N-acetylglucosaminyltransferase V confers hepatoma cells with resistance to anoikis through EGFR/PAK1 activation

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Elevated expression and activity of N-acetylglucosaminyltransferase V (Mgat5) in hepatocellular carcinoma (HCC) is a common early event involved in tumor invasion during hepatocarcinogenesis. A better understanding of the functional role and the molecular mechanism for Mgat5-targeted protein and downstream signaling pathway behind hepatoma invasion and metastasis is urgently needed. Here, we show that Mgat5 overexpression promoted anchorage-independent growth and inhibited anoikis in hepatoma cells. This effect was reversed by glycosyltransferase inactive mutant Mgat5 L188R transfection, α-mannosidase II inhibitor swainsonine treatment and N-acetyl glucosamine (GlcNAc) phosphotransferase (GPT) inhibitor tunicamycin administration. Mgat5 overexpression increased p21-activated kinase 1 (PAK1) expression and shRNA-mediated PAK1 knockdown and kinase inactivation with kinase dead mutant PAK1 K299R coexpression or allosytic inhibitor P21-activated kinase inhibitor III (IPA3) treatment reversed anoikis resistance in Mgat5-overexpressed hepatoma cells. Furthermore, Mgat5 overexpression upregulated β-1,6-GlcNAc branched N-glycosylation and following phosphorylation of epidermal growth factor receptor (EGFR) in hepatoma cells. EGFR tyrosine kinase inhibitors AG1478 and P21-activated kinase inhibitor III (IPA3) treatment reversed anoikis resistance in hepatoma cells, implicating that molecular targeted therapeutics against Mgat5/EGFR/PAK1 might open a new avenue for personalized medicine in advanced-stage HCC patients.

Keywords: Anoikis / EGFR / HCC / Mgat5 / PAK1

Introduction

Primary liver cancer, which consists predominantly of hepatocellular carcinoma (HCC), is currently the fifth most common solid tumor worldwide and the third leading cause of cancer-related death globally, accounting for ~500,000 new cases per year and >60,000 deaths annually (Llovet et al. 2003; El-Serag and Rudolph 2007). This high mortality rate of HCC is mainly related to hepatoma metastasis, which is also one of the major causes of tumor recurrence in HCC patients after surgical resection. Despite advances in many aspects of HCC treatment, including liver transplantation, surgical resection and locoregional therapies, no effective therapeutic options except one chemotherapeutic agent sorafenib are available for the most majority of HCC patients with advanced-stage disease (Thomas et al. 2010). These challenges highlight an urgent need for the identification of relevant molecular pathways and novel therapeutic targets for this malignant lesion.

As a hallmark of tumor phenotype, altered glycosylation patterns during tumorigenesis include both the under- and overexpression of naturally occurring glycans, as well as neoexpression of glycans normally restricted to embryonic tissues (Dube and Bertozzi 2005). These tumor-associated glycans most often arise from changes in the expression levels of glycosyltransferases in the Golgi compartment of cancerous cells (Dube and Bertozzi 2005). One of the most common changes is an increase in the size and branching of N-linked glycans mainly mediated by increased activity of N-acetylglucosaminyltransferase V (Mgat5) (Dennis et al. 1987). The Mgat5-encoded N-acetyl glucosamine (GlcNAc-T-V) glycosyltransferase generates a β-1,6-linked GlcNAc branch from the α-1,3-linked mannose (Lowe and Marth 2003). Mgat5 and its corresponding branched glycans have been studied extensively in the context of malignant transformation.

N-glycans bearing a β-1,6-linked GlcNAc branch are consistently elevated in concert with increased expression of Mgat5, and numerous direct correlations have been made between the presence of this N-glycan branch and enhanced tumor growth and metastasis (Dennis et al. 1999; Ohtsubo and Marth 2006).
Mgat5 expression was quantitatively analyzed by utilizing a neuroblastoma. High expression levels of Mgat5 were found to be associated with favorable stages. Conversely, the downregulation of Mgat5 expression by small interfering RNA resulted in a decrease in susceptibility to cell apoptosis induced by retinoic acid in a neuroblastoma (Inamori et al. 2006). Loss of Mgat5 suppressed tumor growth and metastasis in the mammary glands of mice carrying the mouse mammary tumor virus polyoma middle T oncogene (Granovsky et al. 2000). Mammary tumor cells expressing Mgat5 were more responsive to growth factors due to enhanced interactions of their growth factor receptors with galectins, leading to reduced endocytosis and prolonged signaling compared with cells lacking Mgat5 (Partridge et al. 2004; Lau et al. 2007). Human cancer cell lines with targeted silencing of the Mgat5 gene also exhibit reduced epidermal growth factor receptor (EGFR) signaling, although apparently by a galectin-independent mechanism (Guo et al. 2009). Increased β-1-6-GlcNAc branched N-glycans due to elevated Mgat5 expression facilitate oncogenesis via promoting surface retention of growth receptors with a reduced dependence on uridine diphosphate (UDP)-GlcNAc (Guo et al. 2009). Her-2-induced mammary tumor onset was significantly delayed in Mgat5 knockout mice, coincident with the reversion of her-2-induced deregulation of acinar morphogenesis and a significantly reduced population of tumor-initiating cells (cancer stem cells) in isolated tumor cells with Mgat5 deletion, resulting in reduced ability to form secondary tumors in nonobese diabetic/severe combined immunodeficiency mice (Guo et al. 2010). Although the oncogenic roles of β-1-6-GlcNAc branching generated by Mgat5 in hepatoma development and progression have been indicated previously (Yao et al. 1998; Shao et al. 1999; Guo et al. 2000; Ito et al. 2001; Yanagi et al. 2001; Liu et al. 2007), the functional role and the molecular mechanism for Mgat5 in hepatocarcinogenesis remain far from fully understood.

Anoikis, defined as a specialized form of apoptosis that occurs on cells due to inadequate or inappropriate cell–matrix interactions, is a critical mechanism for preventing ectopic cell growth or attachment to an inappropriate matrix involving a diversity of tissue developmental, homeostatic and oncogenic processes (Gilmore 2005). Anoikis resistance dictates tumor cell survival during local dissemination and distant colonization, thus endowing malignant cell with anchorage-independent growth and providing a molecular basis for cancer metastasis (Nagaprashantha et al. 2011). The significance of anoikis resistance in liver cancer metastasis was elegantly shown in our previous studies where p21-activated kinase 1 (PAK1) has been identified as a crucial kinase nodule for hepatoma resistance to anoikis (Chen et al. 2003; Xu et al. 2012). However, the potential role of oncogenic Mgat5 for anoikis resistance in hepatocarcinogenesis remains obscure and addresses our research interest herein.

In this study, we found that Mgat5 conferred hepatoma cells with resistance to anoikis via EGFR/PAK1 signal activation, which could be reversed by genetic and pharmacological inhibition of N-glycosylation, PAK1 or EGFR activation. These results identified Mgat5-mediated β-1-6-GlcNAc branched N-glycosylation and following activation of EGFR as a potential novel upstream molecular event for PAK1-induced anoikis resistance in hepatoma cells, implicating that molecular targeted therapeutics against anoikis resistance with Mgat5/EGFR/PAK1 signal intervention might pave a promising way to personalized medicine for advanced-stage HCC patients.

Results

Mgat5 promotes anchorage-independent growth and anoikis resistance of hepatoma cells dependent on its glycosyltransferase activity

To determine the potential function of Mgat5 expression in hepatocarcinogenesis, we selected two hepatoma cell lines (Huh7 and HepG2) with lower Mgat5 expression and glycosyltransferase activity to explore functional alterations after Mgat5 overexpression (Figure 1A). These two cells were transiently transfected by both Mgat5 expression vector plasmid and empty vector plasmid as a control. We also generated a stable BEL7404 Tet-on-Mgat5 cell line in which human Mgat5 can be specifically induced by doxycycline (Dox) to explore functional alterations after Mgat5 overexpression (Figure 1B). Colony formation assay showed that Mgat5 overexpression promoted significantly anchorage-independent growth of hepatoma cells (Figure 1C). Since anoikis resistance is a prerequisite for acquisition of anchorage-independent growth in malignant cells (Nagaprashantha et al. 2011), we propose that anchorage-independent growth obtained from Mgat5 expression might be attributable to Mgat5-induced anoikis resistance. To validate this hypothesis, in vitro anoikis assay for Huh7, HepG2 and Mgat5-inducible BEL7404-Tet-on cells was performed in the presence or absence of Mgat5 overexpression. Elevated cleaved poly ADP ribose polymerase (PARP) after detachment in Huh7, HepG2 and Dox-untransfected Mgat5-inducible cells was significantly rescued by Mgat5 overexpression (Figure 1D). Consistent with this phenomenon, increased apoptosis after detachment in Huh7, HepG2 and Dox-untransfected Mgat5-inducible cells was alleviated significantly by Mgat5 overexpression using Caspase 3/7 activity assay (Figure 1E) and TUNEL assay (Figure 1F). These data suggest that anchorage-independent growth obtained after Mgat5 overexpression is attributed partly to Mgat5-induced anoikis resistance.

To elucidate the pivotal role of Mgat5 in anchorage-independent growth and anoikis resistance, Mgat5 and its catalyzed glycan structure recognized by leukoagglutinating phytohemagglutinin (L-PHA) were knockdown by shRNA in Hep3B cells (Figure 2A). Colony formation assay showed that Mgat5 knockdown decreased anchorage-independent growth (Figure 2B). Furthermore, western blot analysis of cleaved PARP (Figure 2C), Caspase 3/7 activity assay (Figure 2D) and TUNEL assay (Figure 2E) showed that anoikis is increased after detachment in Hep3B cells with Mgat5 knockdown.

To further address the possible role of glycosyltransferase activity in Mgat5-induced anoikis resistance, functional alterations were analyzed after Mgat5 glycosyltransferase inactivation with α-mannosidase II inhibitor swainsonine (SW) treatment, GlcNAc phosphotransferase (GPT) inhibitor tunicamycin (TM) administration and glycosyltransferase inactive mutant Mgat5 L188R transfection in hepatoma cells. Lectin blot analysis with L-PHA recognizing β-1-6-GlcNAc branching provided by Mgat5 revealed that both SW and TM treatment dramatically declined N-glycan branching production in Mgat5-overexpressed hepatoma cells, and glycosyltransferase inactive mutant Mgat5
Fig. 1. Mga5 overexpression confers hepatoma cells with resistance to anoikis. (A) Western blot analysis of Mga5 and GAPDH protein levels and lectin blot analysis with L-PHA for Mga5 glycosyltransferase activity in Huh7, HepG2, BEL7404, BEL7402 and Hep3B cells. (B) Western blot analysis of Mga5 and GAPDH protein levels and lectin blot analysis with L-PHA for Mga5 glycosyltransferase activity in Huh7 and HepG2 cells transiently transfected with empty vector and Mga5 plasmid, respectively (left). Western blot analysis of Mga5 and GAPDH protein levels and lectin blot analysis with L-PHA for Mga5 glycosyltransferase activity in BEL7404 Tet-on vector-alone cells or BEL7404 Tet-on Mga5-inducible cells untreated (−) or treated (+) with Dox, respectively (right). (B) Colony formation assay in Huh7 and HepG2 cells transiently transfected with empty vector and Mga5 plasmid, respectively, and in BEL7404 Tet-on Mga5-inducible cells untreated (−) or treated (+) with Dox, respectively (right). (C–E) Western blot analysis of cleaved PARP and GAPDH (C), Caspase 3/7 activity assay (D) and TUNEL assay (E) for Huh7 and HepG2 cells transiently transfected with empty vector and Mga5, respectively (left), and in BEL7404 Tet-on Mga5-inducible cells untreated (−) or treated (+) with Dox, respectively (right), during attached, serum starvation and detached status for 48 h. The graphic profiles represent the mean of three independent replicates in each group with standard error bars and were statistically analyzed with a t-test (*P < 0.05).
L188R transfection decreased N-glycan branching production compared with wild-type Mgat5 transfection in Huh7 and HepG2 cells (Figure 3A). Colony formation assay showed that increased anchorage-independent growth of hepatoma cells due to Mgat5 expression were reversed by Mgat5 glycosyltransferase inactivation with Mgat5 L188R transfection and SW or TM cotreatment (Figure 3B). Western blot analysis of cleaved PARP (Figure 3C), Caspase 3/7 activity assay (Figure 3D) and TUNEL assay (Figure 3E) in suspended hepatoma cells showed that Mgat5-induced anoikis resistance was reversed by Mgat5 L188R transfection and SW or TM cotreatment. Taken together, these results demonstrate that Mgat5 confers hepatoma cells with resistance to anoikis dependent on its glycosyltransferase activity.

**PAK1 activation involved in resistance of hepatoma cells to anoikis mediated by Mgat5**

Since our previous studies have identified that PAK1 activation is crucial for resistance of hepatoma cells to anoikis (Chen et al. 2003; Xu et al. 2012), we next investigate the possible role of PAK1 activation in resistance of hepatoma cells to anoikis mediated by Mgat5 overexpression. Mgat5 overexpression upregulated PAK1 protein levels in suspended hepatoma cells, indicating the potential significance of PAK1 activation in Mgat5-mediated anoikis resistance (Figure 4A). Moreover, western blot analysis of cleaved PARP showed that shRNA-mediated PAK1 knockdown increased apoptosis of suspended Mgat5-overexpressed hepatoma cells (Figure 4B). Colony formation assay revealed that anchorage-independent growth of Mgat5-overexpressed hepatoma cells was declined by shRNA-mediated PAK1 knockdown (Figure 4C). Furthermore, Caspase 3/7 activity assay (Figure 4D) and TUNEL assay (Figure 4E) showed that decreased anoikis of Mgat5-overexpressed hepatoma cells was also reversed by shRNA-mediated PAK1 knockdown.

To further elucidate the pivotal role of PAK1 kinase activity in Mgat5-induced anoikis resistance, functional alterations were analyzed after PAK1 kinase inactivation with kinase dead mutant PAK1 K299R cotransfection and PAK1 allosteric inhibitor IPA3 administration in Mgat5-overexpressed hepatoma cells. Colony formation assay showed that increased anchorage-independent growth of hepatoma cells due to Mgat5 expression was reversed by PAK1 kinase inactivation with PAK1 K299R cotransfection or IPA3 treatment (Figure 5A). Western blot analysis of cleaved PARP (Figure 5B), Caspase 3/7 activity assay (Figure 5C) and TUNEL assay (Figure 5D) in suspended hepatoma cells showed that Mgat5-induced anoikis resistance was reversed by PAK1 kinase inactivation with PAK1 K299R cotransfection or IPA3 treatment. These data demonstrate that Mgat5-induced anoikis resistance is mediated by hyperactivation of PAK1.

**EGFR/PAK1 signaling arbitrates Mgat5-induced resistance of hepatoma cells to anoikis**

Previous study indicated that siRNA-mediated Mgat5 knockdown caused reduced N-linked β-1,6-branching glycans on EGFR and decreased EGF-mediated downstream signaling,
which consequently suppressed invasiveness-related phenotypes in human breast carcinoma cells (Guo et al. 2007). Lectin immunoprecipitation analysis with L-PHA and wheat germ agglutinin (WGA) recognizing N-glycans revealed that Mgat5 expression increased N-glycosylation of EGFR in Huh7, HepG2 and Mgat5-inducible BEL7404-Tet-on cells (Figure 6A). Although flow cytometry analysis showed no significant effect on EGFR surface retention after Mgat5 expression in Huh7, HepG2 and Mgat5-inducible BEL7404-Tet-on cells (Figure 6B), phosphorylation of EGFR Y1068 indicating EGFR activation status increased remarkably in Mgat5-transfected hepatoma cells and Dox-treated Mgat5-inducible cells, which could be rescued by...
Mgtat5 glycosyltransferase inactivation with Mgtat5 L188R transfection and SW or TM cotreatment (Figure 6C).

Since PAK1 activation has been demonstrated to be a downstream molecular event of EGFR signal, we propose that Mgtat5-induced anoikis resistance might be mediated by EGFR/PAK1 activation. To further characterize the potential role of EGFR/PAK1 signal in Mgtat5-induced anoikis resistance, functional alterations were analyzed after EGFR/PAK1 signal intervention in Mgtat5-overexpressed hepatoma cells. Colony formation assay showed that increased anchorage-independent growth of hepatoma cells due to Mgtat5 expression was reversed by EGFR inhibition with EGFR inhibitor

Fig. 4. PAK1 is involved in Mgtat5-induced anoikis resistance. (A) Western blot analysis of PAK1 and GAPDH for Huh7 and HepG2 cells transiently transfected with empty vector and Mgtat5 plasmid, respectively, and for BEL7404 Tet-on Mgtat5-inducible cells untreated (−) or treated (+) with Dox, respectively, during attached and detached status for 48 h. (B) Western blot analysis of PAK1 and GAPDH for Huh7, HepG2 cells after nonspecific shRNA or PAK1 shRNA transfection, respectively (left). Western blot analysis of PAK1, cleaved PARP and GAPDH for Huh7 and HepG2 cells after Mgtat5 overexpression and for BEL7404 Tet-on Mgtat5-inducible cells untreated (−) or treated (+) with Dox, without or with nonspecific shRNA or PAK1 shRNA cotransfection, respectively (right). (C–E) Colony formation assay (C), Caspase 3/7 activity assay (D) and TUNEL assay (E) for Huh7, HepG2, after Mgtat5 overexpression, and in BEL7404 Tet-on Mgtat5-inducible cells untreated (−) or treated (+) with Dox, without or with nonspecific shRNA or PAK1 shRNA cotransfection undergoing detachment for 48 h, respectively. The graphic profiles represent the mean of three independent replicates in each group with standard error bars and were statistically analyzed with a t-test (*P < 0.05).
AG1478 or Iressa treatment, which could be rescued by PAK1 activation with constitutive active mutant PAK1 T423E cotransfection in hepatoma cells (Figure 7A). Caspase 3/7 activity assay (Figure 7B) and TUNEL assay (Figure 7C) in suspended hepatoma cells revealed that Mgat5-induced anoikis resistance was reversed by EGFR inhibition with AG1478 or Iressa administration, which could also be rescued by PAK1 activation with PAK1 T423E cotransfection in hepatoma cells. Taken together, these data demonstrate that Mgat5 overexpression endows hepatoma cells with resistance to anoikis via EGFR/PAK1 activation.

To further validate the role of EGFR/PAK1 signaling in Mgat5-induced anoikis resistance, we investigated the phosphorylation of EGFR Y1068 indicating EGFR activation status and PAK1 expression in Mgat5-knockdown Hep3B cells. Flow cytometry assay revealed that phosphorylation of EGFR Y1068 was reduced in Mgat5-knockdown Hep3B cells (Figure 8A). Western blot (Figure 8B) revealed that PAK1 expression was decreased in Mgat5-knockdown Hep3B cells. Colony formation assay (Figure 8C) revealed that decreased anchorage-independent growth by Mgat5 knockdown was partially reversed by PAK1 T423E cotransfection. Caspase 3/7 activity assay (Figure 8D) and TUNEL assay (Figure 8E) revealed that increased anoikis by Mgat5 knockdown was partially reversed by PAK1 T423E cotransfection. Taken together, these results suggest that EGFR/PAK1 signaling dominates Mgat5-induced resistance of hepatoma cells to anoikis.

Discussion

In the present study, we found that Mgat5 overexpression conferred hepatoma cells with resistance to anoikis. This effect was reversed by reduction of Mgat5 levels by shRNA or glycosyltransferase inactivation or inhibition, indicating the essential role of glycosyltransferase activity of Mgat5 in anoikis resistance. Consistent with our previous findings characterizing crucial function of PAK1 activation in anoikis resistance (Chen et al. 2003; Xu et al. 2012), Mgat5 overexpression increased PAK1 expression and genetic inhibition or pharmacologic inactivation of PAK1 reversed anoikis resistance in Mgat5-overexpressed hepatoma cells. More importantly, Mgat5 mediated β-1,6-GlcNAc...
Fig. 6. Mgat5 expression promotes N-glycosylation and activation of EGFR. (A) Lectin immunoprecipitation with L-PHA and WGA analysis for N-glycosylation of EGFR in Huh7 and HepG2 cells transiently transfected with empty vector and Mgat5 plasmid, respectively, and in BEL7404 Tet-on Mgat5-inducible cells untreated (−) or treated (+) with Dox, respectively. (B) Flow cytometry analysis for EGFR expression on Huh7 and HepG2 cells transiently transfected with empty vector and Mgat5 plasmid, respectively, and on BEL7404 Tet-on Mgat5-inducible cells untreated (−) or treated (+) with Dox, respectively. (C) Flow cytometry analysis for p-EGFR Y1068 expression on Huh7 and HepG2 cells transiently transfected with empty vector, Mgat5 and Mgat5 L188R plasmid without or with SW or TM treatment, respectively. Flow cytometry analysis for p-EGFR Y1068 expression on BEL7404 Tet-on Mgat5-inducible cells untreated (−) or treated (+) with Dox, without or with SW or TM treatment, respectively.
branched N-glycosylation and following activation of EGFR in hepatoma cells. Pharmacologic inactivation of EGFR declined anoikis resistance, which could be rescued by PAK1 activation in Mgat5-overexpressed hepatoma cells. These findings indicate that Mgat5 expression conferred hepatoma cells with resistance to anoikis via EGFR/PAK1 activation, which could be alleviated by genetic and pharmacologic inactivation of Mgat5/EGFR/PAK1 signal pathway.

Fig. 7. Mgat5-induced anoikis resistance is dependent on EGFR/PAK1 pathway. (A) Colony formation assay, (B) Caspase 3/7 activity assay and (C) TUNEL assay for Huh7 and HepG2 cells after Mgat5 overexpression without or with AG1478 or Iressa treatment in the absence or presence of PAK1 T423E cotransfection undergoing detachment for 48 h, respectively. (A) Colony formation assay, (B) Caspase 3/7 activity assay and (C) TUNEL assay for BEL7404 Tet-on Mgat5-inducible cells untreated (−) or treated (+) with Dox, without or with AG1478 or Iressa treatment in the absence or presence of PAK1 T423E cotransfection undergoing detachment for 48 h, respectively. The graphic profiles represent the mean of three independent replicates in each group with standard error bars and were statistically analyzed with a t-test (*P < 0.05).
Aberrant upregulation of β-1-6-GlcNAc branching provided by Mgat5 is directly associated with metastasis and might serve as marker for tumor invasiveness in HCC patients (Dennis et al. 1987; Yanagi et al. 2001; Blomme et al. 2009). Mgat5-mediated anoikis resistance implicating in our present study might play an essential role for tumor invasion and metastasis in hepatocarcinogenesis. Moreover, we have also identified PAK1 activation as a crucial molecular event for Mgat5-mediated anoikis resistance. Although our previous studies have identified Bcl2 stabilization promoted by mitochondrial-localized PAK1 as a molecular signature for hepatitis B virus X-induced anoikis resistance in hepatitis B virus-associated hepatocarcinogenesis (Chen et al. 2003; Xu et al. 2012), the molecular mechanism underlying Mgat5-mediated anoikis resistance remains far from fully understood. Previous study indicated that anoikis triggered by metabolic defects and reactive oxygen species (ROS) induction caused by loss of matrix attachment could be rescued with antioxidant restoration of adenosine tri-phosphate generation and oncogene activation (Schafer et al. 2009; Soderquist et al. 1988). Loss of Mgat5 in tumor cells elevated ROS induction dependent on glucose metabolism (Lau and Dennis 2008). The potential significance of cancerous glucose metabolism and ROS induction in Mgat5-mediated anoikis resistance dependent on PAK1 activation merits further investigation.

N-glycan branching in medial-Golgi generates ligands for lattice-forming lectins that regulate surface levels of glycoproteins including EGF and transforming growth factor-β receptors via hexosamine/Golgi/lattice (Lau and Dennis 2008). Cytokine and growth factor receptors are generally N-glycosylated transmembrane proteins, and residency at the surface is dependent in part on the dynamics of membrane remodeling (Partridge et al. 2004). Endogeneous lectins, such as galectins, can cross-link glycoproteins at the cell surface forming lattices that enhance residency time at the cell surface (Lau and Dennis 2008). EGF is an important surface receptor with N-glycans in its extracellular domain, and the glycosylation of EGF is essential for its function (Soderquist et al. 1988; Bishayee 2000). The overexpression of Mgat5a in human hepatoma cells caused aberrant N-glycosylation of EGF and increased MAPK signaling mediated by EGF (Guo et al. 2004). Knockdown of Mgat5a by siRNA expression in human breast carcinoma cells caused reduced N-linked β-1-6-branaching on EGF and decreased EGF-mediated activation of the tyrosine phosphatase src homology 2-containing protein-tyrosine phosphatase-2,
which consequently inhibited the dephosphorylation of focal adhesion kinase, consistent with the attenuation of invasiveness-related phenotypes that included decreased actin rearrangement and cell motility (Lowe and Marth 2003). Guo et al. (2010) recently reported that a specific posttranslational modification by deletion of Mga5 disrupted the selective apoptosis of centrally located mammary epithelial cells and delayed her-2-induced mouse mammary tumor onset by downregulating two downstream signaling pathways mediated by her-2 activation, the ERK and PI3K/PKB pathways. In addition, Mga5 expression levels also regulate the expression of members of the Pcdhβ7 cluster by affecting her-2-mediated ERK and PI3K/PKB signaling pathways resulting in mammary tumorigenesis and progression (Guo et al. 2012). Our present study revealed a novel downstream effector and signaling pathway that contributes to the Mga5-induced anoikis resistance. Thus, inhibition of Mga5 might be useful in the treatment of malignancies by targeting their roles in metastasis. Our present revealed that Mga5 mediated β-1-6-GlcNAc branched N-glycosylation and following activation of EGFR, which might be due to increased EGFR residency on cell surface of Mga5-overexpressed hepatoma cells during EGFR endocytosis triggered by ligand stimulation. Inconsistent data herein on EGFR residency on cell surface after Mga5 expression might be attributable to insufficient ligand stimulation after serum starvation. However, potential roles of other transmembrane receptors on cell surface regulated via hexosamine/Golgi/lattice in anoikis resistance remain obscure and await further studies.

In conclusion, current data identified Mga5-mediated β-1-6-GlcNAc branched N-glycosylation and following activation of EGFR as a potential novel upstream molecular event for PAK1-induced anoikis resistance in hepatoma cells, implicating that molecular targeted therapeutics against anoikis resistance with Mga5/EGFR/PAK1 signal intervention might pave a promising way to personalized medicine for advanced-stage HCC patients.

Materials and methods

Cell lines

Human hepatoma cell lines Huh7, HepG2, Hep3B, BEL7404 and Huh7 Tet-on cell were obtained directly from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO2 incubator. The cell lines have been characterized at the bank by DNA fingerprinting analysis using short tandem repeat markers. All cell lines were placed under cryostage after they were obtained from the bank and used within 6 months of thawing fresh vials as described previously (Liu et al. 2011).

Construction of plasmids

Expression plasmids encoding wild-type Mga5 and glycosy trasferase inactive mutant Mga5 L188R (a point mutation that blocks localization of the enzyme to the Golgi apparatus) were kindly provided by Dr. Jianguo Gu (Tohoku Pharmaceutical University, Miyagi, Japan) and constructed as described previously, respectively (Weinstein et al. 1996). The plasmids containing wild-type PAK1, kinase dead mutant PAK1 K299R and constitutive active mutant PAK1 T423E were constructed as described previously (Cheng et al. 2009). All plasmid constructs were confirmed by DNA sequencing.

Plasmid transfection and RNA interference

Transient and stable transfections with various plasmids were performed as previously described (Xu et al. 2010). The shRNA against Gn-T-V gene Gn-T-V shRNA (h), against PAK1 gene PAK1 shRNA (h) and corresponding control shRNA (Santa Cruz Biotechnology, Santa Cruz, CA) were used for RNA interference as described previously (Xu et al. 2012). Gene silencing effect was confirmed by western blot analysis and RT-PCR at 48 h posttransfection.

BEL7404 Tet-on Mga5-inducible cells

Human Mga5 cDNA was subcloned into the p-tetracycline response element (pTRE)-tight vector (Clontech, Palo Alto, CA). BEL7404 Tet-on cells were cotransfected with pTRE-tight Mga5 and a hygromycin resistance gene (Clontech, Palo Alto, CA). Positive clones were selected in the presence of G418 and hygromycin, and the inducibility of Mga5 was then determined by western blot analysis using a mouse antisera to Mga5.

Western blot, immunoprecipitation and lectin blot analyses

Protein extraction from cultured cells, western blot, immunoprecipitation and lectin blot analyses were carried out as previously described (Jiang et al. 2006; Xu et al. 2010; Liu et al. 2011). Briefly, cells cultured were washed with phosphate buffered saline (PBS) and then lysed with lysis buffer (10 mM Tris–HCl, 1% Triton-X100, 150 mM NaCl, aprotinin, leupeptin and 1 mM phenylmethylsulfonyl fluoride). Insoluble materials were removed by centrifugation at 15 000 rpm for 10 min at 4°C. Equal amounts of protein were separated using 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose and probed with the appropriate antibodies or with biotinylated L4-PHA. Primary antibodies used here included those against Gn-T-V (Sigma, St. Louis, MO), PAK1 and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), cleaved PARP and EGFR (Cell Signaling Technology, Beverly, MA).

Colony formation assay

Protein extraction from cultured cells, colony formation assay were carried out as previously described (Xu et al. 2010; Liu et al. 2011). In brief, a bottom layer (0.6% low-melt agarose) was prepared with DMEM medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin. A top layer (0.3% low-melt agarose) was prepared with DMEM medium plus 5000 units of penicillin, 5000 units of streptomycin, 10 mg/mL of amphotericin B, 1% of horse serum and 1% of fetal bovine serum at 37°C in a humidified incubator for ~2 weeks. The plates were then scanned and photographed.
In vitro anoikis assay, Caspase 3/7 activity assay and TUNEL assay

In vitro anoikis assay was performed with cell culture on poly-HEMA-coated six-well tissue culture plates for 48 h as described previously (Gilmore 2005). Caspase 3/7 activity assay and TUNEL assay were performed as described previously (Xu et al. 2012). Briefly, cells were transferred in a white opaque 96-well plate, incubated for 1 h with Caspase-Glo reagent and the enzymatic activity of Caspase 3/7 was measured using a Multiskan Luminometer (Multiscan Ascent, Thermo systems, Belgium). For the TUNEL assay, cells were fixed with 4% paraformaldehyde dissolved in PBS for 1 h at room temperature and were then permeabilized by treating with 0.1% Triton X-100 in 0.1% sodium citrate at 48°C for 30 min. Slides were washed with PBS and TUNEL reaction reagent (10 mL) was placed on the attached sperm. The slide was then covered in parafilm and incubated in a humidified dark box for 48 h at 37°C. After sufficient rinsing with PBS, 1 mg/mL of DAPI (Sigma-Aldrich) solution (10 mL) was added onto the sperm and covered with parafilm. After incubation in a humidified dark box for 30 min, the slide was washed with DW, dried using warm air and scanned and photographed.

Lectin immunoprecipitation

Lectin immunoprecipitation analysis with L-PHA and WGA were carried out as previously described (Jiang et al. 2006). The cells were then washed three times with ice-cold PBS, and solubilized in lysis buffer. Insoluble material was removed by centrifugation at 15 000 rpm for 10 min at 4°C. The supernatant (2 mg protein) was incubated with Streptavidin-agarose (15 mL – PAGE, and transferred to a nitrocellulose membrane. The cells were then washed three times with ice-cold PBS, and resuspended in 0.2 mL of saline; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SW, swainsonine; WGA, wheat germ agglutinin.

Flow cytometry

Flow cytometry analysis for EGFR and phospho-EGFR\textsuperscript{Y1068} expression analysis on hepatoma cells was performed with anti-EGFR–Fluorescein and anti-phospho-EGFR\textsuperscript{Y1068}–FITC (R&D Systems, Minneapolis, MN) as described previously (Zhang et al. 2010; Zhang et al. 2011). For EGFR expression level analysis, 1 × 10\textsuperscript{6} cells were harvested and washed with isotonic PBS buffer (supplemented with 0.5% bovine serum albumin), resuspended in 25 µL of isotonic PBS buffer and Fc-blocked by treatment with 1 µg of human immunoglobulin G/10\textsuperscript{6} cells for 15 min at room temperature prior to staining. Then, 10 µL of CFS-conjugated EGFR reagent was added and the mixture was incubated for 30 min at 4°C. After washing the cells twice with the same PBS buffer, the cells were resuspended in 400 µL of PBS for analysis by flow cytometry. For phospho-EGFR\textsuperscript{Y1068} expression levels analysis, 1 × 10\textsuperscript{6} cells were harvested and washed with cold PBS, resuspended in 0.2 mL of PBS and fixed in cold 4% paraformaldehyde at room temperature for 10 min. The cell pellets were collected by centrifugation, resuspended in 1 mL of SAP buffer (0.1% saponin, 0.05% NaN3 in Hanks’ balanced salt solution). Then, 10 µL of phospho-EGFR\textsuperscript{Y1068} conjugate solution was added and the mixture was incubated for 45 min at room temperature in the dark. Finally, the cells were resuspended in 400 µL of PBS for analysis by flow cytometry. All flow cytometry data were acquired on a BD FACSCalibur (BD Biosciences) and analyzed by the FlowJo software (Tree Star, San Carlos, CA).

Statistical analysis

Experimental data were presented as mean ± SEM of at least three independent replicates through analyzing with GraphPad Prism 5 (GraphPad Software, La Jolla, CA) and assessing comparisons between different groups by Student’s t-test and one-way analysis of variance. Differences were considered significant at values of \( P < 0.05 \).

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Conflict of interest

None declared.

Abbreviations

DMEM, Dulbecco’s modified Eagle’s medium; Dox, doxycycline; EGFR, epidermal growth factor receptor; GlcNAc, N-acetyl glucosamine; GPT, GlcNAc phosphotransferase; HCC, hepatocellular carcinoma; L-PHA, leukoagglutinating phytohemagglutinin; Mgat5, N-acetylglucosaminyltransferase V; PAK1, p21-activated kinase 1; PBS, phosphate buffered saline; ROS, reactive oxygen species; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SW, swainsonine; WGA, wheat germ agglutinin.

References


