




2020

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Recommended Citation

Singer, P. (2020). Treating Carcinomas through Integrin $\alpha6\beta4$ Modification and Inhibition. *The Science Journal of the Lander College of Arts and Sciences*, 14(1), 45-51. Retrieved from <https://touroscholar.touro.edu/sjlcas/vol14/iss1/5>

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Treating Carcinomas through Integrin $\alpha6\beta4$ Modification and Inhibition

Pearl Singer

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Abstract

Integrin $\alpha6\beta4$ is a membrane protein which is expressed in both normal and cancerous epithelia. In normal cells, it is involved in adhesion to the basal membrane. In carcinomas, however, it takes on cell signaling roles related to survival and migration. Due to its key role in cancer progression, it is now being considered as a therapeutic target. This paper examines how we can affect integrins, with a specific focus on integrin $\alpha6\beta4$, to reinforce their ability to prevent migration of cancer cells and progress toward metastasis.

Methods

Data was collected using ProQuest, PubMed databases, and the National Library of Medicine. The images and diagrams can be found in the research articles referenced.

Introduction

Integrin $\alpha6\beta4$ is a membrane protein found on the basal side of epithelial cells. It is a member of the integrin family of proteins. Integrins are heterodimers which are imbedded in cell membranes and signal allosterically; ligand binding to extracellular binding sites causes conformational changes in the integrin which ultimately affect their cytoplasmic domains. These domains link to the cell's cytoskeleton and thereby perform signal transduction. As much as their signaling works outward-inward, their signaling also works inward-outward. Integrins' position in the cell makes them a key component in signaling, cell migration, and cell adhesions. Each integrin has a distinct, specific function, and, therefore, they have diverse ligands. Many integrins, including $\alpha6\beta4$, can be turned on or off and need to be activated by tyrosine kinases or growth factors in order to bind to their ligands.

Integrin $\alpha6\beta4$ is different from other integrins because it has a unique β subunit, with an atypically long cytoplasmic domain which connects to the keratin intermediate filament system, as opposed to the microfilament system. The α subunit binds to its ligand, laminin 332, in the extracellular matrix. Through mediating the interactions with laminin and keratin, integrin $\alpha6\beta4$ is the core component of the hemidesmosome, a protein complex which attaches basal epithelial cells to the basement membrane.

Normally, integrin $\alpha6\beta4$ is involved in hemidesmosomes and contributes to tissue stability. Cancerous cells, however, lack hemidesmosomes and integrin $\alpha6\beta4$ is localized at the leading edges of metastasized carcinoma cells. Integrin $\alpha6\beta4$ interacts with many cancer-related growth factors such as epidermal growth factor receptor (EGFR). Significantly, integrin $\alpha6\beta4$ activates phosphoinositide 3-OH kinase (PI3K), a key signaling molecule for carcinoma invasions.

Because of integrins' unique positioning in the cell and in cell signaling, they are highly involved in development of cancers. At times, integrins can inhibit pathways, and

at times they can activate pathways instead. Therefore, integrins should be considered as a possible target for treating cancers. Integrin $\alpha6\beta4$ can be considered as a model, as it attaches cells to the basement membrane and can contribute to invasion and metastasis of carcinomas. However, before examining cancerous cells, it is necessary to understand the behavior of integrins in normal epithelial tissue.

Integrin $\alpha6\beta4$ in Regular Cells

In healthy epithelial cells, integrin $\alpha6\beta4$ occupies a central place in the hemidesmosome, a protein complex which connects epithelial cells to the basement membrane. There are two kinds of hemidesmosomes: type 1, which is found in stratified and pseudostratified epithelia (such as in the epidermis) and type 2, which is found in simple epithelia (such as in the intestine). In the more complex type 1 hemidesmosomes, integrin $\alpha6\beta4$ facilitates the interactions with most of the proteins involved, including the intracellular proteins plectin and BP180, the transmembrane proteins BP230 and tetraspanin CD151, and the extracellular protein laminin 332, which is part of the basal lamina (Walko et al, 2015). In the simpler type 2 hemidesmosomes, the only components are plectin, laminin, and integrin $\alpha6\beta4$, which mediates the interactions between the other two. Integrin $\alpha6\beta4$'s presence even in these simplified hemidesmosome proves that it is essential for the adhesion of epithelial cells to the basement membrane below (de Pereda et al, 2009). Additionally, mice missing $\beta4$ exhibited pathology similar to junctional epidermolysis bullosa, a human disease characterized by fragile skin that blisters quickly due to the basal layer's separation from the basement membrane. The epithelial tissues of the mice, both simple and stratified, lacked hemidesmosomes and did not adhere normally (Dowling et al, 1996).

The $\alpha6$ subunit is homologous to other integrin α subunits. $\alpha6$, like many other integrin protomers, associates with more than one β subunit. For instance, $\alpha6\beta1$ is an integrin found in the retina and cortex, and is linked to disorders of those tissues (Hynes, 2002). When it is found in epithelial cells, however, it is exclusively associated with $\beta4$ and binds to laminin as its ligand. $\alpha6$ is made up of

1,055 amino acids, with the first 1,011 outside the cell, 36 in the transmembrane region, and 19 in the cytoplasm. (Tamura et al, 1990). The amino domain has 7 homologous repeats, which were found to make up a 7-bladed β propeller (Kamata et al, 2001). It is made up of two polypeptides, a heavy chain and a light chain, which originated from one polypeptide but which were cleaved using post-translational modification. They are joined by a disulfide bridge, similar to $\alpha 5$, αv , $\alpha 11 b$, and $\alpha P52$. However, it does not contain the insert domain near the amino terminus that other α subunits possess (Tamura et al, 1990).

$\beta 4$ has 710 amino acids in its extracellular region, with cysteine conservation of that region the same as that of other β subunits. It has a 23 amino acids in its transmembrane region and has 1089 amino acids in its cytoplasmic region (Tamura et al, 1990). The cytoplasmic domain is made up of 5 globular domains. From the membrane toward the inside of the cell, they are: a Na^+ - Ca^{2+} Calx- β exchange motif, a pair of fibronectin type III domains (FnIII-1,2), and another pair of fibronectin domains (FnIII-3,4). Between the two pairs of FnIII domains, there is a flexible connecting segment (CS), and after the second set, there is a c-terminal tail (Walko et al, 2015). There are 5 possible N-glycosylation sites, all of which are glycosylated (Tamura et al, 1990).

Over time, a clearer picture of $\alpha 6\beta 4$ has been formed. Scanning electron microscopy revealed that both subunits have a large globular head and a stalk portion which then traverses the cell membrane. The two protomers complex by binding their heads together (Luo, 2007). $\alpha 6$'s amino domain contains a 7-bladed β propeller domain, which is made up of 7 four-strand β sheets similar to those found in other integrins, which is topped by a ligand binding site. However, it lacks the "insert" domain, otherwise known as a Von Willebrand Factor A domain, which has a MIDAS (a cation coordination site), that is found imbedded in the propeller of the majority of integrins (Kamata et al, 2001). This makes sense because only integrins that are exclusive to chordates have this domain, and $\alpha 6\beta 4$ is homologous to that of more primitive species. When an integrin has this insert domain, cation coordination to the MIDAS site causes a conformational change in the protein, pulling part of the $\alpha 6$ head further down and allowing the ligand to bind to its binding site.

Ligand binding occurs differently in integrins lacking this insert domain like $\alpha 6\beta 4$. In this integrin, $\beta 4$ has its own insert domain with a MIDAS motif, which, when inactive, is in contact with $\alpha 6$'s propeller. When the C-terminus of $\beta 4$ moves downward, or when a cation binds to the MIDAS, it pulls the rest of the protein, exposing more of the propeller surface to allow for a greater affinity for

ligand binding (Hynes, 2002). When priming and ligand binding occur, a large-scale conformational change overcomes both subunits. In their resting, low-affinity phase, both subunits' stalks are bent downward in the middle in a genuflected position, with their intracellular domains touching. When ligand binds, the subunits straighten in a switchblade-like motion and the legs separate, exposing binding surfaces. This is one instance of allosteric signaling in integrins (Luo, 2007).

The majority of connections in the hemidesmosome occur with the large intracellular domain of $\beta 4$. The four FnIII domains have a conserved hydrophobic core, with 9 out of 11 identical residues, but their outer surfaces, which are exposed to solvent, are entirely different, so they can bind to different groups (Alonso-Garcia et al, 2015). The critical interaction within the hemidesmosome is between $\beta 4$ and plectin. Plectin binds to the IFs in the cell, while $\alpha 6$'s interaction with $\beta 4$ allows it bind to its ligand, laminin. The two proteins join at $\beta 4$'s second Fn-III domain and at plectin's actin-binding domain (ABD). This complex is formed by three sets of ionic interactions: between R1225 of $\beta 4$ and D151 of plectin, between R1281 of $\beta 4$ and E95 of plectin, and between E1242 of $\beta 4$ and R98 of plectin. Missense mutations in one of the residues involved in these salt bridges disturbs the interaction with plectin. The first 7 residues of the connecting segment also play a part in this interaction by extending the binding surface of the Fn-III domain. The first FnIII domain also contributes to the interaction through a hydrophobic pocket made up of I1163, Y1187 and C1190, which interacts with an R-side chain in plectin. When $\beta 4$ binds to plectin, a conformational change is triggered in $\beta 4$; in unbound $\beta 4$, part of the CS is folded over FnIII-2 and engages in antiparallel H-bonding with it. In bound $\beta 4$, that part of the CS is unbent and may reveal binding sites for other proteins in the hemidesmosome, like BP230 and BP180 (de Pereda et al, 2009).

Integrin $\alpha 6\beta 4$ in Wounded Cells

Although integrin $\alpha 6\beta 4$ is associated with cell adhesion, it is also involved in signaling pathways associated with cell migration, differentiation, and proliferation. These processes assist in healing wounds. This is indicated by its localization to the front edge of cells migrating into a wound. Additional proof can be seen in an experiment involving A549 (adenocarcinomic human alveolar basal epithelial) cells. When the $\beta 4$ gene was knocked down, the cells migrated less processively than the controls. When the $\alpha 6$ gene was knocked down, the cells migrated both less processively and more slowly. Examination of keratinocytes has revealed that wild-type keratinocytes

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move on a straight line along their matrix of laminin, while $\beta 4$ -deficient keratinocytes move in a circular fashion. All of this shows that integrin $\alpha 6\beta 4$ plays an essential role in healing wounds (Colburn, Jones, 2017).

In order for cells to migrate toward a wound bed, the hemidesmosomes need to disassemble, a process that begins from the cytoplasmic domain and then continues in the extracellular domain. $\beta 4$ serine residues are first phosphorylated by tyrosine kinases to separate $\beta 4$ from its binding partner, plectin; this is stimulated by epidermal growth factor (EGF) (Mainero et al, 1996), as well as protein kinase C (Rabinovitz, et. al. 1999). After $\beta 4$'s phosphorylation, the rest of the connections in the hemidesmosome are then severed (Walko et al, 2015). At this point, $\alpha 6\beta 4$ detaches from the intermediate filament (keratin) cytoskeleton and instead associates with the actin cytoskeleton. It becomes involved in the formation of lamellae and filopodia, which are protrusions of the cell membrane that give the cell motility.

Epithelial cells involved in wound healing are similar to metastasizing carcinoma cells, allowing comparisons to be drawn between them. In both cases, there is a lack of polarization in the cells and an induction of migration. (Rabinovitz et al, 2015). Importantly, many of the same mechanisms are involved, including the coordination with growth factors, the disassociation from the hemidesmosome, and the formation of lamellae and filopodia.

Integrin $\alpha 6\beta 4$ in Carcinoma

In many types of carcinomas, high levels of integrin $\alpha 6\beta 4$ correlate with the invasiveness of the cancer, as well as the patient prognosis. For example, in basal-type breast cancer, which is a highly aggressive form of breast cancer, a $\beta 4$ "gene signature" was associated with decreased time to tumor recurrence and a smaller chance of patient survival (Lu, 2008). Other research shows similar correlation with elevated $\beta 4$ expression in other invasive phenotypes of squamous cell carcinomas, including some kinds of bladder (Grossman et al, 2000), cervical (Jeffers et al, 1997), head and neck (Kurokawa et al, 2008), lung (Stewart et al, 2016), and pancreatic (Cruz-Montseratte et al, 2007) carcinomas. Interestingly, although thyroid cells do not normally express $\alpha 6\beta 4$, it has been found in thyroid carcinomas (Kitajiri et al, 2002). In addition to overexpression of $\alpha 6\beta 4$, it is also located throughout multiple layers of cells in tumors, as opposed to its normal concentration of the basal side of the epithelial cells (van Waes et al, 1995).

However, in some varieties of cancer, integrin $\alpha 6\beta 4$ expression is correlated with tumor suppression. For example, in prostate carcinomas, $\alpha 6\beta 4$ is downregulated

(Nagle et al, 1995). Also, when $\alpha 6\beta 4$ was added to cells from the RKO cell line, a colon carcinoma cell line which is missing $\beta 4$, the result was partial G1 arrest and apoptosis (Clarke et al, 1995). Because this integrin is associated with both growth and migration, but with suppression as well, it may be hard to target as a treatment for cancer. In order to examine treatment options, an in-depth look at the mechanism of action of integrin-implicated signalling pathways must take place.

Integrin $\alpha 6\beta 4$ Signalling Mechanism

When integrin $\alpha 6\beta 4$ is released from hemidesmosomes, it leads to altered signals promoting tumor cell growth, invasion and metastasis (Lipscomb, Mercurio, 2005). Once free from the hemidesmosome interaction, integrin $\alpha 6\beta 4$ can bind or cooperate with many different molecules or growth factor receptors to activate various cell signalling pathways. Integrin $\alpha 6\beta 4$ can bind directly to its regular ligand, laminin, activating both phosphatidylinositol 3-kinases (PI3K) and RhoA small GTPases. Alternatively, it can cooperate with various growth factor receptors, including those in the EGFR (epidermal growth factor receptor) family, such as ErbB-1,2, and 3 (Guo et al, 2006), as well as c-Met (Chung et al, 2004), Ron, LPA1 and LPA2 (O'Connor et al, 2012). This leads to intensification of signaling through PI3K, AKT, MAPK and the Rho small GTPases, as depicted in Figure 1. Integrin $\alpha 6\beta 4$ can also promote tumor progression through transcriptional regulation. It has been shown to increase the expression of invasive and metastatic proteins such as the epithelial to mesenchymal transition (EMT)-associated protein S100A4 (Stewart, O'Connor, 2015).

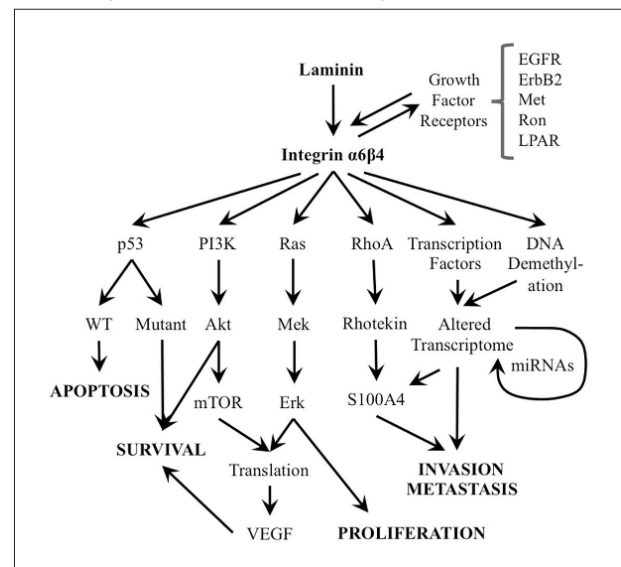


Figure 1 (Stewart, O'Connor, 2015)

Integrin $\alpha 6\beta 4$ is involved in the PI3K pathway, a signaling pathway known for promoting carcinoma progression (Shaw et al, 1997). However, the cytoplasmic domain of the integrin $\beta 4$ subunit does not have a consensus-binding motif for the regulatory subunit of PI3K, so direct activation of the pathway does not seem possible. One possible mechanism for integrin $\beta 4$ -mediated activation of PI3K involves insulin receptor substrate-1 and -2 (IRS-1 and IRS-2), which act as signaling intermediates facilitating integrin $\alpha 6\beta 4$ -mediated PI3K activation. Ligand of integrin $\alpha 6\beta 4$ promotes phosphorylation of IRS-1 and IRS-2, and then activation of PI3K (Shaw, 2001). Additionally, integrin $\alpha 6\beta 4$ cooperates with ErbB-2 to promote PI3K-dependent invasion (Gambaletta et al, 2000). Integrin $\alpha 6\beta 4$ has also been shown to localize to lipid rafts in the membrane, allowing it to recruit other receptor tyrosine kinases, and thus allowing it to activate PI3K (Gagnoux-Palacios et al, 2003).

ErbB-2 is one of the members of the EGFR family which signals for invasion and aggression in breast carcinoma. Integrin $\alpha 6\beta 4$ has been shown to associate with ErbB-2 in multiple breast carcinoma cell lines (Falcioni et al, 1997). Although ErbB-2 is implicated upstream of PI3K, ErbB-2 does not have a binding site for the regulatory subunit of PI3K. ErbB-2, as a receptor tyrosine kinase, must dimerize with another receptor to function. In this case, it dimerizes with an ErbB-3, a different EGFR receptor. ErbB-3 has a binding site for PI3K's regulatory subunit, and this heterodimer is a strong activator of PI3K. There is a positive association between integrin $\alpha 6\beta 4$ and ErbB-3 expression in patient-derived tumors (Folgiro et al, 2008).

Other receptor tyrosine kinases that cooperate with integrin $\alpha 6\beta 4$ include c-Met, which is activated by HGF (hepatocyte growth factor); a complex of $\alpha 6\beta 4$ and c-Met was shown to promote HGF dependent invasion (Trusolino et al, 2001). Ron, a tyrosine kinase receptor closely related to c-Met, has been shown to form a complex with integrin $\alpha 6\beta 4$ that induces hemidesmosome disassembly and the relocation of integrin $\alpha 6\beta 4$ to motility structures. Ron activation is important in pancreatic carcinoma progression, and it interacts with the integrin $\beta 4$ subunit in this setting to disrupt the association between integrin $\beta 4$ and plectin (Yu et al, 2012).

Integrin $\alpha 6\beta 4$ also upregulates the Rho family of small GTPases. The Rho family of small GTPases control the reorganization of the actin cytoskeleton required for cell motility (Machacek, et. al. 2009). The activation of RhoA leads to lamellae formation, as well as the generation of contraction forces that enable cell migration (O'Connor et al, 2000). Significantly, RhoA generally controls the generation of stress fibers rather than lamellae formation,

suggesting that integrin $\alpha 6\beta 4$ changes RhoA's function to facilitate tumor invasion (Stewart, O'Connor, 2015).

Integrin $\alpha 6\beta 4$ can also stimulate angiogenesis by enhancing signaling through the protein kinase ERK and transcription factor NF- κ B. Studies using knockout mice carrying a deletion in the signaling domain of the integrin $\beta 4$ 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 $\beta 4$ subunit could promote both bFGF (basic fibroblast growth factor)- and VEGF (vascular endothelial growth factor)-induced angiogenesis by enhancing signaling through ERK and NF- κ B (Nikolopoulos et al, 2004).

Cell Survival and Tumor Suppression

Returning to the paradox above, integrin $\alpha 6\beta 4$ promotes either cell survival or apoptosis, depending on context. For instance, in normal epithelial cells, integrins promote survival when in contact with the extracellular matrix. However, once separated, the loss of integrin signaling can inhibit cell growth and promote anoikis, a form of apoptosis (Ruoslahti, Reed, 1994). Similarly, in cancer, at times integrin $\alpha 6\beta 4$ acts as a promoter of cell survival, while at times it acts as a tumor suppressant.

To discover why these two different scenarios exist, a group studied two cell lines. In the RKO cell line, $\beta 4$ led to increased apoptosis, while in the MDA-MB-435 cell line, $\beta 4$ did not induce apoptosis. They discovered that the difference lay in the cells' p53, a protein which is often implicated in cancer when mutated. RKO cells have wild-type p53, while MDA-MB-435 cells have mutant p53 (Bachelder et al, 1999). Integrin $\alpha 6\beta 4$ can trigger apoptosis through p53 activation in cells harboring wild-type p53; however, in carcinoma cells deficient in p53, integrin $\alpha 6\beta 4$ promotes cell survival by activating the PI3K pathway and through growth factors such as VEGF (vascular endothelial growth factor). This discovery suggests that tumors expressing high levels of integrin $\alpha 6\beta 4$ in conjunction with mutated p53 are resistant to apoptosis and will therefore display a more aggressive clinical course. Interestingly, an association between p53 mutations and integrin $\alpha 6\beta 4$ 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 (Stewart, O'Connor, 2015).

Treating Carcinomas Through Integrin Modification

Since integrins were discovered as molecules involved in

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cancer, there has been a move to target them in cancer treatment. However, drugs developed to impact integrins have largely been unsuccessful. It's possible that it is because the integrin-targeting drugs may only have moderate affinity in vivo for integrin receptors, as shown by the doses used in clinical trials. Another reason may be that integrin antagonists work best when inhibiting early metastatic spread and not in advanced cancer stages, when cancer cells are already widespread (Paolillo, Schinelli, 2017)

Another issue may be that the wrong integrins have been targeted. Not all integrins have been thoroughly explored as therapeutic targets, including integrin $\alpha 6\beta 4$. That said, a common issue for all the integrins is that cells express multiple integrins at the same time, and if one would be targeted in treatment, the others might compensate for it. That explains why the drugs given were well tolerated with few ill effects, but also did not impact the cancer. The solution would be to try and impact multiple integrins at once, but that might be difficult to achieve in a patient (Alday-Parejo et al, 2019).

The issue may also not be the stage or affinity, but the drug target itself. Most drugs designed for integrins target the integrin-ligand interaction; in this case, they would try to competitively inhibit the interaction between the integrin's extracellular side and laminin 332. This could be a problem because integrins signal allosterically, so if an inhibitor would bind to the integrin's ligand binding pocket, it could actually turn on the integrin and the signalling cascades could begin. The cytoplasmic domain may be a better option (Alday-Parejo et al, 2019).

Thus far, one group has worked on an inhibitor targeting the cytoplasmic side of integrin $\alpha 6\beta 4$. They found that curcumin, a yellow pigment derived from turmeric, inhibited the interaction between the cytoplasmic tail and EGFR, which is implicated in the PI3K pathway related to survival, invasion, and metastasis. EGFR is activated when the residues Y1068 and Y1045 are phosphorylated; when $\alpha 6\beta 4$ was knocked out, there was significantly less phosphorylation, showing a connection between the two structures. They added curcumin to other cells and found that it had a similar effect on EGFR phosphorylation as knocking down $\alpha 6\beta 4$. The hemidesmosomes in cells with curcumin remained stable upon stimulation with EGF (Soung, Chung, 2011).

Discussion/Conclusion

Integrin $\alpha 6\beta 4$ plays an essential part in both regular and cancerous cells. Significantly, it contributes to the PI3K pathway, related to cancer aggression and survival. Integrin $\alpha 6\beta 4$ should definitely be studied, both as a diagnostic

tool and as a target for therapeutic intervention. As discussed above, it is a predictor of the cancer's severity and can be used in a diagnostic setting. More research should be put into targeting its intracellular interaction with growth factors so that these pathways can be inhibited.

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