Xi'an ShiyouDaxueXuebao (ZiranKexue Ban)/
Journal of Xi'an Shiyou University, Natural Sciences Edition
ISSN:1673-064X
E-Publication:Online Open Access
Vol: 66 Issue 02 | 2023
DOI 10.17605/OSF.IO/AYNM8

THE SIGNALING NETWORK THAT UNDERLIES NUCLEAR MET'S ACTIONS IN THE NUCLEUS AND IS MEDIATED BY THE PROTEIN NUCLEAR MET

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Abstract

The expression of "nuclear Met was investigated in both human HCC cell lines and tissue samples from the same patient. It has been demonstrated that nuclear Met is over expressed in HCC tumorous tissues compared to the comparable non-tumorous tissues. Furthermore, it was shown to be expressed more often along the course of HCC development, from non-tumorous liver through cirrhotic liver to early and advanced HCC. Furthermore, increased Met over expression in the nucleus was found to be linked with venous invasion and a shorter overall survival in HCC patients. We then confirmed that nuclear Met, which has a size of approximately 48 kDa and is composed solely of the cytoplasmic fragment of full-length Met, by demonstrating that it could only be detected by a Met antibody directed against the cytoplasmic domain of Met and not the extracellular domain of the protein. Later cellular fractionation and immune fluorescence microscopy revealed that this 48 kDa fragment of Met was present in the nucleus and that it was not dependent on the presence of a ligand for its presence. In addition, we discovered that a region before the tyrosine kinase domain following the juxta membrane region (P1027-L1157) is required for the translocation of nuclear Met to the nucleus. It was necessary to create nuclear Met over expressing stable clones in order to functionally describe the nuclear Met in HCC. These stable clones were subjected to a variety of functional investigations. We have established that nuclear Met has a proliferative effect on in vitro cell proliferation, anchorage independent growth, motility, and invasiveness in a number of experiments. Furthermore, over expression of Met in the nucleus of HCC cells increased the tumorigenicity of the cells in vivo. Our findings provided the first convincing evidence of a tumorigenic role for nuclear Met in HCC, and" they were published in Cancer Research.

Keywords: HCC, HGF, Hepatocarcinogenesis, Tumourigenesis, Genetic Changes.

1. INTRODUCTION

HCC formation is a "multistep process that involves the accumulation of genetic changes over a period of 20 to 40 years, depending on the individual. The vast majority of HCC cases are discovered at an advanced stage since early HCC is usually asymptomatic, making early detection and therapy impossible (Yang et al., 2010). As a result, the therapeutic options for HCC are restricted. Patients with HCC who do not have cirrhosis may be candidates for surgical resection, whereas liver transplantation can treat the illness without compromising liver function in patients with cirrhosis (Forner et al., 2012). Sorafenib is the only multikinase inhibitor approved for the treatment of advanced HCC

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patients with a prolonged survival span of less than 6 months (Llovet et al., 2008).

Following liver damage induced by a variety of causes, a continual process of necrosis followed by proliferation of hepatocytes resulted in chronic liver disease, which eventually progressed to liver cirrhosis and liver failure. The formation of abnormal liver nodules has begun, and hyperplastic nodules have been detected. In the following years, the hyperplastic nodule progressed to become a dysplastic nodule and, eventually, a hepatocellular carcinoma, which may be categorized according to its differentiation status. In advanced stages, the primary tumour may have the ability to spread to other organs and invade them.

Met was discovered as a novel transforming gene in human osteosarcoma cell (HOS) lines that had been exposed to the chemical carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Cooper et al., 1984a). Following the molecular cloning of this MNNG- HOS transforming gene, it was discovered that the gene was located on chromosome 7, and the term Met was proposed to refer to this oncogenic gene as a result (Cooper et al., 1984b). A second round of nucleotide sequencing revealed that Met was more closely related to the tyrosine kinases than it was to the serine/threonine kinases, indicating that Met was more directly related to them (Dean et al., 1985) In addition, successful protein characterization showed that Met belongs to the tyrosine kinase family of growth factor receptors for an as-yet-unidentified ligand at this time (Part et al., 1987; Giordano et al., 1989b). It was later discovered that the activation of the Met oncogene, which had previously been described in MNNG-HOS, was the consequence of a genetic fusion with a translocated promoter region (TPR) located on chromosome 1, rather than a genetic" fusion with a translocated promoter region (TPR) (Park et al., 1986).

Met is synthesised as a 170 kDa single chain precursor protein that is cleaved by furin (Komada et al., 1993), resulting "in a cell surface single-passed heterodimer composed of a glycosylated extracellular subunit that is linked to a transmembrane subunit by a disulfide bond (Gonzatti-Haces et al., 1988; Gior (Figure 1.2). Met's extracellular region is composed of three functional domains: a sema domain that encompasses the entire subunit and a portion of the subunit and is important for ligand binding; a cystine-rich domain that contains four conserved disulfide bonds; and four immunoglobulin-like fold (IPT) domains that act as a "stalk" structure to hold the propeller domain in the correct orientation for ligand binding. Met's extracellular (Gherardi et al., 2003; Trusolino et al, 2010). There are three segments in the intracellular region, which are as follows: a juxtamembrane sequence that is involved in the downregulation of kinase activity following phosphorylation of Ser975; a kinase domain that contains a catalytic region where phosphorylation at Tyr1234 and Tyr1235 positively modulates kinase activity; and a carboxyl-terminal" bidentate docking site that contains two docking ty (Ponzetto et al., 1994).

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2. LITERATURE REVIEW

For the creation of nuclear met "over expression stable clones, the non-metastatic HCC cell lines SMMC7221 and BEL7402, as well as the metastatic HCC cell line MHCC97L, were utilised. pLVX-Tet-On In order to enable tight regulation of nuclear Met expression under doxycycline treatment, an advanced regulator vector was initially introduced into specific HCC cell lines before the expression vector. Co-transfection of 293FT cells with Lenti-Pac HIV mix resulted in the production of recombinant lentivirus using the pLVX-Tight–Puro–Myc–nMet constructs (J1, J3 and T2; Figure 4.1). For the nuclear Met overexpression stable clones, the pLVX-Tight-Puro-Myc vector was employed as the control vector, while the pLVX-Tight-Puro-GFP vector was utilised for the doxycycline treatment control vector. Transduced cells were chosen using a growth media supplemented with Geneticin (700 g/ml) or Puromycin (1 g/ml), respectively. Western" blotting was used to evaluate the expression of Met in the nucleus of resistant clones.

Stable clones were treated with 2 g/ml "doxycycline for 24 hours prior to the test to stimulate nuclear Met expression in the cells. It was decided to undertake cellular fractionation and immunofluorescence of stable clones in order to investigate the localisation of nuclear Met in the stable clones. It was also determined whether stable clones were treated with HGF (20 g/ml) or Leptomycin B (10 g/ml), an inhibitor of CRM1, a protein needed for nuclear export, to determine the effect on the subcellular localization of nuclear" Met.

The incorporation of "bromodeoxyuridine (BrdU) into the adhering stable clones was measured in order to determine the proliferation rate of the clones. Stable clones were treated with 2 g/ml doxycycline for 24 hours prior to the test to stimulate nuclear Met expression in the cells. 3 103 cells per well of a 96-well plate were planted in each well and grown in matching media supplemented with 10% FBS for 24 hours. After 24 hours, the cells were submitted to a BrdU incorporation test (Roche), which was performed in accordance with the manufacturer's instructions. All tests were carried out in duplicate, and three separate experiments were carried out in total.

An experiment using soft agar was used to determine the anchorage independent growth capacity of stable clones. Stable clones were treated with 2 g/ml doxycycline for 24 hours prior to the test to stimulate nuclear Met expression in the cells. Soft agar-supplemented culture media was used to seed cells with a cell count of 2 104 per well. The number of colonies produced after 4 weeks of incubation was determined using a light microscope. All tests were carried out in duplicate, and three separate" experiments were carried out in total.

For cell migration and invasion "experiments, the Transwell® Permeable Support assay (Corning) and the Transwell® Permeable Support assay covered with BD MatrigelTM Basement Membrane Matrix (BD Biosciences) were used (Corning) and (BD Biosciences), respectively. Stable clones were treated with 2 g/ml doxycycline for 24 hours prior to the test to stimulate nuclear Met expression in the cells. For each stable clone, the number of cells and the chemoattractant employed were recorded and reported

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in Table 4.1. After 16 hours and 20 hours of incubation, the cells that had migrating or invaded were fixed and stained, accordingly. Four fields per well were picked at random, and the number of migratory or invaded cells was counted in each field and throughout the well. All tests were carried out in duplicate, and three separate experiments were carried out in total.

Subcutaneous inoculation of stable clones and orthotopic liver implantation in nude mice were used to examine the tumorigenicity and metastatic potential of stable clones. The results showed that stable clones were more tumorigenic" and more metastatic than wild-type animals. Stable clones were "treated with 2 g/ml doxycycline for 24 hours before to the test to promote nuclea r Met expression in the cells. Male BALB/C nude mice were given subcutaneous injections of stable clones (SMMC7721: 2.5 x 10 6; BEL7402: 2.5 x 106; MHCC97L: 1.5 x 106) suspended in 100 l PBS and injected into the flank of the animals at 4 weeks of age. To assess tumour growth, mice were killed at 4-week post-inoculation for MHCC97L stable clones and at 6-week post-inoculation for SMMC7721 and BEL7402 stable clones, respectively. Tumors were meticulously removed and their weights were recorded. Nude mice were implanted with orthotopic livers made from subcutaneous tumours that had been harvested. According to earlier findings, tumours were chopped into 1 mm3 cubes and placed in the liver lobes of 6-week-old nude mice, who were then sacrificed (Yam et al., 2006). Animals were killed 4 - 6 weeks after implantation, depending on the experiment. The organs of the liver and lungs were removed and scanned. For histological inspection, the organs were fixed in 10 percent formalin, followed by 70 percent ethanol, before being embedded in paraffin for preservation. Animals were administered doxycycline (100 g/ml) and sucrose (1 percent w/v) containing drinking" water throughout the experiment in order to constitutively stimulate the expression of nuclear Met in the implanted tumours for the duration of the experiment.

3. RESEARCH GAP

This signalling pathway "interacts with NF-B signalling through signalling intermediates, the PI3K-Akt pathway, and Src to activate IKK and increase cell proliferation, survival, and tubulogenesis, among other things (Fan et al., 2005; Muller et al., 2002). In addition, HGF stimulates NF-B, which regulates HIF-1 activity, which is important for cancer cell survival (Tacchini et al., 2004). Our findings, which were consistent with those of its full-length counterpart, clearly revealed that nuclear Met facilitated NF-B signalling, as evidenced by the increased luciferase activity of the pNF-B reporter upon expression of nuclear Met (Figure 5.1).

When the NF-B signaling pathway was initially identified, it was thought to have a critical function in immune system response. However, it was subsequently shown to play a role in tumor growth, namely through suppression of apoptotic cell death and enhanced metastasis and angiogenesis (Karin et al., 2002). NF-B activation occurs when cells are activated by stimuli such as tumor necrosis factor-alpha (TNF-alpha), which activates the

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inhibitor of NF-B (I-B) kinase (IKK) complex, which in turn activates the nuclear factor-B (NF-B) pathway. Because of its catalytic activity, the IKK complex is primarily responsible for phosphorylating the IB and targeting it for ubiquitin-dependent destruction. Following this, released NF-B dimers, comprised of p65 and p50, translocate into the nucleus and bind to DNA B sites in the promoters and enhancers of target" genes, resulting in their transcriptional activation (Hayden et al., 2012).

Our research focused on "two critical effector molecules, IKK and p65, in order to further demonstrate that nuclear Met was involved in the activation of NF-B signalling. The stimulating impact of nuclear Met on the migration and invasion of HCC cells was eliminated by decreasing the activities of IKK and p65 by treatment with IMD-0354 and temporary knockdown by siRNA, respectively (Figure 5.2 and 5.3). Taken together, we were confident that nuclear Met mediated NF-B signalling was involved in increasing HCC cell motility and invasiveness, which might be implicated in the development of HCC later on in the process.

We went on to investigate the downstream targets that may be implicated in the regulation of NF-B signalling by nuclear Met. We discovered that to examine the mRNA expression of six downstream target genes of NF-B signalling, including the genes IL8, MMP-2, MMP-9, SNAIL, SLUG, and VEGFA, we used real-time quantitative PCR. These genes have been implicated in cancer metastasis and angiogenesis, and our results were published in Cancer Research. Cancer development is characterised by the advancement of epithelial to mesenchymal transition (EMT), and NF-B signalling has been identified as the primary mediator of this transition (Min et al., 2008). SNAIL, SLUG, MMP-2, and MMP-9 were all essential players in the EMT game, and NF-B signaling regulated the expression of these proteins to cause complete EMT. Min et al. (2008) found that the expression of SNAIL and SLUG, two zinc-finger transcription factors, can suppress epithelial phenotype and increase cell migration and invasion. MMP-2 and MMP-9, on the other hand, are type IV collagenases that degrade the basement membrane, allowing for tumor invasion and metastasis (Himelstein et al., 1997). As a result of its regulation of an enzyme called membrane type metalloprotease (MT-MMP), NF-B indirectly regulates MMP-2 activity. This enzyme is responsible for cleaving the N terminus of pro-MMP-2, resulting in active MMP-2 (Han et al., 2001). Another essential characteristic of tumorigenesis is the presence of ongoing angiogenesis, and both IL8 and VEGFA play a role in the induction of angiogenesis. It has been demonstrated that activation of the "nuclear factor-B (NF-B) induces the production of IL8 and VEGF, both of which are known to increase" angiogenesis (Huang et al., 2000). Using real-time qPCR, we were able to identify the MMP-2 gene as a potential increased downstream target that confers aggressiveness to HCC" cells in response to nuclear Met expression in our study.

Collectively, our findings provided the "first evidence that nuclear Met-mediated NF-B signaling in HCC cells is involved in the promotion of cell motility and invasiveness. Inhibition of IKK and p65 activity was shown to be effective in removing the encouraging impact of nuclear Met on the aggressiveness of HCC cells. We also discovered MMP-2

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as a potential downstream target of nuclear Met-mediated NF-B signaling, and that enhanced MMP-2 expression in HCC cells might improve the invasiveness and metastatic potential of the cancer cells. Given that nuclear Met is comprised solely of the cytoplasmic portion of full-length Met, it is possible that the molecular mechanism of activation of NF-B signaling will change. Instead of interacting with signaling intermediates such as the PI3K-Akt pathway and Src, nuclear Met may form complexes with additional binding partners or transcription factors within the nucleus to regulate NF-B signaling, as opposed to wild-type Met. In order to unravel the whole signaling cascades" that are involved in nuclear Met signaling shortcuts, a thorough investigation is required. Nonetheless, further extensive research into MMP-2 activity is required in order to have a better understanding of the overall physiological alterations.

4. RESEARCH OBJECTIVE & METHODOLOGY

We have three main objectives in this paper:

- To "understand the signalling network underpinning nuclear Met's activities inside the nucleus, which is mediated by the protein nuclear" Met.

To achieve these "objectives, we seek to uncover the functional implications and molecular basis of nuclear Met in HCC, which sets it different from its full-length cousin in terms of length. Afterwards, these results will provide a solid" foundation for future studies, which will disclose other new functions for nuclear Met in cancer cells.

The proportion of nuclei stained positively with "Met staining was represented as a continuous variable in the analysis. In order to get the most amount of information feasible, a cutoff threshold was chosen for further categorising the patients into groups with high and low nuclear Met expression levels, respectively. A study was conducted to determine the relationship between nuclear Met expression and clinic pathological parameters in the patients, including gender, age, non-tumorous liver status, cirrhosis status, tumour size, number of tumour nodules, tumour encapsulation, cellular differentiation according to the Edmondson grading system, stage of pTMN, serum hepatitis B surface antigen status, venous invasion without differentiation into portal or hepatic veins, tumour microsatel It is a categorization system established by Edmondson and Steiner (Edmondson and Steiner, 1954) that divides tumours into those with better (Grade I and II) and those with worse (Grade III and IV) cellular differentiation. An investigation was conducted on the relationship between nuclear Met expression and the overall survival rate (death as the endpoint regardless of recurrence status) and disease-free survival rate (recurrence or death as the endpoint) of patients. The Kaplan-Meier technique was used to analyse the survival rate data, and the log-rank test was performed to evaluate the differences between the two groups. All statistical analyses were carried out using IBM SPSS Statistics 19 (SPSS Inc., Chicago, IL, USA) in accordance with the procedures specified. In order" to be deemed statistically significant, a P-value less than 0.05 (P 0.05) has to be obtained. The media was aspirated, and "adhering cells were washed with ice-cold PBS to remove any remaining culture medium before being

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harvested using a cell scrapper, as previously described. Detached cells were transferred to a 1.5 ml micro-centrifuge tube and centrifuged at maximum speed for 1 minute at 4°C after which they were collected. The supernatant was removed, and whole cell lysates were obtained using either Radio-Immunoprecipitation Assay (RIPA) (50mM Tris, pH 7.4, 150mM NaCl, 1 percent NP-40, 0.25 percent sodium deoxycholate, 1 M EDTA) or NETN (50mM Tris, pH 8.0, 150mM NaCl, 5 mM EDTA, 0.2 percent NP-40) lysis buffer (Roche). The suspension was incubated on ice for 30 minutes, after which it was centrifuged at maximum speed for 20 minutes at 4 degrees Celsius. Cell lysate was collected and kept at -80°C in preparation for future use in the" experiment.

In accordance with the "manufacturer's instructions, nuclear and cytoplasmic fractions of cells were collected using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Waltham, MA, USA). For the most part, adhering cells were extracted with trypsin and then washed with PBS. It was necessary to remove the supernatant after centrifuging at 500 g for 5 minutes. A 15-second vortex on the maximum setting was used to aggressively vortex the cell pellet after it had been exposed to ice-cold CER I for 15 seconds. After 10 minutes of incubation on ice, ice-cold CER II was added and vortexed for 5 seconds on the maximum setting before incubating on ice for another 1 minute, before repeating the process. For 5 minutes at 4°C, a cell suspension was centrifuged at its highest speed, and the supernatant (cytoplasmic extract) was collected in a clean, pre-chilled tube. It was necessary to wash the residual insoluble fraction pellet two or three times with PBS before to suspending it in ice-cold NER and vortexing it on the highest setting for 15 seconds. The suspension was incubated on ice for 40 minutes at 4°C, during which time it was exposed to continuous vortexing for 15 seconds every 10 minutes for a total of 40 minutes. At the end, the solution was centrifuged at maximum speed for ten minutes" before the supernatant (nuclear extract) was transferred to a clean, pre-chilled tube for storage. Before being utilized for extraction, both CER I and NER "were treated with protease inhibitor cocktail and phosphatase inhibitor cocktail, respectively. The cytoplasmic and nuclear lysates were kept at -80°C in preparation for future experiments.

Using the Bradford technique (Bio- Rad Laboratories, Hercules, CA, USA), the protein content of the cell lysate was determined in accordance with the manufacturer's instructions. To summarise, BSA standards were produced in order to establish a standard curve that could be used to determine the protein content of cell lysates. 96-well plate with 1 Bradford reagent (Bio-Rad Laboratories) was filled with one millilitre of BSA standards (0, 0.7, 1.4, 2.1, 2.8 mg/ml) or cell lysates, each of which was mixed well. With the Infinite® F200 microplate reader, we were able to get an absorbance reading at 595 nm (Tecan, Maennedorf, Switzerland). The relative protein concentrations of various cell lysates were determined, and 30 g of proteins were combined with 6 g of protein loading dye to produce the final product (0.35 M Tris-HCl, pH 6.8, 30 percent glycerol, 20 percent SDS, 9.3 percent DTT and 0.05 percent bromophenol blue). The mixture was cooked" for 5 minutes at 95°C and then kept at -20°C for a short period of time before being subjected to western blot analysis.

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5. DATA ANALYSIS & FINDINGS

The design of the N-terminally "shortened cytoplasmic segments of Met (J1, J3, and T2), which were used in the study. In SMMC7221, BEL7402, and MHCC97L HCC cells, a Myc-tagged nuclear Met overexpressing stable clone was generated and was found to be effective. In order to differentiate between the exogenous nuclear Met protein and the native nuclear Met protein, the Myc-tag was used. Western blotting was used to investigate the conditions under which doxycycline induction of nuclear Met expression occurred. As a general rule, the expression of nuclear Met could be observed starting at 4 hours after the addition of doxycycline (1 g/ml) and increased significantly with the lengthening of the induction time (Figure 4.2). Furthermore, the increase of nuclear Met expression by doxycycline was shown to be dose-dependent. An increase in expression was found when the concentration of doxycycline was increased (Figure 4.3). The smallest dosage of doxycycline tested, 0.25 g/ml, was shown to be capable of triggering the expression of nuclear Met after 24 hours of treatment with the antibiotic. Despite this, expression of nuclear Met was observed even after doxycycline was removed after 144 hours from the cells (Figure 4.4). The use of 2 g/ml doxycycline treatment for 24 hours before to any experiment was determined to be the most effective method for maintaining the expression" of nuclear Met throughout the functional assays based on these characterizations of inducible expression of nuclear Met.

To investigate the subcellular location of "nuclear Met in the created stable clones, cellular fractionation was conducted followed by western blotting analysis to determine the presence of nuclear met. Both J1 and J3 were identified in the nuclear lysate of stable clones, but T2 was only found in the cytoplasmic portion of the same stable clones (Figure 4.6). The presence of Met expression in the nucleus was confirmed by immunofluorescence microscopy, which provided further confirmation. In the stable clones, immunofluorescence labelling indicated that both J1 and J3 were accumulating in the nucleus, whereas T2 was being excluded from the nucleus according to the results of the experiment (Figure 4.7, 4.8 and 4.9). Due to the fact that HGF causes Met to become activated, we wondered if HGF had an effect on the expression and subcellular location of nuclear Met in cells. The treatment of SMMC7721 cells with HGF after they had been transfected with nuclear Met constructs had no effect on the nuclear localization of the Met protein (Figure 4.10). We also wanted to know if nuclear Met shuttles back and forth between the cytoplasm and the nucleus on a continual basis. In order to answer this question, we treated the cells with LMB, a nuclear exporter inhibitor that targets the CRM1 nuclear exporter. Interestingly, it was discovered that LMB had no influence on the subcellular" distribution of Met.

The incorporation of BrdU into the DNA of nuclear Met overexpression stable clones was measured in order to determine "their proliferation rate. SMMC7721, BEL7402, and MHCC97L were the only stable clones of construct J1 that demonstrated a statistically significant increase in proliferation rate when compared to the vector control (Figure 4.12). As a last check to ensure that the promoting effect seen in J1 stable clones was due to

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the increased expression of J1, a BrdU incorporation experiment was carried out using SMMC7721 and BEL7402 stable clones treated with or without doxycycline, respectively. The J1 stable clone treated with doxycycline was found to have a higher proliferation rate than the J1 stable clone that was not treated with doxycycline, as well as the vector control stable clone that was treated with doxycycline, indicating that induced J1 expression is the only factor that promotes cell proliferation in this model.

With the use of a soft agar test, the anchorage independent growth capacity of stable clones was determined. In order to establish that the induced J1 expression was indeed responsible for the boosting impact on anchorage "independent growth, a soft agar experiment was carried out using SMMC7721 and BEL7402 stable clones treated with or without doxycycline for another round of testing. Using doxycycline treatment, it was discovered that the J1 stable clone formed more colonies than either the J1 stable clone without treatment or the vector control stable clone treated with doxycycline indicating that J1 expression has an impact on promoting cell anchorage" independent growth..

6. CONCLUSION

The unique findings "generated from our work offered a thorough knowledge of the critical function of nuclear Met in the progression of HCC tumorigenesis and metastasis, which was previously unknown. The existence of nuclear Met and its oncogenic impact in HCC have been shown; however, the processes of nuclear translocation of the cytoplasmic fragment of Met are still being investigated. It has been shown that a number of receptor tyrosine kinase (RTK) enzymes undergo regulated intramembrane proteolysis (RIP) to liberate a cytoplasmic area that is then translocated into the nucleus. In the context of cell surface membrane receptors, RIP is a highly controlled proteolytic processing event in which the receptor is cleaved within the transmembrane region to generate a shortened protein domain (Brown et al., 2000). This freed protein domain may be able to function as a signal messenger in and of itself at specific cellular compartments. We hypothesised that the full-length Met could be digested by RIP to release the cytoplasmic fragment, which we found to be true. In fact, it has been revealed that the Met receptor uses presenilin-dependent regulated intramembrane proteolysis (PS-RIP) to degrade Met in the absence of ligand stimulation.1 (Foveau et al., 2009). PS- RIP of Met is structurally and mechanistically comparable to the processing of ErbB-4, which required two cleavage stages to be completed. Before the cleavage of the membrane-anchored fragment by metalloproteinase, which results in the release of a labile intracellular domain, extracellular domain shedding mediated by metalloproteinase must take place, resulting in the generation of a soluble N-terminal fragment (Foveau et al., 2009). Despite the fact that the study reported that this labile intracellular domain is the result of a downregulation approach to Met signalling, we believed that nuclear Met could be produced through this PS-RIP as well, and we believe that further research is needed to gain a better understanding of how this happens.

What is the mechanism by which the cytoplasmic fragment of Met is transported into the

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nucleus once it has been produced? In order to translocate into the nucleus, it was commonly assumed that the cytoplasmic portion of Met contained a nuclear localization signal (NLS). However, this was never confirmed. Previous" investigations, on the other hand, were unable to locate the NLS inside the cytoplasmic portion of Met. In order for nuclear localization to occur, it has been anticipated that a region (P1027-I1084) will be required (Pozener-Moulis et al., 2006; Gomes et al., 2008). "Furthermore, it has been revealed that Met is localised in the nucleus and is responsible for the initiation of calcium signals (Gomes et al., 2008). The association of Met with the adaptor protein Gab-1, which contains a nuclear localization signal (NLS), may be able to begin nuclear translocation with the assistance of importin 1, which directs importin-cargo complexes through the nuclear pores (Gomes et al., 2008). An experimentally confirmed pH-dependent NLS in the juxtamembrane region of Met (H1068-H1079) with two histidine residues separated by a 10-amino-acid spacer has been effectively mapped in a recent research (Chaudhary et al., 2014). The deletion of these sequences or the replacement of the histidines with alanines prevented Met from translocating into the nucleus and from interacting with importin. This NLS was shown to be pH-dependent, with decreasing cytosolic pH substantially increasing the amount of nuclear Met and the interaction of the Met fragment with importin (Chaudhary et al., 2014). This suggests that the cytoplasmic component of Met" may use its own NLS in conjunction with importin to reach the nucleus, as previously suggested.

In conclusion, even though we were unable to demonstrate Met cleavage, our research has offered fresh insights into the functional significance of nuclear Met in HCC and other cancers (Figure 6.1). We hypothesised that full-length Met could be processed by PS-RIP to release a cytoplasmic domain of Met, which could then be translocated into the nucleus with the assistance of importin, as previously reported. This cytoplasmic portion of Met, located within the nucleus, facilitates NF-B signalling, which modulates target genes such as MMP-2, therefore promoting the invasiveness and aggressiveness of HCC tumours. Our findings have established a strong foundation for further inquiry, which may lead to the identification of other underlying mechanisms of nuclear Met signalling in cancer cells.

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ISSN:1673-064X
E-Publication:Online Open Access
Vol: 66 Issue 02 | 2023
DOI 10.17605/OSF.IO/AYNM8

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