Axl glycosylation mediates tumor cell proliferation, invasion and lymphatic metastasis in murine hepatocellular carcinoma

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Abstract

AIM: To investigate the effects of Axl deglycosylation on tumor lymphatic metastases in mouse hepatocellular carcinoma cell lines.

METHODS: Western blotting was used to analyze the expression profile of Axl glycoprotein in mouse hepatocellular carcinoma cell line Hca-F treated with tunicamycin and PNGase F 3-(4,5)-dimethylthiazol(2-yl)-3,5-diphenyltetrazolium bromide (MTT) assay, extracellular matrix (ECM) invasion assay (in vitro) and tumor metastasis assay (in vivo) were utilized to evaluate the effect of Axl deglycosylation on the Hca-F cell proliferation, invasion and lymphatic metastasis.

RESULTS: Tunicamycin and PNGase F treatment markedly inhibited Axl glycoprotein synthesis and expression, proliferation, invasion, and lymphatic metastasis both in vitro and in vivo. In the MTT assay, proliferation was apparent in untreated Hca-F cells compared with treated Hca-F cells. In the ECM invasion assay (in vitro), treated cells passed through the ECM gel in significantly smaller numbers than untreated cells (tunicamycin 5 μg/mL: 68 ± 8 vs 80 ± 9, P = 0.0222; 10 μg/mL: 50 ± 6 vs 80 ± 9, P = 0.0003; 20 μg/mL: 41 ± 4 vs 80 ± 9, P = 0.0001); (PNGase F 8 h: 66 ± 7 vs 82 ± 8, P = 0.0098; 16 h: 49 ± 4 vs 82 ± 8, P = 0.0001; 24 h: 34 ± 3 vs 82 ± 8, P = 0.0001). In the tumor metastasis assay (in vivo), average lymph node weights of the untreated Hca-F group compared with treated Hca-F groups (tunicamycin 5 μg/mL: 0.84 ± 0.21 g vs 0.72 ± 0.19 g, P = 0.0001; 10 μg/mL: 0.84 ± 0.21 g vs 0.54 ± 0.11 g, P = 0.0113; 20 μg/mL: 0.84 ± 0.21 g vs 0.42 ± 0.06 g, P = 0.0008); (PNGase F 8 h: 0.79 ± 0.15 g vs 0.63 ± 0.13 g, P = 0.0766; 16 h: 0.79 ± 0.15 g vs 0.49 ± 0.10 g, P = 0.0022; 24 h: 0.79 ± 0.15 g vs 0.39 ± 0.05 g, P = 0.0001). Also, average lymph node volumes of the untreated Hca-F group compared with treated Hca-F groups (tunicamycin 5 μg/mL: 815 ± 61 mm³ vs 680 ± 59 mm³, P = 0.0613; 10 μg/mL: 815 ± 61 mm³ vs 580 ± 29 mm³, P = 0.0001; 20 μg/mL: 815 ± 61 mm³ vs 395 ± 12 mm³, P = 0.0001); (PNGase F 8 h: 670 ± 56 mm³ vs 581 ± 48 mm³, P = 0.0532; 16 h: 670 ± 56 mm³ vs 412 ± 22 mm³, P = 0.0001; 24 h: 670 ± 56 mm³ vs 323 ± 11 mm³, P = 0.0001). CONCLUSION: Alteration of Axl glycosylation can attenuate neoplastic lymphatic metastasis. Axl N-glycans may be a universal target for chemotherapy.

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INTRODUCTION

The receptor tyrosine kinases (RTKs) constitute a large family of transmembrane proteins that relay signals from extracellular growth factors into the cell.[7,8] The Tyro-Axl-Mer (TAM) subfamily shares the vitamin K-dependent ligand Gas6 (growth arrest specific 6). TAM receptors contain a combination of two immunoglobulin-like domains and dual fibronectin type III repeats in the extracellular region, and a cytoplasmic kinase domain.[5,9] The TAM receptors regulate a diverse range of cellular responses including cell survival, proliferation, autophagy, migration, angiogenesis, platelet aggregation, and natural killer cell differentiation.[5]

The Axl receptor (also called UFO, Tyro7, and Ark) is a RTK originally identified as a transforming gene in chronic myeloid leukemia.[10] Axl is expressed in various organs, including the brain, suggesting its involvement in mesenchymal and neural development.[11] Axl has been shown to have transforming potential when overexpressed during development. Axl overexpression is clearly associated with invasiveness and metastasis in several cancer cell types, including myeloid leukemia,[12,13] esophageal[14], metastatic lung,[15] metastatic colon,[16] renal cell[17], prostate[18], breast[19], gastric[20], and thyroid[21] cancers. Axl also affects multiple pathways in angiogenesis.[22] Thus, Axl may play an important role in tumor progression, although its mechanism remains unknown.

Protein glycosylation is one of the major types of posttranslational modifications that have profound biological implications.[23,24] Specific changes in the glycosylation pattern of cell surface glycoproteins have been shown to correlate with metastatic efficiency in tumor cells.[25] In particular, protein N-glycosylation is one of the most prominent biochemical alterations in tumorigenesis and metastatic spread.[26,27] A cell surface transmembrane glycoprotein, little is known about the mechanism of Axl deglycosylation.

The mouse hepatocellular carcinoma cell line Hca-F is highly aggressive, with a metastasis rate over 80%. Hca-P, on the other hand, has a lymphatic metastasis rate of less than 30%. Both cell lines are derived from 615-mice ascites-type hepatocellular carcinoma cells. Hca-F and Hca-P cells metastasize only to lymph nodes, and not extrahepatic organs. However, the relationship between Axl glycosylation and lymphatic metastasis of mouse hepatocellular carcinoma cells remains unclear.

Our aim was to investigate whether Axl glycosylation regulates lymphatic metastasis. We demonstrated a possible correlation, based upon regulation of Axl glycosylation in mouse hepatocellular carcinoma cells.

MATERIALS AND METHODS

Cell culture and animals

Mouse hepatocellular carcinoma cell lines Hca-F and Hca-P, grown and stored in our institution (Department of Pathology, Dalian Medical University) were cultured in 90% Roswell Park Memorial Institute (RPMI)-1640 (Gibco) and supplemented with antibiotics (1 × penicillin/streptomycin 100 U/mL, Gibco) and 10% fetal bovine serum (FBS) (Gibco). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. 615-mice (8 wk old males) were obtained from the Experimental Animal Center of Dalian Medical University.

Whole protein extract

10⁴ cells were centrifuged at room temperature at 1000 × g for 10 min. Cells were rinsed twice with phosphate buffered saline (PBS) at 1000 × g for 5 min, and lysed with a protease inhibitor cocktail (whole protein extraction kit KGP2100, KeyGEN). Cells were suspended on a swing bed at 4 °C for 15 min, and centrifuged at 4 °C at 14 000 × g for 15 min. Protein concentration of the whole cells was measured with a bicinchoninic acid protein assay kit (KGPBCA, KeyGEN).

Western blotting analysis

Western blotting analysis was performed to evaluate Axl (with or without tunicamycin or PNGase F treatment) protein levels. Extracted proteins were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto polyvinylidene fluoride membranes (Pall Corporation). After blocking for 2 h with 5% skimmed milk in PBS containing 0.1% Tween 20 (PBST), membranes were incubated with rabbit anti-mouse Axl polyclonal antibody (Santa Cruz Biotech Inc., 1/200 diluted) overnight in 5% powdered skim milk buffer, washed thrice with PBS with 0.1% Tween 20, and then incubated with secondary antibody anti-rabbit-HRP (Santa Cruz Biotech Inc., 1/3000 diluted). Glyceraldehyde-3-phosphate dehydrogenase antibody (Santa Cruz Biotech Inc., 1/200 diluted) was used as controls. All blot analysis was performed with a ECL Western blotting kit (Amersham Biosciences, United Kingdom).

Tunicamycin treatment

To inhibit N-linked glycosylation of newly synthesized proteins, Hca-F cells were washed once with PBS and cultivated for 12 h in fresh culture media (90% RPMI 1640 supplemented with antibiotics) with or without tunicamycin (Sigma Aldrich, St. Louis, MO) in a dose-dependent manner (0 μg/mL, 5 μg/mL, 10 μg/mL, or 20 μg/mL). Cells were washed with PBS and subjected to Western blotting analysis, 3-(4,5)-dimethylthiazol-(2-yl)-2,5-diphenyltetrazolium bromide (MTT), migration in...


**Figure 1** Expression profile of Axl glycoprotein in mouse hepatocellular carcinoma cell lines. A: Axl glycoprotein levels by Western blotting analysis in Hca-P and Hca-F cell lines. Relative signal intensities of Axl protein were compared with GAPDH by LabWorks (TM ver.6.0, UVP; Biolimaging Systems, *P < 0.05 vs untreated Hca-F cells); B: Hca-F cells were treated with 0 μg/mL, 5 μg/mL, 10 μg/mL, and 20 μg/mL tunicamycin for 12 h. Total protein extracts were loaded for each sample; C: Hca-F cell protein was deglycosylated with 12 units of PNGase F in lysis buffer. Probes were incubated at 37 °C in a time-dependent manner (0 h, 8 h, 16 h, 24 h). Protein was separated on a gel for Western blotting Analysis. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins were transferred to a polyvinylidene fluoride membrane, and were detected by rabbit anti-mouse Axl polyclonal antibody. GAPDH blotting was used as the control. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

**RESULTS**

**Expression profile of Axl glycoprotein in mouse hepatocellular carcinoma cell lines**

Axl glycoprotein relative expression was determined by Western blotting analysis using whole-cell extracts (Figure 1A). Axl expression varied among cell lines, with higher and lower levels in Hca-F and Hca-P cells, respectively (Figure 1A, *P < 0.05*).

Tunicamycin, an inhibitor of endogenous N-linked glycosylation of newly synthesized proteins, was used to inhibit Axl glycosylation of Hca-F cells. Treatment in a dose dependent manner (0 mg/mL, 5 mg/mL, 10 mg/mL, and 20 mg/mL) for 12 h showed N-linked glycosylation to be highly sensitive to tunicamycin inhibition (Figure 1B).

Axl appears as broad bands, with molecular weights ranging from 60 kDa to 140 kDa. With tunicamycin treatment, 130 kDa Axl band density decreased, 60 kDa
To further evaluate whether Axl deglycosylation was essential for tumor lymphatic metastasis in vivo, we tested the effect of Axl deglycosylation on the metastatic ability of Hca-F cells in mice peripheral lymph nodes. Treated and untreated Hca-F cells were injected in the footpads of 615-mice. After 3 wk’ inoculation, a significant reduction in positive lymph nodes in the deglycosylation groups was observed, compared with untreated controls (Figure 4). Average lymph node weights of the untreated Hca-F group compared with dose-adjusted tunicamycin treated Hca-F groups (5 μg/mL: 0.84 ± 0.21 g vs 0.72 ± 0.19 g, P = 0.3237; 10 μg/mL: 0.84 ± 0.21 g vs 0.54 ± 0.11 g, P = 0.0113; 20 μg/mL: 0.84 ± 0.21 g vs 0.42 ± 0.06 g, P = 0.0008) (Figure 4A right). The average lymph node volumes of these groups were 815 ± 61 mm$^3$ vs 680 ± 59 mm$^3$, P = 0.0613; 815 ± 61 mm$^3$ vs 580 ± 29 mm$^3$, P = 0.0001; 815 ± 61 mm$^3$ vs 395 ± 12 mm$^3$, P = 0.0001 (Figure 4A left). The average lymph node volumes of these groups were: 815 ± 61 mm$^3$ vs 680 ± 59 mm$^3$, P = 0.0613; 815 ± 61 mm$^3$ vs 580 ± 29 mm$^3$, P = 0.0001; 815 ± 61 mm$^3$ vs 395 ± 12 mm$^3$, P = 0.0001 (Figure 4B right). These results demonstrate Axl deglycosylation may reduce Hca-F cells to peripheral lymph nodes in vivo.

**DISCUSSION**

Axl has garnered attention because of its high expression in many tumor cells, and its key role in neoplastic invasion and metastasis. In this study, we demonstrated Axl protein expression varied based on antineoplastic treatment of mouse hepatocellular carcinoma cell lines Hca-F and Hca-P. We found Axl protein expression to be higher in Hca-F cells, which have high lymphatic metastasis potential compared with Hca-P cells, which have low lymphatic metastasis potential. This confirms previ-
ously reported findings of Axl overexpression in highly invasive lung adenocarcinoma cell lines, compared with their less invasive counterparts. This suggests that high Axl expression may be associated with tumor lymphatic metastasis, and that Axl may be associated with tumor metastatic potential.

In our study, we achieved Hca-F deglycosylation with two methods. First, we inhibited N-glycan biosynthesis with tunicamycin; secondly, we extracted protein in the presence of PNGase F enzyme, which digests N-glycans. Both treatments resulted in significant effects on cell surface N-glycans by Western blotting assays.

Among post-translational modification reactions involving proteins, glycosylation is the most common; nearly 50% of all proteins are glycosylated. Alterations of glycan structures are frequently observed in various cancer cells, and this appears to be one association in cancer invasion and metastasis. We found Axl deglycosylation to be a possible factor in tumor progression, including cell proliferation, invasion, and lymphatic metastasis. In this study, we detected a significant inhibition of proliferation and invasion in Axl deglycosylated Hca-F cells in vitro, by both MTT and extracellular matrix assays. These results confirmed prior reports that cell proliferation requires growth factors signalling through cell surface glycoprotein receptors, which may be inactive when underglycosylated. Although our findings support the role of Axl deglycosylation in reducing cell proliferation and invasion in vitro, its mechanism had not been elucidated. Further experiments showed that Axl deglycosylation led to a significant reduction in metastatic lymph node burden in vivo. These results were consistent with previous reports of changes in N-linked oligosaccharide branching associated with malignancy and metastasis.

Figure 3 Axl deglycosylation alters the invasive ability of Hca-F cells in vitro. In vitro ECM Matrix gel analysis. Wright-Giemsa staining results of the lower surface filter showed that the cells passed through the filter and attached to the lower side (400 ×). The average number of cells invading the filter was counted. Cells treated with tunicamycin (A) or PNGase F (B) were significantly less invasive (P < 0.05 vs untreated Hca-F cells) than untreated Hca-F cells. Data was obtained in triplicate.
Some authors reported that the addition of exogenous Gas6 mediated the migration and invasion of Hca-F cells both in vitro and in vivo through the Axl pathway\cite{28}. RNAi-mediated knockdown of Axl expression decreased the ability of YAP-expressing MIHA cells and of the primary HCC cell line to proliferate and invade\cite{29}. In our study, we were unable to elucidate the mechanism by which Axl deglycosylation inhibits lymphatic metastasis in murine Hca-F cells. However, in many glycoproteins, N-linked oligosaccharides contribute to the folding, stability, and biological function of adhesion molecules and growth factor receptors on cell surfaces\cite{30-32}. An increasing body of evidence indicates that glycoprotein glycans are involved in the regulation of cellular functions, including cell-cell communication and signal transduction\cite{33,34}. The products of N-acetylglucosaminyltransferase (GnT)-IV, GnT-V and 1,6-fucosyltransferase (1,6-FucT) are all increased in hepatocellular carcinoma\cite{35}. The presence of 1,6-GlcNAc structures in N-glycans and the expression of GnT-V, which catalyzes the addition of the 1,6-branching, were shown to promote metastasis\cite{36-39}. At the very least, these reports demonstrate the relationship between metastasis and N-glycans to be extremely complicated. This area requires additional research.

In conclusion, we have found a role of Axl glycosylation in mediating tumor cell proliferation and invasion, and have provided the first evidence that Axl deglycosylation is required for lymphatic metastasis in murine Hca-F cells. Tunicamycin (μg/mL) and PNGase F (25 U, h) were used to inhibit N-glycosylation and remove N-linked sugars, respectively. Unjected and treated Hca-F cells were injected into the footpads of 615-mice. After 3 wk of infection, the mice were sacrificed and axillary lymph nodes isolated, weighed, measured, and photographed. A significant reduction in mean tumor weight (n = 6) of Axl tumor deglycosylation was observed, as compared with untreated Hca-F cells (P < 0.05 vs untreated Hca-F cells).

**Figure 4** Axl deglycosylation inhibits the ability of Hca-F cells to metastasize to peripheral lymph nodes in vivo. Untreated and treated Hca-F cells were injected into the footpads of 615-mice. After 3 wk of infection, the mice were sacrificed and axillary lymph nodes isolated, weighed, measured, and photographed. A significant reduction in mean tumor weight (n = 6) of Axl tumor deglycosylation was observed, as compared with untreated Hca-F cells (P < 0.05 vs untreated Hca-F cells).
hepatocellular carcinoma cell lines. These results may at least partially explain the role of Axl glycosylation in the promotion of lymphatic metastasis. This study may provide new insights into regulatory mechanisms of mouse hepatocellular carcinoma with lymphatic metastasis.

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