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Plexin A1 signaling confers malignant phenotypes in lung cancer cells

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ABSTRACT

Aberrant changes to several signaling pathways because of genetic mutations or increased cytokine production are critical for tumor cells to become malignant. Semaphorin 3A (SEMA3A) acts as a bivalent factor that suppresses or promotes tumor development in different pathological backgrounds. Previously, we showed that SEMA3A positively regulated the proliferative and glycolytic activities of mouse-derived Lewis lung carcinoma (LLC) cells. Plexins A1-A4 (PLXNA1-PLXNA4) are SEMA3A receptors; however, it is not known which subtype is critical for oncogenic SEMA3A signaling. We used LLC cells to investigate the role of PLXNA1 in oncogenic SEMA3A signaling. Using short hairpin RNA-mediated knockdown, we investigated the effects of constitutive inhibition of *Plxna1* on cell proliferation, metabolic dependency, and epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) sensitivity. We found that *Plxna1* knockdown did not affect apoptosis but resulted in decreased cell proliferation and reductions in mRNA expression levels of proliferation-marker genes, such as *Ccnd1*, *Pcna*, and *Myc*. In addition, we found decreased mRNA expression levels of glycolysis-associated genes, such as *Pkm2* and *Ldha*, and decreased lactate production. In contrast, we found no changes in the mRNA expression levels of oxidative phosphorylation-associated genes, such as *Cycs*, *Cox5a*, and *Atp5g1*. We found that *Plxna1* knockdown conferred resistance to glucose starvation but increased cytotoxicity to oligomycin. *Plxna1* or *Sema3a* knockdown caused an increased sensitivity to the EGFR-TKIs gefitinib and erlotinib, in Lewis lung carcinoma (LLC) cells. These findings demonstrate that PLXNA1 mediates the acquisition of malignant phenotypes induced by autocrine SEMA3A signaling.

1. Introduction

Despite recent improvements in treatment and diagnosis, lung cancer, particularly non-small-cell lung cancer (NSCLC), is the leading cause of cancer-related death. Mutations or increased expression in the epidermal growth factor receptor gene (*EGFR*) are frequently found in patients with NSCLC. EGFR-specific tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, provide the best initial therapeutic response in patients with NSCLC, but this therapeutic effect is reduced because of acquired resistance [1]. EGFR activates several downstream signaling pathways shared by other cytokines. Previous studies found that hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and interleukin 6 (IL6) stimulate these downstream pathways, including PI3K/AKT/mTOR and JAK/STAT signaling that result in EGFR-TKI resistance [2]. Although multiple mechanisms of acquired resistance to EGFR-TKIs have been reported, the molecular machinery that causes the decreased therapeutic response has not been fully defined.

Semaphorins are membrane-anchored or secreted proteins, which were originally discovered as axon guidance factors during the development of the central nervous system. Recently, associations between semaphorins and their receptors, plexins, have been associated with various pathophysiological disorders, including lung cancer and bone diseases [3]. In addition, there is increasing evidence that semaphorins, in particular Class 3 semaphorins, have critical roles in tumor progression by regulating angiogenesis, metastasis, and tumor cell survival [4,5]. Semaphorin 3A (SEMA3A) is a secreted semaphorin that exerts its effects by binding to its co-receptor neuropilin1 (NRP1) or to the plexin family of receptors,

Abbreviations: CSC, cancer stem cell; EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor; LLC, Lewis lung carcinoma; NSCLC, non-small-cell lung cancer; shRNA, short hairpin RNA.

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plexin A1-A4 (PLXNA1-PLXNA4). SEMA3A functions not only as a regulator of axonal extension and bone metabolism, but also as a tumor suppressor by inhibiting angiogenesis and metastasis [6–9]. However, some studies found that SEMA3A promoted tumor progression by increasing tumor angiogenesis and invasion [10,11]. In addition to the complicated role of SEMA3A in cancer, it remains unclear through which PLXNA receptor SEMA3A exerts its oncogenic or tumor-suppressive effects.

In cancer research, cell metabolism including glycolysis has been widely analyzed owing to its critical role in tumorigenesis [12,13]. Although a positive correlation of cancer stem cell (CSC) aggressiveness with glycolytic activity has been reported [14], targeting glycolysis is also demonstrated to spare CSCs that are less glycolytic than differentiated cells [15,16]. Thus, there is the need to identify the regulatory factors involved in cell metabolism to understand fully the role of glycolysis in cancer.

Previously, we reported that *Sema3a* knockdown caused decreased cell proliferation and induced a metabolic shift from glycolysis to oxidative phosphorylation in mouse-derived Lewis lung carcinoma (LLC) cells [17,18]. To identify the critical receptor responsible for oncogenic SEMA3A signaling, we inhibited PLXNA1 function by knockdown using short hairpin RNA (shRNA), and investigated the effects on proliferative capacity, metabolic dependency, and EGFR-TKI sensitivity.

2. Material and methods

2.1. Cell culture

LLC cells were provided from AntiCancer Japan. Cells were maintained in Dulbecco's modified Eagle Medium (DMEM) (Sigma-Aldrich) containing 10% fetal bovine serum (FBS) (JRH Biosciences) and 50 U/ml penicillin/0.5% streptomycin (Life Technologies) at 37 °C in a humidified 5% CO_2 atmosphere. Following 4 days of culture, cells were trypsinized using 0.05% Trypsin/EDTA (Life Technologies) and re-seeded on collagen-coated culture plates (Iwaki).

2.2. Lentiviral infection

pLKO.1/TRC1-based MISSION short hairpin RNA (shRNA) Lentiviral Transduction Particles were purchased from Sigma-Aldrich. The following clones were used: TRCN0000067328 (shSema3a#1), TRCN0000067329 (shSema3a#2), TRCN0000079188 (shPlxna1#1), and TRCN0000079189 (shPlxna1#2). Lentivirus encoding a scrambled shRNA was used as a control. LLC cells were incubated with medium containing shRNA-encoding lentivirus for 24 h, followed by a 24-h incubation in lentivirus-free medium. LLC cells were selected using 50 ng/ml puromycin (Wako). shRNA-expressing LLC cell lines identified as LLC/shSema3a, LLC/shPlxna1, and LLC/scramble, were maintained using the same culture medium containing 10 ng/ml puromycin.

2.3. Growth curve

LLC cells (1×10^3) were seeded and cultured on collagen-coated 96-well plates (Iwaki) at 37 °C in a humidified 5% CO_2 atmosphere. Cells were trypsinized following 2, 4, and 6 days of culture. Trypan blue (Sigma-Aldrich) negative cells were counted as viable cells.

2.4. TUNEL assay

Cells (5×10^3) were seeded on Geltrex (Life Technologies)-coated chamber slides (Matsunami, Japan) and cultured at 37 °C in a humidified 5% CO_2 atmosphere for 24 h. TUNEL staining was performed in accordance with the manufacturer's instructions (Promega). All photomicrographs were taken using a LSM700 (ZEISS). The number of TUNEL-positive cells was counted to measure apoptotic cell death.

2.5. Lactate measurement

LLC cells (1×10^3) were seeded and cultured on collagen-coated plates (Iwaki) at 37 °C in a humidified 5% CO_2 atmosphere for 24 h. Medium lactate levels were measured using the L-Lactate assay kit (Cayman). To monitor lactate levels, fluorescent products were measured using an excitation wavelength of 530–540 nm and an emission wavelength of 590 nm with an Infinite F200 Pro (TECAN).

2.6. Quantitative RT-PCR

Total RNA was extracted using TRIZOL (Life Technologies) and 1 μg was reverse-transcribed to cDNA using PrimeScript Reverse Transcriptase (Takara). Quantitative real-time PCR (RT-PCR) was performed using GoTaq® qPCR Master Mix (Promega) on a Mini-Opticon RT-PCR system (BioRad). The following cycle parameters were used: denaturation at 95 °C for 30 s followed by annealing for 30 s at 58 °C, and elongation for 30 s at 72 °C. The following sense and antisense primers were used:

Plxna1 sense 5′-GGGTGTGTGGATAGCCATCA-3′, *Plxna1* antisense 5′- GCCAACATATACCTCTCCTGTCT-3′, *Plexa2* sense 5′-AACCTGTCTGTGGTTCTGCTC-3′, *Plxna2* antisense 5′-TCCAGTCACGATTCTCAGAGT-3′, *Plxna3* sense 5′- CAGATACCACTCTGACTCACCT-3′, *Plxna3* antisense 5′-GGCCCGTAGCTCAGTTAGG-3′, *Plxna4* sense 5′-ACAGGGCACATTTATTTGGGG-3′, *Plxna4* antisense 5′-CACTTGGGGTTGTCCTCATCT-3′, *Nrp1* sense 5′-GACAAATGTGGCGGGACCATA-3′, *Nrp1* antisense 5′-TGGATTAGCCATTCACACTTCTC-3′, *Ccnd1* sense 5′-GCGTACCCTGACACCAATCTC-3′, *Ccnd1* antisense 5′-CTCCTCTTCGCACTTCTGCTC-3′, *Pcna* sense 5′-TTTGAGGCACGCCTGATCC-3′, *Pcna* antisense 5′-GGAGACGTGAGACGAGTCCAT-3′, *Myc* sense 5′-ATGCCCCTCAACGTGAACTTC-3′, *Myc* antisense 5′-CGCAACATAGGATGGAGAGCA-3′, *Pkm2* sense 5′-GCCGCCTGGACATTGACTC-3′, *Pkm2* antisense 5′-CCATGAGAGAAATTCAGCCGAG-3′, *Ldha* sense 5′-TGTCTCCAGCAAAGACTACTGT-3′, *Ldha* antisense 5′-GACTGTACTTGACAATGTTGGGA-3′, *Actb* sense 5′-GGCTGTATTCCCCTCCATCG-3′, and *Actb* antisense 5′-CCAGTTGGTAACAATGCCATGT-3′.

2.7. Glucose starvation assay

DMEM without glucose (-) (Life Technologies) containing 10% dialyzed FBS (Thermo Fisher Scientific) and 50 U/ml penicillin/0.5% streptomycin was used as the standard medium for the glucose starvation assay. Glucose was added to reach final concentrations of 0.045, 0.45, or 4.5 g/L. Cells (1×10^3) were seeded onto collagen-coated 96-well plates (Iwaki) and cultured for 4 days. Cell viability was assessed using the Cell Counting Kit (Dojindo, Japan) following the manufacturer's instructions. Briefly, cells were incubated with WST-8 at 37 °C in a humidified 5% CO_2 atmosphere for 30 min, followed by measuring optical density at 450 nm using an Infinite F200 Pro (TECAN). Values were determined as relative percentages normalized to that found in 4.5 g/L glucose-treated samples.

2.8. Oligomycin or EGFR-TKI sensitivity assay

LLC cells (1×10^3) were seeded and cultured on collagen-coated plates (Iwaki) at 37 °C in a humidified 5% CO_2 atmosphere. Cells were treated with oligomycin (Cell signaling technologies), gefitinib (Sigma-Aldrich), or erlotinib (LC laboratories) for 72 h. Drugs

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