2015

CLINICAL AND PATHOLOGIC SIGNIFICANCE OF INTEGRIN α6β4 EXPRESSION IN HUMAN MALIGNANCIES

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CLINICAL AND PATHOLOGIC SIGNIFICANCE OF INTEGRIN α6β4 EXPRESSION IN HUMAN MALIGNANCIES

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

Rachel Lauren Stewart, D.O.

Lexington, Kentucky

Director: Dr. Kathleen L. O'Connor, Professor of Molecular and Cellular Biochemistry

2015

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ABSTRACT OF DISSERTATION

CLINICAL AND PATHOLOGIC SIGNIFICANCE OF INTEGRIN α6β4 EXPRESSION IN HUMAN MALIGNANCIES

Integrins are cellular adhesion molecules that bind cells to the extracellular matrix. The integrin α6β4, a receptor for laminins, is predominantly expressed on epithelial cells where it is present at the basal surface adjacent to the basement membrane. This integrin plays a critical role in maintaining normal cellular functions, yet has also been implicated in promoting invasion and metastasis in human malignancies. While overexpression of the integrin α6β4 has been detected in select human cancers, the clinical significance of integrin α6β4 expression in a number of malignancies has not been determined. The purpose of this study was to examine integrin α6β4 expression as it relates to clinical variables and patient outcomes in tumors of the lung, breast, and central nervous system. In order to study integrin α6β4 protein expression in patient-derived tumors, tissue microarrays were constructed and sections were stained using immunohistochemistry for the integrin β4 subunit. Integrin β4 mRNA levels in patient-derived tumors were also examined using publicly available gene expression datasets. Integrin β4 expression was found to be elevated in lung squamous cell carcinoma, and its overexpression was associated with venous invasion and decreased overall survival among patients with non-small cell lung cancer. In gliomas, integrin β4 was highly expressed in glioblastomas when compared to lower grade gliomas and non-neoplastic brain tissue. Integrin β4 expression was shown to be an adverse prognostic marker in gliomas, and furthermore, integrin β4 expression was reduced in gliomas with mutations in IDH1. In breast cancer, integrin α6β4 expression was found to be elevated in triple negative tumors, and in one cohort, elevated integrin β4 expression was associated with HER2 overexpression. In summary, I have shown that integrin β4 expression is elevated in a number of aggressive human malignancies, and that its expression associates with poor prognosis in these tumors.

KEYWORDS: Pathology, cancer, integrin, glioma, tissue microarray

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July 24, 2015
Date
CLINICAL AND PATHOLOGIC SIGNIFICANCE OF INTEGRIN α6β4 EXPRESSION IN HUMAN MALIGNANCIES

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For Jean and Lillian
ACKNOWLEDGEMENTS

I would first like to acknowledge Dr. Joseph Pulliam for inspiring me to pursue research and for being an outstanding mentor. Joe was an incredible physician, teacher and friend, and he was an inspiration to all of those that worked with him. Without him, I would not have found this path.

I would like to express my deepest gratitude to my mentor Dr. Kathleen O’Connor, for giving me the opportunity to join her lab and for nurturing my development as a scientist. Her guidance, caring, patience, and enthusiasm were critical throughout this process. I would like to thank my clinical mentor, Dr. Craig Horbinski, for his support and practical advice. He patiently taught me fundamental techniques and was always there to provide guidance on everything from molecular testing to fellowship decisions. I would like to thank my department Chairman, Dr. C. Darrell Jennings, for his unwavering support, enthusiasm, and guidance over the past five years. I would like to thank Dr. Luis Samayoa for teaching me everything that I know about breast pathology and for making it a fun process. Huge thanks to Dr. Min Chen, who has been a great friend and mentor, and to Dr. Thomas Kelly, for being a kind and supportive member of my committee. I would like to thank Dr. Jennifer Harris for joining me on this journey and for being a great friend throughout the process. Thanks to Dr. Edward Romond for teaching me about breast cancer and clinical oncology, and thanks to Dr. Philip Westgate for agreeing to serve as an external reader. I would also like to thank Dr. B. Mark Evers for providing the opportunity for me to pursue full-time research, and for his continued support.

I would like to thank all of the members of the University of Kentucky Department of Pathology, and I would like to acknowledge the many faculty members that have helped with my training and research over the past few years. In particular, I would like to thank Dr. Dava West, Dr. William O’Connor, and Dr. Yolanda Brill for their superb editing and diagnostic skills. I would like to specifically acknowledge Dr. Janna Neltner for being a righteous dude. Dr. Greg Davis has been a trusted friend and mentor since I joined the residency program. Thanks to Dr. Michael Cibull for asking tough questions and for keeping me in line. Dr. Peter Nelson and Dr. Charles Lutz have been extremely supportive of my research goals. Huge thanks to Lissa Holland-Morris and Betsy Meredith for their encouragement and kind words.

I would like give special thanks to Dana Napier and the Markey Biospecimen Core Facility. This work would not have been completed without Dana’s patience, expertise, and great sense of humor. Thanks to Dr. Chi Wang and Dr. Heidi Weiss in
Biostatistics for their help with TMA design, and thanks to Dr. Tamas Gal and Dr. Eric Durbin at the Cancer Research Informatics Facility. I would also like to thank Dr. Zobeida Cruz-Monserrate for developing the integrin β4 immunohistochemistry protocol, and for helping me get it to work. Many thanks to Teresa Knifley, Brittany Carpenter, and Brittany Metts for their patience, support, and comic relief.

Finally, I would like to thank my friends and family for their tireless support over the years. My loving husband Shadow has been there for me every step of the way. He helped me keep it together. My sister Tara is a talented scientist, and though her chosen field is evolutionary ecology, I believe that she has become an accidental integrin expert over the past few years. Thanks to my brother Ian for inspiring me to take up martial arts – it really helped. Thanks to my parents for believing in me, and special thanks to my dad, for giving me an anatomy book when I was young, and for learning to rock climb with me.
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CHAPTER 1: INTRODUCTION*

Integrins are cellular adhesion molecules that serve as receptors for extracellular matrix (ECM) components and select cell adhesion molecules. Named for their ability to integrate signals from the extracellular environment to the inside of the cell, integrins are responsible for securing cells to the surrounding adhesion molecules while amplifying and potentiating signals from growth factor receptors and other extracellular stimuli.\(^1\) These transmembrane proteins permit cells to sense and respond to their environment and thus play critical roles in maintaining normal cellular functions, yet have also been implicated in promoting invasion and metastasis in human malignancies.\(^2\)

Integrins are heterodimeric receptors that consist of paired α and β subunits. In the human genome, there are 18 α and 8 β subunits that combine in a limited combination to provide 24 integrin receptors, each with its own specificity for select ECM or cellular adhesion proteins (for review, see \(^1,3\)). Integrins containing the α6 subunit are laminin receptors in which the α6 subunit can pair with either the β1 or β4 subunit. In contrast, the integrin β4 subunit can only pair with the α6 subunit,\(^1,2,4\) thus making β4 subunit expression predictive of integrin α6β4 expression.

Integrin α6β4 is predominantly expressed on epithelial cells where it is present at the basal surface adjacent to the basement membrane where it nucleates the formation of hemidesmosomes. These stable adhesions are critical for the integrity of epithelial monolayers. In contrast to this function, integrin α6β4 signaling in various cancers promotes an invasive and metastatic phenotype. This functional change is mediated by phosphorylation of the cytoplasmic tail of the integrin β4 subunit that releases integrin α6β4 from hemidesmosomes and allows the integrin to promote invasive signaling through cooperation with growth factor receptors and alteration of the transcriptome, which in turn facilitates tumor progression.\(^2,4-9\)

*ADAPTED FROM: Stewart RL, O’Connor KL. Clinical significance of the integrin α6β4 in human malignancies. Laboratory Investigation. 2015.
Structure and normal function of integrin α6β4

The α6β4 integrin is a specialized integrin that is expressed in various normal epithelia, Schwann cells and endothelial cells. The integrin β4 subunit is distinct from other integrin subunits in that it has a particularly long cytoplasmic signaling domain. Whereas the cytoplasmic domains of other integrin subunits are less than 50 amino acids in length, the integrin β4 subunit is over 1000 amino acids in length.10,11 As depicted in Figure 1.1, the cytoplasmic domain of the integrin β4 subunit is characterized by two pairs of fibronectin type III domains, a Calxβ domain and a connecting segment.12 The fibronectin repeats and the connecting segment are necessary for hemidesmosome assembly.13-15

At the basal surface of normal cells adjacent to the basement membrane, integrin α6β4 binds to laminins in the ECM and facilitates stable adhesion through the formation of hemidesmosomes. Hemidesmosomes are large adhesion complexes that anchor the basal layer of epithelial cells to the basement membrane.13-15 In these junctions, the α6β4 integrin nucleates the connection between cytokeratin intermediate filaments in the cell and laminins in the basement membrane through its interactions with plectin, collagen XVII/BP180 and BP230, as depicted in Figure 1.1B.16,17 The importance of these junctions is highlighted by the fact that mutations in the integrin β4 gene (ITGB4) can cause lethal forms of epidermolysis bullosa with pyloric atresia, a disorder characterized by blistering and ulceration of the skin and mucosal tissues.18 While it has been suggested that other integrins may be able to compensate for a loss of integrin α6β4, studies in mouse models have demonstrated that this is not the case.19 Integrin β4 knockout mice (−/−) are born with severe epidermal blistering, exhibit widespread separation of the epithelial-mesenchymal junction, and die shortly after birth. These observations emphasize the importance of the integrin β4 subunit in maintaining the integrity of the epithelial-ECM junction.19
Figure 1.1: Integrin α6β4 structure and hemidesmosome assembly. A) The integrin β4 subunit only pairs with the α6 subunit. The long cytoplasmic domain of β4 is structurally distinct from other known receptors but contains several distinct domains including a Calx-β domain, four fibronectin type III repeats, a connecting segment and C-terminal tail. B) Integrin α6β4 nucleates hemidesmosomes by binding to multiple hemidesmosomal associated proteins including Plectin (HD1), BP180 (also known as BPAG2 or collagen XVII) and BP230 (also known as BPAG1).
Hemidesmosomes are dynamic structures. During wound healing, hemidesmosomes must be dismantled to allow the leading edge cells to migrate into the wound.\textsuperscript{14,20} This functional change is mediated by phosphorylation of the cytoplasmic tail of the integrin $\beta 4$ subunit that releases integrin $\alpha 6\beta 4$ from hemidesmosomes. This process occurs through stimulation by growth factor receptors such as the epidermal growth factor receptor (EGFR), and by direct phosphorylation of the integrin $\beta 4$ cytoplasmic tail by protein kinase C.\textsuperscript{7,8,21} Upon release from hemidesmosomes, integrin $\alpha 6\beta 4$ relocalizes from the keratin cytoskeleton to the actin cytoskeleton.\textsuperscript{22} When bound to F-actin, the integrin $\alpha 6\beta 4$ signaling domain promotes the formation of motility structures, such as filopodia and lamellae, by cooperating with growth factor receptors and stimulating key signaling pathways\textsuperscript{6,7,22-24} that in turn facilitate migration and wound closure.

Integrin $\alpha 6\beta 4$ and hemidesmosomes are also suggested to play a role in the contextual orientation of cells. When cells are not adhered to the proper extracellular matrices, they will undergo a specialized form of apoptosis known as anoikis. Notably, when caspases are activated, the $\beta 4$ cytoplasmic tail is cleaved leading to apoptosis.\textsuperscript{25,26}

**Integrin $\alpha 6\beta 4$ signaling in malignant cells**

Release of integrin $\alpha 6\beta 4$ from hemidesmosomes can lead to altered signals that promote tumor cell growth, invasion and metastasis.\textsuperscript{2,4-9} Under conditions where hemidesmosomes are disassembled, integrin $\alpha 6\beta 4$ binding directly to laminin has been shown to activate both phosphoinositide 3-OH kinase (PI3K) and RhoA small GTPases.\textsuperscript{6,24} Alternatively, the integrin can cooperate with multiple different growth factor receptors including those in the EGF receptor family (ErbB-1,2,3), c-Met, Ron, LPA1 and LPA2\textsuperscript{23,27-30} to enhance signaling through PI3K, AKT, MAPK and the Rho small GTPases,\textsuperscript{6,23,24,31,32} as depicted in Figure 1.2. In cells with mutant p53, the $\alpha 6\beta 4$ integrin
promotes cell survival through activation of AKT/PKB, and stimulates cell cycle progression and proliferation by interacting with Shc to activate the Ras-MAPK pathway. As shown in breast and pancreatic cancers, this integrin can promote tumor progression through transcriptional regulation and has been shown to increase the expression of invasive and metastatic proteins such as the epithelial to mesenchymal transition (EMT)-associated protein S100A4 (also known as metastasin/FSP). In the next several sections, I discuss our current understanding of how integrin α6β4 signaling promotes a malignant phenotype with an emphasis on its impact on invasion, cell survival and angiogenesis.
Figure 1.2: Cancer progression-associated signaling pathways activated by integrin α6β4. Integrin α6β4 can activate multiple signal transduction cascades either directly by binding its ligand laminin or by amplifying signals from multiple growth factors. Enhanced signaling through these pathways contributes to tumor progression in terms of enhanced proliferation, cell survival, invasion and metastasis. Notably, integrin α6β4 can act as a tumor suppressor by promoting apoptosis in the presence of a wild-type p53. For this reason, integrin α6β4 is often overexpressed primarily in tumor types where p53 mutations are prominent such as pancreatic and basal-like breast cancers.
Invasive signaling functions

The finding that integrin α6β4 mediates stable adhesive complexes that anchor cells to the basement membrane would seem to argue against the participation of integrin α6β4 in cell migration. Despite this apparent contradiction, numerous studies have confirmed that integrin α6β4 is responsible for promoting migratory and invasive behavior in carcinoma cells. A potential explanation for this phenomenon comes from its normal role in wound healing, as described above.

Early studies in colon and breast carcinoma lines demonstrated that expression of integrin α6β4 contributes to tumor cell invasiveness. These studies lead to the pivotal discovery that integrin α6β4 can promote invasive properties in carcinoma cells by activating the PI3K pathway, a signaling pathway that is now well known for its role in promoting carcinoma progression. Notably, this was the first time that PI3K had been implicated in carcinoma invasion. This finding was subsequently confirmed in a number of additional reports, although the exact mechanism by which integrin α6β4 activates PI3K initially remained elusive. The cytoplasmic domain of the integrin β4 subunit does not contain a consensus-binding motif for the regulatory subunit of PI3K, making direct activation of this pathway by integrin β4 subunit unlikely. One mechanism for integrin β4-mediated activation of PI3K was found to involve insulin receptor substrate-1 and -2 (IRS-1 and IRS-2), which act as signaling intermediates that facilitate integrin α6β4-mediated PI3K activation. Ligation of integrin α6β4 promotes phosphorylation of IRS-1 and IRS-2, leading to subsequent activation of PI3K. An additional mechanism has been described wherein integrin α6β4 cooperates with ErbB-2 to promote PI3K-dependent invasion. Finally, integrin α6β4 has been shown to localize to lipid rafts in the plasma membrane, which may allow it to activate PI3K by facilitating close interactions with other receptor tyrosine kinases.

The involvement of integrin α6β4 in cell polarization and the formation of F-actin
rich motility structures such as filopodia and lamellae lead to investigations into the Rho family of small GTPases. The Rho family of small GTPases largely control the reorganization of the actin cytoskeleton needed for cell motility.43 Initial studies by Shaw and colleagues on the activation and function of PI3K in invasion found that the small GTPase Rac1 was required for invasion downstream of PI3K,6 a finding confirmed by others.44 Notably, integrin α6β4 can cooperate with growth factor receptors45 and other integrins23 to activate Rac1. Further studies examining the impact of integrin α6β4 on migration and invasion found that integrin α6β4 increased the activity of cAMP-specific phosphodiesterase, thereby resulting in decreased cAMP concentrations and subsequent RhoA activation.9,24 The activation of RhoA downstream of integrin α6β4 subsequently leads to the formation of RhoA-dependent membrane ruffling and lamellae formation, as well as the generation of contraction forces that enable cell migration.24,46 Our group found that the metastasis associated protein S100A4, which is regulated by integrin α6β435 interacts with Rho effector Rhotekin to promote cell membrane ruffling.47 Notably, the traditional function for RhoA is the generation of stress fibers rather than membrane ruffling, suggesting that integrin α6β4 can change the function of RhoA to facilitate tumor invasion.

Integrin α6β4 amplifies signaling through multiple receptor tyrosine kinases and G-protein coupled receptors in order to promote tumor cell proliferation and invasion. Notably, cooperative signaling has been identified between integrin α6β4 and multiple members of the EGFR family. EGFR and integrin α6β4 have also been shown to co-localize at the leading edge of carcinoma cells subjected to EGF stimulation, and notably, their interaction is inhibited by curcumin, a compound present in turmeric.48 Integrin α6β4 has also been shown to associate with ErbB-2 in multiple breast carcinoma cell lines,30,49 though reports are conflicting as to whether integrin α6β4 expression correlates with ErbB-2 protein overexpression in patient-derived carcinoma
tissues.\textsuperscript{50-53} Using a mouse model of ErbB-2 mediated tumorigenesis, loss of integrin β4 signaling was shown to reduce breast tumor invasive growth and metastasis, and deletion of the integrin β4 signaling domain was shown to enhance the efficacy of ErbB2-targeted therapy.\textsuperscript{27} This study also demonstrated that the integrin β4 subunit forms a complex with ErbB-2 and amplifies ErbB-2 signaling. In addition, integrin α6β4 has been shown to regulate the expression of ErbB-2 by influencing its translation.\textsuperscript{54} These findings are particularly notable as ErbB-2 amplification promotes invasion in human malignancies such as breast carcinoma, and is associated with aggressive behavior.\textsuperscript{55,56}

While an interaction with ErbB-2 may be necessary in order for integrin α6β4 to activate PI3K mediated invasion in select cell types, ErbB-2 lacks a consensus-binding site for the regulatory subunit of PI3K. Furthermore, ErbB-2 must dimerize with an EGFR family member in order to function.\textsuperscript{30,57} A potential solution to this issue was suggested by the finding that integrin α6β4-mediated PI3K activation is dependent on ErbB-2/ErbB-3 heterodimerization.\textsuperscript{30} The ErbB-2/ErbB-3 heterodimer is a strong activator of PI3K,\textsuperscript{30,58} and the ErbB-3 cytoplasmic domain contains binding sites for the regulatory subunit of PI3K.\textsuperscript{59} Integrin α6β4 can regulate the expression of ErbB-3, leading to an increase in ErbB-2/ErbB-3 heterodimerization and subsequent PI3K activation,\textsuperscript{30} and notably, a positive association has been identified between integrin α6β4 and ErbB-3 expression in patient-derived tumors.\textsuperscript{51}

Integrin α6β4 can also cooperate with c-Met, a receptor tyrosine kinase that is activated by the hepatocyte growth factor (HGF).\textsuperscript{28,29} In one study, integrin α6β4 was shown to form a direct complex with c-Met that promotes HGF dependent invasion.\textsuperscript{28} Additional studies have shown that while an interaction with integrin α6β4 may contribute to c-Met dependent invasion, integrin α6β4 and c-Met are also able to promote invasion independently.\textsuperscript{29} Evidence for a physical association between integrin α6β4 and c-Met is
controversial;\textsuperscript{4,29} however, this does not preclude the probability that integrin α6β4 can cooperate with c-Met without physical association.

Ron ("recepteur d'origine nantais"), a tyrosine kinase receptor closely related to c-Met, has been shown to form a complex with integrin α6β4 that induces hemidesmosome disassembly and the relocation of integrin α6β4 to motility structures.\textsuperscript{36,60} Further studies have shown that Ron activation is important in pancreatic carcinoma progression,\textsuperscript{61,62} and have confirmed that Ron interacts with the integrin β4 subunit in this setting to disrupt the association between integrin β4 and plectin.\textsuperscript{60}

**Cell survival and apoptosis**

Integrin α6β4 promotes either cell survival or apoptosis, depending on the cellular context. In normal epithelia, integrins are critical to maintaining cellular growth and survival as long as they maintain proper contact with the ECM.\textsuperscript{63} If anchorage to the ECM is lost, the subsequent loss of integrin signaling can inhibit cell growth and promote a specialized form of apoptosis referred to as anoikis.\textsuperscript{63} While tumor suppressive functions of the integrin β4 subunit have been identified in bladder,\textsuperscript{64} colon,\textsuperscript{65} and breast carcinoma\textsuperscript{66} cell lines, a number of additional studies have indicated that the β4 integrin promotes cell survival.\textsuperscript{19,41,66-68} This dichotomy appears to hinge on the expression and mutation status of the p53 tumor suppressor.

Early experiments demonstrated that expression of the integrin β4 subunit in the colon cancer cell line RKO led to increased apoptosis, thus lending support to the notion that integrin α6β4 functions as a tumor suppressor.\textsuperscript{65} Conversely, expression of the β4 subunit was unable to induce apoptosis in the cell line MDA-MB-435.\textsuperscript{69} The Mercurio group investigated the mechanism underlying this apparent contradiction and observed that the RKO and MDA-MB-435 cell lines differed in their p53 mutation status. While RKO cells harbor a wild-type p53, the MDA-MB-435 cell line expresses mutant p53.\textsuperscript{69}
This group demonstrated that integrin α6β4 can trigger apoptosis through p53 activation in cells harboring wild-type p53, however, in carcinoma cells deficient in p53, integrin α6β4 promotes cell survival by activating AKT/PKB and through translational regulation of VEGF expression. These findings suggest that tumors expressing high levels of integrin α6β4 in conjunction with loss of p53 function are resistant to apoptosis and will therefore display a more aggressive clinical course. Interestingly, an association between p53 mutations and integrin α6β4 overexpression is present in a number of aggressive human malignancies, including basal-like breast cancer, head and neck squamous cell carcinoma, and pancreatic ductal adenocarcinoma.

**Angiogenesis**

In addition to promoting invasive properties in carcinoma cells, the integrin α6β4 can stimulate invasion and migration of endothelial cells, processes that are necessary for pathologic angiogenesis (for review, see ). Studies using knockout mice carrying a deletion in the signaling domain of the integrin β4 subunit displayed reduced angiogenesis in a retinal neovascularization model, and developed smaller and less vascularized tumors after subcutaneous implantation. The same study demonstrated that the integrin β4 subunit could promote both bFGF- and VEGF-induced angiogenesis by enhancing signaling through ERK and NF-κB.

**The role of α6β4 in transcriptional regulation**

Integrin α6β4 regulates the expression of molecules important for carcinoma invasion and metastasis. The best studied of these include NFAT1, NFAT5, S100A4, ErbB-2, ErbB-3 and autotaxin. The NFATs, or Nuclear Factors of Activated T-cells, are transcriptionally regulated by the α6β4 integrin and drive carcinoma invasion. As shown in breast cancer, integrin α6β4-mediated upregulation of NFAT1 leads to
increased expression of autotaxin \((ENPP2)\), an enzyme that acts as a motility factor by promoting LPA production.\(^{34,79}\) Integrin \(\alpha 6\beta 4\) can also regulate the expression of ErbB-2 and ErbB-3\(^{30,54}\) as mentioned above.

Interestingly, expression of the \(\alpha 6\beta 4\) integrin in MDA-MB-435 cells leads to altered expression of over 500 genes.\(^{35}\) One of the most regulated and clinically relevant of these genes is S100A4, a calcium binding protein also known as metastasin-1.\(^{80}\) S100A4 promotes tumor metastases\(^{80}\) and is regulated by integrin \(\alpha 6\beta 4\) through NFAT5 activation in conjunction with DNA demethylation of the S100A4 promoter.\(^{35}\) S100A4 interacts with Rhotekin to promote the formation of an S100A4/Rhotekin/RhoA complex, thus allowing RhoA to promote invasion through membrane ruffling.\(^{47}\) Integrin \(\alpha 6\beta 4\) has also been shown to negatively regulate the expression of miR-92ab and miR-99ab/100 miRNA families that impact target genes implicated in promoting cell motility.\(^{81}\)

**Integrin \(\alpha 6\beta 4\) expression in human malignancies**

Integrin \(\alpha 6\beta 4\) was originally identified as a tumor progression antigen by two separate groups, one who termed it tumor-specific antigen-180 (TSP-180)\(^{82}\) and the other who referred to it as the A9 complex.\(^{83}\) Subsequently, the TSP-180 and A9 complexes were shown to be identical to integrin \(\alpha 6\beta 4\).\(^{84,85}\) During the invasive process, integrin \(\alpha 6\beta 4\) is released from hemidesmosomes where it can then participate in many of the most aggressive properties of advanced carcinomas. Immunohistochemical staining in patient-derived tissues confirms that in many cancers, expression and localization of integrin \(\alpha 6\beta 4\) are altered (as demonstrated in Fig. 3).

Studies examining integrin \(\beta 4\) expression in patient-derived tissue, in some cancer types, have obtained conflicting results. It is unclear whether these contradictory findings relate to sample size, cancer subtype examined or the method of detection. Some investigators have reported inconsistent immunohistochemical staining for integrin
Our group found that immunohistochemistry for the integrin β4 subunit is particularly sensitive to the antigen retrieval process. In addition, different studies have used a variety of clonal antibodies to detect integrin β4 expression, which may partially explain the variability in staining patterns that has been reported. Below, I discuss the clinical associations of integrin α6β4 in various human malignancies, noting where there is disagreement in the literature.
Figure 1.3: Altered localization of integrin α6β4 expression in invasive breast carcinoma. In a dilated duct with benign columnar cell change (left), integrin α6β4 is expressed in myoepithelial cells surrounding the duct, but is absent in luminal cells. Adjacent nests of invading carcinoma cells (right) display elevated expression of the integrin β4. Immunohistochemical staining was performed using the 439-9B antibody as described in Chapter 2. Brown staining represents positive expression of the integrin β4.
**Malignancies with strong evidence that integrin β4 expression is pathologically significant**

**Breast cancer**

In breast cancer, integrin β4 overexpression is associated with aggressive behavior and poor prognosis. Given the challenges described with immunohistochemistry, gene expression profiling provides an excellent alternative that allows for quantitative assessment of integrin β4 expression. One study used gene expression profiling and immunohistochemistry of tissue microarray (TMA) sections to demonstrate that integrin β4 is overexpressed in basal-like breast cancer.50 This finding is particularly notable as basal-like breast cancer is an aggressive subtype that is associated with a notoriously poor prognosis.71 This group further developed a 65-gene signature that included the top genes whose expression was found to correlate with that of integrin β4. This integrin “β4 signature” was shown to be a prognostic indicator that could predict both decreased survival and decreased time to recurrence in four breast cancer cohorts.50 In other studies, integrin β4 mRNA expression was found to positively correlate with nuclear grade and tumor size,53 and elevated integrin α6β4 protein expression has been found to associate with decreased survival.86 Co-expression of integrin α6β4 and Net1, a RhoA guanine nucleotide exchange factor, has also been associated with decreased distant metastasis-free survival.52

In contrast to the findings described above, a number of early reports indicated that integrin β4 expression is absent or rare in breast cancers. This observation may be due to difficulties with immunohistochemistry on frozen specimens, or may be related to the fact that these studies were performed before the modern sub-classification of breast cancers was developed. As integrin β4 overexpression is more frequent in triple negative breast cancers (TNBCs), and these tumors represent a minority of breast cancers, these studies may not have included an adequate number of TNBCs to detect integrin β4
overexpression. Two of these early investigations report that integrin β4 expression was absent in all invasive breast carcinomas examined,\textsuperscript{82,87} while another found strong integrin β4 staining in only a small subset of breast tumors.\textsuperscript{88} Others have reported that integrin β4 is redistributed over the cell surface in select breast carcinomas.\textsuperscript{89} Integrin β4 expression in ductal carcinoma \textit{in situ} (DCIS) is also reportedly rare, with expression identified in only 20\% of cases in one study.\textsuperscript{90} According to another report, integrin β4 expression was absent in the neoplastic cells of DCIS and detected only in residual myoepithelium.\textsuperscript{87} Given more recent evidence using gene expression profiling, it is reasonable to conclude that at least a certain subset of breast tumors overexpress integrin β4, including basal-like breast cancers.

\textbf{Bladder cancer}

Studies investigating integrin β4 expression in bladder cancer demonstrate that it is overexpressed in a proportion of transitional cell carcinomas, and suggest its use as a prognostic marker. An early study reported that in normal urothelium integrin α6β4 is expressed in the basal layer of urothelial cells where this expression is highly polarized and localized to the lamina propria junction. The authors then examined integrin α6β4 expression in ten low stage bladder cancers, where they found increased, non-polarized expression in 80\% of tumors.\textsuperscript{91} A subsequent study by the same group examined integrin α6β4 expression in bladder tumors from 57 patients; each case was categorized as having negative, weak, or strong expression of integrin α6β4, where weak was defined as expression that most closely resembled that of normal urothelium.\textsuperscript{92} They found that patients with weak integrin α6β4 expression had improved survival compared to patients with either strong or negative expression.\textsuperscript{92} Another study examined integrin β4 expression in a cohort of patients with non-muscle invasive bladder cancer and found
that integrin β4 expression levels were an independent predictor of intravesical recurrence after transurethral resection.\textsuperscript{93}

**Cervical cancer**

Integrin β4 expression in cervical lesions has been examined primarily in cervical intraepithelial neoplasia (CIN) and squamous cell carcinoma. Multiple reports have confirmed that the integrin β4 is strongly expressed in invasive squamous cell carcinomas of the cervix.\textsuperscript{94-96} Interestingly, integrin β4 expression positively correlates with the degree of squamous atypia in CIN.\textsuperscript{94,95} In a study of cervical biopsies from 35 patients, integrin β4 expression was present only in cells of the basal and parabasal layers of normal ectocervical mucosa, and this pattern was maintained in flat condylomas, hyperplastic epithelium, and in CIN I lesions.\textsuperscript{94} Interestingly, in CIN II-III, integrin β4 expression was present throughout the entire thickness of the epithelium, with strong staining observed toward the superficial surface. Furthermore, expression of integrin β4 was identified in 90% of cervical carcinomas studied, where expression was diffusely present in the invasive nests.\textsuperscript{94}

A larger study investigated integrin β4 expression in 40 cervical biopsies. The authors describe that in normal ectocervical mucosa, integrin β4 expression was localized to the basal aspect of cells in the basal layer of epithelium.\textsuperscript{95} They found that in CIN, expression of the integrin β4 followed the distribution of atypia: for example, in CIN II, β4 was expressed throughout the lower 2/3 of the epithelial thickness, while in CIN III, β4 was expressed throughout the full epithelial thickness. These findings suggest that the integrin β4 may play an early role in promoting the survival and growth of pre-invasive neoplasms.

A third study examined expression of the integrin β4 in 20 cases of invasive cervical carcinoma and 23 cases of CIN III.\textsuperscript{96} The invasive cervical carcinomas included
examples of well, moderately, and poorly differentiated squamous cell carcinoma, as well as three endocervical adenocarcinomas. Diffuse expression of the integrin β4 was identified in all 20 cases of invasive carcinoma. In addition, integrin β4 expression was identified in all epithelial layers in 65% of CIN III lesions.96

**Head and neck cancer**

In squamous cell carcinomas of the head and neck, integrin β4 is commonly overexpressed. An early study of 82 patients with SCC of the head and neck found that strong expression of the UM-A9 antigen (later identified as the α6β4 integrin) was associated with early relapse and decreased patient survival.97 In another study, integrin β4 expression was found to be upregulated in SCCs when compared to normal squamous mucosa, although an association was found between loss of integrin β4 expression and the presence of nodal metastases.98

Multiple studies using gene expression profiling have confirmed that integrin β4 gene expression levels are prognostically significant in SCCs of the head and neck. One of these reports demonstrated that integrin β4 gene expression was associated with decreased survival in a cohort of 66 patients.99 In a larger study, increased integrin β4 gene expression was associated with the presence of lymph node metastases, distant metastases and patient death on univariate analysis, and was an independent predictor of distant metastases on multivariate analysis.100

**Lung cancer**

Integrin β4 is overexpressed in non-small cell lung cancers, and expression is particularly high in pulmonary squamous cell carcinomas. An early study investigated expression of the integrin α6β4 in a series of patient-derived lung cancers and found moderate to strong expression in all of the squamous cell carcinomas (N = 36) and
adenocarcinomas \((N = 23)\) tested, though expression was notably absent in all \((N = 10)\) of the small cell carcinomas examined.\(^{101}\) Integrin \(\beta 4\) expression was identified in a number of non-small cell carcinoma cell lines (A431, A549, DG3), but was absent in the single small cell carcinoma cell line tested (AE2).\(^{101}\)

In a different study that included uninvolved normal lung tissue, normal alveolar epithelial cells were found to be negative for integrin \(\beta 4\) expression, and instead exhibited expression of the \(\alpha 1\beta 1\) and \(\alpha 3\beta 1\) laminin receptors. They further found that bronchial and bronchiolar epithelium exhibited weak and inconsistent integrin \(\beta 4\) expression that was localized to the basement membrane interface. In squamous cell carcinomas of the lung, integrin \(\beta 4\) expression was intense and localized to the tumor-stroma interface.\(^{102}\) In this same study, integrin \(\beta 4\) expression was identified in large cell carcinomas of the lung, but was found to be absent in neuroendocrine carcinomas. Patriarca et al.\(^{103}\) also found that in normal bronchial epithelium, integrin \(\beta 4\) expression was localized to the basal surface of cells in a linear pattern. They found strong and extensive staining for the integrin \(\beta 4\) in 85% of squamous cell carcinomas \((N = 20)\), but found positive staining in only 25% pulmonary adenocarcinomas studied \((N = 20)\). In a complementary study using molecular profiling, integrin \(\beta 4\) was upregulated in lung squamous cell carcinomas when compared to adenocarcinomas, and this was confirmed using both immunohistochemistry and in-situ hybridization.\(^{104}\) Additional studies will be needed to determine the clinical and prognostic significance of integrin \(\beta 4\) expression in non-small cell lung cancer.

**Pancreatic cancer**

Integrin \(\beta 4\) is overexpressed in pancreatic carcinomas, and is also a marker of poor prognosis. Using gene expression profiling, Logsdon et al. first determined that integrin \(\beta 4\) is upregulated in pancreatic adenocarcinoma when compared to normal
pancreatic tissue and chronic pancreatitis tissue samples, a finding that was confirmed by others. In order to validate these findings, the Logsdon group performed immunohistochemistry for the integrin β4 subunit and a number of other candidate genes in 28 pancreatic adenocarcinomas, where they found that all cases had strong integrin β4 expression. In another report, Gleason et al. found moderate to strong integrin β4 staining in 92% (N = 48) of pancreatic adenocarcinomas that were evaluated using immunohistochemistry; they also found that in normal pancreas, integrin β4 staining was weak and expressed only along the basement membranes of large ducts.

Integrin β4 expression has also been studied in pancreatic intraepithelial neoplasia, a non-invasive precursor lesion to pancreatic adenocarcinoma. In a comprehensive study of pancreatic lesions, Cruz-Monserrate et al. determined that integrin β4 overexpression is present in the early stages of pancreatic adenocarcinoma development. As reported previously, they found that in normal pancreas, integrin β4 expression is localized to the interface between ductal epithelial cells and the basement membrane. Upregulation of integrin β4 expression was observed in 92% (N = 113) of pancreatic adenocarcinomas studied, and distinguished pancreatic cancer from pancreatitis. Furthermore, overexpression and altered localization of the integrin β4 was identified in all pancreatic intraepithelial neoplasia lesions ranging from Grade 1A to Grade 3.

Recently, elevated integrin β4 expression was shown to associate with reduced overall survival among pancreatic adenocarcinoma patients (N = 134), where it was found to have independent prognostic significance on multivariate analysis. Interestingly, elevated integrin β4 expression was also found to correlate with a number of EMT hallmarks, including solitary cell infiltration, reduced expression of E-cadherin, and increased expression of vimentin. Pancreatic adenocarcinoma has one of the poorest prognoses of all epithelial malignancies; the fact that integrin β4 is highly expressed in
these tumors provides further evidence for its role in aggressive neoplasms.

**Thyroid cancer**

Thyroid carcinomas are unique in that they are one of the few malignancies to exhibit neoexpression of integrin α6β4 during cancer progression. While expression of integrin α6β4 is absent in normal and adenomatous follicular cells, strong expression has been observed in both follicular and papillary thyroid carcinomas.\(^{110}\) Similar findings have been reported using flow cytofluorometry, where expression of integrins such as α1β1 and α6β1 was found in normal thyroid and tumor specimens, integrin α6β4 expression was found only in thyroid carcinomas and carcinoma cell lines.\(^{111}\) Others have confirmed neoexpression of integrin α6β4 in thyroid carcinoma tissue\(^ {112,113}\) and have also found that it is expressed in anaplastic thyroid carcinoma, an aggressive and poorly differentiated malignancy.\(^ {112}\)

**Malignancies in which integrin β4 expression has controversial or undetermined significance**

**Colon cancer**

One of the earliest studies to investigate α6β4 expression in human malignancies found that the α6β4 integrin was expressed in colon cancer.\(^ {82}\) However, additional reports have been controversial. One study investigating integrin β4 expression in colorectal carcinomas reported that integrin β4 expression was reduced during malignant transformation.\(^ {114}\) This group found that while expression of the integrin β4 subunit was maintained at the basal epithelial cell membrane in normal colonic mucosa and in colonic adenomas, expression of the integrin β4 subunit was reduced or absent in most colorectal carcinomas, but was maintained in well-differentiated colon cancer.\(^ {114}\) Similar findings were reported in another study, where expression of the integrin β4
subunit was reduced or absent in most colon carcinomas examined.  

A contrasting study demonstrated that integrin β4 is overexpressed in a majority of colon carcinomas and that its expression is elevated in high stage, poorly differentiated cancers. These findings are supported by subsequent work examining integrin β4 expression in colorectal carcinomas using double immunofluorescence and RT-QPCR, where integrin β4 protein and transcript levels were increased in colorectal carcinoma when compared to normal tissue. Data from our laboratory also confirms the observation that integrin β4 levels are particularly high in colon cancer cell lines and patient-derived tissues (unpublished observation).

**Ovarian cancer**

In benign ovary, integrin β4 is basally located in surface and cyst lining epithelium. One of the few studies investigating integrin β4 expression in ovarian cancer found strong basal expression in all of the epithelial ovarian tumors studied, and also found that integrin β4 was expressed in malignant cells within the ascitic fluid in three out of nine cases. A second report found basally polarized integrin β4 expression in normal ovary and in 40% of serous ovarian carcinomas examined. The authors further noted that expression of both integrin α6 and β4 subunits were positively correlated with laminin expression. Interestingly, serous ovarian cancer has a similar genomic profile to basal-like breast cancer, with both subtypes displaying frequent loss of TP53, BRCA1, and RB1, suggesting that integrin β4 may play an important role in both types of cancer. Further work will be needed to fully characterize integrin β4 expression in ovarian neoplasia and to determine how expression associates with prognosis.
Prostate cancer

Early reports indicated that integrin β4 is downregulated in prostatic adenocarcinoma, and in one investigation, expression of integrin β4 was absent in all prostate cancers examined (N = 20).\textsuperscript{118} Multiple additional studies have reported that integrin β4 expression is lost during the transition from benign epithelium to prostatic adenocarcinoma.\textsuperscript{119-122} A potential explanation for this phenomenon is that androgen receptor expression has been reported to cause downregulation of integrin α6β4.\textsuperscript{123} The assertion that integrin β4 is downregulated in prostate cancer was challenged by a report demonstrating that integrin β4 mRNA is overexpressed in a subset of prostate carcinomas using gene expression datasets and a DNA microarray.\textsuperscript{124} The authors of this study also investigated integrin β4 protein expression using immunohistochemistry and found overexpression in 35% of invasive cancers and in a number of metastatic lesions.\textsuperscript{124} More recently, a population of integrin β4 positive circulating tumor cells was identified in the peripheral blood of patients with castration resistant prostate cancer.\textsuperscript{125} Overall, prostate cancer is one cancer in which integrin α6β4 is suggested to be downregulated. However, given recent findings, it will be important to determine how residual or enhanced integrin α6β4 expression, in the minority of cases that overexpress it, associates with clinical parameters.

Tumors of the central nervous system

Expression of integrin α6β4 has not been extensively studied in glial tumors; however, there is evidence demonstrating that integrin α6β4 is expressed in astrocytomas, oligodendrogliomas, glioblastomas, and a number of glioma cell lines. Integrin β4 expression has been identified in reactive astrocytes\textsuperscript{126} as well as in subependymal glia, choroid plexus and meningothelial cells.\textsuperscript{127} One study found that integrin β4 expression is higher in astrocytomas and glioblastomas when compared to
benign astrocytes. A larger study investigated expression of the integrin β4 subunit in a series of astrocytomas and oligodendrogliomas where they found that integrin β4 expression was slightly higher in oligodendrogliomas. Further studies will be needed to determine how integrin α6β4 relates to glioma stage and prognosis.

**Sarcomas**

Integrin β4 overexpression has been described in high-grade osteosarcomas and there is evidence it may play a role in promoting a metastatic phenotype by interacting with ezrin. While integrin β4 is expressed in benign endothelial cells, its expression appears to be reduced in angiosarcomas and other vascular tumors. Integrin β4 staining is also reportedly absent in rhabdomyosarcomas, ganglioneuroblastomas, primitive peripheral neuroectodermal tumors and Ewing's sarcomas.

**Conclusions**

Integrin β4 is commonly overexpressed in high-grade malignancies. Notably, there is strong evidence that integrin β4 is overexpressed in tumors of the bladder, cervix, lung, pancreas and thyroid. In addition, integrin β4 overexpression has been identified as an adverse prognostic marker in tumors of the bladder and pancreas, and in squamous cell carcinomas of the head and neck. The reason for these poor prognoses stems from the ability of the integrin α6β4 to promote several key hallmarks of cancer, including the capacity to sustain proliferative signaling, evade apoptosis, promote tissue invasion and metastasis, and stimulate angiogenesis.

In a number of malignancies, it is still unclear whether expression of integrin β4 is elevated or reduced. In tumors of the breast, prostate and colon, a number of studies examining integrin β4 expression have obtained conflicting results, with some authors reporting that integrin β4 is overexpressed and others reporting a reduction in integrin β4
expression. These disparate findings may relate to differences in sample size, antibody usage, tissue processing or antigen retrieval process. Because integrin β4 is highly expressed at the basal aspect of normal epithelial cells but is redistributed over the cell surface during malignant transformation, it may appear in some cases that integrin β4 expression is reduced. It is likely that altered localization of the α6β4 integrin and its concurrent release from hemidesmosomes are as important in carcinoma progression as overexpression, thus altered integrin α6β4 localization should be studied carefully in these malignancies.

Study rationale and hypothesis

Our lab previously demonstrated that integrin β4 is highly expressed in pancreatic adenocarcinomas, a tumor type with a high frequency of TP53 mutations.\textsuperscript{72} Notably, mutation or inactivation of TP53 is one mechanism that allows integrin α6β4 to promote cell survival and amplify signaling through a number of invasive and proliferative pathways. Integrin α6β4 can trigger apoptosis in cells harboring wild-type TP53; however, in carcinoma cells with mutant TP53, integrin α6β4 promotes cell survival.\textsuperscript{31,68,69} Interestingly, tumors with a high frequency of TP53 mutations (pancreatic adenocarcinoma, basal-like breast cancer, squamous cell carcinomas of the head and neck) tend to also display integrin β4 overexpression. In these tumor types, there is evidence that integrin β4 expression is clinically significant and associates with a poor prognosis. This observation may partially explain why integrin β4 expression is prognostically significant in some, but not all tumor types.

Non-small cell lung cancer (NSCLC) and malignant gliomas are both aggressive malignancies that have a high frequency of TP53 mutations. While preliminary studies have investigated integrin β4 expression in these tumor types, the pathologic and prognostic significance of integrin β4 expression in these cancers remains
undetermined. I therefore sought to investigate integrin β4 expression in patient-derived lung cancers and in malignant gliomas. In order to examine integrin β4 expression in a large number of patient-derived tumors, I performed immunohistochemistry of tissue microarray sections and analyzed integrin β4 gene expression using gene expression databases.

While a number of studies have investigated integrin β4 expression in breast cancer, the significance of integrin β4 overexpression in these tumors remains controversial. In addition, integrin β4 expression in ductal carcinoma in situ (DCIS) and its clinical significance has not been thoroughly investigated. For these reasons, I sought to investigate integrin β4 expression in benign breast, DCIS, and invasive breast cancer. Basal-like breast cancer has a high frequency of TP53 mutations and integrin β4 overexpression has previously been demonstrated in this tumor type. In order to study integrin β4 expression in these aggressive tumors, a TMA enriched for TNBCs was constructed. The hypothesis of this study is that in tumors of the lung, breast, and central nervous system, integrin β4 overexpression associates with aggressive behavior and reduced patient survival.
CHAPTER 2: GENERAL MATERIALS AND METHODS

Immunohistochemistry

As described previously, the integrin α6 subunit can pair with either the β1 or β4 subunit. In contrast, the integrin β4 subunit can only pair with the α6 subunit, therefore integrin β4 subunit expression is predictive of integrin α6β4 expression.1,2,4 Immunohistochemistry was performed on formalin fixed paraffin embedded (FFPE) tissue sections (4 μm) that had been baked at 60°C for 1 hour. First, sections were deparaffinized by washing in xylene three times. The sections then went through three washes each in 100% ethanol followed by 95% ethanol, each for 1 minute. Slides were then rinsed in a water bath for three minutes. The activity of endogenous peroxidases was blocked by placing slides in 3% hydrogen peroxide in phosphate buffered saline (PBS, pH 7.4) (P3813) (Sigma-Aldrich, Saint Louis, MO) for 10 minutes. Slides were then rinsed with water, and antigen retrieval was then performed by placing slides in a container filled with 1 X Dako Target Retrieval Solution (S1699) (Dako Corporation, Carpinteria, CA, USA), which had been brought to temperature in a steamer at 100°C. The slides were kept at 100°C for 20 minutes, and then removed and allowed to cool for an additional 20 minutes. After rinsing in water and 1 X PBS with 0.05% Tween-20 (PBST, pH 7.4) (P3563) (Sigma-Aldrich, Saint Louis, MO, USA), blocking was performed using an Avidin-Biotin Blocking kit (SP-2001) (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Sections were then blocked against non-specific binding by placing them in 1 X PBST and 0.3% casein for 30 minutes. Next, the sections were incubated with a rat monoclonal primary antibody to the integrin β4 subunit (CD 104) (clone 439-9B; BD Pharmingen, San Jose, CA), at a concentration of 1:200 in antibody diluent solution (S3022) (Dako Corporation). Slides were incubated with the primary antibody for one hour at room temperature, after which
they underwent three rinses in PBST. Next, the slides were incubated with a biotinylated rat secondary antibody (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature at a concentration of 1:500 in antibody diluent solution (S3022) (Dako Corporation). After incubation with the secondary antibody, slides were again rinsed three times in PBST. Slides were then incubated for 20 minutes with Streptavidin HRP (P0397) (Dako Corporation) at a concentration of 1:500 in PBS. Detection was accomplished using a 3′3 diaminobenzidine (DAB) developer from a kit manufactured by Dako Corporation (K3468). Sections were counterstained with Hematoxylin, rinsed in water, and washed three times in 95% ethanol, 100% ethanol, and xylene. The slides were then coverslipped using Cytoseal 60 mounting media (Richard-Allan Scientific, Kalamazoo, MI). This immunohistochemistry protocol for integrin β4 staining was developed by Cruz-Monserrate (14).

**Statistical analysis**

Differences between groups were analyzed using chi-square or Fisher’s exact test for categorical variables, as appropriate. For continuous variables, two-tailed t-test, two-tailed t-test with Welch’s correction, Mann-Whitney test, one-way ANOVA with post hoc Tukey’s test, or Kruskal-Wallis test with Dunn’s Multiple Comparison Test were used, as appropriate. Survival differences were assessed via log-rank tests for univariate analyses and Cox proportional hazards for multivariate. Significance was reached when P < 0.05. Statistical analyses were performed using GraphPad software (La Jolla, CA).
CHAPTER 3: INTEGRIN α6β4 EXPRESSION IN LUNG CANCER

Introduction

Lung cancer epidemiology

Lung cancer is the leading cause of cancer-related mortality in the United States and is a significant problem globally.\textsuperscript{132} Patients diagnosed with lung cancer have a poor prognosis, and over 80% succumb to disease within 5 years of diagnosis.\textsuperscript{132} Lung cancer is most often diagnosed between the ages of 40 – 70, with a peak incidence at 50 – 60 years of age.\textsuperscript{133} The state of Kentucky is severely affected by lung cancer, having both the highest incidence and mortality rates for lung cancer in the United States. This phenomenon is at least partially explained by the high prevalence of tobacco use in this state.

Evidence demonstrating a link between cigarette smoking and lung cancer is overwhelming. Exposure to cigarette smoke is responsible for approximately 80-90% of lung cancers in the United States,\textsuperscript{134} though exposure to a number of additional agents can also contribute to the development of lung cancer. These include uranium, asbestos, radon, polycyclic aromatic hydrocarbons, arsenic, nickel, vinyl chloride, radiation and air pollution.\textsuperscript{135} In the southeastern United States, occupational exposure related to coal-mining may also elevate lung cancer risk.\textsuperscript{136} A small number of lung cancers develop in never-smokers, and although rare, these tumors may be attributable to inherited mutations such as the T790M mutation in \textit{EGFR}.\textsuperscript{137,138}

Lung cancer pathobiology

Lung cancer can be categorized into a number of groups based on histologic features, immunophenotype and clinical behavior. Broadly, lung cancer can be divided into two main categories: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC is an epithelial tumor that is notable for its aggressive behavior and neuroendocrine phenotype. It is distinct from other lung cancers in both clinical behavior...
and response to chemotherapy, and is often diagnosed at an advanced stage. Morphologically, SCLC is characterized by small, round to oval cells with scant cytoplasm (Fig. 3.1). The nuclei are hyperchromatic with finely granular chromatin, leading to the common descriptor “salt and pepper” chromatin. SCLC exhibits neuroendocrine differentiation, and typically expresses chromogranin, synaptophysin, CD56, and other neuroendocrine markers. In accordance with their notoriously aggressive behavior, the proliferation rate of these tumors reaches nearly 100% as measured using Ki-67 immunohistochemistry. The presence of zonal necrosis is another characteristic feature of SCLC.

The pathogenesis of SCLC includes aberrations in Notch and Hedgehog signaling that mimic processes found in early lung development. Genomically, SCLC is characterized by a high frequency of TP53 mutations, inactivation of RB, deletion of chromosome 3 (p14-p23) and upregulation of BCL-2. Unfortunately, study of these tumors is limited somewhat by the fact that pathologic material is difficult to obtain. Because of the aggressive nature of SCLC, surgical resection is rarely attempted, and hence, only small biopsies or fine needle aspiration specimens are available for analysis. These factors limited the examination of integrin β4 expression in SCLC in this study.

NSCLC is the most common form of lung cancer and accounts for up to 80% of lung cancer diagnoses. NSCLC can be further divided into a variety of histologic subtypes, the most common of which include adenocarcinoma (ADC), squamous cell carcinoma (SCC) and large cell carcinoma (LCC). There are a few additional histologic subtypes that are less frequently encountered; these include adenosquamous carcinoma, sarcomatoid carcinoma, giant cell carcinoma, and carcinoid tumors. Although exceedingly rare, analogues of salivary gland tumors are occasionally observed in the lung and bronchus.
Figure 3.1: Histologic features of SCLC and NSCLC.
SCLC demonstrating small, round to oval cells with hyperchromatic nuclei and scant cytoplasm (A). NSCLC with eosinophilic cytoplasm, pleomorphic vesicular nuclei and prominent nucleoli (B). Magnification = 200x, all images.
Adenocarcinoma is the most common type of lung cancer in the United States. Histologically, adenocarcinoma is characterized by glandular differentiation and the presence of intracytoplasmic mucin. Poorly differentiated adenocarcinomas may lack characteristic morphologic features thus requiring the use of immunohistochemistry to arrive at a pathologic diagnosis. Adenocarcinomas are often positive for expression of TTF-1, Napsin A, and CK7 by immunohistochemistry. Genomic alterations frequently observed in lung adenocarcinoma include mutations in KRAS, EGFR, and TP53. Fusions involving ALK, ROS1, and EML4 have also been identified. Although rare, HER2 mutations also occur in lung adenocarcinomas and clinical trials targeting these mutations are ongoing.

Squamous cell carcinomas (SCC) of the lung frequently display intracellular bridges, and in well to moderately differentiated tumors, distinctive keratin pearls may be noted. Poorly differentiated SCCs can often be distinguished from lung adenocarcinoma by positive immunohistochemical staining for CK5/6, p63, and p40, and by negative staining for TTF-1. SCCs have a high frequency of TP53 mutations, with some sources reporting these mutations in greater than 80% of cases. Lung SCC carries a poor prognosis and is typically treated with surgical resection, radiation and traditional cytotoxic chemotherapy. While a number of targeted therapies have recently been developed for the treatment of NSCLC, these agents target genomic alterations that occur more frequently in lung adenocarcinoma, such as mutations in EGFR and rearrangements of ALK and ROS1. Squamous cell carcinomas lack targeted therapies and display notoriously aggressive behavior.

In carcinoma cells with mutant TP53, the integrin β4 promotes cell survival by activating AKT. Interestingly, TP53 mutations and integrin β4 overexpression co-occur in many aggressive malignancies, including basal-like breast cancer, serous ovarian carcinoma, and pancreatic ductal adenocarcinoma. Given that lung SCC has a
high frequency of TP53 mutations, I predicted that integrin β4 expression in this tumor type would associate with aggressive behavior and poor prognosis. While preliminary investigations into integrin β4 expression in NSCLC have been published previously, an association has not been demonstrated between integrin β4 overexpression and clinical outcomes. We therefore sought to investigate integrin β4 expression as it relates to histologic subtype, clinicopathologic features and patient survival in lung cancer. Here, I report that integrin β4 is highly expressed in lung SCC, and that its overexpression is associated with decreased overall survival in NSCLC patients.

**Materials and Methods**

**Lung cancer tissue microarray construction**

Institutional Review Board Approval (13-0692-P6H) was obtained prior to initiating the project. Surgical resections for NSCLC performed at the University of Kentucky from 2006-2010 were identified using natural language searches in CoPath (Cerner Corporation, Kansas City, MO). Cases were excluded if the primary tumor was not of lung origin, or if adequate pathologic material was not available. A total of 216 cases were selected, including 83 adenocarcinomas, 102 squamous cell carcinomas, 12 adenosquamous carcinomas, 12 poorly differentiated carcinomas, 2 large cell neuroendocrine carcinomas, 1 giant cell carcinoma, 1 pleomorphic carcinoma, 1 sarcomatoid carcinoma and 2 tumors with mixed histology (mixed adenocarcinoma and large cell neuroendocrine). For each case, I reviewed the original hematoxylin and eosin (H&E) stained slides and selected representative tumor blocks. Fresh H&E stained sections were then cut from each selected block. These fresh sections were again reviewed, and tumor areas were selected for inclusion in the arrays. Pathologic features (tumor grade, tumor size, histologic type, pTNM staging, presence of lymphovascular, venous, and pleural invasion) were abstracted from pathology records using CoPath.
(Cerner Corporation, Kansas City, MO). Treatment and outcome data were collected by the Cancer Research Informatics Shared Resource Facility. Listing of randomly-sorted samples for allocation into the recipient TMA blocks was generated by the MCC Biostatistics and Bioinformatics Shared Resource Facility. Assembly of TMA blocks (12 in total) was performed by the MCC Biospecimen and Tissue Procurement Shared Resource Facility. Three 2 mm diameter tissue cores were obtained from each tumor specimen, which were transferred to recipient paraffin blocks using a TMArayer (Pathology Devices, Westminster, MD). TMA sections used for integrin β4 immunohistochemistry had interpretable tissue cores in 211/216 cases. Patient characteristics for these 211 cases are summarized in Table 3.1.

**Immunohistochemistry Scoring and Analysis**

Immunohistochemistry was performed according to the protocol described in Chapter 2. Specifically, the primary antibody (439-9B) was used at a concentration of 1:200 and the secondary antibody was used at 1:500. A semi-quantitative scale was used to score integrin β4 expression as follows: negative (0), weak (1), moderate (2), strong (3). Results from each of the three tissue cores were averaged to produce a final score for each tumor. As integrin β4 was expressed at moderate (2) levels in benign bronchial epithelium, we defined integrin β4 overexpression as an average score of ≥ 2.5.

**Data Mining**

Multiple lung cancer gene expression datasets were analyzed for ITGB4 expression. The first of these was a NSCLC dataset generated by the Cancer Genome Atlas Research Network (TCGA, [http://cancergenome.nih.gov/](http://cancergenome.nih.gov/)) containing 155 SCC samples and 32 ADC samples that were analyzed using a custom Agilent microarray.
The second was a dataset generated by Hou et al. containing 91 NSCLCs and 65 adjacent normal lung samples that had been analyzed using a Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA). These datasets were accessed and downloaded using The Oncomine™ Platform (Life Technologies, Ann Arbor, MI). The UCSC Cancer Browser (https://genome-cancer.ucsc.edu/proj/site/hgHeatmap/) was used to download a processed lung SCC gene expression dataset ($N = 155$) in order to study genes correlated with $ITGB4$. In addition, cBioPortal (http://www.cbioportal.org/) was used to generate a network map showing the 50 most highly altered genes neighboring $ITGB4$ in the TCGA lung SCC dataset.
Table 3.1: Lung tissue microarray patient and tumor characteristics.

**Patient Characteristics**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis</td>
<td>63</td>
<td>39-84</td>
</tr>
</tbody>
</table>

**Gender**

<table>
<thead>
<tr>
<th>Gender</th>
<th>Count</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>88</td>
<td>42%</td>
</tr>
<tr>
<td>Male</td>
<td>123</td>
<td>58%</td>
</tr>
</tbody>
</table>

**Smoking Status**

<table>
<thead>
<tr>
<th>Smoking Status</th>
<th>Count</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with data</td>
<td>159</td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>154</td>
<td>97%</td>
</tr>
<tr>
<td>Never smoker</td>
<td>5</td>
<td>3%</td>
</tr>
</tbody>
</table>

**Residence**

<table>
<thead>
<tr>
<th>Residence</th>
<th>Count</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appalachian</td>
<td>145</td>
<td>69%</td>
</tr>
<tr>
<td>Non-Appalachian</td>
<td>59</td>
<td>28%</td>
</tr>
<tr>
<td>Out of state</td>
<td>7</td>
<td>3%</td>
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**Vital Status**

<table>
<thead>
<tr>
<th>Status</th>
<th>Count</th>
<th>Percentage</th>
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<tbody>
<tr>
<td>Alive</td>
<td>89</td>
<td>42%</td>
</tr>
<tr>
<td>Deceased</td>
<td>122</td>
<td>58%</td>
</tr>
</tbody>
</table>

**Tumor characteristics**

**Histology**

<table>
<thead>
<tr>
<th>Histology</th>
<th>Count</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma</td>
<td>81</td>
<td>38%</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>99</td>
<td>47%</td>
</tr>
<tr>
<td>Other</td>
<td>31</td>
<td>15%</td>
</tr>
</tbody>
</table>

**Differentiation**

<table>
<thead>
<tr>
<th>Differentiation</th>
<th>Count</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well</td>
<td>12</td>
<td>6%</td>
</tr>
<tr>
<td>Moderate</td>
<td>87</td>
<td>41%</td>
</tr>
<tr>
<td>Poor</td>
<td>112</td>
<td>53%</td>
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**AJCC Stage**

<table>
<thead>
<tr>
<th>Stage</th>
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<th>Percentage</th>
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</thead>
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<tr>
<td>I</td>
<td>108</td>
<td>51%</td>
</tr>
<tr>
<td>II</td>
<td>37</td>
<td>18%</td>
</tr>
<tr>
<td>III</td>
<td>44</td>
<td>21%</td>
</tr>
<tr>
<td>IV</td>
<td>13</td>
<td>6%</td>
</tr>
<tr>
<td>Unknown</td>
<td>9</td>
<td>4%</td>
</tr>
</tbody>
</table>

**Total** | 211  |
Results

Patient and tumor characteristics

The mean age at diagnosis for patients in this study was 63.4 (range 39-84), with a median of 63 years. Lung cancer was more frequently diagnosed in males (58%) than in females (42%). Of patients with data available on tobacco use, 97% had a smoking history while only 3% were never smokers. Squamous cell carcinoma was the most common histologic type and accounted for 47% of cases, while adenocarcinoma accounted for 38% of cases. Most tumors were moderate (41%) or poorly (53%) differentiated, with only a small number of well differentiated tumors (6%). The well differentiated tumors tended to be bronchoalveolar carcinomas (92%). As patients with localized disease are more frequently treated with surgical resection, there was a predominance of American Joint Committee on Cancer (AJCC) Stage I and II cases, with only 6% Stage IV cases.

Comparison of integrin \(\beta_4\) expression in benign and neoplastic lung

In an external gene expression dataset, NSCLCs were found to have elevated integrin \(\beta_4\) expression when compared to normal lung tissue (Fig. 3.2A; \(P < 0.0001\)). Although normal lung tissue was not specifically selected for inclusion in the tissue microarrays, benign bronchial epithelium was identified in a subset of tissue cores. In pseudostratified columnar epithelium, integrin \(\beta_4\) was primarily expressed in basal cells and along the basement membrane (Fig. 3.2B). Weak staining was also present at the apical surface of ciliated columnar cells, while goblet cells and immune cells in the bronchial epithelium were negative for integrin \(\beta_4\) expression. In a few cases, benign bronchial epithelium was identified adjacent to invasive carcinoma cells. Here, integrin \(\beta_4\) expression was higher in the invasive carcinoma (Fig. 3.2C-D). In addition, basal polarization of integrin \(\beta_4\) expression was lost in invasive carcinoma.
Expression and localization of the integrin β4 in NSCLC

Integrin β4 expression was found to be highly variable in NSCLCs: some cases exhibited strong and diffuse staining while others were completely negative for integrin β4 expression (Fig. 3.3A-D). In some tumors, staining was predominantly membranous, while others exhibited a mixture of cytoplasmic and membranous immunoreactivity (Fig. 3.4A-B). Integrin β4 staining intensity was elevated at the tumor-stromal interface in some tumors, a phenomenon that has been previously described by others (Fig. 3.4C). Individual infiltrating tumor cells and nests at the invasive front also tended to have elevated integrin β4 expression compared to cells at the center of the tumor (Fig. 3.5).

Integrin β4 expression and association with clinical features

In our TMA cohort, integrin β4 expression was elevated in squamous cell carcinomas when compared to adenocarcinomas (Fig. 3.6A; $P < 0.0001$); this finding was confirmed in two external gene expression datasets (Fig. 3.6B-C; $P < 0.0001$). Integrin β4 protein expression was also elevated in a subset of adenosquamous carcinomas and poorly differentiated tumors that were evaluated in our TMA (Table 3.2). Using data abstracted from pathology reports, integrin β4 overexpression was found to associate with the presence of venous invasion (Fig. 3.7; $P = 0.0037$). To date, no data have been published that demonstrate an association between integrin β4 expression and patient outcome in NSCLC. In our TMA cohort, integrin β4 overexpression was significantly associated with shorter overall survival (Fig. 3.8A; hazard ratio 1.457, 95% confidence interval 1.013 to 2.094, $P = 0.0422$). This relationship was also significant when using a higher cutoff point to define integrin β4 overexpression (Fig. 3.8B; hazard ratio 1.714, 95% confidence interval 1.171 to 2.510, $P = 0.0056$). Furthermore, elevated integrin β4 gene expression in the Hou dataset was associated with reduced one year
survival (Fig. 3.8C; $P = 0.0062$), and showed a trend towards reduced overall survival (Fig. 3.8D; hazard ratio 1.674, 95% confidence interval 0.8977 to 3.120, $P = 0.1051$). In the TCGA dataset, higher levels of integrin β4 gene expression were associated with reduced overall survival (Fig. 3.8E-F; hazard ratio 1.714, 95% confidence interval 1.052 to 2.792, $P = 0.0305$). cBioportal was used to generate a list of genes most positively correlated with $ITGB4$ in squamous cell lung cancers. $ITGB4$ expression was highly correlated with expression of stem cell markers $ITGA6$ and $CD44$ (Figure 3.9A-B; $P < 0.0001$). Expression of $EGFR$ was also highly correlated with $ITGB4$ expression (Fig. 3.9C). In addition, a network map was generated using cBioPortal. This map demonstrates highly altered genes neighboring $ITGB4$ (Fig. 3.10). These genes included various laminins, collagens, CD151, the Myc pathway, EGFR, Met, Yes and genes in the PI3K-AKT pathway.
Figure 3.2: Expression of the integrin β4 in benign and neoplastic lung. In the Hou dataset, NSCLCs had greater average levels of ITGB4 mRNA than normal lung, \( P < 0.0001 \) via two tailed t-test with Welch's correction (A). In benign bronchial epithelium, integrin β4 had moderate expression in basal cells and along the basement membrane, with weak expression at the apical surface of ciliated columnar cells (B). However, in invasive carcinoma, integrin β4 was overexpressed and basal polarization was lost (C, D).
Figure 3.3: Integrin β4 staining intensity in non-small cell lung carcinoma. Examples of negative (A), weak (B), moderate (C), and strong (D) integrin β4 expression in NSCLCs. Left panels show integrin β4 staining, right show H&E. Magnification = 200x, all images.
Figure 3.4: Localization of integrin β4 staining in NSCLC. Some cases exhibited predominantly membranous staining (A), while others had strong membranous and cytoplasmic expression of the integrin β4 (B). In some cases, integrin β4 was elevated predominantly at the tumor-stroma interface (C). Magnification = 200x, all images.
Figure 3.5: Integrin β4 expression at the invasive front. Integrin β4 expression is elevated in infiltrating cells at the invasive front of a poorly differentiated NSCLC.
Table 3.2: Integrin β4 expression in NSCLC by histologic subtype.

<table>
<thead>
<tr>
<th></th>
<th>Integrin β4 High</th>
<th>Integrin β4 Low</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total</strong></td>
<td>101/211 (48%)</td>
<td>110/211</td>
</tr>
<tr>
<td><strong>Histologic Type:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>77/99 (78%)</td>
<td>22/99 (22%)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>11/81 (14%)</td>
<td>70/81 (86%)</td>
</tr>
<tr>
<td><strong>Other histologic types:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>5/12 (42%)</td>
<td>7/12 (58%)</td>
</tr>
<tr>
<td>Adenosquamous</td>
<td>7/12 (58%)</td>
<td>5/12 (42%)</td>
</tr>
<tr>
<td>Mixed histology</td>
<td>1/2 (50%)</td>
<td>1/2 (50%)</td>
</tr>
<tr>
<td>Large cell neuroendocrine</td>
<td>0/2 (0%)</td>
<td>2/2 (100%)</td>
</tr>
<tr>
<td>Pleomorphic carcinoma</td>
<td>0/1 (0%)</td>
<td>1/1 (100%)</td>
</tr>
<tr>
<td>Giant cell carcinoma</td>
<td>0/1 (0%)</td>
<td>1/1 (100%)</td>
</tr>
<tr>
<td>Sarcomatoid carcinoma</td>
<td>0/1 (0%)</td>
<td>1/1 (100%)</td>
</tr>
</tbody>
</table>
Figure 3.6: Integrin β4 expression in NSCLC by histologic subtype. SCCs had a higher proportion of cases with integrin β4 overexpression than did adenocarcinomas, as measured using semi-quantitative IHC, \( P < 0.0001 \) via Mann-Whitney test (A). In a TCGA NSCLC dataset, average \( ITGB4 \) mRNA expression was higher in SCCs than in adenocarcinomas, \( P = 0.0001 \) via two-tailed t-test (B), and in the Hou dataset, average \( ITGB4 \) mRNA expression was higher in SCCs when compared to both normal lung tissue and adenocarcinomas, \( P < 0.0001 \) via one-way ANOVA with post hoc Tukey’s test (C).
Figure 3.7: Integrin β4 is elevated in tumors with venous invasion. Integrin β4 overexpression is associated with the presence of venous invasion, $P = 0.0037$ via Mann-Whitney test (A). An example of a tumor from the TMA with venous invasion, stained for integrin β4 and elastic trichrome (B). In the elastic trichrome, black staining highlights elastic fibers of the vein wall, and tumor fills the vessel.
Figure 3.8: Integrin β4 and survival in NSCLC. In our TMA cohort, elevated expression of integrin β4 was associated with shorter median overall survival, $P = 0.0422$ (A) and $P = 0.0056$ (B). In the Hou cohort, average $ITGB4$ mRNA levels were higher in patients that were deceased at one year (C), and there was a trend towards reduced overall survival for patients with elevated $ITGB4$ mRNA expression (D). In the TCGA cohort, elevated $ITGB4$ mRNA expression was associated with reduced overall survival depending on the cutoff point used, $P = 0.0305$ (E and F). Survival analyses in (A), (B), and (D) were done via log-rank (Mantel-Cox) tests.
Figure 3.9: *ITGB4* expression correlates with *ITGA6*, *CD44*, and *EGFR* in SCC.

By linear regression, *ITGB4* mRNA expression levels were positively correlated with *ITGA6* (A), *CD44* (B), and *EGFR* (C).
Figure 3.10: Network map illustrating highly altered genes associated with ITGB4.

In this network map generated using cBioPortal, ITGB4 is connected to laminins (LAMC2, LAMA1, LAMB1, LAMA2, etc), genes in the EGFR family (EGFR, ERBB3), genes in the PI3K pathway (PIK3CA, AKT1), other integrins (ITGA6, ITGB1) and the tetraspanin CD151.
**Discussion**

In this study, I found that integrin β4 is highly expressed in lung SCC, and that its overexpression is associated with venous invasion and reduced overall survival in patients with NSCLC. These results are in accordance with findings in other cancers that demonstrate an association between integrin β4 overexpression and poor prognosis.50,100,109 Previous studies have demonstrated that the integrin β4 is highly expressed in SCC, however, this is one of the first studies to demonstrate that it has prognostic significance. Early reports have shown that expression of the integrin α6β4 (previously described as TSP-18084) is elevated in murine Lewis lung carcinoma variants with high metastatic potential.154,155 As mentioned previously, integrin β4 has been shown to form a complex with and to amplify ErbB-2 signaling, and notably, ErbB-2 and integrin α6β4 co-localization have been identified in the lung cancer cell line Calu-3.156 Multiple studies investigating integrin β4 expression in patient-derived tissues have shown that it is expressed in NSCLC, with high levels observed in squamous cell carcinomas.101-103 A more recent study using gene expression profiling identified differentially expressed genes in samples of pulmonary ADC, SCC, and normal bronchus.104 In this study, integrin β4 was found to be significantly upregulated in SCC.104 Notably, the integrin β4 gene (ITGB4) is upregulated in the basal molecular subtype of lung SCC as defined using unsupervised clustering of gene expression microarray data.157

Tumor metastases are responsible for the majority of cancer related deaths.158 In order for cancer cells to metastasize, they must acquire access to the circulation through a process termed intravasation.159 Integrin β4 has been shown to enhance tumor cell binding to the vascular endothelium, and can also mediate vascular permeability through its effects on vascular endothelial growth factor (VEGF) expression.160,161 Notably, integrin β4 mediated signaling has been shown to enhance VEGF protein expression by
upregulating its cap-dependent translation. VEGF expression leads to the disruption of endothelial junctions, thus allowing tumor cells to enter the circulation where they can travel to distant sites, extravasate, and metastasize. We have shown that integrin β4 overexpression is associated with venous invasion in NSCLC, and its effects on VEGF expression may be partially responsible for this phenomenon. In addition, integrin β4 has been shown to promote the expression of a number of pro-metastatic and pro-invasive proteins including S100A4, which also contributes to its ability to promote vascular invasion and tumor metastases.

In this study, expression of the integrin β4 gene was positively correlated with expression of the cancer stem cell maker, CD44. CD44 is a transmembrane glycoprotein that has been shown to facilitate many aspects of tumor progression and is important in promoting resistance to therapy. Interestingly, integrin β4 has been implicated in promoting stem cell like properties in breast cancer, and has been identified in the cancer stem cell population in NSCLC. Integrin β4 gene expression was found to positively correlate with expression of EGFR, and in the network map, integrin β4 was connected to genes in the EGFR signaling pathway. These findings are notable in light of evidence demonstrating a functional relationship between EGFR and integrin β4. In particular, integrin β4 has been shown to interact with EGFR in lipid rafts where it enhances cell growth and proliferation. Furthermore, our lab has demonstrated that in pancreatic cancer, integrin α6β4 promotes autocrine EGFR signaling (unpublished data). In the network map, integrin β4 was connected to a number of laminins (LAMA3, LAMC2, LAMB4), which is consistent with the fact that integrin β4 binds laminins in the extracellular matrix. Also notable was the connection between integrin β4 and COL17A1, (Collagen, Type XVII, Alpha 1), as this gene encodes the BP180 protein that is necessary for hemidesmosome assembly. Finally, expression of integrin β4 gene was correlated with that of its binding partner, integrin α6.
In summary, I have demonstrated that integrin β4 is elevated in NSCLCs compared to normal lung tissue, and that it is overexpressed in lung SCC. Furthermore, a positive association was demonstrated between the presence of venous invasion and integrin β4 overexpression. Integrin β4 overexpression was associated with reduced overall survival in patients included in the TMA, as well as in external cohorts. While difficult to quantify on tissue microarray sections, integrin β4 expression was noted to be elevated at the invasive front of numerous tumors.
CHAPTER 4: INTEGRIN α6β4 IS DOWNREGULATED BY MUTANT IDH1 AND ASSOCIATES WITH A WORSE CLINICAL OUTCOME IN GLIOMAS

Gliomas account for 30% of all primary tumors of the central nervous system (CNS) and represent about 80% of malignant CNS tumors. In adults, this class of tumors tends to have a poor prognosis, with 5-year survival rates for high-grade gliomas ranging from 4%-52%. Glioblastoma multiforme (GBM) accounts for over 50% of gliomas and carries an extremely poor prognosis, with a median survival of around only 14 months even when treating with temozolomide and radiation therapy. A key reason for this dismal prognosis is the diffusely infiltrative nature of these tumors, which renders complete surgical excision impossible.

Tumors of the central nervous system can be classified into four categories using criteria developed by the World Health Organization (WHO). These categories include tumors that range from those with low proliferative potential (Grade I) to tumors that are cytologically malignant and display increased mitotic activity and areas of necrosis (Grade IV). This histologic grading system provides a mechanism that can assist with the prediction of biologic behavior and response to therapy.

Mutations in isocitrate dehydrogenases types 1 and 2 (IDH1/2) have been identified in a number of human malignancies, including gliomas, chondroid tumors, and acute myeloid leukemia (AML). These mutations are present in the majority of WHO grade II and III gliomas, and are also characteristic of secondary GBMs. Both enzymes normally oxidize isocitrate to alpha-ketoglutarate, but point mutations at R132 in IDH1 or R172 in IDH2 produce neoenzymatic activity, reducing alpha-ketoglutarate to D-2-hydroxyglutarate (2-HG). 2-HG may contribute to gliomagenesis by promoting histone and DNA hypermethylation and suppressing cellular differentiation. Although IDH1/2 mutations have been shown to contribute to gliomagenesis, they do not appear to be sufficient for tumor induction. It is likely
that IDH1/2 mutations are early events in gliomagenesis that, when coupled with either TP53 or 1p/19q co-deletion, can lead to tumor development.\textsuperscript{181,183} In particular, these mutations are powerful favorable prognostic markers, though the reasons for this have not been fully elucidated.\textsuperscript{181}

Expression of the integrin $\beta_4$ subunit has not been extensively studied in gliomas, and it is not known whether integrin $\beta_4$ has clinical or prognostic significance in these tumors. Furthermore, the effect of IDH1 mutations on the expression of cellular adhesion molecules such as the integrin $\beta_4$ is unknown. We therefore investigated integrin $\beta_4$ expression in a cohort of grade II-IV gliomas with and without IDH1 mutations, integrated results with outcome, and evaluated the effect of R132H IDH1 overexpression on integrin $\alpha_6\beta_4$ levels in human glioma cells. Here, we report that integrin $\alpha_6\beta_4$ is associated with progression and reduced overall survival in diffuse gliomas and is inversely correlated with mutant IDH1 status, potentially identifying a factor contributing to the more favorable prognosis associated with mutant IDH1.

**Materials and Methods:**

**Glioma tissue microarray**

Formalin-fixed, paraffin-embedded (FFPE) glioma tissues were retrieved from the pathology archives at the University of Kentucky. Deidentified tissue microarrays (TMAs) were constructed from the gliomas as previously described.\textsuperscript{184} A total of 104 cases comprised the TMAs, including 9 nonneoplastic controls (cortical dysplasia), 9 grade II astrocytomas, 11 grade III astrocytomas, 12 anaplastic oligodendrogliomas, 16 grade II oligodendrogliomas, and 47 grade IV GBMs. Treatment and outcomes were obtained via the Kentucky Cancer Registry. Institutional Review Board approval was obtained prior to collecting the archival tissues for TMA construction and patient retrieval data.
Immunohistochemistry scoring and analysis

Immunohistochemistry for the integrin β4 subunit was performed as described in Chapter 2. Specifically, the primary antibody was used at 1:100 and the secondary antibody was used at 1:500. Integrin β4 expression was scored using a semiquantitative scale as follows: negative (0), minimal staining (1), weakly positive (2), moderately positive (3), strongly and diffusely positive (4). Endothelial cells were used as internal positive controls while non-neoplastic tissue cores served as negative controls. Scoring was done while blinded to WHO grade and IDH1 mutation status. Results from all three tissue cores were averaged together to produce a final score for each tumor.

R132H IDH1 immunohistochemistry of TMAs was performed as previously described.\textsuperscript{184} Of note, 5 TMA cases were immunonegative for R132H IDH1 but were still suspected of having a less common IDH1 or IDH2 mutation based on mutation-associated variables like WHO grade and younger patient age. Of those 5, a single anaplastic astrocytoma turned out to have R132S IDH1 via pyrosequencing. Pyrosequencing was performed in the Molecular Pathology Department at the University of Kentucky according to a clinically validated protocol.

Immunoblotting

LN18-GFP, LN18-GFP WT IDH1 and LN18-GFP IDH1 mutant cell lines were generated as previously described.\textsuperscript{184} Cells were harvested in RIPA buffer (150 mM NaCl, 0.5 mM EGTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 50 mM Tris-HCl pH 7.4, 15 μg/ml protease inhibitor cocktail, 1 mM PMSF). Total cell lysates (80 μg) were subjected to 8% SDS-PAGE, transferred and immunoblotted with mouse anti-human integrin β4 antibody (clone 7/CD104; BD Bioscience, San Jose, CA). β-actin was used as the loading control. Immunoblotting was performed by Min Chen, MD, PhD.
Bioinformatics analysis of external cohorts

To validate TMA data with external cohorts, the relationship of integrin β4 to tumor type and GBM patient survival was done using the Oncomine microarray database (Oncomine, Compendia Bioscience, Ann Arbor, MI http://www.oncomine.org). Specific correlations between ITGB4 mRNA and IDH1 status, or ITGB4 mRNA and other mRNAs, were done via direct query of The Cancer Genome Atlas (TCGA Research Network, http://cancergenome.nih.gov/). Additional survival data in a separate cohort of GBMs was obtained via the National Cancer Institute REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT, https://caintegrator.nci.nih.gov/rembrandt/).

Results

To determine the range of integrin α6β4 expression in gliomas, immunohistochemistry for the β4 subunit was performed on a set of glioma TMA blocks from 104 cases (see Materials and Methods). Integrin β4 expression was uniformly weak in nonneoplastic control tissues, but showed a greater range of staining intensities in grades II-III gliomas (Figs. 4.1 and 4.2A). However, the overall mean intensity was significantly greater only in grade IV GBMs (Fig. 4.2A; P < 0.0001). After adjusting for WHO grade, no significant difference in β4 expression was seen between astrocytic and oligodendroglial tumors (Fig. 4.3A). External cohorts confirmed this association, showing elevated ITGB4 mRNA in GBMs compared to nonneoplastic brain (Figs. 4.2B and 4.3B). Patient-derived nonneoplastic neural progenitor cells also showed significantly lower ITGB4 mRNA in vitro compared to primary patient-derived GBM cells and some other commonly-used GBM cell lines (Fig. 4.3C).

In carcinomas, integrin β4 is known to promote more invasive and aggressive behaviors50,53,72,86,99,105,110 and has been shown to upregulate S100A4,172 a gene encoding for a pro-metastatic protein that is involved in the epithelial to mesenchymal
Gene expression profiling has identified multiple molecular subtypes of GBM, including a mesenchymal phenotype. In the TCGA set of GBMs, increasing levels of ITGB4 mRNA correlate with increasing levels of CHI3L1 and S100A4 ($P < 0.0001$), both of which are robust mesenchymal markers (Fig. 4.4A-B), and average ITGB4 mRNA levels were the highest in the mesenchymal sub-type of GBMs (Fig. 4.4C).

Mutant IDH1 has been shown to be a strong favorable prognostic factor in diffusely infiltrative gliomas, and high integrin β4 expression can contribute to cancer invasiveness and aggression. Interestingly, a strong inverse relationship between IDH1 mutations and integrin β4 expression was observed in our TMA cohort ($P < 0.0001$) (Figs. 4.5 and 4.6A-B). Likewise, TCGA GBMs that had IDH1 mutations showed lower ITGB4 mRNA levels than IDH1 wild-type tumors ($P = 0.005$) (Fig. 4.6C). In the TCGA, IDH1-mutant GBMs also showed a slight increase in ITGB4 promoter methylation ($P = 0.0008$) (Fig. 4.6D). To test whether IDH1 mutations can drive the reduction in integrin β4 expression, we analyzed LN18 GBM cells overexpressing either wild-type IDH1 or R132H IDH1 mutant. We found that R132H IDH1 overexpression downregulated integrin β4 protein levels compared to either the control vector transfected or wild-type IDH1 expressing cells ($P < 0.0001$) (Figs. 4.6E-F). These data suggest that mutant IDH may suppress integrin β4.

To date, no data on integrin β4 and outcome in gliomas has been published. In our TMA cohort, high integrin β4 expression significantly associated with shorter overall survival (OS) in all grade II-IV gliomas (median survival not reached for integrin β4 IHC score < 1; 63.5 months for 1-1.9; 18.1 months for 2+, $P = 0.0005$) (Fig. 4.7A), as well as when considering only high-grade (III-IV) gliomas (30.7 months for integrin β4 IHC score <2; 17.8 months for 2+, $P = 0.02$) (Fig. 4.7B). When focusing only on GBMs, however, no significant difference was seen (10.9 months for integrin β4 IHC score <2; 9.3 months
for 2+, $P = 0.97$). However, in both the TCGA and NCI Rembrandt GBM datasets, increasing levels of $ITGB4$ mRNA correlated with worse OS (Figs. 4.7C-D) This indicates that integrin β4 may have an effect on outcome in GBMs as has been seen in other cancers, but that larger cohorts are needed to demonstrate this. Moreover, in our TMA cohort, integrin β4 was not an independent prognostic factor when including variables like WHO grade and IDH1 status (Table 4.1).
**Figure 4.1: Integrin β4 expression in gliomas.** Average integrin β4 levels are significantly increased in grade IV GBMs compared to nonneoplastic brain tissues and grade II and III gliomas. The inset in the grade IV integrin panel highlights membranous staining of integrin β4 in GBM cells (arrowheads). Magnification = 400x for all images.
Figure 4.2: Integrin β4 expression is higher in grade IV GBMs than lower grades of gliomas. (A) GBMs had a higher level of integrin β4 expression than lower grade tumors and nonneoplastic brain tissue, as measured by semiquantitative immunohistochemistry. No significant differences were seen between grades II and III gliomas, or between II-III gliomas and nonneoplastic tissue. Overall $P < 0.0001$ via one-way ANOVA, *$P < .0001$ between grade IV GBMs and each other group via post-hoc Tukey’s test. (B) Likewise, TCGA GBMs showed greater average levels of ITGB4 mRNA than nonneoplastic brain. $P = 0.01$ via two-tailed t-test.
Figure 4.3: Integrin β4 expression by glioma histotype and cell lines. (A) There was no significant difference between mean integrin β4 IHC scores among astrocytomas (astro) or oligodendrogliomas (oligo), either at the grade II level or grade III level. $P = 0.51$ via one-way ANOVA. (B) In the Bredel 2 glioma cohort obtained through Oncomine (see Methods), there was a strong trend toward higher $ITGB4$ mRNA in GBMs versus nonneoplastic control brain and grades II-III gliomas ($P = 0.05$ via one-way ANOVA). (C) In the Lee data obtained through Oncomine (see Methods), nonneoplastic human neural progenitor cells (NPCs) had lower $ITGB4$ mRNA compared to primary cultures of patient-derived GBMs and three GBM cell lines: U373 MG, U251, and U387. Overall $P < 0.0001$ via one-way ANOVA, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ via post-hoc Tukey’s test. For the sake of simplicity, other statistically significant differences that were found (e.g. between U373 MG and U188 MG) are not shown. Data in this figure analyzed by Craig Horbinski, MD, PhD.
Figure 4.4: Integrin β4 expression correlates with mesenchymal markers in TCGA GBM dataset. By linear regression, ITGB4 mRNA positively correlates with mesenchymal markers CHI3L1 (A) and S100A4 (B) in TCGA GBMs. N = 424. (C) By GBM expression subtype, average ITGB4 mRNA levels were the highest in mesenchymal GBMs, with proneural tumors showing the lowest ITGB4 levels. Overall P < 0.0001, *P < 0.05, **P < 0.01, ***P < 0.001. N = 434. Data in this figure analyzed by Craig Horbinski, MD, PhD.
Figure 4.5: Integrin β4 immunoreactivity in IDH1-mutant versus wild type gliomas. Compared to a grade IV GBM that is wild-type for IDH1 (left panels), a GBM with R132H IDH1 has less intense integrin β4 staining (right panels). Magnification = 400x for all images.
Figure 4.6: Integrin β4 expression is lower in IDH1-mutant gliomas. Tumors with mutant IDH1 showed significantly less integrin β4 expression via immunohistochemistry, either by pooling all grade II-III gliomas together (A) or by focusing only on high-grade (III & IV) gliomas (B). Likewise, IDH1-mutant GBMs from the TCGA had lower levels of ITGB4 mRNA compared to wild-type GBMs (C) and tended to have more methylation of cg23913400, a CpG island in the ITGB4 promoter (D). Overexpression of R132H IDH1 markedly downregulated integrin β4 in LN18 glioma cells (E), densitometric data from 5 independent blots were combined and quantified in (F). Statistical analyses in (A-D) were done via two-tailed t-tests; (F) was done via one-way ANOVA. *P < 0.05 versus vector, **P < 0.001 versus vector and wtIDH1 as determined by post hoc Tukey’s test. Data in E and F generated by Min Chen, MD, PhD.
**Figure 4.7: Integrin β4 and survival in gliomas.** When pooling all gliomas together (A), increased expression of integrin β4 (categorized by the semiquantitative data as shown in Figures 1 and 2A) correlated with shorter median OS. *P = 0.004 versus 1-1.9 and *P = 0.0003 versus < 1. There was no significant difference between < 1 and 1-1.9 (P = 0.14). Even when focusing on just high-grade (III & IV) gliomas (B), expression levels of 2 or more still correlated with worse OS, P = 0.02. (C) In TCGA GBMs, increasing ITGB4 mRNA levels significantly correlated with decreased proportion of patients surviving 5 years. Overall P = 0.002 via Kruskal-Wallis test; post-hoc values were derived via Dunn’s multiple comparison test. (D) In the NCI Rembrandt dataset, GBMs with upregulation of ITGB4 mRNA (as defined by > 3-fold increased mRNA levels compared to nonneoplastic control tissue) had a 30% shorter median survival compared to GBMs with less than threefold increase in ITGB4 mRNA (13.2 versus 17.1 months). Survival analyses in (A), (B), and (D) were done via log-rank (Mantel-Cox) tests.
Table 4.1: Multivariate survival analysis of gliomas. Via Cox proportional hazards survival regression (N = 88), significant independent prognostic factors included high WHO grade, IDH1 mutations, adjuvant radiation, and adjuvant temozolomide (TMZ), but not integrin β4 expression (2+ versus < 2 as described in Figures 1 and 2). All tumors underwent extensive resection, not just biopsy. Multivariate analysis performed by Craig Horbinski, MD, PhD.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Relative risk</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
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<td>integrin β4 moderate to high</td>
<td>1.3</td>
<td>0.61—2.6</td>
<td>0.55</td>
</tr>
<tr>
<td>grade III-IV vs II</td>
<td>21.3</td>
<td>4.4—104.6</td>
<td>0.0002</td>
</tr>
<tr>
<td>age &lt; 45 years</td>
<td>0.81</td>
<td>0.41—1.6</td>
<td>0.55</td>
</tr>
<tr>
<td>astrocytic morphology</td>
<td>1.3</td>
<td>0.48—3.4</td>
<td>0.62</td>
</tr>
<tr>
<td>IDH1 status</td>
<td>0.21</td>
<td>0.069—0.63</td>
<td>0.0052</td>
</tr>
<tr>
<td>adjuvant radiation</td>
<td>0.42</td>
<td>0.19—0.91</td>
<td>0.029</td>
</tr>
<tr>
<td>adjuvant TMZ</td>
<td>0.32</td>
<td>0.16—0.67</td>
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</tr>
</tbody>
</table>
Discussion

The results of this study demonstrate that integrin β4 is highly expressed in GBMs compared to lower grade gliomas and non-neoplastic tissue. We found that high integrin β4 expression is an adverse prognostic marker, which is consistent with work done in other cancers showing that integrin α6β4 overexpression imparts more aggressive, invasive behavior.\textsuperscript{50,53,72,86,99,105,110} We also found that integrin β4 was significantly lower in tumors with IDH1 mutations, which was confirmed in external gene expression databases, and that overexpressing R132H IDH1 sharply suppresses integrin β4 expression in glioma cells. This may help explain why IDH1/2-mutant gliomas tend to be less aggressive than their wild-type counterparts.\textsuperscript{187,188}

To date, very few papers have been published on the subject of integrin α6β4 in gliomas. Prior work has shown that integrin β4 expression in gliomas is variable, though these studies examined smaller numbers of patient-derived glioma tissues. Previtali et al.\textsuperscript{126} found that the integrin β4 expression is present in reactive astrocytes, and that expression is elevated in astrocytomas. A larger study investigated expression of the β4 subunit in a series of astrocytomas and oligodendrogliomas, and found that β4 expression was slightly higher in oligodendrogliomas.\textsuperscript{128} Previtali et al\textsuperscript{189} found that the α6β4 integrin was diffusely expressed in both neoplastic and reactive astrocytes in an animal model of ethynitrosourea (ENU) induced glioblastoma. Interestingly, they describe that β4 staining was identified early in tumor development, and that staining was more intense within the neoplastic cells of proliferative centers.\textsuperscript{189} Integrin β4 expression has been identified in multiple astrocytoma cell lines (SF-767, NCE G-22 and NCE G-112) by RT-PCR,\textsuperscript{190} and was identified in the oligodendroglial cell line HS-683.\textsuperscript{128} More recently, a study was published describing the interaction between the β4 integrin and netrin-4 (NTN4) in glioblastoma progression.\textsuperscript{191} The current study is the first to
show that integrin α6β4 is higher in GBMs than lower-grade gliomas, that it is an adverse prognostic marker, and that mutant IDH1 suppresses its expression.

Within GBMs, integrin β4 had no prognostic significance in the TMA cohort, although it did in external GBM databases (Figure 5). This is likely due to the fact that the external cohorts were larger. Moreover, integrin β4 was not an independent prognostic marker on multivariate analysis (Table 1), which may be due to the fact that integrin β4 is so tightly correlated with IDH1 mutation status and tumor grade (Figures 2-4). Thus, it is very unlikely that lower integrin β4 expression is the sole reason why mutant IDH1 is a positive biomarker, but rather that it contributes to the overall favorable prognosis in such tumors. For example, mutant IDH1 also leads to MGMT promoter hypermethylation, thus reducing the ability of the tumor to repair DNA damage done by alkylating chemotherapeutic agents. It is therefore not surprising that a mutation that triggers global hypermethylation would accidently make a few changes that are somewhat deleterious to the tumor. Epigenetic regulation of integrin β4 through promoter hypermethylation and histone modification has been previously described. Whether hypermethylation is the mechanism by which mutant IDH1 suppresses integrin β4 mRNA and protein expression is the subject of ongoing research.

The role of integrins in glioma development and progression is a growing component of neurooncology research. For example, integrin α6 is a marker for GBM stem cells, and its interaction with laminin α2 in the extracellular matrix is an important mediator of stem cell properties like self-renewal and tumor formation. Integrin β8 is also expressed in gliomas, positively correlates with overexpression of EGFR and PDGFRA, and may promote FAK and ERK activity in regions rich in extracellular matrix. The αvβ3 and αvβ5 integrins have been implicated in tumor-induced angiogenesis, which is a common feature of GBMs. The αvβ3 and αvβ5 integrins increase with increasing histologic grade in gliomas, and tumor cells at the periphery of
high-grade gliomas have higher αvβ3 expression.\textsuperscript{198} Attempts to target αvβ3 and αvβ5 in GBM led to multiple clinical trials with cilengitide, a small molecule inhibitor of the αvβ3, αvβ5 and α5β1 integrins.\textsuperscript{199} While a phase II clinical trial (NABTT 0306) demonstrated a modest improvement in median survival for patients treated with cilengitide, further studies in patients with newly diagnosed GBM have not been successful.\textsuperscript{169,200}

In summary, we have demonstrated that expression of the β4 integrin is decreased in tumors with mutations in \textit{IDH1}, which we confirmed using a glioma tissue microarray, gene expression databases, and overexpression of R132H IDH1 \textit{in vitro}. This study is the first to demonstrate that mutant IDH1 can decrease the expression of a cell adhesion molecule. Further work is needed to clarify the mechanism(s) by which mutant IDH1 alters the expression of cellular adhesion molecules, and to examine the role of integrin α6β4 in gliomagenesis and invasion. Gaining a deeper understanding of why mutant IDH1 gliomas are less aggressive than their wild-type counterparts will not only help develop ways of targeting IDH1-mutant gliomas, but also of making wild-type tumors behave more like mutant tumors.
CHAPTER FIVE: INTEGRIN α6β4 EXPRESSION IN NORMAL AND NEOPLASTIC BREAST

Breast cancer is the most common form of cancer diagnosed among women in the United States, and is also a leading cause of cancer related death among women in this country. Breast cancer is responsible for over 40,000 deaths annually, and incidence continues to rise. It is now estimated that 1 in 8 women will be diagnosed with breast cancer during their lifetime. While advances in clinical care have resulted in improved survival for many women with breast cancer, there are still subtypes that carry a poor prognosis and remain difficult to treat effectively. Importantly, breast cancer does not represent one disease, but instead represents a number of different disease processes, all with unique morphologies and molecular phenotypes.

Clinical and molecular subtypes of breast cancer

From a clinical perspective, breast cancer can be divided into three main categories: hormone receptor positive, HER2 positive, and triple negative. These clinical subtypes of breast cancer are defined by expression of the estrogen receptor, progesterone receptor, and HER2 (ERBB2). Assessment of these three markers is routine in clinical practice. While expression of ER and PR are assessed using immunohistochemistry, HER2 expression can be assessed using immunohistochemistry, fluorescence in situ hybridization (FISH), or chromogenic in situ hybridization (CISH). Hormone receptor positive tumors generally express the estrogen receptor (ER) and have variable expression of the progesterone receptor (PR). Patients whose tumors are hormone receptor positive are generally candidates for therapy with hormone receptor antagonists, selective estrogen receptor modulators (SERMs), aromatase inhibitors, and drugs that suppress ovarian function. Patients with amplification or overexpression of HER2 can now be treated with a wide array of anti-HER2 therapies including monoclonal
antibodies such as trastuzumab and tyrosine kinase inhibitors such as Lapatinib. While these advances have significantly improved the survival of patients with breast cancer, triple negative tumors lack expression of ER, PR, and HER2, and so are not amenable to treatment with the therapies described above. Patients with TNBC tend to have reduced survival when compared to patients with hormone receptor positive or HER2 amplified disease\textsuperscript{201,202}.

The clinical classification of breast cancer based on expression of ER, PR, and HER2 helps to inform and streamline clinical decision making, however, it does not reflect the true heterogeneity of this disease. The WHO histologic classification of breast tumors recognizes at least 37 different entities, not including primary mesenchymal and lymphoid neoplasms of the breast\textsuperscript{203}. The epithelial subtypes of breast cancer range from commonly encountered tumors such as invasive ductal, lobular, and tubular carcinomas to rare entities including adenoid cystic carcinoma, secretory carcinoma, and glycogen-rich clear cell carcinoma. While pathologists have long recognized these diverse histologic subtypes, recent studies using molecular profiling have uncovered specific subtypes that are based on differences in gene expression, mutation profile, copy number alterations, and epigenetic modifications. One of the more widely accepted classification schemes divides breast cancer into four main molecular subtypes: Luminal A, Luminal B, HER2 and basal-like\textsuperscript{70,204,205}.

Luminal A breast cancers are characterized by elevated mRNA expression of genes such as $ESR1$, $GATA3$, $FOXA1$, $XBP1$, and by mutations in $PIK3CA$, $GATA3$ and $FOXA1$.\textsuperscript{70} Luminal A tumors tend to express both ER and PR, and so are amenable to treatment with estrogen receptor modulating agents. These tumors have the best prognosis out of the molecular subtypes. Luminal B tumors are characterized by a higher proliferation index than Luminal A tumors, and by mutations in $PIK3CA$, $GATA3$, and $TP53$.\textsuperscript{70} The third molecular subtype is the HER2 enriched; clinically, these tumors tend
to have amplification and/or overexpression of HER2. Mutations in PIK3CA and TP53 are common in HER2 enriched tumors. The final molecular subtype is the basal-like. Basal-like breast cancer tends to be triple negative, though the two categories are not mutually exclusive. Many, but not all, basal-like tumors are triple negative, though there are some exceptions. The basal-like subtype is marked by a high frequency of TP53 mutations, and loss of TP53 function is present in the vast majority of basal-like breast cancers. Basal-like breast cancer is also characteristic of basal-like breast cancers. These tumors tend to express basal cytokeratins such as CK5 and CK6, and also have elevated expression of genes associated with proliferation. Correspondingly, these tumors tend to be aggressive and highly proliferative, leading them to have the poorest prognosis of all breast cancer subtypes. Basal-like breast cancer is also common in African-American women and in young women, which partially explains the poor prognosis associated with these patient populations.

Ductal carcinoma in situ (DCIS) is a pre-invasive neoplasm of the breast that accounts for up to 20% of newly diagnosed breast cancers. This neoplastic change takes place in the ductal epithelium, where a proliferation of cells fills and expands the ductal system. In order for a lesion to be classified as DCIS, the neoplastic cells must remain confined to the duct, without invasion through the basement membrane. Once neoplastic cells escape from the confines of the basement membrane and invade into the stroma, the lesion is termed invasive breast cancer. While pure DCIS has a better prognosis when compared to invasive breast cancer, it is a marker of increased risk for the development of subsequent invasive carcinoma, and when diagnosed on core needle biopsy, DCIS may be associated with the presence of an underlying invasive breast cancer. In particular, high-grade DCIS is associated with a worse clinical outcome and may be linked to the development of high-grade invasive breast cancer.
been shown that DCIS can be categorized into molecular subtypes similar to those identified in invasive breast carcinoma,\textsuperscript{209} and while low-grade DCIS is often positive for the hormone receptors estrogen and progesterone, high-grade DCIS is commonly hormone-receptor negative.\textsuperscript{206} DCIS has a variety of morphologic subtypes, many of which have distinct molecular profiles and clinical outcomes.\textsuperscript{209} Included among these subtypes are solid, cribriform, papillary, micropapillary, basal-like, apocrine and comedo-type DCIS. A variety of clinical classification systems have been developed for DCIS, though consensus guidelines focus on the evaluation of nuclear grade.\textsuperscript{210} This system divides DCIS into low grade (grade 1), intermediate grade (grade 2), and high-grade (grade 3) based on features such as nuclear atypia and nuclear size.\textsuperscript{210} This grading scheme provides prognostic information, as high-grade DCIS is associated with an increased risk for the later development of invasive breast cancer.

**Materials and Methods**

**Breast Translational Group (BTG) TMA:**

Institutional Review Board Approval (13-0608-P2H) was obtained prior to initiating the project. Surgical resections for breast cancer performed at the University of Kentucky from January 1, 2000 to December 31, 2012 were identified using natural language searches in CoPath (Cerner Corporation, Kansas City, MO). A total of 343 invasive breast cancers were selected for inclusion, including 165 TNBCs, 74 HER2 positive tumors, and 104 hormone receptor positive tumors. Original hematoxylin and eosin (H&E) stained slides were reviewed and appropriate tumor blocks were selected from each case. Fresh H&E stained sections were then cut from each selected tumor block. These fresh sections were then reviewed and tumor areas were selected for inclusion in the TMA. Pathologic features, treatment and outcome data were collected by the Cancer Research Informatics Shared Resource Facility and the Kentucky Cancer
Registry. Tissue core placement was randomized by the Biostatistics and Bioinformatics Shared Resource Facility. Three 2 mm diameter tissue cores were obtained from each tumor specimen, which were transferred to recipient paraffin blocks using a TMArrayer (Pathology Devices, Westminster, MD). The completed TMAs contained 1029 tissue cores spread over 15 blocks. TMA sections used for integrin β4 immunohistochemistry had interpretable tissue cores in 336/343 cases.

**DCIS and Benign Breast Tissue**

Institutional Review Board Approval (14-0236-P6H) was obtained prior to initiating the project. In order to identify cases of normal breast and DCIS, CoPath (Cerner Corporation, Kansas City, MO) was used to conduct natural language searches. Individual cases were then reviewed and appropriate cases were selected for inclusion. Benign breast tissue was obtained from leftover reduction mammoplasty cases, and patients were excluded from the benign category if they had a history of atypia, in situ disease, or invasive carcinoma. A total of 78 tissue blocks were identified, including 20 benign breast samples, 16 cases of low-grade DCIS, 18 cases of intermediate-grade DCIS, and 24 cases of high-grade DCIS.

**India Breast TMA**

Breast cancers obtained from patients in India were used by the Markey Biospecimen and Tissue Procurement Shared Resource Facility to construct tissue microarrays by the method describe above. A total of 56 invasive breast cancers comprised the Indian Breast TMA.
Immunohistochemistry and TMA Scoring

For BTG TMA sections, immunohistochemistry for the integrin β4 was performed according to the protocol described in Chapter 2. Specifically, the primary antibody was used at 1:200 and the secondary antibody was used at 1:500. Due to differences in tissue processing between this cohort and the India breast cancer cohort, sections from the India TMA were stained for the integrin β4 subunit using the protocol described in Chapter 2, but at a primary antibody concentration of 1:100 and a secondary antibody concentration of 1:500. Immunohistochemical staining for ER, PR, and HER2 was performed using the antibodies and protocols listed in Table 5.1 (Dako Corporation, Carpinteria, CA, USA). Integrin β4 expression was scored using a semiquantitative scale as follows: negative (0), minimal staining (1), weakly positive (2), moderately positive (3), strongly and diffusely positive (4). Scoring for ER, PR, and HER2 was performed according to guidelines published jointly by the College of American Pathologists (CAP) and the American Society for Clinical Oncology (ASCO). Results from each of the three tissue cores were averaged to produce a final score for each tumor. Scoring was performed by while blinded to clinical and pathologic data.
Table 5.1: Antibodies used for ER, PR, and HER2 immunostaining.

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<th>Marker</th>
<th>ER</th>
<th>PR</th>
<th>HER2</th>
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<td>IR06861-5</td>
<td>A048529-1</td>
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<td>AR Solution</td>
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<td>EnVision FLEX Target Retrieval Solution, High pH</td>
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<tr>
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</tr>
<tr>
<td>AR Temperature</td>
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<td>97°</td>
<td>N/A</td>
</tr>
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<td>1° Antibody Conc.</td>
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<td>Pre-diluted</td>
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</tr>
<tr>
<td>1° Incubation Time</td>
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<td>25 minutes</td>
<td>20 minutes</td>
</tr>
<tr>
<td>2° Antibody</td>
<td>Anti-rabbit (Envision+ kit)</td>
<td>Anti-mouse (Envision+ kit)</td>
<td>Anti-rabbit (Envision+ kit)</td>
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<tr>
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<td>Envision+</td>
<td>Envision+</td>
</tr>
<tr>
<td>DAB Time</td>
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All reagents are from Dako Corporation. AR = Antigen retrieval.
Results

**Integrin β4 expression in benign breast and DCIS**

In benign breast tissue, integrin β4 was strongly expressed in myoepithelial cells, while staining in luminal epithelial cells was absent (Fig. 5.1A). This pattern was preserved in fibrocystic change and apocrine metaplasia (Fig. 5.1B). In usual ductal hyperplasia and columnar cell change without atypia, integrin β4 expression was limited to myoepithelial cells (data not shown). Microglandular adenosis is a benign proliferation that is believed to be a non-obligate precursor to invasive TNBC.\(^{213}\) Interestingly, integrin β4 expression was elevated in the single example of microglandular adenosis studied (Fig. 5.1B). Benign stroma, fibroblasts, adipose, and infiltrating lymphocytes were all negative for expression of the integrin β4 (Fig. 5.1). In benign breast tissue, endothelial cells and peripheral nerves had weak to moderate expression as has been previously described (Fig. 5.2).\(^{72}\)

Expression of the integrin β4 in DCIS was variable. In some cases, integrin β4 expression was present only in the residual myoepithelial cells lining DCIS (Fig. 5.3), whereas in other cases, integrin β4 expression was present in both neoplastic and myoepithelial cells. Within DCIS, integrin β4 expression was heterogeneous. There was a tendency for neoplastic cells in the outer layers of DCIS (adjacent to myoepithelial cell) to have elevated integrin β4 expression (Fig. 5.3).

**Integrin β4 in invasive breast cancer**

Integrin β4 expression in invasive breast cancer was also variable (Figs. 5.4 and 5.6B-C). In some cases, infiltrating tumor cells exhibited strong and diffuse expression of the integrin β4, while others were completely negative for integrin β4 expression (Fig. 5.4). In the India TMA cohort, we found that integrin β4 expression was elevated in TNBCs and HER2 positive breast cancers (Fig. 5.4). Although the number of HER2
positive tumors in this cohort was relatively small (N =14), integrin β4 overexpression was identified in the majority of these HER2 positive tumors (Fig. 5.5). In addition, integrin β4 overexpression was associated with high tumor grade in this cohort (Table 5.2). An association was not identified between tumor size or the presence of lymph node metastasis in the India TMA cohort (Table 5.2).

In the BTG TMA cohort, low-grade breast cancers such as tubular and cribriform carcinoma had low to absent expression of the integrin β4 (Fig. 5.6B). In the few tumors examined with residual benign ductal epithelium, integrin β4 was still present in myoepithelial cells surrounding the ducts (Figs. 5.6B and 5.7). In high-grade breast cancers, integrin β4 was expressed strongly and diffusely throughout infiltrating tumors cells (Fig. 5.6C). Integrin β4 expression was elevated in TNBCs and HER2 positive tumors when compared to hormone receptor positive breast cancers, with the highest levels of integrin β4 expression observed in TNBCs (Fig. 5.8A). Furthermore, integrin β4 expression was associated with high tumor grade in the BTG TMA cohort (Fig. 5.8B).
Figure 5.1: Integrin β4 expression in benign breast. Integrin β4 expression in benign breast with fibrocystic change (A). Integrin β4 is absent in cells with apocrine metaplasia (center lesion), however, expression is elevated in adjacent benign microglandular adenosis (B).
Figure 5.2: Integrin β4 expression in endothelial cells and peripheral nerve. In a medium sized vessel, integrin β4 is expressed in endothelial cells lining the lumen (A) and is also expressed in peripheral nerves (B).
Figure 5.3: Integrin β4 expression in DCIS. Low grade DCIS with integrin β4 expression in myoepithelial cells surrounding neoplastic duct (A). Intermediate grade DCIS with elevated integrin β4 expression in myoepithelial and neoplastic cells (B). All images = 200x.
**Figure 5.4: Integrin β4 expression patterns in clinical breast cancer subtypes.** In the India TMA cohort, integrin β4 levels were significantly higher in triple negative and HER2 positive tumors. Paraffin-embedded breast cancer tissues were stained with antibodies to the integrin β4 subunit, ER, PR, and HER2. Representative images of triple negative, hormone receptor positive, and HER2 overexpressing tumors are shown.
Figure 5.5: Integrin β4 expression is elevated in HER2 positive tumors. Integrin β4 expression was elevated in a subset of HER2 positive tumors in the India TMA. Panels A-D each show an example of a tumor stained for ER, PR, HER2 and integrin β4.
Table 5.2: Integrin β4 expression and clinicopathologic features in breast cancer.

<table>
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<th>β4 High (n= 23)</th>
<th>β4 Low (n=31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average tumor size</td>
<td>3.99 cm</td>
<td>3.91 cm</td>
</tr>
<tr>
<td></td>
<td>$P = 0.8617$</td>
<td></td>
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<tr>
<td>Nodal disease</td>
<td></td>
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<tr>
<td>Positive</td>
<td>17/40 (42.5%)</td>
<td>23/40 (57.5%)</td>
</tr>
<tr>
<td>Negative</td>
<td>6/14 (43%)</td>
<td>8/14 (57%)</td>
</tr>
<tr>
<td></td>
<td>$P = 1.000$</td>
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</tr>
<tr>
<td>Tumor Grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>2/15 (13%)</td>
<td>13/15 (87%)</td>
</tr>
<tr>
<td>III</td>
<td>21/39 (54%)</td>
<td>18/39 (46%)</td>
</tr>
<tr>
<td></td>
<td>$P = 0.0125$</td>
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<tr>
<td>Positive</td>
<td>10/14 (71%)</td>
<td>4/14 (29%)</td>
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<tr>
<td>Negative</td>
<td>13/39 (33%)</td>
<td>26/39 (66%)</td>
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<tr>
<td></td>
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<tr>
<td>Subtype</td>
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<tr>
<td>Triple negative</td>
<td>7/10 (70%)</td>
<td>3/10 (30%)</td>
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<tr>
<td>Hormone receptor +</td>
<td>8/34 (24%)</td>
<td>26/34 (76%)</td>
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<tr>
<td></td>
<td>$P = 0.0187$</td>
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Figure 5.6: Examples of integrin β4 staining in the BTG TMA.

The top panel demonstrates integrin β4 expression in benign ductal epithelium. The myoepithelial cells display strong staining for the integrin β4, whereas luminal epithelial cells are negative for integrin β4 expression (A). In this low-grade invasive breast cancer, integrin β4 remains positive in the myoepithelial cells of benign ducts, but is absent in adjacent invasive carcinoma (B). In this high-grade invasive breast cancer, integrin β4 is strongly and diffusely expressed (C).
Figure 5.7: Example of integrin β4 relocalization. In the benign ducts and lobules (right) integrin β4 is highly expressed in myoepithelial cells, and at the basal aspect of luminal cells. In the invasive carcinoma (left), integrin β4 expression appears reduced, however, this relocalization of integrin β4 may be clinically significant due to a change in β4 signaling after release from hemidesmosomes.
Figure 5.8: Elevated integrin β4 expression is associated with clinical subtype and tumor grade. In the BTG TMA, integrin β4 levels were higher in triple negative and HER2+ breast cancers when compared to hormone receptor positive (HR+) breast cancers, $P < 0.0001$ via Kruskal-Wallis test with Dunn’s Multiple Comparison Test (A). Average integrin β4 levels were also elevated in high-grade breast cancer, $P = 0.0001$ (B).
Discussion

In this study, I examined integrin β4 expression in invasive breast cancers from 390 patients. This is the largest study to date of integrin β4 protein expression in human breast cancer. Here, I found that integrin β4 is highly expressed in basal-like and HER2 positive tumors, and that it associates with tumor grade. Furthermore, I characterized integrin β4 staining patterns in benign breast, where I found that expression is absent in the luminal cells of benign breast tissue, but is strongly expressed in the myoepithelial cells surrounding benign ducts.

A major finding of this study is that integrin β4 expression is elevated in HER2 positive tumors. Early studies demonstrated that integrin β4 expression correlates with HER2 expression in breast carcinoma cell lines, and furthermore, the integrin β4 subunit has been shown to form a complex with HER2 that can amplify HER2 signaling. However, in patient-derived tissues, the relationship between integrin β4 and HER2 expression is controversial. The Mercurio group analyzed breast cancer gene expression datasets using linear regression but did not find a positive correlation between ITGB4 and HER2 mRNA expression. The same group also analyzed patient-derived tumors using immunohistochemistry, but again was unable to demonstrate a relationship between integrin β4 and HER2 protein expression. I analyzed expression of the integrin β4 in 72 HER2 positive tumors from two different patient cohorts where I found that integrin β4 levels were elevated in HER2 positive tumors when compared to hormone receptor positive tumors. While these data provide further evidence for a possible relationship between HER2 and integrin β4, study of additional cohorts will be necessary to make a definitive determination.

A second important finding in this study is that integrin β4 expression is elevated in TNBCs. TNBC is the most aggressive breast cancer subtype and it carries a particularly poor prognosis. In this study, integrin β4 expression levels were highest in
TNBC. This observation may be partially explained by the fact that many TNBCs are immunohistochemically and phenotypically similar to myoepithelial cells, which have very high expression of the integrin β4. The finding that integrin β4 is highly expressed in TNBC is not surprising given its association with other high-grade, aggressive cancers such as lung SCC (Chapter 3) and glioblastoma (Chapter 4).

Microglandular adenosis is a rare but benign breast lesion that is characterized by a proliferation of small glands infiltrating through the stroma.\textsuperscript{214} This lesion can be mistaken for invasive breast cancer due to its infiltrative appearance and because it often produces a mass.\textsuperscript{214} We identified an incidental case of microglandular adenosis in the BTG TMA, and interestingly, the infiltrating glands were strongly and diffusely positive for expression of the integrin β4. Recently, a number of studies have suggested that microglandular adenosis may be a precursor lesion to invasive TNBC. Specifically, one group identified a pattern of recurrent gains and losses (2q+, 5q-, 8q+, and 14q-) in microglandular adenosis which is similar to that observed in basal-like breast cancer.\textsuperscript{215} Further investigation will be needed to determine whether integrin β4 plays a role in microglandular adenosis and the transition to invasive breast cancer.
CHAPTER SIX: SUMMARY AND FUTURE DIRECTIONS

Summary

The purpose of this study was to examine integrin α6β4 expression as it relates to clinical variables and patient outcomes in aggressive human malignancies. *The hypothesis of this study was that in tumors of the lung, breast, and central nervous system, integrin β4 overexpression associates with aggressive behavior and reduced survival.* In order to study integrin α6β4 protein expression in patient-derived tumors, tissue microarrays were constructed and sections were stained using immunohistochemistry for the integrin β4 subunit. Integrin β4 mRNA levels in patient-derived tumors were also examined using external gene expression datasets. I found that integrin β4 expression was elevated in a subset of NSCLCs, and that overexpression associated with pathologic features. Specifically, integrin β4 was highly expressed in squamous cell carcinoma, and overexpression associated with venous invasion. Elevated integrin β4 protein and mRNA expression were also associated with decreased overall survival in patients with NSCLC. In gliomas, integrin β4 overexpression was associated with tumor grade, and was also an adverse prognostic marker. Furthermore, integrin β4 expression was reduced in gliomas with mutations in *IDH1*, thus partially explaining the better prognosis observed for *IDH1* mutant tumors. In breast cancer, integrin β4 expression was found to be highest in triple negative tumors, and elevated integrin β4 expression was associated with HER2 overexpression and tumor grade. In summary, I have shown that integrin β4 expression is elevated in a number of human malignancies, and that its expression associates with tumor aggressiveness and patient prognosis.
Future Directions

Can the integrin β4 be a therapeutic target?

Integrins have been the target of a variety of therapeutic agents, and notably, abciximab, eptifibatide and tirofiban, all of which target the integrin αIIbβ3, are important anti-platelet agents in clinical use.\textsuperscript{216} Thus far, attempts at targeting integrins in cancer have been less successful, though cilengitide, an inhibitor of the αvβ3, αvβ5 and α5β1 integrins demonstrated modest success in early clinical trials.\textsuperscript{199,200,217} The αvβ3 and αvβ5 integrins have been implicated in tumor-induced angiogenesis, an important mechanism of tumor growth and survival.\textsuperscript{196} Results of a phase II trial of cilengitide combined with chemoradiation therapy in patients with the brain tumor glioblastoma (NABTT 0306) demonstrated a modest improvement in median survival (19.7 months) when compared to patients in the EORTC trial of temozolomide and radiation therapy (14.6 months).\textsuperscript{169,200} In addition, a recent phase II clinical trial of cilengitide as a second-line treatment for patients with advanced lung cancer found that at a dose of 600 mg/m^2, median progression free survival was similar to that of patients treated with the current standard of care, docetaxel.\textsuperscript{218} Importantly, in this limited phase II clinical trial, cilengitide was associated with fewer grade 3/4 treatment-related adverse events as compared to docetaxel.\textsuperscript{218} These findings suggest that integrin targeting may be an efficacious and potentially safe treatment for cancer patients. Interestingly, a function-blocking antibody to the integrin α6 subunit has also been developed, and treatment with this antibody was able to halt the progression of osteolytic metastases in a xenograft model of metastatic prostate cancer.\textsuperscript{219}

While targeting the α6β4 integrin may be difficult due to the necessity of this integrin for integrity of the epithelial-mesenchymal junction, it may be possible to target the cytoplasmic signaling domain of the integrin β4 subunit in order to prevent invasive and proliferative signaling.\textsuperscript{77} Notably, mice carrying a targeted deletion of the β4
cytoplasmic signaling domain develop smaller and less vascularized tumors than wild-type controls upon subcutaneous tumor implantation.\textsuperscript{5} This targeted deletion has also been introduced into a mouse model of mammary carcinoma (MMTV-Neu), where mice with the targeted deletion exhibit impaired tumor growth and metastases compared to controls.\textsuperscript{27} It has been suggested that targeting of the integrin α6β4 could lead to severe adverse effects such as skin and mucosal blistering. However, in normal epithelial tissues, the integrin β4 is present only at the basal aspect of cells where they are bound to the extracellular matrix, thus protecting this integrin from targeted therapies in normal cells. However, during carcinoma progression, the integrin β4 is released from hemidesmososomes and relocated over the entire cell surface where it can be more easily targeted. This change in integrin localization and binding may make it possible to preferentially target integrin β4 in malignant cells. Targeting could be accomplished using monoclonal antibodies, small molecule inhibitors, or through nanoparticle delivery systems that recognize and bind to the integrin β4.

\textit{Where does the integrin β4 fit in with molecular subtypes of TNBC?}

As was previously described, the major molecular classification scheme for breast cancer divides tumors into Luminal A, Luminal B, HER2 enriched, and basal-like. Additional subtypes including claudin-low and normal-like have also been described.\textsuperscript{220} In addition to these classification schemes, the Pietenpol group has developed a molecular classification scheme specifically for TNBC.\textsuperscript{221} This classification includes the following subtypes: basal-like 1, basal-like 2, immunomodulatory, mesenchymal, mesenchymal stem-like, and luminal androgen receptor.\textsuperscript{221} As these TNBC subtypes demonstrate unique chemosensitivity patterns and clinical outcomes, we would like to determine which of these specific subtypes display integrin β4 overexpression. This will
help further delineate the role of integrin β4 in specific subtypes of breast cancer, and will allow for further mechanistic studies within specific molecular subtypes.

**What is the role of the integrin β4 in the cancer stem cell population?**

In benign epithelia, integrin β4 is expressed in basal progenitor cells. For example, integrin β4 expression is strong in the basal cells of stratified squamous epithelium and in the basal cell layer of bronchial epithelium (Chapter 3). The binding partner for the integrin β4 subunit is the integrin α6 subunit (CD49f), a known mediator of stem-cell like properties in breast cancer. However, the role of integrin β4 in cancer stem cells remains unclear. I have shown that integrin β4 expression correlates with the expression of stem-cell markers such as CD44, and furthermore, we have demonstrated that integrin β4 is upregulated in the glioblastoma cancer stem-cell population (unpublished data). In breast cancer, expression of the integrin β4 is highly correlated with expression of FOXC1, a transcription factor implicated in cancer stem-cell development. Further investigations will be needed to clarify the role of the integrin β4 in the cancer stem cell population.
REFERENCES


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Markey Cancer Center Research Day, University of Kentucky

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American Society for Clinical Pathology

2014 Engaged Leadership Academy Scholarship
College of American Pathologists

2015 First Place for Best Post-Doctoral Poster in Clinical Science
Markey Cancer Center Research Day, University of Kentucky

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ABSTRACTS AND PRESENTATIONS


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* Presenting author