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Collagen type XI $\alpha 1$ facilitates head and neck squamous cell cancer growth and invasion

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Background: Although it is well established that the extracellular matrix affects tumour progression, not much is known about the various components and their effect on head and neck squamous cell carcinoma (HNSCC) progression. Levels of collagen type XI $\alpha 1$ (colXI $\alpha 1$), a minor fibrillar collagen, have been shown to be increased in tumour compared with normal tissue in several cancers, including colorectal, breast, and non-small cell lung cancer. Currently, the functional significance of colXI $\alpha 1$ is not understood.

Methods: We examined the expression levels of colXI $\alpha 1$ mRNA and elucidated the functional role of colXI $\alpha 1$ in HNSCC. Cell proliferation, invasion, and migration were examined with and without colXI $\alpha 1$ knockdown with siRNA in HNSCC cells.

Results: Our data demonstrate that colXI $\alpha 1$ expression is increased in tumour samples compared with levels in normal adjacent tissue in 16/23 HNSCC patients. In addition, colXI $\alpha 1$ is increased in HNSCC cell lines compared with normal immortalised epithelial cells and is increased in tumour-derived fibroblasts compared with normal fibroblasts. Using an siRNA approach, we demonstrate that colXI $\alpha 1$ contributes to proliferation, migration, and invasion of HNSCC.

Conclusion: Our cumulative findings suggest that colXI $\alpha 1$ contributes to HNSCC tumorigenesis and may serve as a potential therapeutic target.

Worldwide, head and neck cancer is the 6th leading cause of cancer mortality, with 350 000 deaths per year due to cancers of the oral cavity, pharynx, and larynx (Ferlay *et al*, 2008). Over 630 000 new cases of head and neck cancer are diagnosed annually, of which 90% are squamous cell carcinoma (HNSCC). Treatment with standard modalities, including chemotherapy, radiation, and surgery, is associated with a 5-year survival rate of only 50%, emphasising the need for newer, more effective approaches (Cognetti *et al*, 2008). Although cumulative evidence supports the epidermal growth factor receptor (EGFR) as a therapeutic target in HNSCC, where increased expression correlates with worse prognosis (Rubin Grandis *et al*, 1998), treatment with the EGFR-targeted monoclonal antibody cetuximab has not been shown to prevent metastasis (Bonner *et al*, 2006).

In a previous study, we used a 12 000 gene oligonucleotide microarray of paired tumour and normal tissue from nine HNSCC patients to determine differential gene expression in order to identify potential therapeutic targets (Sok *et al*, 2003). Gene expression changes were found in 227 genes, including collagen type XI subunit $\alpha 1$ (colXI $\alpha 1$), which was found in all nine tumours with virtually no expression in normal tissue.

Type XI collagen, a minor fibrillar collagen, is a heterotrimer composed of $\alpha 1$, $\alpha 2$, and $\alpha 3$ chains that co-polymerise with type II and IX collagen to form the cartilage (Spranger, 1998). The $\alpha 1$ subunit produced by non-cartilaginous tissues, such as bone, skin, and arterial smooth muscle cells, is located on chromosome 1 and regulates the promoter of the major collagen type V (COL5a2) (Yoshioka *et al*, 1995). Although type XI collagenopathies at birth

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are known to produce phenotypes that include facial anomalies, cleft palate, and hearing defects (Spranger, 1998), relatively little is known about the function of type XI collagen in the adult.

A 2001 study of colorectal tumours first identified an association between colXI $\alpha 1$ and cancer (Fischer *et al*, 2001b). ColXI $\alpha 1$ mRNA expression was found in 20 of 24 colorectal tumours, whereas no apparent expression was found in the 4 normal tissue samples. In addition, COL5a2, which is normally expressed in human fetal gut and not expressed in the normal adult colon tissue, was correlated with the expression of colXI $\alpha 1$ in the colorectal tumours. Recent studies in several other areas of cancer research, including gastric (Vecchi *et al*, 2007; Zhao *et al*, 2009), pancreatic (Badea *et al*, 2008), breast (Ellsworth *et al*, 2009), and non-small lung cancer (Chong *et al*, 2006), have also demonstrated increased levels of colXI $\alpha 1$ in tumour tissue compared with less-diseased or normal tissue. In a multi-cancer computational analysis of four data sets of ovarian and colorectal cancer, the authors concluded that colXI $\alpha 1$ overexpression is a proxy for 'metastasis-associated fibroblast (MAF)' signature (Kim *et al*, 2010).

Our initial observation of increased expression of colXI $\alpha 1$ in HNSCC was corroborated by another group whose findings also implicated colXI $\alpha 1$ in the pathogenesis of HNSCC metastasis to the lymph nodes (Schmalbach *et al*, 2004). Primary tumours that had metastasised to the lymph nodes demonstrated over seven-fold higher expression of colXI $\alpha 1$ than tumours that had not metastasised and over 25-fold higher expression of colXI $\alpha 1$ than levels in normal oral cavity mucosa samples. Thus far, the precise role of colXI $\alpha 1$ in proliferation and invasion remains incompletely understood, and few functional data have been reported.

In the present study, we examined colXI $\alpha 1$ expression in a panel of HNSCC cell lines and a cohort of paired tumour/normal samples derived from HNSCC patients. We then used an siRNA approach to determine the role of colXI $\alpha 1$ in cellular proliferation, migration, and invasion.

MATERIALS AND METHODS

Cell lines, tumours, and reagents. Human HNSCC cell lines included UMSCC-1 (Krause *et al*, 1981), UMSCC-10A (Grenman *et al*, 1991), UMSCC-10B (Somers *et al*, 1992), UMSCC-22A (Lansford *et al*, 1999), and UMSCC-22B (Grenman *et al*, 1991), which were kind gifts from Dr. Thomas Carey (University of Michigan); UPCI-15B (Snyderman *et al*, 1994) from Dr. Theresa Whiteside (University of Pittsburgh); 1483 (Sacks *et al*, 1988) from Dr. Peter Sacks (New York University); 686LN (Sturgis *et al*, 1994) from Dr. Georgia Chen (Emory University); and FaDu (Rangan, 1972) from Dr. Jeffrey Myers (The University of Texas MD Anderson Cancer Center). The Het1A (Stoner *et al*, 1991) cell line, derived from mucosal epithelial cells, was obtained from American Type Culture Collection (Manassas, VA, USA). All cell lines were validated through genotyping with an AmpF/STR Identifier PCR Amplification Kit (Applied Biosystems, Carlsbad, CA, USA).

UMSCC-1 and FaDu cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) and 0.4 $\mu\text{l ml}^{-1}$ hydrocortisone. 686LN cells were maintained in Dulbecco's modified Eagle's medium/F12 (1:1) (Invitrogen) and 10% FBS. Het1A cells were maintained in Airway Epithelial Cell Growth Media (PromoCell, Heidelberg, Germany) with a supplement provided by the company. All other cell lines including cancer-associated fibroblasts (CAFs), and normal fibroblasts were maintained in DMEM with 10% FBS. All cells were incubated at 37 °C in the presence of 5% CO₂. Fibroblast cultures were growth from HNSCC tissue or from

uvulopalatoplasty or tonsil explants from cancer-free subjects. Written consent was collected from patients, using a University of Pittsburgh, Institutional Review Board approved protocol. In brief, tissue pieces were minced and placed in 10% serum containing media. Fibroblast cultures free of epithelial cells were used in these studies.

Primary HNSCC tumour samples, as well as normal mucosal samples adjacent to the tumour site, were obtained under an institutional review board-approved protocol from 23 HNSCC patients undergoing surgical excision with curative intent (Table 1). Signed informed consent was obtained from each subject. Human papilloma virus (HPV) status was not determined for majority of the patients (58%). All evaluated patients were HPV negative.

Table 1. Summary of HNSCC patient and tumour characteristics

Characteristic	HNSCC cases (N = 23)
Age, years	
Median (range)	63 (32–88)
Sex, N (%)	
Men	17 (73.9)
Women	6 (26.1)
Smoking, N (%)	
Never	2 (8.7)
Former Smoker	8 (34.8)
Active Smoker	13 (56.5)
Alcohol, N (%)	
Never	8 (34.8)
Ever	15 (65.2)
Cancer site, N (%)	
Oral cavity	16 (79.6)
Pharynx	1 (4.3)
Larynx	4 (17.4)
Other	2 (8.7)
Cancer type, N (%)	
Primary	20 (87.0)
Recurrence	3 (13.0)
Tumour path stage, N (%)	
T1-T2	8 (34.8)
T3-T4	12 (52.2)
Unstaged recurrent tumour	3 (13.0)
Nodal path stage, N (%)	
N0-N1	12 (52.2)
N2-N4	8 (34.8)
Unstaged recurrent tumour	3 (13.0)
Perineural invasion, N (%)	
Yes	14 (60.9)
No	7 (30.4)
Not evaluated	2 (8.7)
HPV, N (%)	
Positive	0 (0.0)
Negative	9 (39.1)
Not evaluated	14 (60.9)

RT-PCR analysis for colXI α 1. Total RNA was isolated from 30 mg of snap-frozen tumour tissue as described previously using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) (Sok *et al*, 2003). One nanogram of total RNA was added to a final reaction volume of 25 μ l. To detect colXI α 1, standard RT-PCR was done using a One-Step RT-PCR kit (Qiagen) with primers (5'-GGATCAAA TGATGAGGAGATGTCCTATG-3' and antisense sequence as 5'-CTAAATGGTACCTGTATATGCAGCGTTG-3') designed to amplify a 345-bp fragment. Actin primers were used as previously described (Sok *et al*, 2006). Primers were diluted to a final concentration of 0.6 μ M. All other reagents were used according to the manufacturer's protocol. Reverse transcription was performed at 50 °C for 30 min, followed by enzyme inactivation and PCR hot start at 95 °C for 15 min. Denaturation, annealing, and extension were performed at 94 °C, 55 °C, and 72 °C, respectively, for 1 min each for a total of 35 cycles. The reaction was completed with an extension period at 72 °C for 10 min. PCR products were visualised on a 1% agarose gel containing ethidium bromide. Expression levels of colXI α 1 and actin in the paired tumour and normal epithelial samples were compared using ImageJ densitometry software.

Total RNA was isolated from 5×10^6 cells from each of the nine representative HNSCC cell lines (UMSCC-1, UMSCC-10A, UMSCC-10B, UMSCC-22A, UMSCC-22B, UPCI-15B, 1483, 686LN, and FaDu), normal mucosal epithelial cell line Het1A (5×10^6 cells), and fibroblasts, using an RNeasy Mini kit (Qiagen). RT-PCR to detect colXI α 1 was performed as described above.

ColXI α 1 siRNA transfection. Four different siRNA duplexes (A-D) targeted against human colXI α 1 mRNA were generated according to the Assays-on-Demand system (Dharmacon, Lafayette, CO, USA): duplex (A) GCAAAUUGGUGUUGAGGUU UU, duplex (B) GAACGUGGGUCAGCAGGUUUU, duplex (C) AAAGGACAUCUGGUUUUUUUU, duplex (D) UCUGGUAGA UGGAGAUUUUUUUU. Pooled siRNA-colXI α 1 was formed by combining all four duplexes. One day before transfection, UPCI-15B cells were plated at 0.5×10^4 cells into 24-well plates in order to attain 80–90% confluency at the time of transfection. Twenty-picomole siRNA was transfected in cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in Opti-MEM media.

In vitro growth inhibition by colXI α 1 siRNA. To examine the effects of colXI α 1 on growth kinetics, UMSCC1 cells were treated with colXI α 1 siRNA B or control non-targeting siRNA. Around 42 h post transfection, the cells were trypsinised and plated in xCelligence E-plates Roche (Indianapolis, IN, USA) as previously described (Hickok *et al*, 2011). In brief, after baseline measurements, 2000 cells were plated in 200 μ l DMEM+10% FBS in quadruplicates into 16-well E-plates. Cell index (difference between the impedance at a given time point and the background, divided average resistance across the plate in media only using 10 kHz frequency of current) measurements were taken every 15 min. The growth rates of the cells were assessed in real-time over 40 h on the xCelligence DP system. The fold-change in growth relative to the measurement at 5 h post plating was determined. The experiment was repeated three independent times.

Matrigel invasion assay and migration assay. Cell invasion was evaluated *in vitro* using Matrigel-coated semipermeable-modified Boyden inserts with a pore size of 8 μ m (Becton Dickinson/Biocoat, Bedford, MA, USA). Cell migration was evaluated *in vitro* using semipermeable-modified Boyden inserts with a pore size of 8 μ m (Becton Dickinson/Biocoat). Cells from HNSCC lines (UMSCC-1 and FaDu) and the normal cell line Het1A were transfected with siRNA-colXI α 1 duplex B or non-targeting control siRNA (siRNA-NTC) as described above and plated in duplicate at a density of 1.25×10^4 cells per well in serum-free media in the insert. The insert contained serum-free media, while the holding well

contained 10% FBS that served as a chemoattractant. After 24 h of treatment at 37 °C in a 5% CO₂ incubator, the cells in the insert were removed by gentle wiping with a cotton swab. Cells on the reverse side of the insert were fixed and stained with Hema 3 (Fisher Scientific, Hampton, NH, USA) according to the manufacturer's instructions. Cells plated in 24-well plates were subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, and the cell numbers across the groups were normalised. A ratio of the number of invading/migrating cells to the OD from the MTT assay was determined in order to adjust for changes in cell number across wells.

Statistical analysis. Non-parametric, Wilcoxon–Mann–Whitney exact tests were used to compare effects on cell proliferation, invasion, and migration in cells treated with control or colXI α 1 siRNA. In order to analyse the differences between levels of colXI α 1 in the paired tumour and normal adjacent mucosa tissue samples from 23 patients, the exact Wilcoxon Signed rank test was used. All statistical analyses were performed with Cytel Studio 9 (Cytel, Inc., Cambridge, MA, USA).

RESULTS

Increased expression of colXI α 1 in HNSCC tumours compared with levels in adjacent normal mucosa. Previous cDNA microarray analysis demonstrated that, although colXI α 1 was present in tumour tissue from all nine HNSCC tumours examined, it was virtually undetectable in corresponding normal adjacent tissue (Sok *et al*, 2003). The high incidence of synchronous and metachronous tumours of the upper aerodigestive tract that characterises HNSCC carcinogenesis suggests that molecular changes that are found in both the tumour and adjacent normal mucosa may represent relatively early genetic changes (Bedi *et al*, 1996; Scholes *et al*, 1998). We sought to determine colXI α 1 mRNA levels in tumour tissue compared with levels in paired adjacent normal tissue in 23 HNSCC patients using RT-PCR. In 16 of 23 pairs, colXI α 1 expression was significantly higher in the tumour compared with normal tissue ($P=0.0006$) with a median fold increase of 6.63 (range 1.8–169), suggesting that increased colXI α 1 represents a later event in HNSCC carcinogenesis (Figure 1). In the absence of specific antibodies of sufficiently high quality, we could not validate these expression results at the protein level. However, our results confirm earlier studies demonstrating increased expression of colXI α 1 in HNSCC tumours.

Increased expression of colXI α 1 in HNSCC cell lines compared with normal immortalised mucosal epithelial cells. Tumour lysates represent both epithelial and stromal cells in the tumour microenvironment. There are no reports of colXI α 1 expression in cancer cell lines compared with corresponding normal cells. To determine if the transformed epithelial cells were the likely source of colXI α 1 lines, we compared colXI α 1 mRNA levels in nine genotypically validated, representative HNSCC cell lines (UMSCC-1, UMSCC-10A, UMSCC-10B, UMSCC-22A, UMSCC-22B, UPCI-15B, 1483, 686LN, and FaDu) with expression levels in immortalised normal mucosal epithelial cells (Het1A) by RT-PCR. Expression of colXI α 1 was detected in all nine HNSCC cell lines examined compared with the corresponding normal cell line, which did not express colXI α 1 transcript (Figure 2). These results suggest that colXI α 1 overexpression is, at least in part, due to increased expression in the transformed epithelial cell component of tumours.

Increased expression of colXI α 1 in tumour-derived fibroblasts compared with fibroblasts from cancer-free subjects. It is well documented that, in response to wounds, normal fibroblasts secrete several types of collagen including colXI α 1. Stromal

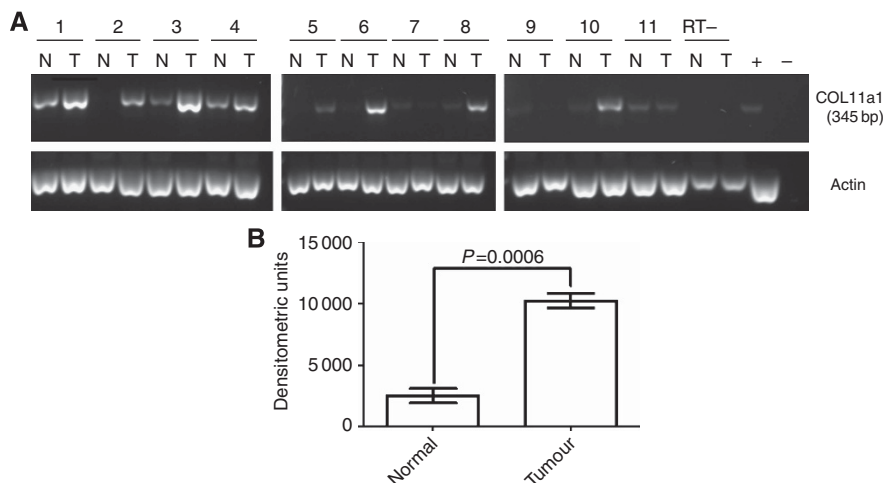


Figure 1. Increased expression of colXI $\alpha 1$ expression in HNSCC tumours compared with levels in normal mucosa. ColXI $\alpha 1$ mRNA levels were examined in 23 HNSCC tumour/normal pairs by RT-PCR. (A) Representative gel images from 11 patients are depicted. (B) Cumulative densitometric analyses of ColXI $\alpha 1$ and actin levels from 23 paired samples demonstrates significantly higher colXI $\alpha 1$ expression in 16 tumours compared with the paired normal adjacent mucosa ($P=0.0006$). Error bars represent \pm s.e.m.

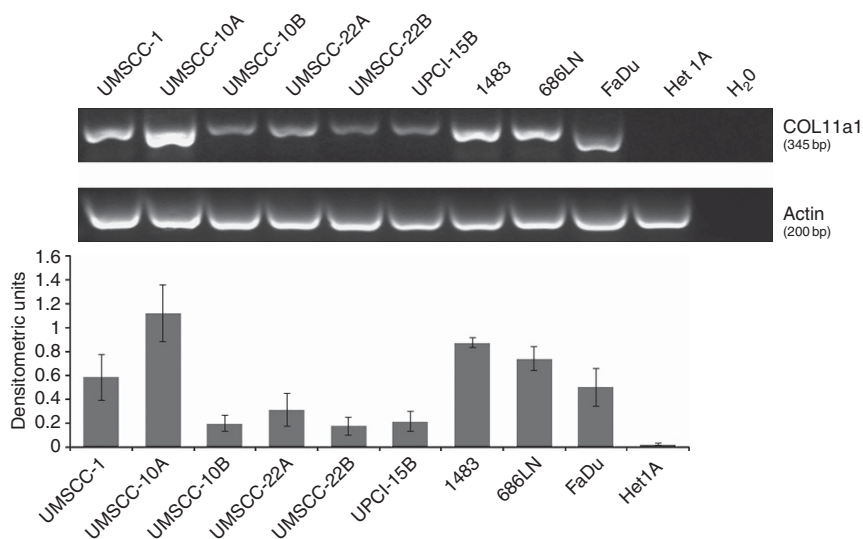


Figure 2. Increased expression of colXI $\alpha 1$ in HNSCC cell lines compared with normal immortalised mucosal epithelial cells. ColXI $\alpha 1$ mRNA levels were examined in nine representative HNSCC cell lines (UMSCC-1, UMSCC-10A, UMSCC-10B, UMSCC-22A, UMSCC-22B, UPCI-15B, 1483, 686LN, and FaDu) by RT-PCR. Expression of colXI $\alpha 1$ was increased in all nine cell lines compared with immortalised normal mucosal epithelial cells (Het1A). The experiment was repeated twice with similar results.

expression of colXI $\alpha 1$ is associated with malignancy in colorectal cancer (Fischer *et al*, 2001b). Higher levels of colXI $\alpha 1$ were reported in fibroblasts associated with lung cancer compared with normal fibroblasts (Navab *et al*, 2011). HNSCC tumours are closely associated with fibroblasts. There are no reports on the levels of colXI $\alpha 1$ expressed by stromal fibroblasts in the head and neck region. Primary HNSCC cancer-associated fibroblasts and fibroblasts from cancer-free subjects were grown out of tissue explants. We examined the levels of colXI $\alpha 1$ in primary fibroblasts isolated from cancer-free patients and HNSCC explants. Our data demonstrate that the cancer-associated fibroblast express higher levels of colXI $\alpha 1$ compared with fibroblasts from cancer-free patients (Figure 3).

ColXI $\alpha 1$ contributes to HNSCC proliferation, migration, and invasion. Although colXI $\alpha 1$ has been shown to correlate with tumour size in non-small cell lung cancer (Chong *et al*, 2006), the role of colXI $\alpha 1$ in cell growth has not been examined in cancer

cells. We hypothesised that, if colXI $\alpha 1$ contributes to HNSCC proliferation, then knocking down colXI $\alpha 1$ with siRNA would decrease cell proliferation *in vitro*. We transfected HNSCC cells with pooled siRNA targeting colXI $\alpha 1$ or GFP as a control, collected cells on days 2, 3, 4, and 5, and performed RT-PCR. Gene inactivation was observed on day 5 (Figure 4A). Next, we tested individual siRNA and determined that siRNA B demonstrated efficient knockdown of colXI $\alpha 1$ up to 48 h post transfection (Figure 4B).

The growth kinetics of cells transfected with siRNA- colXI $\alpha 1$ duplex B was compared with cells transfected with non-targeting siRNA. colXI $\alpha 1$ on transfection with siRNA duplex B significantly reduced cell proliferation compared with cells transfected with non-targeting control siRNA ($P=0.014$) (Figure 5A). These results suggest that colXI $\alpha 1$ contributes to cell proliferation in HNSCC cells *in vitro*.

ColXI $\alpha 1$ expression has been reported to be 7.61-fold higher in the primary HNSCC tumour in the setting of metastasis to the

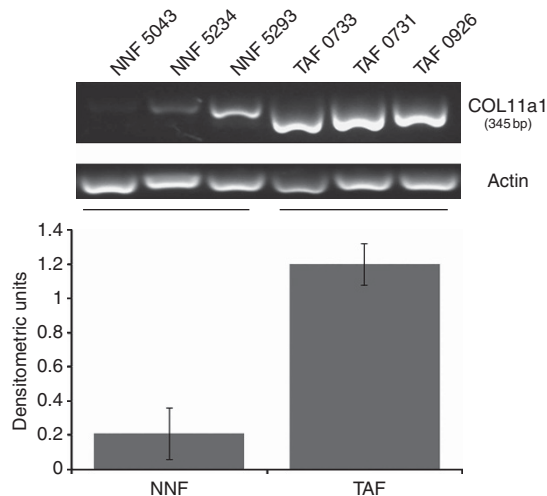


Figure 3. Increased expression of colXI $\alpha 1$ in tumour-derived fibroblasts compared with normal fibroblasts. Primary cancer-associated fibroblasts (TAF) isolated from HNSCC and fibroblasts isolated from oral mucosa of cancer-free patients (NNF) were assessed for colXI $\alpha 1$ mRNA levels by RT-PCR. Expression of colXI $\alpha 1$ was higher in the TAF lines compared with the NNF lines.

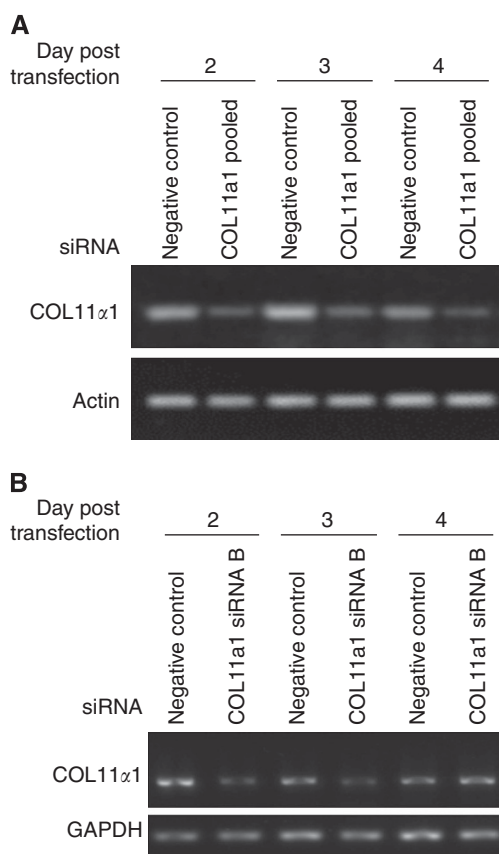


Figure 4. ColXI $\alpha 1$ siRNA effectively reduces mRNA levels of colXI $\alpha 1$. **(A)** HNSCC cells were transfected with pooled siRNA-targeted against the colXI $\alpha 1$ gene or with control siRNA, which does not share sequences with the colXI $\alpha 1$ gene. Cells were collected on days 2, 3, 4, and 5 and subjected to RT-PCR. Maximum gene inactivation was observed on day 5. RT-PCR amplification of the β -actin gene (lower panel) was used in each sample to monitor RNA levels and nonspecific RNA template degradation. **(B)** Cells were transfected with colXI $\alpha 1$ duplex B siRNA. Efficient knockdown of colXI $\alpha 1$ was achieved up to 3 days post transfection.

lymph nodes compared with levels in a primarily HNSCC tumour that remains confined to the head and neck mucosa (Schmalbach *et al*, 2004). The role of colXI $\alpha 1$ in cell migration or invasion has not been studied. To determine if colXI $\alpha 1$ contributes to HNSCC migration *in vitro*, representative HNSCC cell lines (UMSCC-1 and FaDu) were transfected with colXI $\alpha 1$ duplex B siRNA or the non-targeting control siRNA (siRNA-NTC). We also assessed invasion using a Boyden Matrigel chamber assay. ColXI $\alpha 1$ knockdown in UMSCC-1 and FaDu cells resulted in reduced migration ($P = 0.05$) and invasion ($P = 0.05$), respectively (Figure 5B and C). Thus, colXI $\alpha 1$ is an important facilitator of HNSCC progression.

DISCUSSION

Type XI collagen, a minor fibrillar collagen, is a heterotrimer made up of $\alpha 1(XI)$ -, $\alpha 2(XI)$ -, and $\alpha 3(XI)$ chains (Yoshioka *et al*, 1995) and accounts for $\sim 3\%$ of the adult cartilage matrix (Eyre *et al*, 2006). Molecules of collagen IX and XI stabilise collagen II, a major collagen, and limit its lateral growth (Blaschke *et al*, 2000). The role of type XI collagen in the adult remains unclear, although recent evidence in numerous types of cancer implicates colXI $\alpha 1$ in carcinogenesis (Vecchi *et al*, 2007; Badea *et al*, 2008; Ellsworth *et al*, 2009; Kim *et al*, 2010). Further, expression of colXI $\alpha 1$ is reported to be accompanied expression of genes associated with epithelial-to-mesenchymal transition (EMT) (Anastassiou *et al*, 2011).

We previously reported that colXI $\alpha 1$ was present in tumour tissue and was undetectable in corresponding normal adjacent tissue in a cDNA microarray analysis of tissue from nine HNSCC patients (Sok *et al*, 2003). Microarray of paired tumour and normal tissue from 36 patients with pancreatic ductal adenocarcinoma revealed greater than two-fold overexpression of colXI $\alpha 1$ in tumour tissue compared with normal tissue (Badea *et al*, 2008). RT-PCR of paired tumour and normal tissue from 70 pairs of patients with non-small cell carcinoma demonstrated 3.4-fold higher expression of colXI $\alpha 1$ in tumour tissue (Chong *et al*, 2006). Similarly, RT-PCR of paired malignant and premalignant tissue from 21 patients with gastric cancer showed increased levels of colXI $\alpha 1$ in malignant tissue (Zhao *et al*, 2009), and RT-PCR of polyps and normal tissue from 1 patient with familial adenomatous polyposis showed increased colXI $\alpha 1$ expression in the polyps (Fischer *et al*, 2001a). Most of the reports on colXI $\alpha 1$ in cancer represent correlative studies; thus, to our knowledge, this is the first investigation of the functional significance of colXI $\alpha 1$ overexpression in any type of cancer.

Collagens in general are important components of the extracellular matrix. It has been shown that collagen cross linking increases ECM stiffness and promotes tumorigenesis in breast cancer and that reduction in collagen cross linking leads to a decrease in tumour incidence (Blaschke *et al*, 1994). We undertook the present study to determine the role of colXI $\alpha 1$ in proliferation, migration, and invasion of head and neck cancer. Our results suggest that colXI $\alpha 1$ expression is increased in HNSCC tumours and cell lines compared with levels in normal tissue or immortalised epithelial cells. Further investigation demonstrated that colXI $\alpha 1$ contributes to the proliferation, migration, and invasion of head and neck cancer cells *in vitro*.

HNSCC carcinogenesis is characterised by a high incidence of synchronous and metachronous tumours, suggesting that molecular changes that are found in both the tumour and adjacent normal mucosa represent relatively early genetic changes (Bedi *et al*, 1996; Scholes *et al*, 1998). In this study, we found that colXI $\alpha 1$ expression by RT-PCR was higher in tumour compared with normal tissue in paired samples in 16 of 23

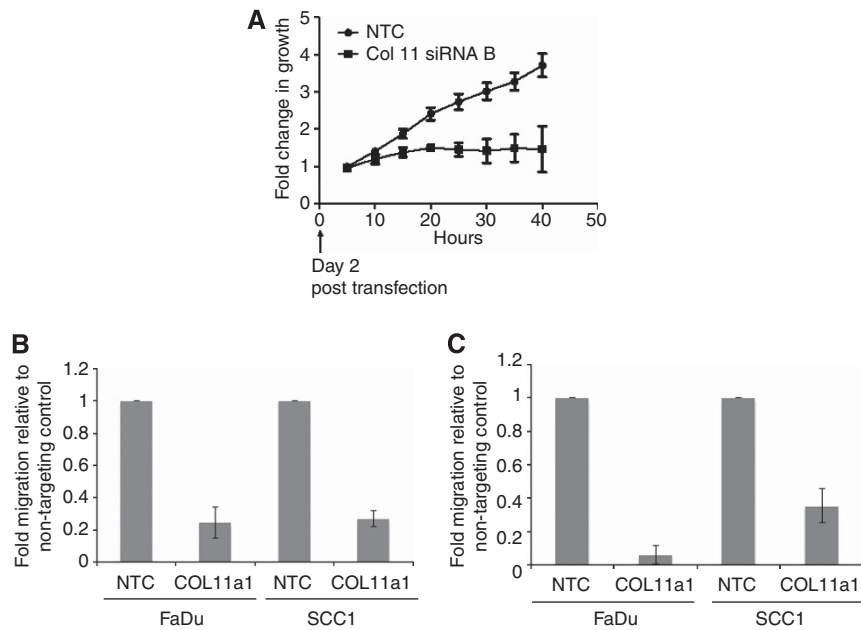


Figure 5. ColXI α 1 contributes to HNSCC proliferation, migration, and invasion. **(A)** HNSCC cells UMSSC-1 were transfected with non-targeting control siRNA (NTC) or colXI α 1 siRNA. Cells were plated on day 2 post transfection, in the xCelligence DP E-plate and assessed for cell growth in real-time over 40 h. The cells were treated in quadruplicates. The line graph depicts cumulative data from four separate experiments. Error bars represent \pm s.e.m. UMSSC-1 and FaDu cells transfected with siRNA-colXI α 1 duplex B or non-targeting control siRNA (NTC) was determined using a Boyden chamber **(B)** migration and **(C)** invasion assay. Migrated cells were counted 24 h post plating in four separate fields. Cumulative results are shown for wells treated in triplicate from three independent experiments ($P=0.05$). Error bars represent \pm s.e.m. **(C)** The invasion of UMSSC-1 and FaDu cells transfected with siRNA-colXI α 1 duplex B compared with cells transfected with non-targeting control siRNA (siRNA-NTC) was determined using a Boyden chamber Matrigel assay. Cumulative results from three independent experiments demonstrate a significant reduction in HNSCC invasion up on colXI α 1 siRNA treatment compared with the NTC siRNA control ($P=0.05$).

HNSCC patients, indicating that increased colXI α 1 expression is a later event in HNSCC carcinogenesis. Our findings are consistent with previous findings of increased colXI α 1 in tumour compared with normal tissue in paired samples from 70 patients with non-small cell lung carcinoma and in malignant tissue compared with pre-malignant tissue in paired specimens from 21 patients with gastric cancer (Zhao *et al*, 2009). Our attempts at generating a polyclonal antibody to colXI α 1 were not successful primarily because of the paucity of unique antigenic epitopes. Lack of specific antibodies limited our ability to assess protein levels of colXI α 1 in tissue and cell lines.

Because changes in the stromal tumour microenvironment are thought to contribute to invasion and spread of tumours (De Wever and Mareel, 2003), we compared the expression levels of colXI α 1 in cancer-associated fibroblasts and normal oral fibroblasts derived from cancer-free patients. We found that cancer-associated fibroblasts had higher levels of colXI α 1 compared with normal oral fibroblasts. The cancer-associated fibroblasts and normal oral fibroblasts used in these studies were primary cells derived from fresh tissue. These cells are cultured for up to 10 passages and retain their fibroblast morphology. However, it cannot be ruled out that HNSCC cells that have undergone epithelial-to-mesenchymal transition are present among the cancer-associated fibroblast cultures. The data suggest that changes in mesenchymal cells in the HNSCC microenvironment are associated with increased levels of colXI α 1, a finding that is at odds with Halsted *et al* (2008), who found that colXI α 1 was downregulated in stroma surrounding breast cancer. In another study of breast cancer, however, higher levels of colXI α 1 expression in primary breast tumours than in corresponding metastatic lymph node tumours were reported and concluded that the overexpression of genes in the primary tumours were involved with extracellular matrix degradation and contributed to metastatic

spread of the tumour (Ellsworth *et al*, 2009). Further, it has been proposed that interaction between breast and colon cancer cells with the microenvironment triggers expression of mesenchymal signature genes including colXI α 1 (Cheng *et al*, 2013).

We sought to determine if increased colXI α 1 expression was derived from the transformed epithelial cells and/or stromal cells in the tumour microenvironment. To date, there is no literature on colXI α 1 expression in cancer cell lines compared with normal cells. We compared colXI α 1 mRNA levels in nine validated, representative HNSCC cell lines using RT-PCR and found that expression was increased in all nine HNSCC cell lines, whereas the corresponding normal cells did not express the colXI α 1 transcript. Thus we conclude that both the tumour and stromal fibroblast cells contribute to the high levels of colXI α 1 in HNSCC tissue.

Although studies of gastric, lung, ovarian, and colorectal carcinomas have implicated the role of colXI α 1 overexpression in more advanced disease (Schmalbach *et al*, 2004; Vecchi *et al*, 2007; Zhao *et al*, 2009; Kim *et al*, 2010), only one study has correlated colXI α 1 and tumour size (Chong *et al*, 2006). Further, no direct investigation has been undertaken to examine the role of colXI α 1 in cellular proliferation of cancerous or normal cells. We postulated that by knocking down colXI α 1 in HNSCC cells *in vitro*, proliferation would decrease. We transfected a representative HNSCC cell line (UMSSC-1) with siRNA directed against colXI α 1 and demonstrated decreased colXI α 1 expression by RT-PCR. We then demonstrated that cellular proliferation of UMSSC-1 cells decreased in the siRNA-colXI α 1-transfected cells in comparison with the control. In contrast, normal Het1A cells did not display a decrease in proliferation after transfection with siRNA-colXI α 1. Therefore, these results suggest that colXI α 1 overexpression does, in fact, contribute to cellular proliferation in cancer cells and that knockdown of colXI α 1 abrogates cell growth in cancer cells but not normal cells.

ColXI $\alpha 1$ has been implicated in metastasis, with one study demonstrating higher levels of colXI $\alpha 1$ in primary tumours of the head and neck that have metastasised to the lymph node compared with tumours that remain confined to the primary site (Schmalbach *et al*, 2004). Cellular migration and invasion are thought to contribute to metastasis. No studies to date have examined the role of colXI $\alpha 1$ on cell motility of any type. We assessed the role of colXI $\alpha 1$ in invasion and migration in UMSSC-1 and Het1A cells that had been transfected with siRNA-colXI $\alpha 1$. We demonstrated that knocking down colXI $\alpha 1$ decreases migration and invasion in the HNSCC cell lines UMSSC-1 but not in the normal cell line Het1A. Thus, colXI $\alpha 1$ not only contributes to proliferation but also to invasion and migration as well in cancer cells, with no effect on normal cells.

Patients with HNSCC, like many epithelial cancers, succumb to disease that has metastasised. The precise mechanisms of HNSCC metastasis, including migration and invasion remain incompletely understood. The results of the present study suggest that colXI $\alpha 1$ has a role in mediating the proliferation, invasion, and migration of cancer cells, underscoring the need for further investigation of this collagen in carcinogenesis.

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