Lin28A/let-7 oncogenic circuit is a potential therapeutic target in neurocutaneous melanosis-associated CNS tumors in children

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This study aims to describe the initial findings on the implication of Lin28A oncoprotein in rare and currently difficult to cure CNS tumors associated with neurocutaneous melanosis in children. We provide evidence, for the first time, that both pharmacological inhibition and knockdown of Lin28A leads to increase in tumor suppressor let-7, and suppression of pro-tumorigenic markers such as PI3K, IGF-1R, and PD-L1, triggering loss of tumor viability and growth in vitro. Our findings indicate that Lin28A may be a critical contributor to the oncogenesis of these tumors and targeting the Lin28/let-7 axis could potentially lead to effective future therapeutics for these patients.

Neurocutaneous melanosis (NCM) is a rare pediatric cancer-predisposing syndrome characterized by the presence of large or numerous congenital melanocytic nevi (LCMN) and malignant growth of melanocytes in the CNS. It predominantly manifests in infants and young children. Patients with symptomatic NCM have an extremely poor prognosis and there are no universally accepted treatments available for these patients. The preclinical evidence to support an effective therapeutic regimen for patients with NCM associated tumors are extremely limited to case studies. Current treatment options, such as surgery, chemotherapy, and radiation, have not advanced the outcome of high risk and symptomatic young children. Therefore, there is an urgent unmet need to understand the critical oncogenic driver pathways to identify effective druggable targets for future therapeutic options.

It has been demonstrated that several somatic missense mutations of NRAS, including the Q61K mutation observed in LCMN, are responsible for the overexpression of Lin28 oncogene and negative regulation of the lethal-7 (let-7) microRNA precursor through MYCN transcription. Lin28A is an RNA binding protein and a putative regulator of oncogenic processes that include increased cell proliferation and invasion, resulting in poor treatment response and decreased survival outcome in a number of malignancies. Mechanistically, it has been shown that Lin28A binds to the conserved sequences of the tumor suppressor microRNA pre-let-7 and blocks its maturation into let-7 and further cellular differentiation. The downregulation of let-7 is significantly associated with elevated expression of major oncogenic circuits and poor prognosis in human cancers. Recently, a number of compounds have been identified to inhibit Lin28-related functions, including the small-molecule-inhibitor, c1632. It was originally developed as an anxiolytic agent, and later described as a functional blocker of interaction between Lin28/pre-let-7, leading to the rescue and maturation of let-7. In this study, we investigate the active involvement of Lin28/let-7 oncogenic axis in NCM associated CNS tumor cells and evaluate the potential to perturb this circuit and its downstream tumor cell-driven targets. We demonstrate that both the pharmacological inhibition of Lin28 using c1632 and siRNA mediated knockdown of Lin28A leads to the maturation of let-7 and further suppression of PI3K and IGF-1R, and potentially prevents tumorigenic evasion of immune surveillance as evidenced by downregulated PD-L1. These results reveal a targetable Lin28/let-7 pathway as an upstream mediator of NCM tumor cell proliferation and immune evasion.

The cell line YP-MEL was derived from the malignant melanoma transformation of a child with NCM. We also used primary NCM tumor cells derived from a 2-year-old male with multiple, large congenital nevi who initially presented with increased hydrocephalus, intracranial pressure, probable seizures, Dandy-Walker variant and classic meningeal enhancement. Relapse occurred after 6 months of initial treatment with cyclophosphamide, temozolomide, and sorafenib. Single-cell suspension of the tumor was prepared by gentle dissociation and filtration through nylon section, cells were transferred to culture medium without delay. Cell lines, KCCF1, BT12, and BT16 that were derived from the pediatric
**Brief Communication**

**Assay:** has-let-7a: 000377) in NCM (YP-MEL and patient cells) and ETMR (BT-183) cells compared to treatment with non-targeted vector control.

**miRNA (Taqman MicroRNA**

Lentivirus transiently transfected into cells using Lipofectamine 2000 (Invitrogen), both leads to increase in the expression of neurocutaneous melanosis (NCM); NCM patient cells: derived from a 2-year old male with multiple, large congenital nevi (B) IC50 concentrations BT16: pediatric atypical-teratoid rhabdoid tumor (AT/RT) (negative controls for Lin28A expression); YP-MEL: malignant melanoma derived from tumors with multilayered rosettes (ETMR) (positive control for Lin28A expression); IMR5 and LAN1: pediatric neuroblastoma; KCCF1, BT12, and antibody (#8706; Cell Signaling Technology) at 1:2000 dilution. T47D: adult breast cancer (positive control for Lin28A expression); BT-183: embryonal treated cells harvested at 80%–90% confluency. Samples loaded with volumes for 30

**Figure 1.** (A) Lin28A expression profile in pediatric CNS cancer cell lines. SDS-PAGE on 10% polyacrylamide gel of total cell lysates from untreated cells harvested at 80%–90% confluency. Samples loaded with volumes for 30 μg protein. Lin28A protein was detected using the anti-Lin28A antibody (#8706; Cell Signaling Technology) at 1:2000 dilution. T47D: adult breast cancer (positive control for Lin28A expression); BT-183: embryonal tumors with multilayered rosettes (ETMR) (positive control for Lin28A expression); IGR1: pediatric neuroblastoma; KCCF1, BT12, and BT16: pediatric atypical-teratoid rhabdoid tumor (AT/RT) (negative controls for Lin28A expression); YP-MEL: malignant melanoma derived from neurocutaneous melanosis (NCM); NCM patient cells: derived from a 2-year old male with multiple, large congenital nevi (B) IC50 concentrations of compound c1632 in panel of CNS cancer cell lines. Lin28A expression in the presence of c1632 correlates with the sensitivity of cells to Lin28A-specific inhibitor c1632. Cells were cultured in the presence of the c1632 at increasing concentrations and cell viability was measured after 96 h. All the data shown are representative of three replicates. (C) Effect of compound c1632 (#6688; Tocris Bioscience) on Lin28A protein levels, 96 h post-treatment. Lin28A positive cell lines (T47D, BT-183 and YP-MEL) demonstrated successive reduction in Lin28A protein in the presence of c1632. Lin28A expression levels in the presence of c1632 in each cell line were calculated by normalizing (β Actin protein loading controls. (D) Pharmacological inhibition of Lin28A using c1632 and siRNA targeting Lin28A (OnTarget Plus SMARTpool L-028584-01; Thermo Scientific) transiently transfected into cells using Lipofectamine 2000 (Invitrogen), both leads to increase in the expression of let-7a miRNA (Taqman MicroRNA Assay; has-let-7a: 000377) in NCM (YP-MEL and patient cells) and ETMR (BT-183) cells compared to treatment with non-targeted vector control.
brain tumor atypical-teratoid rhabdoid tumor (AT/RT) and two neuroblastoma cells lines, IMR5 and LAN1, pediatric embryonal tumor with multilayered rosettes (ETMR) cell line BT-183 (a gift from Dr. Jennifer Chan) exhibiting tumor neurospheres, and an adult breast cancer cell line (T47D) with known high expression of Lin28A were used as controls.6,7 Western blotting was used to identify Lin28A expression in the NCM tumor cell line YP-MEL and NCM patient primary tumor cells as well as the control cells (Figure 1A). The absence or very low expression of Lin28A in the panel of pediatric AT/RT and neuroblastoma cell lines were correlated with the lack of sensitivity towards c1632 in vitro (Figure 1B). However, the Lin28A-positive cells, YP-MEL, BT-183, and T47D, showed sensitivity to c1632 under the same experimental conditions. The capability of c1632 to reduce Lin28A protein and subsequently release matured let-7 miRNA,3,8 was assessed by treating YP-MEL, T47D and BT-183 positive control cells with 0, 60, and 100 µM of c1632 (Figure 1C and D). The pharmacological inhibition using c1632 at 100 µM and knockdown of Lin28A by siRNA also increased the matured miRNA let-7a in YP-MEL, BT-183, and NCM patient cells (Figure 1D). Furthermore, the depletion of Lin28A in YP-MEL and BT-183 lead to the downregulation of phosphorylated PI3-Kinase p85 subunit, IGF-1R and PD-L1 (Figure 1E and F). Whereas, the inhibition of PI3K using its known inhibitors PIK-75 and GDC-0980 at 10 µM did not significantly affect the endogenous expression of Lin28A (Figure 1E).

Data presented in this study show a novel and critical finding that the Lin28A/let-7 tumorigenic pathway may be an important contributor to the oncogenesis of NCM and perturbation of this axis could potentially hold promise to delineate effective future therapeutics. Lin28 is a positive regulator and activator of oncogenic signaling mechanisms such as PI3K, IGF-1R, and PD-L1 pathways.7,10 The inhibition or knockdown of Lin28A causing an increase in let-7 miRNA in the NCM tumor cells is a significant therapeutic event because let-7 is a known differentiation marker and tumor suppressor, and is directly involved in the downregulation of tumorigenic markers such as PI3K, IGF-1R and PD-L1 in these cells. This is in agreement with findings from other malignant tumors.7,8 Although there is a possibility that the NCM tumors harbor multiple sets of oncogenes,1 we observed that a direct disturbance in the Lin28/let-7 oncogenic circuit caused a disruption in multiple tumorigenic events, which could be exploited for therapeutic benefits. The previously known sensitivity of NCM and ETMR tumor cells to PI3K and IGF-1R inhibitors,17 correlates with the reduction in PI3K observed in our study but failed to inhibit Lin28A suggesting the upstream role of this oncoprotein.

In conclusion, we present that the direct targeting of this Lin28/let-7 pathway in NCM tumor cells can efficiently rescue the maturation of miRNA let-7 tumor suppressor, causing significant reduction of the oncogenic hallmarks such as tumor initiation and progression, tumorigenic evasion of immune surveillance and survival. Future investigations into a new class of therapeutics based on c1632 modeling could potentially improve the therapeutic efficacy for Lin28/let-7 targeting.3,8

Keywords

CNS cancer | Lin28A | let-7 | malignant melanoma | neurocutaneous melanosis

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