TPL-2, TAK1, etc. HBV and HCV are the two foremost etiological factors for developing human HCC. The HBV X protein encoded by HBx gene and the truncated envelope protein pre-S2/S encoded by HBV S gene had been reported as bona fide activators for multiple intracellular kinases, scaffold proteins and transcription factors, such as Src tyrosine kinases, PKC, PKR, c-Raf, COP9 (Jab1), CREB, RXR, AR, and TBP [90]. Many of these HBV-activated kinases are direct activators or integrators of the JNK signaling pathway. Expression of the HBV X protein in mouse hepatocytes induced a sustained activation of JNK kinase [41,91], which correlated with a persistent AP-1 activation in these cells. The progression of human HCC from chronic HBV infection and cirrhosis was believed to be mediated by the persistent activation of JNK that shifts the function of the TGFβ signaling from tumor suppression to tumor promotion through Smad3 phosphorylation at the linker region [92]. This appears to be also the case for the HCC resulting from infection of HCV that expresses nucleocapsid (core), envelop (E1 and E2) and several nonstructural proteins in the infected host cells [93]. Both the core protein and some nonstructural proteins of HCV had been shown to be able to activate JNK in hepatocytes [94–96]. In the cells without JNK activation, the Smad3 is c-terminal phosphorylated and plays a role in cell apoptosis in response to TGFβ [97]. In HBV or HCV infection, activated JNK phosphorylates the linker region of Smad3 to minimize the c-terminal phosphorylation. The linker phosphorylated Smad3 is predominantly localized in nuclei to facilitate transcription of genes important for cell growth, migration and transformation mediated by AP-1 and other transcription factors [97–100].

5.4. How ROS activate JNK1

5.4.1. Oxidation of the cellular proteins

In concert with viral infection and chronic liver inflammation, ROS are the additional crucial regulators for the activation and activity of JNK during the pathogenesis of HCC. Typical ROS include superoxide ($O_2^{\cdot -}$), hydroxyl radical (·OH) and hydrogen peroxide (H_2O_2). Mitochondria are the predominant source for the formation of superoxide by ubiquinone-dependent reduction. The second source of superoxide is the NADPH oxidase that catalyzes the one-electron reduction of oxygen to superoxide [101]. The superoxide can be spontaneously or enzymatically dismutated into hydrogen peroxide that is further converted into the hydroxyl radical. The mechanisms by which ROS alter the function of the important cellular proteins include but not limited to oxidation, nitrosylation, di-sulphide bond formation, and glutathionylation on the thiol groups of amino acids, mainly

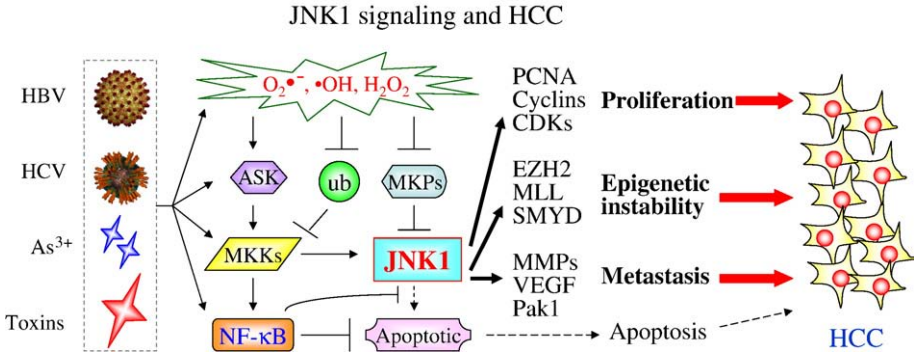

Fig. 2. Molecular mechanisms of JNK1 activation and HCC development. HCC etiological factors including viral infection and exposure to environmental hazards induce ROS generation and upstream kinase activation, leading to a sustained activation of the JNK1. The pro-apoptotic function of the JNK1 might be minimized by the accompanying anti-apoptotic signalings, such as oncogenes, growth factors and NF-κB. Consequently, the tumorigenic effect of JNK1 in the liver is dominating through positive regulations on genes or signaling molecules for cell proliferation, epigenetic perturbation and cancer cell metastasis. ub: protein ubiquitination.

cysteine (Cys, C). The majority of protein phosphatases, include tyrosine phosphatases and dual specificity MAPK phosphatases (DUSPs) contain an essential Cys residue in the signature active site motif, (I/V)HCXAGXXR(S/T/G), (where X is any amino acid). Well-established evidence suggests that the catalytic Cys can be oxidized to form sulphenic acid (Cys-SOH) that can be reversed through the formation of a sulphenyl-amide intermediate in the presence of reducing agents [102,103]. Under the circumstance when the cellular reducing capacity is exhausted, such as the case of HCC, the sulphenic acid can be further oxidized to form irreversible sulphinic (Cys-SO_2H) and sulphonic (Cys-SO_3H) species, leading to permanent inactivation of the phosphatases.

5.4.2. ROS generation in HCC

There are several mechanisms for the generation of ROS in HCC. First, the viral proteins, including HBV X protein and the core protein and nonstructural protein NS5A of HCV, are capable of inducing accumulation of ROS in the hepatocytes [104–106]; secondly, certain inflammatory cytokines, such as TNFα, IL-1 and IL-6, released during the chronic liver inflammation and cirrhosis, as well as some environmental factors, mainly alflatox B1 and arsenic, are potent inducers of tissue oxidative stress [84,107–110]; lastly, due to the progressive deterioration of the liver function during the transition of inflammation to cirrhosis and HCC, the expression of the genes for antioxidant defense, such as those for SOD, catalase, MGSTs, EPHX, and metallothionines, was severely diminished, leading to a further elevation of ROS [16].

5.4.3. ROS and ubiquitination in JNK1 activation

The fact that ROS inactivate JNK phosphatases and stimulate ASK1 or other upstream kinases of JNK to boost the JNK activity and its activation has been well-documented [101]. Potential role of ROS on the protein ubiquitination signaling that linked to JNK activation has not been adequately addressed so far. Many important cellular proteins can be conjugated with poly-ubiquitin chains catalyzed by ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) [111]. A large proportion of the ubiquitinated proteins will be targeted to proteasome for degradation. Several lines of evidence indicate that protein ubiquitination is one of many regulatory mechanisms in the activation of JNK. First, ASK1, a physiological JNK activating kinase, has been shown to be ubiquitinated by ubiquitin ligase c-IAP1 in response to TNFα receptor 2 (TNFR2) signaling, followed by proteasomal degradation [112]. Accordingly, ASK1 ubiquitination can serve as a controlled terminating signal for JNK activation. Secondly, MEKK1, the MAP3K for JNK as well as p38 signaling, contains an N-terminal PHD domain with an ubiquitin ligase activity for self ubiquitination [113,114]. The ubiquitination of MEKK1 appears to be not for proteasomal degradation, but rather, for the inhibition of its kinase activity. This could explain why the N-terminal truncated MEKK1 without the PHD domain is constitutively active [115]. MEKK2, another important JNK activating kinase, has been demonstrated to be a substrate for Smurf1 E3 ubiquitin ligase that targets MEKK2 ubiquitination and degradation [116]. The protein level as well as JNK activation was substantially elevated in the cells with a genetic disruption of smurf1 gene. Lastly, the JNK scaffold protein plenty of SH3s protein (POSH) can be self-ubiquitinated for proteasomal degradation [117]. POSH is important for the assembly of JNK activation complex containing mixed-lineage kinases (MLKs), MKK4/7, JNK, and c-Jun. Removal of the N-terminal Zn ring finger domain exhibiting putative E3 ubiquitin ligase activity in the POSH protein stabilized POSH and enhanced JNK activation [117]. Taken together, the JNK activation can be negatively regulated by the ubiquitination for ASK1, MEKK1 MEKK2, and POSH, which requires a sequential activation of E1, E2 and E3 for ubiquitin activation, conjugation and ligation. When the cells undergo oxidative stress, the activity of E1 and E2 is compromised pronouncedly due to a rapid S-thiolation of Cys at the active site of the enzymes following the formation of GSSG induced by ROS through glutathione (GSH) oxidation [118,119]. Inhibition of the E1 and E2 activity by ROS will delay or ablate protein ubiquitination for these JNK activating kinases, and consequently result in a prolonged activation of JNK (Fig. 2).

6. JNK1 is potentially an ideal therapeutic target for HCC

The only curative treatment for HCC is still the surgical resection based on a careful assessment of the pathological and clinical conditions of the patients at present. Unfortunately, very few HCC patients are eligible for surgical resection because of large tumor size, advanced stage and severe damage to the liver function [48]. In addition, there is a high frequency of tumor recurrence after resection, which is largely attributed to the early intravascular metastasis of the tumor cells [48]. Numerous studies have confirmed that conventional chemotherapy or radiotherapy is ineffective in achieving any meaningful clinical improvement due to inherent resistance of HCC or overpowering systemic side effects [39]. Recent advancements in mechanistic understanding of the HCC have provided new opportunities in HCC intervention through molecular targeting strategies. A number of phase II and phase III clinical trials have been conducted during the past several years using the newly introduced molecular targeting agents aimed towards growth factor receptors and/or specific signaling pathways [39]. The agents currently under investigation include Sorafenib, Erlotinib, Cetuximab, Lapatinib, Sunitinib, Bevacizumab, etc., that are assumed to be able to abrogate signals of Raf, VEGF, PDGFR, EGFR, or the ubiquitin–proteasome system. Except Sorafenib that showed a nearly 3-month survival advantage over placebo, limited success has been accomplished for most of the other molecular targeting agents in those randomized clinical trials [38]. Thus, new perspectives that may reshape the existing therapeutic regimens or clinical trials for HCC are urgently needed. While the promise or peril of growth factor receptor-based targeting strategies remains in question, the potential of JNK1 inhibition in molecular targeting warrants extensive investigation to circumvent the limitations of the existing methods or trials in battling HCC.

Encouraging results of JNK inhibition in HCC amelioration have been obtained in some experimental HCC models or HCC cell lines recently. An earlier study using HepG2 cell line clearly indicated that blockage of JNK activation by SP600125, a small molecule inhibitor of JNK, or silencing JNK expression by siRNA, could augment CD95-induced apoptosis and G2/M phase cell cycle arrest [120]. This observation was most recently validated by two independent studies that showed inhibition of JNK amplifies not only CD95-induced, but also TRAIL- or WWOX-induced cell apoptosis in a manner of p53 independency [121,122]. Intriguingly, the anti-apoptotic or tumorigenic effect of JNK was only limited in the cancer cells with no effect on the primary normal hepatocytes. These experimental results may form a basis for a feasible therapeutic option for treating human HCC through JNK inhibition [121]. More compelling evidence showing advantages of JNK targeting in HCC intervention was established by the studies in JNK1 gene knockout mice. As discussed earlier, the HCC incidence and tumor size induced by a chemical carcinogen were substantially reduced in the mice with genetic disruption of jnk1, but not jnk2 gene [17,18]. The HCC development in response to the chemical carcinogen was significantly delayed in the wild-type mice that were given a peptide JNK inhibitor for 3 months [17].

Undoubtedly, the findings implicating pivotal contribution of JNK1 to HCC initiation and progression have offered opportunities in designing new therapeutic strategies. A number of small molecular compounds that either interferes with JNK activation signaling or blocks interaction of JNK with its scaffold proteins or substrates have been developed and used in both bench-top experiments and pre-clinical trials. The first and most commonly used JNK inhibitor is SP600125, an anthrapyrazolone derivative that exhibited high efficacy

in blocking the kinase activity of JNK by competing with the ATP-binding site with an IC_{50} of 40 nM for JNK1 and JNK2, and 90 nM for JNK3 in vitro, respectively [123]. In an animal model, SP600125 has been shown to be able to inhibit inflammatory response and showed promise as a potential therapeutic agent for rheumatoid arthritis and asthma in human [124]. A number of other small molecules with inhibitory effect on JNK have been developed by several pharmaceutical companies through high throughput screening of the small molecule libraries [125]. A common problem for these JNK inhibitors is the poor water solubility and the off-targeting effect on other kinases that limited their suitability in pre-clinical and clinical trials [126,127], despite their reasonable efficacy in JNK inhibition and inflammatory amelioration in cell culture or animal disease models. A cell permeable JIP peptide fused with HIV-TAT peptide had been shown to be capable of disrupting interaction between JIP and JNK to inhibit JNK activity [128]. In mouse HCC model, this peptide JNK inhibitor suppressed HCC induction by chemical carcinogen substantially, suggesting great promise for this type of JNK inhibitor in HCC therapy [17]. The challenges for the clinical use of peptide JNK inhibitor are the peptide instability, potential immunogenicity and nondiscrimination between tumor cells and normal cells.

7. Conclusions

HCC is the third leading cause of cancer-related death and the fourth most common malignant tumor worldwide. The tumor burden of HCC caused considerable loss in Mongolia, Southeast Asia and sub-Saharan Africa for several decades [129]. An alarming sharp increase in HCC incidence in Europe, North American and Japan has been noted in recent years, which is very likely attributed to the epidemic of HCV infection, alcoholic liver diseases or exposure to environmental factors [130]. Because of lack of earlier detection and effective therapeutic regimens, the 5-year survival rate of HCC is extremely poor. It is estimated that more than 600,000 deaths per year occur globally. Recent data revealed a clear correlation between HCC and aberrant JNK1 in the pathogenesis of HCC, including gene expression and prognostic outcomes. Therefore, targeting JNK1 should be viewed as a new avenue for HCC therapy. While current euphoria in molecular targeting for HCC is largely focused on the identified HCC signature genes, there is no clear-cut about which gene or gene sets are truly targetable because of the large quantity of the so-called signature genes. On the other hand, molecular intervention to a specific signaling pathway, such as JNK1, might be highly achievable and clinically feasible. The hurdles need to be tackled in this regard are discoveries of effective JNK1 inhibitors that are relatively specific with a very limited off-targeting effect and can be easily administrated clinically. When such inhibitors are available, a new therapeutic era seems to be close at hand.

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