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Full-length Mst1 exhibits growth promoting function in human hepatocellular carcinoma cells

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ABSTRACT

The putative tumor suppressor Mst1, when cleaved to its 36 kDa cleaved form, amplifies apoptotic signals. We found that Mst1 was predominantly expressed in its full-length form in 76% (17/25 cases) of hepatocellular carcinoma (HCC) tumors. Mst1 cleavage was basically absent in HCC cells. Ectopic full-length Mst1 expression increased the growth of HCC cells by 55–80% within 3 days after transfection. Expression of exogenous NORE1B, a tumor suppressor commonly lost in HCC tumors (~56% of our cohort), was sufficient to suppress the growth promotion of full-length Mst1. Hence, Mst1 exhibits a growth promoting activity in HCC cells upon NORE1B downregulation.

1. Introduction

Recent studies on multiple solid cancers indicate that the Mammalian Sterile 20-like Kinase 1 (Mst1/Stk4) is a putative tumor suppressor [1,2]. Conditional Mst1 ablation studies in mice have already demonstrated the tumor suppressor role of Mst1 against spontaneous liver cancer development [3,4]. Mst1 was originally identified as a proapoptotic cytoplasmic kinase important for amplifying the apoptotic signals when cleaved to its 36 kDa cleaved form by caspases [5]. A recent study by Zhou et al. showed that the full-length Mst1 is the predominant form expressed in HCC patient tumors, while the pro-apoptotic cleaved form is frequently absent [3]. At present, the functional role of this widely expressed full-length Mst1 has not been studied yet and the biologic implication of full-length Mst1 in HCC tumorigenesis remains undefined.

Phosphorylation of Mst1 by Akt has been implicated in limiting the pro-apoptotic activity of Mst1 in human ovarian and prostate cancer cells [6,7]. In addition, the pro-apoptotic activity of Mst1 is also regulated by the RAS Association Family members, RASSF1 and NORE1 (RASSF5) [8]. Due to the frequent absence of RASSF1A/NORE1B expression in HCC tumors [9], it is highly possible that the normal biological functions of Mst1 may have been altered in HCC tumor cells.

In this study, we have reported for the first time that the full-length Mst1 harbors a growth promoting activity in human HCC models. We found that ectopic expression of full-length Mst1 did not induce apoptosis in multiple HCC cell lines. Surprisingly, the full-length Mst1 enhanced the growth of HCC cells particularly in high cell density. Furthermore, we also demonstrated that the absence of NORE1B expression in HCC provided a permissive environment for the full-length Mst1 to promote HCC cellular growth.

Abbreviations: HCC, hepatocellular carcinoma; Mst, mammalian sterile 20-like kinase; Stk, sterile 20-like kinase; PAK, p21 activated kinase; RASSF, RAS Association domain family; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; PARP, poly ADP-ribose polymerase; FL, full length form; NT, amino terminal cleaved form

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2. Materials and methods

2.1. Patients samples

The patient tissue collection and research study protocol was approved by the Joint Chinese University of Hong Kong and Hospital Authority New Territories East Cluster (CUHK-NTEC) Clinical Research Ethics Committee. Twenty-five tumor and adjacent normal liver tissue pairs were randomly selected from our primary HCC tissue collections. Clinical characteristics of the selected samples are listed in Supplementary Table 1.

2.2. Chemicals and plasmids

Mst1 and its kinase dead mutant (Mst1KD) expression plasmids were obtained from Addgene (Cambridge, MA, USA) and NORE1B mammalian expression plasmid was from Origene (Rockville, MD, USA). Mst1 full-length cDNA was subcloned with the hemaglutinin (HA) tag into pcDNA3.1 (Invitrogen, Carslbad, CA, USA). Myc-tagged Mst1 mammalian expression plasmid was a gift from Dr. Yukiko Gotoh (University of Tokyo, Tokyo, Japan). Cell culture reagents were obtained from Invitrogen (Carslbad, CA, USA). General chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA).

2.3. Mst1 mRNA expression meta-analysis

Cancer microarray meta-analysis was performed on multiple HCC RNA expression datasets using the Nextbio Public software (https://www.nextbio.com/b/nextbio.nb).

2.4. Cell culture and transfection

HEK293T cells (ATCC, Manassas, VA, USA), HCC cell lines (Hep3B (ATCC, Manassas, VA, USA), PLC/PRF/5(ATCC, Manassas, VA, USA), Huh-7 (JCRB Cell Bank, Japan)), and immortalized normal human hepatocytes MIHA (Brown et al., 2000) [10] were cultured in DMEM + 10% FBS with penicillin and streptomycin. The gastric cancer cell line AGS (ATCC, Manassas, VA, USA) was cultured in RPMI1640 + 10% FBS with penicillin and streptomycin. Transfection was done with Lipofectamine 2000 (Invitrogen, Carslbad, CA, USA) according to the manufacturer's instructions. For stable clone selection, the transfected cells were selected and maintained with 700 μg/ml G418.

2.5. Western blotting

Aliquots of cell/tissue lysates containing 50 μg protein were resolved on 10% SDS–PAGE and transferred to nitrocellulose membranes. Detailed procedures can be found in the Supplementary Methods.

2.6. Cell viability assays

Detailed procedures have been described in the Supplementary Methods.

2.7. Statistical analysis

Data were expressed as mean ± S.E.M. Statistical analyses were performed with GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego California USA). Statistical significance in each experiment was determined by unpaired *t* test for two group comparison, or one-way ANOVA followed by Dunnett's test/Bonferroni's test for multiple group comparison, with a 95% confidence level (i.e., $P < 0.05$).

Other methods are described in the Supplementary Method.

3. Results

3.1. Mst1 mRNA is upregulated in HCC tumors and cell lines

To investigate the role of Mst1 in HCC, we first examined Mst1 mRNA expression by meta-analysis of published RNA expression microarray data from three published studies on HCC: (1) Chen et al. (2002) (76 adjacent normal liver tissues and 104 HCC liver tissues; disease samples were HBV and/or HCV associated samples) [11], (2) Wurmbach et al. (2007) (10 normal, 35 HCC, and 13 cirrhotic livers; disease samples were HCV-associated) [12], and (3) Mas et al. (2008) (19 normal livers, 38 HCC livers, and 58 cirrhotic livers; disease samples were HCV-associated) [13] (Table 1). Our analysis showed that Mst1 was upregulated in HCC and in cirrhotic livers by ~1.3 to 1.75-fold. RT-PCR showed that increased Mst1 mRNA was increased on average by 1.59 ± 0.52 folds in 15/16 tumor samples (except for Patient 6) from our patient tumor cohort (Supplementary Fig. 1). Upregulation of Mst1 mRNA expression was also detected in 3 HCC cell lines with various p53 and HBV genomic integration statuses [14]: Hep3B (HBV genome integrated, p53-null), PLC/PRF/5 (HBV genome integrated, p53 mutated), Huh7 (HBV negative, p53 mutated) when compared with normal liver tissues (Supplementary Fig. 2).

3.2. Full-length Mst1 is the predominant form in HCC patient tumors

Next, we examined the expression of Mst1 protein in human HCC tumors by Western blotting using an antibody which can detect both the full length (FL) and the proapoptotic 36 kDa cleaved form (NT) [3] (Validation was done with HEK293T fibroblasts (293T) overexpressing Mst1 as shown in Supplementary Fig. 3). Full-length Mst1 protein was overexpressed in 60% of the HCC patient tumor tissues (15 out of 25 samples) (Fig. 1). Similar to the previous findings of Zhou et al. (2009), 17 sample pairs (76% of

Table 1
Upregulation of Mst1 RNA in cirrhotic livers and HCC livers revealed by RNA expression array meta-analysis.

RNA expression dataset	GEO series	Mst1		References
	Accession No.	Fold change	*P* value	
HCC from HBV^+ patient vs. adjacent non-tumor tissue	GSE3500	1.37	0.0122	[11]
Liver from patients with HCV cirrhosis vs. normal liver	GSE14323	1.66	1.4×10^{-15}	[13]
Liver from patients with HCV HCC and cirrhosis vs. normal liver	GSE14323	1.75	3.8×10^{-7}	[13]
Cirrhotic liver without HCC vs. normal liver	GSE6764	1.43	0.002	[12]
Low-grade dysplastic liver tissue vs. normal liver	GSE6764	1.28	0.0217	[12]
Liver from very early HCC patients vs. normal liver	GSE6764	1.4	0.002	[12]
Liver from early HCC patients vs. normal liver	GSE6764	1.56	0.0043	[12]
Liver from advanced HCC patients vs. normal liver	GSE6764	1.58	0.0005	[12]
Liver from very advanced HCC patients vs. normal liver	GSE6764	1.54	0.0092	[12]