Lecture synopsis Targeting oral cancer invasion: direct and indirect consequences of impairing integrin function.

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Integrins are a family of heterodimeric, cation-dependent transmembrane glycoproteins which are found on virtually all cells, and constitute the largest family of ECM-adhesion receptors. As well as mediating cell adhesion they are involved in many cell-signalling pathways regulating dynamic processes such as proliferation, migration, cell survival and differentiation.

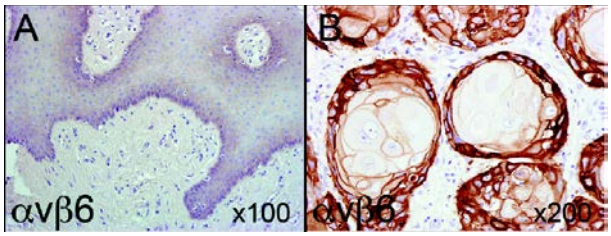
Each integrin consists of an α and a β subunit which associate in a non-covalent manner to form functional heterodimers. Sixteen α and eight β subunits have been identified and these combine to form more than twenty receptors. Most cells express at least one, and usually several integrin heterodimers.

The epithelial-specific integrin αvβ6 is not usually detectable on normal adult epithelia but is upregulated during tissue remodelling, including during wound healing and carcinogenesis[1]. Several ligands, which include the matrix proteins fibronectin and tenascin[1] have been identified for αvβ6, and it also binds to the latency associated peptide (LAP) of TGF-β1 and TGF-β3 resulting in activation of the cytokines, a process implicated in pathogenic organ fibrosis[1].

Increasingly, αvβ6 over-expression has been reported in numerous types of carcinoma, particularly oral squamous cell carcinoma (OSCC), where it appears to have a tumour-promoting effect, and studies have shown that αvβ6 may modulate several cell functions associated with tumour progression[1,2]. High expression in colon[3], lung[4] and cervical[5] carcinomas correlate with poor patient survival. Recently we have examined over 900 cases of breast carcinoma and identified αvβ6 as an independent prognostic marker, high expression correlating with poor patient prognosis[6].

We, and others, have shown that αvβ6 is up-regulated early in the development of OSCC and expression in epithelial dysplasia has been correlated with malignant transformation[7,8]. High expression of the integrin occurs in around 90% of OSCC (Figure 1), and is maintained as tumours progress and metastasise.

Figure 1

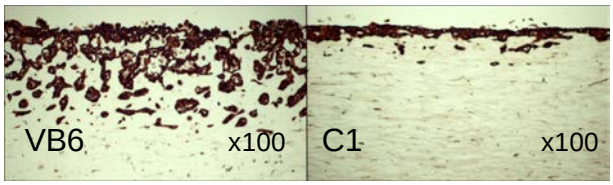


Immunohistochemistry showing αvβ6 expression in hyperplastic oral epithelium (A) and OSCC (B). Expression in OSCC often was most prominent at the periphery of invading tumour islands

To study the function of αvβ6 in OSCC cells, we used cDNA-transfer techniques to generate a panel of OSCC cell lines expressing varying levels of αvβ6, including αvβ6-negative, low-expressing and high-expressing cells. The H357 cell line, which is αv negative, was transfected with αv cDNA to create the V3 cell line which expresses low levels of αvβ6. This was further transfected using a retroviral construct with β6 cDNA creating the VB6 cell line, which has high αvβ6 expression. A control cell line C1 contained the empty vector only[9,10].

To study OSCC invasion we used Transwell assays, organotypic cultures (Figure 2) and an *in vivo* xenograft model. In all these techniques we found that high αvβ6 expressing VB6 cells were more invasive than control (C1) cells), and that the increased invasion was αvβ6-dependent and modulated through the protease MMP-9[7,9,10]. We also identified the region of the β6 cytoplasmic tail responsible for generating pro-invasive signals[11].

Figure 2



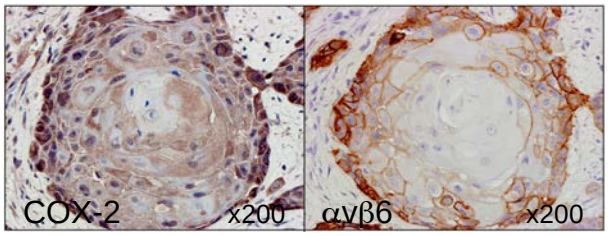
OSCC cells were grown in organotypic culture for 14 days then stained for cytokeratin. VB6 cells were significantly more invasive than C1 control cells

These early data suggested that αvβ6 is an attractive tumour target; it is a cell-surface molecule expressed by OSCC cells but not by normal oral epithelium, it has a high level of expression, which is maintained in metastasis, and it is tumour-promoting.

Over the last 5 years we have developed several αvβ6-specific reagents to target tumour cells. These include antibodies (scFv and monoclonal), peptides and genetically modified adenoviruses. These currently are being tested as possible therapeutic or imaging agents in pre-clinical models. However, an alternative method for inhibiting αvβ6-dependent tumour cell functions is to abrogate αvβ6 signalling. Recently we described a novel link between αvβ6 and the cyclooxygenase pathway and showed that αvβ6-dependent invasion could be inhibited by non-steroidal anti-inflammatory drugs (NSAIDs)[7].

Cyclo-oxygenases (COXs) catalyse the key step in prostanoid biosynthesis, and are targets of NSAIDs[12]. Two human isoforms exist: COX-1, expressed constitutively in most mammalian cells, generates prostaglandins necessary for normal physiological function, while COX-2, normally undetectable, is induced rapidly by various inflammatory or oncogenic stimuli, and consequently is upregulated in many carcinomas including OSCC (Figure 3)[12]. Similar to αvβ6, COX-2 is expressed in premaligant oral dysplasia where expression has been reported to correlate with malignant transformation[13]. NSAIDs inhibit COX–1 and –2, and also have been shown to exert a number of their effects through integrins. For example, the selective COX-2 inhibitor NS-398 suppresses function of αvβ3 on endothelial cells[14]. This integrin, although not expressed by epithelial cells, has similar functions to αvβ6, raising the possibility that αvβ6 may also be inhibited by NSAIDs.

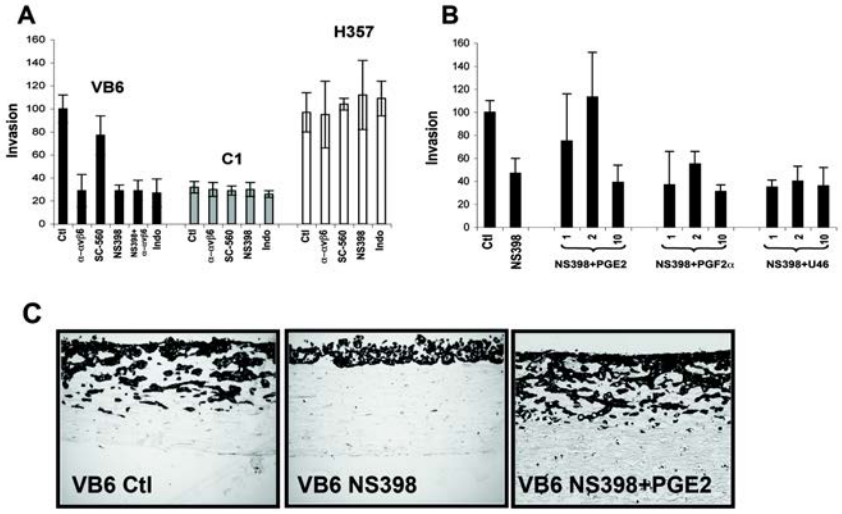
Figure 3



Immunohistochemistry showing COX-2 and αvβ6 expression in OSCC. COX-2 and αvβ6 often co-localised at the periphery of invading tumour islands

To test this hypothesis we examined the effect of COX inhibitors on αvβ6-dependent cell functions using the panel of cell lines generated previously. We found that COX-2 inhibition specifically blocked αvβ6-dependent invasion in Transwell assays (Figure 4A,B), organotypic culture (Figure 4C) and tumours *in vivo*. COX-2 inhibition however, did not inhibit the invasion of cells lacking αvβ6 (Figure 4A).

Figure 4

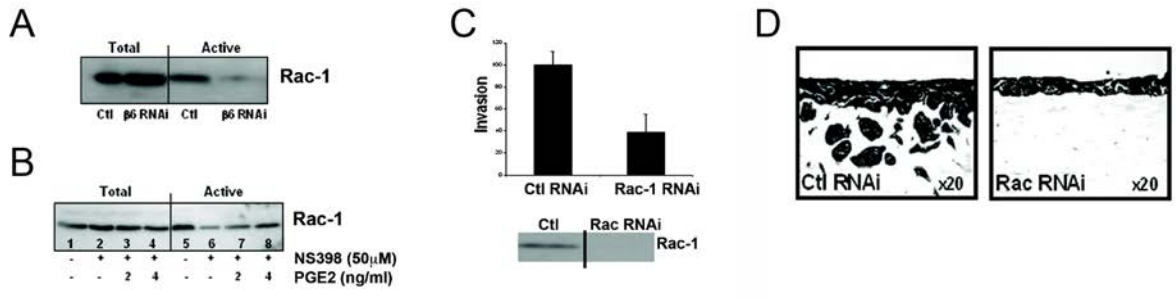


(A) Transwell invasion of VB6 cells was inhibited by blockade of αvβ6 integrin and by the COX-2 inhibitor, NS398. Indomethacin produced a similar level of inhibition, while a COX-1 inhibitor, SC-560, had no effect. H357 and C1 cell invasion were not affected by anti-αvβ6 antibody or any of the NSAIDs. **(B)** Inhibition of VB6 Transwell invasion by NS398 was abrogated completely by PGE_2 at 2ng/ml, while $PGF_{2\alpha}$ and U46619 had no effect. Titration of PGE_2 produced a bell-shaped curve. **(C)** OSCC cells were grown in organotypic culture for 14 days, then invasion in cytokeratin-stained sections was examined. NS398 inhibited invasion of VB6 cells and, similar to Transwell assays, treatment with PGE_2 restored invasion in the presence of NS398.

Levels of COX-2 protein and the COX metabolite PGE_2 were similar in the cell lines regardless of αvβ6 expression, and were not affected by αvβ6 inhibition. The mechanism by which NS398 suppressed αvβ6-dependent invasion was through

inhibition of the small GTPase, Rac-1. Rac-1 regulates cell migration and spreading following integrin-binding, and may also modulate tumour cell invasion. Rac-1 was activated by αvβ6 ligand-binding, and activation was suppressed by the COX-2 inhibitor, NS398, suggesting that PGE_2 is required for Rac-1 activation (Figure 5A,B). Down-regulation of Rac-1 expression by RNAi inhibited αvβ6-dependent invasion in Transwell assays and organotypic culture to the same degree as COX-2 inhibition (Figure 5C and D respectively). Conversely, expression of constitutively active Rac-1 (V12Rac-1-GFP) or addition of the COX-2 metabolite, PGE_2 (2ng/ml), abrogated the anti-invasive effect of NS398 (Figure 4B, C),

Figure 5



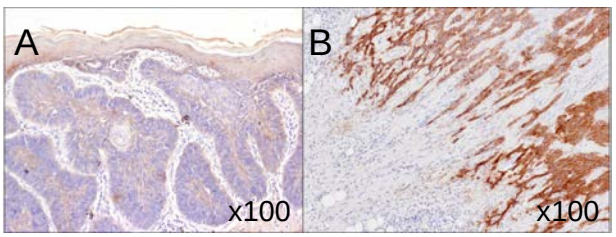
(A) RNAi β6 knockdown in VB6 cells significantly reduced Rac-1 activation on LAP (69%). **(B)** NS398 suppressed αvβ6-mediated Rac-1 activation in VB6 cells on LAP. Addition of PGE_2 restored Rac-1 activation in the presence of NS398. **(C)** Rac-1 RNAi inhibited VB6 Transwell invasion. Western blot verified protein knock-down. **(D)** RNAi knockdown of Rac-1 inhibited invasion of VB6 cells in six-day organotypic culture.

Thus a novel link between the COX-2 enzyme, αvβ6 and Rac-1 exists in OSCC invasion, raising the possibility that long-term treatment with NSAIDs has a rational basis for the prevention of the development and progression of OSCC[7]. We are currently seeking ethical approval to conduct a study on the preventive effect of aspirin mouthwash on OSCC development in patients with oral epithelial dysplasia.

As well as promoting tumour invasion, αvβ6 modulates several other cell functions. We recently have demonstrated in basal cell carcinomas (BCC), that αvβ6 may also promote tumour invasion indirectly through stromal modulation, a process requiring αvβ6-dependent activation of the cytokine, TGF-β1[15]. Most studies of neoplastic progression have tended to focus on the tumour cell. However, there is abundant evidence to suggest that the tumour stroma actively contributes to malignant progression. A common finding in many types of solid tumour, is that stromal fibroblasts become 'activated' myofibroblasts and express a number of contractile proteins, particularly α-smooth muscle actin (SMA)[16]. The importance of tumour stroma is emphasised by the absolute requirement of myo(fibroblasts) to promote invasion in our organotypic invasion model[17], and we have demonstrated previously a pro-invasive, paracrine interaction between myofibroblasts and head and neck SCC cells[18].

BCC is the most common cancer in the Western world and its incidence is increasing[19]. The pathogenesis of BCC involves deregulated sonic hedgehog signalling (shh), leading to activation of the Gli transcription factors[20]. Most BCC have a nodular growth pattern, are indolent, slow-growing and considered 'low risk' lesions[19]. However, several aggressive histological variants exist, which may cause significant morbidity. The 'high risk' morphoeic variant accounts for around 6% of BCCs[19] and is so-called because of its fibrotic (desmoplastic) stroma. Unlike the more common nodular BCC variant, morphoeic BCC are aggressively infiltrative resulting in greater depth of invasion, tissue destruction and thus greater frequency of recurrence[19]. Since 95% of these tumours are located on the face or head this causes significant morbidity. We found that 77% of morphoeic BCC expressed αvβ6 strongly. This was similar to expression levels in cutaneous SCC (66% high expression; 13/19 tumours; unpublished data), but was significantly higher than nodular BCC (7%)(Figure 6).

Figure 6

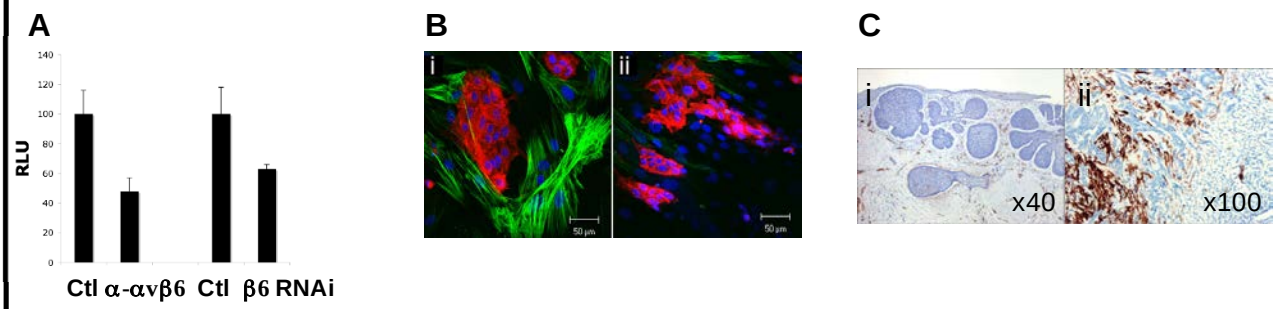


Immunohistochemistry showing low level αvβ6 expression in nodular BCC (A) and high level expresion in morphoeic BCC (B).

model, we examined the effect of αvβ6 expression in these cells. Surprisingly, given our previous results in OSCC, inhibition of αvβ6 had no direct effect on cell invasion. However, activation of TGF-β1 in NTGli1 cells was αvβ6-dependent (Figure 7A). The role of TGF-β1 in tumour biology is complex, having both suppressive and promoting effects, however one of the mechanisms by which TGF-β1 may promote tumour progression is through stromal modulation, since the cytokine is considered to have a central role in inducing the myofibroblastic phenotype.

We found, in co-culture assays, that NTGli1 cells modulated myofibroblast transdifferentiation through αvβ6-dependent activation of TGF-β1 (Figure 7B), and also confirmed, by immunochemistry, that the stroma of morphoeic BCCs is myofibroblastic-rich compared with nodular BCCs (Figure 7C).

Figure 7

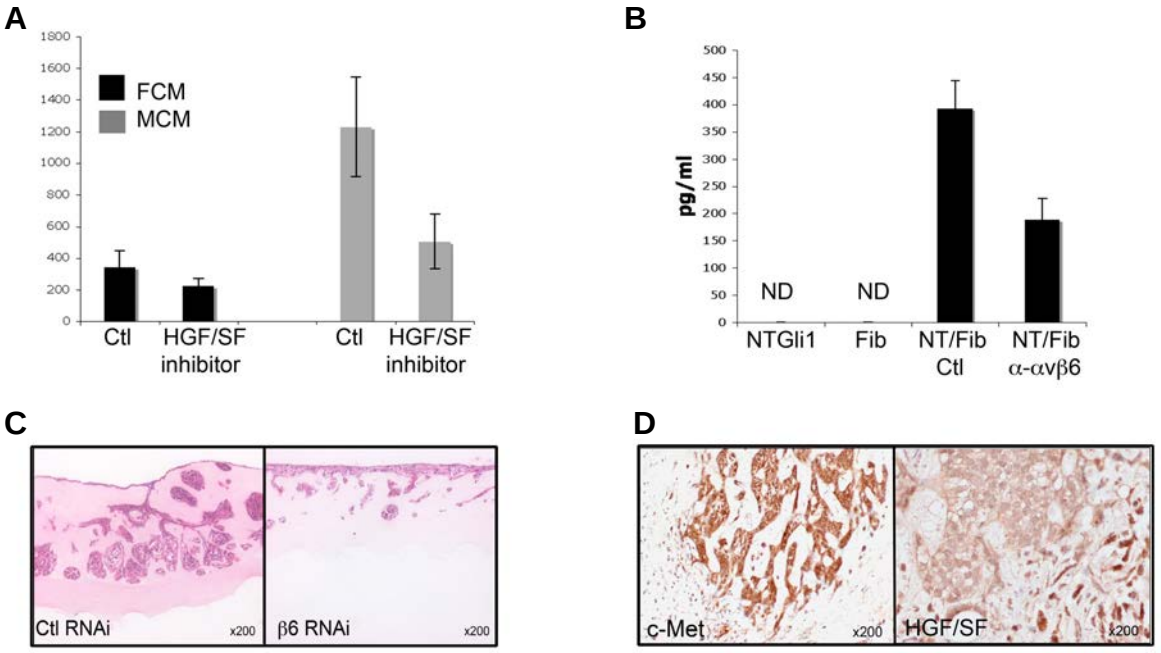


(A) TGF-β activation co-culture assay. Co-culture of NTGli1 with MLEC promoted TGF-β activation (RLU), which was suppressed by antibody blockade of αvβ6 integrin (10D5) or using β6 RNAi. **(B)** Confocal micrograph showing co-cultures of fibroblasts and NTGli1 cells treated with control (i) or anti-αvβ6 (ii) antibodies. Images shows cell nuclei (blue), αvβ6-positive NTGli1 cells (red) and SMA-positive myofibroblasts (green). When co-cultured with NTGli1, fibroblasts upregulated SMA indicating myofibroblastic transdifferentiation (i). When αvβ6 was inhibited, no myofibroblastic phenotype was produced (ii). **(C)** Immunohistochemistry showing representative SMA expression in nodular BCC (i) and morphoeic BCC (ii). SMA expression was significantly higher in morphoeic BCC.

Myofibroblasts may promote tumour progression in a number of different ways including secretion of proteases, matrix proteins and cytokines[16]. We found that conditioned medium from myofibroblasts promoted NTGli1 Transwell invasion, suggesting that myofibroblasts secrete an invasion-promoting soluble factor (Figure 8A). We found that co-culture of NTGli1 and fibroblasts (HFFF2) resulted in upregulated secretion of HGF/SF, which was suppressed when αvβ6 was inhibited (Figure 8B). Inhibition of HGF/SF signaling suppressed the invasion-promoting effect of myofibroblast-conditioned medium in Transwell assays (Figure 8A), and also inhibited invasion in organotypic culture. Consistent with these findings we found that, although inhibition of αvβ6 had no anti-invasive effect in Transwell assays, when NTGli1 cells were admixed with fibroblasts in organotypic culture, β6 RNAi knockdown markedly reduced invasion, suggesting that this effect was modulated through suppression of myofibroblast transdifferentiation (Figure 8C). We confirmed by immunochemistry that morphoeic BCC express both c-Met receptor and stromal HGF/SF (Figure 8D). These data suggest that αvβ6 also may promote invasion indirectly through stromal modulation, and that αvβ6-dependent TGF-β1 activation may explain both the infiltrative growth pattern and fibrotic stroma of morphoeic BCC.

In summary, αvβ6 is not detectable in normal epithelia but is upregulated in numerous carcinoma types. We have shown previously that the integrin directly promotes tumour invasion, and more recently described an indirect invasion-pomoting effect through modulation of tumour stroma. We have generated several αvβ6-specific reagents, which are being tested for tumour therapy or imaging, and our pathological studies have indicated that αvβ6 is an important prognostic marker in breast carcinoma. These data suggest that αvβ6 is an attractive target for therapy and it is our intention to translate our laboratory findings into clinical applications in the near future.

Figure 8



(A) Transwell invasion assay following treatment of NTGli1 cells with a HFF/SF inhibitor and using conditioned medium from fibroblasts (FCM) or myofibroblasts (MCM) as a chemoattractant. MCM significantly promoted invasion of NTGli1 cells. Inactivation of HGF/SF signalling suppressed significantly the invasion-promoting effect of MCM. **(B)** ELISA showed that HGF/SF was not detectable in NTGli1 or Fibroblasts (fibs) when cultured alone. HGF/SF was upregulated when the NTGli1 and fibs were co-cultured and this was suppressed when αvβ6 was inhibited. **(C)** NTGli1 cells were transfected transiently with random or β6 RNAi and then grown in organotypic culture with fibroblasts for 6 days. In contrast to Transwell invasion, inhibition of αvβ6 suppressed invasion of NTGli1 cells. **(D)** Immunohistochemistry showing c-Met and HGF/SF expression in morphoeic BCC. Strong c-Met expression was observed in most tumours. HGF/SF expression was present in stromal cells.

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