L1-CAM knock-down radiosensitizes neuroblastoma IMR-32 cells by simultaneously decreasing MycN, but increasing PTEN protein expression

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Abstract. Childhood neuroblastoma is one of the most malignant types of cancers leading to a high mortality rate. These cancerous cells can be highly metastatic and malignant giving rise to disease recurrence and poor prognosis. The proto-oncogene myelocytomatosis neuroblastoma (MycN) is known to be amplified in this type of cancer, thus, promoting high malignancy and resistance. The L1 cell adhesion molecule (L1-CAM) cleavage has been found upregulated in many types of malignant cancers. In the present study, we explored the interplay between L1-CAM, MycN and PTEN as well as the role played by PDGFR and VEGFR on tumorigenicity in neuroblastoma cells. We investigated the effect of L1-CAM knock-down (KD) and PDGFR/VEGFR inhibition with sunitinib malate (Sutent®) treatment on subsequent tumorsphere formation and cellular proliferation and migration in the MycN-amplified IMR-32 neuroblastoma cells. We further examined the effect of combined L1-CAM KD with Sutent treatment or radiotherapy on these cellular functions in our cells. Tumorsphere formation is one of the indicators of aggressiveness in malignant cancers, which was significantly inhibited in IMR-32 cells after L1-CAM KD or Sutent treatment, however, no synergistic effect was observed with dual treatments, rather L1-CAM KD alone showed a greater inhibition on tumorsphere formation compared to Sutent treatment alone. In addition, cellular proliferation and migration were significantly inhibited after L1-CAM KD in the IMR-32 cells with no synergistic effect observed on the rate of cell proliferation when combined with Sutent treatment. Again, L1-CAM KD alone exhibited greater inhibitory effect than Sutent treatment on cell proliferation. L1-CAM KD led to the simultaneous downregulation of MycN, but the upregulation of PTEN protein expression. Notably, radiotherapy (2 Gy) of the IMR-32 cells led to significant upregulation of both L1-CAM and MycN, which was abrogated with L1-CAM KD in our cells. In addition, L1-CAM KD radiosensitized the cells as exhibited by the synergistic effect on the reduction in cell proliferation compared to radiotherapy alone. Taken together, our data show the importance of L1-CAM interplay with MycN and PTEN on the MycN amplified neuroblastoma cell radioresistance, proliferation and motility.

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Introduction

Neuroblastoma is an embryonal tumor of the sympathetic nervous system, arising during fetal or early postnatal life from sympathetic cells derived from the neural crest (1). In children, it is the most common extracranial solid tumor, representing approximately 7% of childhood malignancies and up to 15% of childhood cancer mortality (2). The median age of diagnosis is 22 months (3) and it rarely presents in adolescence and adulthood (4). Neuroblastoma is an extremely heterogeneous disease (5); tumors can spontaneously regress or mature, regardless of therapy, or display a very aggressive, malignant phenotype that is poorly responsive to current intensive, multimodal therapy (1). Management of this malignancy remains a challenge. Recently, we have witnessed a rise in the incidence of neuroblastoma with a very poor prognosis in children diagnosed after the age of 2 years, where the 5-year survival rate is only 38% (2). The heterogeneous nature of this disease makes treatment options tricky because different treatment strategies are indicated on a case by case basis, depending on the nature of the disease. Neuroblastoma prognosis depends on various factors, including the child's age, histological characteristics of the tumor, magnitude of genetic abnormalities and disease stage at diagnosis (6).

Various transcription factors have been implicated in neuroblastoma pathogenesis contributing to its uncontrolled cell proliferation, including MycN (2). The MycN phosphoprotein is a member of the MYC family of transcription factors, encoded by the MYCN oncogene (2), which drives cell proliferation. While its expression is very abundant in early embryonic development and in early neonatal life (7),

its expression in adult cells becomes generally confided to B lymphocytes.

MycN expression arises again in many cancers and is found to be amplified in up to 20% of neuroblastoma tumors (8), and is associated with advanced stages of disease, rapid tumor progression and poor treatment outcome. As such and since its first identification in 1983 (5), MycN has gained a reputation as being a powerful predictor of disease prognosis and mortality in highly aggressive, malignant tumors, and therefore, been the attractive target for therapeutic intervention in numerous cancers including childhood neuroblastomas.

Another interesting molecule found to be expressed in numerous tumors is the L1-cell adhesion molecule (L1-CAM). First identified in 1984, the glycoprotein's role in the development of the nervous system has been well-established (9). L1-CAM participates in two different but important physiological processes: on the one hand, it can act as a cell adhesion molecule that forms the 'glue' between cells; and on the other hand, it can promote cellular motility that drives cell migration during neural development, but unfortunately also induces metastasis of human cancers (10). L1-CAM feeds into the MAPK signal transduction pathway to achieve its functions (11), and it also associates with casein kinase 2 (12) which inhibits the functions of the tumor suppressors PTEN and p53, implicated in neurodegeneration and functional recovery after injury (13,14). Upregulation of L1-CAM protein expression subsequently leads to the downregulation of PTEN and p53 protein expression, thereby promoting neurite outgrowth and neuronal survival (15).

Various cancers expressing high levels of L1-CAM also exhibit enhanced survival and proliferation potential of the cancer cells (16). L1-CAM works together with various receptor tyrosine kinases to promote tumor growth, metastasis and angiogenesis. In fact, the vascular endothelial growth factor receptor (VEGFR) has been reported to associate with L1-CAM and induce proliferation, migration and angiogenesis (tube formation) in bovine aortic endothelial cells (17). Furthermore, Schröder *et al* (18), reported that overexpression of L1-CAM in the high grade breast cancers correlated with overexpression of VEGFR, human epidermal growth receptor 2 (Her-2) and the plasminogen activator inhibitor 1 (PAI-1) and was a negative prognostic factor. In addition to stimulating cancer proliferation and migration, L1-CAM has also been reported to induce the maintenance of self-renewal and pluripotency, two properties typical of stem cells (19).

Bao *et al* (20) demonstrated that the $CD133^{+}$ glioma stem cells also expressed higher levels of L1-CAM compared to the $CD133^{-}$ non-stem glioma cells and that targeting of L1-CAM for transcriptional knock-down led to the decreased expression of the transcription factor Olig2 and upregulated the expression of the $p21^{WAF1/CIP1}$ tumor suppressor. To determine its role on *in vivo* malignancy, the authors injected the L1-CAM knocked-down cells into mice or targeted L1-CAM for knock-down in mice with established *in vivo* tumors. In both groups, tumor growth was inhibited and survival in tumor-bearing mice was increased with L1-CAM knock-down (20). Furthermore, L1-CAM was reported in many cancers, to be concentrated in the peripheral cells, sustaining invasion and metastasis. An abundance of L1-CAM, therefore, is associated with poor patient prognosis (17).

In neuroblastomas of children, in contrast to most adult cancers, the expression of L1-CAM induces improvement and not worsening of prognosis (21). However, other pediatric cancers such as osteosarcoma, showed a negative correlation between the expression of L1-CAM and prognosis, where higher expression of L1-CAM correlated with disease progression and poorer prognosis in these children (22).

In the present study, we investigated the role of L1-CAM and the receptor tyrosine kinase inhibitor, sunitinib malate (Sutent), on neuroblastoma cell migration, tumorsphere formation and proliferation. We specifically explored its expression and bio-function in the malignant, MycN-amplified human neuroblastoma IMR-32 cells. We examined the effect of L1-CAM knock-down on the expression of MycN and PTEN and the subsequent effect on radio-resistance, cell proliferation, migration and tumorsphere formation and self-renewal. Tumorsphere self-renewal in a limited dilution assay is a characteristic of a cancer 'initiating cell' (23,24). In addition, these anchorage-independent cells are usually resistant to conventional antitumor therapy and can regrow large tumors both *in vitro* and *in vivo* regardless of aggressive radio- or chemotherapies (25). The MycN-amplified human IMR-32 neuroblastoma cell line normally grows as lightly adhered clusters of cell colonies and if cultured in NeuroCult neural stem-cell enriching media, they would detach from the culture flask and become fully anchorage-independent, forming large tumorspheres that resist chemo- and radiotherapy (26). Chakrabarti *et al* (27) previously reported that neuroblastoma cells grown in serum-free, NeuroCult stem cell media supplemented with growth factors (EGF and FGF) underwent phenotypic transformation from anchorage-dependent, adhered cells to anchorage-independent floating tumorspheres that overexpressed tumorigenic proteins and were treatment-resistant. The authors reported that these cells use their plastic adaptive phenotypic transformation as a tool to survive unfavorable selection pressure. We attempted herein, to determine whether L1-CAM played a significant role on the IMR-32 cells' capability to undergo anchorage-independence and form large tumorspheres that can self-renew in a limited dilution assay. We further attempted to determine if L1-CAM played a significant role on the IMR-32 cell proliferation and migration.

Materials and methods

Human cell lines. The human neuroblastoma/neuro-epithelioma cell lines IMR-32 (MycN-amplified) and SK-N-SH (non-MycN-amplified) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in EMEM containing 10% fetal bovine serum (FBS), 0.5% penicillin/streptomycin, 10% L-glutamine.

Derivation of anchorage-independent tumorspheres

Media. NeuroCult complete media was used to grow the IMR-32 cells as anchorage-independent tumorspheres. NeuroCult complete is composed of NeuroCult neural stem cell (NSC) basal medium, 1/10 with NeuroCult NSC proliferation supplements, plus 20 ng/ml rh EGF, 10 ng/ml rh FGF-b and 2 μg/ml Heparin (StemCell Technologies, Inc., Vancouver, BC, Canada). EMEM (Sigma-Aldrich, St. Louis, MO, USA) with 10% FBS was used to grow cells in a monolayer of anchorage-dependent, adhered phenotype.

Tumorsphere formation and self-renewal assay. Cells were grown in NeuroCult complete media until large tumorspheres formed. Pelleted tumorspheres were dissociated into a single-cell suspension, and cell viability determined on a hemocytometer using trypan blue. Single cells were plated into 96-well plate and cultured in NeuroCult Complete or EMEM + 10% FBS for 10-14 days. Self-renewal capacity was determined if a single cell grown in NeuroCult media formed a large tumorsphere starting as early as day 4-5 after reseeding and reaching full capacity by days 10-14. Tumorsphere sizes were measured in several random field images taken using a Zeiss camera mounted onto an inverted microscope and the AxioVision Systems software (Carl Zeiss, Oberkochen, Germany).

siRNA transfection. Four L1-CAM siRNA oligonucleotides were purchased from Qiagen and used to knock-down L1-CAM protein expression in the IMR-32 cells using Opti-MEM (Lonza, Bassel, Switzerland) and HiPerFect (Qiagen, Hilden, Germany) transfection reagent. Cells were grown in a 6-well plate until they reached 70-80% confluency after which they were transfected with either L1-CAM siRNA at a concentration of 2 μM for 6 h or with mock-transfection reagent. The transfection media was then removed and cells were cultured in their respective media for the length of the experiment.

Radiotherapy. Cells were grown to ~70-80% confluency in their respective media, then collected and pelleted by centrifugation. Cells were then irradiated using a single cycle of 2 Gy and then reseeded in their respective media and grown in the standard culture conditions. For western blot analysis of protein expression, cells were lysed 48 h after radiotherapy. For viability and proliferation studies, cells were plated in 96-well plates at a density of $3x10^3$ in triplicates and analyzed for the respective measures at 24, 48, 72 and 96 h post-radiotherapy.

Sunitinib malate (Sutent) treatment. Cells were grown to ~70-80% confluency in their respective media, and then treated with varying doses of Sutent (0.1, 0.2, 0.4, 0.8 and 1 μM) for 24, 48, 72 and 96 h to test cell proliferation rate and tumorsphere formation over time. For cell proliferation, the cells were seeded as described below in a 96-well plate and the absorbance of WST-1 was measured at the indicated time-points. For tumorsphere renewal, 6 h after Sutent treatment, cells were dissociated and reseeded in a limited-dilution assay (LDA) in NeuroCult neural stem-cell media with Sutent to determine tumorsphere self-renewal from single cells over 7-14 days.

Cell proliferation and viability assays. Proliferation rate was determined by the colorimetric absorbance of the WST-1 assay (cat # ab155902; Abcam, Cambridge MA, USA), at 24, 48, 72 and 96 h post-treatment. This assay measures the cleavage of the tetrazolium salt WST-1 into formazan by cellular mitochondrial dehydrogenases. This leads to more formazan dye formation which can be quantified by measuring the absorbance at 450 nm. The average absorbance of each group was graphed using Microsoft Excel at 24, 48, 72 and 96 h post-radiation. Cell viability was determined by hemocytometer cell counting using trypan blue. The mean ± the standard deviation of the mean of multiple experiments was graphed.

Cell migration assays. IMR-32 cells with or without L1-CAM KD were plated in a tissue culture treated 6-well plate at a density of ~70-80% confluence of semi-adhered monolayer, serum-deprived overnight and the next day a cell 'wound' was created in the middle of the plate using a 200 μl pipette. The cells scraped off were washed out (1x) using serum-free media and fresh media was replenished containing either 0 or 5% FBS. Images of 6-8 random fields in the scraped 'wound' were taken at the time of 'wound' induction (0 h) and again 8 h after. The area of the 'wound' in these fields was traced and measured in μM^2 using AxioVision systems. The average area in μM^2 of the 'wound' 8 h after induction was subtracted from that at time of induction (0 h) and graphed using Microsoft excel.

Immunoblot analysis. L1-CAM, PTEN and MycN protein expression was confirmed by immunoblot as previously described (28). Briefly, whole cell lysates were prepared from cells lysed in 200 μl of 1X cell lysis buffer (Cell Signaling Technology). The protein concentration was determined using the Bradford dye-binding assay (Bio-Rad Laboratories, Hercules, CA, USA). An aliquot of the lysate was mixed with an equal volume of 2X Laemmli sample buffer and heated at 97˚C for 10 min. Between 20-40 μg of total protein concentration was electrophoresed on a stain-free easy-cast 10% SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad Laboratories). Target proteins were detected using primary antibodies for L1-CAM (rabbit polyclonal ab123990), MycN (rabbit polyclonal ab24193) and PTEN (rabbit polyclonal ab31392) with reactivity to mouse, rat and human (dilution of 1:500; 1:250 and 1:500, respectively) (Abcam). Beta-actin antibody (rabbit polyclonal anti-actin, sc-130656; Santa Cruz Biotechnology, Dallas, TX, USA) was used as a loading control. The blots were incubated with the primary antibody overnight at 4˚C, washed (3x) 5 min each in TBS-Tween (0.1%) and incubated with secondary antibody 1 h at room temperature using the anti-rabbit or anti-mouse IgG HRP-conjugated (Bio-Rad Laboratories) at a dilution of 1:2,000. The blots were then washed (4x) 15 min each with 1x TBS-Tween (0.1%), incubated in Clarity Western ECL substrate (cat# 1705060; Bio-Rad Laboratories) and quantified by densitometric analysis using Image Lab software from Bio-Rad Laboratories. Protein expression of L1-CAM, PTEN and MycN was determined using western blot analysis 24 h after L1-CAM siRNA transfection. Whereas protein expression after radiotherapy was conducted 48 h after the radiotherapy (2 Gy) treatment.

Statistical analysis. Experiments were conducted in triplicates and the mean ± SD of all three experiments was calculated and plotted. A two-sided Student's t-test was used to determine statistical significance between groups. The mean ± SD of three or more experiments was derived and graphed using Microsoft Excel. Statistical significance was set at $P<0.05$.

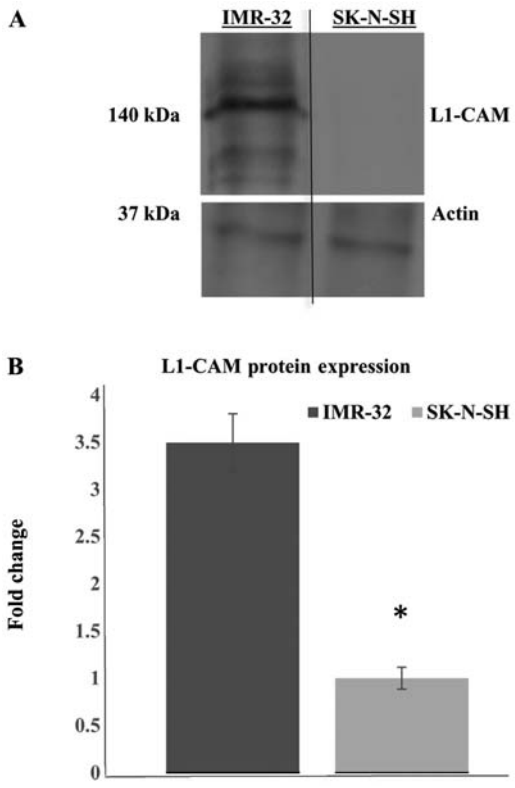

Figure 1. Western blot analysis revealed the significant upregulation of L1-CAM protein expression in IMR-32 cells. (A) Western blot images showing upregulation of L1-CAM in the IMR-32 cells compared to the SK-N-SH cells. (B) Graphical representation of the densitometric analysis of multiple western blot experiments verifying the statistically significant (*P<0.05) upregulation of L1-CAM protein expression in IMR-32 cells compared to SK-N-SH cells. Results represent the mean ± the SDM of multiple experiments.

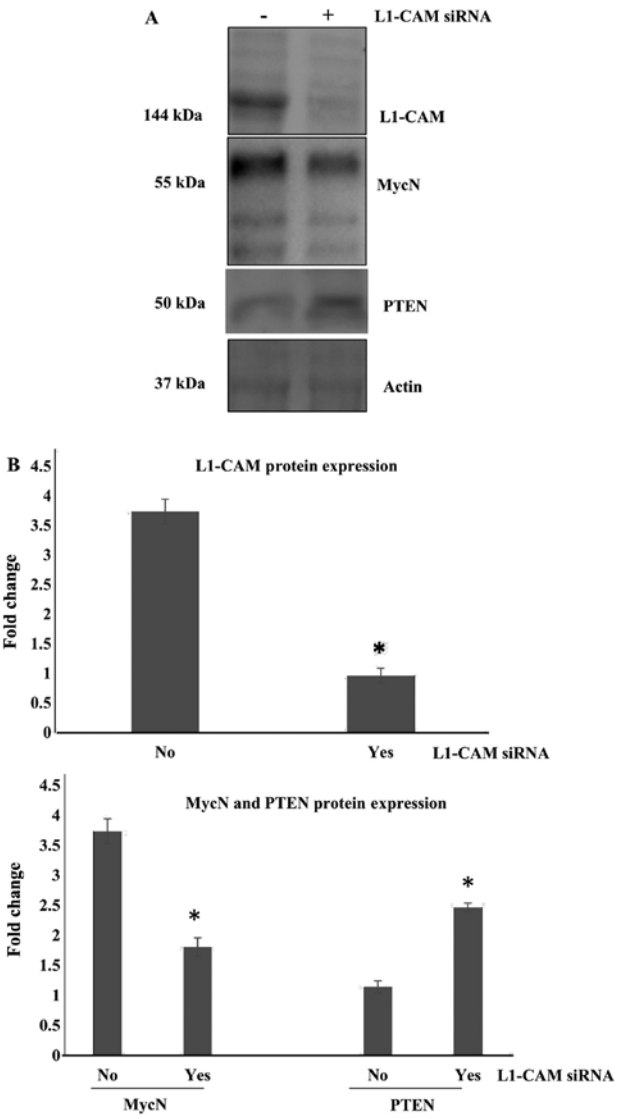

Figure 2. L1-CAM knock-down (KD) abrogates MycN, but enhances PTEN expression in IMR-32 cells. (A) Representative western blot images showing siRNA transfection of L1-CAM constructs downregulates both L1-CAM and MycN protein expressions, but upregulates PTEN protein expression in the IMR-32 cells compared to mock-transfected controls. (B) Graphical representation of the densitometric analysis showing L1-CAM siRNA transfection led to a statistically significant (*P<0.05) downregulation of both the L1-CAM and MycN, but upregulation of the PTEN protein expression in the IMR-32 cells compared to mock-transfected controls. Results represent the mean ± the SDM of multiple experiments.

Results

L1-CAM is upregulated in the MycN-amplified IMR-32 cells compared to the non-MycN amplified SK-N-SH cells. Western blot analysis revealed the protein expression of L1-CAM to be upregulated in the MycN-amplified and highly malignant IMR-32 human neuroblastoma cells, compared to the less malignant, non-MycN amplified SK-N-SH human neuroblastoma cells (Fig. 1A). Actin antibody was used as a loading control. Multiple western blot analyses and densitometric quantification of the protein bands showed a statistically significant (P<0.05) upregulation of L1-CAM protein expression (2.5-fold increase) in the IMR-32 compared to the SK-N-SH cells (Fig. 1B).

L1-CAM protein expression correlates with MycN, but inversely correlates with PTEN protein expression in IMR-32 cells. In an attempt to determine the role of L1-CAM expression in our MycN-amplified IMR-32 cells, we sought to knock-down (KD) the protein expression of L1-CAM in these cells and examine the subsequent effect on cell behavior. Small interfering RNA (siRNA) was used to KD the protein expression of L1-CAM in our IMR-32 cells which was successfully inhibited compared to the mock-transfected control cells (Fig. 2A). Notably, protein expression of MycN in these cells was also abrogated after the siRNA KD of L1-CAM (Fig. 2).

The phosphatase and tensin homolog gene PTEN is one of the most frequently mutated tumor suppressor genes in many human cancers. We previously reported that PTEN expression is negatively regulated by the protein expression and activation of the platelet-derived growth factor receptor beta (PDGFRβ) in childhood medulloblastomas (28). We therefore sought to determine whether PTEN expression would also be negatively regulated by the protein expression of L1-CAM in our cells. We found that siRNA KD of the L1-CAM protein expression in the IMR-32 cells, led to a statistically significant (P<0.05) upregulation of PTEN protein expression (Fig. 2).

L1-CAM KD significantly inhibits the radiotherapy-induced upregulation of L1-CAM and MycN protein expression.

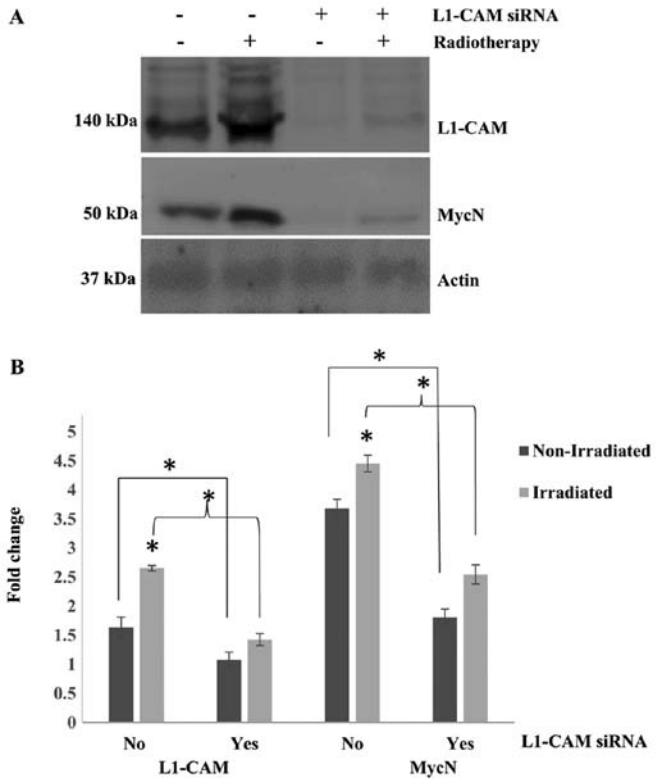

Figure 3. Radiotherapy significantly upregulated the expression of L1-CAM and MycN, which was reversed by L1-CAM KD. (A) Representative western blot images of IMR-32 cells with L1-CAM siRNA (+) or mock-transfection (-) with (+) and without (-) radiotherapy showing upregulation of L1-CAM and MycN protein expression after radiotherapy, which was abolished with L1-CAM siRNA knock-down. (B) L1-CAM and MycN protein expression showed a statistically significant upregulation (*P<0.05) after 2 Gy radiotherapy compared to non-irradiated IMR-32 cells (first and third light grey bars). siRNA transfection of L1-CAM and its statistically significant transcriptional inhibition (second and fourth dark grey bars) led to the simultaneous statistically significant (*P<0.05) downregulation of the radiotherapy-induced upregulation of L1-CAM and MycN in these cells (second and fourth light grey bars) compared to mock-transfected controls. Results represent the mean ± the SDM of multiple experiments.

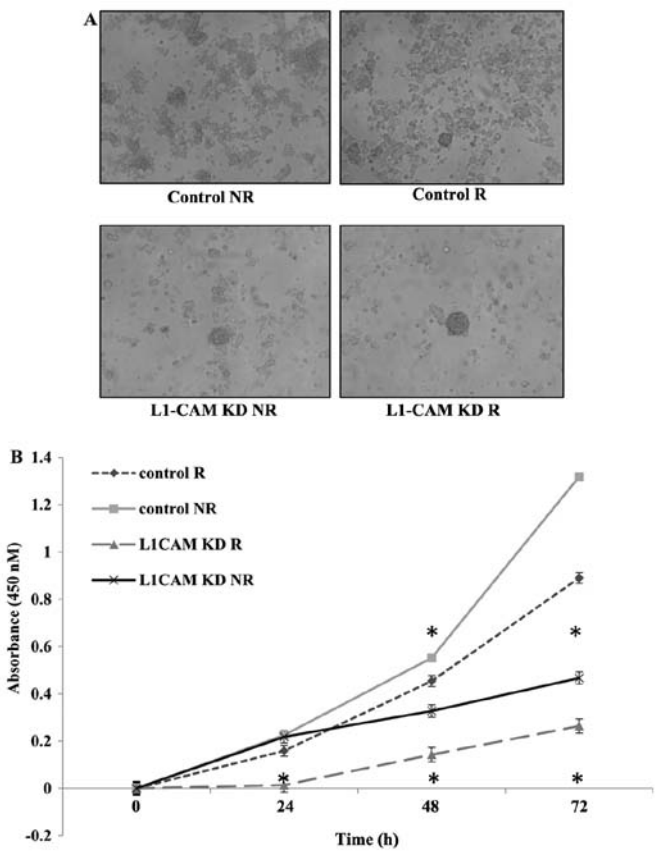

Figure 4. L1-CAM knock-down (KD) radiosensitizes IMR-32 cells by inducing a synergistic inhibitory effect on the rate of cell proliferation over 72 h. (A) Micrographic images of IMR-32 cells non-irradiated (NR) and irradiated (R) with a single dose of 2-Gy radiotherapy in L1-CAM siRNA transfected (L1-CAM KD) and mock transfected (control) cells. Images taken 48 h post-radiotherapy. (B) Radiotherapy resulted in a statistically significant inhibition (*P<0.05) in the rate of cell proliferation at 48 and 72 h in the control group. However, L1-CAM KD alone (L1-CAM KD NR) showed a more potent anti-proliferative effect than radiotherapy alone (control R). Furthermore, L1-CAM KD combined with radiotherapy (L1CAM KD R) led to a statistically significant synergistic inhibition (*P<0.001) on the rate of cell proliferation compared to radiotherapy or L1-CAM KD alone and observed as early as 24 h post radiotherapy. Results represent the mean ± the SDM of multiple experiments.

Notably, exposing our cells to a single cycle of 2-Gy radiotherapy led to statistically significant upregulation (P<0.05) of L1-CAM and MycN expression in the IMR-32 cells (Fig. 3). This effect was observed 48 h after radiotherapy treatment. The radiotherapy-induced overexpression of L1-CAM and MycN in our cells was abrogated after L1-CAM siRNA KD of protein expression. Cells with L1-CAM KD were treated with a single cycle of 2-Gy radiotherapy and then allowed to grow in culture for an additional 48 h after radiotherapy treatment. Western blot analysis 48 h after radiotherapy revealed a statistically significant inhibition (P<0.05) in the radiotherapy-induced upregulation of L1-CAM and MycN protein expression in these cells after L1-CAM KD (Fig. 3).

L1-CAM KD radiosensitizes IMR-32 cells by inducing a synergistic inhibitory effect on cell proliferation. Next we aimed to measure the rate of cell proliferation in IMR-32 cells after L1-CAM siRNA transfection (L1-CAM KD) with (R) and without (NR) radiotherapy to determine if L1-CAM KD would radiosensitize the cells. One cycle of radiotherapy (2 Gy) in IMR-32 cells led to a statistically significant inhibition (P<0.05) in the rate of cell proliferation 48 and 72 h post-treatment as measured using a WST-1 cell proliferation assay. L1-CAM KD alone led to greater anti-proliferative effect in our cells compared to radiotherapy alone. More importantly, L1-CAM KD in combination with a single cycle of 2-Gy radio-therapy, led to a statistically significant synergistic (P<0.001) inhibition on the rate of cell proliferation compared to radio-therapy alone that was evident as early as 24 h (P<0.05) post-radiotherapy. There was no statistically significant difference in the rate of cell proliferation between the treatment groups at 24 h post-therapy except when L1-CAM KD was combined with radiotherapy (Fig. 4).

L1-CAM plays a more prominent role than PDGFRβ or VEGFR on tumorsphere self-renewal in IMR-32 cells. To determine whether L1-CAM plays a significant role on the formation of tumorspheres in the anchorage-independent IMR-32 cells, we conducted a tumorsphere self-renewal assay after L1-CAM KD in these cells. Cells were transfected with

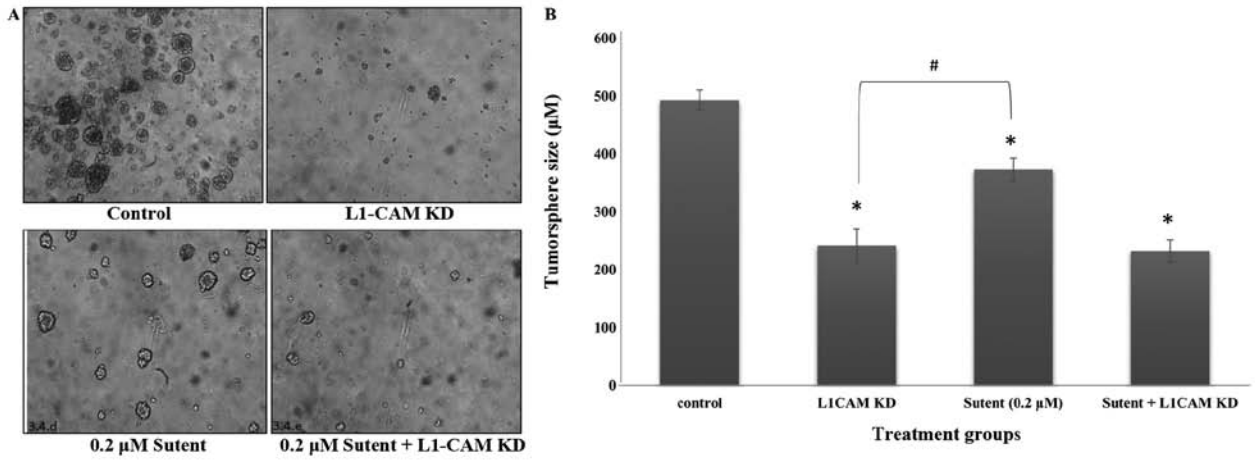

Figure 5. Tumorsphere self-renewal was inhibited after L1-CAM siRNA knock-down (KD) or Sutent treatment in IMR-32 cells. Both L1-CAM KD (*P<0.001) or Sutent treatment (*P<0.05) or both (*P<0.001) resulted in a statistically significant reduction in IMR-32 tumorsphere self-renewal compared to mock-transfected control cells. However, L1-CAM KD showed a more significant (*P<0.05) inhibition on tumorsphere self-renewal than Sutent treatment alone, as evidenced in the micrograph images (magnification, x4) (A) and in the representative graph comparing the tumorsphere sizes in μM between the treatment groups (B). Results represent the mean ± the SDM of multiple experiments.

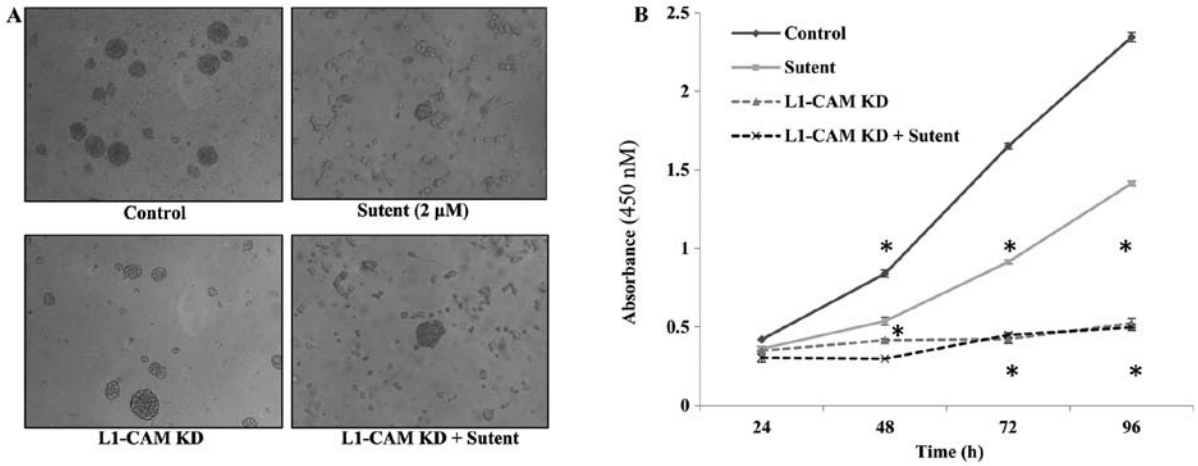

Figure 6. L1-CAM knock-down (KD) in IMR-32 cells is more potent at inhibiting cell proliferation than Sutent treatment. (A) Micrographic images of IMR-32 cells treated with vehicle (control), Sutent (0.2 μM), L1-CAM siRNA (L1-CAM KD) or both (L1-CAM KD + Sutent), showing a reduction in cell confluency 72 h post treatment. (B) WST-1 assay was used to determine the rate of cell proliferation overtime after L1-CAM KD, Sutent treatment, or both. The rate of cell proliferation was significantly reduced (*P<0.01) from 48 to 96 h after L1-CAM KD or Sutent treatment. L1-CAM KD alone led to a statistically significant (*P<0.005) reduction in the rate of cell proliferation compared to Sutent treatment alone. There was no added effect of dual L1-CAM KD and Sutent treatment compared to L1-CAM KD alone on the rate of cell proliferation. The results represent the mean ± the SDM of multiple experiments.

L1-CAM siRNA and 6 h later, reseeded in a limited dilution assay in NeuroCult, stem-cell enriching media supplemented with EGF and FGF. Tumorsphere self-renewal was determined if a single cell recapitulated a large tumorsphere within 5-10 days. IMR-32 cells transfected with L1-CAM siRNA formed smaller and fewer tumorspheres compared to the mock-transfected control cells, which formed more and larger tumorspheres (Fig. 5A) with a statistically significant (P<0.001) difference compared to the L1-CAM siRNA transfected cells (Fig. 5B). In an attempt to determine whether tumorsphere self-renewal is equally inhibited by inactivation of the RTKs PDGFRβ and VEGFR, we treated the cells with Sutent, the selective inhibitor of both PDGFR and VEGFR, and tested tumorsphere self-renewal. Tumorsphere self-renewal capacity was significantly (P<0.05) inhibited after Sutent treatment (Fig. 5), but the inhibition induced by L1-CAM KD was more significant (P<0.05) compared to Sutent treatment. The combination of the two treatments yielded the same outcome as that seen with L1-CAM KD alone (Fig. 5B).

L1-CAM plays a more prominent role than PDGFRβ or VEGFR on the rate of cellular proliferation in IMR-32 cells. The rate of cell proliferation was assessed after L1-CAM KD, Sutent treatment or both in the IMR-32 cells compared to mock-transfected or vehicle-treated control cells. L1-CAM KD led to a statistically significant (P<0.01) inhibition in the rate of cell proliferation at 48, 72 and 96 h post-transfection compared to mock-transfected cells as assessed using a WST-1 cell proliferation assay (Fig. 6). Sutent treatment was used to determine whether the rate of cell proliferation was dependent on the activity of the RTKs PDGFRβ and VEGFR. A single dose of 0.2 μM Sutent led to a statistically significant inhibition

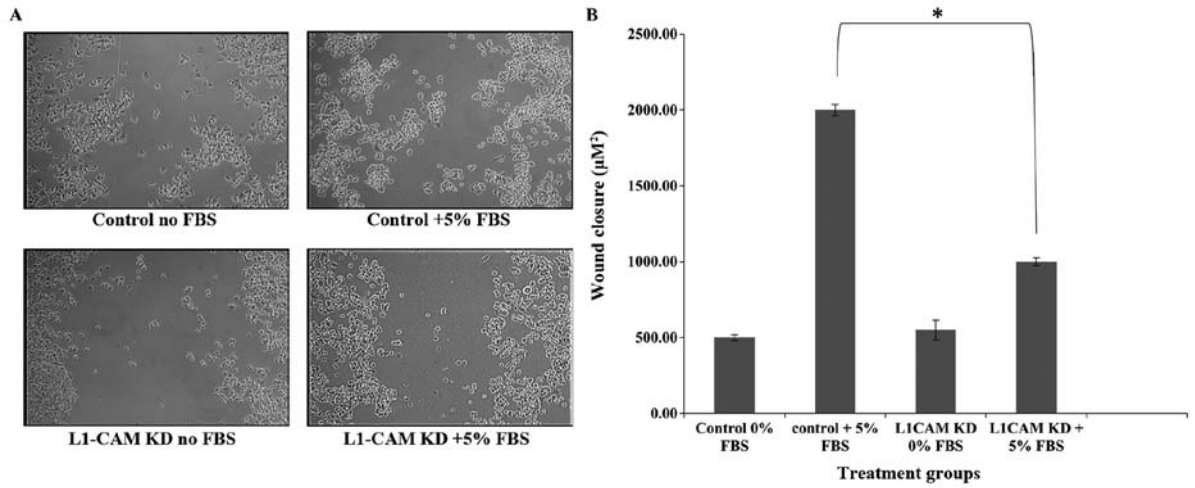

Figure 7. L1-CAM knock-down (KD) significantly inhibits IMR-32 cell migration. (A) micrographic images of 'wound-healing' assay in IMR-32 cells 8 h after 'wound-induction' with and without L1-CAM knock-down (KD). Cells were serum-starved overnight after which 'wound-healing' was induced in the absence (no FBS) or presence of 5% FBS. (B) Graphic representation of cell migration in IMR-32 cells with and without L1-CAM KD showed a statistically significant reduction (*P<0.005) in 'wound-healing' between the groups. Results represent the mean ± the SDM of multiple experiments.

in the rate of cell proliferation (Fig. 6B); however, L1-CAM KD alone induced a statistically more significant (P<0.005) reduction in the rate of cell proliferation compared to Sutent treatment. There was no added effect of dual L1-CAM KD and Sutent treatment on the rate of cell proliferation compared to L1-CAM KD alone.

L1-CAM KD significantly inhibits migration in IMR-32 cells. To determine whether L1-CAM expression is important in IMR-32 cell migration, we used the 'wound-healing' assay to measure IMR-32 cell migration before and after L1-CAM KD in the presence of 5% FBS. L1-CAM KD led to a statistically significant (P<0.005) inhibition in the migratory capability of IMR-32 cells compared to mock-transfected controls as assessed by their migration into and closure of the wound (Fig. 7). This effect was not due to a decrease in the rate of cell proliferation because 'wound-healing' was assessed within 8 h of 'wound-induction' and only 24 h after L1-CAM KD, while the rate of cell proliferation was not significantly different between L1-CAM KD and mock-transfected control cells until 48 h post-L1-CAM KD (Fig. 5).

Discussion

Of all the childhood malignancies, neuroblastoma takes center stage, being the most common extracranial solid tumor, with an incidence rate of approximately 10 cases per million each year and a spectrum of stages ranging from very mild to very severe. Children diagnosed with the mild, low-risk disease have an excellent prognosis with a 5-year survival rate >95%, whereas those diagnosed with the intermediate and high-risk disease have a less favorable prognosis with a 5-year survival rate of 90-95 and 40-50%, respectively (29). MycN-amplification in neuroblastoma is affiliated with poor prognosis and treatment failure. In the present study, we sought to determine the role played by L1-CAM in the MycN-amplified human neuroblastoma cell line IMR-32. We found, using western blot analysis, L1-CAM expression in the IMR-32 cells to be significantly overexpressed compared to the non-MycN-amplified human SK-N-SH cells. Originally identified in the nervous system, L1-CAM was shown to be expressed in various human tumors and is involved in cancer cell proliferation, progression and poor prognosis (30-32). One of the first reports that demonstrated the involvement of L1-CAM in cancer was the detection of L1-CAM expression in high and low metastatic B16 melanoma cells (33). Other studies showed the expression of L1-CAM only in metastatic melanoma cells compared to non-metastatic cells, which correlated with high levels of αv-integrin involved in tumor migration (34,35). Using an antibody against the L1-CAM extracellular domain, L1-CAM inhibition decreased the migration and invasion of melanoma cells (36).

We report herein that transcriptional downregulation of L1-CAM in IMR-32 cells using siRNA transfection led to a statistically significant reduction in the rate of proliferation, migration and tumorsphere formation. This effect may be partly due to the simultaneous abrogation of MycN, and upregulation of PTEN in these cells after L1-CAM KD. MycN is an onco-protein very well known for its tumorigenic role in various cancers and particularly neuroblastomas (8) where it has been shown to drive uncontrolled cellular proliferation. PTEN, on the other hand, is a powerful tumor suppressor whose expression and activity are commonly found to be disrupted in various cancers (37). PTEN acts a tumor suppressor by negatively regulating the activity of PI3K/AKT/mTOR pathway as well as promoting chromosomal stability and DNA repair. Thus, the inhibition of proliferation in our cells after L1-CAM KD may be due, in part, to the dysregulation of this pathway.

Previous studies in colon cancer cells showed that L1-CAM overexpression increased proliferation and migration *in vivo*, and metastasis formation upon injection of these cells into nude mice (38). L1-CAM was found overexpressed in the invasive front of the cancer (39) and correlated with poor prognosis and formation of distant metastasis (39,40). Other studies have shown that inhibiting L1-CAM in Capan-2 pancreatic cancer cells inhibited cell proliferation and invasion and blocked the

cell cycle (41), while overexpressing L1-CAM in PT45-P1 pancreatic cells activated proliferation and induced tumor growth in xenograft models (11). L1-CAM was also detected in breast and ovarian cancers, gastrointestinal stromal tumors, renal carcinomas and Schwannomas (42).

Most interestingly, we report that a single cycle of radiotherapy (2 Gy) led to a statistically significant upregulation of both L1-CAM and MycN in our cells. Transcriptional KD of L1-CAM in the IMR-32 cells abrogated this radio-therapy induced upregulation of L1-CAM and MycN. Thus, there seems to be cross-talk communication between L1-CAM and MycN in our cells. In support of our findings, Keerthikumar *et al* (43) recently reported the co-expression of L1-CAM among other highly tumorigenic proteins in the MycN-amplified SK-N-BE2 neuroblastoma cells.

In glioma cells, a subpopulation of $CD133^+$ stem cells promotes high resistance to radio- and chemotherapy. Bao *et al* (20) showed that L1-CAM expression co-segregated with CD133. Knocking down L1-CAM in this tumor subpopulation significantly decreased growth and neurosphere formation and induced apoptosis of cells, showing that L1-CAM could be potentially used to therapeutically target glioma cancer stem cells. Cheng *et al* (44) have also demonstrated that these stem cells activate the DNA damage checkpoint through the translocation of L1-CAM intracellular domain into the nucleus when exposed to radiation therapy. The translocated domain activates the transcription of c-Myc, a member of the Myc family, which upregulates the expression of NBS1, an important factor involved in the checkpoint response. Inhibiting L1-CAM using siRNA decreased c-Myc expression and the checkpoint activation sensitizing the cells to radiation. Conversely, NBS1 overexpression rescued this decreased activation and radioresistance. L1-CAM KD in our IMR-32 cells led to a statistically significant synergistic inhibition on the rate of cell proliferation when combined with radiotherapy compared to the effect of radiotherapy alone in these cells.

We next sought to determine whether L1-CAM KD would exert a synergistic effect on cell behavior when combined with the tyrosine kinase inhibitor sunitinib malate (Sutent®), a potent inhibitor of PDGFRβ and VEGFR. To that end, cells with and without L1-CAM KD were treated with vehicle or 0.2 μM Sutent and then seeded in a 96-well plate for the analysis of cell proliferation and tumorsphere self-renewal over time. L1-CAM KD alone was more prominent than Sutent treatment alone or combined treatment on inhibiting tumorsphere self-renewal in our cells. There was no synergistic effect of dual L1-CAM KD and Sutent treatment on tumorsphere self-renewal capacity. Furthermore, there was a greater reduction in the rate of cell proliferation after L1-CAM KD compared to Sutent treatment alone, again demonstrating the more prominent role played by L1-CAM on the rate of cell proliferation compared to PDGFRβ and VEGFR. The combination of L1-CAM KD and Sutent treatment yielded the same effect as L1-CAM KD alone. Again we report that the lack of synergy when the two treatments were combined highlights the prominent role played by L1-CAM on these tumorigenic cellular behaviors. Cell migration, as assessed using a 'wound-healing' assay was also significantly inhibited after L1-CAM KD in our malignant, MycN-amplified neuroblastoma cells.

Although the mechanism by which L1-CAM promotes neuroblastoma growth is still not clear, the present study shows an important interplay between L1-CAM, PTEN and N-MYC. L1-CAM has been shown to mediate its signaling either through association with receptor tyrosine kinases, activation of NF-κB inducing cell proliferation and metastasis, or through intramembrane proteolysis. The latter involves the release of L1-CAM extracellular domain to interact with integrins promoting cell migration, and nuclear translocation of its intracellular domain to activate gene transcription (11). Other studies have determined the involvement of L1-CAM in the MAPK-ERK pathway where it activates ERK and interacts with Src protein kinase and Ran-binding protein M (45-47). It has been particularly found expressed on the invasive front edge of many malignant tumors, thus, indicating its role in malignant neoplasm invasion and metastasis, which coincide with poor patient outcomes and increased mortality rates (47).

We report herein an important interaction between L1-CAM, PTEN and MycN in the aggressive, MycN-amplified neuroblastoma IMR-32 cells. Transcriptional downregulation of L1-CAM led to the concurrent downregulation of MycN and upregulation of PTEN protein expression. Cellular tumorigenic behavior was inhibited after L1-CAM KD to a greater extent than that observed with radiotherapy or Sutent treatment alone. Moreover, L1-CAM KD led to a synergistic effect on radiotherapy-induced inhibition of cell proliferation. We conclude that L1-CAM KD radiosensitizes our IMR-32 cells partly by downregulating MycN and upregulating PTEN protein expression. In our future direction, we plan to interrogate the interplay between PTEN, MycN and L1-CAM in our cells and investigate the molecular mechanism of this interplay to determine the tumorigenic pathways activated. We plan to concurrently knock-down the expression of MycN and L1-CAM and examine the effect of this dual KD on cellular tumorigenic behavior, radioresistance and PTEN expression and activation. Moreover, we will examine if overexpression of L1-CAM and MycN in the non-MycN-amplified SK-N-SH cells would render them more malignant as assessed by their cellular behavior and radioresistance.

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