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A study to investigate the expression of nuclear Met and its clinical significance in HCC

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Abstract

In the world, hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality and the fifth most common cancer overall. As long as the prognosis for HCC remains poor, better therapeutic options will be available if the underlying mechanisms contributing to hepatocarcino-genesis can be elucidated. Human tumours are drawn to the Met/HGF signalling pathway because it has been linked in several types of cancer, including HCC. Current clinical trials are testing treatment techniques that target the Met surface receptor. Because receptor tyrosine kinase signalling has long been believed to occur at the membrane surface, the discovery of Met inside the nucleus raises the possibility of a new "signalling shortcut."

Using three key objectives as a focus, we aimed to provide a thorough knowledge of nuclear Met's role in HCC tumorigenesis and development, as well as the underlying signalling network mediated by nuclear Met in this study.

Keywords: HCC, HGF, hepatocarcinogenesis, tumourigenesis

Introduction

A primary liver "cancer, of which 85-90 percent is hepatocellular carcinoma, is the third leading cause of cancer death worldwide and the fifth most common cancer in people (El-Serag et al., 2007). In underdeveloped nations, such as Southeast Asia and Sub-Saharan Africa, where infection with hepatitis B virus (HBV) is common, most of the burden of HCC is documented (El-Serag, 2011). HCC was the fourth most common cancer and the third major cause of cancer death in Hong Kong in 2012, according to cancer data. HCC incidence and mortality rates are three times higher in men than in women in Hong Kong, a finding that may be explained by the role played by sex hormones" in cancer formation (Yu et al., 2003).

Hepatitis B and C virus (HBV and HCV) chronic infection, alcoholism, and nonalcoholic fatty liver disease are all known risk factors for HCC development. Most HBV and HCV infections result in cirrhosis, but even without cirrhosis, they can still induce HCC. It's possible that alcoholism and nonalcoholic fatty liver disease, which are linked to both diabetes and obesity, have something to do with the onset of hepatocellular carcinoma (El-Serag et al., 2007; El-Serag, 2011). For HCC, a slew of preventative strategies have been devised. In Asian nations like "Taiwan, where the prevalence of HCC has decreased due to universal HBV vaccine delivered to newborns, the most beneficial technique has been found (Chang et al., 1997). While in western regions and Japan, the HCC incidence rate has doubled in the last two decades due to increased HCV infection. This has resulted in an intensified effort to avoid HCV infection in these" locations.

Literature Review

Metabolic regulation of Met activity has long been an important therapeutic goal for many receptor tyrosine kinase diseases (Gherardi et al., 2012). HGF antagonists, which interfere with recognition by competing with ligands for the Met receptor, have turned the interaction between Met and HGF into their principal target. "Several HGF/Met biological antagonists, such as decoy Met, uncleavable HGF and Sema, prevent the activation of Met, hence inhibiting biological responses such as invasion, angiogenesis and migration both in vitro and in vivo (Mazzone et al., 2004; Michieli et al., 2004; Kong-Beltran et al., 2004). Antibodies that neutralise Met or HGF, such as DN30 and L2G7, limit cell and xenograft growth in a similar

manner (Kim et al, 2006; Petrelli et al., 2006). Met kinase activity has been attempted to be blocked by small-molecule Met inhibitors such as SU11274 and PHA665752 (Sattler et al., 2003; Christensen et al., 2003). Several small-molecule inhibitors have been studied in vitro and briefly in vivo, showing considerable promise in the prevention of cancer cell proliferation, migration, and invading" abilities However, only a small number of clinical trials using Tivantinib (ARQ197), SGX523, and Crizotinib (PF2341066) have been conducted, and the results are still ambiguous.

Even while these proteins usually create essential signalling responses when stimulated when membrane-bounded, research has shown an ever-expanding number of RTKs residing in the nucleus. The fact that RTK was being transported to the nucleus raised the question of what role they "would play in an unusual cellular compartment. These proteins were eventually found to translocate to the nucleus as whole receptors or a shortened fragment, initiating transcription of target genes. In most RTKs, the ectodomain is shed, releasing an intracellular domain (ICD) that is then localized in the nucleus (Wells et al., 2002; Krolewski, 2005); this is known as ectodomain" shed.

Research Gap

In different cell contexts and human tissues, scarce investigations have reported the occurrence of a cytoplasmic Met fragment in the nucleus, although nuclear Met's functional relevance has only been demonstrated in a limited way We found in Chapter 3 that nuclear Met expression in HCC was associated with an increased risk of the cancer progressing to metastasis, which was corroborated by our complete clinic pathological investigation. In this chapter, we used in vitro and in vivo models to show conclusively that nuclear Met has a functional role in HCC. "Nuclear Met overexpression stable clones were produced in non-metastatic HCC cell lines SMMC7721 and BEL7402, as well as the metastatic cell line MHCC97L, to functionally evaluate the protein in HCC. In the juxtamembrane region of Met, the N-terminal deletion positions at D972 (J1) and P1027 (J3) as well as L1157 (T2) in the tyrosine kinase domain were created as three constructs, referred to as juxtamembrane deletion 1 (J1), juxtamembrane deletion 3 (J3) and tyrosine kinase deletion 2 (T (Figure 4.1). Doxycycline was used to tightly control the expression of these constructs after they were subcloned into an inducible Myc-tagged expression vector (Figure 4.2, 4.3 and 4.4). We were able to induce the necessary nuclear Met product expression for a follow-up functional research using 2 g/ml doxycycline for" 24 hours.

Research Objective & Methodology

Themajoraimsinour study:

- To find out if nuclear Met is expressed in HCC and if it has any clinical significance.

We expect to uncover the functional consequences and mechanical nature of nuclear Met in HCC by fulfilling these objectives, which will set it apart from its full-length companion and reveal new information. As a result of these findings, future studies will be able to build on them to uncover even more new functions for nuclear Met in cancer cells.

There were generous gifts from "the Department of Surgery at The University of Hong Kong (MIHA and MHCC97L), the Shanghai Institute of Cell Biology at the Chinese Academy of Sciences in the People's Republic Of China (BEL7402 and SMMC7721), and the Japan Research Bioresources Collection (JRCB, Japan) for HEK293FT, PLC, and MHCCLM3 cell lines from the American Type Culture Collection in Manassas, Virginia, USA (HLE). All cells were grown at 37°C in a humidified incubator with" 5% CO2 for the entire time.

Before becoming fully confluent, all cell lines were passaged. Incubation at 37°C for 5 minutes was followed by rinsing adherent cells with "phosphate-buffered saline (PBS) and trypsinizing them with a suitable

volume of 0.05 percent trypsin (0.05 percent EDTA and 10.6 g/ml phenol red). Overtreatment of trypsin can induce cell death, thus isolated cells were suspended in fresh culture media to inactivate the enzyme before further use in the study. Particle Counter (Beckman Coulter, Pasadena, CA, USA) was used to count the desired number of cells in accordance with the manufacturer's instructions" and the seeds were then transferred to the suitable culture plate (TPP, Trasadingen Switzerland) for further culture or testing.

For the introduction of foreign "plasmid DNA into the cells, transfection was carried out according to the manufacturer's protocol with Lipofectamine 2000 (Invitrogen Gaithersburg, MD, USA) or FuGENE®6 (Roche Basel, Switzerland) transfection reagent. Transfection reagent was diluted in serum-free culture media at a 1:2.5 ratio with the desired amount of DNA. Before applying the mixture to the cells, it was incubated for 20 minutes at room temperature. A minimum of 24" hours after transfection, transfected cells were collected and exposed to a variety of tests.

103 Chinese patients with primary HCC had their tumours surgically removed at Queen Mary Hospital in Hong Kong, and paired samples of tumour and normal liver tissue were acquired. Cases were chosen at random for the investigation. Tissue samples were formalin-fixed, paraffin-embedded, and stained for histological analysis after being sectioned. Because the use of human materials was approved by both HKU/HA HKW IRB and the Hospital Authority Hong Kong West Cluster's institutional review board (IRB).

Data Analysis & Findings

The fraction of the nucleus that stained positively for Met was recorded as a constant. "Cutoff point was considered to further classify the cases into high and low nuclear Met expression groups in order to obtain as much information as feasible. Tumor size, tumour nodule count, number of tumour encapsulations, Edmondson grading, pTMN staging, serum Hepatitis B surface antigen status, venous invasion without differentiation into portal or hepatic venules, tumour microsatellite formation, and direct liver invasion were all associated with nuclear Met expression. These associations were analgesic. Grading tumours according to their degree of cellular differentiation is done using the Edmonson-Steiner system (Edmonson and Steiner, 1954), which was established by Edmondson and Steiner. An correlation was found between nuclear Met expression and both overall survival (death regardless of recurrence status) and disease-free survival (recurrence or death as an endpoint). The Kaplain Meier technique was used to process the survival rate data, and the log-rank test was employed to assess if the two groups differed. IBM SPSS Statistics 19 (SPSS Inc., Chicago, IL, USA) was used to conduct all statistical analyses. Statistical significance" was defined as a P-value less than or equal to 0.05.

Thermo Scientific, Waltham, MA, "USA, used NE-PER® Nuclear and Cytoplasmic Extraction Reagents (according to the manufacturer's protocol) to extract cellular fractions from cells. Trypsin was used to extract adherent cells, and the cells were then washed in PBS. After a five-minute centrifugation at 500 g, the supernatant was discarded. Once the cell pellet was cooled, it was immersed in ice-cold CER I for 15 seconds before being vortexed. It was incubated on ice for 10 minutes before adding CER II, which was vortexed on high for 5 seconds before being incubated on ice for an additional 1 minute. Five minutes of maximum speed centrifugation at 4°C yielded a cytoplasmic extract, which was collected in a clean, pre-chilled tube. Insoluble pellets were rinsed" twice with PBS before being suspended in ice-cold NER and vortexed for 15 seconds on the highest setting. The suspension was incubated on ice for 40 minutes at 4°C with 15-second vortexing intervals every 10 minutes. Finally, the suspension was centrifuged for 10 minutes at high speed before the supernatant (nuclear extract) was transferred to a fresh, previously refrigerated tube for further analysis (see below). The protease and phosphatase inhibitor cocktails were added to both CER I and NER before extraction. The nuclear and cytoplasmic lysates were kept at -80°C for potential future use.

SDS-PAGE was performed "on protein samples previously prepared with the Mini-PROTEAN® II device using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Laboratories). SDS-polyacrylamide separation gel with a 10% acrylamide concentration was used to separate protein samples. The molecular weight was calculated using the protein marker KaleidoscopeTM (Bio-Rad Laboratories). In 1 protein running buffer, SDS-PAGE was carried out at a constant voltage of 100 V for 10 minutes and then 160 V for an hour (25 mMTris-base, 192 mM glycine" and 0.1 percent SDS).

We used an AmershamHybond-P PVDF membrane (Little Chalfont, UK) to transfer the resolved protein from the gel to the membrane after running phase. The membrane was pre-wetted in 100 per-cent methanol and equilibrated with 1 protein transfer buffer before usage. Two filter sheets and filter pads were used to sandwich the separating gel and membrane before being combined into a plastic cassette. Using a transfer tank, the cassette was submerged in protein transfer buffer for an hour, with the voltage held at 100 V.

The AperioScanScope CS "System was used to analyses high-quality digital pictures for analysis after IHC was done on formalin-fixed, paraffin-embedded liver tissue sections. A 5 m tissue piece was deparaffinized in xylene, and it was then rehydrated using an alcohol gradient and distilled fluids. The portion was heated for 15 minutes at 100°C in antigen retrieval solution to remove antigens (10 mM citrate buffer, pH 6.0, 1 mM EDTA, pH 8.0). The endogenous peroxidase was blocked with 10% hydrogen peroxidase (H2O2) in TBS for 20 minutes before using 10% normal goat/mouse/rabbit serum for 30 minutes to complete the experiment. Prior to secondary antibody incubation, specimens were placed in a freezer at 40°C. Sections were washed three times with TBST and then incubated with a secondary antibody for 30 minutes at room temperature after the primary antibody had been removed. Finally, hematoxylin and eosin was used to counterstain" the sections, and the DAKO EnVisionTM system (based in Carpinteria, California, USA) was used to display the signal.

Cells sown in 6-well culture plates were homogenized with one ml of Invitrogen's TRIzol® reagent (Invitrogen) and "incubated at room temperature for five minutes. 200 l of chloroform was added to the homogenized mixture in a micro centrifuge tube, and the liquid was vortexed violently for 15 seconds to ensure thorough mixing. The tube was incubated at room temperature for three minutes before being centrifuged at 4°C for 15 minutes at maximum speed. In order to preserve the pinkish bottom phase, only the aqueous phase (which contained RNA) was moved to a new micro centrifuge tube. The RNA was precipitated at 4°C using 500 l isopropanol and 10 minutes of high-speed centrifugation. In this experiment, the supernatant was removed and the RNA pellet was washed twice with 70 percent ethanol before being used. At 4°C, the supernatant was removed and the pellet air-dried for 10 minutes before being suspended in DEPC-treated water after centrifugation at maximum speed. Assays were performed with BioDrop LITE" to determine the amount and quality of RNA, utilizing absorbance measurements at wavelengths of 260 nm (A260/A280 ratio = 2.0), as well as a single-stranded RNA concentration of 1OD260 = 40 g/ml (Biochrom Ltd., Cambridge, UK). Up to its intended usage, the RNA was kept cold by being refrigerated at -80°C.

One microgram of RNA and two microliters of the SUperScript ® VILOTM Master Mix (Invitrogen) were combined in a volume of 10 microliters to create the reaction mixture. Using a 25°C/10 min, 42°C/1hr, 85°C/5min cycle, and reverse transcription was carried out. While waiting for its first usage, complementary DNA was kept at -20°C in a freezer.

It was decided to create three Met cytoplasmic segments, each with an N-terminal truncation (J1, J3, and T2). In SMMC7221, BEL7402, and MHCC97L HCC cells, a Myc-tagged nuclear Met overexpression stable clone was effectively created. With the help of the Myc tag, scientists were able to discriminate between foreign and native nuclear Met proteins. Western blotting was used to determine whether or not

doxycycline stimulation of nuclear Met expression was working as expected. When doxycycline (1 g/ml) was added, nuclear Met expression could usually be detected 4 hours later and increased with time. Doxycycline induced nuclear Met expression but only at a certain dose. Increasing the concentration of doxycycline resulted in greater expression. After 24 hours of treatment, the lowest dose of doxycycline, 0.25 g/ml, was able to induce nuclear Met expression. Despite this, even after removing doxycycline for 144 hours, nuclear Met expression was still observed. Based on these findings, a 24-hour doxycycline treatment at a concentration of 2 g/ml was chosen to ensure nuclear Met expression throughout the functional assays.

Cellular fractionation and western blotting were used to determine the subcellular distribution of nuclear Met in the previously produced stable "clones. Stable clones had J1 and J3 discovered in the nuclear lysate, whereas T2 was only found in the cytoplasmic fraction of the cells tested. The presence of increased Met expression in the nucleus was confirmed further using immunofluorescence microscopy. Both J1 and J3 were found to accumulate in the nucleus, but T2 was excluded from the nucleus of stable clones by immunofluorescence labelling. Since HGF activates Met, we wondered if HGF" had an impact on nuclear Met expression and subcellular location. HGF administration had no influence on Met's nuclear localization in SMMC7721 cells transfected with nuclear Met constructs. Nuclear Met was also examined to see if it shuttled back and forth between the cytoplasm and nucleus on a regular basis we used LMB, a CRM1 nuclear exporter inhibitor, to treat the cells to find out. In this study, it was discovered that LMB had no impact on Met's subcellular location.

An experiment for BrdU incorporation was carried out on nuclear Met overexpression stable clones to determine their proliferative potential. There were substantial increases in the proliferation rate of construct J1 only in the stable clones "developed in SMMC7721, BEL7402 and MHCC97L when compared to vector controls. BrdU incorporation assay was repeated with SMMC7721 and BEL7402 stable clones treated with or without doxycycline to confirm that the promoting effect seen in J1 stable clones was caused by the induced expression of J1. A higher proliferation rate in the doxycycline-treated J1 clone, as well as in the vector control clone with doxycycline, indicates that inducing J1" expression is the only factor that promotes cell proliferation.

Soft agar experiment was used to examine the anchorage independent growth ability of stable clones. SMMC7721 and BEL7402 stable J1 developed considerably more colonies than vector control J3 and T2. To demonstrate that the induced J1 expression was indeed responsible for the growth promotion observed in anchorage independent SMMC7721 and BEL7402 stable clones treated with or without doxycycline, a soft agar assay was carried out once more. This shows that J1 expression promotes cell anchorage independent growth in the J1 stable clone treated with doxycycline, as well as in the vector control stable clone treated with the drug.

Conclusion

According to research, several nuclear-localized receptor tyrosine kinases have a role in physiological responses. Several carcinomas had nuclear Met, a shortened version of full-length Met, whereas HCC did not. Met/HGF signalling played an important part in HCC tumorigenesis, invasiveness, and metastasis, thus we decided to look into whether or not nuclear Met was present in the tumour cells of patients with HCC. When this nuclear Met is found in HCC, we hypothesised it could play a similar role to the full-length Met. Perhaps it indicates a new way for carcinoma cells to progress via signalling modulation. This nuclear Met study is enticing because it will focus on "three major aspects: the profile and clinical relevance of nuclear Met expression in HCC; the functional implications for HCC tumourigenesis and progression; and finally, the

underlying mechanisms mediated by this nuclear Met study will help us answer our questions.

Overexpression of Nuclear Met in HCC tumorous tissues compared to non-tumorous tissues was found. The expression of this gene was found to rise with the progression of HCC from non-tumorous liver to cirrhotic liver, and finally early and advanced HCC. Nuclear Met overexpression has also been linked to venous invasion in HCC patients, as well as a worse prognosis. This led" us to conclude that nuclear Met is just the cytoplasmic fraction of full-length Met, as only Met antibodies targeting the cytoplasmic domain but not the extracellular domain could be used to identify it. Immunofluorescence microscopy followed by cell fractionation demonstrated the presence of this ligand-independent, 48-kDa fragment of Met within the nucleus there was also a region before to the tyrosine kinase domain (P1027-L1157) that is critical for the nuclear translocation of the cytoplasmic portion of Met, which we discovered.

The overexpression technique "was used to describe the nuclear Met in HCC, and three N-terminally shortened cytoplasmic Met fragments were created (J1, J3 and T2). In HCC cell lines, we used the doxycycline inducible expression technique to create nuclear Met overexpressing stable clones. In vitro functional assays and in vivo tumorigenicity experiments were carried out on the stable clones that had been established. J1 promoted cell proliferation, anchorage independent growth, motility, and invasiveness in vitro, as described in Chapter 4. Furthermore, J1 overexpression boosted the cancer-causing potential of human colorectal cancer (HCC) cells in vivo. Our" findings are the first to demonstrate that nuclear Met has an oncogenic role in HCC.

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