

High α Integrin Level of Cancer Cells Is Associated with Development of Brain Metastasis in Athymic Rats

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Abstract. *Background/Aim:* Brain metastases commonly occur in patients with malignant skin, lung and breast cancers resulting in high morbidity and poor prognosis. Integrins containing an α subunit are cell adhesion proteins that contribute to cancer cell migration and cancer progression. We hypothesized that high expression of α integrin cell adhesion protein promoted metastatic phenotypes in cancer cells. *Materials and Methods:* Cancer cells from different origins were used and studied regarding their metastatic ability and intetumumab, anti- α integrin mAb, sensitivity using *in vitro* cell migration assay and *in vivo* brain metastases animal models. *Results:* The number of brain metastases and the rate of occurrence were positively correlated with cancer cell α integrin levels. High α integrin-expressing cancer cells showed significantly faster cell migration rate *in vitro* than low α integrin-expressing cells. Intetumumab significantly inhibited cancer cell migration *in vitro* regardless of α integrin expression level. Overexpression of α integrin in cancer cells with low α integrin level accelerated cell migration *in vitro* and increased the occurrence of brain metastases *in vivo*. *Conclusion:* α integrin promotes brain metastases in cancer cells and may mediate early steps in the metastatic cascade, such as adhesion to brain vasculature. Targeting α integrin with intetumumab could provide clinical benefit in treating cancer patients who develop metastases.

Brain metastases from systemic cancers occur in approximately 20% of lung cancer, 8% of melanoma, and 5% of breast cancer patients (1, 2). Patients with symptomatic central nervous system lesions that do not receive local treatment have a median overall survival of 2-4 months. Though prophylactic cranial irradiation is an approved prevention strategy in patients with small cell lung cancer, radiation is not appropriate for cancer types carrying a lower risk of metastatic brain disease due to long-term cognitive impairments (3, 4). Therefore, more effective approaches to prevent and treat brain metastases are needed to improve the quality of life and survival of cancer patients.

Integrins are a family of cell adhesion proteins that mediate adhesion between cells and between cells and their extracellular environment (5). These molecules consist of dimers of 18α and 8β subunits and are known to promote the survival, proliferation and motility of tumor cells and tumor vascular endothelial cells (6-9). Intetumumab, anti- α integrin monoclonal antibody, has demonstrated anti-migratory and anti-proliferative effects *in vitro* (10-12) as well as therapeutic and preventive effects in preclinical models (11, 13, 14). However, these promising benefits have not yet been transferred to the clinical setting (15-17).

Using a hematogenous breast cancer brain metastasis model, we previously found that treating rats with intetumumab intravenously before or after cell infusion, or mixing cells with intetumumab prior to intracarotid infusion, significantly decreased the number of brain metastases (14). The objective of the current study was to investigate the potential role of α integrin in the development of brain metastases among primary cancer cell lines of different origins.

Materials and Methods

Cell culture and reagents. Human cancer cell lines used in this study were human melanoma (A375 and A2058), small cell lung carcinoma (SCLC; LX-1, H146 and DMS79), non-small cell lung carcinoma (NSCLC; H2126, A549, H460 and H520) and breast

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cancer cells (MDA-MB-231BR-HER2). Human brain-seeking metastatic breast cancer cells (MDA-MB-231BR-HER2) were kindly provided by Dr. Pat Steeg (NCI, Bethesda, MD, USA) and LX-1 was originally obtained from Mason Research Institute (Worcester, MA, USA). The other cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). All cells were cultured with proper medium supplemented with serum and antibiotics. Cells were confirmed mycoplasma-free, used at low passage, harvested immediately prior to implantation and used only if viability exceeded 90%. Intetumumab (fully human anti- αv integrin monoclonal antibody) was provided by Ortho Biotech Oncology R&D (Radnor, PA, USA). Antibodies for immunohistochemistry were anti-human αv , $\alpha 1$, $\beta 5$ integrin (Cell Signaling Technology, Danvers, MA, USA), anti-EphA2 (Proteintech, Rosemont, IL, USA), anti-human mitochondrial antigen (Chemicon/Millipore Temecula, CA, USA), and anti-tubulin (Sigma, St. Louis, MO, USA). Cyclophosphamide (Cytosan[®] Bristol-Myers Squibb, Princeton, NJ, USA) was dissolved in sterile saline before use.

In vitro cell labeling with quantum dot for cell trafficking. To monitor or track the location of human cancer cells after intracarotid infusion, 10^6 metastatic MDA-MB-231BR-HER2 breast cancer cells were labeled *in vitro* using a quantum dot (Q-dot) Qtracker Cell Labeling kit (ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's protocol. Rats were perfused with saline and euthanized at 3 and 7 days after cancer cell infusion (n=3 per time point). Brain vasculature-associated Q-dot-labeled cancer cells were analyzed from three random fields of view using a fluorescent microscope after counterstained with Hoechst nuclei stain.

Cellular αv integrin overexpression (knock-in). For αv integrin overexpression, pTag-integrin expression vector (Axxora LLC; San Diego, CA, USA) and empty vector (pTag without αv integrin) control were used in knock-in experiments. Stably-transfected human cancer cells, overexpressing αv integrin, were selected by neomycin (G418) resistance. Cellular integrin proteins expression was characterized using immunoblotting and immunofluorescence microscopy. *In vitro* cell migration was assessed using a 0.5-mm gap closure wound healing and migration assay (Ibidi LLC, Verona, WI, USA). *In vitro* cell viability WST assay (ThermoFisher Scientific, Waltham, MA, USA) was used to measure cell proliferation. Western immunoblotting was performed as described previously (18).

Rat xenografted tumor models. The care and use of the animals was approved by the Institutional Animal Care and Use Committee (IACUC) and was under the supervision of the Department of Comparative Medicine at Oregon Health and Science University (OHSU). Female (200-250 g) athymic (*rnu/rnu*) and heterozygous (*rnu/wt*) rats from the OHSU breeding colony were housed in a room with a 12-h light: dark cycle maintained at $22 \pm 2^\circ\text{C}$. Food and water were supplied to all rats ad libitum. Rats were euthanized with CO₂ inhalation at 20% weight loss or poor clinical condition as per IACUC regulations as a surrogate for survival.

Hematogenous metastases model. For the hematogenous metastases model, rats were pre-treated intraperitoneally with cyclophosphamide (100 mg/kg) 24 h before and 2 weeks after tumor cell inoculation. We have shown that cyclophosphamide decreases innate immunity and increases VEGF production in rat tumor models (19), and is

essential for optimal brain metastases in the rat model. Rats (n=4 to 30 per each different cancer cell line) were anesthetized with isoflurane (5% induction, 2% maintenance Aerrane; Anaquest, Inc., Madison WI, USA). A catheter filled with heparinized saline was tied into the right external carotid artery and human cancer cells (10^6 cells or number as indicated, suspended in 1 ml saline) were infused retrograde into the right internal carotid artery (19).

For the *in vivo* melanoma metastases study, intetumumab (30 mg/kg) was given intravenously 3 times at 1 h prior to, 7 and 14 days after intracarotid fusion of 10^6 A2058 cells. Rat brains (n=7 per treatment group) were harvested for immunohistochemical analysis at 21 days after infusion.

Intracerebral tumor model. Rats were anesthetized with ketamine (60 mg/kg) and diazepam (7.5 mg/kg) intraperitoneally. Rats (n=8 per each clone of H520 or A375 cell) received 10^6 human cancer cells in a volume of 15 μl , stereotactically injected (20) in the right caudate putamen (vertical, bregma 6.5 mm; lateral, bregma 3.1 mm). Rats were euthanized and brains were harvested for immunohistochemical analysis at 56 days after inoculation or when neurological symptoms occurred.

Histology and immunohistochemistry. Brains were excised and fixed in 10% buffered formalin for vibratome sectioning, 100 μm in the coronal plane. For intracerebral tumor volumetrics, every 5th brain section was stained for human mitochondrial antigen and then imaged at high resolution (30 μm pixel diameter) on an Epson 1640XL flatbed scanner using Adobe Photoshop software. The volume of individual metastases and total tumor burden was assessed using NIH ImageJ software. Immunohistochemistry was performed by incubating brain slides with primary antibodies (1:200-1:1,000 dilution) in blocking buffer overnight at 4°C followed by biotinylated secondary antibody and visualization with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) using diaminobenzidine (19).

Data analysis and statistical analysis. Immunoblotting signal of proteins was quantified using UN-SCAN-IT Gel software (Silk Scientific, Inc., Orem, UT, USA). An un-paired Student's *t*-test was used to compare quantum-dot labeled cells in brains. For the *in vitro* wound healing assay, different cell lines and treatments were compared using a one-way ANOVA and Tukey's test. For the *in vivo* animal studies, the difference of tumor occurrence rate was measured using Fisher's exact test. The difference in tumor volumes between treatment groups was compared using a non-parametric Wilcoxon sum test. All analyses were performed with Graphpad Prism version 6.04 or Microsoft Excel. Statistical significance between treatment and control (or vehicle) group or any two other groups was indicated by $p < 0.05$ or $p < 0.01$.

Results

To better understand the brain metastases cascade occurring after intra-carotid infusion, we tracked the location of metastatic human cancer cells by Quantum dot labeled MDA-MB-231BR-HER2 breast cancer cells *in vitro*. All labeled breast cells were found associated with brain vasculature at 3 days (either adhered to blood vessel walls or still in the circulation). About 50% of the labeled breast

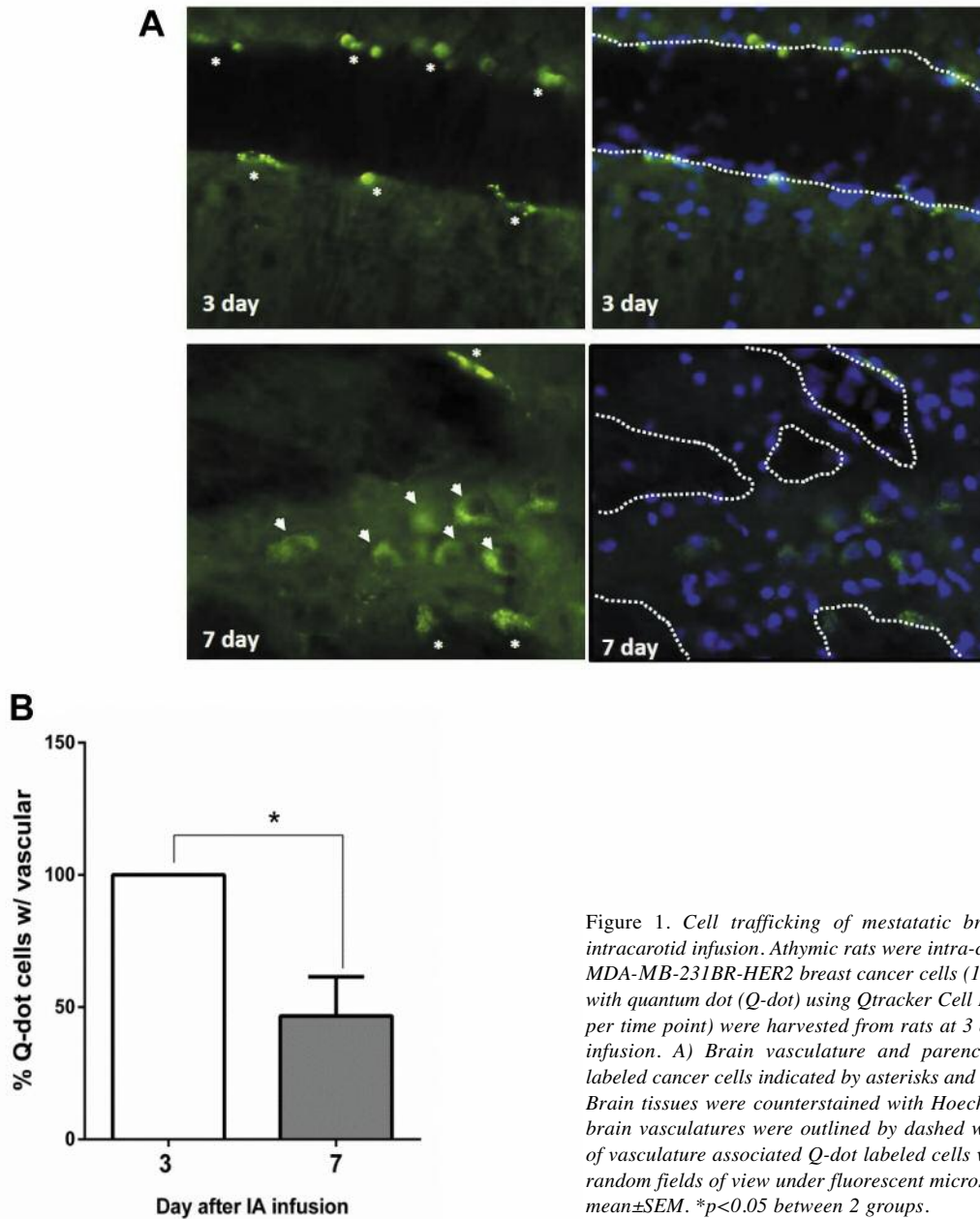


Figure 1. Cell trafficking of metastatic breast cancer cells after intracarotid infusion. Athymic rats were intra-carotid infused metastatic MDA-MB-231BR-HER2 breast cancer cells (10^6) after *in vitro* labeling with quantum dot (Q-dot) using Qtracker Cell Labeling kit. Brains ($n=3$ per time point) were harvested from rats at 3 and 7 days after 10^6 cell infusion. A) Brain vasculature and parenchyma associated Q-dot labeled cancer cells indicated by asterisks and arrowheads, respectively. Brain tissues were counterstained with Hoechst nuclei stain. Putative brain vasculatures were outlined by dashed white line. B) Percentage of vasculature associated Q-dot labeled cells were analyzed from three random fields of view under fluorescent microscopy and represented as mean \pm SEM. * $p<0.05$ between 2 groups.

cells transmigrated into brain parenchyma at 7 days after infusion (Figure 1A and B). We next assessed the role of α v integrin cell adhesion protein in the metastatic phenotype of a broad range of human cancer cell lines expressing different level of α v, α 1, and β 5 integrin and EphA2 from lung, melanoma and breast primary cancer origins (Figure 2A and B). The brain metastasis occurrence rate, approximate number of metastases, total tumor volume and survival time in rats with hematogenous brain metastases is summarized in Table I. With the pool of 10 cell lines tested, we found that the incidence and number of brain metastases formation

was positively correlated ($R^2=0.81$) to cellular α v integrin level normalized by tubulin expression after intra-carotid infusion (Figure 2C). There was no significant correlation of brain metastasis incidence found with other adhesion proteins investigated.

Cells that expressed high α v integrin levels (A549 NSCLC and A2058 melanoma) showed a higher cell migration rate than cells expressing low α v integrin levels (H520 NSCLC and A375 melanoma), as assessed by rate of a 0.5-mm gap closure in an *in vitro* wound healing migration assay (Figure 3). In 4 different cancer cell lines, the time needed for gap closure was

Table I. Summary of brain metastasis development among different cancer cell lines in athymic rats.

Cancer type	Cell line	α_v integrin ¹	Metastasis incidence ²	Tumor no. ³	Tumor vol. ⁴	Survival ⁵ (d)	
Lung carcinoma	Small cell	LX1	Medium	3/30 (10%)	1±0	0.1-78	36
		H146	Low-medium	0/4 (0%)	0	0	–
		DMS79	Low	0/4 (0%)	0	0	–
	Non-small	H460	High	4/4 (100%)	13±6	0.05-55	34
		H2126	Medium-high	0/4 (0%)	0	0	–
		A549	High	6/6 (100%)	9±5	0.05-126	62
Melanoma	A375	Low	3/4 (75%)	1±0	3.2-63	67	
		High	4/4 (100%)	140±70	0.05-7.8	29	
	A2058 ⁶	High	14/14 (100%)	96±66	0.02-24	–	
		High	9/9 (100%)	15±9	91-316	52	
Breast cancer	MDA-MB-231BR-HER2	High	9/9 (100%)	15±9	91-316	52	

Brain metastases were produced by intra-carotid infusion of 10⁶ human cancer cells in nude rats pre-treated intraperitoneally with cyclophosphamide (100 mg/kg) 1 day before and 14 days after cell inoculation. ¹Cancer cell α_v integrin level was measured by immunoblotting after normalization with β -tubulin. ²Brain metastasis incidence was calculated by no. of rats with brain metastasis/no. of rat injected. ³Number of tumors per brain in rats with brain metastases is indicated as mean±SD. ⁴Tumor volume indicates range in mm³. ⁵Survival (mean day after cell infusion) is shown for animals that were euthanized at 20% body weight loss for tumor burden; all non-tumor bearing rats were euthanized at 84 days after cell infusion or 3 weeks post infusion in A2058 melanoma. ⁶10⁵ A2058 melanoma cells were given by intra-carotid infusion.

negatively correlated ($R^2=0.75$) to their α_v integrin level (Figure 3D). Additionally, this time was also negatively correlated ($R^2=0.72$) to the number of brain metastases formed (Figure 3E) and mimicked the metastatic phenotype and characteristics of these cells *in vivo* (Figure 2B and Table I). In particular, A2058 melanoma cells had the highest migration rate, closing the 0.5-mm gap within 12 h (Figure 3C), and these cells also induced brain metastases in 100% of rats after intracarotid inoculation, with the highest number of metastases (140±70 metastases per brain) and the shortest median survival (29 days; n=26). In contrast, low α_v integrin-expressing A375 melanoma cells took 6 days to close the 0.5-mm gap (Figure 3B), and only formed one brain metastasis per brain in 3 of 4 test animals with a median survival of 67 days. In the NSCLC models, A549 (high α_v) and H520 (low α_v) cells closed the 0.5-mm gap after 3 days and 7 days *in vitro*, and developed 9±5 and zero brain metastases, respectively, after intra-carotid infusion of 10⁶ cells.

Intetumumab treatment (50 μ g/ml) significantly inhibited *in vitro* cell migration of 3 out of 4 tested cell lines (A549, H520 and A375) by delaying the time to gap closure in both the high and low α_v integrin cells (Figure 3A-C). However, high α_v integrin-expressing A2058 melanoma cells were found to be resistant to intetumumab with an effective inhibitory concentration of 10 times or higher (>0.5 mg/ml; Figure 3C). Similarly, rats treated 30 mg/mL intetumumab intravenously (n=7 per treatment group) failed to reduce the number of A2058 melanoma induced brain metastases (Figure 3F) and tumor volume (Figure 3G) when compared to control.

To validate the role of α_v integrin in cell invasion and migration, we over-expressed α_v integrin in A375 melanoma and H520 NSCLC cells (parental cells with low α_v) using a TagRFP-integrin vector. In the A375 melanoma cells, α_v integrin was overexpressed about 2.5-fold by pTagRFP-integrin vector without affecting tubulin level (Figure 4A). Compared to the empty vector control, A375 α_v integrin-overexpressing cells showed accelerated cell migration *in vitro*, with decreased time for gap closure (4 vs. 6 days; $p<0.05$; Figure 4B) without affecting cell proliferation rate (data not shown). After intracerebral inoculation, α_v integrin overexpressing A375 melanoma significantly ($p=0.007$) increased the brain tumor occurrence rate (100%; 8/8 vs. 25%; 2/8) by Fisher's exact test, without affecting tumor volume (76.7±75.6 vs. 114.7±104.4 mm³; Figure 4C and D). Similar results were obtained when H520 NSCLC were transfected with the same TagRFP-integrin vector (Figure 5). There was no statistical significance in brain tumor volume (36.3±22.6 vs. 61.4±53.5 mm³) after intracerebral inoculation, even with 5-fold α_v integrin upregulation (clone #2). Intracerebral inoculation with α_v integrin knockin H520 NSCLC cells doubled the brain tumor occurrence rate (75%; 6/8 vs. 37.5%; 3/8) compared to the empty vector control, though this result was not statistically significant ($p=0.315$), possibly due to our small sample size. Overall, our results suggest that α_v integrin plays more important roles in cancer cell adhesion to brain vasculature than cell proliferation for the development of brain tumor in both hematogenous and intracerebral brain metastases models.

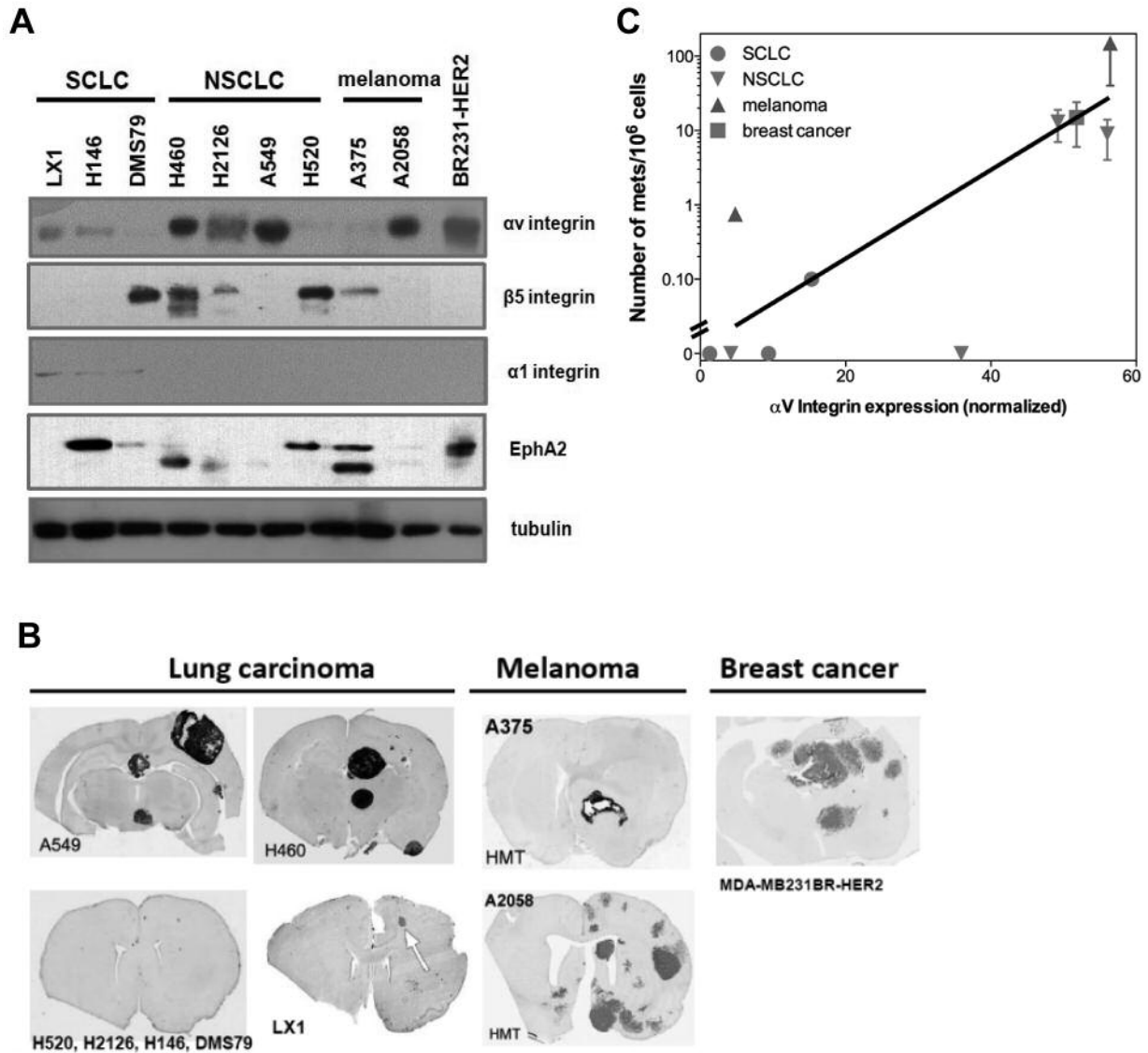


Figure 2. Human cancer cell α v integrin protein level correlates with brain metastasis formation in vivo. A) Immunoblot showing variable α v, α 1, β 5 integrin and EphA2 protein levels in different metastatic human tumor cells from multiple origins. B) Brain immunohistochemistry of one representative rat from each of the indicated hematogenous metastasis models. Sections were stained for human mitochondrial antigen (hematoxylin in LX1 SCLC) as a marker of human tumor growth. C) Correlation of α v integrin and number of brain metastases ($R^2=0.81$). The immunoblotting band intensity was normalized to tubulin as a loading control.

Discussion

Brain metastases is an inefficient process with less than 0.1% of cancer cells eventually developing brain tumors after intra-carotid infusion in the hematogenous metastasis model. It requires that cancer cells leave their primary site, travel and survive inside blood circulation, recognize, bind to, and migrate through brain vasculature, infiltrate into brain parenchyma, and thrive in the brain microenvironment (21-

24). The mechanisms involved in this cascade remain unclear. Experimental brain metastases models using intracardiac, intra-carotid, or orthotopic inoculation of cancer cells have been established previously (14, 19, 25). In this study of 10 human cancer cell lines that have been confirmed to form tumors after intracerebral implantation, several cell lines formed no brain metastases after intra-carotid infusion. Overall, we found an average of only 10 brain metastases (ranging from 0 to 150) after infusing 10⁶ cells. We

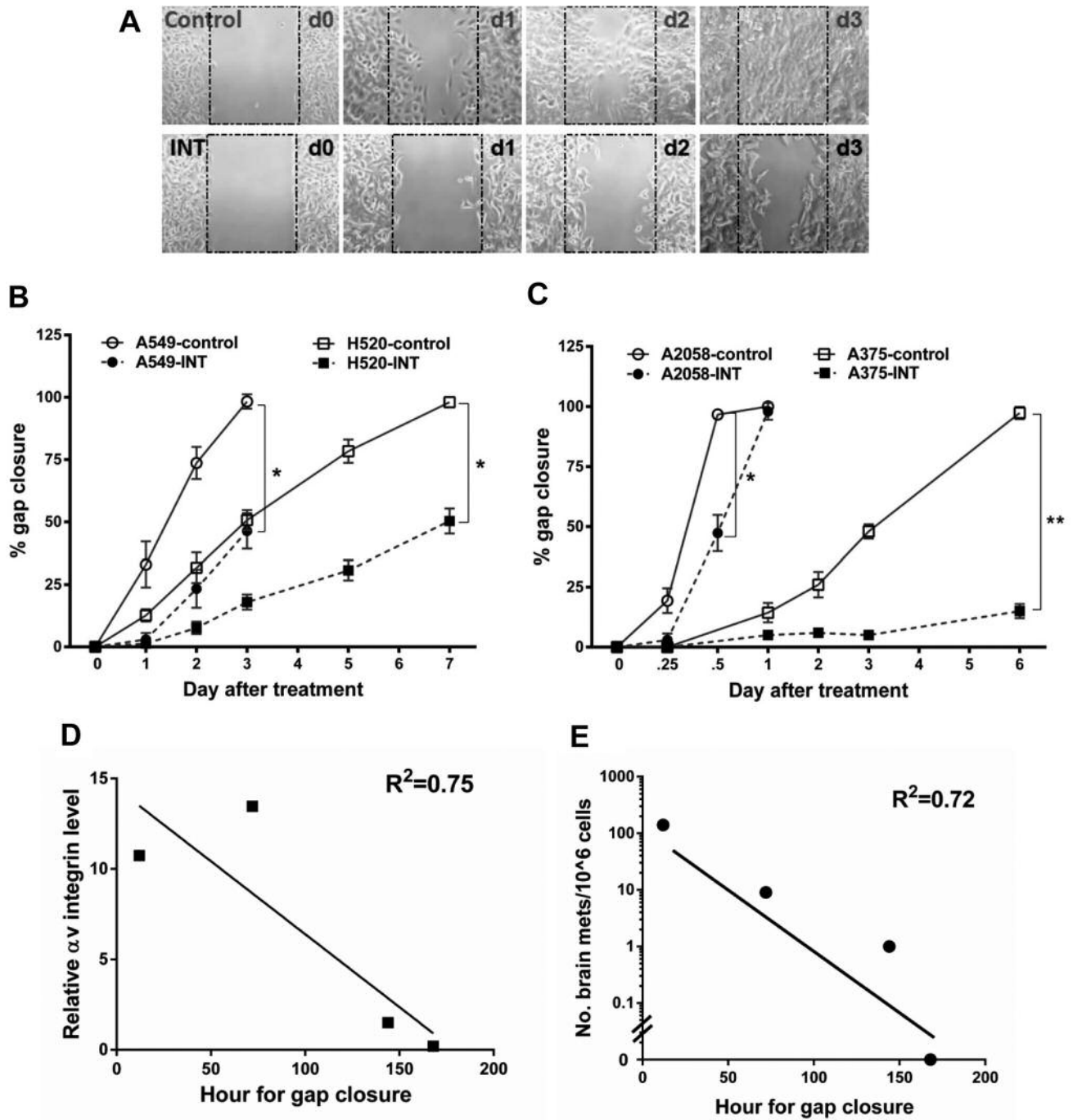


Figure 3. Continued

demonstrated that the incidence and number of brain metastases formation was positively correlated ($R^2=0.81$) to cellular αv integrin level after intra-carotid inoculation even with a small ($n=10$) pool of cell lines tested. Our data suggest that αv integrin plays important roles in cancer cell recognition and adhesion to brain vasculature and

parenchyma infiltration for the development of brain tumors. Our results support the previous findings that integrins are overexpressed in malignant cells, and promote invasion and metastasis (7, 9). Vogetseder *et al.* (26) found elevated levels of $\alpha v\beta 3$, $\alpha v\beta 6$, and $\alpha v\beta 8$ integrin in human breast cancer brain metastases. The αv integrins are expressed on the

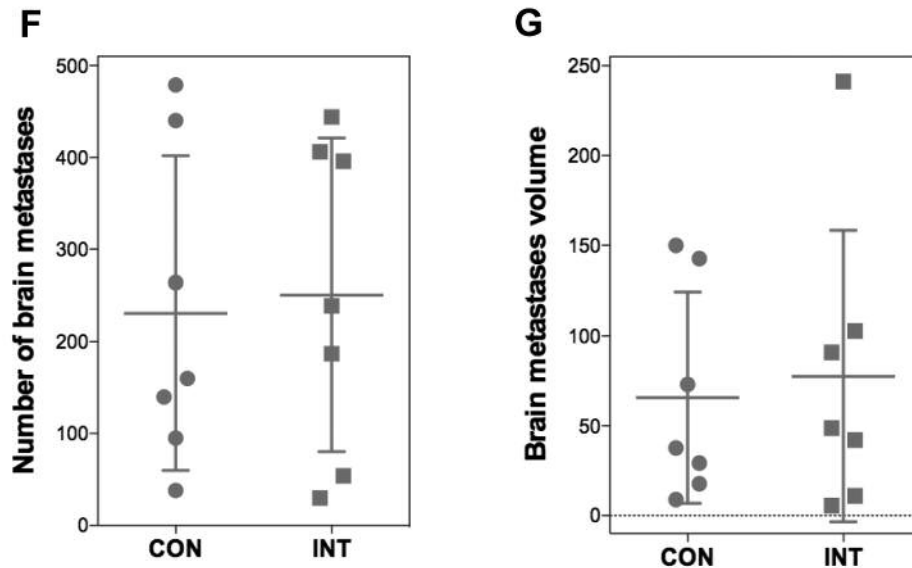


Figure 3. Role of αv integrin in cell migration *in vitro* and effect of intetumumab in cell migration and melanoma brain metastases *in vivo*. A) Representative micrographs of A549 non-small cell lung carcinoma (NSCLC) cells that were untreated (control) or treated with intetumumab (50 $\mu\text{g/ml}$; bottom) in the *in vitro* cell migration assay (ibidi® μ -dish culture insert, Verona, WI, USA); B) Quantification of NSCLC *in vitro* cell migration assay. A549 (high αv) cells are more invasive than A375 cells with shorter time (3 vs. 7 days) to close the 0.5 mm gap; C) Quantification of melanoma *in vitro* cell migration assay. A2058 (high αv) melanoma is more invasive than A375 cells. Intetumumab dose used to treat A2058 and A375 melanoma is 750 and 50 $\mu\text{g/ml}$, respectively. Error bars show SD and * and ** indicates values $p < 0.05$ and $p < 0.01$ in comparison to control group, respectively; D) Correlation of hour needed for gap closure and cellular αv integrin level ($R^2 = 0.75$) among 4 cancer cell lines studied; E) Correlation of hour needed for gap closure and number of brain metastases *in vivo* ($R^2 = 0.72$); F, G) Compared to control (CON), 3 times treatment of intetumumab (INT, 30 mg/ml, IV) failed to reduce number of A2058 melanoma brain metastases F) and tumor volume G).

surface of most epithelial tumors and expression is altered in tumor progression and metastasis (27, 28). In contrast, Hoshino *et al.* (29) found that tumor exosomes rich in integrin $\alpha 2$ are more brain-tropic and αv integrin is more involved in liver metastases. Recently, Chen *et al.* (30) demonstrated that connexin 43 and protocadherin 7 are over-expressed in brain- compared to bone- or lung-tropic clones of MDA-MB-231 human breast cancer cell. Brain-tropic H2030 NSCLC also has elevated levels of these two proteins compared to its parental counterpart.

Cancer cells with low αv integrin will loosely bind to extracellular matrix (ECM) of brain parenchyma after intracarotid or intracerebral inoculation and few of them will further develop brain lesions (31). Stapack *et al.* (32) found that the absence of $\alpha v \beta 1$ and $\alpha v \beta 3$ mediated attachment to the ECM can trigger anoikis (apoptosis due the absence of cell-matrix interaction) and apoptosis. Integrin signaling through growth factors and cytokines induces tumor cell growth and motility and blocks apoptosis (33, 34). Morozevich *et al.* (35) showed that $\alpha v \beta 3$ integrin expression of human intestinal carcinoma cells became resistant to anoikis. Moreover, expression of $\alpha v \beta 3$ integrin in glioma cells increases survival in response to stress (36) and

cilengitide, an $\alpha v \beta 3$ and $\alpha v \beta 5$ antagonist, treatment blocked the fibroblast senescence without affecting proliferation (37) and inhibits pulmonary metastasis in an intramuscular osteosarcoma xenograft mouse model (38). Xiong *et al.* (39) found that transforming growth factor- $\beta 1$ induces endometrial cancer cell adhesion and migration by up-regulating $\alpha v \beta 3$ integrin. Knockdown of integrin αv induced loss of the metastatic phenotype in human prostate and breast cancer cells (40-43). In support of this, we found that cell migration, as measured by the gap closure time *in vitro*, was negatively correlated to their αv integrin level and number of brain metastases *in vivo* (Figure 3D and E). Overall, our findings suggested that cellular αv integrin level and *in vitro* migration assay data could potentially be used to predict the *in vivo* brain metastasis outcome.

Chen *et al.* showed that intetumumab prevents integrin binding to ECM molecules (11), which prevents downstream molecular interactions for cancer cell migration, invasion, and successful metastasis (6, 34). In the current study, we found that intetumumab (50 $\mu\text{g/ml}$) reduced cell migration of A549, H520 and A375 but not A2058 cells regardless of αv integrin level. Compared to A549 NSCLC, highly invasive A2058 with similar αv integrin level is extremely

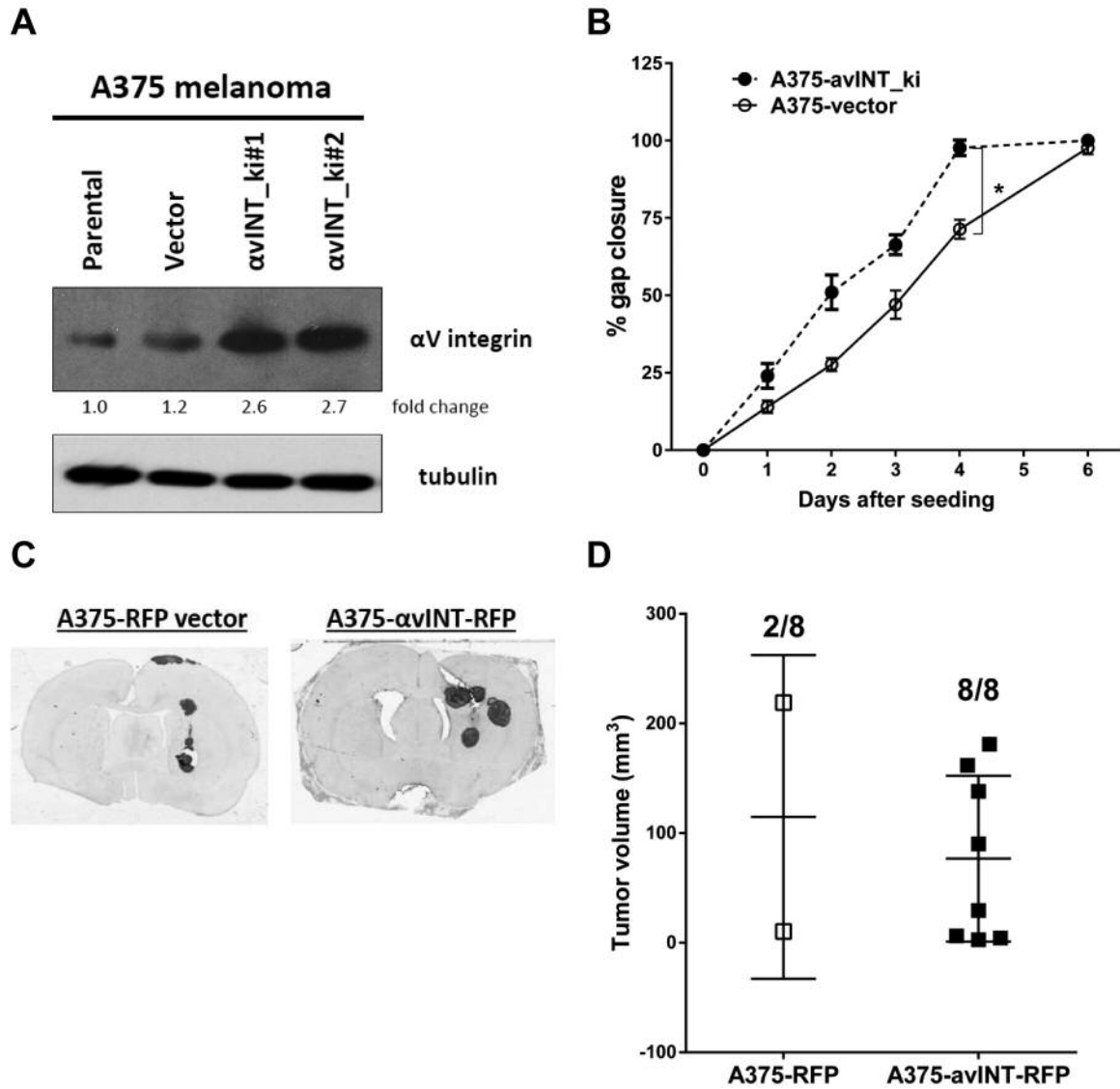


Figure 4. Overexpression of α v integrin increases A375 melanoma cell migration *in vitro* and tumor occurrence *in vivo*. A) α v integrin protein level in A375 cells including parental, vector only or α v integrin knock-in (avINT_ki) clones with tubulin as loading control; B) Quantification of *in vitro* cell migration assay among A375 clones. A375-avINT_ki cells are more invasive than A375 parental and vector cells with shorter time (4 vs. 6 days) to close the 0.5 mm gap. Error bars show SD and * $p < 0.05$ in comparison to control group; C) Brain immunohistochemistry of representative rats after intracerebral inoculation with 10^6 A375 avINT-RFP#2 and -RFP alone. Brains were stained with human mitochondrial antigen after harvesting at 56 days after inoculation or when neurological symptom occurred; D) Dot plot chart of individual brain tumor volume and number of rats with tumor after inoculation shown in Figure 3C.

resistant to intetumumab both *in vitro* (>500 μ g/ml) and *in vivo* (30 mg/kg, IV) treatment. Previously, we found single or weekly administrations of intetumumab at lower dosage (10 mg/kg, IV) reduced the incidence and number of breast cancer brain metastases in athymic rats (14). Liu *et al.* (44) found that STK11, KRAS mutation, ZEB1 upregulation and E-cadherin down-regulation was associated with intetumumab resistance in 23 lung cancer cell lines.

However, the molecular mechanism(s) involved in regulating integrin levels and intetumumab resistance are not defined and need further investigation.

We believe that α v integrin of brain metastatic cells predominantly regulate cell adhesion, invasion and metastasis rather than cell proliferation and tumor growth. To evaluate the role of α v integrin in invasion and proliferation, we demonstrated that overexpression of α v integrin in A375

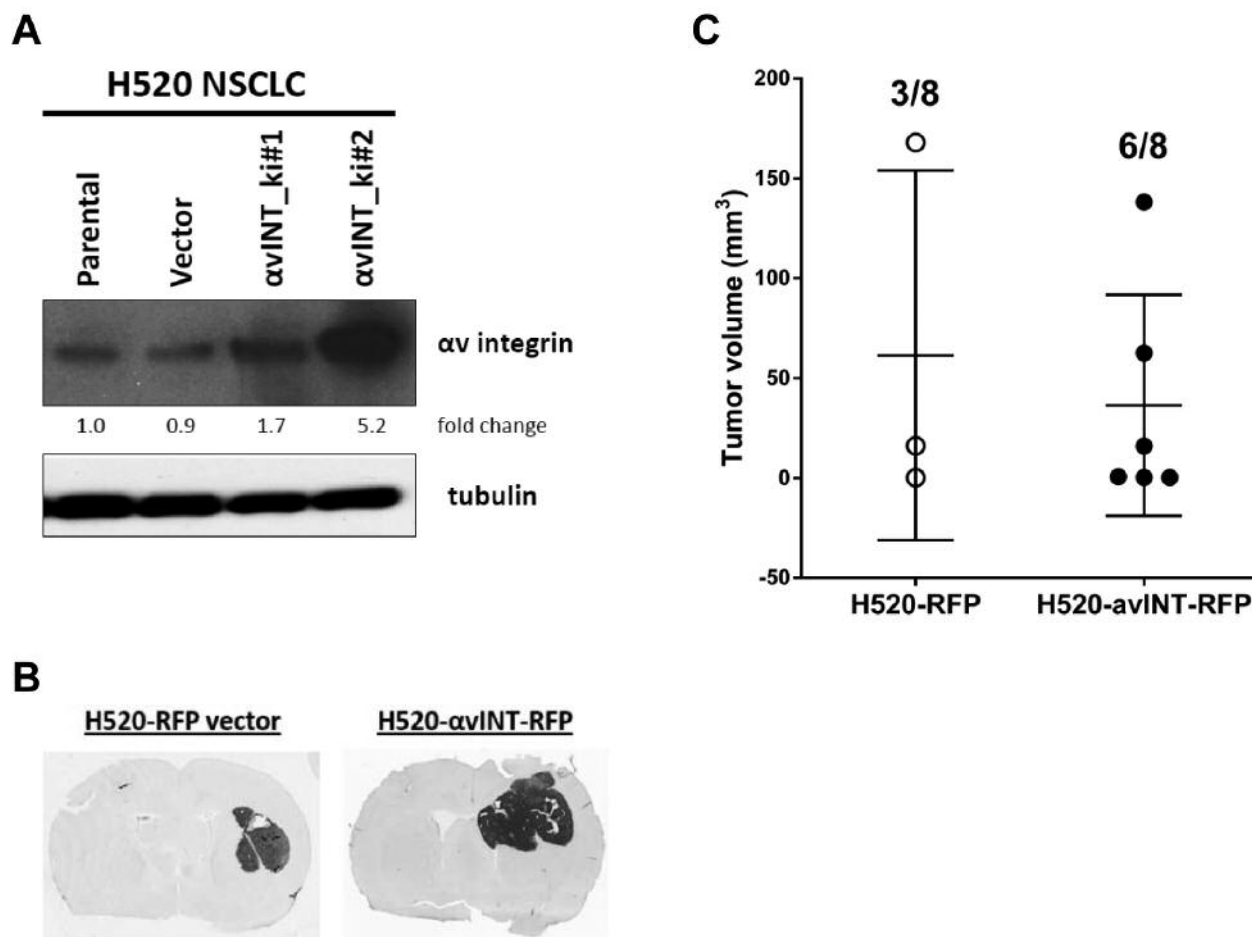


Figure 5. Overexpression of α v integrin in H520 NSCLC *in vitro* increases tumor occurrence after intracerebral inoculation. A) α v integrin protein level in H520 cells including parental, vector only or α v integrin knock-in (α vINT_ki) clones with tubulin as loading control. B) Brain immunohistochemistry of representative rats stained with human mitochondrial antigen; Athymic rats were intracerebrally inoculated with 10^6 H520 α vINT-RFP#2 and -RFP alone, brains were harvested at 56 days after inoculation. C) Dot plot chart of individual brain tumor volume and number of rats with tumor after inoculation shown shown in Figure 4B.

increase cell invasion and motility *in vitro* without affecting proliferation rate (Figure 4). In the orthotopic metastasis model, we only found the difference of tumor occurrence rate (A375 and H520) but not the mean brain tumor volume after directly intracerebral inoculation. Thus, the observed increase in cell adhesion and tumorigenic occurrence did not result from higher cell numbers, but rather was a direct reflection of α v integrin overexpression. Similarly, we found that α v integrin-knockdown MDA-MB-231BR clones lose their invasiveness without affecting cell proliferation *in vitro* and *in vivo* (42). Our results are in agreement with Weber *et al.* who identified the activated α v β 3 integrin in cancer cells and its interaction with platelets to promote extravasation as critical for early steps during hematogenous metastasis (45). McCarroll *et al.* (46) also showed that β III-tubulin regulates lung cancer metastasis without altering growth of tumor cells

in vivo in an anchorage-dependent manner. Parental (MDA-MB-231) and brain metastatic cells (MDA-MB-231BR) do not exhibit differences in proliferation, although MDA-MB-231BR showed a 2.5-fold increase in β III-tubulin protein levels (47). Jiang *et al.* (48) also demonstrated that targeting α v integrin with abitumumab inhibited prostate cancer cell adhesion, migration and invasion, but not proliferation *in vitro*. MicroRNA-124 inhibited hepatocarcinoma cell invasion and migration by suppressing α v integrin (49). Inhibition of α v β 3 integrin by RGD disintegrin also induces loss of cell directionality and reduction of migration speed in oral squamous carcinoma cells (50). Cellular integrin regulation, trafficking and recycling is extremely complicated processes highly dependent upon the activation and ligand bound state of integrin (28, 51-53). In metastasizing cancer cells, where different integrin dimers are activated by different ECM

ligands as these cells encounter multiple distinct microenvironments including brain (54). Therefore, switching between active and inactive states, proper trafficking and cellular localization of integrin dimers are critical to regulate their tumorigenic properties after cancer cells arriving at pre-metastatic niches such as brain neural-vascular unit.

A major limitation of this study is the use of intracerebral xenografts in the αv integrin-overexpressing cancer cells. Intracerebral implantation does not recapitulate the metastatic cascade and does not fully mimic the heterogeneity of human brain metastasis localization or progression. We tested the transfected clones *in vivo* in the hematogenous metastasis paradigm. Unexpectedly, no animals receiving the cancer cells with modified αv integrin survived after intra-carotid injection. This was apparently due to stroke caused by cell clumping and/or larger cell size in a portion of the transfected cells. Attempts to filter clumped cells or dilute the cell infusate were unsuccessful. A second limitation of this study is that the detailed molecular mechanism(s) of *in vitro* intetumumab resistance of A2058 melanoma compared to A549 NSCLC remain unclear and need to be delineated.

In conclusion, our study demonstrated the concept that i) αv integrin plays more important roles in regulating cancer cell adhesion to brain vasculature than cell proliferation for the development of brain tumor; ii) cancer αv integrin levels from biopsy of primary tumor and their *in vitro* migration ability could potentially be used to predict the *in vivo* metastasis outcome; and iii) targeting αv integrin including intetumumab may be used as an adjuvant to prevent and improve the survival in brain metastasis patients regardless of αv integrin level.

Conflicts of Interest

The Authors have no conflicts of interest to disclose.

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